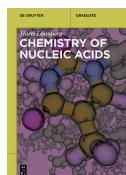


Ramon Eritja (Ed.)

**Nucleic Acids Chemistry**

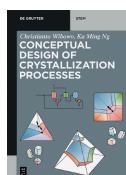
## Also of Interest



*Chemistry of Nucleic Acids*

Lönnberg, 2020

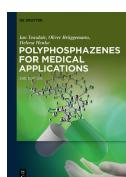
ISBN 978-3-11-060927-1, e-ISBN 978-3-11-060929-5



*Conceptual Design of Crystallization Processes*

Wibowo, Ng, 2021

ISBN 978-1-5015-1987-1, e-ISBN 978-1-5015-1990-1



*Polyphosphazenes for Medical Applications*

Teasdale, Brüggemann, Henke, 2020

ISBN 978-3-11-065253-6, e-ISBN 978-3-11-065418-9



*Bioinorganic Chemistry*

Rabinovich, 2021

ISBN 978-3-11-049204-0, e-ISBN 978-3-11-049443-3



*Climate Change and Marine and Freshwater Toxins*

Botana, Louzao, Vilarino (Eds.), 2021

ISBN 978-3-11-062292-8, e-ISBN 978-3-11-062573-8

# **Nucleic Acids Chemistry**

---

Modifications and Conjugates for Biomedicine and  
Nanotechnology

Edited by  
Ramon Eritja

**DE GRUYTER**

**Editor**

Ramon Eritja

Institute for Advanced Chemistry of Catalonia  
(IQAC-CSIC)

Networking Center on Bioengineering,  
Biomaterials and Nanomedicine (CIBER-BBN)  
Jordi Girona 18-26  
E-08034 Barcelona  
Spain  
[recgma@cid.csic.es](mailto:recgma@cid.csic.es)

ISBN 978-3-11-063579-9

e-ISBN (PDF) 978-3-11-063953-7

e-ISBN (EPUB) 978-3-11-063648-2

**Library of Congress Control Number: 2020949537**

**Bibliographic information published by the Deutsche Nationalbibliothek**

The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie;  
detailed bibliographic data are available on the Internet at <http://dnb.dnb.de>.

© 2021 Walter de Gruyter GmbH, Berlin/Boston

Cover image: ktsimage / iStock / Getty Images Plus

Typesetting: VTeX UAB, Lithuania

Printing and binding: CPI books GmbH, Leck

[www.degruyter.com](http://www.degruyter.com)

# Contents

## List of Contributing Authors — VII

Anna Aviñó, Carme Fàbrega, and Ramon Eritja

- 1      **Methods for the synthesis of oligonucleotides** — 1

Carme Fàbrega and Ramon Eritja

- 2      **Synthesis of oligonucleotides carrying DNA lesions for DNA repair studies** — 45

Carme Fàbrega and Ramon Eritja

- 3      **Synthesis of oligonucleotides carrying modified bases for DNA and protein recognition** — 87

Anna Aviñó, Carme Fàbrega, and Ramon Eritja

- 4      **Nonradioactive labeling of oligonucleotides and postsynthetic modification of oligonucleotides** — 143

Ramon Eritja

- 5      **Nucleic acids triple helices** — 187

Raimundo Gargallo, Carlos González, Carme Fàbrega, Anna Aviñó, and Ramon Eritja

- 6      **Nucleic acids quadruplex** — 231

Andreia F. Jorge, Santiago Grijalvo, Anna Aviñó, Carme Fàbrega, and Ramon Eritja

- 7      **Advances in therapeutic oligonucleotide chemistry** — 273

Andreia F. Jorge, Santiago Grijalvo, Anna Aviñó, Carme Fàbrega, and Ramon Eritja

- 8      **Oligonucleotide conjugates and DNA nanotechnology** — 331

**Index** — 359



# List of Contributing Authors

## **Anna Aviñó**

Institute for Advanced Chemistry of Catalonia  
(IQAC-CSIC)  
Networking Center on Bioengineering,  
Biomaterials and Nanomedicine (CIBER-BBN)  
Jordi Girona 18-26  
E-08034 Barcelona  
Spain  
E-mail: aaagma@cid.csic.es

## **Raimundo Gargallo**

Dept. of Chemical Engineering and Analytical  
Chemistry  
University of Barcelona  
Martí i Franquès 1  
E-08028 Barcelona  
Spain  
E-mail: raimon\_gargallo@ub.edu

## **Ramon Eritja**

Institute for Advanced Chemistry of Catalonia  
(IQAC-CSIC)  
Networking Center on Bioengineering,  
Biomaterials and Nanomedicine (CIBER-BBN)  
Jordi Girona 18-26  
E-08034 Barcelona  
Spain  
E-mail: recgma@cid.csic.es

## **Carlos González**

Instituto de Química Física “Rocasolano”  
CSIC  
Serrano 119  
E-28006 Madrid  
Spain  
E-mail: cgonzalez@iqfr.csic.es

## **Carme Fàbrega**

Institute for Advanced Chemistry of Catalonia  
(IQAC-CSIC)  
Networking Center on Bioengineering,  
Biomaterials and Nanomedicine (CIBER-BBN)  
Jordi Girona 18-26  
E-08034 Barcelona  
Spain  
E-mail: carme.fabrega@iqac.csic.es

## **Santiago Grijalvo**

Institute for Advanced Chemistry of Catalonia  
(IQAC-CSIC)  
Networking Center on Bioengineering,  
Biomaterials and Nanomedicine (CIBER-BBN)  
Jordi Girona 18-26  
E-08034 Barcelona  
Spain  
E-mail: santiago.grijalvo@iqac.csic.es

## **Andreia F. Jorge**

Institute for Advanced Chemistry of Catalonia  
(IQAC-CSIC)  
Networking Center on Bioengineering,  
Biomaterials and Nanomedicine (CIBER-BBN)  
Jordi Girona 18-26  
E-08034 Barcelona  
Spain  
E-mail: andreiajorge09@gmail.com



Anna Aviñó, Carme Fàbrega, and Ramon Eritja

# 1 Methods for the synthesis of oligonucleotides

## Contents

1.1	Introduction —	1
1.2	Historical development of the synthesis of oligonucleotides —	3
1.2.1	Phosphodiester method —	3
1.2.2	Phosphotriester method —	4
1.2.3	Introduction of the solid-phase —	5
1.2.4	Phosphite-triester —	5
1.2.5	H-phosphonate —	6
1.2.6	Enzymatic synthesis of oligonucleotides —	6
1.3	The protection of the 5'- and 3'-hydroxyl functions —	7
1.4	The protection of the 2'-OH group of RNA —	10
1.5	The protection of the nucleobases —	13
1.6	The protection of the phosphate group —	16
1.7	The solid-support and linking units —	18
1.8	The synthesis cycle in solid-phase phosphoramidite chemistry —	21
1.8.1	The basic steps —	21
1.8.2	Modifications in the basic steps —	23
1.9	Synthesis of DNA and RNA protected fragments —	25
	Bibliography —	30

## 1.1 Introduction

Nucleic acids are the molecules of life, responsible for the transmission of the genetic information. Oligonucleotides are small fragments of nucleic acids that can be produced by chemical synthesis. The development of chemical methods for the synthesis of oligonucleotides was addressed to fulfill an important needs encounter during the study of the structure and function of nucleic acids. It was the description of the double helical model for DNA by Watson and Crick [1], the event that triggered the interest for the development of the methodology for the preparation of synthetic oligonucleotides. The first synthesis of a nucleotide (5'-TT-3') was published in August 1955 by the group of Dr. Alexander Todd with the aim of demonstrating the nature of the 5'-3' phosphate bonds present in DNA [2]. This dinucleotide is the simplest oligonucleotide as thymidine does not need the protection of the nucleobase. For the formation of the

---

**Anna Aviñó, Carme Fàbrega, Ramon Eritja,** Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Networking Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Jordi Girona 18-26, E-08034 Barcelona, Spain, e-mails: aaagma@cid.csic.es, carme.fabrega@iqac.csic.es, recgma@cid.csic.es

5'-3' phosphate bond, the authors used H-phosphonate chemistry that was rediscovered 30 years later [3].

Gobind Khorana's group was the first lab that took a systematic study for the development of a method to produce defined synthetic oligonucleotides. The method, known as the phosphodiester method, took near 20 years of intensive research to reach the aim of producing a synthetic gene that was able to direct the synthesis of the corresponding tRNA [4]. In addition, the synthesis of DNA triplets, which were used for deciphering the genetic code, was also accomplished using the phosphodiester method [5]. The Nobel Prize in Physiology or Medicine was awarded to Dr. Khorana together with Drs. Holley and Nirenberg in 1968 for this important contribution.

The increasing need of synthetic genes and oligonucleotides such as adapters, primers and probes used in molecular biology triggered the development of the phosphotriester method and solid-phase methods. These were soon replaced by the phosphite-triester or phosphoramidite method which is the method of choice today.

At this time, the availability of oligonucleotides with natural or mutated DNA sequences allowed the production of specific point mutations on cloned DNA using oligonucleotide-directed mutagenesis [6, 7]. Synthetic oligonucleotides were also useful in the clinical diagnosis of point mutations found in several human genetic diseases [8]. The discovery of the Polymerase Chain Reaction (PCR) for *in vitro* amplification of nucleic acids [9] and the use of oligonucleotides as antisense inhibitors of gene expression in the 1990s [10] triggered a large demand for synthetic oligonucleotides. These advances were accomplished thanks to the full development of the phosphoramidite chemistry and the generalized use of DNA synthesizers.

Oligonucleotides for structural studies were crucial to elucidate the molecular details of DNA-DNA, RNA-RNA and DNA-RNA interactions as well as DNA-protein and RNA-protein interactions [11]. The synthetic methodology soon was able not only to produce natural oligonucleotides but also oligonucleotides carrying modified nucleobases. These modified oligonucleotides are described in the next chapters showing the large impact that these compounds had in the study of proteins with DNA as a substrate. These include transcription factors, DNA polymerases, DNA repair enzymes, restriction enzymes, DNA methylases [12] as well as to study proteins that have RNA as a substrate such as the RNA splicing machinery [13]. In addition, synthetic oligoribonucleotides were crucial in the discovery of ribozymes and the enzymatic activity of RNA [14].

The therapeutic potential of synthetic oligonucleotides, especially after the discovery of the RNA interference mechanism [15] and noncoding RNA [16] triggered a large research effort in the development of a large number of nucleic acid mimics and inhibitors that will be reviewed in Chapter 7.

The success in DNA sequencing of full genomes activated the need for the simultaneous synthesis of large arrays of oligomers (DNA microarrays or DNA chips) for diagnostic purposes and for the analysis of gene expression profiles [17, 18]. These arrays opened new fields such as molecular computation in where DNA molecules are

used for storing information [19, 20] and the development of artificially-designed new species [21].

Finally, DNA nanobiotechnology has emerged a new research field in which the synthetic oligonucleotides are the precise bricks for the construction of large architectures thanks to the base-pairing properties of DNA [22, 23]. Moreover, oligonucleotides have resulted as key elements in the development of the new detection systems of several diseases. The contribution of synthetic oligonucleotides in the development of these fields will be summarized in Chapter 8.

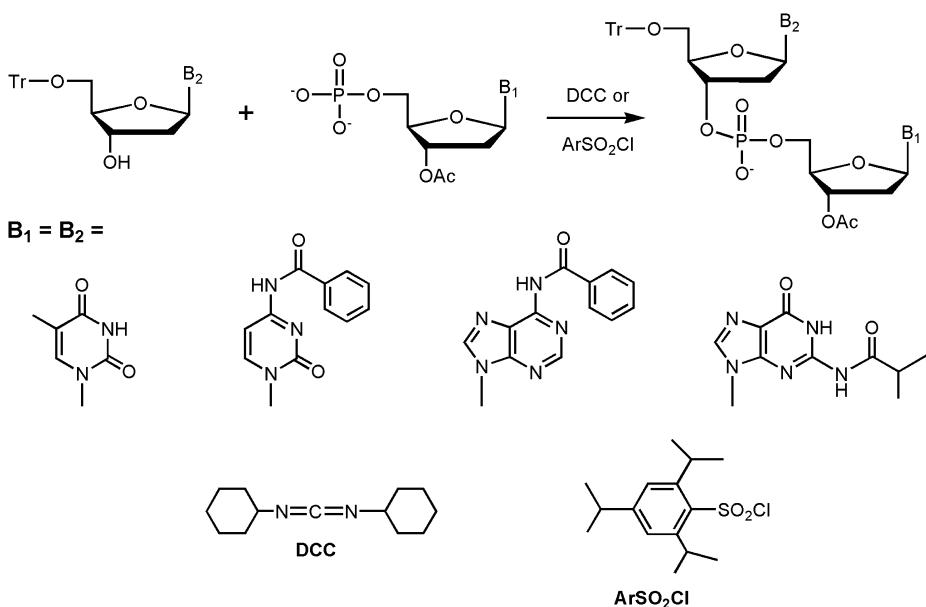
The progressive increment of synthetic oligonucleotides applications has been possible thanks to the continuous efforts in preparing modified phosphoramidites, solid supports and methodologies for an efficient synthesis of oligonucleotides. These advances have been accomplished over the years and will be summarized in the following chapters.

## 1.2 Historical development of the synthesis of oligonucleotides

The preparation of oligonucleotides is complex due to the 3'-5' phosphate group and the presence of several groups including hydroxyl and amino functions that may cross-react with phosphate activated derivatives. In order to synthesize defined oligonucleotides, a complete strategy of the protecting group as well as phosphate activating methods have to be developed [24, 25]. The first solution to the synthetic problem was developed during the 1960s by the group of Dr. H. G. Khorana and it is known as the phosphodiester method [4].

### 1.2.1 Phosphodiester method

The chemical methodology used for the synthesis of these oligomers involved the condensation of a nucleoside having a free 3'-hydroxyl group with a nucleoside 5'-phosphate (Figure 1.1). The coupling reaction was catalyzed by dicyclohexylcarbodiimide (DCC) [26] or by an aromatic sulfonyl chloride ( $\text{ArSO}_2\text{Cl}$ ) [27]. The method was named as phosphodiester because of the intermediate produced during the coupling reaction is a phosphodiester. The 3' and the 5' functions that were not involved in the reaction were protected with the base labile acetyl group and the acid labile trityl group, respectively. In this way, further elongation of the chain was achieved by removal of the 3'-acetyl group and the subsequent coupling of the following nucleoside 5'-phosphate. This methodology produced small oligonucleotides which were joined by T4 DNA ligase to give synthetic genes [4, 5]. The major drawbacks of this methodology were the ionic nature of the products, which had to be separated by tedious

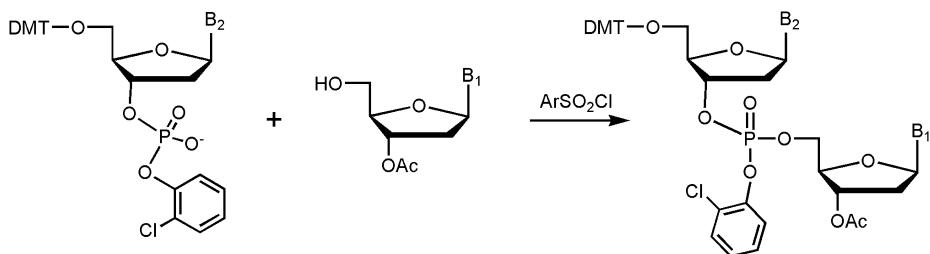


**Figure 1.1:** Protecting groups and coupling reaction used in the phosphodiester method. Tr: trityl; Ac: acetyl; DCC: dicyclohexylcarbodiimide; ArSO<sub>2</sub>Cl: arenesulfonyl chloride.

ion-exchange chromatography and the low efficiency of the condensation reactions. Importantly, the phosphodiester methodology set the grounds of the oligonucleotide synthesis field and the protecting groups developed for the 5'-hydroxyl and the amino functions are still used nowadays.

### 1.2.2 Phosphotriester method

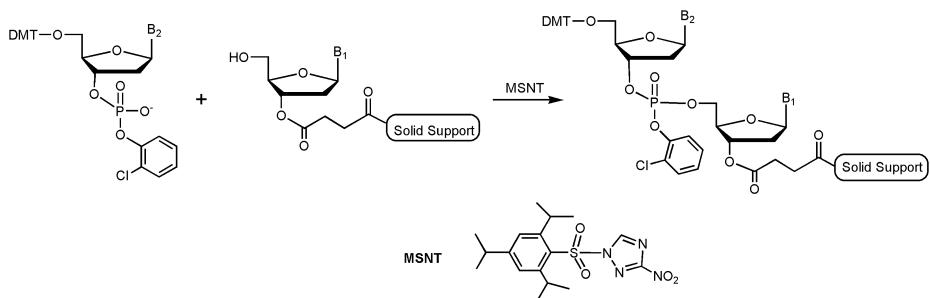
The next step was the development of the phosphotriester methodology. In this method, a protected 3'-O-phosphodiester was reacted with the 5'-hydroxyl group of the next nucleotide (Figure 1.2). However, two main problems had to be solved: first, which group was adequate for the protection of the phosphate group, and second, the activation of the phosphodiester. Aryl groups, especially the 2-chlorophenyl, were found the most suitable for the protection of phosphate group [28]. They could be removed by aldoximate ions without chain degradation. Other phosphate protecting groups such as 2-cyanoethyl and methyl were also developed at this time and they were of extreme importance during the development of the phosphite-triester method. Arenesulfonylazoles were among the most popular coupling agents together with a nucleophilic catalyst such as *N*-nitrotriazole, *N*-methylimidazole and 4-substituted pyridine-*N*-oxides [29, 30]. Oligoribonucleotides were also prepared using the phosphotriester method in spite of the complexity added by the presence of an extra hydroxyl in the 2' position [31].



**Figure 1.2:** Coupling reaction in the phosphotriester method. DMT: dimethoxytrityl; Ac: acetyl; ArSO<sub>2</sub>Cl: arenenesulfonyl chloride.

### 1.2.3 Introduction of the solid-phase

Another important milestone during the development of the phosphotriester method was the use of solid supports for the synthesis of oligonucleotides (Figure 1.3). The first successful synthesis was reported in 1965 [32], shortly after Merrifield's description of the synthesis of the first peptide on solid-phase. The newly developed phosphotriester methodologies were suitable for solid-phase synthesis and, for this reason, linkers and supports were developed. In the mid-1970s, automation of the synthetic cycles started and HPLC began to be used for the purification of oligonucleotides. DNA synthesizers appeared and oligonucleotides started to be available for biochemical and molecular biology studies.

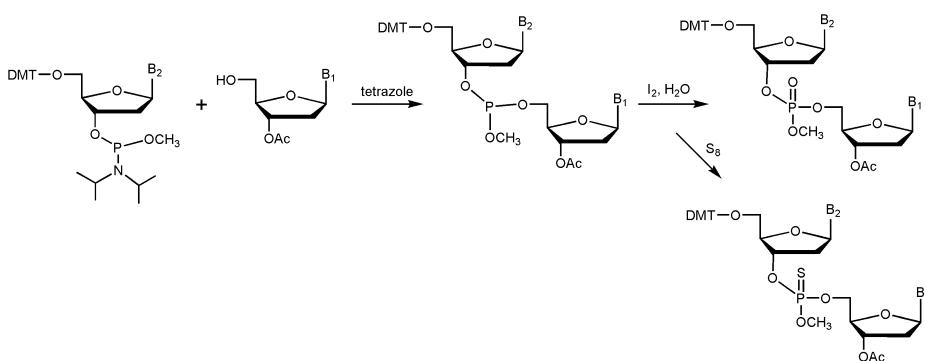


**Figure 1.3:** Solid-phase coupling reaction between 5'-O-DMT-3'-O-2-chlorophenyl phosphate-protected nucleoside and the protected nucleoside attached to the solid support using MSNT as a coupling agent.

### 1.2.4 Phosphite-triester

At the same time that automation of the DNA synthesis was being developed, the group of Letsinger realized the potential of phosphorous III derivatives [33] that lead to the development of the phosphite-triester method. Shortly afterwards, Beaucage

and Caruthers reported the nucleoside phosphoramidites [34] that could be synthesized, stored and activated by  $1H$ -tetrazole to yield phosphite-triester intermediates. Finally, these intermediates were transformed to phosphate-triester by oxidation (Figure 1.4). Nowadays, phosphoramidite derivatives are the most widely used derivatives for oligonucleotide synthesis allowing the preparation of oligonucleotides with natural phosphodiester bonds and also with modified phosphates such as phosphorothioates. A large number of the advances on nucleic acid chemistry achieved by the phosphoramidite approach can be found in the excellent reviews of Beaucage and Iyer [35–38].



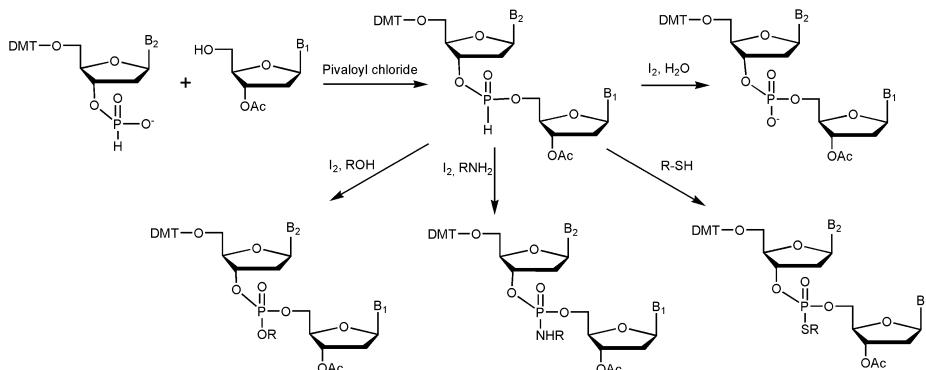
**Figure 1.4:** Coupling reaction between 5'-DMT-3'-O-methylphosphoramidites and the 3'-O-protected nucleoside to obtain phosphite-triester intermediates. The intermediate can be oxidized with iodine solution to produce oligonucleotide methyl phosphates or with sulfur to produce oligonucleotide methyl phosphorothioates. DMT: dimethoxytrityl; Ac: acetyl.

### 1.2.5 H-phosphonate

Soon after the development of phosphoramidites, the *H*-phosphonate method was introduced [3, 39]. In this case, the protected nucleosides 3'-*O*-hydrogen phosphonate were activated with acid chlorides such as pivaloyl chloride or, preferably, adamantoyl chloride to yield *H*-phosphonate diester intermediates (Figure 1.5). Oxidation of *H*-phosphonate diesters to phosphodiester (or as well to phosphorothioate and phosphoramidates) was performed at the end of the synthesis in one single step. The *H*-phosphonate method is as rapid as the phosphoramidite one although it is less efficient. Therefore it is limited to shorter oligonucleotides and for the synthesis of some modified oligonucleotides.

### 1.2.6 Enzymatic synthesis of oligonucleotides

As alternative to chemical methods, it is possible to use enzymatic processes to prepare defined oligonucleotides from cloned DNA and using nucleoside triphosphates



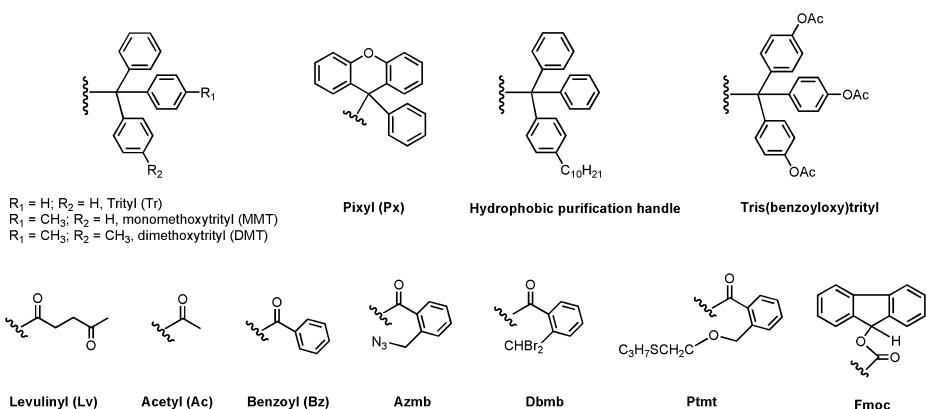
**Figure 1.5:** The *H*-phosphonate method to obtain oligonucleosides phosphate diesters, phosphoroxyates and phosphoramidates. The oxidation takes place at the end of the synthesis. DMT: dimethoxytrityl; Ac: acetyl.

as building blocks. This is especially relevant for the synthesis of RNA using SP6 [40] and T7 [41, 42] RNA polymerases. The enzymatic method is frequently used for the preparation of RNA larger than 50 bases as well as for the preparation of <sup>13</sup>C and <sup>15</sup>N labeled RNAs for structural studies [43, 44].

Recently, the use of terminal deoxynucleotidyl transferase (TdT) for the preparation of oligonucleotides has been described [45]. This enzyme is a template-independent polymerase that has been profusely used for the addition of labeled nucleotides at the 3'-end of synthetic oligonucleotides [46]. In the work of Palluk et al., TdT is linked to a single 2'-deoxynucleotide triphosphate which is transferred to the 3'-end of a single-stranded DNA that remains tethered to the TdT molecule avoiding the incorporation of an undesired extra nucleotide. Then the cleavage of the one-base extended oligonucleotide from the TdT molecule allow the oligonucleotide undergo to another cycle of addition of a nucleotide at the 3'-end by a novel nucleotide-loaded TdT conjugate. Although the actual size of oligonucleotides made by this methodology are only 10 bases long, the authors demonstrate the potential of this environmental friendly method triggering a large interest in the field of digital information storage [47].

### 1.3 The protection of the 5'- and 3'-hydroxyl functions

From the very beginning, the acid labile trityl groups (Figure 1.6) were selected for the protection of the 5'-hydroxyl group [48]. These groups have the advantage of being voluminous and for this reason they can react selectively with the 5'-primary hydroxyl functions which are one order of magnitude more reactive than the secondary 3'-hydroxyl groups. Trityl groups are removed in acidic conditions and, for



**Figure 1.6:** Groups used in the protection of the 3'- and 5'-OH functions of nucleosides.

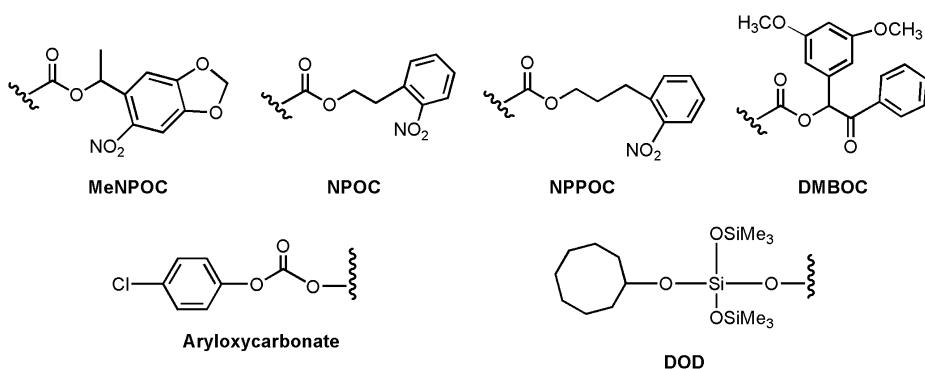
this reason, they are orthogonal to ester groups that are labile to bases. However, as the 2'-deoxypyrimidine nucleosides can undergo nucleobase cleavage (depurination) during acid treatments; the plain trityl group was found too stable. The acid lability of the trityl groups was increased by the introduction of one or two electro withdrawing methoxy groups. The dimethoxytrityl (DMT) was the group selected for oligonucleotide synthesis as it can be easily removed with diluted solutions of dichloroacetic or trichloroacetic acid [49, 50]. In the past, the monomethoxytrityl (MMT) group was used for the protection of 2'-deoxyguanosine (dG) as the DMT group was considered too labile, but finally the use of rapid synthesis protocols in the automatic synthesizers implemented the DMT group for the four natural bases.

The DMT group has also the advantage to produce an orange color due to the release of the DMT cation during its removal. The colored solution is used to monitor the coupling reactions in solid-phase methods. Other trityl groups with different colored cations were developed to control the coupling reactions [51]. The pixyl group [52] was proposed as alternative for the DMT group as pixyl-nucleoside derivatives produced crystalline solids. Nevertheless, despite their potential advantages, these groups have no longer been used. Moreover, trityl groups carrying long hydrocarbon linear chains have been used as “purification handles” to facilitate the purification of tritylated oligonucleotides by reverse-phase chromatography [53]. Another interesting trityl derivative was the 4,4',4''-tris(benzyloxy)trityl group [54] that is removed under basic conditions instead of the usual acid conditions. Saponification of the benzoyl groups generates the labile trihydroxytrityl group that decomposes generating the free OH group.

Other groups (Figure 1.6) that were developed for the protection of the 5'-OH in oligonucleotide synthesis in solution were: levulinyl (Lev) [55], 2-(dibromomethyl)benzoyl (Dbmb) [56, 57], 2-(isopropylthiomethoxymethyl)benzoyl (Ptmt) [58], 2-(azidomethyl)benzoyl (AZMB) [59] and the 9-fluorenylmethoxycarbonyl (Fmoc) [60].

These groups were mostly used for solution synthesis although the Fmoc group has also been described for RNA solid-phase synthesis [61]. Silyl groups such as the *bis*(trimethylsiloxy)-cyclooctyloxysilyl and the *bis*(trimethylsiloxy)-cyclododecyloxysilyl (DOD) groups have also been reported for the protection of the 5'-OH in a singular protocol for RNA solid-phase synthesis [62, 63] that will be detailed in Section 1.4.

The preparation of DNA microarrays introduced several changes in the protection of the 5'-hydroxyl group. In some cases, the DMT group was removed by acid generated at the required site by electrochemistry [64]; however, in most of the cases, photolabile groups (Figure 1.7) were developed for the protection of the 5'-hydroxyl groups [17, 18]. The  $\alpha$ -methyl-*o*-nitropiperonyloxycarbonyl (MeNPOC) [65] was the first photolabile group used in the fabrication of DNA microarrays. This group was removed by irradiation with UV light of 365 nm wavelength [66]. The 2-nitrophenylethoxycarbonyl (NPOC) [67], the 2-nitrophenylpropoxycarbonyl (NPPOC) group [68] and other related derivatives were developed by the laboratory of Dr. Pfeiderer [69]. The NPPOC group and the benzoyl derivative of the NPPOC (BzNPPOC) group have been also used for the preparation of DNA microarrays by reverse 5' → 3' direction with similar high yields than conventional synthesis [70]. Another photolabile group was the 3',5'-dimethoxybenzoinoxycarbonyl (DMBOC) [71].



**Figure 1.7:** Groups described for the protection of the 5'-hydroxyl functions of nucleosides in the synthesis of DNA microarrays.

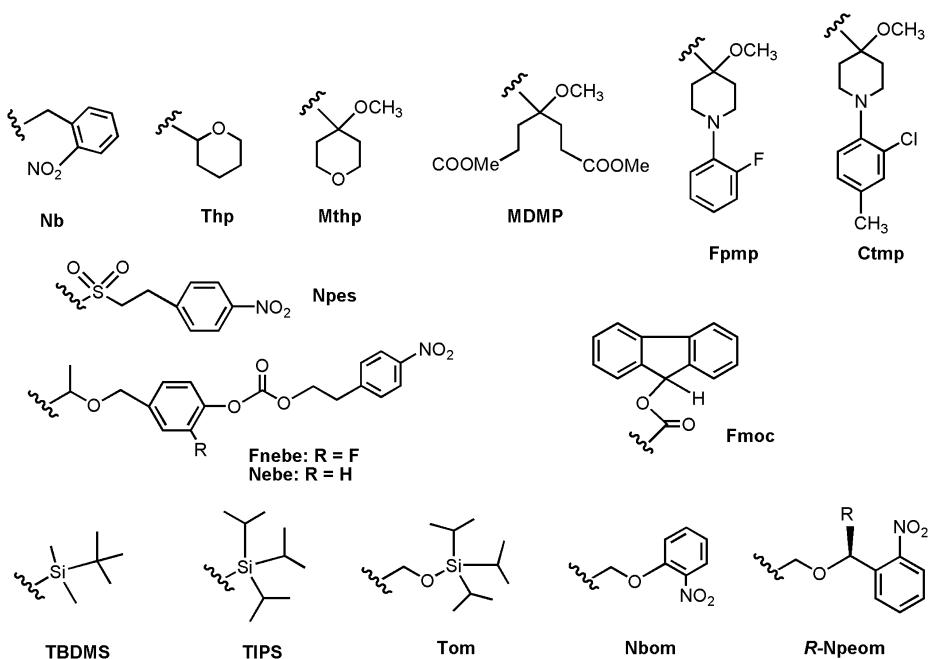
Another technique for the preparation of DNA microarray uses high-resolution printers to deliver the reagents. To this purpose, a novel protecting group for the 5'-OH was developed to substitute the acid labile trityl ones. 5'-Aryloxy carbonates (Figure 1.7) were selected as they are easily removed by peroxy anions buffered at pH 9.6 [72]. These oxidative conditions were also able to oxidize the phosphite to phosphate groups, avoiding the use of the standard iodine solution, and consequently converting the classical three-step to a faster two-step synthesis cycle.

In the solution synthesis, the terminal 3'-hydroxyl function is usually protected with a base labile group. Esters are the most used especially acetyl (Ac) and benzoyl (Bz) groups [73]. Other ester groups such as the levulinyl (Lev) and the silyl groups were also used. These protecting groups are detailed in the next section (1.4).

## 1.4 The protection of the 2'-OH group of RNA

One of the major challenges in RNA oligonucleotide synthesis is the protection of the 2'-hydroxyl function. Although a large number of potential protecting groups have been developed [74], there is still a large interest in the discovery of new groups [75]. The protection of the 2'-OH not only has to be orthogonal to the rest of protective groups but also it has to be stable to potential 2'-3' migration observed for some protective groups. In addition, unprotected oligoribonucleotides are not stable to strong basic conditions and they are highly susceptible to degradation by ribonucleases.

One of the first groups to be successfully used was the o-nitrobenzyl (Nb) ether (Figure 1.8) that can be removed by photolysis [76]. Ethers are not prone to 2'-3' migration and its removal was done in slightly acid conditions. Unfortunately, the photolytical removal of these groups becomes ineffective for the synthesis of long RNA sequences.



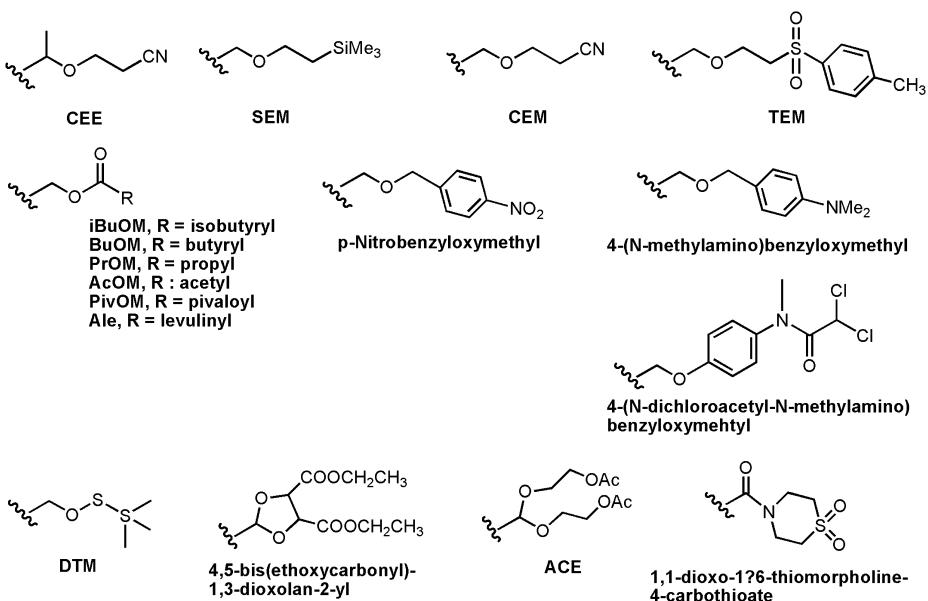
**Figure 1.8:** Groups described for the protection of the 2'-hydroxyl functions of ribonucleotides.

Next, acid labile acetal groups (Figure 1.8) were developed including the tetrahydropyran-2-yl (Thp) [77], the 4-methoxytetrahydropyran-4-yl (Mthp) [78], the 3-methoxy-1,5-dicarbomethoxypentan-3-yl (MDMP) [79], the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) [80, 81] and the 1-(2-chloro-4-tolyl)-4-methoxypiperidin-4-yl (Ctmp) [82] groups. All were removed in strong aqueous acidic conditions (pH 1.2) but still were orthogonal to 5'-O-DMT group that was removed in diluted trichloroacetic acid solutions in dichloromethane. The most popular of the acetal groups was the Fpmp and the corresponding Fpmp-protected phosphoramidite were produced commercially until the beginning of the twenty-first century. This allowed the synthesis of oligoribonucleotides carrying modified bases [83, 84].

Silyl ethers protecting groups are another alternative because they are stable to acid and basic conditions but they can be removed using fluoride solutions due to the strong stability of the F-Si bond. The *t*-butyldimethylsilyl (TBDMS) and the triisopropylsilyl (TIPS) groups were developed in the nucleotide field by the laboratory of Dr. Ogilvie [85–87] being the TBDMS group profusely used for solid-phase RNA synthesis. Although it is the most popular protecting group for the 2'-OH, some disadvantages have been described such as potential 2'-3' migration, incomplete removal during fluoride deprotection of long RNA sequences and low coupling yields due to steric hindrance of the bulky TBDMS group. Some of these inconveniences have been solved using triethylamine hydrofluoride at 50 °C instead of tetrabutylammonium fluoride [88, 89] and using more reactive tetrazole catalysts [89]. However, there is an intense research effort in the search of new protective groups with less steric hindrance. One of the best solutions is the use of (triisopropylsilyl)oxymethyl (Tom) [90, 91] groups. In this protecting group, the bulky triisopropylsilyl function is connected to the 2'-OH position through an oxymethyl linker that reduces the steric effect of the silyl group. The oxymethyl moiety decomposes after the elimination of the silyl group by fluoride yielding the free 2'-OH. The advantages of the addition of the oxymethyl moiety were first demonstrated in the development of the photolabile (2-nitrobenzyl)oxymethyl (Nbm) [92] group that carries the oxymethyl moiety linked to the photolabile 2-nitrobenzyl group. A variation of these derivatives is the photolabile (*R*)-1-(2-nitrophenyl)ethoxymethyl (*R*-Npeom) [93] group.

Other hydroxyl protecting groups without silicon are the groups that are removed with bases by a  $\beta$ -elimination process such as the *p*-nitrophenylethylsulfonyl (Npes) [94], the 4-[2-(4-nitrophenylethoxycarbonyl)oxybenzyloxyethyl (Nebe) [95] and the 3-fluoro-4-[2-(4-nitrophenylethoxy)carbonyl]oxybenzyloxyethyl (Fnebe) [95] groups.

The combination of the oxymethyl acetals with base and fluoride labile derivatives generate a large number of new protecting groups for the 2'-OH functions (Figure 1.9) including the 2(trimethylsilyl)ethoxymethyl (SEM) [96], the 1-(2-cyanoethoxy)ethyl (CEE) [97], the 2-cyanoethoxymethyl (CEM) [98], the 2-(4-Tolylsulfonyl)ethoxymethyl (TEM) [99] and the *p*-nitrobenzyloxymethyl groups [100]. Between all of them, the CEM group generated large interest in the synthesis of large RNA molecules [101]. Other oxymethyl acetal derivatives are the acetal esters including isobutyryloxymethyl



**Figure 1.9:** Oxyacetal derivatives as protecting groups of hydroxyl groups of nucleosides.

(iBuOM) [102], the butyryloxymethyl (BuOM) [102], propionyloxymethyl (PrOM) [102], acetyloxymethyl (AcOM) [103], pivaloyloxymethyl (PvOM) [104] and levulinylloxymethyl (Ale) [105]. In addition to basic conditions, some of these groups can be cleaved by cellular esterases and for this reason they can be used to generate the RNA molecules inside cells acting as RNA prodrugs [102, 103].

Other oxymethyl acetals have been designed to be cleaved at acid aqueous conditions such as the 4-(*N*-methylamino)benzyloxymethyl [106] and the 4-(*N*-dichloroacetyl-*N*-methylamino)benzyloxymethyl [107]. Finally, in the *tert*-butyldithiomethyl (DTM) [107] group, a disulfide bond is connected to the oxymethyl acetal moiety so this group can be removed using reductive conditions such as thiols or phosphines [108].

A special case of the oxymethyl acetals are the ones based on *ortho*-esters such as the 4,5-*bis*(ethoxycarbonyl)-1,3-dioxolan-2-yl [109] and the *bis*(2-acetoxyethoxy)methyl (ACE) group [62, 63]. A special combination of the ACE with the DOD silyls at the 5'-OH [62] has been used to generate RNA molecules with high purity [62]. Moreover, the use of the benzhydroxy-*bis*(trimethylsilyloxy)silyl (BzH) for the protection of the 5'-OH was also described in combination with the ACE group for the protection of the 2'-OH [110].

Finally, another interesting group is the thionocarbamate 1,1-dioxo-1*λ*<sup>6</sup>-thiomorpholine-4-carbothioate [111]. This group is very stable during the RNA synthesis but is readily removed using 1,2-diamines such as ethylenediamine.

## 1.5 The protection of the nucleobases

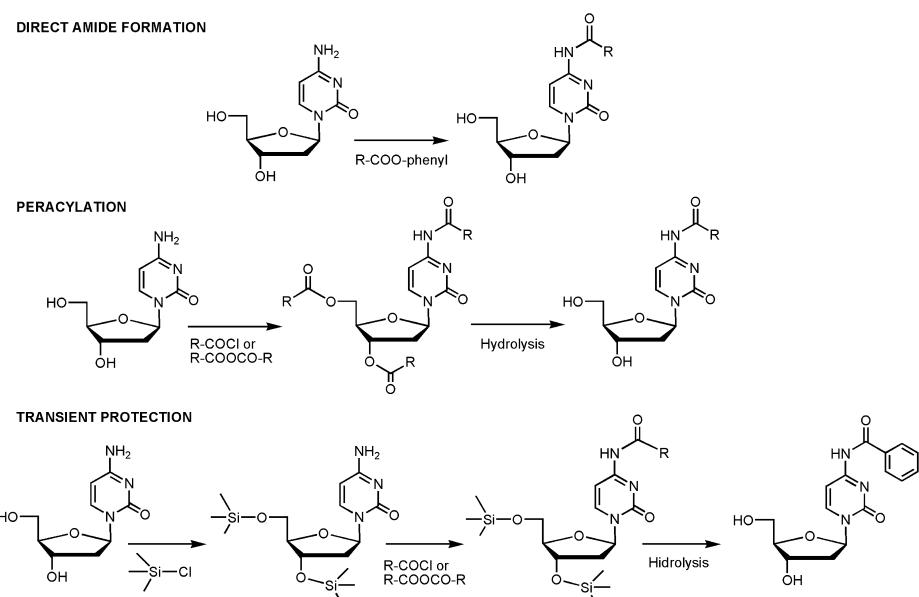
Nucleobases have amino and lactam groups that require protection as they may interfere in the coupling reactions. The most nucleophilic amino group is the 4-amino group of the cytosine (C) that is more reactive than the alcohol functions. In this case, the introduction of the protecting group could be done without interference with the alcohol groups. The amino groups of adenine (A) and guanine (G) are less reactive nevertheless they have to be protected. Although there have been reports using H-phosphonates which describes the synthesis of short oligonucleotides without the protection of the nucleobases [112–114].

In general, the lactam groups of thymine (T) and G are left unprotected. These groups may react with activated phosphotriester [115] and phosphoramidites [116] derivatives; however, some of these side reactions can be reversed in the capping step of the solid-phase synthesis protocols by replacing *N,N*-dimethylaminopyridine for *N*-methylimidazole [116]. In addition, T modification was observed during the removal of the methyl phosphate protecting groups [117, 118]. In spite of these potential side reactions, the lactam groups of T and G are usually left unprotected [119], although there are several protective groups [120] that may be used for the protection of T and G lactams such as the *O*<sup>6</sup>-diphenylcarbamoyl (Dpc) [121] and the *O*<sup>4</sup>-phenyl [122] groups.

The most frequent amino protecting groups are the benzoyl (Bz) for the protection of A and C and the isobutyryl (ibu) for G. These groups, developed by Khorana for the phosphodiester method (Figure 1.1), are removed by a treatment with concentrated aqueous ammonia at 55 °C for at least 6 hours [120]. The benzoyl group was also studied for the protection of G but it was considered too stable and, for this reason, the ibu group is used. However, still the isobutyryl group is considered too stable for the phosphotriester and phosphoramidite chemistries [120].

The introduction of the protecting groups in the nucleobases can be done by three different strategies. As mentioned above, cytosine nucleosides can be reacted directly with active esters to generate the *N*-4-protected derivatives without reaction with the alcohol functions (Figure 1.11) [123, 124]. In the classical peracylation method, the A, C or G nucleosides reacted with an excess of the corresponding acid anhydride or acid chloride [48]. This generates the introduction of the protecting group in both amino and alcohol functions. Then the ester functions are hydrolyzed selectively with an aqueous sodium hydroxide solution that generates the desired amino-protected nucleoside (Figure 1.10). In the transient protection method, the nucleoside is reacted first with a silylating reagent and this derivative is reacted with the corresponding acid anhydride or acid chloride [125]. In these conditions, the acid derivative reacts with the amino function to generate the desired amide functions. Then the *O*-silyl bonds are hydrolyzed to produce the desired protected nucleoside (Figure 1.10).

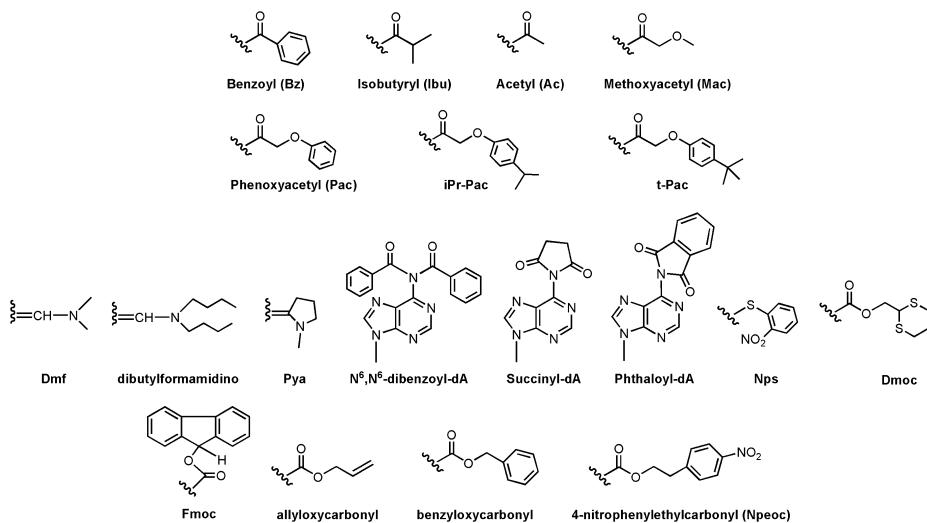
The introduction of solid-phase methodology increase the speed of the production of oligonucleotides and the Khorana's protecting groups were considered too stable.



**Figure 1.10:** Strategies reported for the protection of the *N*-4 amino group of dC.

The search for rapid deprotection protocols implemented the search for more labile amino-protecting groups and the use of methylamine instead of the ammonia [126].

The combined use of the phenoxyacetyl (Pac) and the methoxyacetyl (Mac) groups for the protection of dA and dG and the isobutyryl (Ibu) group for dC (Figure 1.11) was found to be an excellent strategy for the fast-deprotection of oligodeoxynucleotides [127, 128]. This allowed the reduction of the time needed for the deprotection of the amino groups as well as the use of very soft deprotection conditions such as potassium carbonate/methanol [129] reducing the deprotection time as well as allowing the introduction of ammonia-labile nucleobases such as *O*<sup>4</sup>-alkylthymidines [130]. Some of the Pac-protected nucleoside phosphoramidites such as G-derivatives had low solubility in acetonitrile. The 4-isopropylphenoxyacetyl (iPr-Pac) and the 4-*tert*-butylphenoxyacetyl (*t*Pac) were developed as the isopropyl and *tert*-butyl groups increase the solubility of the derivatives without affecting the lability of the group in very mild conditions [131]. The use of corresponding anhydride for the capping step, instead of acetic anhydride, is needed to avoid exchange of the *t*PAC group to less labile acetyl group [131, 132]. Another concern of the use of more reactive amines for the deprotection instead of ammonia is the modification of cytosine nucleosides as the nucleophilic attack of the amines to the position 4 of cytosine generates *N*-alkylcytosine derivatives [133]. For this reason, the benzoyl group is not compatible to the use of methylamine or ethylenediamine in the final deprotection step as it generates 5–10 % of the *N*-alkylcytosine derivatives [133]. The use of more labile groups such as the acetyl, the isopropoxyacetyl (Ipa) or the *t*Pac groups minimizes this side reaction [134].



**Figure 1.11:** Groups for the protection of the exocyclic amino functions of nucleobases.

Other labile amino-protecting groups are the formamidine and acetamidine groups [135–137]. In an excellent study, the *N,N*-dimethylformamidine (dmf) group was found to be the more appropriate for G derivatives, the *N,N*-dibutylformamidine (dnf) for A and the *N*-methylpyrrolidine (pya) group for C [135]. These groups are profusely used for the protection of G and A phosphoramidite derivatives in the synthesis of DNA oligonucleotides [138] as well as RNA oligonucleotides [139].

The depurination of dA derivatives during the acid conditions used for the removal of the DMT group is a potential side reaction. The standard *N*<sup>6</sup>-benzoyl-dA derivative is more susceptible to depurination than the unprotected dA nucleoside. Luckily, in addition of a faster deprotection rates, the dmf, dnb and tPac groups for the protection of 2'-deoxyadenosine derivatives are also more stable to depurination than the corresponding *N*<sup>6</sup>-benzoyl-dA derivative. Other groups that have been developed to increase stability in acid conditions are the bis-amide derivatives such as; *N*<sup>6</sup>,*N*<sup>6</sup>-dibenzoyl [140], succinyl [141] and phthaloyl [141, 142] derivatives. These last groups have no use as they are too stable to ammonia deprotection.

The 4-nitrophenylethoxycarbonyl (Npeoc) [143, 144] and the fluorenylmethoxycarbonyl (Fmoc) [145, 146] are carbamate protecting groups that are removed using nonnucleophilic bases by a  $\beta$ -elimination mechanism. These carbamates are appropriate for the synthesis of oligonucleotides carrying ammonia-labile groups [145, 147, 148]. Other protecting groups that are removed in mild conditions are: allyloxycarbonyl (Aoc) [149–151] that are removed by palladium reagents, benzyl and benzylloxycarbonyl [152, 153] that are removed by hydrogenation, 2-nitrophenylsulfenyl (Nps) that are removed by thiols [154] and 1,3-dithian-2-yl-methoxycarbonyl (Dmoc) [155, 156] that are removed under nonnucleophilic oxidative conditions.

## 1.6 The protection of the phosphate group

In the phosphotriester method, the protection of the phosphate group was an important issue in order to speed the coupling time when using reactive condensing reagents such as sulfonyl chlorides and sulfonyl arenes. The search for the optimal protecting group led to the development of phenyl groups [157, 158] and specially the 2-chlorophenyl one [159, 160] that was considered the best choice for the protection of phosphates in the phosphotriester method (Figure 1.12). These groups were removed with tetramethylguanidinium aldoxymate to ensure the removal of the 2-chlorophenyl moieties without cleavage of the internucleotide linkages [161, 162]. The 2-chlorophenyl was used together with the 2-cyanoethyl group for the preparation of dimer and trimer building blocks [163, 164] as the 2-cyanoethyl was stable to the acid conditions needed to remove the DMT group but it was easily removed with a triethylamine solution. During the development of the phosphoramidite method, the methyl group was selected for the protection of the phosphates [34, 165, 166]. The methyl group was removed by a short treatment with thiophenol before the final deprotection step with ammonia [117, 118]. Later, the methyl group was replaced by the 2-cyanoethyl group which can be removed at the same time than the nucleobases [167, 168]. The use of 2-cyanoethyl group is preferred as it shortens the deprotection step, avoids the use of the bad smelling thiophenol and decreases the extension of a potential side reaction that generated  $N^3$ -alkyl-T derivatives [118].

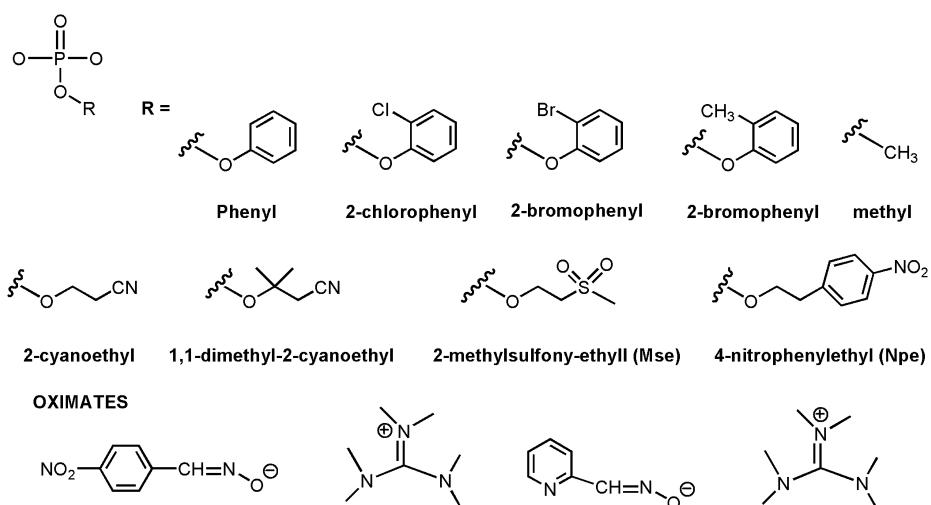
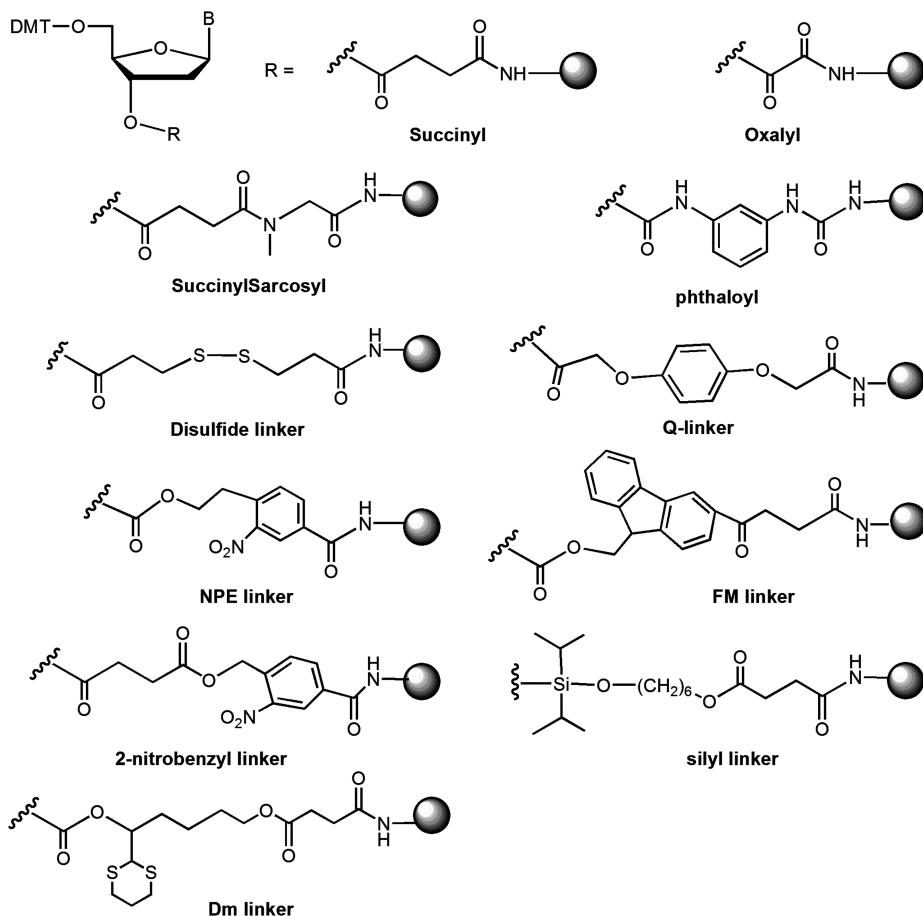


Figure 1.12: Protecting groups for the phosphate functions.

Phenyl groups such as 2-chlorophenyl [169], and a series of phenyls with electrodonating and electrowithdrawing groups [170] have also been used for the protection of

phosphoramidites. In general, phenyl phosphoramidites are less reactive than alkyl phosphoramidites but the resulting phenyl phosphites can be used as intermediates to produce H-phosphonates, phosphoramidates or methylphosphonates [170]. Similarly, oligonucleotide carrying phosphoramidates or methylphosphonates linkages can be produced [171] modifying the oxidation step when using phosphoramidites protected with the 2-cyano-1,1-dimethylethyl group [172].

Other phosphate groups that have been employed for the synthesis of oligonucleotides in mild deprotection conditions are the allyl group [149, 150] which is removed with palladium reagents and the 2-methylsulfonylethyl (Mse) [173] and the 4-nitrophenylethyl (Npe) [143, 174, 175] groups (Figure 1.12) that are removed using mild basic conditions.



**Figure 1.13:** Linkers developed for anchoring the first nucleoside to the solid support.

## 1.7 The solid-support and linking units

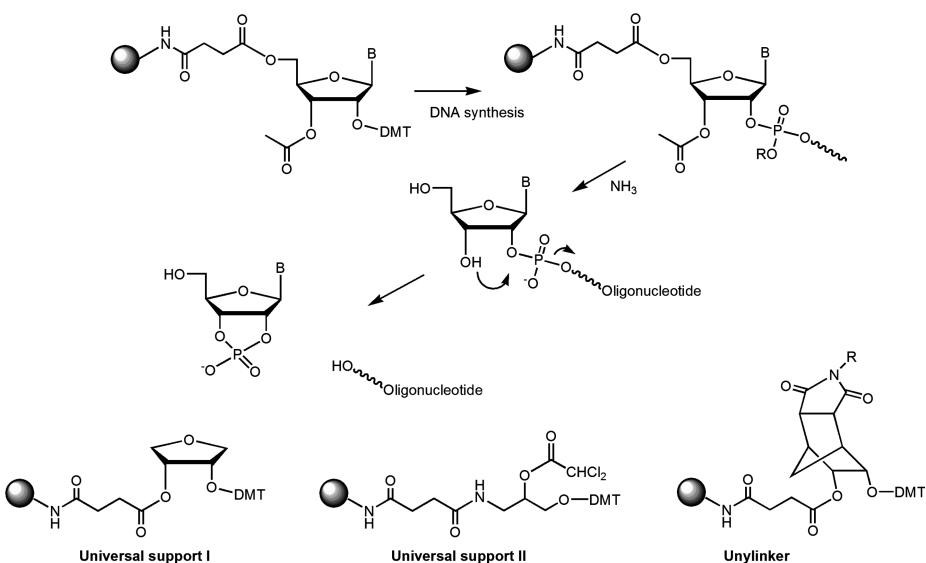
Researchers used the solution-phase methods for the synthesis of oligonucleotides for almost 20 years [4, 28]. At the end of the 1970s, solid phase methods were implemented and after some improvements they became the method of choice for the preparation of oligonucleotides. In solid phase methods the 3'-end of the first nucleoside is attached covalently to a polymeric support. In this way, the oligonucleotide chain is growing on the polymeric support and the excess of the chemicals used during the synthesis are washed out, simplifying the synthetic process. In addition, solid-phase techniques made possible the automation of the synthetic process and oligonucleotide synthesizers or “gene machines” became available to most laboratories.

The first supports used in solid-phase phosphotriester synthesis were similar to the polymers used in solid-phase peptide synthesis including polystyrene-1%-divinylbenzene [176] and polydimethylacrylamide-kieselguhr [177]. These were high loaded (0.1-0.5 mmol/gr) but the volume of the polymer changed depending on the solvent. In pyridine, dichloromethane and dioxane/tetrahydrofuran the supports had good swelling properties; however, in acetonitrile (commonly used in phosphoramidite coupling reactions) the supports were condensed generating bad coupling yields. For this reason, the use of silica-based supports was preferred. These had lower loadings (20–60 nmol/gr) and were more convenient when using acetonitrile as no swelling properties were observed [178, 179]. Most of the machine-assisted syntheses of oligonucleotides are performed on silica-derived supports especially on controlled-pore glass (CPG). A pore size of 500 Å is generally used for oligonucleotides smaller than 50 bases and a pore size of 1000 Å for oligonucleotides greater than 50 bases [25, 49, 180]. It is also important for an efficient synthesis, at least in silica-based supports, to separate the growing chain from the surface of the solid support. This is achieved by adding a “long chain alkylamine” spacer between the CPG and the succinyl linker [165]. In addition, new solid supports were introduced. Highly crosslinked polystyrene (20 % divinylbenzene) is used for small scales (40–200 nmol) due to its hydrophobic properties and the lack of reactivity of the surface [181]. For large scale synthesis, several alternative supports have been described such as polystyrene-1%-divinylbenzene used in peptide synthesis [182] or polystyrene-polyethyleneglycol [183] supports. The low swelling properties of the polystyrene support utilized in peptide synthesis in acetonitrile can be overcome by dissolving the phosphoramidites in dichloromethane and 1*H*-tetrazole in acetonitrile or tetrahydrofuran. In this way, the coupling reactions are performed in mixtures of more apolar solvents in where the swelling of the support is improved [170, 182]. Using polystyrene-polyethyleneglycol supports coupling reactions can be done in acetonitrile [183]. High loaded supports have been recently developed for large scale oligonucleotide synthesis by several manufactures such as the polystyrene NittoPhase support [184], the polyvinylacetate OligoPrep support [185], the primer support 5G and the silica-based HybCPG support [186].

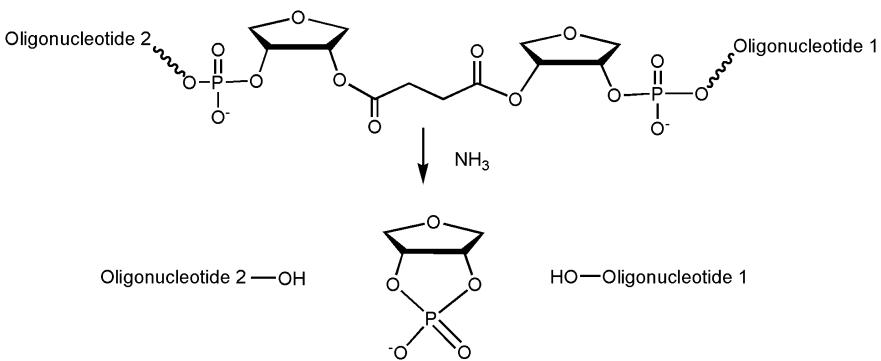
The first nucleoside is linked to the solid support through a succinic acid molecule between the 3' hydroxyl and an amino group on the support [187]. The succinyl linker (Figure 1.13) is used in most of the cases but also other linkers have been described [188]. The hydrolysis of the succinyl ester between the oligonucleotide and the solid support to generate free 3'-OH can be done in relatively mild conditions (30–60 min at room temperature using concentrated ammonia). Some authors consider the succinyl linker too labile describing premature release during the phosphotriester method. They suggested a new more stable urethane linker that was prepared in one-pot reaction [189]. The sarcosyl-succinyl and the phthaloyl linkers were designed to remain stable to 1,8-diazabicyclo [5.4.0]undecene-7-ene (DBU) solutions used in oligonucleotide synthesis when 5'-O-Fmoc – and 5'-O-Npeoc-protected nucleosides phosphoramidites [190, 191] are employed. On the other hand, new linkers have been developed to release oligonucleotides carrying ammonia sensitive molecules from the support in very mild conditions such as the oxanyl linker [192], the Q-linker [193, 194]. Other alternative linkers are the *o*-nitrophenylethyl (NPE) [195, 196] and the fluorenylmethyl (Fm) linkers [197] that are removed with mild bases, the photolabile *o*-nitrobenzyl [198, 199], the fluoride labile silyl [200] and disiloxyl [201] linkers and the 1,3-dithian-2-yl-methoxy (Dm) [155] linker that is removed under nonnucleophilic oxidative conditions.

Another important issue is the possibility of using one single support for the synthesis of any oligonucleotide. Instead of preparing one solid support for each of the four natural bases it is possible to use the so called universal linkers that contain *cis*-diol or *cis*-aminool functions designed to remove the 3'-phosphate in the final deprotection step to yield oligonucleotides with free 3'-OH function (Figure 1.14). One of the first universal linkers was a uridine derivative that was attached to the support by the 5'-end and carry an acetyl and a DMT group (Figure 1.14). The DMT group allows the assembly of the DNA sequence. At the end of the synthesis, the ammonia treatment unblocks the neighboring OH group that participates in an intramolecular attack to the phosphate groups generating a cyclic phosphate and the free 3'-OH (Figure 1.14). Due to the 2' to 3' migration properties of the acetyl group the compound was prepared and attached to the support as a mixture of the 2' and 3'-isomers [202, 203]. In a next step, the uridine derivative was replaced by tetrahydrofuranol [204, 205] or 1,4-*anhydro*-D-ribitol [206] derivatives (Figure 1.14). One of the alcohol functions was used to connect the linker to the supports via succinyl bonds and the second alcohol was functionalized with the DMT group to allow the assembly of the desired DNA sequence [205, 206]. A third approach used 3-amino-propan-1,2-diol derivatives [207–209]. Finally, a bicyclo[2.2.1] diol derivative functionalized with a cyclic imine (Unylinker) was developed [210–213]. The geometry of the molecule (Figure 1.14) allows a fast and clean cleavage followed by and efficient 3'-dephosphorylation.

Another interesting issue is the possibility of synthesizing two oligonucleotides in one solid support. In this way, the two primers needed for a PCR reaction can be prepared one after the other. After ammonia treatment an equimolar amount of the two primers is obtained. In order to obtain the two primers from one synthesis, cleavable



**Figure 1.14:** One single solid support for the four nucleotides. Universal linkers.

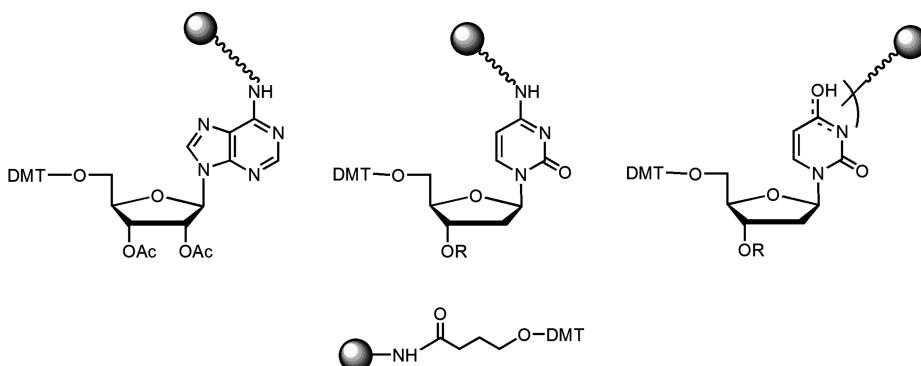


**Figure 1.15:** Synthesis of two oligonucleotides using one single support.

linkers are needed to generate the two primers with the free 3'-OH during the final deprotection step. Figure 1.15 shows the chemical structure of the cleavable linkers that combine succinyl esters and *cis*-diol groups for the liberation of the two primers and subsequent 3'-dephosphorylation [214].

The study of the protein-DNA interactions triggered the interest for the development of permanent linkers. The permanent linkers are stable to synthesis and final deprotection conditions generating the oligonucleotide-supports that can be used for affinity purification of DNA-binding proteins [215, 216]. Oligonucleotide-supports have been used for purification of DNA-binding proteins [217] but also to screen G-quadruplex ligands [217] and DNA hybridization properties [218]. Some of these sup-

ports contain a nucleoside linked by the nucleobase (Figure 1.16). Special attention was paid to provide a special solid support that can be used for oligonucleotide synthesis using anhydrous organic solvents and still allow the access of biomolecules in aqueous solutions. Some of the matrices studied included polyacrylmorpholide [215] silica and controlled pore glass [215, 217], Teflon [216] and polyethyleneglycol (PEG) [218]. An additional application of the ammonia stable linkers is the removal of impurities generated during the final deprotection. Grajkowski et al. have described the 3-hydroxypropyl-thiophosphoryl linker [219]. This molecule allows the synthesis and final deprotection; however, the release of the oligonucleotides is done by a thermolytic intramolecular reaction in aqueous buffer.



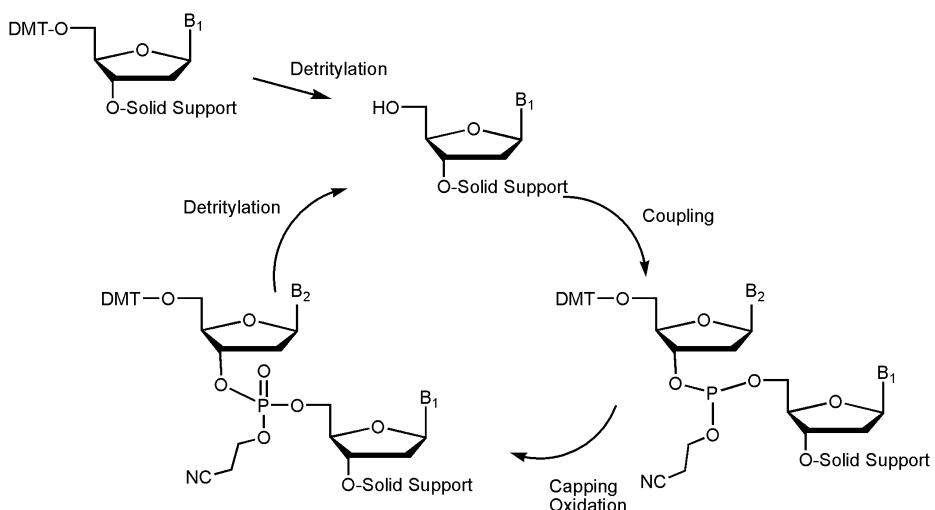
**Figure 1.16:** Linkers for the preparation of oligonucleotide affinity supports.

## 1.8 The synthesis cycle in solid-phase phosphoramidite chemistry

### 1.8.1 The basic steps

The synthesis of an oligonucleotide on solid-phase is accomplished by a cycle of reactions that are repeated for each nucleotide until the desired oligonucleotide has been assembled on the solid support. The oligonucleotide is then cleaved from the support for subsequent purification. The phosphoramidite synthesis cycle requires four basic reactions: detritylation, coupling, capping and oxidation (Figure 1.17).

- (1) **Detritylation.** In the first step, the support-bound nucleoside is deprotected to provide a free 5'-hydroxyl group for the incorporation of the next nucleotide. The 5'-hydroxyl group is blocked with the acid labile dimethoxytrityl group which is removed with several treatments of the support with a 2–3% dichloroacetic or trichloroacetic solution in dichloromethane. The dimethoxytrityl cation is re-



**Figure 1.17:** Solid-phase oligonucleotide synthesis cycle.

leased from the support and its absorbance can be measured at 500 nm to control the performance of the synthesis. The amount of DMT groups present in the detritylation solutions is a direct measurement of the amount of phosphoramidite that has been incorporated during the coupling reaction. A decline on the DMT absorbance indicates a poor coupling reaction at the previous step.

- (2) **Coupling.** An excess of the next nucleoside phosphoramidite protected at the 5'-position is added together with a large excess of *1H*-tetrazole that activates the phosphoramidite function. Usually, in low scales a large excess of phosphoramidite gives a 99 % coupling yield in 30 seconds.
- (3) **Capping.** The remaining of the 5'-hydroxyl groups that did not react with the phosphoramidite is blocked to avoid further additions of incoming phosphoramidites. In this way, deleted sequences are avoided facilitating the purification of the desired oligonucleotide. In addition, the capping step has been shown to reverse phosphorylation in the guanine residues [116]. This is accomplished by a 30 second treatment of the support with a solution of acetic anhydride and *N*-methylimidazole in tetrahydrofuran and lutidine. The reagents are separated in two different bottles to avoid darkening of the solution: Capping A solution with acetic anhydride, lutidine and tetrahydrofuran and Capping B with *N*-methylimidazole in tetrahydrofuran.
- (4) **Oxidation.** The polymeric phosphite-triester formed during the coupling reaction is oxidized to phosphate-triester by a 30-second treatment of the support with an iodine solution in tetrahydrofuran, pyridine and water (80/20/2). The complete cycle for the addition of one nucleoside unit takes between 3-5 minutes on an oligonucleotide synthesizer (see Table 1.1).

**Table 1.1:** Basic steps involved during the addition of one nucleoside using the phosphoramidite approach.

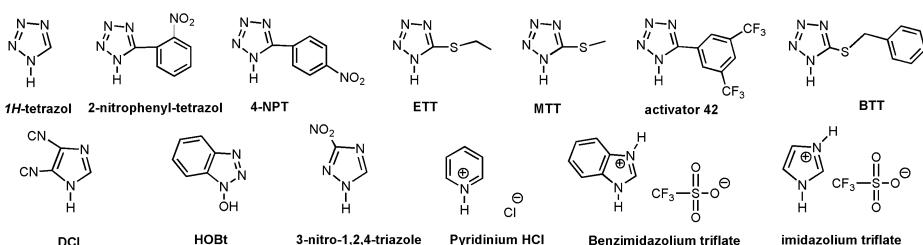
Operation	Reagent/solvent	Time (seconds)
1. Wash	acetonitrile	20
2. Detritylation	3 % trichloroacetic acid in dichloromethane	40
3. Wash	acetonitrile	30
4. Coupling	0.1 M phosphoramidite in acetonitrile 0.4 M 1 <i>H</i> -tetrazole in acetonitrile	30
5. Acetylation	acetic anhydride/lutidine/tetrahydrofuran 1:1:8 0.1 M <i>N</i> -methylimidazole in tetrahydrofuran	20
6. Oxidation	0.05 M iodine in tetrahydrofuran/pyridine/water	30
7. Wash	acetonitrile	40

After the assembly of the desired sequence, the support is treated with concentrated ammonia to cleave the succinate linkage and to eliminate the phosphate and amino protecting groups. The succinyl ester linkage cleaves after treatment with concentrated ammonia or methylamine at room temperature for 1–2 hours, while the amide protecting groups of the bases require at least a 6 hr treatment at 50–60 °C. Some oligonucleotide synthesizers have the capability of treating the supports with ammonia at room temperature to release the oligonucleotide from the support. The oligonucleotide solution is then placed at 50–60 °C for 6 hours to complete deprotection.

### 1.8.2 Modifications in the basic steps

In the phosphotriester protocols, the removal of the DMT was performed with a ZnBr<sub>2</sub> solution in nitromethane [220, 179] or dichloromethane/isopropanol (85:15) [176] to avoid dA depurination. The use of this Lewis acid was considered not suitable for DNA synthesizers due to potential precipitation of the salts.

In addition to 1*H*-tetrazole, there are an important number of catalysts that have been described in the phosphoramidite coupling reactions (Figure 1.18) [221]. These include more acidic tetrazoles such as; 5-(2-nitrophenyl)-1*H*-tetrazole [222], 5-(4-nitrophenyl)-1*H*-tetrazole (4-NPT) [223], 5-ethylthio-1*H*-tetrazole (ETT) [224], 5-methylthio-1*H*-tetrazole (MTT) [225] 5-(Bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole (activator 42) [226] and 5-benzylthio-1*H*-tetrazole (BTT) [227] as well as chiral tetrazoles for stereo-controlled synthesis [228]. Other azoles such as 4,5-dicyanoimidazole (DCI) [229] are less acidic but better nucleophiles. Similarly, other azoles such as 1-hydroxybenzotriazole (HOBT) activators [230] and 3-nitro-1,2,4-triazole activators [231] have been reported. Other important coupling activators are salts such as pyridinium hydrochloride [232], benzimidazolium triflate [233, 234] and imidazolium triflate [235] as well as some organic carboxylic acids [236].



**Figure 1.18:** Representative coupling agents or activators used in phosphoramidite chemistry.

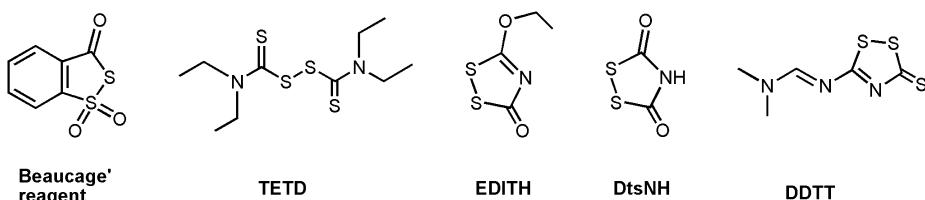
Phosphoramidites are commercially available as are produced in large scale by reaction of the corresponding 5'-O-DMT-*N*-protected-nucleosides with either a chlorophosphine [166] in the presence of a base such as; diisopropylethylamine or a bisphosphoramidite catalyzed by 0.5 equivalents of 1*H*-tetrazole or by amine salts [237]. In this last method, phosphoramidites can be obtained “*in situ*” and the solution can be used directly for solid-phase synthesis without the isolation of the corresponding phosphoramidites [231, 238, 239].

During the use of labile protecting groups such as Pac or *t*Pac acetylation of the nucleobases has been observed [131, 132]. It has been suggested that this acetylation may be the result of the acetylation of the amide groups. As the acetyl groups are less labile than Pac or *t*Pac groups, the presence of acetyl groups can be observed after mild deprotection conditions [131, 132]. In order to reduce the acetyl side products, it is needed to change the acetic anhydride solution for the corresponding Pac or *t*Pac anhydrides.

In some cases, the aqueous iodine solution used in the oxidation step is harmful for the stability of some functions. For example, the *North*-pseudonucleosides are not stable to regular iodine solution. In these cases, it is recommended the use a *tert*-butyl peroxide solution [240]. Other alternative oxidation reagents suitable for nonaqueous oxidation of phosphite triesters include bis(trimethylsilyl)peroxide [241, 242], cumene hydroperoxide [243], *m*-chloroperbenzoic acid [243] tetrabutylammonium periodate [244] and 10-camphorsulfonyl oxaziridine [245, 246].

The synthesis of oligonucleoside phosphorothioates implies the substitution of the iodine oxidation solution by a sulfurizing solution (Figure 1.19). The most frequently employed sulfurizing reagents are: 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage's reagent) [247, 248], tetraethylthiuram disulfide (TETD) [249], 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) [250], 1,2,4-dithiazoline-3,5-dione (DtsNH) [250] and 3-((dimethylaminomethylidene)amino-3*H*-1,2,4-dithiazole-3-thione (DDTT) [251]. The sulfurizing reaction is done prior de capping step. The reversal of the order between capping and oxidation is due to the interference of the capping step in the sulfurizing reaction [251].

In addition to the use of concentrated ammonia for the final deprotection several alternatives have been described. A fast cleavage may be achieved using ammonia/

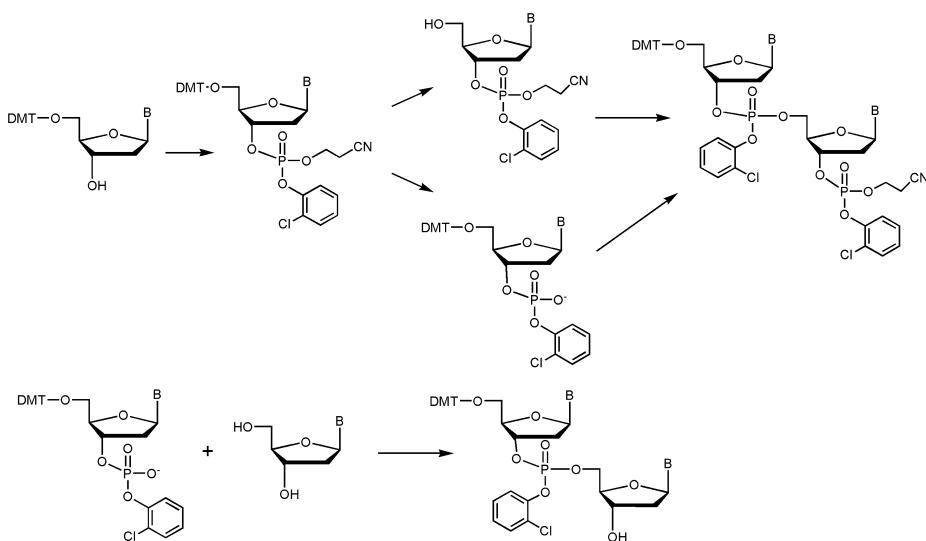


**Figure 1.19:** Representative sulfurizing reagents used in the synthesis of oligonucleotide phosphorothioates by the phosphoramidite chemistry.

methylamine mixtures at 65 °C [252]. In this method, dC should be protected with acetyl group to avoid the formation *N*-4-methyl-dC. Other conditions designed to avoid concentrated ammonia solutions are the use of potassium carbonate in methanol and labile protecting groups such Pac or iPac [129].

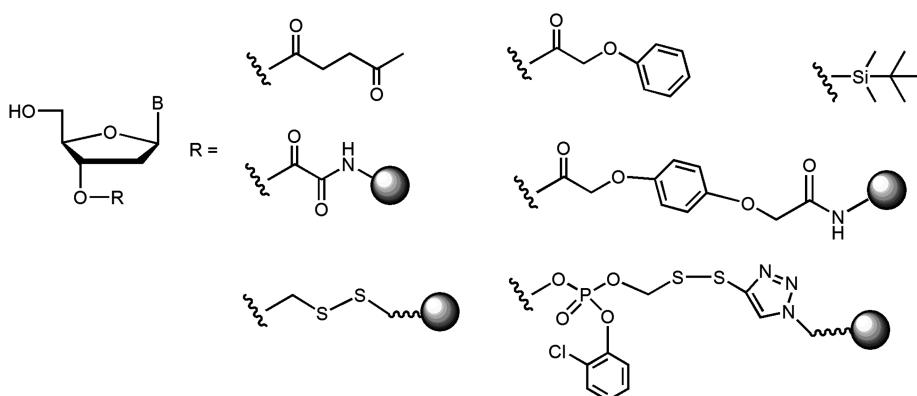
## 1.9 Synthesis of DNA and RNA protected fragments

The assembly of oligonucleotides in solid-phase is performed by sequential addition of the appropriate monomer units. The high efficiency of the phosphoramidite couplings facilitates the assembly of long oligonucleotides. This was not the case for the solid-phase synthesis using the phosphotriester method. At the beginning of the 1980s, the way to reach long oligonucleotides was the use of DNA-protected dimer and trimers that were produced in solution [253, 254]. The 2-chlorophenyl-protected dinucleotides and trinucleotides were produced commercially facilitating the rapid synthesis of long oligonucleotides by solid-phase phosphotriester methods. Longer blocks such as 7mer and 9mer were also produced using solid-phase phosphotriester methods followed by block condensation to obtain longer oligonucleotides [255]. The development of the phosphoramidites made the synthesis of the DNA-protected dimer and trimers unpractical. Later, some authors developed methods for the preparation of DNA dimers using phosphoramidites. For example, Kumar and Poonian described the synthesis of DNA dimeric phosphoramidites and applied them to the synthesis of oligonucleotides demonstrating that the coupling yields were similar to the monomeric phosphoramidites so they were useful for the preparation of long oligonucleotides [256]. For the synthesis of the dimer phosphoramidite units, they condensed 5'-O-DMT-*N*-protected 3'-O-methyl-*N,N*-dialkylamino phosphoramides with nucleosides protected in the 3' with the levulinyl or TBDMS groups. Orthogonal removal of the 3'-protecting groups generated the free 3'-OH dimer units that were phosphorylated to yield the appropriate dimer units [256] (Figure 1.20). In a similar study, the TT dimer was prepared using 3'-levulinyl protection and 2-cyanoethyl phosphate protecting groups. The TT dimer was used for the synthesis of a 101-bases long oligonucleotide [257].



**Figure 1.20:** Synthesis of protected a dinucleotide DNA block by the phosphotriester chemistry.

Advances in oligonucleotide-directed mutagenesis triggered the interest for the preparation of trinucleotide phosphoramidites to be used for random mutagenesis. The generation of random libraries made by addition of monomeric phosphoramidites was not appropriate for random mutagenesis as they could incorporate stop and nonsense codons. One of the synthetic methods for the preparation of trinucleotide phosphoramidites used the phenoxyacetyl (Pac) group for the protection of the 3'-position (Figure 1.21) and phosphoramidites carrying the methyl phosphate protecting groups [258]. These authors considered that 2-cyanoethyl group was labile to the conditions of removal of the Pac groups while the methyl group was found to be orthogonal to the Pac group. The use of the Fmoc group for the protection of the 3'-position was not appropriate [258]. In a different approach 20 trinucleotide phosphoramidite units were prepared using a combined strategy. The assembly of the trinucleotide was performed using the phosphotriester method in solution [259]. The 2-chlorophenyl group was selected for the protection of the phosphate. There was no protection of the 3'-OH (Figure 1.20) as the coupling reaction was selective for the most reactive 5'-OH [259]. Following this strategy, an optimized method was described later showing the full characterization of the triplet codons for all 20 amino acids [260]. Later, a new method was described using the phosphotriester method in solution [261]. In this protocol, the 3'-OH was protected with 2-azidomethylbenzoyl (Azmb) group that was removed under very mild conditions. Recently, trinucleotide phosphoramidites were prepared using 2-cyanoethyl phosphoramidites and TBDMS groups (Figure 1.21) for the protection of the 3'-OH [262, 263]. The synthesis of the trimers was made from 3' to 5' di-



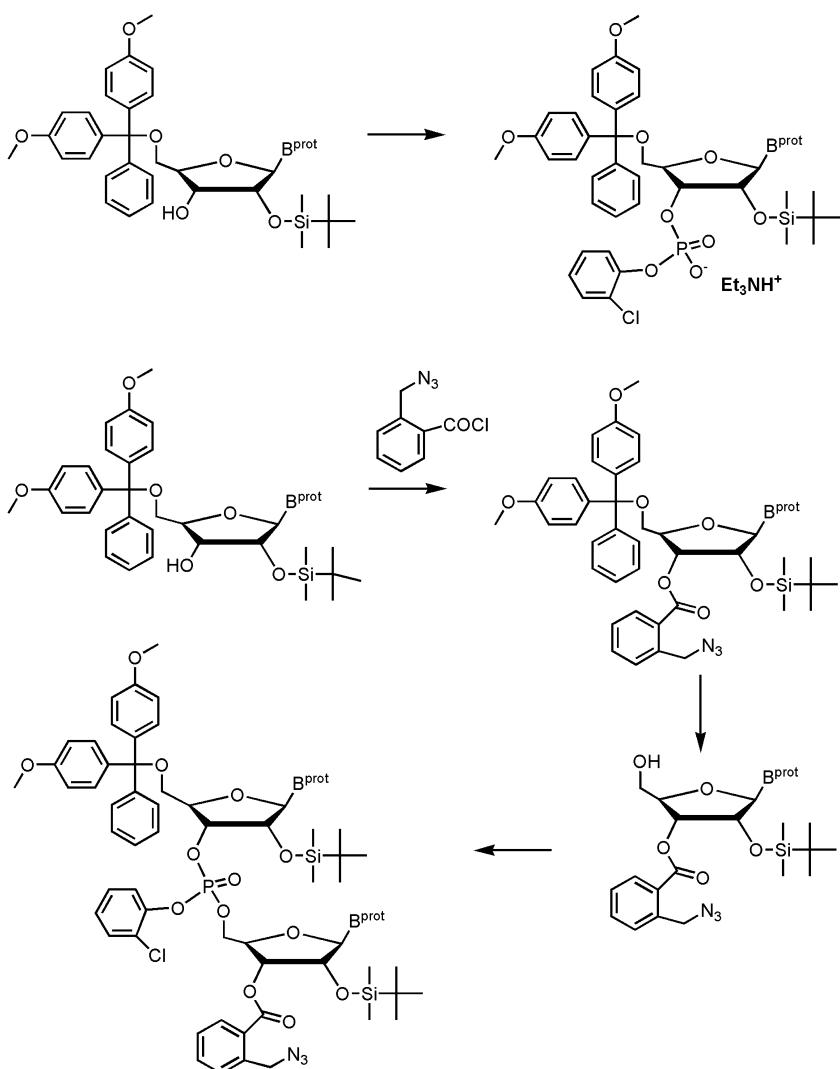
**Figure 1.21:** The protection of the 3'-function used for the synthesis of protected DNA fragments using both solution and solid-phase methods.

rection in order to avoid TBDMS removal until the trinucleotide was assembled. The removal of TBDMS was done with triethylammonium hydrofluoride in the presence of 2-cyanoethyl phosphate groups [262, 263].

Trimer building blocks were also prepared using solid-phase [264]. In a first method, controlled pore glass (CPG) supports together with the labile oxalyl linker (Figure 1.21) and *o*-chlorophenyl nucleotides as monomeric units. The release of the trinucleotide building blocks was performed with pyridine/methanol mixtures [264]. A similar approach was used for the synthesis of trimer building blocks on precipitative soluble supports [265, 266]. In one case, an ethyldisulfide linker was used [265]. The reductive cleavage of the disulfide linker generated 2-mercaptopethyl phosphate (Figure 1.21) that decompose to generate trinucleotide-3'-*o*-chlorophenyl phosphates [265]. In a second approach, the labile Q-linker was used to attach the first nucleotide to the precipitative soluble supports [266]. Recently, a solid-phase method was described that uses a special disulfide linker (Figure 1.21) and phosphoramidites as monomeric units [263, 267]. These methods have been reviewed recently [268, 269].

Recently, it has been proposed that large scale synthesis of therapeutic oligonucleotides or small scale of large RNA molecules will benefit of the use of dimer and trimer building blocks units. The synthesis of the trimer RNA building blocks follows two main strategies that are inspired in the synthesis of trimer DNA building blocks. The method described by Huang and Xi [270] uses phosphotriester intermediates, (Figure 1.22) while the method used by Hassler [271] uses phosphite-triester intermediates (Figure 1.23).

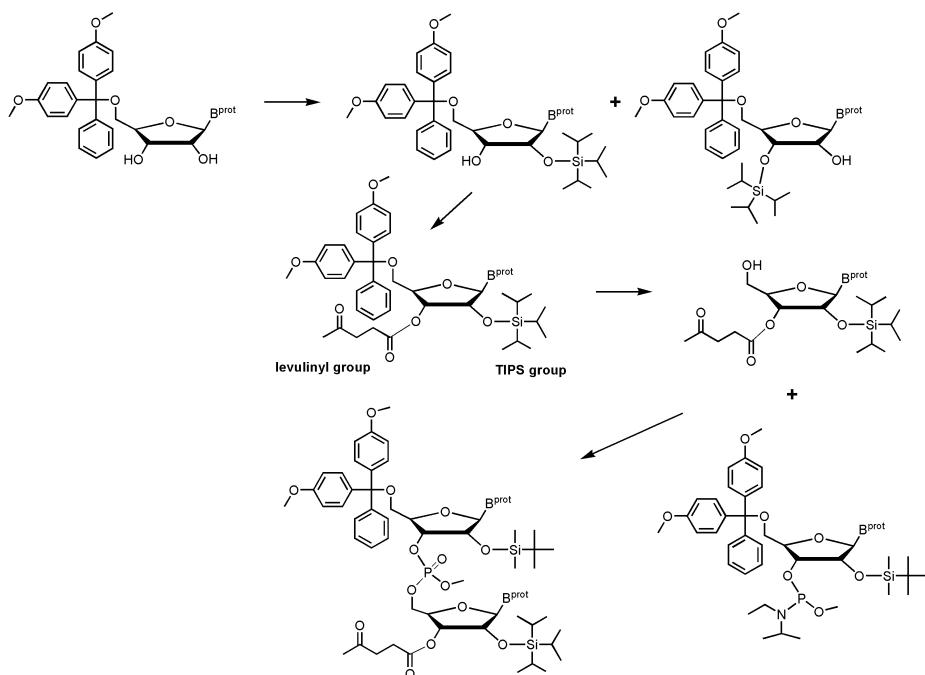
The method described by Huang and Xi [270] uses the TBDMS group for the protection of the 2'-OH (Figure 1.22). The levulinyl group is usually used for the protection of the 3'-OH in the solution synthesis of DNA dimer and trimers. This group is removed using hydrazine in pyridine-acetic acid solutions. In RNA units using the TBDMS group, the levulinyl group is not convenient because during the hydrazine treatment



**Figure 1.22:** Synthesis of a protected RNA dimer by the phosphotriester chemistry.

it has been observed the migration of the 2'-TBDMS group to the 3'-position. For these reasons, these authors suggested the utilization of the 2-(azidomethyl)benzoyl (Azmb) for the protection of the 3'-OH instead of the levulinyl. This group is removed using triphenylphosphine. This reagent reduces the azido group to amino and the amino derivative undergoes spontaneous cleavage by an intramolecular reaction. These conditions are very mild and do not cause migration of the TBDMS group. The internucleotide bond is made using phosphotriester derivatives and using the *o*-chlorophenyl group for the protection of the phosphate.

The study reported by Hassler [271] demonstrates that in RNA units the combined use of TBDMS and the levulinyl group is not convenient because during the hydrazine treatment the migration of the 2'-TBDMS group to the 3'-position was observed. To solve this problem, they propose two modifications: (a) the synthesis started with the nucleoside at the 3'-end moving toward the addition of phosphoramidites in the 5'-direction (Figure 1.23). (b) The nucleoside at the 3'-position contains the levulinyl group at the 3'-OH but the TBDMS group at the 2' is changed for the triisopropylsilyl (TIPS) group. The TIPS group does not migrate to the 3'-position during the hydrazine/pyridine/acetic acid treatment employed for the removal of the levulinyl group. The internucleotide bond is made using phosphoramidites derivatives and using the methyl group for the protection of the phosphate. This group is resistant to the hydrazine/pyridine/acetic acid treatment utilized for the removal of the levulinyl group. The classical 2-cyanoethyl group used for the protection of the phosphate in the phosphoramidite method cannot be applied as this group is labile to hydrazine.



**Figure 1.23:** Synthesis of a protected RNA dimer by the phosphoramidite chemistry.

Recently, the synthesis of protected dinucleotides and dinucleotide phosphorothioates using mechanochemical synthesis has been reported [272]. These protocols together with the liquid phase synthesis using membranes for nanofiltration of or-

ganic solvents [273] will facilitate the industrial production of DNA and RNA oligonucleotides for therapeutic use.

## Bibliography

- [1] Watson JD, Crick FH. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature*. 1953;171:737–8.
- [2] Michelson AM, Todd A. Synthesis of a dithymidine dinucleotide containing a 3',5'-internucleotidic linkage. *J Chem Soc*. 1955;2632–38.
- [3] Garegg PJ, Lindh I, Regberg T, Stawinski J, Stromberg R, Henrichson C. Nucleoside H-phosphonates. Chemical synthesis of oligodeoxyribonucleotides by the hydrogen phosphonate approach. *Tetrahedron Lett*. 1986;27:4051–4.
- [4] Khorana HG. Total synthesis of a gene. *Science*. 1979;203:614–25.
- [5] Khorana HG. Synthesis in the study of nucleic acids. The fourth jubilee lecture. *Biochem J*. 1968;109:709–25.
- [6] Razin A, Hirose T, Itakura K, Riggs AD. Efficient correction of a mutation by use of chemically synthesized DNA. *Proc Natl Acad Sci USA*. 1978;75:4268–70.
- [7] Gillam S, Smith M. Site-specific mutagenesis using synthetic oligodeoxyribonucleotide primers: I optimum conditions and minimum oligodeoxyribonucleotide length. *Gene*. 1979;8:81–97.
- [8] Conner BJ, Reyes AA, Morin C, Itakura K, Teplitz RL, Wallace RB. Detection of sickle cell  $\beta$ -globin allele by hybridization with synthetic oligonucleotides. *Proc Natl Acad Sci USA*. 1983;80:278–82.
- [9] Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific-enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symp Quant Biol*. 1986;51:263–73.
- [10] Stephenson ML, Zamecnik PC. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci USA*. 1978;75:285–8.
- [11] Wahl MC, Sundaralingam M. New crystal structures of nucleic acids and their complexes. *Curr Opin Struct Biol*. 1995;5:282–95.
- [12] Basu AK, Essigmann JM. Site-specifically modified oligodeoxynucleotides as probes for the structural and biological effects of DNA-damaging agents. *Chem Res Toxicol*. 1988;1:1–18.
- [13] Lamond AI, Sproat BS. Antisense oligonucleotides made of 2'-O-alkylRNA: their properties and applications in RNA biochemistry. *FEBS Lett*. 1993;325:123–7.
- [14] Christoffersen RE, Marr JJ. Ribozymes as human therapeutic agents. *J Med Chem*. 1995;38:2023–37.
- [15] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998;391:806–11.
- [16] He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*. 2004;5:522–31.
- [17] Pease AC, Solas D, Sullivan EJ, Cronin MT, Holmes CP, Fodor SPA. Light-generated oligonucleotides arrays for rapid DNA sequence analysis. *Proc Natl Acad Sci USA*. 1994;91:5022–26.
- [18] Singh-Gasson S, Green RD, Yue Y, Nelson C, Blattner F, Sussman MR, Cerrina F. Maskless fabrication of light-directed oligonucleotide microarrays using a digital micromirror array. *Nat Biotechnol*. 1999;17:974–8.

- [19] Adelman LM. Molecular computation of solutions to combinatorial problems. *Science*. 1994;266:1021–24.
- [20] Lipton RJ. DNA solution of hard computational problems. *Science*. 1995;268:542–5.
- [21] Hutchison CA, Chuang RY, Noskov VN, Assad-Garcia N, Deering TJ, Ellsman MH, Gill J, Kannan K, Karas BJ, Ma L, Pelletier JF, Qi ZQ, Richter RA, Strychalski EA, Sun L, Suzuki Y, Tsvetanova B, Wise KS, Smith HO, Glass JI, Merryman C, Gibson DG, Venter JC. Design and synthesis of a minimal bacterial genome. *Science*. 2016;351:aad6253.
- [22] Seeman NC. DNA nanotechnology: novel DNA constructions. *Annu Rev Biophys Biomol Struct*. 1998;27:225–48.
- [23] Aldaye FA, Palmer AL, Sleiman HF. Assembling materials with DNA as the guide. *Science*. 2008;321:1795–9.
- [24] Bannwarth W. Gene technology: a challenge for a chemist. *Chimia*. 1987;41:302–17.
- [25] Narang SA. Synthesis and applications of DNA and RNA. Orlando, Florida: Academic Press; 1987.
- [26] Smith M, Moffat JG, Khorana HG. Carbodiimides VIII. Observations on the reactions of carbodiimides with acids and some new applications in the synthesis of phosphoric acid esters. *J Am Chem Soc*. 1958;80:6204–12.
- [27] Jacob TM, Khorana HG. Studies on polynucleotides. XXX. A comparative study of reagents for the synthesis of the C3'-C5' internucleotide linkage. *J Am Chem Soc*. 1964;86:1630–5.
- [28] Reese CB. The chemical synthesis of oligo-and poly-nucleotides by the phosphotriester approach. *Tetrahedron*. 1978;34:3143–79.
- [29] Narang SA. DNA synthesis. *Tetrahedron*. 1983;39:3–22.
- [30] Sonveaux E. The organic chemistry underlying DNA synthesis. *Bioorg Chem*. 1986;14:274–325.
- [31] Ohtsuka E, Ikehara M, Söll D. Recent developments in the chemical synthesis of polynucleotides. *Nucleic Acids Res*. 1982;10:6553–70.
- [32] Letsinger RL, Mahadevan V. Oligonucleotide synthesis on a polymer support. *J Am Chem Soc*. 1965;87:3526–27.
- [33] Letsinger RL, Lunsford WB. Synthesis of thymidine oligonucleotides by phosphite triester intermediates. *J Am Chem Soc*. 1976;98:3655–61.
- [34] Beaucage SL, Caruthers MH. Deoxynucleoside phosphoramidites – a new class of key intermediates for deoxypolynucleotide synthesis. *Tetrahedron Lett*. 1981;22:1859–62.
- [35] Beaucage SL, Iyer RP. Advances in the synthesis of oligonucleotides by the phosphoramidite approach. *Tetrahedron*. 1992;48:2223–311.
- [36] Beaucage SL, Iyer RP. The functionalization of oligonucleotides via phosphoramidite derivatives. *Tetrahedron*. 1993;49:1925–63.
- [37] Beaucage SL, Iyer RP. The synthesis of modified oligonucleotides by the phosphoramidite approach and their applications. *Tetrahedron*. 1993;49:6123–94.
- [38] Beaucage SL, Iyer RP. The synthesis of specific ribonucleotides and unrelated phosphorylated biomolecules by the phosphoramidite method. *Tetrahedron*. 1993;49:10441–88.
- [39] Froehler BC, Ng PG, Matteucci MD. Synthesis of DNA via deoxynucleoside H-phosphonate intermediates. *Tetrahedron Lett*. 1986;27:5399–407.
- [40] Krieg PA, Melton DA. In vitro RNA synthesis with SP6 RNA polymerases. *Methods Enzymol*. 1987;155:397–415.
- [41] Milligan JF, Groebe DR, Witherell GW, Uhlenbeck OC. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res*. 1987;15:8783–98.
- [42] Milligan JF, Uhlenbeck OC. Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol*. 1989;180:51–62.

- [43] Nikonowicz EP, Sirr A, Legault P, Jucker FM, Baer LM, Pardi A. Preparation of  $^{13}\text{C}$  and  $^{15}\text{N}$  labeled RNAs for heteronuclear multi-dimensional NMR studies. *Nucleic Acids Res.* 1992;20:4507–13.
- [44] Dayie KT. Key labeling technologies to tackle sizeable problems in RNA structural biology. *Int J Mol Sci.* 2008;9:1214–40.
- [45] Palluk S, Arlow DH, de Rond T, Barthel S, Kang JS, Bector R, Baghdassarian HM, Truong AN, Kim PW, Singh AK, Hillson NJ, Keasling JD. De novo DNA synthesis using polymerase-nucleotide conjugates. *Nat Biotechnol.* 2018;36:645–50.
- [46] Motea EA, Berdis AJ. Terminal deoxynucleotidyl transferase: the story of a misguided DNA polymerase. *Biochim Biophys Acta.* 2010;1804:1151–66.
- [47] Lee HH, Kalhor R, Goela N, Bolot J, Church GM. Enzymatic DNA synthesis for digital information storage. <https://doi.org/10.1101/348987>.
- [48] Schaller H, Weimann G, Lerch B, Khorana HG. Studies on polynucleotides. XXIV. The stepwise synthesis of specific deoxyribopolynucleotides (4). Protected derivatives of deoxyribonucleosides and new syntheses of deoxyribonucleoside-3' phosphates. *J Am Chem Soc.* 1963;85:3821–7.
- [49] Gait MJ. Oligonucleotide synthesis. A practical approach. Oxford: IRL Press; 1984.
- [50] Seliger H. Protection of 5'-hydroxy functions of nucleosides. *Curr Prot Nucl Acids Chem.* 2000;2.3.1–34.
- [51] Fischer EF, Caruthers MH. Color coded triarylmethyl protecting groups useful for deoxypolynucleotide synthesis. *Nucleic Acids Res.* 1983;11:1589–99.
- [52] Chattopadhyaya JB, Reese CB. The 9-phenylxanthen-9-yl protecting group. *J Chem Soc, Chem Commun.* 1978;639–40.
- [53] Seliger H, Holupirek M, Görtz HH. Solid-phase oligonucleotide synthesis with affinity – chromatographic separation of the product. *Tetrahedron Lett.* 1978;19:2115–18.
- [54] Sekine M, Hata T. 4,4',4''-Tris(benzyloxy)trityl as a new type of base-labile groups for protection of primary hydroxyl group. *J Org Chem.* 1983;48:3011–14.
- [55] Van Boom JH, Burgers PMJ. Use of levulinic acid in the protection of oligonucleotides via the modified phosphotriester method: synthesis of the decaribonucleotide UAUUAUUAUA. *Tetrahedron Lett.* 1976;17:4875–78.
- [56] Chattopadhyaya JB, Reese CB, Todd AH. 2-Dibromobenzoyl: an acyl protecting group removable under exceptionally mild conditions. *J Chem Soc, Chem Commun.* 1979;987–8.
- [57] Brown T, Christodoulou C, Jones SS, Modak AS, Reese CB, Sibanda S, Ubasawa A. Synthesis of the 3'-terminal half of yeast alanine transfer ribonucleic acid (tRNA<sup>Ala</sup>) by the phosphotriester approach in solution. Part I. Preparation of nucleoside building blocks. *J Chem Soc, Perkin Trans I.* 1989;1735–50.
- [58] Brown T, Christodoulou C, Modak AS, Reese CB, Halina T. Synthesis of the 3'-terminal half of yeast alanine transfer ribonucleic acid (tRNA<sup>Ala</sup>) by the phosphotriester approach in solution. Part 2. *J Chem Soc, Perkin Trans I.* 1989;1751–67.
- [59] Wada T, Ohkubo A, Mochizuki SM. 2-(Azidomethyl)benzoyl as a new protecting group in nucleosides. *Tetrahedron Lett.* 2001;42:1069–72.
- [60] Gioeli C, Chattopadhyaya J. The fluorenyl-9-ylmethoxycarbonyl group for the protection of hydroxy groups; its application in the synthesis of an octathymidylic acid fragment. *J Chem Soc, Chem Commun.* 1982;672–4.
- [61] Leumann C, Xu YZ, Christodoulou C, Tan ZK, Gait MJ. Solid-phase synthesis of oligoribonucleotides using 9-fluorenylmethoxycarbonyl (Fmoc) for 5'-hydroxyl protection. *Nucleic Acids Res.* 1989;17:2379–90.
- [62] Scaringe SA, Wincott FE, Caruthers MH. Novel RNA synthesis method using 5'-O-silyl-2'-O-orthoester protecting groups. *J Am Chem Soc.* 1998;120:11820–1.

- [63] Scaringe SA. Advanced 5'-silyl-2'-orthoester approach to RNA oligonucleotide synthesis. *Methods Enzymol.* 2000;317:1–18.
- [64] Egeland RD, Southern EM. Electrochemically directed synthesis of oligonucleotides for DNA microarray fabrication. *Nucleic Acids Res.* 2005;33:e125.
- [65] McGall GH, Barone AD, Diggelmann M, Fodor SPA, Gentalen E, Ngo N. The efficiency of light-directed synthesis of DNA arrays on glass substrates. *J Am Chem Soc.* 1997;119:5081–90.
- [66] Pease AC, Solas D, Sullivan EJ, Cronin MT, Holmes CP, Fodor SPA. Light generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc Natl Acad Sci USA.* 1994;91:5022–26.
- [67] Bühler S, Giegrich H, Pfeiderer W. New photolabile protecting groups of the 2-(2-nitrophenyl)ethoxycarbonyl and the 2-(2-nitrophenyl)ethoxysulfonyl type for the oligonucleotide synthesis. *Nucleosides Nucleotides.* 1999;18:1281–3.
- [68] Giegrich H, Eisele-Bühler S, Hermann C, Kvassuk E, Charubala R, Pfeiderer W. New photolabile protecting groups in nucleoside and nucleotide chemistry synthesis. Cleavage mechanisms and applications. *Nucleosides Nucleotides.* 1998;17:1987–96.
- [69] Bühler S, Lagoja I, Giegrich H, Stengele KP, Pfeiderer W. New types of very efficient photolabile protecting groups based upon the [2-(2-nitrophenyl)propoxy]carbonyl (NPPOC) moiety. *Helv Chim Acta.* 2004;87:620–59.
- [70] Hölz K, Hoi JK, Schaudy E, Somoza V, Lietard J, Somoza MM. High-efficiency reverse (5'-3') synthesis of complex DNA microarrays. *Sci Rep.* 2018;8:15099.
- [71] Pirrung MC, Fallon L, McGall G. Proofing of photolithographic DNA synthesis with 3',5'-dimethoxybenzoinyloxycarbonyl protected deoxynucleoside phosphoramidites. *J Org Chem.* 1998;63:241–6.
- [72] Sierzchala AB, Dellinger DJ, Betley JR, Wyrzykiewicz TK, Yamada CM, Caruthers MH. Solid-phase oligodeoxynucleotide synthesis: a two-step cycle using peroxy anion deprotection. *J Am Chem Soc.* 2003;125:13427–41.
- [73] Ralph RK, Connors WJ, Schaller H, Khorana HG. Studies on polynucleotides. XVIII. Experiments on the polymerization of mononucleotides. The synthesis and characterization of deoxyguanosine oligonucleotides. *J Am Chem Soc.* 1963;85:1983–8.
- [74] Reese CB. Protection of 2'-hydroxy functions of ribonucleotides. *Curr Prot Nucl Acids Chem.* 2000;2,2.1–24.
- [75] Somoza A. Protecting groups for RNA synthesis: an increasing need for selective preparative methods. *Chem Soc Rev.* 2008;37:2668–75.
- [76] Ohtsuka E, Tanaka S, Ikebara M. Synthesis of the heptanucleotide corresponding to a eukaryotic initiator tRNA loop sequence. *J Am Chem Soc.* 1978;100:8210–3.
- [77] Griffin BE, Reese CB. Oligoribonucleotide synthesis via 2,5-protected ribonucleoside derivatives. *Tetrahedron Lett.* 1964;5:2925–31.
- [78] Reese CB, Safhill R, Sulston JE. A symmetrical alternative to the tetrahydropyranyl group. *J Am Chem Soc.* 1967;89:3366–8.
- [79] Sandström A, Kwiatkowski M, Chattopadhyaya J. Chemical synthesis of a pentaribonucleoside tetraphosphate constituting the 3'-acceptor stem sequence of *E. coli* tRNA<sup>Ile</sup> using 2'-O-(3-methoxy-1,5-dicarbomethoxypentan-3-yl)ribonucleoside building blocks. *Acta Chim Scand B.* 1985;39:273–90.
- [80] Reese CB, Thompson EA. A new synthesis of 1-arylpiperidin-4-ols. *J Chem Soc, Perkin Trans I.* 1988;2881–5.
- [81] Lloyd W, Reese CB, Song Q, Vandersteen AM, Visintin C, Zhang PZ. Some observations relating to the use of 1-aryl-4-alkoxypiperidin-4-yl groups for the protection of the 2-hydroxy functions in the chemical synthesis of oligoribonucleotides. *J Chem Soc, Perkin Trans I.* 2000;165–76.

- [82] Reese CB, Serafinowska HT, Zappia G. An acetal group suitable for the protection of 2'-hydroxy functions in rapid oligoribonucleotide synthesis. *Tetrahedron Lett.* 1986;27:2291–4.
- [83] Pieles U, Beijer B, Bohmann K, Weston S, O'Loughlin S, Adam V, Sproat B. New and convenient protection system for pseudouridine, highly suitable for solid phase oligoribonucleotide synthesis. *J Chem Soc, Perkin Trans I.* 1994;3423–9.
- [84] Aviñó A, Güimil-García R, Eritja R. Synthesis of oligoribonucleotides containing 4-thiouridine using the convertible nucleoside approach and the Fmp group. *Nucleos Nucleot Nucleic Acids.* 2004;23:1767–77.
- [85] Ogilvie KK, Sadana KL, Thompson AE, Quillian MA, Westmore JB. The use of silyl groups in protecting the hydroxyl functions of ribonucleotides. *Tetrahedron Lett.* 1974;2861–3.
- [86] Usman N, Pon RT, Ogilvie KK. Preparation of ribonucleotide 3'-O-phosphoramidites and their application to the automated solid phase synthesis of oligonucleotides. *Tetrahedron Lett.* 1985;26:4567–70.
- [87] Wu T, Ogilvie KK, Pon RT. Prevention of chain cleavage in the chemical synthesis of 2'-silylated oligoribonucleotides. *Nucleic Acids Res.* 1989;17:3501–17.
- [88] Westman E, Strömbärg R. Removal of t-butyldimethylsilyl protection in RNA synthesis. Triethylamine hydrofluoride (TEA·3HF) is a more reliable alternative to tetrabutylammonium fluoride (TBAF). *Nucleic Acids Res.* 1994;22:2430–1.
- [89] Wincott F, DiRenzo A, Shaffer C, Grimm S, Tracz D, Workman C, Sweedler D, Gonzalez C, Scaringe S, Usman N. Synthesis, deprotection, analysis and purification of RNA and ribozymes. *Nucleic Acids Res.* 1995;23:2677–84.
- [90] Pitsch S, Weiss PA, Jemmy L, Stutz A, Wu X. Reliable chemical synthesis of oligoribonucleotides (RNA) with 2'-O-[(triisopropylsilyl)oxy]methyl (2'-O-tom)-protected phosphoramidites. *Helv Chim Acta.* 2001;84:3773–95.
- [91] Pitsch S, Weiss PA. Preparation of 2'-O-[(triisopropylsilyl)oxy]methyl-protected ribonucleotides. *Curr Prot Nucleic Acid Chem.* 2001;2.9.1–14.
- [92] Schwartz ME, Breaker RR, Asteriadis GT, deBear JS, Gough GR. Rapid synthesis of oligoribonucleotides using 2'-O-(o-nitrobenzyloxymethyl)-protected monomers. *Bioorg Med Chem Lett.* 1992;2:1019–24.
- [93] Pitsch S, Weiss PA, Wu X, Ackermann D, Honegger T. Fast and reliable automated synthesis of RNA and partially 2'-O-protected precursors ('caged RNA') based on two novel, orthogonal 2'-O-protecting groups. *Helv Chim Acta.* 1999;82:1753–61.
- [94] Pfister M, Schirmeister H, Mohr M, Farkas S, Stengele KP, Reiner T, Dunkel M, Gokhale S, Charuhala R, Pfleiderer W. Nucleotides, part LIV. The 2-(4-Nitrophenyl)ethylsulfonyl (Npes) group: a new type of protection in nucleoside chemistry. *Helv Chim Acta.* 1995;78:1705–37.
- [95] Matysiak S, Pfleiderer W. Nucleotides, part LXVIII. Acetals as new 2'-O-protecting functions for the synthesis of oligoribonucleotides: synthesis of monomeric building units and oligoribonucleotides. *Helv Chim Acta.* 2001;84:1066–85.
- [96] Wada T, Tobe M, Nagayama T, Furusawa K, Sekine M. Regioselective protection of the 2'-hydroxyl group of N-acyl-3',5'-O-di(t-butyl)silanediylnucleoside derivatives by use of t-BuMgCl and 2-(trimethylsilyl)ethoxymethyl chloride. *Tetrahedron Lett.* 1995;36:1683–4.
- [97] Umehoto T, Wada T. Oligoribonucleotide synthesis by the use of 1-(2-cyanoethoxy)ethyl (CEE) as a 2'-hydroxy protecting group. *Tetrahedron Lett.* 2004;45:9529–31.
- [98] Ohgi T, Masutomi Y, Ishiyama KH, Shiba Y, Yano J. A new RNA synthetic method with a 2'-O-(2-cyanoethoxymethyl) protecting group. *Org Lett.* 2005;7:3477–80.
- [99] Zhou C, Honcharenko D, Chattopadhyaya J. 2-(4-Tolylsulfonyl)ethoxymethyl (TEM) – a new 2'-OH protecting group for solid-supported RNA synthesis. *Org Biomol Chem.* 2007;5:333–43.

- [100] Gough GR, Miller TJ, Mantick NA. p-Nitrobenzyloxymethyl: a new fluoride-removable protecting group for ribonucleotide 2'-hydroxyls. *Tetrahedron Lett.* 1996;37:981–2.
- [101] Shiba Y, Masuda H, Watanabe N, Ego T, Takachika K, Ishiyama K, Ohgi T, Yano J. Chemical synthesis of a very long oligoribonucleotide with 2-cyanoethoxymethyl (CEM) as the 2'-O-protecting group: structural identification and biological activity of a synthetic 110mer precursor-microRNA candidate. *Nucleic Acids Res.* 2007;35:3287–96.
- [102] Martin AR, Lavergne T, Vasseur JJ, Debart F. Assessment of new 2'-O-acetal ester protecting groups for regular RNA synthesis and original 2'-modified proRNA. *Bioorg Med Chem Lett.* 2009;19:4046–9.
- [103] Parey N, Baraguey C, Vasseur JJ, Debart F. First evaluation of acyloxymethyl or acylthiomethyl groups as biolabile 2'-O-protections of RNA. *Org Lett.* 2006;8:3869–72.
- [104] Lavergne T, Bertrand JR, Vasseur JJ, Debart F. A base-labile group for 2'-OH protection of ribonucleosides: a major challenge for RNA synthesis. *Chem Eur J.* 2008;14:9135–8.
- [105] Lackey JG, Mitra D, Somoza MM, Cerrina F, Damha M. Acetal levulinyl ester (ALE) groups for 2'-hydroxyl protection of ribonucleosides in the synthesis of oligoribonucleotides on glass and microarrays. *J Am Chem Soc.* 2009;131:8496–502.
- [106] Cieslak J, Kauffman JS, Kolodziejksi MJ, Lloyd JR, Beauchage SL. Assessment of 4-nitrogenated benzyloxymethyl groups for 2'-hydroxyl protection in solid-phase RNA synthesis. *Org Lett.* 2007;9:671–4.
- [107] Cieslak J, Grajkowski A, Kauffman JS, Duff RJ, Beauchage SL. The 4-(N-dichloroacetyl-N-methylamino)benzyloxymethyl group for 2'-hydroxyl protection of ribonucleosides in the solid-phase synthesis of oligoribonucleotides. *J Org Chem.* 2008;73:2774–83.
- [108] Semenyuk A, Foldesi A, Johansson T, Estmer-Nilsson C, Blomgren P, Brannvall M, Kirsebom LA, Kwiatkowski M. Synthesis of RNA using 2'-O-DMT protection. *J Am Chem Soc.* 2006;128:12356–7.
- [109] Karwowski B, Seio K, Sekine M. 4,5-Bis(ethoxycarbonyl)-[1,3]dioxolan-2-yl as a new orthoester-type protecting group for the 2'-hydroxyl function in the chemical synthesis of RNA. *Nucleos Nucleot Nucleic Acids.* 2005;24:1111–4.
- [110] Scaringe SA, Kitchen D, Kaiser RJ, Marshall WS. Preparation of 5'-silyl-2'-orthoester ribonucleotides for use in oligoribonucleotide synthesis. *Curr Prot Nucleic Acid Chem.* 2004;2.10.1–16.
- [111] Dellinger DJ, Timar Z, Myerson J, Sierzchala AB, Turner J, Ferreira F, Kupihar Z, Dellinger G, Hill KW, Powell JA, Sampson JR, Caruthers MH. Streamlined process for the chemical synthesis of RNA using 2'-O-thionocarbamate-protected nucleoside phosphoramidites in the solid phase. *J Am Chem Soc.* 2011;33:11540–6.
- [112] Kung PP, H-phosphonate JRA. DNA synthesis without amino protection. *Tetrahedron Lett.* 1992;33:5869–72.
- [113] Wada T, Honda F, Sato Y, Sekine M. First synthesis of H-phosphonate oligonucleotides bearing N-unmodified bases. *Tetrahedron Lett.* 1999;40:915–8.
- [114] Sekine M. DNA synthesis without base protection. *Curr Prot Nucleic Acid Chem.* 2004;3.10.1–15.
- [115] Reese CB, Ubasawa A. Reaction between 1-arenesulfonyl-3-nitro-1,2,4-triazole and nucleoside residues. Elucidation of the nature of side-reactions during oligonucleotide synthesis. *Tetrahedron Lett.* 1980;21:2265–8.
- [116] Eadie JS, Davidson DS. Guanine modification during chemical DNA synthesis. *Nucleic Acids Res.* 1987;15:8333–49.
- [117] Gao X, Gaffney BL, Senior M, Riddle RR, Jones RA. Methylation of thymine residues during oligonucleotide synthesis. *Nucleic Acids Res.* 1985;13:573–84.

- [118] McBride LJ, Eadie JS, Efcavitch JW, Andrus WA. Base modification and the phosphoramidite approach. *Nucleosides Nucleotides*. 1987;6:297–300.
- [119] Nielsen J, Taagaard M, Marugg JE, van Boom JH, Dahl O. Application of 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphordiamidite for *in situ* preparation of deoxyribonucleoside phosphoramidites and their use in polymer-supported synthesis of oligodeoxyribonucleotides. *Nucleic Acids Res.* 1986;14:7391–403.
- [120] Iyer RP. Nucleobase protection of deoxyribo- and ribonucleosides. *Curr Prot Nucleic Acid Chem.* 2000;2,1.1–17.
- [121] Kamimura T, Tsuchiya M, Koura K, Sekine M, Hata T. Diphenylcarbamoyl and propionyl groups. A new combination of protecting groups on the guanine residue. *Tetrahedron Lett.* 1983;24:2775–8.
- [122] Jones SS, Reese CB, Sibanda S, Ubasawa A. The protection of uracil and guanine residues in oligonucleotide synthesis. *Tetrahedron Lett.* 1981;22:4755–8.
- [123] Igolen J, Morin C. Rapid synthesis of protected 2'-deoxycytidine derivatives. *J Org Chem.* 1980;45:4802–4.
- [124] Finlay M, Debiard JP, Guy A, Molko D, Teoule R. An efficient one-pot synthesis of a fully protected 2'-deoxycytidine 3'-monophosphate. *Synthesis*. 1983;303–4.
- [125] Ti GC, Gaffney BL, Jones RA. Transient protection: efficient one-flask synthesis of protected deoxynucleosides. *J Am Chem Soc.* 1982;104:1316–9.
- [126] Boal JH, Wilk A, Harindranath N, Max EE, Kempe T, Beaucage SL. Cleavage of oligodeoxyribonucleotides from controlled-pore glass supports and their deprotection by gaseous amines. *Nucleic Acids Res.* 1996;24:3115–7.
- [127] Schulhof JC, Molko D, Teoule R. Facile removal of new protecting groups useful in oligonucleotide synthesis. *Tetrahedron Lett.* 1987;28:51–4.
- [128] Schulhof JC, Molko D, Teoule R. The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. *Nucleic Acids Res.* 1987;15:397–416.
- [129] Zhu Q, Delaney MO, Greenberg MM. Observation and elimination of N-acetylation of oligonucleotides prepared using fast-deprotecting phosphoramidites and ultra-mild deprotection. *Bioorg Med Chem Lett.* 2001;11:1105–7.
- [130] Xu YZ, Swann PF. A simple method for the solid phase synthesis of oligodeoxynucleotides containing O<sup>4</sup>-alkylthymine. *Nucleic Acids Res.* 1990;18:5729–34.
- [131] Sinha ND, Davis P, Usman N, Pérez J, Hodge R, Kremsky J, Casale R. Labile exocyclic amine protection of nucleotides in DNA, RNA and oligonucleotide analog synthesis facilitating N-deacylation, minimizing depurination and chain degradation. *Biochimie.* 1993;75:12–23.
- [132] Fàbrega C, Eritja R, Sinha ND, Dosanjh M, Singer B. Synthesis and properties of oligonucleotides containing the mutagenic base O4-benzylthymidine. *Bioorg Med Chem.* 1995;3:101–3.
- [133] MacMillan AM, Verdine GL. Engineering tethered DNA molecules by the convertible nucleoside approach. *Tetrahedron.* 1991;47:2603–13.
- [134] Polushin NN, Morocho AM, Chen BC, Cohen JS. On the rapid deprotection of synthetic oligonucleotides and analogs. *Nucleic Acids Res.* 1994;22:639–45.
- [135] McBride LJ, Kierzek R, Beaucage SL, Caruthers MH. Amidine protecting groups for oligonucleotide synthesis. *J Am Chem Soc.* 1986;108:2040–8.
- [136] McBride LJ, Caruthers MH. *N*<sup>6</sup>-(*N*-methyl-2-pyrrolidinemethyl) deoxyadenosine. A new deoxynucleoside protecting group. *Tetrahedron Lett.* 1983;2953–6.
- [137] Froehler BC, Matteucci MD. Dialkylfomamidines: depurination resistant *N*<sup>6</sup>-protecting group for deoxyadenosine. *Nucleic Acids Res.* 1983;11:8031–6.

- [138] Vu H, McCollum C, Jacobson K, Theisen P, Vinayak R, Spiess E, Andrus A. Fast oligonucleotide deprotection phosphoramidite chemistry for DNA synthesis. *Tetrahedron Lett.* 1990;31:7269–72.
- [139] Vinayak R, Anderson P, McCollum C, Hampel A. Chemical synthesis of RNA using fast oligonucleotide deprotection chemistry. *Nucleic Acids Res.* 1992;20:1265–9.
- [140] Takaku H, Morita K, Sumiuchi T. Selective removal of terminal dimethoxytrityl groups. *Chem Lett.* 1883;1661–4.
- [141] Kume A, Iwase R, Sekine M, Hata T. Cyclic diacyl groups for protection of the  $N^6$ -amino group of deoxyadenosine in oligodeoxynucleotide synthesis. *Nucleic Acids Res.* 1984;12:8525–38.
- [142] Kume A, Iwase R, Sekine M, Hata T. Phthaloyl group: a new amino protecting group of deoxyadenosine in oligonucleotide synthesis. *Tetrahedron Lett.* 1982;23:4365–8.
- [143] Himmelsbach F, Schulz BS, Trichtinger T, Charubala R, Pfleiderer W. The *p*-nitrophenylethyl group. A versatile new blocking group for phosphate and aglycone protection in nucleotides and nucleotides. *Tetrahedron.* 1984;40:59–72.
- [144] Pfleiderer W. Universal 2-(*p*-nitrophenyl)ethyl and 2-(*p*-nitrophenyl)-ethoxycarbonyl protecting groups for nucleosides and nucleotides. *Curr Prot Nucleic Acid Chem.* 2007;2.13.1–25.
- [145] Webb TR, Matteucci MD. Hybridization triggered cross-linking of deoxyoligonucleotides. *Nucleic Acids Res.* 1986;14:7661–74.
- [146] Koole LH, Moody HM, Broeders NLHL, Quaedflieg PJLM, Kuijpers WA, van Genderen MP, Coenen AJJM, Wal S Buck HM. Synthesis of phosphate-methylated DNA fragments using 9-fluorenylmethoxycarbonyl as transient base protecting groups. *J Org Chem.* 1989;54:1657–64.
- [147] Moody HM, Genderen MHP, Koole LH, Kocken HJM, Meijer EM, Buck HM. Regiospecific inhibition of DNA duplication by antisense phosphate-methylated oligodeoxynucleotides. *Nucleic Acids Res.* 1989;17:4769–82.
- [148] Fernandez-Forner D, Palom Y, Ikuta S, Pedroso E, Eritja R. Synthesis and characterization of oligonucleotides containing the mutagenic base analogue 4-O-ethylthymidine. *Nucleic Acids Res.* 1990;18:5729–34.
- [149] Hyodo M, Hayakawa Y. Nucleobase protection with allyloxycarbonyl. *Curr Prot Nucleic Acid Chem.* 2005;2.12.1–26.
- [150] Hayakawa Y, Wakabayashi S, Kato H, Noyori R. The allylic protection method in solid-phase oligonucleotide synthesis. An efficient preparation of solid-anchored DNA oligomers. *J Am Chem Soc.* 1990;112:1691–96.
- [151] Hayakawa Y, Kato H, Uchiyama M, Kajino H, Noyori R. Allyloxycarbonyl group: a versatile blocking group for nucleotide synthesis. *J Org Chem.* 1986;51:2400–2.
- [152] Watkins BE, Rapoport H. Synthesis of benzyl and benzyloxycarbonyl base-blocked 2'-deoxyribonucleosides. *J Org Chem.* 1982;47:4471–7.
- [153] Watkins BE, Kiely JS, Rapoport H. Synthesis of oligodeoxyribonucleotides using N-benzyloxycarbonyl-blocked nucleosides. *J Am Chem Soc.* 1982;104:5702–8.
- [154] Heikkila J, Balgobin N, Chattopadhyaya J. The 2-nitrophenylsulfenyl (Nps) group for the protection of amino functions of cytidine, adenosine, guanosine and their 2'-deoxysugar derivatives. *Acta Chim Scand B.* 1983;37:857–84.
- [155] Lin X, Chen J, Shahsavari S, Green N, Goyal D, Fang S. Synthesis of oligodeoxynucleotides containing electrophilic groups. *Org Lett.* 2016;18:3870–3.
- [156] Halami B, Shahsavari S, Nelson Z, Prehoda L, Eriyagama DNAM, Fang S. Incorporation of sensitive ester and chloropurine groups into oligodeoxynucleotides through solid-phase synthesis. *ChemistrySelect.* 2018;3:8857–62.

- [157] Reese CB, Saffhill R. Oligonucleotide synthesis via phosphotriester intermediates: the phenyl-protecting group. *J Chem Soc, Chem Commun.* 1968;767–8.
- [158] Stabinsky Y, Sakata RT, Caruthers MH. Metal ions as selective triggers for removing oligodeoxynucleotide phosphotriester protecting groups. *Tetrahedron Lett.* 1982;23:275–8.
- [159] Van Boom JH, Burgers PMJ, van Deursen PH, Arentzen R, Reese CB. Internucleotide cleavage during unblocking in oligonucleotide synthesis by the phosphotriester approach. *Tetrahedron Lett.* 1974;15:3785–8.
- [160] Chattopadhyaya JB, Reese CB. Some observations relating to phosphorylation methods in oligonucleotide synthesis. *Tetrahedron Lett.* 1979;20:5059–62.
- [161] Reese CB, Titmas RC, Yau L. Oximate ion promoted unblocking of oligonucleotide phosphotriester intermediates. *Tetrahedron Lett.* 1978;19:2727–30.
- [162] Reese CB, Zard L. Some observations relating to the oximate ion promoted unblocking of oligonucleotide aryl esters. *Nucleic Acids Res.* 1981;9:4611–26.
- [163] Cusack NJ, Reese CB. Block synthesis of oligonucleotides by the phosphotriester approach. *Tetrahedron Lett.* 1973;14:2209–12.
- [164] Itakura K, Bahl CP, Katagiri N, Michniewicz JJ, Wightman RH, Narang SA. A modified triester method for the synthesis of deoxyribopolynucleotides. *Can J Chem.* 1973;51:3649–51.
- [165] Adams SP, Kavka KS, Wykes EJ, Holder SB, Galluppi GR. Hindered dialkylamino nucleoside phosphite reagents in the synthesis of two DNA 51-mers. *J Am Chem Soc.* 1983;105:661–3.
- [166] McBride LJ, Caruthers MH. An investigation of several deoxynucleoside phosphoramidites useful for synthesizing deoxyoligonucleotides. *Tetrahedron Lett.* 1983;24:245–8.
- [167] Sinha ND, Biernat J, Köster H.  $\beta$ -Cyanoethyl, *N,N*-dialkylamino/*N*-morpholinomonochloro phosphoramidites, new phosphitylating agents facilitating ease of deprotection and work-up of synthesized oligonucleotides. *Tetrahedron Lett.* 1983;24:5843–6.
- [168] Sinha ND, Biernat J, McManus J, Köster H. Polymer support oligonucleotide synthesis XVIII: use of  $\beta$ -cyanoethyl-*N,N*-dialkylamino-/ *N*-morpholino phosphoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. *Nucleic Acids Res.* 1984;12:4539–57.
- [169] Fourrey JL, Varenne J. A new and general procedure for the preparation of deoxynucleoside phosphoramidites. *Tetrahedron Lett.* 1983;24:1963–6.
- [170] Eritja R, Smirnov V, Caruthers MH. *O*-Aryl phosphoramidites: synthesis, reactivity and evaluation of their use for solid-phase synthesis of oligonucleotides. *Tetrahedron.* 1990;46:721–30.
- [171] Nielsen J, Directed CMH. Arbusov-type reactions of 2-cyano-1,1-dimethylethyl deoxynucleoside phosphites. *J Am Chem Soc.* 1988;110:6275–6.
- [172] Marrug JE, Dreef CE, van der Marel GA, van Boom JH. Use of 2-cyano-1,1-dimethylethyl as a protecting group in the synthesis of DNA via phosphite intermediates. *Recl Trav Chim Pays-Bas.* 1984;103:97–8.
- [173] Claes C, Tesser GI, Dreef CE, Marugg JE, vam der Marel GA, van Boom JH. Use of 2-methylsulfonylethyl as a phosphorous protecting group in oligonucleotide synthesis via a phosphite triester approach. *Tetrahedron Lett.* 1984;25:1307–10.
- [174] Uhlmann E, Pfleiderer W. Nucleotide XIV. Substituted  $\beta$ -phenylethyl groups. New blocking groups for oligonucleotide syntheses by the phosphotriester approach. *Helv Chim Acta.* 1981;64:1688–703.
- [175] Beiter AH, Pfleiderer W. Solution synthesis of protected di-2'-deoxynucleoside phosphotriesters via the phosphoramidite approach. *Tetrahedron Lett.* 1984;25:1975–8.
- [176] Ito H, Ike Y, Ikuta S, Itakura K. Solid phase synthesis of polynucleotides. VI further studies on polystyrene copolymers for the solid support. *Nucleic Acids Res.* 1982;10:1755–69.

- [177] Gait MJ, Mattes HWD, Singh M, Sproat BS, Titmas RC. Rapid synthesis of oligodeoxyribonucleotides VII. Solid phase of oligodeoxyribonucleotides by a continuous flow phosphotriester method on a kieselguhr-polyamide support. *Nucleic Acids Res.* 1982;10:6243–54.
- [178] Matteucci MD, Caruthers MH. The synthesis of oligodeoxypyrimidines on a solid support. *Tetrahedron Lett.* 1980;21:719–22.
- [179] Matteucci MD, Caruthers MH. Synthesis of deoxyoligonucleotides on a polymer support. *J Am Chem Soc.* 1981;103:3185–91.
- [180] Gait MJ. Oligonucleotide synthesis. A practical approach. Oxford: IRL Press; 1985.
- [181] McColumn C, Andrus A. An optimized polystyrene support for rapid, efficient oligonucleotide synthesis. *Tetrahedron Lett.* 1991;32:4069–72.
- [182] Montserrat FX, Grandas A, Eritja R, Pedroso E. Criteria for the economic large scale solid-phase synthesis of oligonucleotides. *Tetrahedron.* 1994;50:2617–22.
- [183] Wright P, Lloyd D, Rapp W, Andrus A. Large scale synthesis of oligonucleotides via phosphoramidite nucleosides and a high-loaded polystyrene support. *Tetrahedron Lett.* 1993;34:3373–6.
- [184] Putta MR, Yu D, Kandimalla ER. Synthesis, purification, and characterization of immune-modulatory oligodeoxynucleotides that act as agonists of Toll-Like Receptor 9. *Methods Mol Biol.* 2011;764:263–77.
- [185] Aitken S, Oligoprep AE. PVA support for oligonucleotide synthesis in columns on a scale up to 10 micromol. *Nucleoside Nucleotide Nucleic Acids.* 2007;26:931–4.
- [186] Rothstein DM, Rothstein ML. Solid-phase supports for oligo synthesis. *Genetic Eng Biotech News.* 2012;32:42–3.
- [187] Yip KF, Tsou KC. A new polymer-support method for the synthesis of ribooligonucleotide. *J Am Chem Soc.* 1971;93:3272–6.
- [188] Sproat BS, Brown DM. A new linkage for solid phase synthesis of oligodeoxyribonucleotides. *Nucleic Acids Res.* 1985;13:2979–87.
- [189] Pon RT. Solid-phase supports for oligonucleotide synthesis. *Curr Proct Nucleic Acids Chem.* 2000;3.1.1–28 and 3.2.1–23.
- [190] Brown T, Pritchard CE, Turner G, Salisbury SA. A new base-stable linker for solid-phase oligonucleotide synthesis. *J Chem Soc, Chem Commun.* 1989;891–3.
- [191] Stengele K, Pfeiderer W. Improved synthesis of oligodeoxyribonucleotides. *Tetrahedron Lett.* 1990;31:2549–52.
- [192] Alul RH, Singman CN, Zhang G, Letsinger RL. Oxalyl-CPG: a labile support for synthesis of sensitive oligonucleotide derivatives. *Nucleic Acids Res.* 1991;19:1527–32.
- [193] Pon RT, Yu S. Hydroquinone-*O,O'*-diacetic acid ('Q-linker') as a replacement for succinyl and oxalyl linker arms in solid phase oligonucleotide synthesis. *Nucleic Acids Res.* 1997;25:3629–35.
- [194] Pon RT. Attachment of nucleosides to solid-phase supports. *Curr Proct Nucleic Acids Chem.* 2000;3.2.1–23.
- [195] Eritja R, Robles J, Fernández-Forner D, Albericio F, Giralt E, Pedroso E. NPE-resin, a new approach to the solid-phase synthesis of protected peptides and oligonucleotides I: synthesis of the supports and their application to oligonucleotides synthesis. *Tetrahedron Lett.* 1991;32:1511–4.
- [196] Eritja R, Robles J, Aviñó A, Albericio F, Pedroso E. A synthetic procedure for the preparation of oligonucleotides without using ammonia and its application for the synthesis of oligonucleotides containing O-4-alkyl thymidines. *Tetrahedron.* 1992;48:4171–82.
- [197] Aviñó A, Güimil García R, Díaz D, Albericio F, Eritja R. A comparative study for the synthesis of oligonucleotides without using ammonia. *Nucleosides Nucleotides.* 1996;15:1871–89.

- [198] Greenberg MM, Gilmore JL. Cleavage of oligonucleotides from solid-phase supports using o-nitrobenzyl photochemistry. *J Org Chem.* 1994;59:746–53.
- [199] Venkatesan H, Greenberg MM. Improved utility of photolabile solid phase synthesis supports for the synthesis of oligonucleotides containing 3'-hydroxyl termini. *J Org Chem.* 1996;61:525–9.
- [200] Routledge A, Wallis MP, Ross KC, Fraser W. A new deprotection strategy for automated oligonucleotide synthesis using a novel silyl-linked solid support. *Bioorg Med Chem Lett.* 1995;5:2059–64.
- [201] Kwiatkowski M, Nilsson M, Landegren U. Synthesis of full-length oligonucleotides; cleavage of apurinic molecules on a novel support. *Nucleic Acids Res.* 1996;24:4632–8.
- [202] Gough GR, Brunden MJ, Gilham PT. 2'(3')-O-benzyluridine 5' linked to glass: an all-purpose support for solid phase synthesis of oligodeoxyribonucleotides. *Tetrahedron Lett.* 1983;24:5321–4.
- [203] Schwartz ME, Breaker RR, Asteriadis GT, Gough GR. A universal adapter for chemical synthesis of DNA or RNA on any single type of solid support. *Tetrahedron Lett.* 1995;36:27–30.
- [204] Nelson PS, Muthini S, Vierra M, Acosta L, Smith TH. Rainbow universal CPG: a versalite solid support for oligonucleotide synthesis. *BioTechniques.* 1997;22:752–6.
- [205] Kumar P, Mahajan S, Gupta KC. Universal reusable polymer support for oligonucleotide synthesis. *J Org Chem.* 2004;69:6482–5.
- [206] Scheuer-Larsen C, Rosenbohm C, Jorgensen TJD, Wengel J. Introduction of a universal solid support for oligonucleotide synthesis. *Nucleosides Nucleotides.* 1997;16:67–80.
- [207] Lyttle MH, Hudson D, Cook RM. A new universal linker for solid phase DNA synthesis. *Nucleic Acids Res.* 1996;24:2793–8.
- [208] Yodokkin A, Azhayev A. Universal linker phosphoramidite. *Arkivoc.* 2009;187–97.
- [209] Azhayev A, Antopolksky ML. Amide group assisted 3'-dephosphorylation of oligonucleotides synthesized on universal A-supports. *Tetrahedron.* 2001;57:4977–86.
- [210] Guzaev AP, Manoharan M. A conformationally preorganized universal solid support for efficient oligonucleotide synthesis. *J Am Chem Soc.* 2003;125:2380–1.
- [211] Kumar RK, Guzaev AP, Rintel C, Ravikumar VT. Efficient synthesis of antisense phosphorothioate oligonucleotides using a universal solid support. *Tetrahedron.* 2006;62:4528–34.
- [212] Wang Z, Olsen P, Ravikumar VT. A novel universal linker for efficient synthesis of phosphorothioate oligonucleotides. *Nucleosides Nucleotides Nucleic Acids.* 2007;26:259–69.
- [213] Ravikumar VT, Kumar RK, Olsen P et al. Unylinker: an efficient and scaleable synthesis of oligonucleotides utilizing a universal linker molecule: a novel approach to enhance the purity of drugs. *Org Proc Res Devel.* 2008;12:399–410.
- [214] Hardy PM, Holland D, Scott S, Garman AJ, Newton CR, McLean MJ. Reagents for the preparation of two oligonucleotides per synthesis (TOPS). *Nucleic Acids Res.* 1994;22:2998–3004.
- [215] Pochet S, Huynh-Dinh T, Igolen J. Synthesis of DNA fragments linked to a solid support. *Tetrahedron.* 1987;43:3841–90.
- [216] Duncan CH, Cavalier SL. Affinity chromatography of a sequence-specific DNA binding protein using Teflon-linked oligonucleotides. *Anal Biochem.* 1988;169:104–8.
- [217] Platella C, Musumeci D, Arciello A, Doria F, Freccero M, Randazzo A, Amato J, Pagano B, Montesarchio D. Controlled pore glass-based oligonucleotide affinity support: towards high throughput screening methods for the identification of conformation-selective G-quadruplex ligands. *Anal Chim Acta.* 2018;1030:133–41.

- [218] Mazzini S, García-Martín F, Alvira M, Aviñó A, Manning B, Albericio F, Eritja R. Synthesis of oligonucleotide derivatives using ChemMatrix supports. *Chem Biodivers.* 2008;5:209–18.
- [219] Grajkowski A, Cieslak J, Kauffman JS et al. Thermolytic release of covalently linked DNA oligonucleotides and their conjugates from controlled-pore glass at near neutral pH. *Bioconjug Chem.* 2008;19:1696–706.
- [220] Matteucci MD, Caruthers MH. The use of Zinc Bromide for removal of dimethoxytrityl ethers from deoxynucleosides. *Tetrahedron Lett.* 1980;21:3243–6.
- [221] Wei X. Coupling activators for the oligonucleotide synthesis via phosphoramidite approach. *Tetrahedron.* 2013;69:3615–37.
- [222] Pon RT. Enhanced coupling efficiency using 4-dimethylaminopyridine (DMAP) and either tetrazole, 5-(*o*-nitrophenyl)tetrazole or 5-(*p*-nitrophenyl)tetrazole (NPT) in the solid phase synthesis of oligoribonucleotides by the phosphoramidite procedure. *Tetrahedron Lett.* 1987;28:3643–6.
- [223] Froehler BC, Matteucci MD. Substituted 5-phenyltetrazoles; improved 5-activators of deoxynucleoside phosphoramidites in deoxyoligonucleotide synthesis. *Tetrahedron Lett.* 1983;24:3171–4.
- [224] Wright P, Lloyd D, Rapp W, Andrus A. Large scale synthesis of oligonucleotides via phosphoramidite nucleosides and a high-loaded polystyrene support. *Tetrahedron Lett.* 1993;34:3373–6.
- [225] Yao O. 5'-Methylthio-1*H*-tetrazole. *Encyclopedia of reagents for organic synthesis.* Wiley; 2003.
- [226] Wolter A, Leuck M. Activators for Oligonucleotide and Phosphoramidite Synthesis. Sigma-Aldrich Co. [7897758]. 2011. Missouri, USA. 2006. US patent 0,247,431, 2006.
- [227] Welz R, Müller S. 5-(Benzylmercapto)-1*H*-tetrazole as activator for 2'-O-TBDMS phosphoramidite building blocks in RNA synthesis. *Tetrahedron Lett.* 2002;43:795–7.
- [228] Hirose M, Kawai R, Hayakawa Y. Preparation of (*R*)-(−)-2-methoxy-2'-(1*H*-tetrazol-5-yl)-1, 1'-binaphthyl and (1*R*,2*R*)-(+) -1-trimethylsilyl-2-(1*H*-tetrazol-5-yl)ferrocene: optically active tetrazoles with axial or planar chirality. *Synlett.* 2000;1997:495–7.
- [229] Vargeese C, Carter J, Yegge J et al. Efficient activation of nucleoside phosphoramidites with 4, 5-dicyanoimidazole during oligonucleotide synthesis. *Nucleic Acids Res.* 1998;26:1046–50.
- [230] Seio K, Kumura K, Bologna JC, Sekine M. Enhanced stereoselectivity in internucleotidic bond formation by the use of the chiral ribose moiety of thymidine. *J Org Chem.* 2003;68:3849–59.
- [231] Zhang Z, Tang JY. A novel phosphitylating reagent for in situ generation of deoxyribonucleoside phosphoramidite. *Tetrahedron Lett.* 1996;37:331–4.
- [232] Rajwanshi VK, Håkansson AE, Dahl BM, Wengel J. LNA stereoisomers: xylo-LNA ( $\beta$ -D-xylo configured locked nucleic acid) and  $\alpha$ -L-LNA ( $\alpha$ -L-ribo configured locked nucleic acid). *Chem Commun.* 1999;1395–6.
- [233] Hayakawa Y, Kataoka M, Noyori R. Benzimidazolium triflate as an efficient promoter for nucleotide synthesis via the phosphoramidite method. *J Org Chem.* 1996;61:7996–7.
- [234] Hayakawa Y, Kawai R, Hirata A et al. Acid/azole complexes as highly effective promoters in the synthesis of DNA and RNA oligomers via the phosphoramidite method. *J Am Chem Soc.* 2001;123:8165–76.
- [235] Hayakawa Y, Kataoka M. Facile synthesis of oligodeoxyribonucleotides via the phosphoramidite method without nucleoside base protection. *J Am Chem Soc.* 1998;120:12395–401.
- [236] Hayakawa Y, Iwase T, Nurminen EJ, Tsukamoto M, Kataoka M. Carboxylic acids as promoters for internucleotide-bond formation via condensation of a nucleoside phosphoramidite and a nucleoside: relationship between the acidity and the activity of the promoter. *Tetrahedron.* 2005;61:2203–9.

- [237] Caruthers MH, Barone AD, Beaucage SL, Dodds DR, Fischer EF, McBride LJ, Matteucci M, Stabinsky Z, Tang JY. Chemical synthesis of deoxyoligonucleotides by the phosphoramidite method. *Methods Enzymol.* 1987;154:287–313.
- [238] Barone AD, Tang JY, Caruthers MH. In situ activation of bis-alkylaminophosphines – a new method for synthesizing deoxyoligonucleotides on polymers support. *Nucleic Acids Res.* 1984;12:4051–61.
- [239] Moore MF, Beaucage SL. Conceptual basis of the selective activation of bis(dialkylamino)methoxyphosphines by weak acids and its application toward the preparation of deoxynucleoside phosphoramidites in situ. *J Org Chem.* 1985;50:2019–25.
- [240] Maier MA, Choi Y, Gaus H, Barchi JJ, Marquez VE, Manoharan M. Synthesis and characterization of oligonucleotides containing conformationally constrained bicyclo[3.1.0]hexane pseudosugar analogs. *Nucleic Acids Res.* 2004;32:3642–60.
- [241] Hayakawa Y, Uchiyama M, Noyori R. Solid-phase synthesis of oligodeoxyribonucleotides using the bis(trimethylsilyl)peroxide oxidation of phosphites. *Tetrahedron Lett.* 1986;27:4195–6.
- [242] Hayakawa Y, Uchiyama M, Noyori R. Nonaqueous oxidation of nucleoside phosphites to the phosphates. *Tetrahedron Lett.* 1986;27:4191–4.
- [243] Ogilvie KK, Nemer MJ. Nonaqueous oxidation of phosphites to phosphates in nucleoside chemistry. *Tetrahedron Lett.* 1981;22:2531–2.
- [244] Fourrey JL, Varenne J. Introduction of a nonaqueous oxidation procedure in the phosphite triester route for oligonucleotide synthesis. *Tetrahedron Lett.* 1985;26:1217–20.
- [245] Ugi I, Jacob P, Landgraf B, Rupp C, Lemmen P, Verfürth U. Phosphite oxidation and the preparation of five-membered cyclic phosphorylating reagents via the phosphites. *Nucleosides Nucleotides.* 1988;7:605–8.
- [246] Gianolio DA, McLaughlin LW. Synthesis and triplex forming properties of pyrimidine derivative containing extended functionality. *Nucleosides Nucleotides.* 1999;18:1751–69.
- [247] Iyer RP, Egan W, Regan JB, Beaucage SL. 3H-1,2-benzodithiol-3-one-1,1-dioxide as an improved sulfurizing reagent in the solid phase synthesis of oligodeoxyribonucleoside phosphorothioates. *J Am Chem Soc.* 1990;112:1253–4.
- [248] Iyer RP, Phillips LR, Egan W, Regan JB, Beaucage SL. The automated synthesis of sulfur-containing oligodeoxyribonucleotides using 3H-1,2-benzodithiol-3-one-1,1-dioxide as a sulfur-transfer reagent. *J Org Chem.* 1990;55:4693–9.
- [249] Vu H, Hirschbein BL. Internucleotide phosphite sulfurization with tetraethylthiuram disulfide. Phosphorothioate oligonucleotide synthesis via phosphoramidite chemistry. *Tetrahedron Lett.* 1991;32:3005–8.
- [250] Xu Q, Musier-Forsyth M, Hammer RP, Barany G. Use of 1,2,4-dithiazolidine-3,5-dione (DtsNH) and 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) for synthesis of phosphorothioate-containing oligodeoxyribonucleotides. *Nucleic Acids Res.* 1996;24:1602–7.
- [251] Guzaev AP. Reactivity of 3H-1,2,4-dithiazole-3-thiones and 3H-1,2-dithiole-3-thiones as sulfurizing agents for oligonucleotide synthesis. *Tetrahedron Lett.* 2011;52:434–7.
- [252] Reddy MP, Hanna NB, Farooqui F. Fast cleavage and deprotection of oligonucleotides. *Tetrahedron Lett.* 1994;35:4311–14.
- [253] Hirose T, Crea R, Itakura K. Rapid synthesis of trideoxyribonucleotide blocks. *Tetrahedron Lett.* 1978;28:2449–52.
- [254] Broka C, Hozumi T, Arentzen R, Itakura K. Simplification in the synthesis of short oligonucleotide blocks. *Nucleic Acids Res.* 1980;8:5461–71.
- [255] Iwai S, Koizumi M, Ikehara H, Ohtsuka E. Solid-phase synthesis of protected oligonucleotide blocks. Applications to block condensation on a polymer support and synthesis of 3'-modified oligonucleotides. *Tetrahedron.* 1987;43:59–67.

- [256] Kumar G, Poonian MS. Improvements in oligodeoxyribonucleotides synthesis: methyl N,N-dialkylphosphoramidite dimer units for solid support phosphite methodology. *J Org Chem.* 1984;49:4905–12.
- [257] Wolter A, Biernat J, Köster H. Polymer support oligonucleotide synthesis of a henhectacosa deoxynucleotide by use of a dimeric phosphoramidite synthon. *Nucleosides Nucleotides.* 1986;5:65–77.
- [258] Virnekäs B, Ge L, Plückthun A, Schneider C, Wellnhofer G, Moroney SE. Trinucleotide phosphoramidites: ideal reagents for the synthesis of mixed oligonucleotides for random mutagenesis. *Nucleic Acids Res.* 1994;22:5600–7.
- [259] Ono A, Matsuda A, Zhao J, Santi DV. The synthesis of blocked triplet-phosphoramidites and their use in mutagenesis. *Nucleic Acids Res.* 1995;23:4677–82.
- [260] Kayushin AL, Korosteleva MD, Miroshnikov AI, Kosch W, Zubov D, Piel N. A convenient approach to the synthesis of trinucleotide phosphoramidites-synthons for the generation of oligonucleotide/peptide libraries. *Nucleic Acids Res.* 1996;24:3748–55.
- [261] Yagodkin A, Azhayev A, Roivainen J et al. Improved synthesis of trinucleotiotide phosphoramidites and generation of randomized oligonucleotide libraries. *Nucleosides Nucleotides Nucleic Acids.* 2007;26:473–97.
- [262] Janczyk M, Appel B, Springstubb D, Fritz HJ, Muller S. A new and convenient approach for the preparation of  $\beta$ -cyanoethyl protected trinucleotide phosphoramidites. *Org Biomol Chem.* 2012;10:1510–3.
- [263] Suchsland R, Appel B, Müller S. Synthesis of trinucleotide building blocks in solution and on solid phase. *Curr Prot Nucleic Acid Chem.* 2018;75:e60.
- [264] Kayushin AL, Korosteleva MD, Miroshnikov A. Large-scale solid-phase preparation of 3'-unprotected trinucleotide phosphotriesters. Precursors for synthesis of trinucleotide phosphoramidites. *Nucleosides Nucleotides.* 2000;19:1967–76.
- [265] Jabgunde AM, Gimenez Molina A, Virta P, Lönnberg H. Preparation of a disulfide-linker precipitative soluble support for solution-phase synthesis of trimeric oligodeoxyribonucleotide 3'-(2-chlorophenylphosphate) building blocks. *Beilstein J Org Chem.* 2015;11:1553–60.
- [266] Kungurtsev V, Lönnberg H, Virta P. Synthesis of protected 2'-O-deoxyribonucleotides on a precipitative soluble support: a useful procedure for the preparation of trimer phosphoramidites. *RSC Adv.* 2016;6:105428.
- [267] Suchslan R, Appel B, Janczyk M, Müller S. Solid phase assembly of fully protected trinucleotide building blocks for codon based gene synthesis. *Appl Sci.* 2019;9:2199.
- [268] Arunachalam TS, Wichert C, Appel B, Müller S. Mixed oligonucleotides for random mutagenesis: best way of making them. *Org Biomol Chem.* 2012;10:4641–50.
- [269] Suchslan R, Appel B, Müller S. Preparation of trinucleotide phosphoramidites as synthons for the synthesis of gene libraries. *Beilstein J Org Chem.* 2018;14:397–406.
- [270] Huang J, Xi Z. Chemical synthesis of bioactive siRNA in solution phase by using 2-(azidomethyl)benzoyl as 3'-hydroxyl group protecting group. *Tetrahedron Lett.* 2012;53:3654–7.
- [271] Hassler M, Wu YQ, Reddy M, Chan TH, Damha MJ. RNA synthesis via dimer and trimer phosphoramidite block coupling. *Tetrahedron Lett.* 2011;52:2575–8.
- [272] Thorpe JD, O'Reilly D, Friscic T, Damha MJ. Mechanochemical synthesis of short DNA fragments. *Chem Eur J.* 2020;2:8857–61.
- [273] Kim JF, Gaffney PRJ, Valtcheva IB, Williams G, Buswell AM, Anson MS, Livingston AG. Organic solvent nanofiltration (OSN): a new technology platform for liquid-phase oligonucleotide synthesis (LPOS). *Org Process Res Dev.* 2016;20:1439–52.



Carme Fàbrega and Ramon Eritja

## 2 Synthesis of oligonucleotides carrying DNA lesions for DNA repair studies

### Contents

2.1	Alkylating agents —	45
2.1.1	Synthesis of oligonucleotides carrying alkylpurines —	46
2.1.2	Synthesis of oligonucleotides carrying alkylpyrimidines —	50
2.1.3	Synthesis of oligonucleotides carrying double crosslinks —	52
2.2	Photodimers —	53
2.3	Oxidation and radiation products —	56
2.3.1	Oxidized purines and their degradation products —	56
2.3.2	Cyclonucleosides —	60
2.3.3	Oxidized pyrimidines, their degradation products and epigenetic nucleobases —	60
2.4	Oligonucleotides carrying abasic sites —	64
2.5	DNA adducts with bifunctional reagents and bulky substituents —	66
2.5.1	DNA adducts with bifunctional reagents —	66
2.5.2	DNA adducts with bulky aromatic compounds —	68
	Bibliography —	72

The major role of nucleic acids is to store and transfer genetic information. This information is coded in the form of DNA sequence which is translated into proteins by decoding the DNA sequence in groups of three nucleobases (triplets). The genetic information transfer relies in the integrity of the nucleobases and in the maintenance of the specific pairing properties of the nucleobases. However, DNA as most organic compounds can undergo to chemical reactions that modify their structure. These DNA decomposition products may cause mutations if they are not repaired properly [1–3]. A large effort has been made in order to provide modified oligonucleotides carrying DNA lesions as substrates to study DNA repair and DNA mutagenesis [4–7].

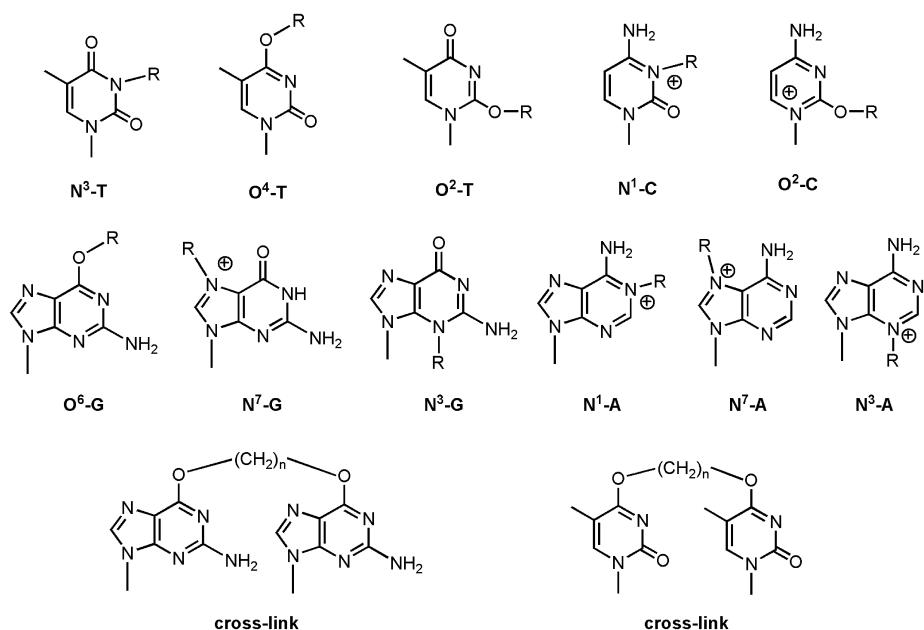
### 2.1 Alkylating agents

Alkylating agents are a chemical compounds that react with the nucleobases of DNA introducing alkyl modifications in the nucleophilic centers. These include chemical warfare such as mustard gas, nitrites, epoxides, lactones, alkyl sulphates, alkyl sulfonates and *N*-nitroso compounds such as *N*-nitrosamines, *N*-nitrosoguanidines

---

**Carme Fàbrega, Ramon Eritja,** Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Networking Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Jordi Girona 18-26, E-08034 Barcelona, Spain, e-mails: carme.fabrega@iqac.csic.es, recgma@cid.csic.es

(*N*-methyl-*N*-nitro-*N*-nitrosoguanidine, MNNG) and *N*-nitrosoureas (carmustine, BCNU) used as chemotherapeutic agents. Bifunctional alkylating agents such as nitrogen mustards (e. g., chlorambucil and cyclophosphamide) led to the formation of double interstrand crosslinks, which are more complex and highly cytotoxic. Simple alkylating agents form adducts at the *N* or the *O* atoms in DNA bases with a higher formation of *N*-alkylated bases such *N*<sup>7</sup>-alkylguanine and *N*<sup>3</sup>-alkyl adenine because of their higher reactivity [2–4] (Figure 2.1). But the mutagenic effect of the alkylating agents is mostly due to the formation *O*<sup>6</sup>-alkylguanine and *O*<sup>4</sup>-alkylthymidines that are produced in lower amounts but they exhibit higher stabilities as well higher possibility of mispairing [8]. For these reasons, most of synthetic studies were centered in the preparation of oligonucleotides carrying *O*<sup>6</sup>-alkylguanine and *O*<sup>4</sup>-alkylthymidines, although other related DNA lesions have also been studied [5].



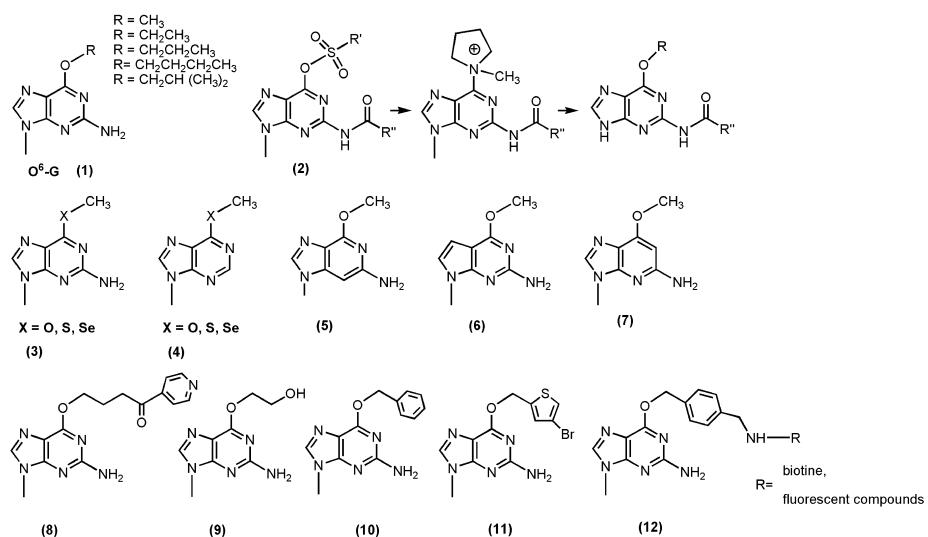
**Figure 2.1:** Chemical structures of the most common DNA nucleobase products produced by the reaction of DNA with alkylating agents.

### 2.1.1 Synthesis of oligonucleotides carrying alkylpurines

The interest started with the synthesis of oligonucleotides of defined sequence carrying *O*<sup>6</sup>-alkylguanine derivatives, as DNA lesions. In order to obtain these modified oligonucleotides, two methodological problems have to be solved: first, the preparation of the monomer unit carrying the modified nucleotide, and second, the com-

patibility of the modified nucleobases with the chemical treatments used during the synthesis. In an initial approach,  $O^6$ -methyl-2'-deoxyguanosine was prepared by condensation of 2-amino-6-chloropurine with the chloro sugar (2-deoxy-3,5-di-*O*-toluoyl-D-*erithro*-pentosyl chloride) followed by methanolysis [9]. The resulting nucleoside was functionalized and a short oligonucleotide sequence was prepared on solution phase using the phosphotriester method [9]. Some side products were observed because the authors selected the benzoyl group for the protection of the amino group, which probably is too stable. Starting from 2-deoxyguanosine, a simpler method for the preparation of  $O^6$ -alkyl-2'-deoxyguanosines was described by Gaffney and Jones [10]. The key intermediate of this method is the formation of an  $O^6$ -triisopropylsulfonyl intermediate followed by the displacement of the sulfonyl group by trimethylamine that react with alcohols and phenols, to yield  $O^6$ -alkyl-2'-deoxyguanosine derivatives [10]. In this case, authors used the isobutyryl group for the protection of the amino group and they observed that the deprotection was too slow and with the potential formation of 2,6-diaminopurine [11]. For this reason, the acetyl group was selected for the protection of the amino group of  $O^6$ -methyl-2'-deoxyguanosine [12]. Alternatively, the  $O^6$ -alkylguanine derivatives can be prepared by reaction of DMT-dG(*N*-isobutyryl) with the corresponding diazoalkane although the authors recommended a variation of the method described by Gaffney and Jones [10] using mesitylene sulfonic chloride instead of triisopropylsulfonyl chloride [13]. In a detailed study of potential side reactions, the authors confirmed the potential formation of 2,6-diaminopurine derivatives if the ammonolysis, used for the removal of the base protecting groups, was performed in extremely vigorous conditions such as 60–70 °C and 72 hrs [13]. Following these results, Pauly et al. described the synthesis of oligonucleotides carrying  $O^6$ -methyl-,  $O^6$ -ethyl- and  $O^6$ -benzyl-2'-deoxyguanosines by the solid-phase phosphoramidite method [14]. Likewise, Li and Swann described an improved protocol for the synthesis of  $O^6$ -alkyl-2'-deoxyguanosine [15]. In this protocol, they employed mesitylene sulfonic chloride instead of triisopropylsulfonyl chloride, and *N*-methylpyrrolidine instead of trimethylamine. This change avoids the use of trimethylamine that has a low boiling point. In addition, these authors described the use of the phenylacetyl group that is more labile to ammonia than the isobutyryl group. In this way, the ammonolysis for the removal of the protecting groups can be done at shorter and milder conditions avoiding the formation of 2,6-diaminopurine [15]. These authors recommend exploiting zinc bromide solutions instead of dichloroacetic acid to avoid potential depurination of  $O^6$ -alkyl-G derivatives [15]. The group of Dr. Jones reported a simple method for the synthesis of  $O^6$ -substituted dG derivatives including  $O^6$ -methyl-dG. In one pot reaction, dG is treated with a mixture of trifluoroacetic anhydride and pyridine generating a pyridinium intermediate that can be displaced by amines, alcohols and phenols [16]. In more detailed studies, the application of 2-cyanoethyl phosphoramidites and solid-phase protocols are recommended [17–20]. Methylphosphoramidites were not appropriate as the removal of the methyl group in the phosphates with thiophenol may as well remove the alkyl group of the  $O^6$ -alkyl-G derivatives [18].

The human DNA repair protein  $O^6$ -alkylguanine-DNA alkyltransferase (hAGT) is able to remove the alkyl group of  $O^6$ -alkylguanines via alkyl transfer to a highly conserved cysteine residue of the enzyme. hAGT behaves as a suicidal enzyme as the formation of the S-alkylcysteine results in the functional inactivation of the enzyme. In order to study the interactions between  $O^6$ -methyl-G analogues (Figure 2.2) were synthesized [21] including S and Se derivatives and several deazaguanine derivatives (Figure 2.2). All  $O^6$ -methyl-G analogues except 3-deazaguanine derivative were substrates of hAGT. The substitution of oxygen by S and Se gave a large decrease of the reaction rates as well as the removal of the nitrogen 1, indicating that these sites are implicated in the interactions with the hAGT protein [21]. The implication of these atoms in the contacts with the hAGT protein suggests the opening of the duplex and the flipping of  $O^6$ -methyl-G out of the duplex to reach the active center of hAGT [21].



**Figure 2.2:**  $O^6$ -alkylguanines (1). (2) Optimized synthetic protocol using  $N$ -methylpyrrolidine for the displacement of the  $O^6$ -sulfonyl-G intermediates; (3) S and Se analogues of  $O^6$ -G derivatives; (4) S and Se derivatives of hypoxanthine; (5), (6), (7) deazaguanine derivatives; (8)  $O^6$ -pyridyloxobutyl-G isolated from the reaction with tobacco nitrosamines; (9)  $O^6$ -2-hydroxyethyl-G; (10)  $O^6$ -benzyl-G; (11)  $O^6$ -(4-bromothienyl)-G; (12)  $O^6$ -(4-aminomethylbenzyl)-G.

The  $O^6$ -pyridyloxobutylate guanine (Figure 2.2) derivative was detected in the reaction of duplex DNA with the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and  $N'$ -nitrosonornicotine (NNN). In order to evaluate the mutagenic contribution of the  $O^6$ -pyridyloxobutylguanine adduct [22], the synthesis of oligonucleotides carrying this derivative was undertaken [23, 24]. A similar strategy as the one used in the synthesis of other  $O^6$ -alkylguanine derivatives was followed

for the synthesis of these oligonucleotides [21]. In this case, the labile phenoxyacetyl protecting group was employed for the protection of the  $N^2$ -amino function of guanine and the ketone function was protected with the 1,3-dithiopropyl group [22]. These protein activity studies demonstrated that  $O^6$ -pyridyloxobutylguanine adduct is repaired by hAGT in a similar way than other  $O^6$ -alkylguanine derivatives [22].

Overexpression of hAGT is one of the factors that facilitate the resistance to chemotherapy, due to its capacity to repair DNA damage generated by alkylating agents. For this reason, hAGT is a key target for inhibition in cancer treatment. Some  $O^6$ -alkylguanine nucleobases such as benzyl or 4-bromophenyl have shown a good activity as hAGT inhibitors. Their incorporation in a duplex DNA may generate inhibitors with higher efficiency. To this end, Shibata et al. developed a post-synthetic method to generate oligonucleotides carrying a series of  $O^6$ -alkylguanine derivatives [25]. The method relies on the use of a methylsulfonylpurine derivative that can be converted in several  $O^6$ -alkylguanine derivatives by nucleophilic displacement of the appropriate alcohol [25]. The introduction of modifications in oligonucleotides with post-synthetic methods will be explained in more detail in Sections 2.5.2 and 4.3.

The transfer of the alkyl group of the  $O^6$ -alkylguanine to the cysteine residue at the active site of hAGT is the base for the covalent labeling of fusion proteins [26]. Keppler et al. prepared oligonucleotides carrying an  $O^6$ -(4-aminomethyl-benzyl) 2'-deoxyguanosine derivative. The 4-amino function was functionalized with reporter compounds such as fluoresceine and biotine molecules (Figure 2.2). These oligonucleotides were able to transfer the reporter compounds to hAGT by the alkyl group transfer reaction. This labeling protocol was successfully applied for the tagging of hAGT produced inside the cells [26]. In another application, oligonucleotides carrying  $O^6$ -(4-aminomethyl-benzyl) 2'-deoxyguanosine derivatives were functionalized with fluorescent-quencher systems. The preparation of the  $O^6$ -(4-aminomethyl-benzyl) 2'-deoxyguanosine protected derivatives was done by the Mitsonobu reaction [27, 28] instead of the arylsulfonyl displacement, shown in Figure 2.2 [27]. These responsive elements light up as a result of the enzymatic activity of hAGT [27, 28] as the fluorescent compound move away of the quencher as result of the alkyl transfer to hAGT cysteine. These fluorescent-quencher systems allowed the *in vitro* measurement of the enzymatic activity of hAGT [27, 28], which is an important step for the screening and characterization of hAGT inhibitors required for improving cancer treatments.

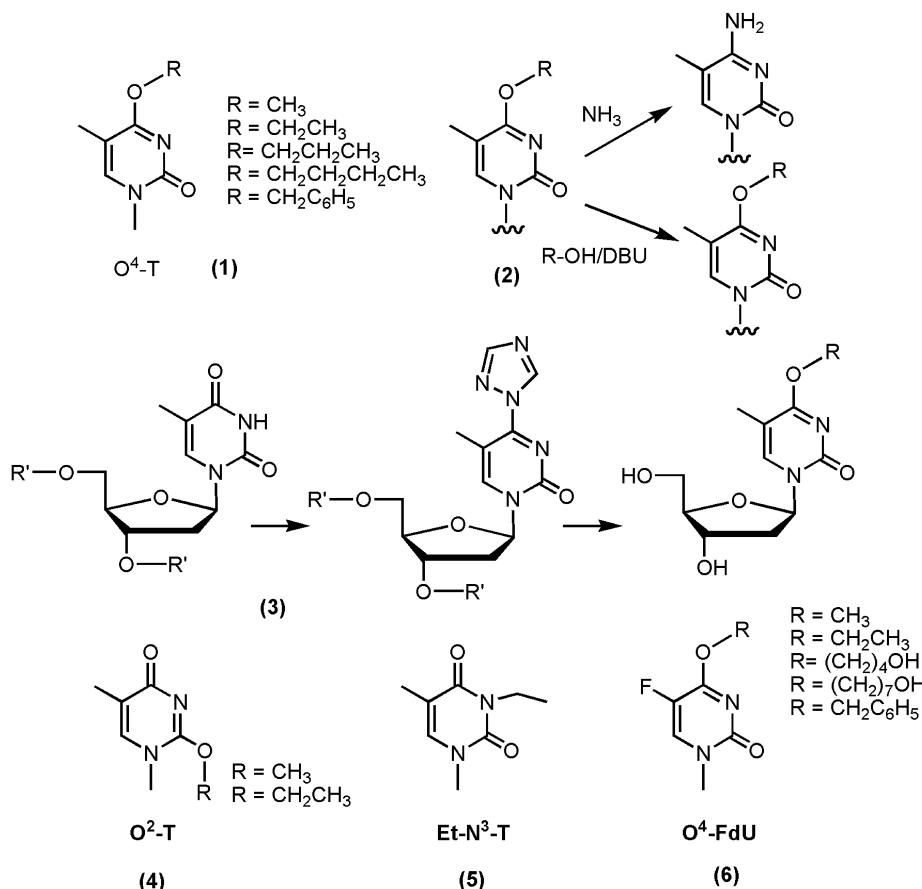
The formation of  $N^7$ -alkylguanine in DNA by nitrosoureas, nitrosoguanidines and diazomethane has been assessed [29].  $N^7$ -alkylguanine lesions were detected by piperidine treatment to convert the  $N^7$ -alkylguanine residues in strand breaks. In a clever enzymatic method,  $N^7$ -methyl-2'-deoxyguanosine residues have been incorporated into synthetic oligonucleotides by using  $N^7$ -methyl-2'-deoxyguanosine triphosphate, T7 DNA polymerase and DNA ligase [30]. Thermal denaturation experiments show a small decrease in the melting temperature of duplex DNA carrying  $N^7$ -alkylguanine residues. The stability of this DNA lesion was found to be much

higher than the one reported by the natural nucleoside justifying the observed persistence of these lesions in genomic DNA [30].

### 2.1.2 Synthesis of oligonucleotides carrying alkylpyrimidines

The synthesis of oligonucleotides carrying  $O^2$ -alkyl- and  $O^4$ -alkylthymidines is especially complex due to the extreme lability of these derivatives to nucleophiles. In the synthesis of  $O^6$ -alkylguanines, it was possible to use ammonia for the deprotection of the  $O^6$ -alkylguanine modified oligonucleotides, if utilized at room temperature and for a short time. This is not possible with the synthesis of oligonucleotides carrying  $O^4$ -alkylthymidines as it will generate 5-methyl-cytosine derivatives (Figure 2.3) [31]. In the synthesis of  $O^4$ -methylthymine oligonucleotides, the ammonia solution was replaced by a solution of 1,8-diaza[5.4.0]bicyclo[4.1.0]hept-7-ene (DBU) in methanol that generate methoxide ions that do not modify  $O^4$ -methylthymine residues (Figure 2.3) [31]. The preparation of  $O^4$ -methylthymidine derivative was accomplished by the synthesis of the 4-(1,2,4-triazol) thymidine derivatives followed by reaction with methoxide solutions (Figure 2.3) [30]. In a similar way, other  $O^4$ -alkylthymidines can be synthesized [32]. Alternatively,  $O^4$ -alkylthymidines can be prepared by a silver ion catalyzed reaction with alkyl halides [32]. Mixtures of  $O^4$ -alkylthymidines and  $O^2$ -alkylthymidines can be prepared by reaction of protected derivatives of thymidine with diazoalkanes [33]. Both isomers can be separated by silica gel chromatography allowing the preparation of oligonucleotides carrying  $O^2$ -alkylthymidines [33]. Oligonucleotides carrying  $O^4$ -ethylthymine residues were prepared replacing the ammonia solution used in the final deprotection with DBU/ethanol solutions [34]. Otherwise, the use of *p*-nitrophenylethyl (NPE) and *p*-nitrophenylethoxy carbonyl (NPEOC) base protecting groups instead of the acyl protecting groups for the nucleobases was recommended [35]. Oligonucleotides carrying  $O^4$ -propylthymine and  $O^4$ -butylthymine were obtained using NPE protecting groups and DBU in aprotic solvents [36, 37]. The synthesis of  $O^4$ -benzylthymine oligonucleotides was accomplished by protection of the amino groups of the natural nucleobases with *t*-butylphenoxyacetyl (*t*Pac) [38]. These labile protecting groups are removed in very mild conditions such as benzylalcohol/DBU solutions. However, acetic anhydride has to be replaced by *t*-butylphenoxyacetyl anhydride during the synthesis to avoid exchange of *t*Pac by acetyl groups, which are more stable to ammonia [38]. Another improvement was the use of the phosphoramidite of 4-(1,2,4-triazol) thymidine. This derivative was employed as a common intermediate for the synthesis of several thymine derivatives including several  $O^4$ -alkylthymidines [39, 40]. This strategy will be explained in more detail at the end of this chapter.

The synthesis of oligonucleotides carrying  $O^2$ -alkylthymidines (Figure 2.3) follows a similar strategy of using labile protecting groups for the natural nucleobases and



**Figure 2.3:** (1)  $O^4$ -alkylthymidines. (2) Ammonia displacement of  $O^4$ -alkyl group can be prevented by replacing the deprotection solution with a DBU solution in the appropriate alcohol. (3) Synthetic scheme used for the preparation of  $O^4$ -alkylthymidines. Other interesting alkylthymidines:  $O^2$ -alkylthymidines (4),  $N^3$ -alkylthymidines (5), and 5-fluoro- $O^4$ -alkylthymidines (6). DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene.

alcohol/DBU solution for the final deprotection to avoid side reactions. The appropriate  $O^2$ -alkylthymidines are prepared by reaction of DMT-T with diazoalkanes and chromatographic separation of the isomers [33, 41, 42]. Alternatively, the synthesis of  $O^2$ -alkylthymidines can be improved by directing the alkylation towards the 2-position using 5'-tosylthymidine [43]. This compound undergoes to the formation of the intermediate  $O^2,5'$ -cyclothymidine by alcohol/DBU solution treatment. This intermediate generates the desired  $O^2$ -alkylthymidine in one pot reaction [43].

Oligonucleotides carrying  $N^3$ -alkylthymidine s (Figure 2.3) have been also prepared [44]. Furthermore,  $N^3$ -alkylthymidines are prepared by reaction of thymidine with diazaalkanes, and after chromatographic separation, the desired phospho-

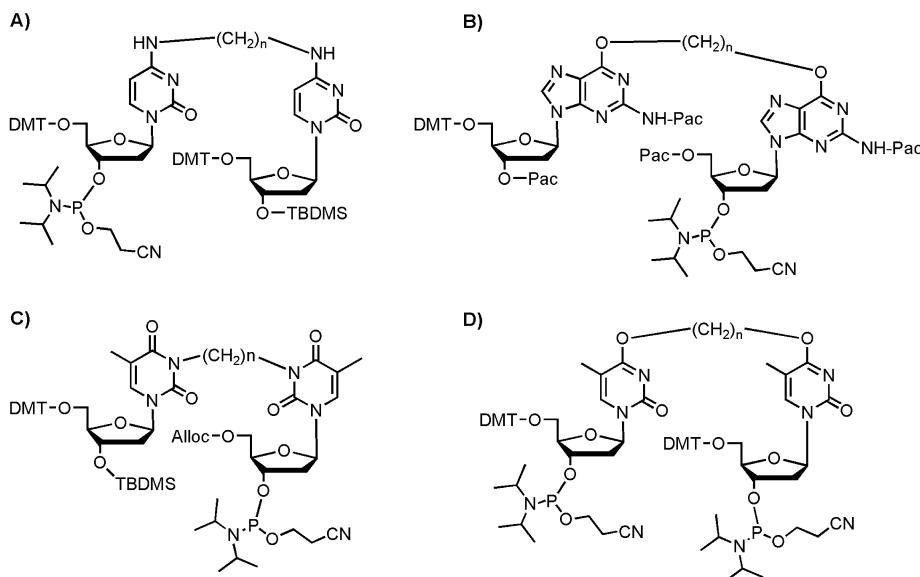
ramidite was obtained [44]. This nucleobase is very stable so the oligonucleotide synthesis is performed using the final standard ammonia deprotection.

Human AGT repaired in a poor manner  $O^4$ -methyl-T, in order to studied the effect of the substitution of the 5-methyl group of thymine by H or F (Figure 2.3) oligonucleotides carrying  $O^4$ -alkyl-uracil derivatives has been prepared [45–47]. These studies revealed that the presence of the fluoro group enhanced the repair of these lesions [45, 46]

### 2.1.3 Synthesis of oligonucleotides carrying double crosslinks

Bifunctional alkylating agents induce the formation of interstrand (ICL) and intrastrand (IaCL) crosslinks. The synthesis of oligonucleotides carrying crosslinked nucleotides has the same difficulties as described by the monoalkylated nucleobases, such as lability to ammonia for the  $O^6$ -guanine and  $O^4$ -thymidine derivatives. In addition, the synthesis of the appropriate dimer units is more complex than the synthesis of monomeric units but follows similar protocols. Oligonucleotides carrying  $N^4$ dC-alkyl- $N^4$ dC [48, 49],  $N^3$ T-alkyl- $N^3$ T [50–52],  $N^2$ G-alkyl- $N^2$ G [53] and  $N^1$ dI- $N^3$ T [54] DNA crosslinks have been prepared as well as other artificial crosslinked mimics [55, 56]. The stability of the  $N$ -crosslinks to ammonia facilitates the final deprotection as no changes from standard conditions are needed. Figure 2.4 shows some examples of the phosphoramidites developed for the synthesis of these modified oligonucleotides. For the preparation of oligonucleotides carrying intrastrand crosslinks, the dimeric phosphoramidites contain a 5'-hydroxyl group protected with the DMT moiety and a 3'-phosphoramidite function in the second nucleoside position (Figure 2.4). The remaining 5' and 3'-hydroxyl functions are protected with base labile protecting groups that are deprotected with ammonia at the end of the synthesis generating a crosslinked position without a phosphate linkage [56]. In the case of the preparation of interstrand crosslink, a combination of orthogonal protecting groups is needed to make the second DNA strand. Usually, allyloxycarbonyl (Alloc) (C, Figure 2.4), levulinyl or a silyl group (A, C, Figure 2.4) is added in the dimer unit (Figure 2.4). In this way, with the DMT group it is possible to elongate one of the strands. When this strand is completed the chain is capped with acetyl group and the orthogonal group (Alloc or silyl o levulinyl) is deprotected allowing the assembly of the second strand [49].

Similar strategies were employed in the synthesis of oligonucleotides carrying  $O^4$ T-alkyl- $O^4$ T [57, 58],  $O^4$ U-alkyl- $O^4$ U [59],  $O^4$ T-alkyl- $O^6$ G [60–62] or  $O^6$ G-alkyl- $O^6$ G [63–66] crosslinks. However, in this case, due to the presence of labile  $O^4$ -alkyl-T and  $O^6$ -alkyl-G groups, extremely labile groups such as phenoxyacetyl and mild conditions (such as DBU/alcohol solutions) are needed to avoid ammonolysis of the alkylated bases.

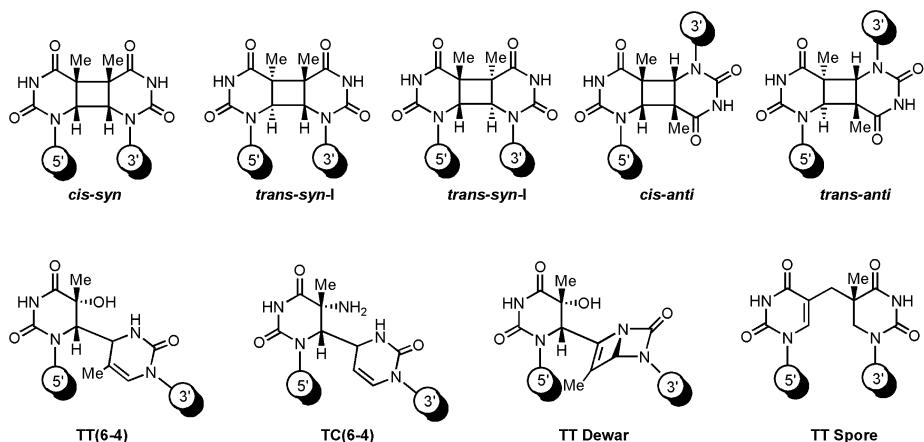


**Figure 2.4:** Chemical structures of several phosphoramidites developed for the synthesis of oligonucleotides carrying artificial crosslinked mimics. Alloc: allyloxycarbonyl; TBDMS: tert-butyldimethylsilyl; Pac: phenoxyacetyl; DMT: dimethoxytrityl.

## 2.2 Photodimers

Ultraviolet irradiation of DNA generates a series of damaged nucleobases that can lead to mutations and cancer if they are not repaired [67, 68]. The pyrimidines are considerably more sensitive to UV irradiation than purines. One of the most abundant compounds isolated from UV-irradiated DNA is the *cis-syn* T-T photodimer (Figure 2.5) [69]. Other cyclobutane pyrimidine dimers (CPD) arising from a [2 + 2] cycloaddition reaction between the C5–C6 double bonds of two pyrimidine nucleobases, which are the *trans-syn*, the *cis-anti* and the *trans-anti* isomers (Figure 2.5) [70, 71]. The formation of cyclobutane pyrimidine dimers is also possible for the all possible doublets of pyrimidines: TT, CT, TC and CC as well as for 5-methylcytosine [70]

A second group of photodimers are the pyrimidine (6-4) pyrimidone and the Dewar valence isomers (Figure 2.5) [72–74]. These are produced by [2+2] cycloaddition involving the C5–C6 double bond of a thymine in the 5'-position and the C4 carbonyl group of a thymine in position 3'. The cyclic intermediate rearranged giving a compound with an OH at position 5 of thymine in the 5'-position of the dimer and a covalent bond between C6 and C4 (6-4) photoproduct, (Figure 2.5). Cytosine can also produce the 6-4 photoproducts but an amino is located at position 5 of thymine in the 5'-position of the dimer (Figure 2.5). The (6-4) photoproducts can rearrange yielding the Dewar valence photoproduct [75].



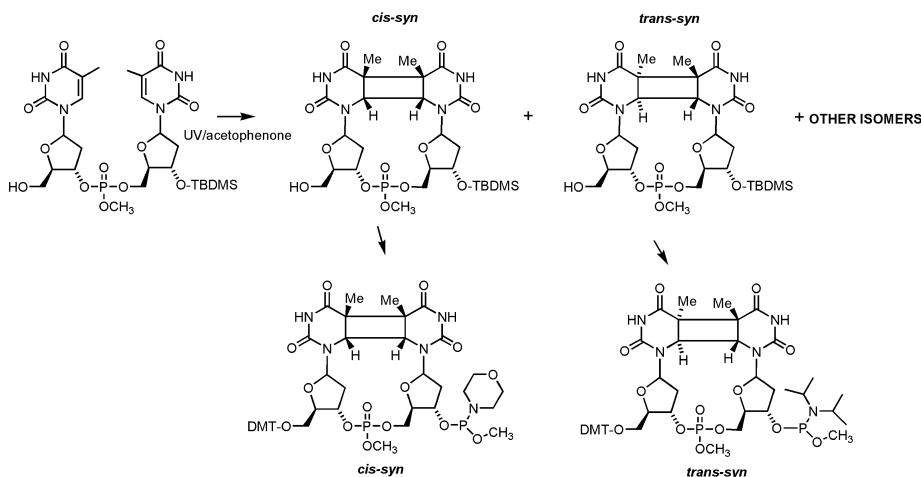
**Figure 2.5:** Structure of the pyrimidine photodimers isolated from UV irradiation of DNA.

Another photoproduct is 5-( $\alpha$ -thyminyl)-5,6-dihydrothymine (Figure 2.5), known as the spore photoproduct as it was found in bacterial spores. Dehydration seems to be the major cause of the formation of the spore photoproduct since irradiation of dry films of isolated DNA leads to the formation of large amounts of this photoproduct [70].

In addition to the pyrimidine photoproducts dimeric photoproducts involving adenine and thymine or two adenine moieties have been characterized [70, 76]. These lesions arise from [2 + 2] cycloadditions; however, they are about two orders of magnitude lower than that of the pyrimidine dimers.

Chemical synthesis of oligonucleotides carrying specific photodimers has been reported. In a simple approach, short oligonucleotides carrying a single TT position have been prepared and irradiated with an UV lamp for a few hours. Preparative HPLC purification was used to isolate the oligonucleotide carrying the *cis*-syn thymine dimer for NMR studies [77]. In another study, the (6-4) and the Dewar photoproducts have also been isolated in low yields by photolysis of a short oligonucleotide carrying a single TT position [78]. The short modified oligonucleotides were ligated to other oligonucleotides to obtain 49-mers required for a comparative characterization of the chemical and enzymatic properties of these lesions. A similar approach has been used recently for the isolation of the TT dimeric pyrimidine (6-4) pyrimidine lesion [79].

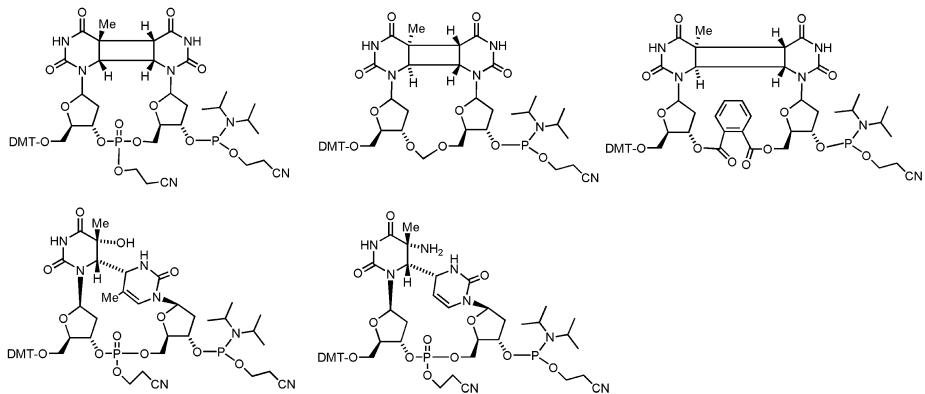
Oligonucleotides carrying photodimers have also been prepared using specific incorporation of dimeric phosphoramidite derivatives. The first synthetic protocols for the preparation of the cyclobutane pyrimidine dimers (CPD) are shown in Figure 2.6. Irradiation of the partially protected TpT dimer gave the *cis*-syn CPD as the major component together with other isomers [80, 81]. Purification of the *cis*-syn CPD [80] and *trans*-syn CPD [81] by preparative HPLC yielded pure CPD that were conveniently functionalized to obtain the DMT-protected photodimer phosphoramidites.



**Figure 2.6:** Synthesis of dimeric phosphoramidites required for the preparation of oligonucleotides carrying photodimers at specific positions.

These compounds were used for the assembly of longer oligonucleotides carrying CPD lesions [82, 83]. Furthermore, an optimized protocol for the synthesis of the phosphorodithioate derivative of the *cis*-syn CPD was described [84, 85]. In this protocol, the levulinyl group was used for the protection of the 3'-position instead of the *t*-butyldimethylsilyl group. A building block for the *cis*-syn cyclobutane dimer dTpdu (Figure 2.7) has also been described [86, 87]. This dimer may result from the deamination of the *cis*-syn cyclobutane dimer dTpdc. Interestingly, crystal structural data from the complex between the dimer and the specific DNA photolyase repair enzymes suggest a base flipping mechanism in where the photodimer is extrahelical [88]. In order to gain more experimental evidence, the negatively charged phosphodiester bond at the cyclobutane dimer was replaced by a neutral formacetal group (Figure 2.7) [89, 90]. Moreover, the phosphodiester bond at the cyclobutane dimer was replaced by an ammonia-cleavable bond such as the phthaloyl group [91]. The resulting oligonucleotide can be enzymatically cleaved by photolyases.

While the cyclobutane photodimers are obtained in good yields by UV irradiation in the presence of acetophenone as sensitizer, the (6-4) photodimer is only obtained in low yields and this lesion is labile to the ammonia treatment conditions. In spite of these difficulties, the TT (6-4) photoproduct was obtained in good yields by irradiation of a partially protected TpT dimer at 254 nm [92]. The (6-4) photodimer was conveniently protected to obtain a phosphoramidite (Figure 2.7) that was used for the successful assembly of oligonucleotides carrying the (6-4) photodimer using the phosphoramidites of the natural bases protected with the *t*-butyl(phenoxy)acetyl groups that are removed in very mild conditions [92]. A similar approach was used for the synthesis of the TC (6-4) photoproduct [93] although in this case some side reac-



**Figure 2.7:** Structures of special phosphoramidites used in the preparation of oligonucleotides carrying complex or artificial photodimers.

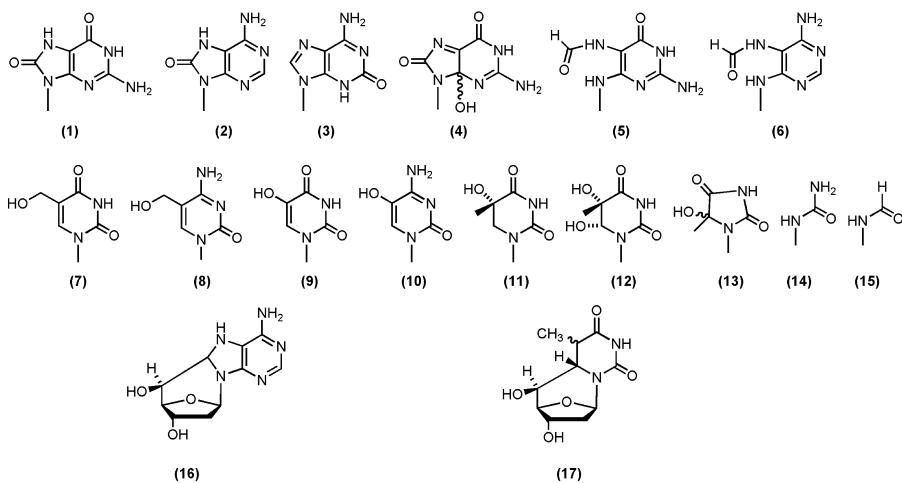
tions were observed during the coupling of the appropriate dimer phosphoramidite (Figure 2.7). These side reactions were prevented using benzimidazolium triflate as coupling reagent [94]. Oligonucleotides carrying the Dewar TT lesion can be obtained by irradiation of the TT (6-4) photoproduct [95]. The synthesis of a TT dinucleotide corresponding to the spore photoproduct has been described [96]. This product has not been still conveniently protected for the assembly of oligonucleotides. Dinucleoside derivatives without the phosphate bond have been prepared demonstrating that the 5S-configured spore lesion is recognized and repaired by the spore photoproduct lyase [97].

## 2.3 Oxidation and radiation products

Reactive oxygen species such as hydroxyl radical, generated endogenously or by ionizing radiation produces a large number of modified bases that provoke DNA degradation [98]. An overview of the main nucleobase lesions is shown in Figure 2.8 including 8-oxopurines, formamidopyrimidine (Fapy), cyclonucleosides as well as pyrimidine oxidation products. Most of these DNA lesions have been incorporated to synthetic oligonucleotides after the preparation of the suitable phosphoramidite derivatives [5, 85] and a careful use of the deprotection conditions to avoid degradation of these labile derivatives [99].

### 2.3.1 Oxidized purines and their degradation products

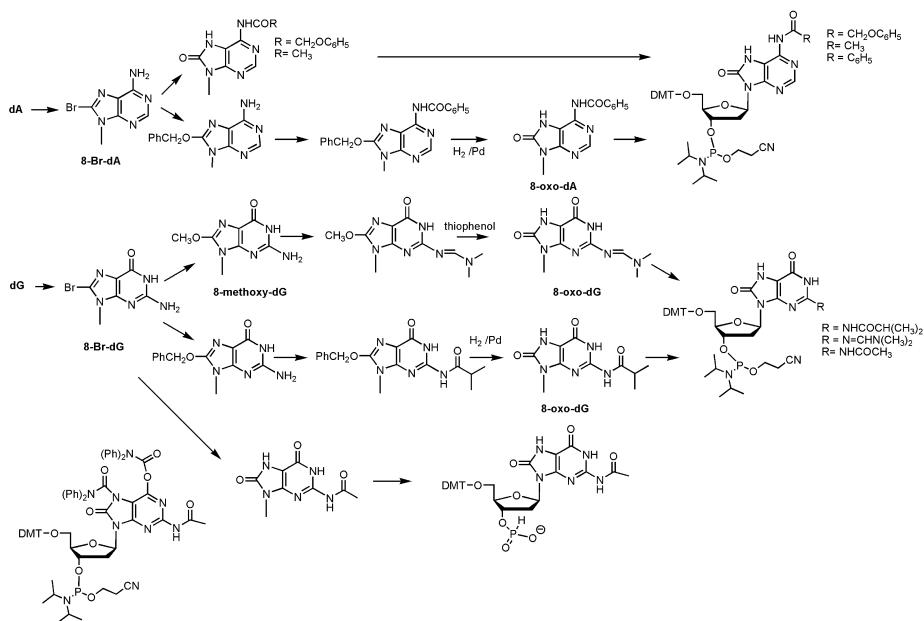
8-Oxopurines are the most abundant modified purines formed by the action of ionizing radiation and reactive oxygen species. Synthetic protocols have been developed for the preparation of the phosphoramidite of 7,8-dihydro-8-oxo-2'-deoxyadenosine



**Figure 2.8:** Oxidation and radiation products. (1) 8-oxo-G; (2) 8-oxo-A; (3) iso-G; (4) 4,8-dihydro-4-hydroxy-8-oxo-G; (5) Fapy-G; (6) Fapy-A; (7) 5-hydroxymethyl-U; (8) 5-hydroxymethyl-C; (9) 5-hydroxy-U; (10) 5-hydroxy-C; (11) 5-hydroxy-5,6-dihydro-T; (12) thymidineglycol; (13) hydantoin; (14) urea; (15) formyl; (16) cyclo-A; (17) cyclo-5,6-dihydro-T.

(8-oxodA) (Figure 2.9). All the protocols started with bromination of dA. Treatment of 8-Br-dA with sodium acetate and phenoxycetic acid anhydride gave the 3',5'-O,N<sup>6</sup>-triphenoxyacetyl 8-oxo-dA derivative [100]. This key compound was further functionalized to obtain the desired 8-oxodA phosphoramidite that was incorporated into oligonucleotides for structural studies [101, 102]. An optimization of this protocol used acetic acid anhydride instead of phenoxycetic acid anhydride with good yields [103]. Another protocol displaces the bromine with sodium benzyloxide. The resulting O<sup>8</sup>-benzyloxy-dA nucleoside was protected with the benzyl group and transformed to the 8-oxo-dA derivative by catalytic hydrogenation [104].

A similar protocol was developed for the synthesis of the 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) phosphoramidite (Figure 2.9). First, bromination of dG gave the 8-bromo-2'-deoxyguanosine (8-Br-dG). The treatment of 8-Br-dG with sodium benzyloxide produced the corresponding O<sup>8</sup>-benzyloxy-dG nucleoside that was protected with the isobutyryl group and transformed to the 8-oxo-dG derivative by catalytic hydrogenation [104, 105]. Later, a simpler protocol was described [106]. In this method 8-Br-dG was treated with sodium methoxide and the 8-methoxy-dG derivative was protected with the dimethylformamidino group (Figure 2.9). The 8-methoxy-dG derivative was transformed to 8-oxo-dG derivative by treatment with thiophenol [106]. In addition, the H-phosphonate derivative of 8-oxo-dG protected with the acetyl group was described [107]. In this protocol, 8-Br-dG was treated with sodium acetate and acetic anhydride to generate the acetyl protected derivative of 8-oxo-dG [107]. A variation of this method was also described for the synthesis of the phosphoramidite of 8-oxo-dG that produced oligonucleotides for the determination of the crystal structure

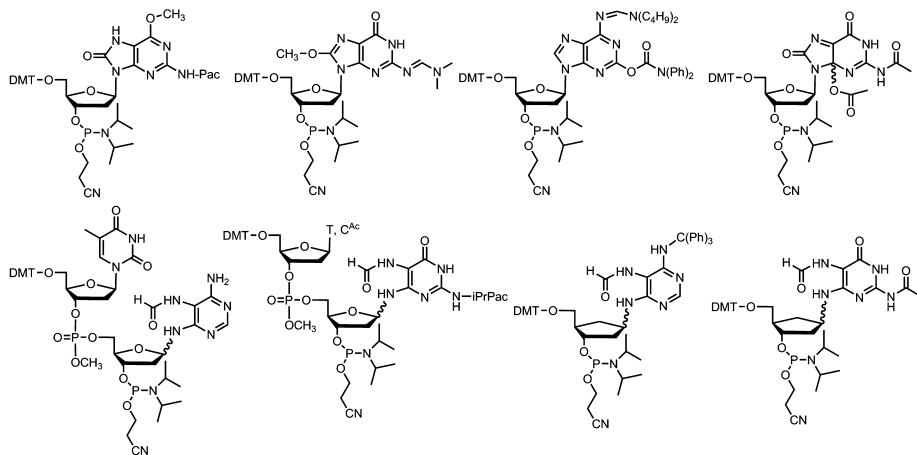


**Figure 2.9:** Synthesis of the phosphoramidite and *H*-phosphonate derivatives of 8-oxo-dA and 8-oxo-dG reported for the preparation of oligonucleotides carrying these DNA oxidative *damage lesions*.

of 8-oxo-dG base pairs [108]. A more complex synthetic scheme was also described [109]. The authors recommended protecting the  $N^7$ - and  $O^6$ -positions of guanosine with the diphenylcarbamoyl group and start with guanosine as bromination followed by bromine displacement was more efficient in the ribo-derivative than with the 2'-deoxyribo derivative. However, later on, the 2'-OH has to be reduced by the Barton procedure in order to produce the desired 8-oxo-dG derivative [109]. In another protocol, the phosphoramidite of  $N^2$ -acetyl-8-methoxy-dG was used for the preparation of oligonucleotides carrying 8-methoxy-dG that were transformed to oligonucleotides carrying 8-oxo-dG by a post-synthetic treatment with thiophenol [110, 111]. Enzymatic methods have also been used for the incorporation of 8-oxo-dG in DNA. Specifically, 8-oxo-dG 3',5'-biphosphate was ligated to synthetic oligonucleotides by utilizing T4 RNA ligase [112].

The phosphoramidites of 8-methoxy-2'-deoxyguanosine and 8-oxo-7,8-dihydro-6-*O*-methyl-2'-deoxyguanosine (Figure 2.10) have been synthesized [113, 114] and used for the preparation of modified DNA to study the substrate specificity of MutY protein, which is a *E. coli* repair enzyme that removes A in front of G. 8-Oxo-dG increased the rate of removal of A but the presence of the methyl group at the 6 position of oxo-G produced a noncleavable substrate [114].

Oxidation of the position 2 of adenine results in the formation of 2-hydroxyadenine residues. This oxidized nucleobase, named as *isoguanine*, has been introduced into



**Figure 2.10:** Special phosphoramidites developed for the preparation of oligonucleotides carrying 8-oxo-6-methyl-G, 8-methoxy-G, *iso*-G, 4,8-dihydro-4-hydroxy-8-oxo-G, Fapy-A, Fapy-G, and the carbocyclic derivatives of Fapy-A and Fapy-G. Pac: phenoxyacetyl, DMT: dimethoxytrityl, iPrPac: iso-propylphenoxyacetyl, Ac: acetyl.

synthetic oligonucleotides (Figure 2.10) [115] showing that it base pairs with *iso*-C with similar stability than G.C [116]. In addition, *iso*-G directs the incorporation of *iso*-C in front of *isoguanine* [117, 118].

4,8-Dihydro-4-hydroxy-8-oxo-2'-deoxyguanine (Figure 2.8) is one of the oxidation products of dG. The introduction of the 4*R* and 5*S* diastereoisomers of this modified bases has been successfully achieved using the corresponding phosphoramidites (Figure 2.10) [119]. Interestingly, oligonucleotides carrying these compounds show an abnormal resistance to degradation to endo and exonucleases [119].

The formamidinopyrimidine (Fapy) derivatives are produced by the imidazole ring opening of the 8-oxopurine derivatives. These are important DNA lesions that are recognized and repaired by the base excision repair enzyme named formamidinopyrimidine glycosylase. The lability and the facile epimerization of these nucleosides difficult the preparation of the corresponding modified oligonucleotides. In spite of these difficulties, oligonucleotides syntheses were described by the Greenberg's group using reversed dinucleotide phosphoramidites [120, 121]. Formamidinopurimidine lesions were introduced as dinucleotides to prevent rearrangement of the furanose to pyranose isomers. An improved method which not requires the utilization of reversed phosphoramidites was also described (Figure 2.10) [121, 122]. In a recent study, oligonucleotides carrying the carbocyclic derivatives of Fapy lesions (Figure 2.10) were prepared [123]. The presence of the carbocyclic moiety avoids the epimerization problem providing stable biomimetic derivatives for these lesions. These oligonucleotides allowed the analysis of the biochemical and structural properties of these lesions bound to a high-fidelity DNA polymerase [123].

### 2.3.2 Cyclonucleosides

Cyclonucleosides and specially purine 5',8-cyclonucleosides are important DNA lesions produced by oxidative radicals and radiation where the 5' carbon makes a C-C bond to the nucleobase [124, 125]. The synthesis of the phosphoramidite of (5'S)-5',8-cyclo-2'-deoxyadenosine (Figure 2.11) was performed by converting *N*<sup>6</sup>-benzoyl-dA to a 5'-thiophenyl derivative, followed by far-UV irradiation to obtain the *N*<sup>6</sup>-benzoyl derivative of 5',8-cyclo-2',5'-dideoxyadenosine. Subsequently, the resulting compound was oxidized with SeO<sub>2</sub>, stereo-selectively reduced with NaBH<sub>4</sub>, protected and phosphorylated [126]. Oligonucleotides carrying this lesion were produced in good yields with some extended coupling times [126]. The conformational changes induced by tandem repetitions of this DNA lesion were recently studied [127]. The phosphoramidite derivatives of the *R* isomer of 5',8-cyclo-2'-deoxyadenosine and the *R* and *S* isomers of 5',8-cyclo-2'-deoxyguanosine (Figure 2.11) were also described by the same group [128], together with the phosphoramidite derivatives of the 5*R* and 5*S* diastereomers of (5'S,6*S*)-5',6-cyclo-5,6-dihydrothymidine [129], the (5'*S*,6*S*)-cyclo-5,6-dihydro-2'-deoxyuridine, the (5'*S*,6*S*)-cyclo-5,6-dihydrothymidine [130] and (5'*S*, 5*S*, 6*S*)-5',6-cyclo-5-hydroxy-5,6-dihydro-2'-deoxyuridine [131].

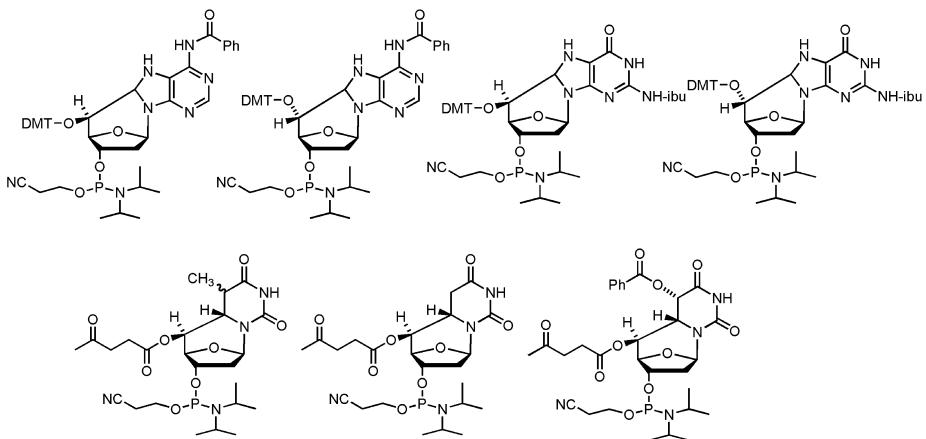


Figure 2.11: Structures of phosphoramidites developed for the preparation of oligonucleotides carrying cyclonucleotides. DMT: dimethoxytrityl, ibu: isobutyryl.

### 2.3.3 Oxidized pyrimidines, their degradation products and epigenetic nucleobases

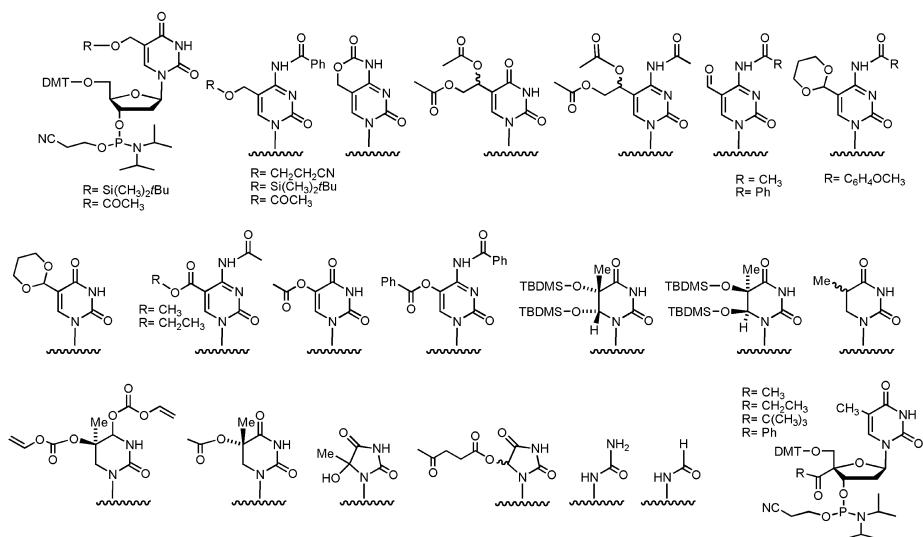
Several oxidized pyrimidines have been identified as damage products of the action of oxidizing chemicals and radiation (Figure 2.8). These products have been introduced

into synthetic oligonucleotides to determine their biological and structural properties [5, 85]. Figure 2.12 shows the phosphoramidite derivatives developed by several authors. 5-Hydroxymethyl-2'-deoxyuridine is the result of the oxidation of the methyl group of thymidine that may result from the action of hydroxyl radicals. There are two methods for the preparation of the 5-hydroxymethyl-dU phosphoramidite depending on the protecting group of the hydroxymethyl function. Conte et al. used the *tert*-butyldimethylsilyl group [132] and Sowers et al. used the acetyl group that was removed using the standard ammonia solution [133].

Endogenous enzymatic action directed to the removal of methyl group of the epigenetic base 5-methyl-dC produces 5-hydroxymethyl-2'-deoxycytidine. Furthermore, this 5-hydroxyl derivative may also be produced by the action of hydroxyl radical over 5-methyl-dC [134]. The detection of this base in the genome led to the discovery of two new cytosine derivatives, 5-formyl-2'-deoxycytidine and 5-carboxy-2'-deoxycytidine [135]. Extensive studies have been done to differentiate the potential oxidative damage from the enzyme-catalyzed formation of these bases, as well as their role in the genome [134, 135]. Several phosphoramidite derivatives have been reported for the synthesis of oligonucleotides carrying 5-hydroxymethyl-dC. First, the 2-cyanoethyl group was selected for the protection of the hydroxymethyl function [136–138]. Moreover, the acetyl [139] and the *tert*-butyldimethylsilyl (TBDMS) [140, 141] groups have also been described. In a different approach, a cyclic carbamate was developed [142]. This cyclic carbamate was removed by a treatment with NaOH solution instead of the standard ammonia, as this solution generates a few side compounds [143].

5-Formyl-2'-deoxyuridine oligonucleotides synthesis were reported by the introduction into oligonucleotides of a modified 5-(1,2-dihydroxyethyl)-2'-deoxyuridine residue (Figure 2.12) followed by postsynthetic oxidation with periodate. The *cis*-diol function is oxidized to the desired 5-formyl-dU derivative [143]. A similar method has been described for the synthesis of oligonucleotides carrying 5-formyl-2'-deoxycytidine [144]. A special phosphoramidite (Figure 2.12) was developed to introduce the 5-(1,2-dihydroxyethyl)-2'-deoxycytidine in oligonucleotides, which was oxidized with periodate to generate 5-formyl-dC [144]. Recently, direct methods were reported for the preparation of 5-formyl-dC oligonucleotides avoiding the use of cys-diol derivatives and the subsequent periodate oxidation [137]. These methods are based in the synthesis of 5-formyl-dC either by Palladium-catalyzed CO insertion on 5-iodo-dC [145, 146] or by oxidation of 5-MedC with  $\text{Na}_2\text{S}_2\text{O}_8\text{-Cu}^{2+}$  [137]. The 5-formyl phosphoramidite can be used for the assembly of oligonucleotides without protection of the formyl group, although acetal protection has been recently developed for 5-formyl-dC [147] and 5-formyl-dU [148]. Oligonucleotides carrying 5-carboxy-dC residues were prepared using a phosphoramidite with the 5-carboxyl group protected as methyl or ethyl ester. The 5-carboxy-dC derivative is prepared by Palladium-catalyzed CO insertion on 5-iodo-dC [141, 142].

Oxidation of cytosine residues generates 5-hydroxyuracil and 5-hydroxycytosine residues. In addition to enzymatic methods [149], the preparation of oligonucleotides



**Figure 2.12:** Structures of phosphoramidites developed for the preparation of oligonucleotides carrying oxidative and radiation DNA lesions. DMT: dimethoxytrityl, TBDMS: tert-butyldimethylsilyl.

containing 5-hydroxyuracil residues has been described [150]. In the preparation of the phosphoramidite derivative, the hydroxyl group in position 5 was protected with the acetyl group [150]. The acetyl was removed in mild conditions because 5-hydroxyuracil residues were found to decompose in hot ammonia solution. For this reason, phenoxyacetyl groups were used for the protection of the natural bases and the final deprotection was done by treatment with a methanolic solution of potassium carbonate at room temperature [150]. The preparation of oligonucleotides containing 5-hydroxycytosine residues has also been reported [151, 152]. In this case, the hydroxyl group in position 5 and the  $N^4$ -amino were protected with the benzoyl group, and the final deprotection was done using ammonia solution at room temperature without decomposition.

Thymine glycol is an important thymine product of the oxidative damage that is capable to block DNA replication [153, 154]. The oxidation of thymine residues can produce 4 different isomers. While oxidizing chemicals such as  $\text{OsO}_4$  and  $\text{KMnO}_4$  produce the *cis*-5*R*, 6*S* isomer,  $\gamma$ -irradiation gives a mixture of the two *cis* isomers, (5*R*, 6*S*) and (5*S*, 6*R*). The synthesis of oligonucleotides carrying the *cis* isomers of thymine glycol has been reported [155, 156]. Thymine glycol nucleosides are obtained by reaction of DMT-protected thymidine with  $\text{OsO}_4$ . For the protection of the *cis*-diol function, the *tert*-butyldimethylsilyl (TBDMS) was selected (Figure 2.12)[155]. In this way, the protected *cis*-diol nucleobase remain stable during the final deprotection treatment. The removal of the TBDMS groups is done after the deprotection with concentrated ammonia solution [155]. An improved method for the production of the *cis*-(5*R*, 6*S*) isomer was described [157]. The key step is the Sharpless asymmetric dihydroxylation that

proceed in good yields when the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate was as a cosolvent [157].

5,6-Dihydrothymine is obtained upon  $\gamma$ -irradiation of DNA in anoxic conditions. The phosphoramidite derivative of 5,6-dihydrothymine has been described [158] and it has been successfully incorporated to synthetic DNA. 5,6-Dihydrothymidine was obtained by catalytic Rhodium-catalyzed hydrogenation of thymidine [159], as 5,6-dihydrothymidine is labile to hot ammonia solutions. For these reasons, the final deprotection was done at room temperature using phenoxyacetyl group for the protection of the natural bases [158]. Hydroxylated-5,6-dihydrothymidines [160, 161] have been also incorporated to synthetic oligonucleotides [162, 163]. As hydroxylated-5,6-dihydrothymidines are alkali labile, in a first approach the nucleobase was protected with allyloxy groups that can be removed with Pd in neutral conditions. The first nucleoside was attached to the solid support by a photolabile 2-nitrobenzyl linker [162]. In this way, short oligonucleotides containing (5*R*)-5,6-dihydro-5-hydroxythymidine were obtained without using bases in the final deprotection [162]. An improved synthesis was described using potassium carbonate in methanol for the final deprotection. As 5-hydroxylated-dihydrothymidine was found to be stable to these conditions, a new phosphoramidite carrying the acetyl group for the protection of the hydroxyl group was developed [163].

Another type of products identified as oxidative damage DNA lesions are the 4'-*C*-modified deoxyribose residues [164, 165]. These lesions have been incorporated in synthetic oligonucleotides by employing special phosphoramidites (Figure 2.12). Several 4'-*C*-acylthymidines were reported as 4'-*C* radical precursors [164, 166]. Other C4'-modified phosphoramidite building blocks such as selenophenyl-dA were also developed [165, 167].

Oxidation caused by ionizing irradiation of DNA produce degradation of pyrimidine nucleobases. When thymidine is degraded 5-hydroxy-5-methylhydantoin is obtained and 5-hydroxy-5-methylhydantoin is produced from the degradation of 2'-deoxycytidine (Figure 2.8). The phosphoramidite derivative of 5-hydroxy-5-methylhydantoin has been prepared [168, 169]. No protection of the tertiary alcohol of the hydantoin is needed due to its lower reactivity (Figure 2.12). The site specific incorporation of 5-hydroxy-5-methylhydantoin in DNA has been also reported [170]. In this case, the hydroxyl group was protected with the levulinyl moiety, as this hydroxyl group is secondary and reactive to oligonucleotide synthesis conditions. The protocol allowed the incorporation of the 5*R* and 5*S* diastereoisomers although ammonia deprotection was performed in mild conditions to avoid epimerization [170].

A more severe degradation of pyrimidines by radiolysis of DNA produces 2'-deoxyribosyl urea and 2'-deoxyribosylformamide (Figure 2.8). The synthesis of oligonucleotides carrying 2'-deoxyribosyl urea [171, 172] and 2'-deoxyribosyl formamidine [173–175] has been described. 2'-Deoxyribosylurea is obtained by oxidation of thymidine with KMnO<sub>4</sub> giving a mixture of isomers [176]. Oligonucleotides containing

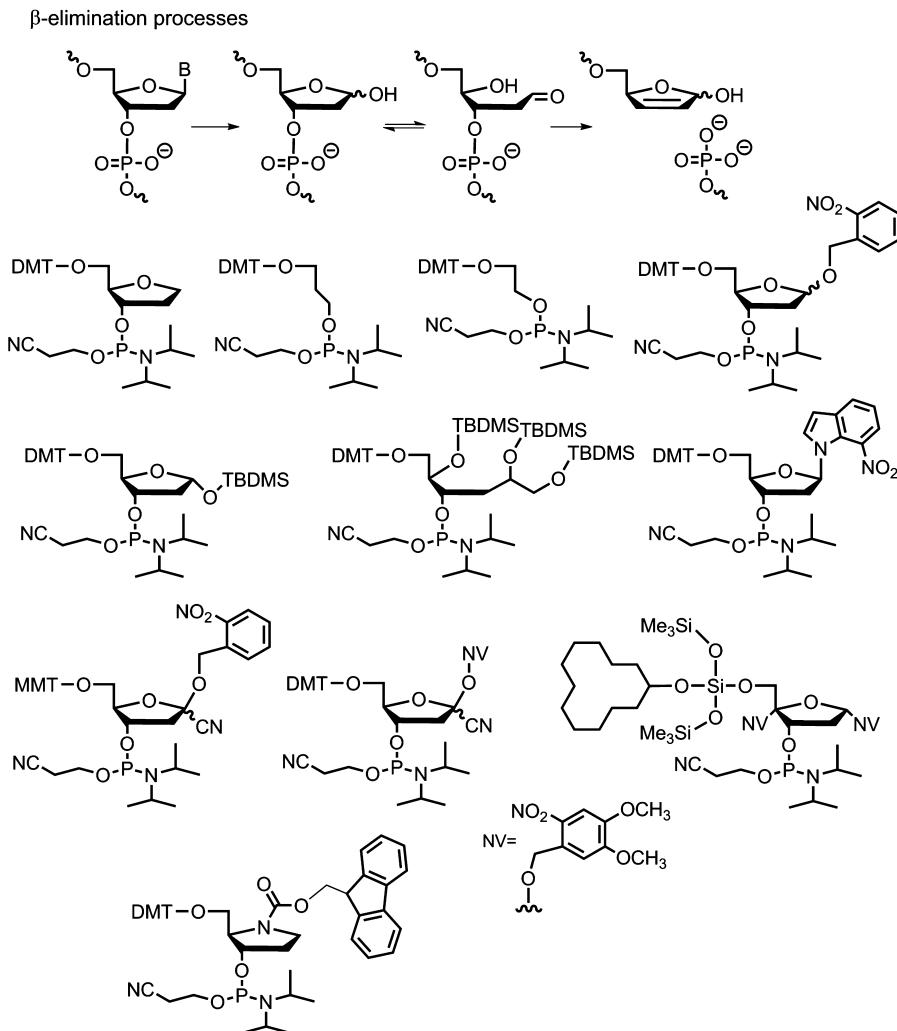
$2'$ -deoxyribosylformamide has also been obtained by oxidation of oligonucleotides carrying thymine glycol [177].

## 2.4 Oligonucleotides carrying abasic sites

The loss of the nucleobase is the most frequent DNA lesion *in vivo*. It is estimated that the production of abasic sites in living mammalian cells is around 2000–10000 per day [178]. These DNA lesions can be produced by spontaneous depurination or by the action of DNA glycosylases during base excision repair. The natural abasic site is in equilibrium between the closed and the open forms. The presence of the hemiacetal or aldehyde function induces instability of the neighboring phosphodiester bonds to bases by  $\beta$ -elimination processes (Figure 2.13). Instead, the reduced form of the 2-deoxyribose (1,4-*anhydro*-2-deoxyribitol) lacks the aldehyde function with the consequent stabilization of the neighboring phosphodiester bond to the basic conditions used in oligonucleotide synthesis. The synthesis of oligonucleotides containing 1,4-*anhydro*-2-deoxyribitol residues allow to have oligonucleotides carrying a mimic of the closed conformation of the natural abasic site for structural purposes [179]. In addition, this mimic had most of the biological properties of the natural abasic site [180]. The synthesis of the 1,4-*anhydro*-2-deoxyribitol building block has been reported by several groups [181–184]. Depurination of 2'-deoxyadenosine derivatives followed by the formation of a thioacetal and desulfurization gave the desired 1,4-*anhydro*-2-deoxyribitol derivative [181]. Reduction of 2-deoxyribose with borohydride followed by acid catalyzed cyclization provide a faster route to this compound [182]. Alternatively, synthesis of the chloro-sugar followed by reduction gave also the corresponding derivatives [183, 184]. Propanediol and ethyleneglycol phosphoramidites (Figure 2.13) were also prepared as potential stable mimics of the open form of abasic sites [183].

Several protocols have also been reported for the preparation of true natural abasic sites. This include mild hydrolysis of oligonucleotides carrying sensitive bases such as 2-pyrimidinone [185] or 8-substituted purines [186] or enzymatic removal of uracil residues by uracil glycosylase [187]. In addition, the phosphoramidite derivatives (Figure 2.13) of 1-*O*-(*tert*-butyldimethylsilyl)-2-deoxy-D-furasone [188], of 1-*O*-(2-nitrobenzyl)-2-deoxy-D-furasone [189] and *tert*-butyldimethylsilyl protected 3-deoxyhexitol [190] have been reported. In this last case, oligonucleotides carrying stable 3-deoxyhexitol are produced and converted in excellent yields to the desired abasic oligonucleotides by mild periodate oxidation of the diol group [190].

2-Deoxyribonolactone is formed by UV irradiation of DNA containing nonadjacent pyrimidines [191]. This oxidative abasic lesion is also produced by the action of drugs such as enediyne antibiotics, and porphyrins, as well as  $\gamma$ -irradiation. The 7-nitroindole nucleoside (Figure 2.13) was found to generate by UV-irradiation



**Figure 2.13:** First line: the abasic sites in oligonucleotides undergo to spontaneous  $\beta$ -elimination reactions resulting in strand breaks. The rest of the figure shows several phosphoramidites which have been reported to generate abasic sites or abasic site mimics. DMT: dimethoxytrityl, MMT: monomethoxytrityl, TBDMS: tert-butyldimethylsilyl.

2-deoxyribonolactone. The incorporation in synthetic oligonucleotides of 7-nitroindole nucleoside allows generating oligonucleotides with 2-deoxyribonolactone by photolysis [192]. Alternatively, a C-1 cyanohydrin 2-nitrobenzyl ether (Figure 2.13) was designed as photolabile precursor of 2-deoxyribonolactone [193]. An optimized C-1 cyanohydrin o-nitroveratryl (NV) phosphoramidite (Figure 2.13) was reported to produce the 2-deoxyribonolactone oligonucleotides in shorter UV-light irradiation times and shorter wavelength (350 nm) [194].

A fragile double 1',4'-oxidized abasic site has also been specifically introduced in synthetic DNA. A complex phosphoramidite carrying two *o*-nitroveratryl (NV)photolabile groups as well as the bis(trimethylsiloxy)-cyclododecyloxysilyl (DOD) group for the protection of the 5'-OH was described [191]. The DOD group was removed with fluoride solution. During the synthesis of the phosphoramidite, two isomers (1*R*,4*S* and 1*S*,4*R*) were isolated generating two different phosphoramidites [195]. In the synthesis, “fast deprotecting” exocyclic amine protecting groups of DMT-phosphoramidites were used for the natural bases before the lesion. After the addition of the lesion, phosphoramidites of the natural nucleotides are 5'-silyl protected to avoid the use of dichloroacetic acid that decomposes the fragile DNA lesion. After the assembly of the sequences and mild final deprotection, photolysis of the NV-protected oligonucleotides was found to be efficient at 350 nm [195].

A pyrrolidine analog of the abasic site has also been reported [196]. The phosphoramidite derivative of (2*R*,3*S*)-2-hydroxymethyl-3-hydroxypyrroline was developed for its incorporation in synthetic DNA (Figure 2.13). The resulting modified oligonucleotides are inhibitors of *E. coli* 3-methyladenine DNA glycosylase II activity, as this compound mimics the transition state of the glycosylase reaction [196].

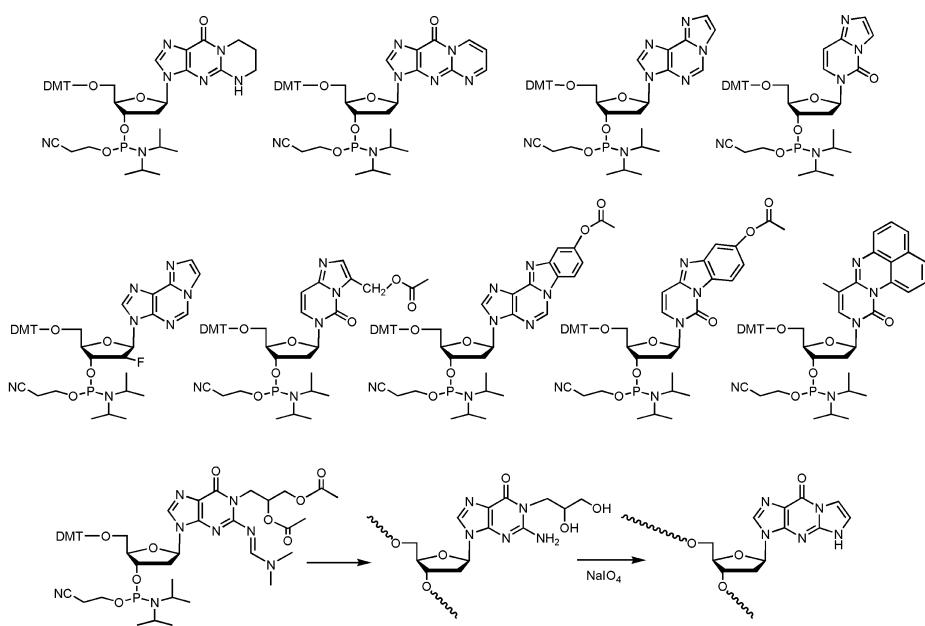
## 2.5 DNA adducts with bifunctional reagents and bulky substituents

The exposition to certain chemicals produces the generation of a large variety of DNA lesions [1–3]. In the previous sections, the effect of the alkylating agents and oxidative damage generated by endogenous free radicals or external radiation was summarized. Environmental exposure to certain chemicals such as malondialdehyde, acrolein, glycidaldehyde, psolarenes, *p*-benzoquinone, 2-acetylaminofluorene and polycyclic aromatic hydrocarbons (PAHs) produce large DNA adducts that are difficult to repair and may initiate cancer processes [197]. Some of the most active chemical tumorigenic compounds have been introduced at predefined positions of oligonucleotides to study the structural and biological properties of these compounds, in order to understand the steps that lead to chemical carcinogenesis. The large variety of potential DNA adducts will be classified in two groups: DNA adducts resulting from bifunctional carcinogens and DNA adducts produced by reaction with bulky aromatic compounds.

### 2.5.1 DNA adducts with bifunctional reagents

Malondialdehyde and acrolein derivatives react with DNA producing a series of cyclic nucleobase derivatives, especially in guanine nucleosides [198]. The synthe-

sis of the phosphoramidite of  $1,N^2$ -(1,3-propano)-2'-deoxyguanosine (Figure 2.14) was reported in order to generate oligonucleotides carrying a stable mimic of the acrolein-guanine adducts [199, 200]. The resulting oligonucleotides were utilized to determine the base pair geometries of  $1,N^2$ -(1,3-propano)guanine with G and A [199]. The unsaturated version of this last compound, (pyrimido[1,2-a]purin-10(3H)-one, Figure 2.14) was also described [201, 202]. This guanine adduct is labile to bases, for this reason the phosphoramidites of the natural bases were protected with the 2-(acetoxymethyl)benzoyl (AMB) group [201] or the *tert*-butylphenoxyacetyl (*t*BuPac) [202] that are removed in mild condition using a potassium carbonate solution in methanol [201, 202].



**Figure 2.14:** Structures of phosphoramidites developed for the preparation of oligonucleotides carrying DNA adducts with bifunctional reagents.

The synthesis of  $1,N^6$ -etheno-2'-deoxyadenosine and  $3,N^4$ -etheno-2'-deoxycytidine phosphoramidites was described [203, 204]. These compounds are formed by reaction of the nucleosides with chloroacetaldehyde. The etheno derivatives are unstable to DNA synthesis conditions. Consequently, the fast deprotection groups are needed for the natural bases [203, 204]. The etheno derivatives (Figure 2.14) are highly fluorescent useful as fluorescent tags in DNA sequencing [203]. These etheno derivatives have also been introduced in DNA enzymatically [205]. The 2'-fluoro derivative of  $1,N^6$ -ethenoadenine (Figure 2.14) was reported as inhibitor of base-

excision DNA repair enzymes [206]. The synthesis of the hydroxymethyl derivative of 3,N<sup>4</sup>-ethenocytosine has been described by reaction of dC with bromoacetaldehyde followed by reduction with sodium borohydride [207]. The hydroxyl extra moiety was protected with the acetyl group (Figure 2.14).

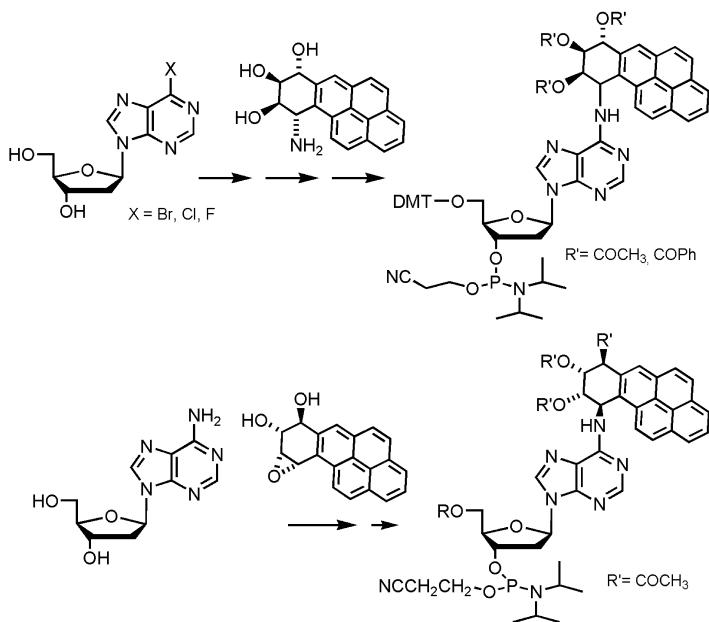
The phosphoramidite derivatives of the *p*-benzoquinone adducts of A and C (Figure 2.14) have also been reported [208]. *p*-Benzoquinone is one of the metabolites of benzene. The potential carcinogenic effects of benzene are known for several years; however, the mechanisms of its toxic effects are unknown. Benzene is metabolized by the liver to phenol and *p*-benzoquinone. Several DNA adducts were isolated by the treatment of DNA with *p*-benzoquinone [3]. In order to determine the structural and biological properties of these adducts, oligonucleotides carrying this lesion were synthesized [208, 209]. The *p*-benzoquinone adducts have a phenol group that was protected with the acetyl group. Special deprotection conditions using a DBU/methanol solution was needed to avoid degradation of the *p*-benzoquinone adducts, which was observed during the ammonia treatment. Other similar polycyclic nucleoside adducts (Figure 2.14) have been described [210]. These adducts are obtained by an unexpected reaction between aromatic diamines with 4-*O*-(triisopropylphenyl)sulfonyl pyrimidine nucleosides. Once incorporated to DNA, these extended nucleobases enhance the binding with the complementary strand, provide a fluorescent tag and may intercalate in DNA gap positions [210].

The synthesis of the phosphoramidite of 1-(3,4-diacetoxypropyl)-2'-deoxyguanosine (Figure 2.14) pursues the incorporation of 1-(3,4-dihydroxypropyl)-dG residues into DNA. Oxidation with sodium periodate generate the aldehyde function that undergoes to a ring closure to form 1,N<sup>2</sup>-etheno-2'-deoxyguanosine oligonucleotides (Figure 2.14) [211]. A similar protocol is described for the synthesis of the phosphoramidite of 1-(3,4-diacetoxybutyl)-2'-deoxyguanosine in order to generate oligonucleotides carrying 1,N<sup>2</sup>-propano-2'-deoxyguanosine [212].

### 2.5.2 DNA adducts with bulky aromatic compounds

Bay-region diol epoxides are oxidative metabolites of polycyclic aromatic hydrocarbons (PAH). Many of these compounds are carcinogenic because they react with the nucleobases giving DNA adducts with bulky aromatic compounds. The most abundant DNA adducts involved the reaction of the epoxide derivatives of PAH with the exocyclic amino groups of dA and dG. The phosphoramidites of some of the most potent PAH carcinogen derivatives have been reported and introduced to model DNA sequences (Figure 2.15).

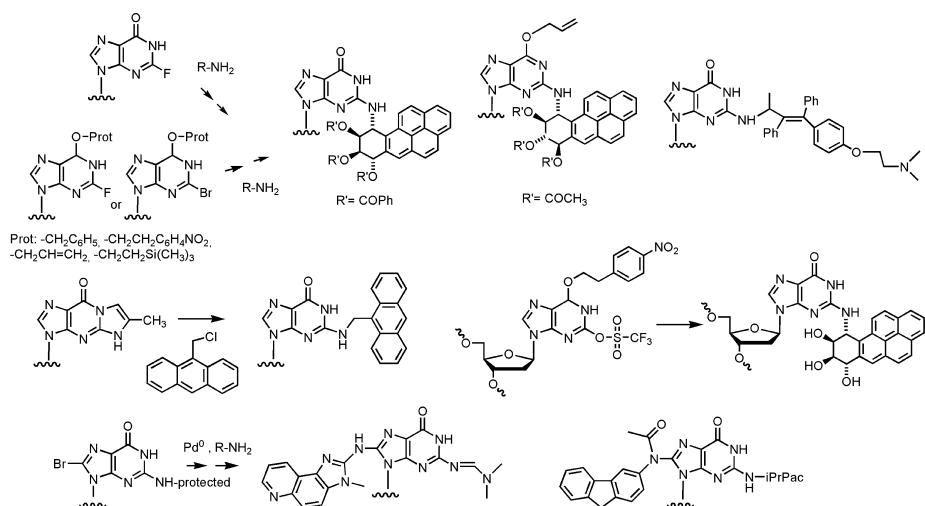
The starting products for the preparation of the N<sup>6</sup> derivatives of 2'-deoxyadenosine are 6-chloro- [213, 214] or 6-fluoropurine 2'-deoxyriboside [215–220] that can be used unprotected or protected with the TBDMS group. In this approach, epox-



**Figure 2.15:** Structures of phosphoramidites developed for the preparation of oligonucleotides carrying DNA adducts with bulky aromatic compounds.

ides are converted to corresponding aminoalcohol derivatives by aminolysis and the amino-PAH derivatives are reacted with the 6-halopurine derivative. If the amino-PAH derivative carries some extra hydroxyl groups, these are left unprotected during the halogen displacement and are protected later on with the acetyl or the benzoyl groups. Postsynthetic protocols for the synthesis of oligonucleotides carrying PAH- $N^6$ -adenine adducts were described [220, 221]. In these methods, the phosphoramidite of 6-fluoropurine [220] or 6-chloropurine 2'-deoxyriboside [221] was incorporated in a specific site of a DNA sequence. The oligonucleotide support was treated with phenylglycinol or amino triol derivatives of tetrahydrobenzopyrene, or tetrahydrobenzoanthracene and then the support was treated with ammonia for the final deprotection step. The desired oligonucleotides were obtained with a moderate yield [220]. Alternatively, 2'-deoxyadenosine can be reacted directly with the epoxide to generate the  $N^6$ -2'-deoxyadenosine adducts [222, 223] that are converted to the appropriate phosphoramidites (Figure 2.15) [224].

The intermediates used in the preparation of the  $N^2$  PAH-derivatives of 2'-deoxyguanosine are 2-fluoro-2'-deoxyinosine derivatives (Figure 2.16). 2-Fluoro-2'-deoxyinosine can be obtained from dG by diazotization and fluorination of  $O^6$ -protected dG. Some authors utilized the benzyl group for the protection of position 6 of dG that is removed by catalytic hydrogenation [225]. Alternatively, the Pd-labile allyl group [223], the base-labile *p*-nitrophenylethyl (NPE) [214, 215] and the fluoride-



**Figure 2.16:** Introduction of bulky aromatic compounds to oligonucleotides by postsynthetic protocols.

labile (trimethyl)silyl [226, 227] have been employed for the preparation of oligonucleotides carrying several  $N^2$ -G adducts (Figure 2.16) such as  $N^2$ -PAH [223, 225] and  $N^2$ -temoxifen (antiestrogen) adducts [226]. 2-Bromo- $O^6$ -benzyl-2'-deoxyinosine and the 6-Bromo-2'-deoxypyrimidine have also been described for the preparation of adducts of dG and dA [228, 229]. 2'-Deoxy-4-desmethylwyoysine (Figure 2.16) has been also used as intermediate for the preparation a 2'-deoxyguanosine phosphoramidite carrying anthracene at  $N^2$  position [230]. Direct alkylation of 2'-deoxy-4-desmethylwyoysine with 9-(chloromethyl)-anthracene gave the  $N^5$ -alkylated 2'-deoxy-4-desmethylwyoysine derivative needed for the regeneration of the desired guanine derivative by treatment with *N*-bromosuccinimide [230].

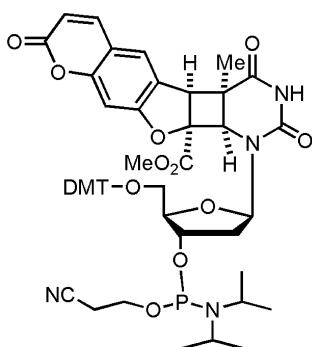
Similar to what has been described for dA, a postsynthetic protocol for the synthesis of oligonucleotides carrying  $N^2$ -guanine adducts was described [221]. In this method, the phosphoramidite of 2-fluoro-2'-deoxyinosine was incorporated in a DNA sequence and reacted with phenylglycinol while the oligonucleotide was still on the solid support. Then the support was treated with ammonia to yield the desired oligonucleotides with the phenylglycidol  $N^2$ -guanine adduct [221]. Moreover, the phosphoramidite of  $O^2$ -triflate- $O^6$ -NPE-2'-deoxyxanthosine (Figure 2.16) was also used for the preparation of  $N^2$ -PAH [231] using a postsynthetic modification strategy. A similar strategy was applied to generate interchain crosslinks mediated by bi-functional pyrroles [227]. In this method, 2-fluoro- $O^6$ -(trimethyl)silyl-2'-deoxyinosine was incorporated into a short DNA sequence and the rest of the natural bases were protected with the phenoxyacetyl group. Mild deprotection of the using 0.1M NaOH yielded the unprotected oligonucleotide carrying the 2-fluoro- $O^6$ -(trimethyl)silyl-2'-

deoxyinosine residue. Treatment of this oligonucleotide with bisamino pyrrol derivatives gave a mixture of monomeric and dimeric adducts that were separated by HPLC [227].

Some carcinogens such as heterocyclic amines (HCAs) and 2-acetylaminofluorene (AAF) form adducts with the position 8 of 2'-deoxyguanosine. The specific introduction of these lesions in oligonucleotides has been reported. The synthesis of the appropriate *C*<sup>8</sup>-dG adducts is usually performed by the Buchwald–Hartwig Palladium catalyzed *N*-arylation between 8-bromo-2'-deoxyguanosine *N*<sup>2</sup>-protected derivatives and the corresponding heterocyclic amine (Figure 2.16) [232–235]. Special care was made during the synthesis of the phosphoramidite of the AAF-dG adducts, as standard ammonia deprotection removed the acetyl group of AAF moiety. To avoid this side reaction, the labile isopropylphenoxyacetyl (iPrPac) group was selected for the protection of the *N*<sup>2</sup>-amino group of dG (Figure 2.16) [232]. The phosphoramidites of the natural bases were also protected with labile (iPrPac, phenoxyacetyl (Pac), acetyl) groups. The optimal final deprotection conditions were found to be an overnight treatment at 55 °C with a 10 % diisopropylamine solution containing 0.25 M of β-mercaptoethanol in MeOH [233]. The phosphoramidite derivative of the guanine *C*-8 adduct of 2-amino-3-methylimidazo[4,5-*f*]quinolone (Figure 2.16) was protected with the dimethylformamidino (dmf) moiety [234]. The phosphoramidites of the natural bases were also protected with labile (iPrPac, Pac, acetyl) groups. However, in this case, argon-degased ammonia solution was used for the final deprotection.

Oligonucleotides carrying pyrenyl [236, 237], anthracenyl [238] and anthraquinonyl [239] groups at the 2' and 5' ribose positions have been also reported. These oligonucleotides are not DNA lesions but oligonucleotides carrying fluorescent tags as well as intercalating agents with potential enhanced hybridization properties.

Psolarens are linear furocoumarins used as drugs for skin and lymphoma treatments. These compounds react with pyrimidines by irradiation via a [2 + 2] cycloaddition reaction yielding cyclobutane psolarens-pyrimidine adducts (Figure 2.17). These adducts can be formed by the furan or the pyrone side of psolarens and these can be monoadducts or cross-linked diadducts. The synthesis of the phosphoramidite of carbomethoxysoralen furan-side thymidine monoadduct has been reported [240–242]. As the psoralen adduct is susceptible to degradation and the carboxymethyl group is sensitive to ammonia, the synthesis of the psolarens-modified oligonucleotides needs especially mild conditions for deprotection. To this end, phosphoramidites of the natural bases were protected with Pac groups and final deprotection was done with an overnight treatment with DBU solution in methanol or ethanol. It was found that the methyl ester is hydrolyzed slowly in water solution so; finally, the ester function was hydrolyzed with sodium carbonate to facilitate the purification.



**Figure 2.17:** Structure of a phosphoramidite developed for the preparation of oligonucleotides carrying DNA-psolarene adducts.

## Bibliography

- [1] Setlow RB. Repair deficient human disorders and cancer. *Nature*. 1978;271:713–7.
- [2] Singer B, Kusmierenk JT. Chemical mutagenesis. *Annu Rev Biochem*. 1982;52:655–93.
- [3] Singer B. DNA damage; chemistry, repair and mutagenic potential. *Reg Toxicol Pharmacol*. 1996;23:2–13.
- [4] Basu AK, Essigmann JM. Site-specifically modified oligodeoxynucleotides as probes for the structural and biological effects of DNA-damaging agents. *Chem Res Toxicol*. 1988;1:1–18.
- [5] Butenandt J, Burgdorf LT, Carell T. Synthesis of DNA lesions and DNA-lesion containing oligonucleotides for DNA-repair studies. *Synthesis*. 1999;1085–105.
- [6] Beaucage SL, Iyer RP. The synthesis of modified oligonucleotides by the phosphoramidite approach and their applications. *Tetrahedron*. 1993;49:6123–94.
- [7] Lartia E. Chemical synthesis of lesion-containing oligonucleotides for DNA repair studies. In: Jurga R et al, editors. *Modified nucleic acids in biology and medicine. RNA technologies*. Springer; 1996. p. 371–402.
- [8] Swann PF. Why do O<sup>6</sup>-alkylguanine and O<sup>4</sup>-alkylthymine miscode? The relationship between the structure of DNA containing O<sup>6</sup>-alkylguanine and O<sup>4</sup>-thymine and the mutagenic properties of these bases. *Mutat Res*. 1990;233:81–94.
- [9] Fowler KW, Büchi G, Essigmann JM. Synthesis and characterization of an oligonucleotide containing a carcinogen-modified base: O-6-methylguanine. *J Am Chem Soc*. 1982;104:1050–4.
- [10] Gaffney BL, Jones RA. Synthesis of O-6-alkylated deoxyguanosine nucleosides. *Tetrahedron Lett*. 1982;23:2253–6.
- [11] Kuzmich S, Marky LA, Jones RA. Specifically alkylated DNA fragments. Synthesis and physical characterization of d[CGC(O<sup>6</sup>Me)GCG] and d[CGT(O<sup>6</sup>Me)GCG]. *Nucleic Acids Res*. 1983;11:3393–404.
- [12] Gaffney BL, Marky LA, Jones RA. Synthesis and characterization of a set of four dodecadeoxyribonucleotide undecaphosphates containing O<sup>6</sup>-methylguanine opposite adenine, cytosine, guanine and thymine. *Biochemistry*. 1984;23:5686–91.
- [13] Borowy-Borowski H, Chambers RW. A study of side reactions occurring during synthesis of oligodeoxynucleotides containing O<sup>6</sup>-alkyldeoxyguanosine residues at preselected sites. *Biochemistry*. 1987;26:2465–71.

- [14] Pauly GT, Powers M, Pei GK, Moschel RC. Synthesis and properties of *H-ras* DNA sequence containing *O*<sup>6</sup>-substituted 2'-deoxyguanosine residues at the first, second and both positions of codon 12. *Chem Res Toxicol.* 1988;1:391–7.
- [15] Li BFL, Swann PF. Synthesis and characterization of oligonucleotides containing *O*<sup>6</sup>-methyl-, *O*<sup>6</sup>-ethyl-, and *O*<sup>6</sup>-isopropylguanine. *Biochemistry.* 1989;28:5779–86.
- [16] Fathi R, Goswami B, Kung PP, Gaffney BL, Jones RA. Synthesis of 6-substituted 2'-deoxyguanosine derivatives using trifluorocetic anhydride in pyridine. *Tetrahedron Lett.* 1990;31:319–22.
- [17] Gaffney BL, Jones RA. Thermodynamic comparison of the base pairs formed by the carcinogenic lesion containing *O*<sup>6</sup>-methylguanine with reference both to Watson-Crick pairs and to mismatched pairs. *Biochemistry.* 1989;28:5881–9.
- [18] Smith CA, Xu YZ, Swann PF. Solid-phase synthesis of oligodeoxynucleotides containing *O*<sup>6</sup>-alkylguanine. *Carcinogenesis.* 1990;11:811–6.
- [19] Roelen HCPF, Brugghe HF, van der Elst H, Klein JC, van der Marel GA, van Boom JH. Solid-phase synthesis of oligodeoxynucleotides containing 6-*O*-alkylguaninoses. *Recl Trav Chim Pay-Bas.* 1992;111:227–34.
- [20] Xu YZ, Swann PF. Solid-phase synthesis of oligodeoxynucleotides containing *O*<sup>6</sup>-alkylguanine and *O*<sup>4</sup>-alkylthymine. *Nucleosides Nucleotides.* 1991;10:315–8.
- [21] Spratt TE, Campbell CR. Synthesis of oligodeoxynucleotides containing analogs of *O*<sup>6</sup>-methylguanine and reaction with *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase. *Biochemistry.* 1994;33:11364–71.
- [22] Mijal RS, Kanugula S, Vu CC, Fang Q, Pegg AE, Peterson LA. DNA sequence context affects repair of the tobacco-specific adduct *O*<sup>6</sup>-[4-oxo-4-(3-pyridyl)butyl]guanine by human *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase. *Cancer Res.* 2006;66:4968–74.
- [23] Wang L, Spratt TE, Liu XK, Hecht SS, Pegg AE, Peterson LA. Pyridyloxobutyl adduct *O*6-[4-oxo-4-(3-pyridyl)butyl]guanine is present in 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone-treated DNA and is a substrate for *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase. *Chem Res Toxicol.* 1997;1997(10):562–77.
- [24] Wang L, Spratt TE, Pegg AE, Peterson LA. Synthesis of DNA oligonucleotides containing site-specifically incorporated *O*<sup>6</sup>-[4-oxo-4-(3-pyridyl)butyl]guanine and their reaction with *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase. *Chem Res Toxicol.* 1999;12:127–31.
- [25] Shibata T, Glynn N, McMurry BH, McElhinney RS, Margison GP, Williams DM. Novel synthesis of *O*<sup>6</sup>-alkylguanine containing oligodeoxynucleotides as substrates for the human DNA repair, *O*<sup>6</sup>-methylguanine DNA methyltransferase (MGMT).
- [26] Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K. A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nat Biotechnol.* 2003;21:86–9.
- [27] Tintoré M, Grijalvo S, Eritja R, Fàbrega C. Synthesis of oligonucleotides carrying fluorescently labelled *O*<sup>6</sup>-alkylguanine for measuring hAGT activity. *Bioorg Med Chem Lett.* 2015;25:5208–11.
- [28] Beharry AA, Nagel ZD, Samson LD, Kool ET. Fluorogenic real-time reporters of DNA repair by MGMT, a clinical predictor of antitumor drug resistance. *PLoS ONE.* 2015;11:e0152684.
- [29] Wurdeman RL, Church KM, Gold B. DNA methylation by N-methyl-N-nitrosourea, N-methyl-*N'*-nitro-*N*-nitrosoguanidine, *N*-nitroso(1-acetoxyethyl)methylamine, and diazomethane: mechanism for the formation of *N*<sup>7</sup>-methylguanine in sequence-characterized 5'-<sup>32</sup>P-end-labeled DNA. *J Am Chem Soc.* 1989;111:6408–12.
- [30] Ezaz-Nikpay K, Verdine GL. Aberrantly methylated DNA: site-specific introduction of *N*<sup>7</sup>-methyl-2'-deoxyguanosine into the Dickerson/Drew dodecamer. *J Am Chem Soc.* 1992;114:6562–3.

- [31] Li BFL, Reese CB, Swann PF. Synthesis and characterization of oligodeoxynucleotides containing 4-*O*-methylthymine. *Biochemistry*. 1987;26:1086–93.
- [32] Kiriasis L, Farkas S, Pfleiderer W. Nucleosides, XLIII. Synthesis and properties of *O*<sup>4</sup>-alkylthymidines. *Nucleosides Nucleotides*. 1986;5:517–27.
- [33] Borowy-Borowsky H, Chambers RW. Solid-phase synthesis and side reactions of oligonucleotides containing *O*-alkylthymine residues. *Biochemistry*. 1989;28:1471–7.
- [34] Xu YZ, Swann PF. A simple method for the solid-phase synthesis of oligodeoxynucleotides containing *O*<sup>4</sup>-alkylthymine. *Nucleic Acids Res.* 1990;18:4061–5.
- [35] Fernandez-Forner D, Palom Y, Ikuta S, Pedroso E, Eritja R. Synthesis and characterization of oligodeoxynucleotides containing the mutagenic base analogue 4-*O*-ethylthymine. *Nucleic Acids Res.* 1990;18:5729–34.
- [36] Eritja R, Robles J, Aviñó A, Albericio F, Pedroso E. A synthetic procedure for the preparation of oligonucleotides without using ammonia and its application for the synthesis of oligonucleotides containing *O*-4-alkyl thymidines. *Tetrahedron*. 1992;48:4171–82.
- [37] Aviñó A, Gümil-Garcia R, Marquez VE, Eritja R. Preparation and properties of oligodeoxynucleotides containing 4-*O*-butylthymine, 2-fluorohypoxanthine and 5-azacytosine. *Bioorg Med Chem Lett*. 1995;5:2331–6.
- [38] Fàbrega C, Eritja R, Sinha ND, Dosanjh M, Singer B. Synthesis and properties of oligonucleotides containing the mutagenic base *O*<sup>4</sup>-benzylthymidine. *Bioorg Med Chem*. 1995;3:101–8.
- [39] Roelen HCPF, Brugghe HF, van der Elst H, van der Marel GA, van Boom JH. Solid-phase synthesis of oligodeoxynucleotides containing 4-alkoxythymidine residues. *Recl Trav Chim Pay-Bas*. 1992;111:99–104.
- [40] Xu YZ, Zheng Q, Swann PF. Synthesis of DNA containing modified bases by postsynthetic substitution. Synthesis of oligomers containing 4-substituted thymine: *O*<sup>4</sup>-alkylthymine, 5-methylcytosine, *N*<sup>4</sup>-(dimethylamino)-5-methylcytosine and 4-thiothymine. *J Org Chem*. 1992;57:3839–45.
- [41] Bhanot OS, Grevatt PC, Donahue JM, Gabrielides CN, Salomon JJ. In vitro DNA replication implicates *O*<sup>2</sup>-ethyldeoxythymidine in transversion mutagenesis by ethylating agents. *Nucleic Acids Res.* 1992;20:587–94.
- [42] Grevatt PC, Salomon JJ, Bhanot OS. In vitro mispairing specificity of *O*<sup>2</sup>-ethylthymidine. *Biochemistry*. 1992;31:4181–8.
- [43] Xu YZ, Swann PF. Oligodeoxynucleotides containing *O*<sup>2</sup>-alkylthymine: synthesis and characterization. *Tetrahedron Lett*. 1994;35:303–6.
- [44] Bhanot OS, Grevatt PC, Donahue JM, Gabrielides CN, Salomon JJ. Incorporation of dA opposite *N*<sup>3</sup>-ethylthymidine terminates in vitro DNA synthesis. *Biochemistry*. 1990;29:10357–64.
- [45] McManus FP, Wilds CJ. *O*(6)-alkylguanine-DNA alkyltransferase-mediated repair of *O*(4)-alkylated 2'-deoxyuridines. *ChemBioChem*. 2014;15:1966–77.
- [46] Sacre L, O'Flaherty DK, Archambault P, Copp W, Peslherbe GH, Muchall HM, Wilds CJ. *O*<sup>4</sup>-Alkylated-2-deoxyuridine repair by *O*<sup>6</sup>-alkylguanine DNA alkyltransferase is augmented by a C5-fluorine modification. *ChemBioChem*. 2018;19:575–82.
- [47] McManus FP, Wilds CJ. *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase-mediated repair of *O*<sup>4</sup>-alkylated 2'-deoxyuridines. *ChemBioChem*. 2014;15:1966–77.
- [48] Noll DM, Webba da Silva M, Noronha AM, Wilds CJ, Colvin M, Gamcsik MP, Miller PS. Structure, flexibility and repair of two different orientations of the same alkyl interstrand DNA cross-link. *Biochemistry*. 2005;44:6764–75.
- [49] Noronha AM, Noll DM, Wilds CJ, Miller PS. *N*<sup>4</sup>C-ethyl-*N*<sup>4</sup>C crosslinked DNA: synthesis and characterization of duplexes with interstrand crosslinks of different orientations. *Biochemistry*. 2002;41:760–71.

- [50] Smeaton MB, Hlavin EM, Noronha AM, Murphy SP, Wilds CJ, Miller PS. Effect of cross-link structure on DNA interstrand cross-link repair synthesis. *Chem Res Toxicol.* 2009;22:1285–97.
- [51] Wilds CJ, Noronha AM, Robidoux S, Miller PS. Synthesis and characterization of DNA duplexes containing an  $N^3$ T-ethyl- $N^3$ T interstrand crosslink in opposite orientations. *Nucleosides, Nucleotides, Nucleic Acids.* 2005;24:965–9.
- [52] Sun G, Noronha A, Wilds C. Preparation of  $N^3$ -thymidine-butylene- $N^3$ -thymidine interstrand cross-linked DNA via an orthogonal deprotection strategy. *Tetrahedron.* 2012;68:7787–93.
- [53] Kowalczyk A, Carmical JR, Zou Y, Van Houten B, Lloyd RS, Harris CM, Intrastrand HTM. DNA cross-links as tools for studying DNA replication and repair: two-, three-, and four-carbon tethers between the N2 positions of adjacent guanines. *Biochemistry.* 2002;41:3109–18.
- [54] Wilds CJ, Xu F, Noronha AM. Synthesis and characterization of DNA containing an  $N^1$ -2'-deoxyinosine-ethyl- $N^3$ -thymidine interstrand cross-link: a structural mimic of the cross-link formed by 1,3-bis-(2-chloroethyl)-1-nitrosourea. *Chem Res Toxicol.* 2008;21:686–95.
- [55] Li HY, Qiu YL, Moyroud E, Kishi Y. Synthesis of DNA oligomers possessing a covalently cross-linked Watson-Crick base pair model. *Angew Chem Int Ed.* 2001;40:1471–5.
- [56] Murata S, Mizumura Y, Hino K, Ueno Y, Ichikawa S, Matsuda A. Modular bent DNAs: a new class of artificial DNAs with a protein binding ability. *J Am Chem Soc.* 2007;129:10300–1.
- [57] McManus FP, O'Flaherty DK, Noronha AM, Wilds CJ.  $O^4$ -Alkyl-2'-de oxythymidine cross-linked DNA to probe recognition and repair by  $O^6$ -alkylguanine DNA alkyltransferase. *Org Biomol Chem.* 2012;10:7078–90.
- [58] O'Flaherty DK, McManus FP, Noronha AM, Wilds CJ. Synthesis of building blocks and oligonucleotides containing [T] $O^4$ -alkylene- $O^4$ [T] interstrand cross-links. *Curr Prot Nucleic Acids Chem.* 2013;5.13.1–5.13.19.
- [59] Denisov AY, McManus FP, O'Flaherty DK, Noronha AM, Wilds CJ. Structural basis of interstrand cross-link repair by  $O^6$ -alkylguanine DNA alkyltransferase. *Org Biomol Chem.* 2017;15:8361–70.
- [60] Copp W, O'Flaherty DK, Wilds CJ. Covalent capture of OGT's active site using engineered human-E. coli chimera and intrastrand DNA cross-links. *Org Biomol Chem.* 2018;16:9053–8.
- [61] McManus FP, Khaira A, Noronha AM, Wilds CJ. Preparation of covalently linked complexes between DNA and  $O^6$ -alkylguanine DNA alkyltransferase using interstrand cross-linked DNA. *Bioconjug Chem.* 2013;24:224–33.
- [62] O'Flaherty DK, Wilds CJ. AGT activity towards intrastrand crosslinked DNA is modulated by the alkylene linker. *ChemBioChem.* 2017;18:2351–7.
- [63] O'Flaherty DK, Wilds CJ. Preparation of intrastrand [G] $O^6$ -alkylene- $O^6$ [G] cross-linked oligonucleotides. *Curr Prot Nucleic Acids Chem.* 2016;5.17.1–5.17.24.
- [64] O'Flaherty DK, Wilds CJ. Synthesis, characterization, and repair of a flexible  $O^6$ -2'-deoxyguanosine-alkylene- $O^6$ -2'-deoxyguanosine intrastrand cross-link. *Chem Eur J.* 2015;21:10522–9.
- [65] McManus FP, Fang Q, Booth JDM, Noronha AM, Pegg AE, Wilds CJ. Synthesis and characterization of an  $O^6$ -2'-deoxyguanosine-alkyl- $O^6$ -2'-deoxyguanosine interstrand cross-link in a 5'-GNC motif and repair by human  $O^6$ -alkylguanine DNA alkyltransferase. *Org Biomol Chem.* 2010;8:4414–26.
- [66] O'Flaherty DK, Wilds CJ.  $O^6$ -Alkylguanine DNA alkyltransferase repair activity towards intrastrand cross-linked DNA is influenced by the internucleotide linkage. *Chem Asian J.* 2016;11:576–83.
- [67] Taylor JS. Unraveling the molecular pathway from sunlight to skin cancer. *Acc Chem Res.* 1994;27:76–82.

- [68] Taylor JS. DNA, sunlight, and skin cancer. *J Chem Educ.* 1990;67:835–41.
- [69] Blackburn GM, Davies RJH. The structure of thymine photo-dimer. *J Chem Soc C.* 1966;2239–44.
- [70] Douki T. The variety of UV-induced pyrimidine dimeric photoproducts in DNA as shown by chromatographic quantification methods. *Photochem Photobiol Sci.* 2013;12:1286–302.
- [71] Su DGT, Kao JLF, Gross ML, Taylor JS. Structure determination of an interstrand-type cis-anti cyclobutane thimine dimer produced in high yield by UVB light in an oligodeoxynucleotide at acidic pH. *J Am Chem Soc.* 2008;130:11328–337.
- [72] Taylor JS, Lu HF, Kotyk JJ. Quantitative conversion of the (6-4) photoproduct of TpdC to its Dewar valence isomer upon exposure to simulated sunlight. *Photochem Photobiol.* 1990;51:161–7.
- [73] Rycyna RE, Alderfer JL. UV irradiation of nucleic acids: formation, purification and solution conformational analysis of the 6-4 lesion of dTpT. *Nucleic Acids Res.* 1985;13:5949–63.
- [74] Taylor JS, Garrett DS, Wang MJ. Models for the solution state structure of the (6-4) photoproduct of thymidylyl-(3'-5')-thymidine derived via a distance- and angle-constrained search procedure. *Biopolymers.* 1988;27:1571–93.
- [75] Taylor JS, Cohrs MP, DNA I. Dewar pyrimidones: the structure and biological significance of TpT3. *J Am Chem Soc.* 1987;109:2834–5.
- [76] Wang Y, Taylor JS, Gross ML. Isolation and mass spectrometric characterization of dimeric adenine photoproducts in oligodeoxynucleotides. *Chem Res Toxicol.* 2001;14:738–45.
- [77] Kemmink J, Boelens R, Koning TMG, Kaptein R, van der Marel, van Boom JH. Conformational changes in the oligonucleotide duplex d(CGCTTGCG). d(CGCAACGC) induced by formation of a cis-syn thymine dimer. A two-dimensional NMR study. *Eur J Biochem.* 1987;162:37–43.
- [78] Smith CA, Taylor JS. Preparation and characterization of a set of deoxyoligonucleotide 49-mers containing site-specific cis-syn, trans-syn-I, (6-4), and Dewar photoproducts of thymidylyl(3'-5').thymidine. *J Biol Chem.* 1993;268:11143–51.
- [79] Wu D, Zhang N, Kong B, Hang H, Wang H. Synthesis and purification of biotinylated oligodeoxynucleotides containing single TpT dimeric pyrimidine (6-4) pyrimidone lesion. *Anal Bioanal Chem.* 2019;411:4123–9.
- [80] Taylor JS, Brockie IR, O'Day CL. A building block for the sequence-specific introduction of cis-syn thymine dimers into oligonucleotides. Solid-phase synthesis of TpT[c,s]pTpT. *J Am Chem Soc.* 1987;109:6735–42.
- [81] Taylor JS, Brockie IR. Synthesis of a trans-syn thymine dimer building block. Solid-phase of CGTAT[t,s]TATGC. *Nucleic Acids Res.* 1988;16:5123–36.
- [82] Taylor JS, O'Day CL. Synthesis of a bacteriophage DNA containing a site-specific cis-syn thymine dimer. *J Am Chem Soc.* 1989;111:401–2.
- [83] Taylor JS, O'Day CL. Cis-syn thymine dimers are not absolute blocks to replication by DNA polymerase I of Escherichia coli in vitro. *Biochemistry.* 1990;29:1624–32.
- [84] Murata T, Iwai S, Ohtsuka E. Synthesis and characterization of a substrate for T4 endonuclease V containing a phosphorodithioate linkage at the thymine dimer site. *Nucleic Acids Res.* 1990;18:7279–86.
- [85] Iwai S. Chemical synthesis of oligonucleotides containing damaged bases for biological studies. *Nucleosides Nucleotides Nucleic Acids.* 2006;25:561–82.
- [86] Taylor JS, Nadji S. Unraveling the origin of the major mutation induced by ultraviolet light, the C-T transition at dTpC sites. A DNA synthesis building block for the cis-syn cyclobutane dimer of dTpU. *Tetrahedron.* 1991;47:2579–90.
- [87] Takasawa K, Masutani C, Hanaoka F, Iwai S. Chemical synthesis and translesion replication of a cis-syn cyclobutane thymine–uracil dimer. *Nucleic Acids Res.* 2004;32:1738–45.

- [88] Vassylyev DG, Kashiwagi T, Mikami Y, Ariyoshi M, Iwai S, Ohtsuka E, Morikawa K. Atomic model of a pyrimidine dimer excision repair enzyme complexed with a DNA substrate: structural basis for damaged DNA recognition. *Cell.* 1995;83:773–82.
- [89] Mees A, Klar T, Gnau P, Hennecke U, Eker APM, Carell T, Essen LO. Crystal structure of a photolyase bound to a CPD-like DNA lesion after *in situ* repair. *Science.* 2004;306:1789–93.
- [90] Butenandt J, Eker APM, Synthesis T. Synthesis, crystal structure, and enzymatic evaluation of a DNA-photolesion isostere. *Chem Eur J.* 1998;4:642–54.
- [91] Nadji S, Wang CI, Taylor JS. Photochemically and photoenzymatically cleavable DNA. *J Am Chem Soc.* 1992;114:9266–9.
- [92] Iwai S, Shimizu M, Kamiya H, Ohtsuka E. Synthesis of a phosphoramidite coupling unit of the pyrimidine (6–4) pyrimidone photoproduct and its incorporation into oligodeoxynucleotides. *J Am Chem Soc.* 1996;118:7642–3.
- [93] Mizukoshi T, Hitomi K, Todo T, Iwai S. Studies on the chemical synthesis of oligonucleotides containing the (6–4) photoproduct of thymine–cytosine and its repair by (6–4) photolyase. *J Am Chem Soc.* 1998;120:10634–42.
- [94] Iwai S, Mizukoshi T, Fujiwara Y, Masutani C, Hanaoka F, Hayakawa Y. Benzimidazolium triflate-activated synthesis of (6–4) photoproduct-containing oligonucleotides and its application. *Nucleic Acids Res.* 1999;27:2299–303.
- [95] Fujiwara Y, Iwai S. Thermodynamic studies of the hybridization properties of photolesions in DNA. *Biochemistry.* 1997;36:1544–50.
- [96] Kim SJ, Lester C, Begley TP. Synthesis of the dinucleotide spore photoproduct. *J Org Chem.* 1995;60:6256–7.
- [97] Friedel MG, Berteau O, Pieck JC, Atta M, Ollagnier-de-Choudens S, Fontecave M, Carell T. The spore photoproduct lyase repairs the 5S- and not the 5R-configured spore photoproduct DNA lesion. *Chem Commun.* 2006;445–7.
- [98] Téoule RR. DNA damage and its repair. *Int J Radiat Biol.* 1987;51:573–89.
- [99] Schulhof JC, Molko D, Teoule E. The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. *Nucleic Acids Res.* 1987;15:397–416.
- [100] Guy A, Duplaa AM, Harel P, Téoule R. Synthesis and characterization of DNA fragments bearing an adenine radiation product: 7,8-dihydroadenin-8-one. *Helv Chim Acta.* 1988;71:1566–71.
- [101] Guschlbauer W, Duplaa AM, Guy A, Téoule R, Fazakerley GV. Structure and *in vitro* replication of DNA templates containing 7,8-dihydro-8-oxoadenine. *Nucleic Acids Res.* 1991;19:1753–8.
- [102] Leonard GA, Guy A, Brown T, Téoule R, Hunter WN. Conformation of guanine. 8-oxoadenine base pairs in the crystal structure of d(CGCGAATT(O8A)GCG). *Biochemistry.* 1992;31:8415–20.
- [103] Wood ML, Esteve A, Morningstar ML, Kuziemko GM, Essigmann JM. Genetic effect of oxidative DNA damage: comparative mutagenesis of 7,8-dihydro-8-oxoguanine and 7,8-dihydro-8-oxoadenine in *Escherichia coli*. *Nucleic Acids Res.* 1992;20:6023–32.
- [104] Bodepudi V, Shibusaki S, Johnson F. Synthesis of 2'-deoxy-7,8-dihydro-8-oxoguanosine and 2'-deoxy-7,8-dihydro-8-oxoadenosine and their incorporation into oligomeric DNA. *Chem Res Toxicol.* 1992;5:608–17.
- [105] Bodepudi V, Iden CR, Johnson F. An improved method for the preparation of the phosphoramidites of modified 2'-deoxynucleotides: incorporation of 8-oxo-2'-deoxy-7H-guanosine into synthetic oligomers. *Nucleosides Nucleotides.* 1991;10:755–61.
- [106] Koizume S, Kamiya H, Inoue H, Ohtsuka E. Synthesis and thermodynamic stabilities of damaged DNA involving 8-hydroxyguanine (7,8-dihydro-8-oxoguanine) in a ras-gene fragment. *Nucleosides Nucleotides.* 1994;13:1517–34.

- [107] Geiger A, Seliger H, Nehls P. A new approach for the efficient synthesis of oligodeoxyribonucleotides containing the mutagenic DNA modification 7,8-dihydro-8-oxo-2'-deoxyguanosine at predefined positions. *Nucleosides Nucleotides*. 1993;12:463–77.
- [108] Lipscomb LA, Peek ME, Morningstar ML, Verghis SM, Miller EM, Rich A, Essigmann JM, Williams LD. X-ray structure of a DNA decamer containing 7,8-dihydro-8-oxoguanine. *Proc Natl Acad Sci USA*. 1995;92:719–23.
- [109] Roelen HCPF, Saris CP, Brugge HF, van der Elst H, Westra JG, van der Marel GA, van Boom JH. Solid-phase synthesis of DNA fragments containing the modified base modification 7,8-dihydro-8-oxo-2'-deoxyguanosine. *Nucleic Acids Res*. 1991;19:4361–9.
- [110] Oda Y, Uesugi S, Ikebara M, Nishimura S, Kawase Y, Ishikawa H, Inoue H, Ohtsuka E. NMR studies of a DNA containing 8-hydroxydeoxyguanosine. *Nucleic Acids Res*. 1991;19:1407–11.
- [111] Kamiya H, Miura K, Ishikawa H, Inoue H, Nishimura H, Ohtsuka E. C-Ha-ras containing 8-hydroxyguanine at codon 12 induces point mutations at the modified and adjacent positions. *Cancer Res*. 1992;52:3483–5.
- [112] Wood ML, Dizdaroglu M, Gajewski E, Essigmann JM. Mechanistic studies of ionizing radiation and oxidative mutagenesis: genetic effects of a single 8-hydroxyguanine (7-hydro-8-oxoguanine) residue inserted at a unique site in a viral genome. *Biochemistry*. 1990;29:7024–32.
- [113] Varaprasad CV, Bulychev N, Grollman AP, Johson F. Synthesis of 8-oxo-7,8-dihydro-6-O-methyl-2'-deoxyguanosine and its use as a probe to study DNA-base excision by MutY enzyme. *Tetrahedron Lett*. 1996;37:9–12.
- [114] Bulychev N, Varaprasad CV, Dormán G, Miller JH, Eisenberg M, Grollman AP, Johson F. Substrate specificity of Escherichia coli MutY protein. *Biochemistry*. 1996;35:13147–56.
- [115] Roberts C, Bandaru R, Switzer C. Synthesis of oligonucleotides bearing the non-standard bases iso-C and iso-G. Comparison of iso-C-iso-G, C-G and U-A base-pair stabilities in RNA/DNA duplexes. *Tetrahedron Lett*. 1995;36:3601–4.
- [116] Roberts C, Bandaru R, Switzer C. Theoretical and experimental study of isoguanine and isocytosine: base pairing in an expanded genetic system. *J Am Chem Soc*. 1997;119:4640–9.
- [117] Switzer C, Moroney SE, Benner SA. Enzymatic incorporation of a new base pair into RNA and RNA. *J Am Chem Soc*. 1989;111:8322–3.
- [118] Chaput JC, Switzer C. Non-enzymatic transcription of an isoG-isoC base pair. *J Am Chem Soc*. 2000;122:12866–7.
- [119] Romieu A, Gasparutto D, Molko D, Ravanat JL, Cadet J. Synthesis of oligonucleotides containing the (4*R*) and (4*S*) diastereoisomers of 4,8-dihydro-4-hydroxy-8-oxo-2'-deoxyguanine. *Eur J Org Chem*. 1999;49–56.
- [120] Haraguchi K, Delaney MO, Wiederholt CJ, Sambandam A, Hantosi Z, Greenberg MM. Synthesis and characterization of oligodeoxynucleotides containing formamidopyrimidine lesions and nonhydrolyzable analogues. *J Am Chem Soc*. 2002;124:3263–9.
- [121] Haraguchi K, Greenberg MM. Synthesis of oligonucleotides containing Fapy-dG (*N*<sup>6</sup>-(2-deoxy- $\alpha$ , $\beta$ -D-erythro-pentofuranosyl)-2,6-diamino-4-hydroxy-formamidopyrimidine). *J Am Chem Soc*. 2001;123:8636–7.
- [122] Jiang YL, Wiederholt CJ, Patro JN, Haraguchi K, Greenberg MM. Synthesis of oligonucleotides containing Fapy-dG (*N*<sup>6</sup>-(2-deoxy- $\alpha$ , $\beta$ -D-erythropentofuranosyl)-2,6-diamino-4-hydroxy-5-formamidopyrimidine) using a 5'-dimethoxytrityl dinucleotide phosphoramidite. *J Org Chem*. 2005;70:141–9.
- [123] Gehrke TH, Lischke U, Gasteiger KL, Schneider S, Arnold S, Müller HC, Stephenson DS, Zipse H, Carell T. Unexpected non-Hoogsten-based mutagenicity mechanism of FaPy-DNA lesions. *Nat Chem Biol*. 2013;9:455–61.

- [124] Cadet J, Wagner JR. DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. *Cold Spring Harb Perspect Biol.* 2013;5:a012559.
- [125] Yoshimura Y, Takahata H. Recent advances in cyclonucleosides: C-cyclonucleosides and spore photoproducts in damaged DNA. *Molecules.* 2012;17:11630–54.
- [126] Romieu A, Gasparutto D, Molko D, Cadet J. Site-specific introduction of (5'S)-5',8-cyclo-2'-deoxyadenosine into oligodeoxyribonucleotides. *J Org Chem.* 1998;63:5245–49.
- [127] Karwowski BT, Gaillard J, Grand A, Cadet J. Effect of (5'S)-5',8-cyclo-2'-deoxyadenosine on the conformation of di and trinucleotides. A NMR and DFT study. *Org Biomol Chem.* 2008;6:3408–13.
- [128] Romieu A, Gasparutto D, Cadet J. Synthesis and characterization of oligonucleotides containing 5',8-cyclopurine 2'-deoxyribonucleosides: (5'R)-5',8-cyclo-2'-deoxyadenosine, (5'S)-5',8-cyclo-2'-deoxyguanosine, and (5'R)-5',8-cyclo-2'-deoxyguanosine. *Chem Res Toxicol.* 1999;12:412–21.
- [129] Romieu A, Gasparutto D, Cadet J. Synthesis and characterization of oligodeoxynucleotides containing the two 5R and 5S diastereomers of (5'S,6S)-5',6-cyclo-5,6-dihydrothymidine; radiation induced tandem lesions of thymidine. *Perkin Trans.* 1999;1:1257–63.
- [130] Muller E, Gasparutto D, Jaquinod M, Romieu A, Cadet J. Chemical and biochemical properties of oligonucleotides that contain (5'S,6S)-cyclo-5,6-dihydro-2'-deoxyuridine and (5'S,6S)-cyclo-5,6-dihydrothymidine, two main radiation-induced degradation products of pyrimidine 2'-deoxyribonucleosides. *Tetrahedron.* 2000;56:8689–701.
- [131] Muller E, Gasparutto D, Cadet J. Chemical synthesis and biochemical properties of oligonucleotides that contain the (5'S, 5S, 6S)-5',6-cyclo-5-hydroxy-5,6-dihydro-2'-deoxyuridine DNA lesion. *ChemBioChem.* 2002;3:534–42.
- [132] Conte MR, Galeone A, Avizonis D, Hsu VL, Mayol L, Kearns DR. Solid phase synthesis of 5-hydroxymethyluracil containing DNA. *Bioorg Med Chem Lett.* 1992;2:79–82.
- [133] Sowers LC, Beardsley GP. Synthesis of oligonucleotides containing 5-(hydroxymethyl)-2'-deoxyuridine at defined sites. *J Org Chem.* 1993;58:1664–5.
- [134] Münz M, Globisch D, Carell T. 5-Hydroxymethylcytosine, the sixth base of the genome. *Angew Chem Int Ed.* 2011;50:6460–8.
- [135] Schiesser S, Pfaffeneder T, Sadeghian K, Hckner B, Steigenberger B, Schröder AS, Steinbacher J, Kashiwazaki G, Höfner G, Wanner KT, Ochsenfeld C, Carell T. Deamination, oxidation, and C-C bond cleavage reactivity of 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxycytosine. *J Am Chem Soc.* 2013;135:14593–9.
- [136] Tardy-Planechaud S, Fujimoto J, Lin SS, Sowers LC. Solid phase synthesis and restriction endonuclease cleavage of oligodeoxynucleotides containing 5-(hydroxymethyl)-cytosine. *Nucleic Acids Res.* 1997;25:553–8.
- [137] Xuan S, Wu Q, Cui L, Zhang D, Shao F. 5-Hydroxymethylcytosine and 5-formylcytosine containing deoxyoligonucleotides: Facile syntheses and melting temperature studies. *Bioorg Med Chem Lett.* 2015;25:1186–91.
- [138] Hansen AS, Thalhammer A, El-Sagheer AH, Brown T, Schofield CJ. Improved synthesis of hydroxymethyl-2'-deoxycytidine phosphoramidite using a 2'-deoxyuridine to 2'-deoxycytidine conversion without temporary protecting groups. *Bioorg Med Chem Lett.* 2011;21:1181–4.
- [139] De Kort M, Visser PC, Kurzeck J, Meeuwenoord NJ, van der Marel GA, Ruger W, van Boom JH. Chemical and enzymatic synthesis of DNA fragments containing 5-( $\beta$ -D-glucopyranosyloxymethyl)-2'-deoxycytidine. A modified nucleoside in T4 Phage DNA. *Eur J Org Chem.* 2001;11:2075–82.

- [140] Dai Q, Song CX, Pan T, He C. Syntheses of two 5-hydroxymethyl-2'-deoxycytidine phosphoramidites with TBDMS as the 5-hydroxymethyl protecting group and their incorporation into DNA. *J Org Chem.* 2011;76:4182–8.
- [141] Dai Q, He C. Preparation of DNA containing 5-hydroxymethyl-2'-deoxycytidine modification through phosphoramidites with TBDMS as 5-hydroxymethyl protecting group. *Curr Protoc Nucleic Acids Chem.* 2011;unit 4.4.718.
- [142] Münzel M, Globisch D, Trindler C, Carell T. Efficient synthesis of 5-hydroxymethylcytosine containing DNA. *Org Lett.* 2010;12:5671–3.
- [143] Sujiyama H, Matsuda S, Kino K, Zhang QM, Yonei S, Saito I. New synthetic method of 5-formyluracil containing oligonucleotides and their melting behavior. *Tetrahedron Lett.* 1996;37:9067–70.
- [144] Karino N, Ueno Y, Matsuda A. Synthesis and properties of oligonucleotides containing 5-formyl-2'-deoxycytidine: in vitro DNA polymerase reactions on DNA templates containing 5-formyl-2'-deoxycytidine. *Nucleic Acids Res.* 2001;29:2456–63.
- [145] Münzel M, Lischke U, Stathis D, Pfaffeneder T, Gnerlich FA, Deiml CA, Koch SC, Karaghiosoff K, Carell T. Improved synthesis and mutagenicity of oligonucleotides containing 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine. *Chem Eur J.* 2011;17:13782–8.
- [146] Dai Q, He CA. Syntheses of 5-formyl- and 5-carboxy-dC containing DNA oligos as potential oxidation products of 5-hydroxymethylcytosine in DNA. *Org Lett.* 2011;13:3446–9.
- [147] Schroder AS, Steinbacher J, Steigenberger B, Gnerlich FA, Schiesser S, Pfaffeneder T, Carell T. Synthesis of a DNA promoter segment containing all four epigenetic nucleosides: 5-methyl-, 5-hydroxymethyl-, 5-formyl-, and 5-carboxy-2'-deoxycytidine. *Angew Chem Int Ed.* 2014;53:315–8.
- [148] Kawasaki F, Murat P, Li Z, Santner T, Balasubramanian S. Synthesis and biophysical analysis of modified thymine-containing DNA oligonucleotides. *Chem Commun.* 2017;53:1389–92.
- [149] Mascareñas JL, Hayashibara KC, Verdine GL. Template-directed interference footprinting of protein-thymine contacts. *J Am Chem Soc.* 1993;115:373–4.
- [150] Fujimoto J, Tran L, Sowers LC. Synthesis and cleavage of oligodeoxynucleotides containing a 5-hydroxyuracil residue at a defined site. *Chem Res Toxicol.* 1997;10:1254–8.
- [151] Morningstar ML, Kreutzer DA, Essigmann JM. Synthesis of oligonucleotides containing two putatively mutagenic DNA lesions: 5-hydroxy-2'-deoxyuridine and 5-hydroxy-2'-deoxycytidine. *Chem Res Toxicol.* 1997;10:1345–50.
- [152] Romieu A, Gasparutto D, Molko D, Cadet J. A convenient synthesis of 5-hydroxy-2'-deoxycytidine phosphoramidite and its incorporation into oligonucleotides. *Tetrahedron Lett.* 1997;38:7531–4.
- [153] Clark JM, Beardsley GP. Thymine glycol lesions terminate chain elongation by DNA polymerase I in vitro. *Nucleic Acids Res.* 1986;14:737–49.
- [154] Clark JM, Beardsley GP. Functional effects of cis-thymine glycol lesions on DNA synthesis in vitro. *Biochemistry.* 1987;26:5398–403.
- [155] Iwai S. Synthesis of thymine glycol containing oligonucleotides from a building block with the oxidized base. *Angew Chem Int Ed.* 2000;39:3874–5.
- [156] Iwai S. Synthesis and thermodynamic studies of oligonucleotides containing the two isomers of thymine glycol. *Chem Eur J.* 2001;7:4343–51.
- [157] Shimizu T, Manabe K, Yoshikawa S, Kawasaki Y, Iwai S. Preferential formation of (5S,6R)-thymine glycol for oligodeoxynucleotides synthesis and analysis of drug binding to thymine glycol-containing DNA. *Nucleic Acids Res.* 2006;34:313–21.
- [158] Schulhof JC, Molko D, Teoule R. Synthesis of DNA fragments containing 5,6-dihydrothymine, a major product of thymine gamma radiolysis. *Nucleic Acids Res.* 1988;16:319–26.

- [159] Molko D, Delort AM, Guy A, Teoule R. Synthesis of a DNA fragment containing dihydro-5,6-thymine. *Biochemie*. 1985;67:801–9.
- [160] Barvian MR, Greenberg MM. Diastereoselective synthesis of hydroxylated dihydrothymidines resulting from oxidative stress. *J Org Chem*. 1993;58:6151–4.
- [161] Barvian MR, Greenberg MM. Independent generation of 5,6-dihydrothymid-5-yl and investigation of its ability to effect nucleic acid strand scission via hydrogen atom abstraction. *J Org Chem*. 1995;60:1916–7.
- [162] Matray TJ, Greenberg MM. Site-specific incorporation of the alkaline labile, oxidative stress product (5*R*)-5,6-dihydro-5-hydroxythymidine in an oligonucleotide. *J Am Chem Soc*. 1994;116:6931–2.
- [163] Sambandam A, Greeberg MM. The effects of 5*R*-5,6-dihydro-5-hydroxythymidine on duplex DNA stability and structure. *Nucleic Acids Res*. 1999;27:3597–602.
- [164] Marx A, Erdmann P, Senn M, Körner S, Jungo T, Petretta M, Imwinkelried P, Dussy A, Kulicke KJ, Macko L, Zehnder M, Giese B. Synthesis of 4'-C-acylated thymidines. *Helv Chim Acta*. 1996;79:1980–94.
- [165] Hess MT, Schwitter U, Petretta M, Giese B, Naegeli H. DNA synthesis arrest at C4'-modified deoxyribose residues. *Biochemistry*. 1997;36:2332–7.
- [166] Giese B, Imwinkelried P, Petretta M. Synthesis of a modified thymidine and preparation of precursors of oligonucleotide radicals. *Synlett*. 1994;1003–4.
- [167] Giese B, Dussy A, Elie C, Erdmann P, Schwitter U. Synthesis abd selective radical cleavage of C-4'-modified oligonucleotides. *Angew Chem Int Ed*. 1994;33:1861–3.
- [168] Guy A, Dubet J, Teoule R. The synthesis of 5-hydroxy-5-methylhydantoin nucleoside and its insertion into oligodeoxyribonucleotides. *Tetrahedron Lett*. 1993;34:8101–2.
- [169] Gasparutto G, Ait-Abbas M, Jaquinod M, Boiteux S, Cadet J. Repair and coding properties of 5-hydroxy-5-methylhydantoin nucleosides inserted into DNA oligomers. *Chem Res Toxicol*. 2000;13:575–84.
- [170] Muller E, Gasparutto D, Lebrun C, Cadet J. Sire-specific insertion of the (5*R*\*) and (5*S*\*) diastereoisomers of 1-[2-deoxy- $\beta$ -D-erythro-pentofuranosyl]-5-hydroxyhydantoin into oligodeoxyribonucleotides. *Eur J Org Chem*. 2001;2091–9.
- [171] Guy A, Ahmad S, Teoule R. Insertion of the fragile 2'-deoxyribosylurea residue into oligodeoxynucleotides. *Tetrahedron Lett*. 1990;31:5745–8.
- [172] Gervais V, Guy A, Teoule R, Fazakerley GV. Solution conformation of an oligonucleotide containing a urea deoxyribose residue in front of a thymine. *Nucleic Acids Res*. 1992;20:6455–60.
- [173] Guy A, Duplaa AM, Ulrich J, Téoule R. Incorporation by chemical synthesis and characterization of deoxyribosylformamide into DNA. *Nucleic Acids Res*. 1991;19:5815–20.
- [174] Shida T, Arakawa M, Sekiguchi J. Properties of DNA duplexes containing an abasic site: effect of nucleotide residue of the opposite part of a 2-deoxyribosylformamide residue. *Nucleosides Nucleotides*. 1994;13:1319–26.
- [175] Shida T, Iwaori H, Arakawa M, Sekiguchi J. Chemical synthesis of an oligodeoxyribonucleotide containing a deoxyribosylformamide residue. *Chem Pharm Bull*. 1993;41:961–4.
- [176] Baillet S, Behr JP. Deoxyribosylurea and deoxyribosylformamide oligonucleotides. *Tetrahedron Lett*. 1995;36:8981–4.
- [177] Toga T, Yamamoto J, Iwai S. Efficient conversion of thymine glycol into the formamide lesion in oligonucleotides. *Tetrahedron Lett*. 2009;50:723–6.
- [178] Lindahl T. Instability and decay of the primary structure of DNA. *Nature*. 1993;362:709–15.
- [179] Ph C, Sowers LC, Eritja R, Kaplan B, Goodman MF, Cognet JAH, LeBret M, Guschlauer W, Fazakerley GV. An abasic site in DNA. Solution conformation determined by proton NMR and molecular mechanics calculations. *Nucleic Acids Res*. 1987;15:8003–22.

- [180] Randall SK, Eritja R, Kaplan BE, Petruska J, Goodman MF. Nucleotide insertion kinetics opposite abasic lesions in DNA. *J Biol Chem.* 1987;262:6864–70.
- [181] Millican TA, Mock GA, Chauncey MA, Patel TP, Eaton MAW, Gunning J, Cutbush SD, Neidle S, Mann J. Synthesis and biophysical studies of short oligodeoxynucleotides with novel modifications: a possible approach to the problem of mixed bases oligodeoxynucleotide synthesis. *Nucleic Acids Res.* 1984;12:7435–53.
- [182] Eritja R, Walker PA, Randall SK, Goodman MF, Kaplan BE. Synthesis of oligonucleotides containing the abasic site model compound 1,4-anhydro-2-deoxy-D-ribitol. *Nucleotides Nucleosides.* 1987;6:803–14.
- [183] Takeshita M, Chang CN, Johnson F, Will S, Grollman AP. Oligodeoxynucleotide containing synthetic abasic sites. Model substrates for DNA polymerases and apurinic/apirimidinic endonucleases. *J Biol Chem.* 1987;262:10171–9.
- [184] Iyer RP, Uznanski B, Boal J, Storm C, Egan W, Matsukura M, Broder S, Zon G, Wilk A, Koziolkiewicz, Stec WJ. Abasic oligodeoxyribonucleoside phosphorothioates: synthesis and evaluation as anti-HIV-1 agents. *Nucleic Acids Res.* 1990;18:2855–9.
- [185] Iocono JA, Gildea B, McLaughlin LW. Mild acid hydrolysis of pyrimidinone-containing DNA fragments generates apurinic/apirimidinic sites. *Tetrahedron Lett.* 1990;31:175–8.
- [186] Laayoun A, Décout JL, Defrancq E, Lhomme J. Hydrolysis of oligonucleotides containing 8-substituted ourine nucleosides. A new route for preparing abasic oligodeoxynucleotides. *Tetrahedron Lett.* 1994;35:4991–4.
- [187] Manoharan M, Ransom SC, Mazumder A, Gerlt JA, Wilde JA, Withka JM, Bolton PH. Enzymatic synthesis of abasic sites in DNA heteroduplexes and their characterization by site specific labeling with  $^{13}\text{C}$ . *J Am Chem Soc.* 1988;110:1620–2.
- [188] Péoc'h D, Meyer A, Imbach JL, Rayner B. Efficient synthesis of oligodeoxynucleotides containing a true abasic site. *Tetrahedron Lett.* 1991;32:207–10.
- [189] Groebke K, Leumann C. A method for preparing oligodeoxynucleotides containing an apurinic site. *Helv Chim Acta.* 1990;73:608–17.
- [190] Shishkina IG, Johnson F. A new method for the postsynthetic generation of abasic sites in oligomeric DNA. *Chem Res Toxicol.* 2000;13:907–12.
- [191] Urata H, Akagi M. Photo-induced formation of the 2-deoxyribonolactone-containing nucleotide for d(ApCpA): effects of neighboring bases and modification of deoxycytidine. *Nucleic Acids Res.* 1991;19:1773–8.
- [192] Kotera M, Bourdat AG, Defrancq E, Lhomme J. A highly efficient synthesis of oligodeoxyribonucleotides containing the 2'-deoxyribonolactone lesion. *J Am Chem Soc.* 1998;120:11810–1.
- [193] Lenox HJ, McCoy CP, Sheppard TL. Site-specific generation of deoxyribonolactone lesions in DNA oligonucleotides. *Org Lett.* 2001;3:2415–8.
- [194] Zheng Y, Sheppard TL. Half-life and DNA strand scission products of 2-deoxyribonolactone oxidative DNA damage lesions. *Chem Res Toxicol.* 2004;17:197–207.
- [195] Kim J, Gil JM, Greenberg MM. Synthesis and characterization of oligonucleotides containing the c-4'-oxidized abasic site produced by bleomycin and other damaging agents. *Angew Chem Int Ed.* 2003;42:5882–5.
- [196] Schärer OD, Ortholand JY, Ganesan A, Ezaz-Nikpay K, Verdine GL. Specific binding of the DNA repair enzyme AlkA to a pyrrolidine-based inhibitors. *J Am Chem Soc.* 1998;120:6623–4.
- [197] Luch A. Nature and nurture. Lessons from chemical carcinogenesis. *Nat Rev Cancer.* 2005;5:113–25.
- [198] Basu AK, O'Hara SM, Valladier P, Stone K, Mols O, Marnett LJ. Identification of adducts formed by reaction of guanine nucleosides with malondialdehyde and structurally related aldehydes. *Chem Res Toxicol.* 1988;1:53–9.

- [199] Kouchakdjan M, Marinelli E, Gao X, Johnson F, Grollman A, Patel D. NMR studies of exocyclic 1,N<sup>2</sup>-propanodeoxyguanosine adducts (X) opposite purines in DNA duplexes: protonated X(syn). A(anti) pairing (acidic pH) and X(syn). G(anti) pairing (neutral pH) at the lesion site. *Biochemistry*. 1989;28:5647–57.
- [200] Marinelli E, Johnson F, Iden CR, Yu PL. Synthesis of 1,N<sup>2</sup>-(1,3-propano)-2'-deoxyguanosine and incorporation into oligodeoxynucleotides: a model for exocyclic acrolein-DNA adducts. *Chem Res Toxicol*. 1990;3:49–58.
- [201] Reddy GR, Marnett LJ. Synthesis of an oligodeoxynucleotide containing the alkaline labile malondialdehyde-deoxyguanosine adduct pyrimido[1,2-a]purin-10(3H)-one. *J Am Chem Soc*. 1995;117:5007–8.
- [202] Schnetz-Boutaud NC, Mao H, Stone MP, Marnett LJ. Synthesis of oligonucleotides containing the alkali-labile pyrimidopurinone adduct, M1G. *Chem Res Toxicol*. 2000;13:90–5.
- [203] Srivastava SC, Raza SK, Misra R. 1,N<sup>6</sup>-etheno deoxy and ribo adenosine and 3,N<sup>4</sup>-etheno deoxy and ribo cytidine phosphoramidites. Strongly fluorescent structures for selective introduction in defined sequence DNA and RNA molecules. *Nucleic Acids Res*. 1994;22:1296–304.
- [204] Zhang W, Rieger R, Iden C, Johnson F. Synthesis of 3,N<sup>4</sup>-etheno, 3,N<sup>4</sup>-ethano, and 3-(2-hydroxyethyl) derivatives of 2'-deoxycytidine and their incorporation into oligomeric DNA. *Chem Res Toxicol*. 1995;8:148–56.
- [205] Basu AK, Wood ML, Niedernhofer LJ, Ramos LA, Essigmann JM. Mutagenic and genotoxic effects of three vinyl chloride-induced DNA lesions: 1,N<sup>6</sup>-ethenoadenine, 3,N<sup>4</sup>-ethenocytosine, and 4-amino-5-(imidazol-2-yl)imidazole. *Biochemistry*. 1993;32:12793–801.
- [206] Schärer OD, Verdine GL. A designer inhibitor of base-excision DNA repair. *J Am Chem Soc*. 1995;117:10781–2.
- [207] Chenna A, Perry A, Singer B. Synthesis of 8-(hydroxymethyl)-3,N<sup>4</sup>-etheno-2'-deoxycytidine, a potential carcinogenic glycidaldehyde adduct, and its site-specific incorporation into DNA oligonucleotides. *Chem Res Toxicol*. 2000;13:208–13.
- [208] Sági J, Chenna A, Hang B, Singer B. A single cyclic *p*-benzoquinone adduct can destabilize a DNA oligonucleotide duplex. *Chem Res Toxicol*. 1998;11:329–34.
- [209] Chenna A, Singer B. Large scale synthesis of *p*-benzoquinone – 2'-deoxycytidine and *p*-benzoquinone-2'-deoxyadenosine adducts and their site-specific incorporation into DNA oligonucleotides. *Chem Res Toxicol*. 1995;8:865–74.
- [210] Bischofberger N, Matteucci MD. Synthesis of novel polycyclic nucleoside analogues, incorporation into oligodeoxynucleotides, and interaction with complementary sequences. *J Am Chem Soc*. 1989;111:3941–6.
- [211] Huang Y, Torres MC, Iden CR, Johnson F. Regioselective synthesis of 1,N<sup>2</sup>-etheno-2'-deoxyguanosine and its generation in oligomeric DNA. *Chem Res Toxicol*. 2003;16:708–14.
- [212] Huang Y, Torres MC, Iden CR, Johnson F. Synthesis of the minor acrolein adducts of 2'-deoxyguanosine and their generation in oligomeric DNA. *Bioorg Chem*. 2003;31:136–48.
- [213] Stezowski JJ, Joos-Guba G, Schönwälder KH, Straub A, Glusker JP. Preparation and characterization in solution of oligonucleotides alkylated by activated carcinogenic polycyclic aromatic hydrocarbons. *J Biomol Struct Dyn*. 1987;5:615–37.
- [214] Lee H, Hinz M, Stezowski JJ, Harvey RG. Syntheses of polycyclic aromatic hydrocarbon-nucleoside and oligonucleotide adducts specifically alkylated on the amino functions of deoxyguanosine and deoxyadenosine. *Tetrahedron Lett*. 1990;31:6773–6.
- [215] Lee H, Luna E, Hinz M, Stezowski JJ, Kiselyov AS, Harvey RG. Synthesis of oligonucleotide adducts of the bay region diol epoxide metabolites of carcinogenic polycyclic aromatic hydrocarbons. *J Org Chem*. 1995;60:5604–13.

- [216] Lakshman MK, Sayer JM, Yagi H, Jerina DM. Synthesis and duplex-forming properties of a nonanucleotide containing an  $N^6$ -deoxyadenosine adduct of a bay-region diol epoxide. *J Org Chem.* 1992;57:4595–90.
- [217] Lakshman MK, Sayer JM, Jerina DM. Synthesis and site-specific incorporation of a bay-region cis ring-opened tetrahydro epoxide-deoxyadenosine adduct into a DNA oligomer. *J Org Chem.* 1992;57:3438–43.
- [218] Lakshman MK, Yeh HJC, Yagi H, Jerina DM. Synthesis of deoxyadenosine adducts from the highly carcinogenic Fjord region diol epoxide of benzo[c]phenanthrene. *Tetrahedron Lett.* 1992;33:7121–4.
- [219] Lakshman MK, Sayer JM, Jerina DM. Chemical synthesis of a bay-region polycyclic aromatic hydrocarbon tetrahydroepoxide-deoxyadenosine adduct and its site-specific incorporation into a DNA oligomer. *J Am Chem Soc.* 1991;113:6589–94.
- [220] Kim SJ, Stone MP, Harris CM, Harris TM. Postoligomerization synthesis of oligodeoxynucleotides containing polycyclic aromatic hydrocarbon adducts at the  $N^6$  position of deoxyadenosine. *J Am Chem Soc.* 1992;114:5480–1.
- [221] Harris CM, Zhou L, Strand EA, Harris TM. New strategy for the synthesis of oligodeoxynucleotides bearing adducts at exocyclic amino sites of purine nucleosides. *J Am Chem Soc.* 1991;113:4328–9.
- [222] Han S, Harris CM, Harris TM, Kim HYH, Kim SJ. Synthesis of deoxyadenosine 3'-phosphates bearing cis and trans adducts of  $7\beta,8\alpha$ -dihydroxy- $9\alpha,10\alpha$ -epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene: standards for  $^{32}\text{P}$ -postlabeling assays. *J Org Chem.* 1996;61:174–8.
- [223] Ramesha AR, Kroth H, Jerina DM. Novel trifluoroethanol mediated synthesis of benzo[a]pyrene 7,8-diol 9, 10-epoxide adducts at the  $N^2$ -position of deoxyguanosine and the  $N^6$ -position of deoxyadenosine. *Tetrahedron Lett.* 2001;42:1003–5.
- [224] Ling H, Sayer JM, Plosky BS, Yagi H, Boudsocq F, Woodgate R, Jerina DM, Yang W. Crystal structure of a benzo[a]pyrene diol epoxide adduct in a ternary complex with a DNA polymerase. *Proc Natl Acad Sci USA.* 2004;101:2265–9.
- [225] Zajc B, Lakshman MK, Sayer JM, Jerina DM. Epoxide and diol epoxide adducts of polycyclic aromatic hydrocarbons at the exocyclic amino group of deoxyguanosine. *Tetrahedron Lett.* 1992;33:3409–12.
- [226] Laxmi YRS, Suzuki N, Dasaradhi L, Johnson F, Shibutani. Preparation of oligodeoxynucleotides containing a diastereoisomer of  $\alpha$ -( $N^2$ -2'-deoxyguanosinyl) tamoxifen by phosphoramidite chemical synthesis. *Chem Res Toxicol.* 2002;15:218–25.
- [227] Tsarouhtsis D, Kuchimanchi S, DeCorte BL, Harris CM, Harris TM. Synthesis of oligonucleotides containing intercahin cross-links of bifunctional pyrroles. *J Am Chem Soc.* 1995;117:11013–4.
- [228] Johnson F, Bonala R, Tawde Torres MC, Iden CR. Efficient synthesis of the benzo[a]pyrene metabolic adducts of 2-deoxyguanosine and 2'-deoxyadenosine and their direct incorporation into DNA. *Chem Res Toxicol.* 2002;15:1489–94.
- [229] Bonala R, Shishkina IG, Johnson F. Synthesis of biologically active  $N^2$ -amine adducts of 2'-deoxyguanosine. *Tetrahedron Lett.* 2000;41:7281–4.
- [230] Casale R, McLaughlin LW. Synthesis and properties of an oligodeoxynucleotide containing a polycyclic aromatic hydrocarbon site specifically bound to the  $N^2$  amino group of a 2'-deoxyguanosine residue. *J Am Chem Soc.* 1990;112:5264–71.
- [231] Cooper MD, Hodge RP, Tamura PJ, Wilkinson AS, Harris CM, Harris TM. Synthesis of oligonucleotides containing bulky adducts at guanine N2 via the phosphoramidite of O<sup>2</sup>-triflate-O<sup>6</sup>-NPE 2'-deoxyxanthosine. *Tetrahedron Lett.* 2000;41:3555–8.

- [232] Gillet LCJ, Schärer OD. Preparation of C8-amine and acetylamine adducts of 2'-deoxyguanosine suitably protected for DNA synthesis. *Org Lett.* 2002;4:4205–8.
- [233] Gillet LCJ, Alzeer J, Schärer OD. Site-specific incorporation of N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-AAF) into oligonucleotides using modified “ultra-mild” DNA synthesis. *Nucleic Acids Res.* 2005;33:1961–9.
- [234] Elmquist CE, Stover JS, Wang Z, Rizzo CJ. Site-specific synthesis and properties of oligonucleotides containing C8-deoxyguanosine adducts of the dietary mutagen IQ. *J Am Chem Soc.* 2004;126:11189–201.
- [235] Bonala R, Torres MC, Iden CR, Johnson F. Synthesis of the PhIP adduct of 2'-deoxyguanosine and its incorporation into oligomeric DNA. *Chem Res Toxicol.* 2006;19:734–8.
- [236] Yamana K, Ohashi Y, Nunota K, Kitamura M, Nakano H, Sangen O, Shimidzu T. Synthesis of oligonucleotide derivatives with pyrene group at sugar fragment. *Tetrahedron Lett.* 1991;32:6347–50.
- [237] Yamana K, Nunota K, Nakano H, Sangen O. A new method for introduction of a pyrene group into a terminal position of an oligonucleotide. *Tetrahedron Lett.* 1994;35:2555–8.
- [238] Yamana K, Aota R, Nakano H. Oligonucleotides having covalently anthracene at specific sugar residue: differential binding to DNA and RNA and fluorescence properties. *Tetrahedron Lett.* 1995;36:8427–30.
- [239] Yamana K, Nishijima Y, Ikeda T, Gokota T, Ozaki H, Nakano H, Sangen O, Shimidzu T. Synthesis and intercalative properties of an oligonucleotide with anthraquinone at the sugar fragment. *Bioconjug Chem.* 1990;1:319–24.
- [240] Kobertz WR, Essigmann JM. Total synthesis of a cis-syn 2-carbomethoxypsoralen furan thymidine monoadduct. *J Am Chem Soc.* 1996;118:7101–7.
- [241] Kobertz WR, Essigmann JM. Solid-phase synthesis of oligonucleotides containing a site-specific psolarene derivative. *J Am Chem Soc.* 1997;119:5960–1.
- [242] Kobertz WR, Essigmann JM. An efficient synthesis of a furan-side furocoumarin thymidine monoadduct. *J Org Chem.* 1997;62:2630–2.



Carme Fàbrega and Ramon Eritja

### 3 Synthesis of oligonucleotides carrying modified bases for DNA and protein recognition

#### Contents

3.1	Hybridization and DNA mutagenesis —	88
3.1.1	Halogenated nucleobases —	88
3.1.2	2-Aminopurine and methoxyamine nucleoside derivatives —	92
3.1.3	Sulfur-containing nucleobases —	94
3.1.4	Disulfide DNA crosslinks —	97
3.2	Dealing with the ambiguities of the genetic code—the “universal” base —	99
3.3	Duplex-stabilizing nucleobases —	103
3.3.1	Modified purines —	103
3.3.2	Modified pyrimidines —	105
3.3.3	Duplex stabilization by modified 5' and 3'-ends —	107
3.4	Influence of size, shape and hydrophobicity of nucleobases —	109
3.4.1	Base-pairs without hydrogen bonds —	109
3.4.2	Extended nucleobases —	112
3.5	Expanding the genetic alphabet—artificial DNA —	114
3.5.1	Orthogonal nucleobases —	114
3.5.2	Enzymatic synthesis of modified DNA—novel DNA polymerases —	115
3.6	DNA-protein contacts —	117
3.6.1	Study of DNA-protein contacts. Restriction endonucleases and bacterial DNA methylation —	117
3.6.2	Inhibitors of cytosine DNA methyltransferases —	120
	Bibliography —	121

Nucleobases are essential elements of the recognition and structural properties of nucleic acids as well as for the protein-nucleic acids interactions. In order to provide tools for structural and biological studies, as well as for the development of oligonucleotide probes with reporting groups a large number of researchers on nucleic acid chemistry have investigated on the chemical modification of nucleobases. Moreover, the search for alternative base-pairing schemes stimulates new views on the origin of life as well as potential building blocks for artificial genetic systems able to run in parallel with the natural one. In this chapter, we will describe the development of this interesting field.

---

**Carme Fàbrega, Ramon Eritja,** Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Networking Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Jordi Girona 18-26, E-08034 Barcelona, Spain, e-mails: carme.fabrega@iqac.csic.es, recgma@cid.csic.es

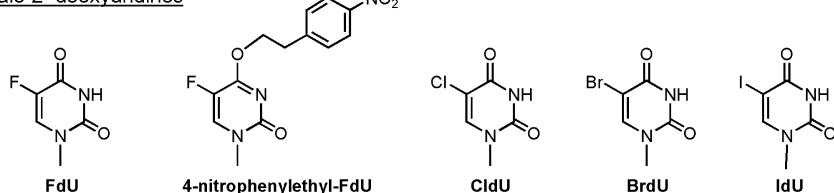
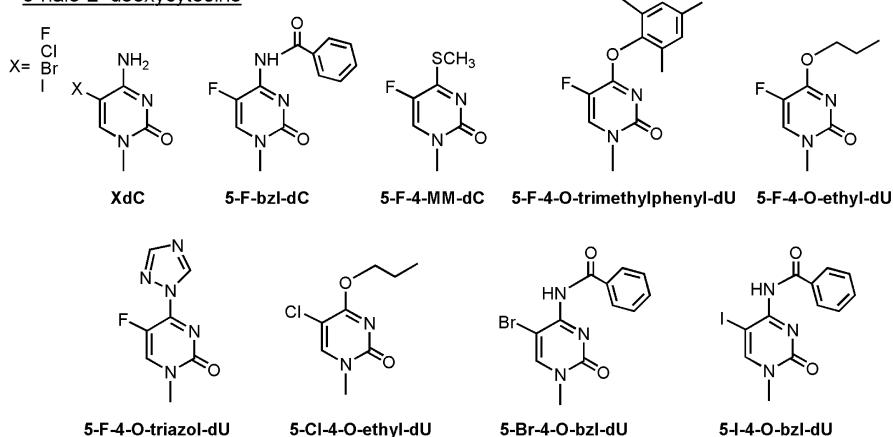
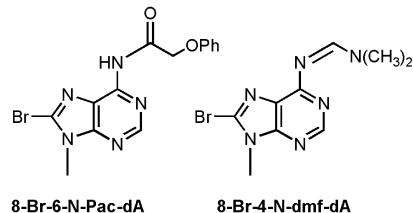
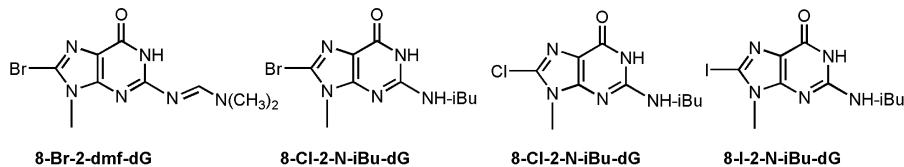
## 3.1 Hybridization and DNA mutagenesis

The description of the double helix structure by Watson–Crick [1, 2] generates the first hypothesis to explain the mutagenic properties of known synthetic nucleosides such as; 2-aminopurine 2'-deoxyriboside, 6-thioguanine 2'-deoxyriboside and 5-fluoro- and 5-bromo-2'-deoxyuridines when incorporated to DNA [3]. These nucleoside derivatives with similar hydrogen-bonding groups than natural nucleobases were able to alter the recognition of the correct complementary base by DNA polymerase and generate a higher rate of mutations. Several potential explanations for this behavior were proposed, including the intervention of rare tautomers [3], ionized species [4] as well as the formation of wobble base-pairs [5]. The synthesis of oligonucleotides carrying mutagenic bases has been critical to understand the role of several factors, including thermodynamic stability of base-pairs, the effect of the neighboring bases and the 3'-exonuclease proof-reading properties of DNA polymerases [6]. Oligonucleotides carrying mutagenic DNA lesions have been already described in previous chapters. In this chapter, we will detail the synthesis of oligonucleotides carrying nonnatural nucleosides with mutagenic properties and related nucleotides such as halogenated and thiolated nucleobases as well as other synthetic nucleosides.

### 3.1.1 Halogenated nucleobases

The introduction of halogens in the nonpairing positions of nucleobases (Figure 3.1) modifies its electronic distribution yielding to changes in the pKa, the hydrogen bonding abilities, tautomerism and reactivity. These changes are followed by dramatic alteration in the biological properties of the modified nucleotides and oligonucleotides.

5-halo-2'-deoxyuridines are between the most studied halogenated nucleobases. 5-Fluorouracil (FU) and derivatives such as 5-fluoro-2'-deoxyuridine or floxuridine are being used as chemotherapeutic agents [7]. In addition to act as inhibitors of thymidine synthetase, it has been shown that 5-fluorouracil nucleoside triphosphate derivatives may be incorporated into both RNA and DNA inducing the formation of base pairs with guanine instead of adenine [7, 8]. The study of the molecular bases of the mutagenic properties of FdU triggered the interest for the synthesis of oligonucleotides carrying FdU in defined positions demonstrating the presence of ionized forms on the FU: G base pairs [10]. Later on, it was confirmed that the efficacy of DNA polymerases to incorporate the wrong nucleotide was pH-dependent supporting a model in which the ionization of 5-halouracils is responsible for the mutagenic activity [11]. The synthesis of the oligonucleotides carrying FdU was done employing the phosphotriester [9, 12] and the phosphoramidite [13, 14] chemistries. Most of these studies use the FdU phosphoramidite without nucleobase protection [14] but it has been described the use of the 4-nitrophenylethyl group (Figure 3.1) for the protection of the  $O^4$  group [13]. Unprotected 5-fluorouracil nucleobase is stable to ammonia and, the deprotection of

5-halo-2'-deoxyuridines5-halo-2'-deoxycytosine8-halo-2'-deoxyadenosine8-halo-2'-deoxyguanosine

**Figure 3.1:** Structures of halogenated nucleobases which have been incorporated into oligonucleotides. Pac: phenoxyacetyl, Bzl: benzoyl; ibu: isobutyryl, dmf: dimethylformamidino.

FdU oligonucleotides is performed using the standard conditions. However, oligonucleotides carrying FdU protected with the 4-nitrophenylethyl group were deprotected using a 1,8-deazabicyclo[5.4.0]undec-7-ene (DBU) solution [13].

Short FdU oligomers have been described to be potent cytotoxic agents presenting less toxicity than FdU, as these oligomers can be hydrolyzed *in vivo* by nucleases to generate the active metabolite of FU therapy, FdU 5'-monophosphate [14, 15]. In addition and based on the stabilities of the FU.G and FU.A base pairs, FdU oligonucleotides have been used to screen gene libraries with A or G ambiguities [13].

Similar to FdU, CldU has been hypothesized to be an inhibitor of thymidine synthetase [16]. 5-Chloro-2'-deoxyuridine (CldU, Figure 3.1) can be produced in DNA by oxidation and chlorination with hypochlorous acid. Oligonucleotides carrying CldU have been synthesized and demonstrated that this lesion is not cleaved by glycosylases [16, 17]. Besides CldU and other halogenated pyrimidines bind with high affinity to methyl-CpG-binding proteins mimicking methylation signals [18]. The synthesis of CldU oligonucleotides starts with the preparation of CldU by the reaction of dU with *N*-chlorosuccinimide [19, 20], followed by protection of the 5'-OH with the dimethoxytrityl (DMT) group and phosphitylation of the 3'-OH. Ammonia deprotection is suggested to be done at room temperature instead of 55 °C [21]. Structural studies showed that CldU.A base pair adopts a Watson–Crick base pair similar to the T.A pair [21]. However, CldU.G base pair is pH-dependent between wobble geometry similar to a G.T base pair at neutral pH and an ionized base pair at alkaline pH [22].

5-Bromo-2'-deoxyuridine (BrdU) and 5-iodo-2'-deoxyuridine (IdU) (Figure 3.1) have been profusely used as thymine analogs for X-ray diffraction experiments [23] and in the study of nucleic acids-protein interactions using photocrosslinking [24]. During the synthesis of oligonucleotides carrying BrdU [25, 26] and IdU [27, 28] the formation of a side product was observed during ammonia deprotection. This side reaction was the displacement of the bromine or iodine by ammonia generating 5-aminouracil-oligonucleotides. This side product is minimized by performing the ammonia deprotection step at room temperature instead of 55 °C [28]. Similar to FdU and CldU, DNA duplexes carrying BrdU.A [29] and BrdU.G [30] base pairs have been studied by NMR obtaining similar results than in the FdU and CldU duplexes. Ionized base pairs observed in BrdU.G duplexes were in agreement with the pH-dependent fidelity of DNA polymerases, supporting the hypothesis in which ionization of 5-halouracils is responsible for their mutagenic activity [11].

Similar to uracil derivatives, 5-halocytosine derivatives have important biological properties but in the cytosine series the presence of the amino group provides an extra step for the preparation of the phosphoramidites. Oligonucleotides carrying 5-fluoro-2'-deoxycytidine (FdC) were first synthesized by enzymatic methods [31, 32] producing small amounts of FdC-oligonucleotides for biological evaluation. The first attempts to prepare the benzoyl derivative of FdC (Figure 3.1) were discontinued due to the instability of FdC in the acidic conditions used during the removal of the DMT group [31]. This instability was reevaluated demonstrating that benzoyl-protected FdC was stable to detritylation [33]. These authors demonstrate also that ammonia deprotection of FdC-oligonucleotides should be performed at room temperature to avoid side reactions observed at 50 °C, for the preparation of benzoyl-protected FdC benzoic

anhydride should be used as benzoyl chloride fails to produce the protected FdC nucleoside [33]. The same authors developed a second phosphoramidite for the preparation of FdC-oligonucleotides. 5-Fluoro-4-methylmercapto-2'-deoxycytidine phosphoramidite (5-F-MM-dC, Figure 3.1) was incorporated to oligonucleotides and was able to generate FdC by displacement of the methylthio group by ammonia in the final deprotection step. Unfortunately, FdC-oligonucleotides prepared by displacement of the methylthio group were impurified with the presence of 5-fluorouracil derivative. These authors provide evidence that this impurity was not formed during the ammonia treatment but during the iodine oxidation step of the synthesis cycle, where was also oxidizing and hydrolyzing the methylthio group [33]. A third method was described by the group of Verdine making use of a convertible nucleoside [34]. In this process, the phosphoramidite derivative of 5-fluoro-4-O-(2,4,6-trimethylphenyl)-2'-deoxyuridine (Figure 3.1) was prepared. This derivative is stable to acid and oxidizing solutions; however, it undergoes rapid and quantitative transformation to FdC during the ammonia treatment. Another method was described using unprotected FdC phosphoramidite (Figure 3.1), which is based on the observation that the reactivity of the amino group of FdC is low enough to use the phosphoramidite without protection of the nucleobase [35]. Alternative methods are based in the use of the phosphoramidite of 5-fluoro-4-O-ethyl-2'-deoxyuridine [36] (5-F-4-O-ethyl-dU, Figure 3.1) and the phosphoramidite of 5-fluoro-4-triazol-2'-deoxyuridine [37] (5-F-4-triazol-dU, Figure 3.1). 5-Fluoro-4-O-ethyl-2'-deoxyuridine phosphoramidite was useful for the preparation of <sup>15</sup>N-enriched-5-fluorouracil oligonucleotides [36]. Oligonucleotides carrying FdC are suicidal inhibitors of DNA methyltransferase as FdC residues react with the catalytic cysteines establishing a covalent crosslink between FdC and the enzyme [38]. Several other nucleosides without halogens are also inhibitors of DNA methyltransferases and they will be commented in other sections.

The synthesis of oligonucleotides carrying 5-chloro-2'-deoxycytosine (CldC) has been reported [39]. In this method the CldC synthon was the phosphoramidite of 5-chloro-4-O-ethyl-2'-deoxyuridine [39] (5-Cl-4-ethyl-dC, Figure 3.1). The amino group at position 4 is introduced during the ammonia treatment by nucleophilic displacement of the ethoxy group as described in the synthesis of FdC-oligonucleotides [36]. However, the authors demonstrated that the hydrolysis product CldU is not formed. Oligonucleotides carrying CldC are not substrates for uracil glycosylases [18, 39], neither bind bacterial DNA methyltransferases [40] even though they have enhanced affinity to methyl-CpG-binding proteins 2 [18].

The synthesis of oligonucleotides carrying 5-bromo- (BrdC) and 5-iodo-2'-deoxycytidine (IdC) (Figure 3.1) have been reported [28]. The corresponding phosphoramidites carrying the benzoyl group for the protection of the 4-amino function are commercially available. The halogenated dC nucleotides are not stable to concentrated ammonia solutions at 60 °C giving 5-amino-2'-deoxycytidine and other side compounds. When the deprotection was performed at room temperature, the formation of these side compounds is minimized [28]. In addition to the use of

5-halocytosines in X-diffraction experiments, oligonucleotides carrying BrdC can be used in photocrosslinking experiments by irradiation with UV light [41].

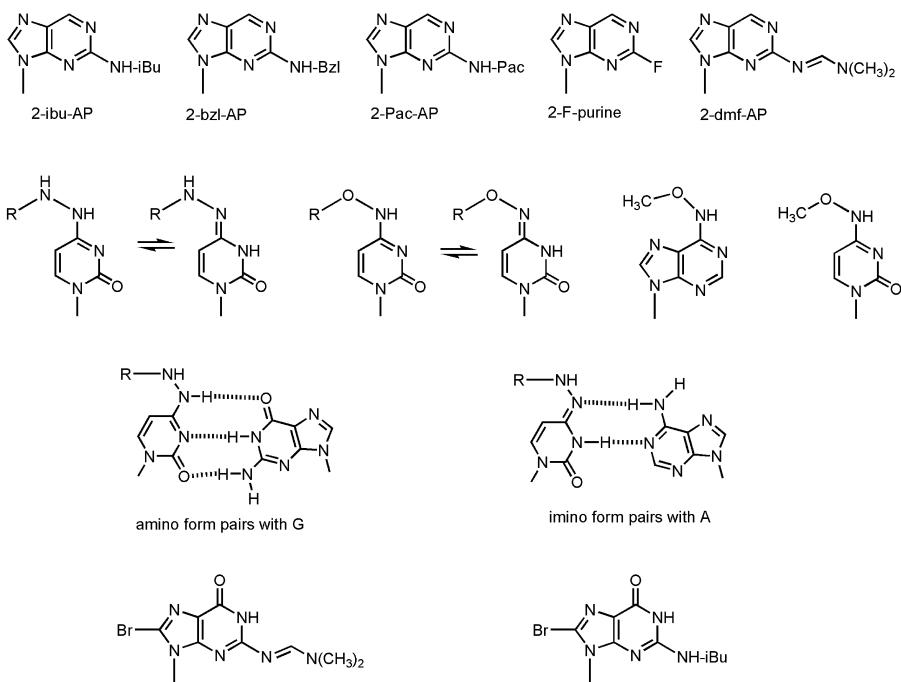
Another photoreactive nucleoside is 8-bromo-2'-deoxyadenosine (8-Br-dA). The synthesis of oligonucleotides carrying 8-Br-dA have been also described [42] using the phosphoramidite of 8-Br-dA carrying the phenoxyacetyl (Pac) group (Figure 3.1) for the protection of the 6-amino function. The natural nucleobases are also protected with labile groups such as Pac and isobutyryl groups. This nucleoside is sensitive to ammonia but it can be deprotected in 2 hr using concentrated ammonia at room temperature. Oligonucleotides carrying 8-Br-dA are also key intermediates for the synthesis of oligonucleotides carrying the photoreactive nucleoside 8-azido-2'-deoxyadenosine [43, 44]. The azido group is incorporated in the DNA by nucleophilic displacement of the bromide with an excess of sodium azide after the assembly of the sequence but before the ammonia treatment that is performed at room temperature to minimize the formation of the hydrolysis product 8-oxo-dA [43]. Oligonucleotides carrying 8-azido-dA have been used for the photocrosslinking of the oligonucleotide to the DNA binding subunit of the NF- $\kappa$ B p50 homodimer [43].

The synthesis of oligonucleotides carrying 8-bromo-2'-deoxyguanosine (8-Br-dG) has been also reported [45, 46]. The isobutyryl (iBu) and the dimethylaminoformamidino (dmf) groups were selected for the protection of the 2-amino group of 8-Br-dG. Oligonucleotides carrying 8-Br-dG stabilize the Z-DNA form [45], and the G-quadruplex [46] as bulky groups in position 8 shift the equilibrium to the *syn* conformation. Oligonucleotides carrying 8-Br-dG were also used to demonstrate that one-electron attachment reaction by  $\gamma$ -radiolysis is enhanced in Z-DNA form [47]. The stabilization of the left-handed Z-DNA form by 8-Br-dG can be enhanced by the incorporation of an ethynyl group at position 2' in addition to the 8-bromo group [48]. Oligonucleotides carrying 8-Br-dG and IdU are also useful intermediates for the introduction of aromatic compounds by post-synthetic methods using palladium catalysts [49]. These processes will be discussed in the next sections.

The synthesis of oligonucleotides carrying 8-chloro- (8-Cl-dG) and 8-iodo-2'-deoxyguanosine (8-I-dG) has been also reported [50] using the appropriate phosphoramidite protected with the isobutyryl group (Figure 3.1). Similar to the observed with 8-Br-dG, the presence of 8-Cl-dG and 8-I-dG destabilize the duplex structure in line with the destabilization of the *anti* conformation of the dG observed for guanine derivatives carrying bulky substituents at position 8.

### 3.1.2 2-Aminopurine and methoxyamine nucleoside derivatives

2-Aminopurine (2-AP, Figure 3.2) is an isomer of adenine which has the exocyclic amino group in position 2 instead of position 6. For this reason, 2-AP can form a base pair with thymine which is very similar to the natural A.T base pair [51] but having one hydrogen bond near the minor groove instead of the major groove. This difference has



**Figure 3.2:** Structures of protected nucleobases which have been used for the incorporation of 2-aminopurine and methoxyamine nucleosides into oligonucleotides. Amino imino tautomerism of methoxyamine nucleosides. Pac: phenoxyacetyl, Bzl: benzoyl; ibu: isobutyryl, dmf: dimethylformamidino.

been used by several authors to characterize DNA-protein interactions [52] and DNA bending properties [53]. 2-AP can also form base pairs with adenine [54] and cytosine [51, 55] but not with guanine [56]. The 2-APC base pair is responsible for the generation of transition mutations during DNA replication [57]. Although it has been suggested that this base pair involved the formation of the imino tautomer of 2-AP, structural studies showed that the 2-APC base pair adopts a wobble geometry at neutral and high pH and protonated species are observed in acid pH [58–61].

The preparation of oligonucleotides carrying 2-AP was first described by Eritja et al. [56]. In this method, 2-aminopurine-2'-deoxyribonucleotide was prepared enzymatically from the free base [62] and the amino function was protected with the isobutyryl (ibu) group [56]. Afterwards, the synthesis of 2-aminopurine phosphoramidite protected with the benzoyl (Bzl) group was described starting from dG [63]. In this method, dG was protected with the Bzl group, converted to the 6-hydrazino derivative which was reduced with silver (I) oxide [63]. This method can be scaled-up and detailed protocols have been published [64, 65]. The Bzl group was also selected for the protection of the 2-amino function during the synthesis of oligoribonucleotides containing 2-aminopurine [66]. The synthesis of the ibu derivative of 2-aminopurine-2'-

deoxyriboside has been described by the reduction of 6-chloroguanosine [67] and 6-thioguanosine [68]. In addition, oligodeoxynucleoside methylphosphonates containing 2-aminopurine have been prepared using the base-labile phenoxyacetyl (Pac) group for the protection of the amino function of 2-aminopurine [69]. A major concern is the high stability to ammonia of the Bzl-protected derivative of 2-aminopurine produced by McLaughlin's protocol [67, 69]. In this sense, the preparation of the Pac-protected derivative of 2-aminopurine solved this problem, however, it implied a long synthetic route in which the Bzl group is first introduced and then removed to allow the introduction of the Pac protecting group [69]. The preparation of oligonucleotides containing  $^{15}\text{N}$ -2-aminopurine has been described using 2-fluoropurine-2'-deoxynucleoside as convertible nucleoside [70]. Currently, the phosphoramidite of 2-AP protected with the dimethylformamidino (dmf) group [71] is commercially available. 2-Aminopurine is one of the most widely used fluorescent probes for DNA structural studies as well as a local probe for DNA-binding enzymes [72] such as DNA polymerases [73, 74], helicases [75] and DNA methyltransferases [76].

The reaction of hydrazine and hydroxylamine derivatives with DNA produces several modified nucleosides that are mutagenic.  $N^4$ -amino-2'-deoxycytidine and  $N^4$ -hydroxy-2'-deoxycytidine (Figure 3.2) are some of the most studied derivatives as they are highly mutagenic and these derivatives can be prepared bisulfite-catalyzed transamination of dC with hydrazine and hydroxylamine [77–80]. The proposed mechanism for the mutagenic properties of these compounds is based on the formation of amino imino tautomers. The main tautomer of  $N^4$ -amino-2'-deoxycytidine is the amino tautomer that will pair with guanine, while the less abundant imino form will pair with adenine (Figure 3.2). Oligonucleotides carrying  $N^4$ -amino-2'-deoxycytidine and  $N^4$ -hydroxy-2'-deoxycytidine have not been prepared because of the lability of these nucleosides to the synthesis conditions but the methoxyamine derivatives (Figure 3.2) can be prepared as they are stable to oligonucleotide synthesis conditions. The synthesis of oligonucleotides carrying  $N^4$ -methoxy-2'-deoxycytidine [81, 82] (Figure 3.2) and  $N^6$ -methoxy-2'-deoxyadenine [83, 84] (Figure 3.2) has been also reported. Thermodynamic, NMR studies and template extension experiments were in agreement with stable base pair with the four natural bases due to the possibility of forming base pairs with both the amino and the imino form of these nucleobases [81–84]. In addition, DNA polymerase incorporates mispaired nucleotides opposite to the modified nucleosides in higher frequencies than the natural bases [81–84]. These properties have suggested the potential use of these analogs as a degenerate base. These properties will be discussed in the next sections.

### 3.1.3 Sulfur-containing nucleobases

Chemical substitution of some oxygen atoms of the nucleobases by sulfur generate a series of modified oligonucleotides with important biological [85] and biophysical

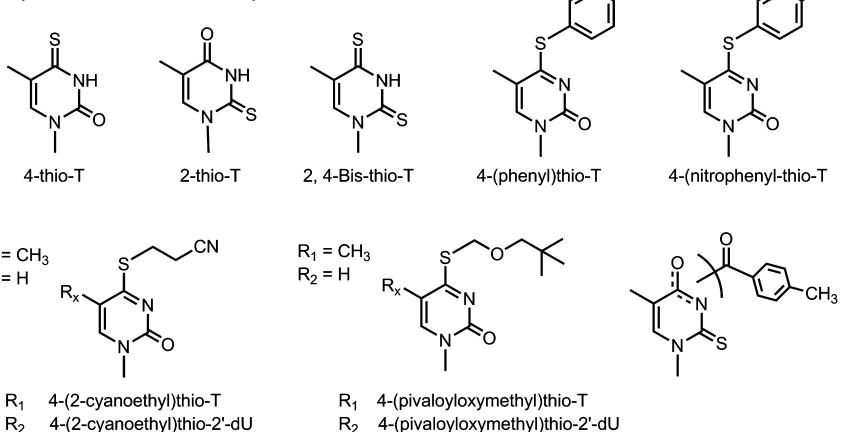
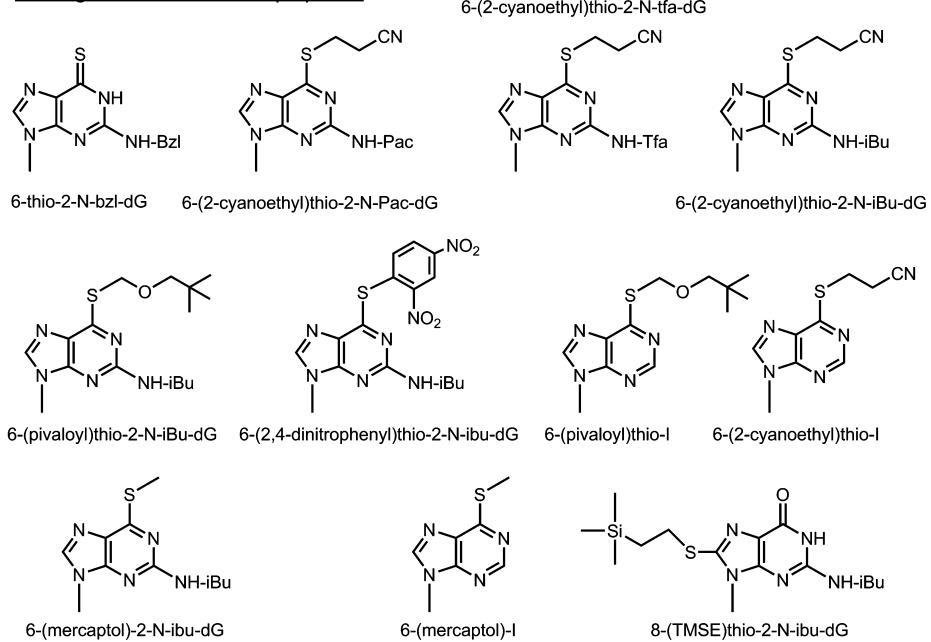
properties, such as enhanced UV absorption, fluorescent [86] properties and photocrosslinking with other nucleic acids and with proteins [87].

The synthesis of oligonucleotides carrying unprotected 4-thiothymidine and 2-thiothymidine (Figure 3.3) was first reported [88–90]. Then *S*-phenyl and *S*-(4-nitrophenyl) 4-thiothymidine (Figure 3.3) phosphoramidite derivatives were produced [91]. Once incorporated into an oligonucleotide the final deprotection was done in two steps. First, the supports were treated with  $\text{CH}_3\text{COK}$  in ethanol at 55 °C to convert the *S*-phenyl to *S*-acetyl derivatives, followed by ammonia deprotection [65, 91]. A more efficient method used the *S*-(2-cyanoethyl)-4-thiothymidine (Figure 3.3) phosphoramidite [92]. Once incorporated into DNA, ammonia deprotection removed the 2-cyanoethyl group generating the desired 4-thiothymidine residue [92]. The 2-cyanoethyl group was also used for the synthesis of oligonucleotides carrying 4-thio-2'-deoxyuridine [93, 94]. Moreover, the pivaloyloxymethyl group (Figure 3.3) has been used for the protection not only of 4-thiouracil and 4-thiothymine but also for the protection of 6-mercaptopurine derivatives [95–97].

The synthesis of oligonucleotides carrying 2'-deoxy-2-thiouridine and 2-thiothymidine (Figure 3.3) was highly improved with the use of the toluoyl group for the protection of the nucleobase [98]. This group is stable to oligonucleotide synthesis conditions and is removed easily in the standard ammonia conditions. The hybridization properties and replication efficiency of thiothymidines have been determined [99, 100] in relation to the potential participation of rare tautomeric forms in the base pairs with natural bases. 4-Thiothymidine and 2-thiothymidine behave as natural thymine except for 2-thiothymidine that do not form base pairs with 2-aminoadenine [101].

6-Thioguanine and 6-mercaptopurine are antimetabolites used in the treatment of leukemia. These bases are incorporated into DNA and their toxicity depends on the *S*-methylation by *S*-adenosylmethionine [102]. The synthesis of oligonucleotides carrying 6-thioguanine and 6-mercaptopurine has been reported by several authors. Rapaport described the preparation of the *o*-chlorophenylphosphate derivative of 6-thio-dG used for the assembly of oligonucleotides by the phosphotriester method [103]. He recommended the use of the *N*-benzoyl group (Figure 3.3) for the protection of the  $N^2$ -amino function as the isobutyryl and the acetyl groups were too labile [103]. The thiol group was not protected and some reaction with the coupling agents was observed. These side compounds were regenerated by treatment with benzenethiol solutions. Some stability problems were also observed during the ammonia treatment which was solved by changing the deprotection conditions. In order to circumvent these problems, the protection of the thiol group with 2-cyanoethyl (Figure 3.3) was recommended together with the use of phenoxyacetyl (Pac) group for the protection of the  $N^2$ -amino function [104].

The phosphoramidite derivative carrying the *S*-(2-cyanoethyl) and the Pac groups was assembled in good yields. For the deprotection, a mixture of sodium hydroxide and sodium hydrogen sulfide was recommended to avoid desulfurization of 6-thioguanine [104]. Alternatively, the synthesis of the phosphoramidite derivative

Thiothymidine and Thio-2'deoxyuridine6-Thioguanine and 6-mercaptopurine

**Figure 3.3:** Structures of protected nucleobases which have been used for the incorporation of sulfur containing nucleosides into oligonucleotides Pac: phenoxyacetyl, Bzl: benzoyl; ibu: isobutyryl.

of 6-thioguanine carrying the *S*-(2-cyanoethyl) and the benzyl groups was also reported [65, 105]. This phosphoramidite has been successfully used for the synthesis of photoreactive substrates of the Eco RV restriction endonuclease [106, 107]. Another method uses the phosphoramidite derivative of 6-thioguanine carrying the

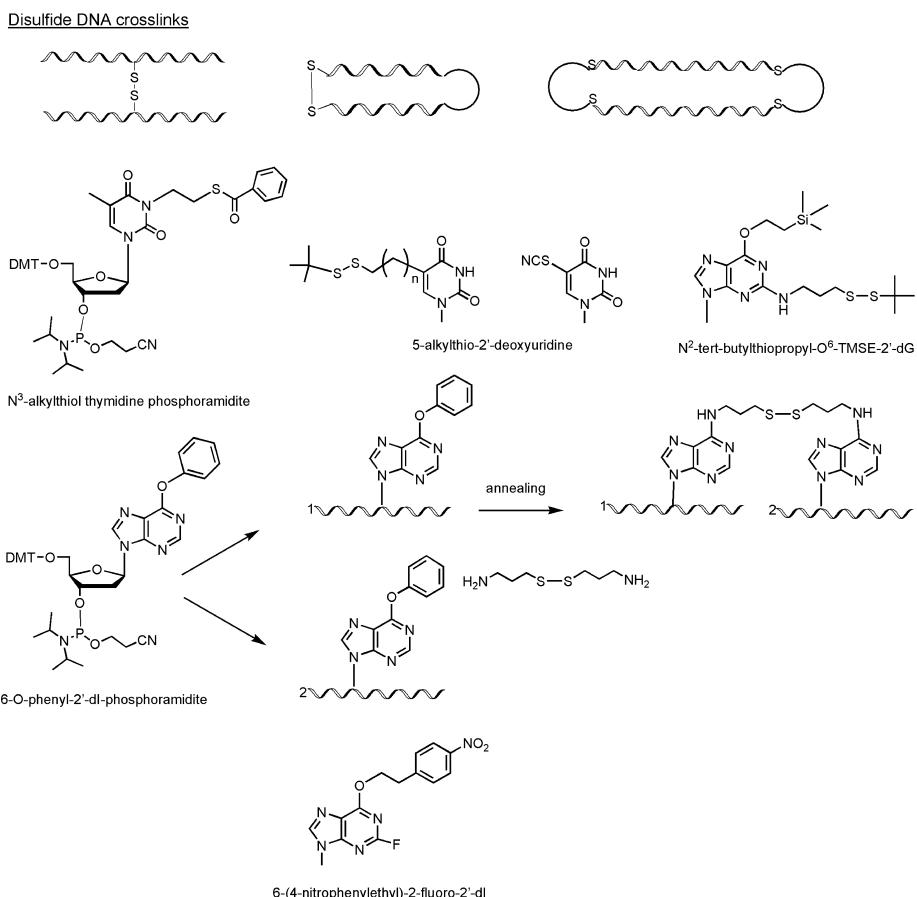
*S*-(2-cyanoethyl) and the trifluoroacetyl groups [108, 109]. The trifluoroacetyl derivative was obtained by an undesired displacement of the isobutyryl group during the introduction of the thiol moiety in the guanine [108]. The phosphoramidite derivative of 6-thioguanine carrying the *S*-(2-cyanoethyl) and the isobutyryl groups was obtained in good yields when the methodology was changed for the synthesis of the 6-thioguanine [108]. Both *N*<sup>2</sup>-trifluoroacetyl and *N*<sup>2</sup>-isobutyryl phosphoramidites were appropriate for DNA synthesis [108, 109]. In addition, the phosphoramidite derivative of 6-thioguanine carrying the *S*-pivaloyl and the *N*<sup>2</sup>-isobutyryl group has been reported [110]. Oligonucleotides carrying 6-thioguanine as well as other *N*<sup>6</sup>- and *O*<sup>6</sup>-derivatives of guanine are also prepared by a post-synthetic protocol using the 6-(2,4-dinitrophenyl)thio-2'-deoxyguanine phosphoramidite protected with the *N*<sup>2</sup>-isobutyryl group [111].

The synthesis of oligonucleotides carrying 2'-deoxy-6-thiinosine (6-mercaptopurine) has been also described [110, 112, 113]. In one case, the thiol group was protected with the pivaloyl group (110) and in the second case with the 2-cyanoethyl group [112, 113]. Both methods proved to be efficient for the production of oligonucleotides carrying 6-mercaptopurine. These oligonucleotides react with complementary oligonucleotides carrying 4-thiothymine forming site-specific disulfide bridges [112]. Oligonucleotides carrying 2'-deoxy-6-methylthiinosine (6-methylthiopurine), 2'-deoxy-6-methylthioguanine (6-methylthioguanine) and 4-(methylthio)-thymine have been prepared to demonstrate that the cytotoxicity of 6-thioguanine is due to the methylation of the thiol group of these nucleosides [102, 114, 115].

The synthesis of oligonucleotides carrying 8-thioguanine (Figure 3.3) has been described [116]. In this case, the thiol group was protected with the (trimethylsilyl)ethyl (TMSE) group which is removed with tetrabutylammonium fluoride (TBAF). The use of a protective group was justified by the difficulty to introduce the 2-cyanoethyl group. The TMSE group was stable to ammonia, generating TMSE protected oligonucleotides which were treated with TBAF to generate the unprotected 8-thioguanine residue [116]. 8-Thioguanine forms base pairs with A that have similar stability to 8-oxo-A [116].

### 3.1.4 Disulfide DNA crosslinks

As mentioned above, oligonucleotides carrying 6-mercaptopurine react with complementary oligonucleotides carrying 4-thiothymine yielding site-specific disulfide bridges [112]. The interest for constrained DNA structures triggered the development of phosphoramidites carrying thiol groups [117, 118] to generate reversible DNA structures, such as cyclic DNA structures or cross-linked duplexes (Figure 3.4). The synthesis of a cyclic stem-loop hairpin using an *N*<sup>3</sup>-alkylthiol thymidine derivative for the crosslinking of both 3' and 5'-ends was reported [119–123]. The phosphoramidite used in this work is shown in Figure 3.4. The thiol group is protected with the benzoyl group being removed with ammonia in the presence of an excess of dithio-



**Figure 3.4:** Structures of protected nucleobases which have been used for the incorporation of disulfide DNA crosslinks.

threitol (DTT) which acts as scavenger of the acrylonitrile formed in the removal of the 2-cyanoethyl phosphate group. A second approach is the use of 5-alkylthio-2'-deoxyuridine derivatives (Figure 3.4). The synthesis of the phosphoramidite implies the use of palladium-catalyzed coupling of 2'-deoxy-5-iodouridine with the appropriate acetylenic alcohol followed by hydrogenation. Alternatively, the reaction of allyl chloride with 5-chloromercury-2'-deoxyuridine provides the route for the preparation of the 5-propylthio dU derivative [124]. The *S*-*tert*-butylthio group is used for the protection of the thiol group (Figure 3.4). The combination of the *N*<sup>3</sup>-ethylthiol thymidine and 5-ethylthio-2'-deoxyuridine phosphoramidites allow the preparation of a crosslinked DNA triplex connecting a terminal thymidine in the Hoogsteen position with the corresponding dU in the Watson–Crick position [125]. Disulfide crosslinks can be also introduced during solid-phase by preparing an *S*-*S* crosslinked dinucleotide

phosphoramidite [126]. The phosphoramidite of 5-thiocyanate 2'-deoxyuridine has been also reported to generate thiol-oligonucleotides that can be used for oligonucleotide functionalization [127].

Another possibility to introduce a thiol group in the nucleobases is the use of the dG phosphoramidite shown in Figure 3.4 [118, 128]. The thiol group is attached to the  $N^2$ -position of guanine through a propyl linker and protected with the *tert*-butylthio group. The  $O^6$  position is protected with the (trimethylsilyl)ethyl (TMSE) group. This group is labile to acids and is removed in the first detritylation reaction after the coupling of the phosphoramidite [128]. Oligonucleotides carrying the  $N^2$ -thiopropyl linker protected with the *tert*-butylthio group were used to crosslink DNA to HIV-1 reversed transcriptase to facilitate structural determination [129].

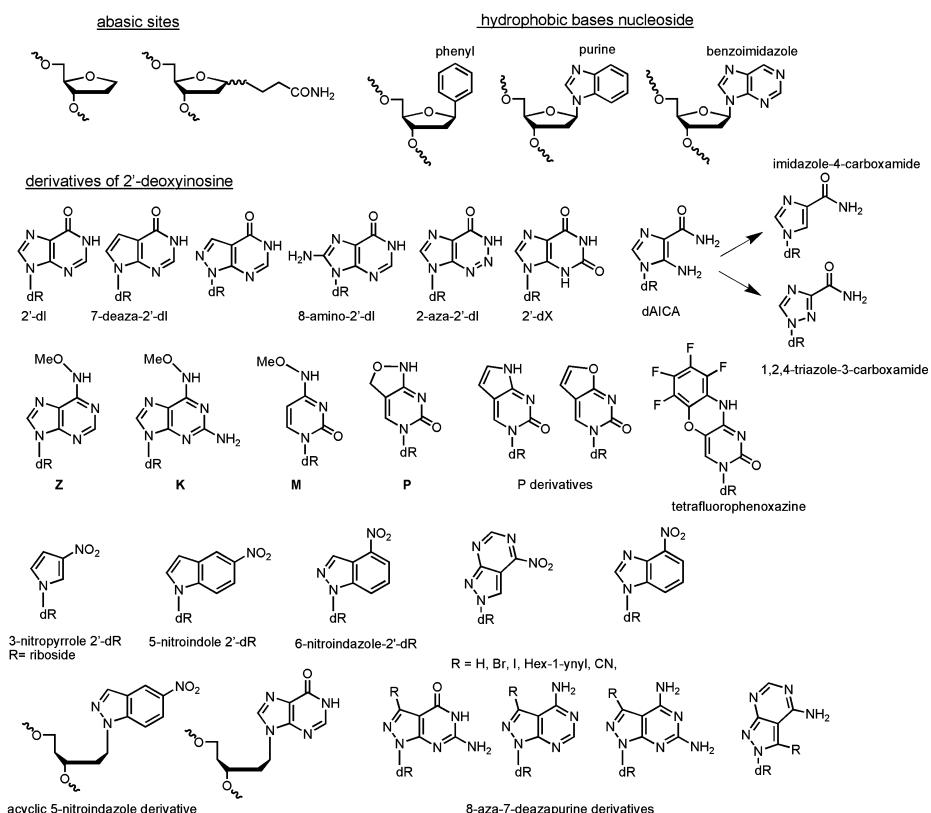
Alternatively, site specific crosslinks have been introduced by reaction of 4-thio-2'-deoxyuridine and  $N^7$ -guanine with bifunctional bromoacetamido compounds [130]. Covalent crosslinking is performed by hybridization of the complementary oligonucleotides carrying 4-thio-2'-deoxyuridine paired with the target guanine followed by the addition of the bromoacetyl derivatives [131].

A simplified method for the formation of specific disulfide crosslinks has been also described [132–134]. The method relies on the use of postsynthetic modification of a common nucleotide that was reported as convertible nucleoside approach. Figure 3.4 shows the main steps of the method. The phosphoramidite of  $O^6$ -phenyl-2'-deoxyinosine was used for the introduction of the  $O^6$ -phenylhypoxanthine residue. This nucleoside is stable to ammonia at room temperature and oligonucleotides carrying  $O^6$ -phenylhypoxanthine residues can be obtained. Treatment with aminoalkylthiol disulfides of the appropriate duplex carrying the reactive nucleotides in opposite strands allows the formation of specific crosslinks [132, 133]. A similar method was described using  $O^6$ -(4-nitrophenylethyl)-2-fluoro-2'-deoxyinosine as convertible nucleoside [134]. Several thiol linkers were studied. Depending on the length of the linker, the modified base pairs were either stabilized or destabilized. When the base-pairs were destabilized, the oligonucleotide was bound by a DNA (cytosine-5)-methyltransferase more tightly than the corresponding unmodified oligonucleotide [134].

## 3.2 Dealing with the ambiguities of the genetic code—the “universal” base

Synthetic oligonucleotides are profusely used as primers for polymerase chain reaction (PCR) [135] as well as probes for the detection of cloned DNA sequences. In the 1980s when a partial protein sequence was available but the DNA sequence not, special degenerate primers and probes were used for the detection or amplification of a particular unknown DNA sequence. Due to the degeneration of the genetic code, the degenerate primers and probes were usually a complex mixture of oligonucleotides,

often containing truncated sequences as a result of the difficulty of the purification. To circumvent this problem, an important effort was made to design bases analogs that potentially could base pair equally well with any of the four natural bases. If successful, this universal base will avoid the use of degenerate probes and it will simplify the synthesis and characterization of the probes. First, a small group of compounds including the abasic sites (Figure 3.5) [136, 137], or nucleoside derivatives carrying hydrophobic bases such as; a phenyl group (Figure 3.5) [136] or a purine (Figure 3.5) [138] or a benzimidazole (Figure 3.5) [139] were described. However, these compounds were found to form duplexes significantly less stable than unmodified oligonucleotides. As mentioned above, oligonucleotides carrying 5-fluorouracil (Figure 3.1) were described to form stable pairs with adenine and guanine [13] with the possibility of being used to solve C/T misambiguities.



**Figure 3.5:** Structures of compounds which have been studied as potential “Universal base.”.

Oligonucleotides carrying 2'-deoxyinosine (dI) were the first and the most appropriate solution for the replacement of degenerated probes [140]. Inosine was known to

be found on the wobble position of the tRNA anticodon where it appears to pair with A, C and U in the decoding process. The thermal stability of hypoxanthine base pairs with the natural bases show a relative preference to base pair with C, as expected for the similarity of this base to G. However, base pairing with A, G and T were favored when compared with guanine [141–143]. Other 2'-deoxyinosine derivatives have been studied. For example, oligonucleotide carrying 7-deaza-2'-deoxyinosine and allopurinol 2'-deoxyribofuranosine (Figure 3.5) have been described [144]. Allopurinol base pairs with natural bases were found to be more stable than hypoxanthine base pairs. Furthermore, the allopurinol was found to have an extraordinary acid stability of its glycosidic bond [144]. Oligonucleotides carrying 8-amino-2'-deoxyinosine were also found to have interesting base-pairing properties [145] besides triplex stabilization properties.

Another possibility to enhance base pairing with the four natural bases is to introduce a hydrogen bond acceptor in position 2. In this sense, the incorporation of one nitrogen atom in position 2 yields 2-aza-2'-deoxyinosine [146] and the incorporation of an oxygen atom produces 2'-deoxyxanthosine [138] (Figure 3.5). These configurations allow drawing two hydrogen bonds for the four natural bases. The synthesis and properties of xanthine oligonucleotides have been reported by several authors [138, 147–149]. The use of the double protection for the xanthine base is reported to be the most efficient method for the production of these oligonucleotides [147–149]. Unfortunately, xanthine-containing oligonucleotides were found to have low melting temperatures at neutral pH because the  $pK_a$  of xanthine is 5.5. For this reason, at neutral pH, xanthine is negatively charged preventing base pairing [138]. Melting temperatures of oligonucleotides carrying 2-aza-2'-deoxyinosine paired with the four natural bases show little variation among different natural bases but they were too low for practical uses.

Another interesting nucleoside that has been suggested as a candidate for an ambiguous base is the 2'-deoxy derivative of the central intermediate in the *de novo* purine biosynthesis, 5-aminoimidazole-4-carboxamide 2'-deoxyriboside (dAICA) (Figure 3.5). The synthesis of the dAICA triphosphate [150] and the solution synthesis of short oligonucleotides carrying dAICA [151] was reported. The AICA nucleobase was protected with the benzyloxycarbonyl group which was removed by catalytic hydrogenation [151]. Furthermore, oligonucleotides carrying dAICA were also obtained by a side reaction during the synthesis of oligonucleotides containing 2-azahypoxanthine protected with the *N,N*-diphenylcarbamoyl group [152]. Other dAICA-related nucleosides such as imidazole-4-carboxamide and 1,2,4-triazole-3-carboxamide (Figure 3.5) were also introduced to oligonucleotides using the corresponding ethyl carboxylate derivatives to avoid phosphorylation of the carboxamido group [153].

In previous sections, it was described that oligonucleotides carrying methoxyamine nucleoside derivatives that have an amino imino tautomeric constant near unity, pair equally well with the corresponding purine or pyrimidine bases [81–84]. This observation was the basis of the development of a series of nucleotides

to deal with ambiguous positions. Oligonucleotides carrying *N*<sup>6</sup>-methoxyadenine (**Z**), 2-amino-6-methoxyaminopurine (**K**), *N*<sup>4</sup>-methoxy cytosine (**M**) and 6*H*, 8*H*-3,4-dihydropyrimidino[5.4-c] [1,2]-oxazin-7-one (**dP**) (Figure 3.5) were prepared and studied for their potential application in the simplification of degenerate oligonucleotides [154–156]. The combination of **P** (for C/T ambiguity) and **K** (for A/G ambiguity) was found to be superior to the use of dI alone in PCR experiments [157]. Furthermore, it has been described that **P**, **Z** and **K** directed nucleotide incorporation by *Taq* DNA polymerase. Adenine was inserted in front of pyrimidine-like **P** and T opposite purines **K** and **Z** [158]. A more detailed study confirmed the insertion of natural bases opposite degenerate bases **P** and **K** by *Taq* DNA polymerase, but it was shown that the pyrimidine-like **P** directed the insertion in the opposite side of 60 % A and 40 % G while purines **K** directed the insertion of 87 % of T and 13 % of C [159]. Other bicyclic derivatives similar to nucleotide **P** were also designed (Figure 3.5) showing interesting hybridization properties [160, 161]. A tricyclic pyrimidine nucleoside, tetrafluorophenoxazine (Figure 3.5), was also incorporated into oligonucleotides showing an enhanced recognition to both adenine and guanine bases [162].

One of the most successful thoughts for the design of new candidates for a universal base was to return to maximizing stacking interactions while avoiding groups forming hydrogen bonds. The first candidate of this generation was 3-nitropyrrole 2'-deoxyriboside (Figure 3.5) [163]. This compound was selected because of the structural and electronic similarity to 4-nitropyridine that generates the smallest known DNA intercalating agents. Oligonucleotides carrying several 3-nitropyrrole units consecutively were successfully used as degenerate primers for DNA amplification by PCR [163]. The success of the 3-nitropyrrole stimulated the search for other nitroaromatic compounds. From these studies, 5-nitroindole 2'-deoxyriboside (Figure 3.5) show the best duplex stability pairing with any of the four natural bases [164]. When several modifications were present at dispersed positions, oligonucleotides carrying 5-nitroindole were performing better in primers for DNA sequencing and PCR than 3-nitropyrrole oligonucleotides [165]. In addition, oligonucleotides carrying 5-nitroindole had enhanced stability when incorporated in bulges or pendant base at either 5'- or 3'-ends [166]. This result was justified for the enhancement of stacking interactions that stabilize DNA secondary structures. Other interesting nucleosides carrying nitro aromatic residues were the regioisomers *N*<sup>1</sup>- and *N*<sup>2</sup>-2'-deoxyribosides of 4-nitroindazole [167], 4-nitrobenzimidazole [168] and the acyclic 5-nitroindazole derivative [169] (Figure 3.5). Especially, the acyclic derivative of 5-nitroindazole has interesting hybridization properties with the four natural bases when compared with a series of imidazole, triazole and hypoxanthine acyclic derivatives [169, 170].

Another series of nucleosides with interesting hybridization properties were the 8-aza-7-deazapurine derivatives including 7-substituted 8-aza-7-deaza-2'-deoxyguanosine [171], 8-aza-7-deaza-2'-deoxyadenosine [172] and 8-aza-7-deaza-purine-2,6-diamine [173, 174] as well as the corresponding 8-aza-7-deazaadenine derivative linked

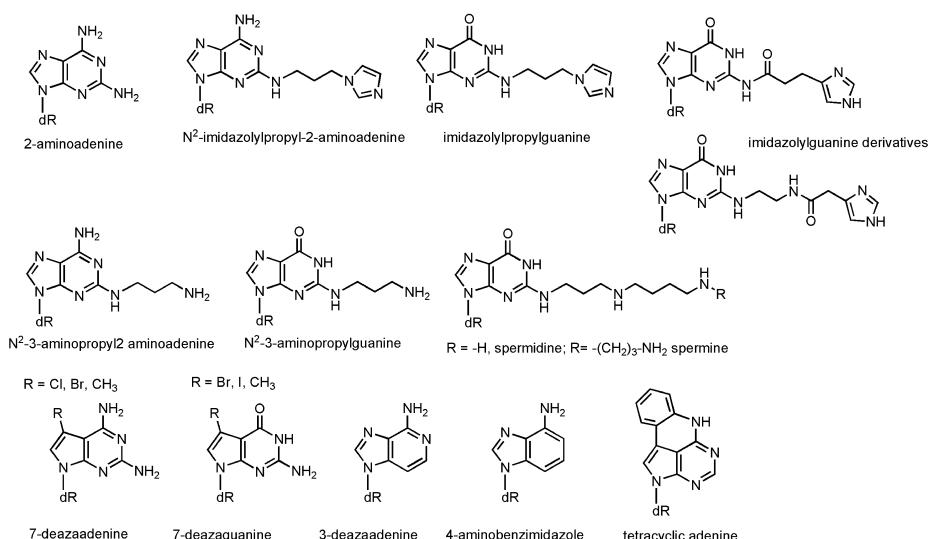
via the 8-position [175, 176] (Figure 3.5). Oligonucleotides carrying these last compounds form duplexes containing base pairs of similar stability with the four natural bases [175]. Specifically, the authors conclude that 8-aza-7-deazaadenine  $N^8$ -2'-deoxyriboside form bidentate base pairs with all four natural bases including Watson–Crick or Hoogsteen geometries. This will lead to base pairs with different shapes but these seem to fit well with the natural Watson–Crick duplex [175].

### 3.3 Duplex-stabilizing nucleobases

Most of the applications of synthetic oligonucleotides rely on its affinity to target complementary nucleic acids. The exquisite recognition properties of natural nucleic acids can also be increased by the addition of functional groups to the nucleobases that can provide extra H-bond and/or hydrophobic interactions yielding modified oligonucleotides with enhanced hybridization properties. These properties are especially relevant in the development of potential therapeutic oligonucleotides as well as in gene analysis detection using PCR primers with improved binding properties. Some of the developments in this area can be found in previous reviews [177, 178]. An especially interesting comparative study on the stabilization properties of modified nucleobases and backbones has also been reported [179].

#### 3.3.1 Modified purines

One of the first modified nucleosides with enhanced hybridization properties was 2,6-diamino purine (Figure 3.6) also known as 2-aminoadenine [180, 181]. Compared with adenine, the presence of an additional amino group at position 2 will allow the formation of a third H-bond with the 2-keto group of thymidine. The protection of the 2,6-diaminopurine nucleobase was found to be more complex than the protection of natural bases. In a first approach the nucleobase was protected with the isobutyryl (at  $N^2$  position) and benzoyl (at  $N^6$  position) groups [182]. However, these protecting groups were found to be very stable to ammonia deprotection conditions as they need between 5–7 days at 60 °C for complete removal. The substitution of the benzoyl group for the *N*-methylpyrrolidine [183] or the di-*n*-butylformamidine [184] group pursued the stabilization of the glycosidic bond but it was also found to be too stable to ammonia deprotection conditions (7 days at 65 °C) [183]. The use of phenoxyacetyl group for the protection of the nucleobase reduced the deprotection time to the standard deprotection conditions used for the natural bases [185–187]. Moreover, phenoxyacetyl groups were also employed for the protection of 2,6-diaminopurine 2'-*O*-alkyl-RNA derivatives [188, 189]. Although most of the studies show that the substitution of adenine by 2,6-diaminopurine induces duplex stabilization [182, 183, 190], in some cases no stabilization or destabilization is described [187, 191, 192].



**Figure 3.6:** Modified purines with duplex stabilization properties.

The second series of purine derivatives with duplex stabilization properties are the 2-substituted guanine and 2-aminoadenine derivatives (Figure 3.6). The synthesis of oligonucleotides carrying 2-substituted guanine was first reported with the aim of introducing imidazole, carboxyl and 2,2'-dipyridyl functions in DNA and generate sequence specific ribonucleases [193–195]. In the case of the introduction of imidazole groups, these were protected with the 2,4-dinitrophenyl moiety that were cleaved with mercaptoethanol [193–195]. Later, it was demonstrated that oligonucleotides containing *N*<sup>2</sup>-imidazolylpropylguanine and *N*<sup>2</sup>-imidazolylpropyl-2-aminoadenine (Figure 3.6) were able to induce an important enhancement of the duplex thermal stability when hybridized with the complementary DNA sequence [196]. These results generate the interest for oligonucleotides carrying polyamines attached at position 2 of guanine such as *N*<sup>2</sup>-3-aminopropylguanine [197–199], *N*<sup>2</sup>-spermineguanine [200–203] *N*<sup>2</sup>-spermidineguanine [198, 199]. The synthesis of these modified oligonucleotides can be done either by using 2-fluoro-*O*<sup>6</sup>-(4-nitrophenyl)ethyl (NPE)-2'-deoxyinosine as a common nucleotide precursor [198–201] or by using a dedicated phosphoramidite [197, 202]. In the latter case, the remaining amino groups of the polyamine are protected with the trifluoroacetyl (TFA) group while the *O*<sup>6</sup> position of guanine was protected either with the diphenylcarbamoyl group [197] or the NPE group [202]. In a comparative study, it was found that the highest stabilization was produced in guanine derivatives carrying at *N*<sup>2</sup> an ethyl or propyl moieties substituted with a group that is protonated under physiological conditions, which is compatible with a potential interaction of the protonable group with the phosphates in the minor groove near the *N*<sup>2</sup> position of guanine [199]. Moreover, oligonucleotides carrying one or sev-

eral  $N^2$ -spermine derivatives not only stabilize duplex but also bind to superhelical double-stranded DNA by strand invasion [200, 203].

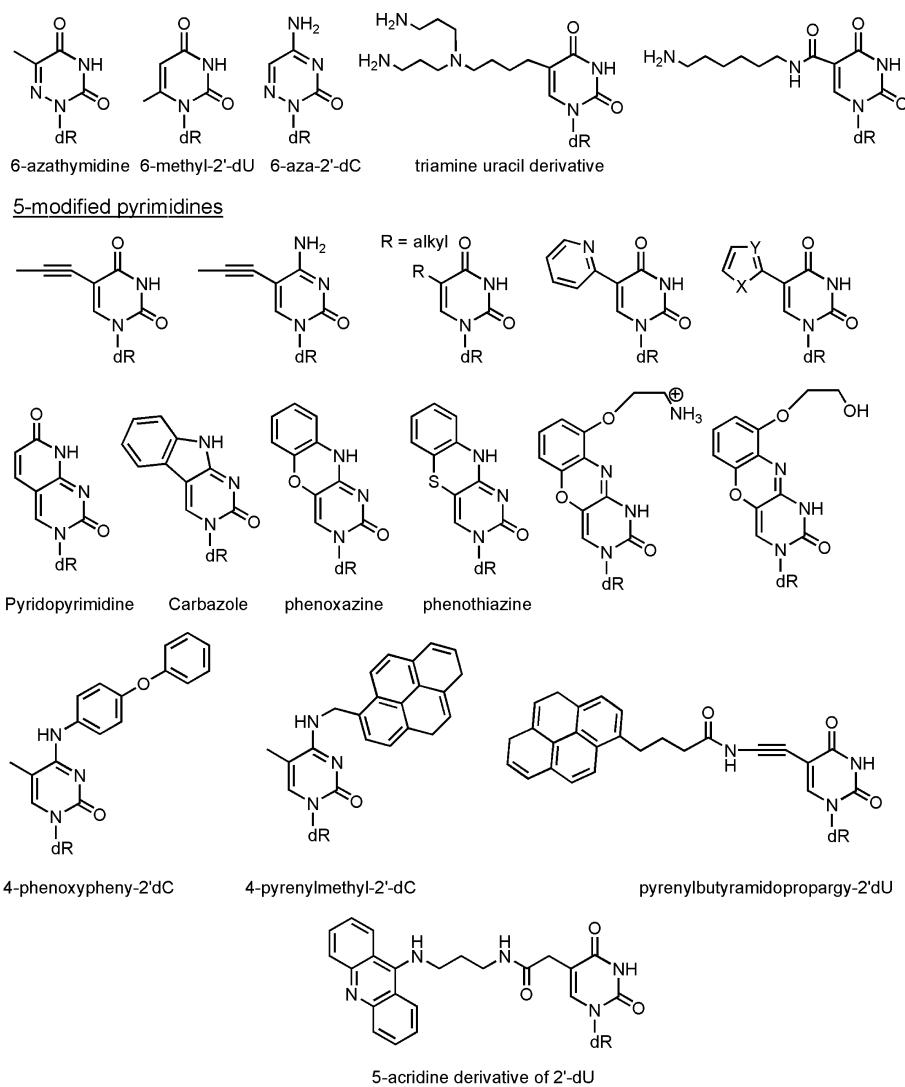
The synthesis of oligonucleotides carrying 7-substituted 7-deazaadenines (Figure 3.6) have been reported [204]. The presence of Br, Cl or methyl groups in position 7 of 7-deazaadenine increases the stability of the duplexes when compared with adenine. Duplex stabilization has been also described in oligonucleotides carrying 7-bromo, 7-iodo and 7-methyl 7-deazaguanine (Figure 3.6) [205, 206]. On the contrary other nucleosides such as 3-deazaadenine [207] or 4-aminobenzimidazole [208] destabilize DNA duplexes. The synthesis of oligonucleotides carrying the tetracyclic adenine analog shown in Figure 3.6 has been reported [209]. The presence of these analogs did not improve de melting temperatures of the corresponding duplexes.

### 3.3.2 Modified pyrimidines

The search for nucleotide derivatives to increase nuclease resistance of antisense oligonucleotides leads to the development of pyrimidines modified at their 5- and/or 6-positions. As position 6 of pyrimidines is required for oligonucleotide degradation by nucleases, some authors focused their interest on 6-azathymidine, 6-aza-2'-deoxycytidine, 6-methyl-2'-deoxycytidine and 5,6-dimethyl-2'-deoxyuridine (Figure 3.7) [176, 210]. Oligonucleotides capped with 6-azathymidine were selected as they have enhanced the stability to degradation by nucleases while maintaining similar hybridization properties than unmodified oligonucleotides [210].

The addition of a *syn*-norspermidine group at position 5 of 2'-deoxyuridine (Figure 3.7) provides a strong duplex stabilization to the corresponding modified oligonucleotides as well as enhanced resistance to degradation by nucleases [211]. Interestingly, oligonucleotides carrying the triamine uracil derivative stabilize also the formation of triplex structures [211]. A similar result was found with the addition of amino-hexyl [212] and other polyamines [213] at position 5 of 2'-deoxyuridine (Figure 3.7) using a carbamoyl linkage.

Oligonucleotides carrying 5-methyl pyrimidines [214] as well as other 5-alkyl pyrimidines [215] (Figure 3.7) have increased affinity to complementary DNA sequences. It has been proposed that this stabilization is mainly entropic and can be assigned to the removal of water molecules from the duplex structure [214]. A large increment on duplex stability was found with oligonucleotides carrying 5-propyne analogs of 2'-deoxyuridine and 2'-deoxycytidine [216]. In addition to an increased hydrophobicity of the propyne group, it has been suggested that the propyne modification allows an increased stacking of the bases. Moreover, 5-propyne pyrimidines stabilize triplexes and they may facilitate passive diffusion of oligonucleotides into cells [216]. The duplex stabilization properties 5-propyne pyrimidine 2'-deoxynucleotides was extended to other nucleotides such as 2'-*O*-allyl- [217, 218] and 2'-*O*-methyl-RNA



**Figure 3.7:** Modified pyrimidines with duplex stabilization properties.

nucleotides [219]. Antisense oligonucleotides carrying 5-propyne pyrimidine 2'-O-alkyl-RNA units were shown to be extraordinarily potent for the inhibition of gene expression in cell culture [218] especially after microinjection into cells [219, 220]. A comparative study of the hybridization properties of several 5-substituted uridine derivatives carrying diverse 5-propyne and 5-thioalkyl groups was described [221]. In the propyne series the duplexes carrying the 5-propyne-dU had the highest melting temperature while the thioether substituents were found to form stable duplexes [221].

The synthesis of oligonucleotides carrying a series of 5-heteroaryl-2'-deoxyuridines including pyridine, thiophene, thiazole and imidazole heterocycles was reported (Figure 3.7) [222, 223]. Thermal denaturation studies showed that duplexes carrying the thiazole group exhibit the highest melting temperature [222]. Oligonucleotides carrying 5-thiazole-2'-deoxyuridines although exhibit the highest *in vitro* binding affinity; they were less potent antisense inhibitors than oligonucleotides carrying 5-propyne-dU [223].

An interesting series of nucleosides are the bicyclic and tricyclic 2'-deoxycytosine derivatives shown in Figure 3.7 including 2,7-dioxopyrido[2,3-d]pyrimidine [224], carbazole [225], phenoxazine [226, 227] and phenothiazine [226] derivatives. Duplexes carrying these analogs paired with guanine have higher melting temperatures than unmodified duplexes, especially when the tricyclic base analogs are clustered. The special stability of duplexes having contiguous modifications has been assigned to  $\pi\text{-}\pi$  overlap of tricyclic bases. In addition, these compounds were created to provide a rigid scaffold for the introduction of appending groups designed to interact with the  $N^7$  Hoogsteen position of the complementary guanine [228]. Several appending groups were studied to achieve the so-called G-clamp effect. The group with the highest stability contained an ammonium group. The authors suggested an interaction of the ammonium group with the  $O^6$ -guanine as it has been observed in Lysine-DNA contacts in protein-DNA complexes studied by X-Ray crystallography [228].

Another strategy to induce duplex stabilization is the addition of intercalating molecules at the nucleobases (Figure 3.7). Some authors demonstrated that the introduction of 4-phenoxyphenyl [229] or pyrenylmethyl [230] group to position 4 of 2'-deoxycytidine generates oligonucleotides with special stabilization properties of three-way junctions as well with some stabilization of triplex structures. The pyrenylbutyramidopropargyl ligand attached to the 5-position of 2'-deoxyuridine residues (Figure 3.7) has also been used to increase the stability of A-T base pair in order to generate DNA probes that are sequence independent (named as isostable DNA probes) [231]. Acridine-modified oligonucleotides at the 5-position of 2'-deoxyuridine have been also demonstrated to increase duplex stability by the intercalation of the acridine ring in the base pairs contiguous at the modification site [232].

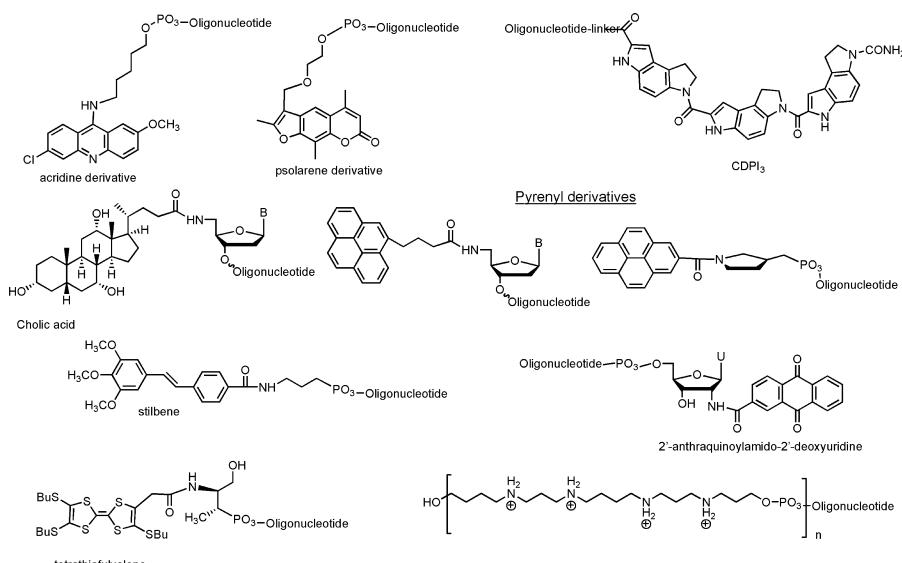
### 3.3.3 Duplex stabilization by modified 5' and 3'-ends

The intercalating agents and polyamines attachment to the nucleobases described in the previous section, if introduced to 5' and 3'-positions can also be used to enhance the affinity of oligonucleotides to their complementary sequence. There are a large number of oligonucleotide conjugates carrying intercalating agents, polyamines and minor groove binders that are reported to have higher affinity than unmodified oligonucleotides. In this section, we will describe a short selection of oligonucleotide

3' or 5'-conjugates with enhanced hybridization properties. A more detailed description is out of the aim of this section but this can be found in reviews elsewhere [233–237].

One of the first oligonucleotide conjugates prepared for the inhibition of gene expression were the oligonucleotides carrying an acridine molecule (Figure 3.8) at the 5'-end as acridine was a known DNA intercalating agent and the resulting oligonucleotides had an increased affinity for single and double stranded DNA via triplex formation [238]. Another strategy was the incorporation of a photoreactive intercalating agent such as psolarene that could form covalent bonds [239, 240].

Some antibiotics are known to bind and stabilize specific double stranded DNA sequences by binding to the minor groove of the double helix. This type of interaction has led to the design of oligonucleotides carrying minor groove binders in order to enhance the affinity to complementary sequences. One of the most successful minor groove binders is the 1,2-dihydro-3H-pyrrolo[3,2-e]indol-7-carboxylate tripeptide (CDPI<sub>3</sub>) (Figure 3.8) that has been introduced in both 5' and 3'-ends [241, 242]. The conjugation of the CDPI<sub>3</sub> tripeptide to oligonucleotides generates DNA probes with enhanced hybridization properties that are able to arrest primer extension by T7 DNA polymerase [243]. The addition of the CDPI<sub>3</sub> tripeptide to molecular probes carrying fluorophore and quencher provides a unique quenching mechanism, where the CDPI<sub>3</sub> tripeptide interacts with the fluorophore causing a very low background [244]. Consequently, these optimized probes can generate a stronger fluorescent signal upon binding with their complementary sequence.



**Figure 3.8:** Modifications introduced at the 3' or 5' position of oligonucleotides with duplex stabilization properties.

Another strategy to increase the duplex stability is the addition of hydrophobic molecules at the 3' or 5'-ends. An important duplex stabilization was observed when cholic acid was attached to short oligonucleotides carrying 5'-amino-thymidine at the 5'-position (Figure 3.8) [245]. Specific interaction of the cholic acid moiety with the terminal A.T base pair was demonstrated by NMR suggesting a capping mechanism in where the hydrophobic cholate group was protecting from water the exposed terminal A.T base pair. This duplex stabilization mechanism was further explored by preparing a series of 5'-acylamino caps. Pyrenyl [246] and stilbene [247] derivatives (Figure 3.8) were among the best compounds in terms of duplex stabilization and base-pairing fidelity. The use of 3'-terminal-2'-acylamido-RNA allowed the study of the optimal 3'-capping compounds [248]. 2'-Pyrenylmethyl amido-2'-deoxyadenosine [249] and 2'-anthraquinoylamido-2'-deoxyuridine [250] (Figure 3.8) were found to have the best duplex stabilization properties.

The addition of two tetrathiafulvalene (TTF) units (Figure 3.8) in a short DNA duplex at the same duplex side has been reported to yield a high increase in melting temperature [251]. When both TTF units were on opposite termini, salt-dependent aggregation is observed, yielding well-defined spherical DNA supramolecular structures [251].

Similar to what has been described in the incorporation of polyamines at the position of guanine, the incorporation of one or several spermine derivatives (Figure 3.8) at the 5'-end of the oligonucleotides increases the stability of duplex structures by removal of the interstrand phosphate repulsion [252–256]. The number of positive charges can be tailored to compensate for the negative charge of phosphates [252, 254]. It has been described that oligospermine-oligonucleotides are specific and efficient primers for PCR and reverse transcription reactions exhibiting faster binding kinetics allowing the use of lower primer concentrations and higher annealing temperatures [253]. Oligospermine-oligonucleotides known as Zip nucleic acids have strand invasion properties [255] and can be transfected to cells without the use of transfecting agents [256].

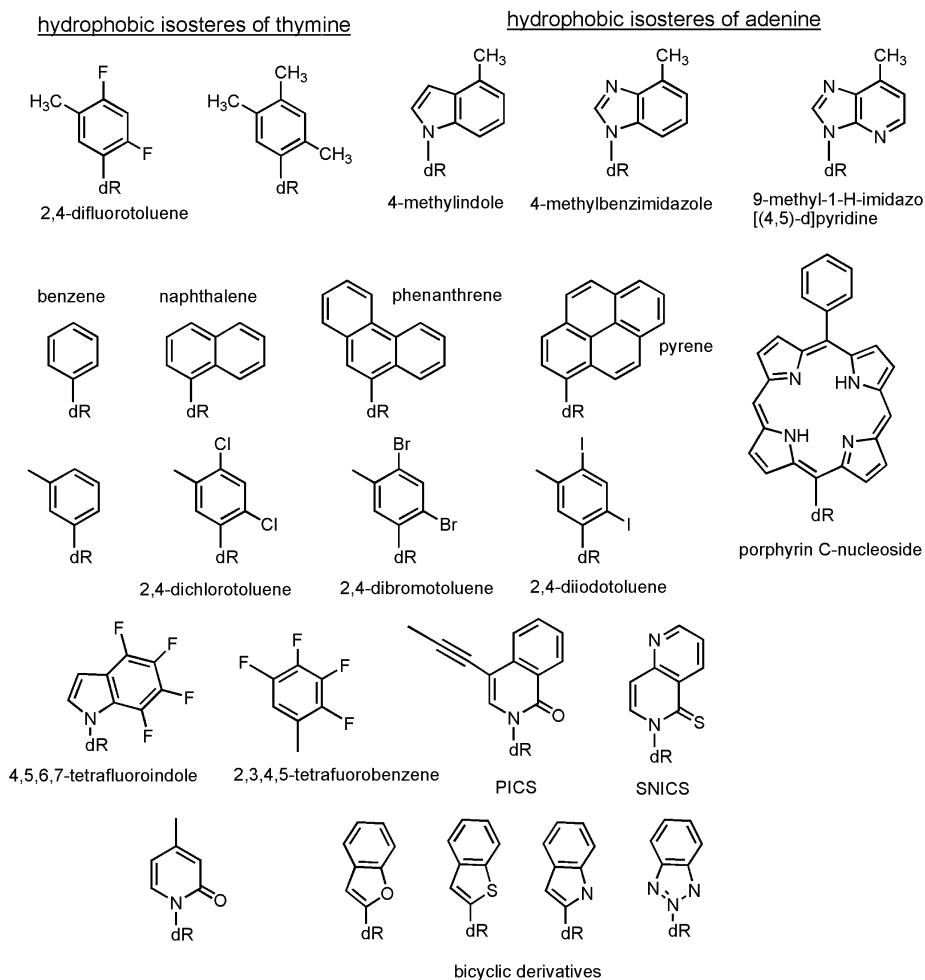
## 3.4 Influence of size, shape and hydrophobicity of nucleobases

### 3.4.1 Base-pairs without hydrogen bonds

The relevance of hydrogen bonds between complementary bases has been an important source of debate. From the biophysical point of view, hydrogen bonds between molecules in water are weak due to the properties of water, but most of the biological properties of nucleic acids are based on the formation of hydrogen bonds. One of the most important contributions to this subject arrived from Dr. Kool's group. The

description of the first hydrophobic, non-hydrogen-bonding bases and the demonstration that specific base pairs can be observed without the formation of H-hydrogen bonds, was very important for the understanding of the relative role of hydrogen bonds and base stacking in the stability and function of DNA [257–259]. The first hydrophobic isosteres of thymine and adenine studied were 2,4-difluorotoluene and 4-methylindole (Figure 3.9). Oligodeoxynucleotides carrying 2,4-difluoro-toluene and 4-methylindole were synthesized and thermal denaturation studies showed that these compounds prefer to base pair with themselves rather than base pair with natural bases [257]. In a separate study, the stacking properties of 2,4-difluorotoluene and 4-methylindole as well as benzene, naphthalene, phenanthrene or pyrene (Figure 3.9) were compared with natural bases demonstrating that natural bases are not very effective at stacking while hydrophobic nucleosides were very efficient [260, 261]. The highly efficient stacking properties of hydrophobic nucleotides have been used to generate terminator nucleosides to improve the homogeneity of the 3'-end of DNA and RNA enzymatic products [262]. In spite of the poor base pairing properties, 2,4-difluoro-toluene was found to promote adenine insertion in primer extension experiments with similar efficacy and selectivity than natural thymine [263–266]. These results led to the hypothesis that the geometry of the base pair rather than stability, even in the absence of hydrogen bonds, is enough to direct nucleotide selection by DNA polymerases [138, 267]. Another interesting application of hydrophobic nucleosides is the incorporation of these in the loops of triplex-forming hairpins. The absence of hydrogen-bond interactions and the hydrophobic nature of these nucleosides induce a strong stabilization of triplexes [268].

Other interesting isosteres for adenosine studied by the group of Dr. Kool were 4-methylbenzimidazole and 9-methyl-1*H*-imidazo[(4,5)-*d*]pyridine 2'-deoxyribosides (Figure 3.9). 4-Methylbenzimidazole has very little tendency to pair with natural bases but surprisingly, it forms relatively stable base pairs with 2,4-difluorotoluene [269, 270]. 9-Methyl-1*H*-imidazo[(4,5)-*d*]pyridine has an additional nitrogen, lacking all the heteroatoms involved in Watson–Crick base pair. The presence of the additional nitrogen in position 3 was used to analyze the minor groove interactions of DNA polymerases during insertion and extension of the templates carrying nonhydrogen-bond hydrophobic nucleosides [271, 272]. In a comparative study of six DNA polymerases, the differences in the insertion and extension of 9-methyl-1*H*-imidazo[(4,5)-*d*]pyridine and 4-methylbenzimidazole were used to classify DNA polymerases in two groups. In the first group, one found DNA polymerases such as the Klenow fragment and HIV reverse transcriptase that efficiently extends nonpolar 9-methyl-1*H*-imidazo-4,5-*b*]pyridine base pairs but not the analog 4-methylbenzimidazole. In the second group, one foundsDNA polymerases such as Pol  $\alpha$  and Pol  $\beta$  that fail to extend all nonhydrogen-bonding base pairs. These differences are due to specific contacts of the DNA polymerases with the minor groove  $N^3$  of 9-methyl-1*H*-imidazo-4,5-*b*]pyridine analog.



**Figure 3.9:** Structures of nucleobases for hydrophobic base pairs without H-bonds.

Other unusual base pair lacking hydrogen bonds were observed in large hydrophobic nucleosides and abasic sites. For example, it has been observed that pyrene can compensate for the absence of a nucleobase in a template having an abasic site model compound [273, 274]. Pyrene nucleoside triphosphate is inserted in front of an abasic site with high efficiency but the modified base pair is not extended by DNA polymerases [273, 274]. Another large hydrophobic nucleoside introduced in synthetic oligonucleotides is a porphyrin C-nucleoside (Figure 3.9). The porphyrin moiety accommodates into DNA duplex preserving its fluorescent properties [275].

An interesting result was obtained when a series of dihalogen toluene derivatives were analyzed [276]. Oligonucleotides carrying 2,4-difluorotoluene, 2,4-dichlorotoluene, 2,4-dibromotoluene and 2,4-diiodotoluene (Figure 3.9) derivatives were pre-

pared and primer extension experiments with the Klenow fragment were performed to analyze the effect of the size of the substituents in the replication efficiency. Bond lengths are increasing with the size of the halogen from Ar-H 1.08 Å, Ar-F 1.36 Å, Ar-Cl 1.74 Å, Ar-Br 1.90 Å, Ar-I 2.10 Å compared with the length of C=O in thymidine 1.22 Å. The highest replication efficiency was observed for the 2,4-dichlorotoluene indicating that the optimal base pair size is larger than natural one. The authors suggested that the allowance of larger size for base pairs may benefit the evolution of DNA polymerases [276]. Interestingly, the analysis of the proofreading selectivity of DNA polymerases indicates that hydrogen-bonds are required for the discrimination between correct and incorrect base pairs as most of the nonhydrogen-bond hydrophobic nucleobases are rapidly removed by DNA polymerases as they were incorrect base pairs [277].

The incorporation of polyfluorinated analogs of hydrophobic nucleobases such as 2,3,4,5-tetrafluorobenzene and 4,5,6,7-tetrafluoroindole (Figure 3.9) in synthetic oligonucleotides increases the stability of base-pairs especially in base-pairs having with two polyfluorinated bases [278, 279]. Triphosphate derivatives of the fluorinated nucleosides were incorporated into DNA by Klenow fragment DNA polymerase more efficiently than natural nucleoside triphosphates especially in front of 2,3,4,5-tetrafluorobenzene. After incorporation of the hydrophobic nucleotide to the DNA template, the extension of the modified base pair was very inefficient [279].

Another hydrophobic nucleoside, 7-propynyl isocarbostyryl or 1-hydroxyisoquinoline, (PINS, Figure 3.9) was found to stabilize duplex especially when paired with itself [280]. The triphosphate derivative of this derivative was able to incorporate in front of natural bases in primer extension experiments with the Klenow fragment DNA polymerase as well as direct the incorporation of natural bases especially T. However, the most significant result was the incorporation of 7-propynyl isocarbostyryl in front of itself with the highest efficiency [280]. Although the extension of the primer beyond the newly synthesized 7-propynyl isocarbostyryl base pair was low, these results led to hypothesize that new nonnatural base pairs can be developed and expansion of the genetic alphabet may be indeed possible [280, 281]. Optimization of the 7-propynyl isocarbostyryl nucleoside leads to the development of 1,6-naphthyridin-5(6H)-one nucleoside derivatives and their corresponding sulfur-containing derivatives [281], from which SNICS (Figure 3.9) was found to represent significant progress toward expansion of the genetic alphabet [282]. Further optimization led to the development of a series of bicyclic derivatives shown in Figure 3.9 with enhanced primer extension properties allowing the full-length extension of primers carrying heteropairs [283].

### 3.4.2 Extended nucleobases

An interesting development was the design and synthesis of nucleobases that were similar to natural bases but they had an extra phenyl ring (xT, xA, xC, xG, Fig-

ure 3.10). DNA carrying these larger nucleobases forms thermodynamically stable right-handed, double-stranded structures due to enhanced base stacking [284–290]. In addition, most of these size-extended nucleobases are highly fluorescent and their fluorescent properties are sensitive to double helix formation so they can be used to detect hybridization [286]. These extended nucleobases are recognized by DNA polymerases such as Klenow fragment and Dpo4 directing the four correct natural bases in front of the size-expanded nucleobase [291], although the efficiency of insertion of the complementary natural base is low compared to that observed with natural bases. The second set of size-expanded nucleobases was also developed (*yT*, *yC*, *yA*, *yyT*, *yyC*, Figure 3.10). In these cases, the substitution of the rings was not aligned but had a twisted geometry [292–294] and an elongated size [295]. Another source of enlargement of base pairs is the elongation of the linker between the sugar moiety and the nucleobase (Figure 3.10). These derivatives include the methyl [296], ethyl [297] thymine alkane 2-deoxyribosides as well as the methylcarboxamido derivative [298]. These compounds were designed to stabilize triplexes with short polypurine–polypyrimidine tracts, to be used as a wild card in the interruptions of the polypurine–polypyrimidine tracts [296, 297] and as flexible nucleosides in modified siRNAs [298].

size expanded nucleobases

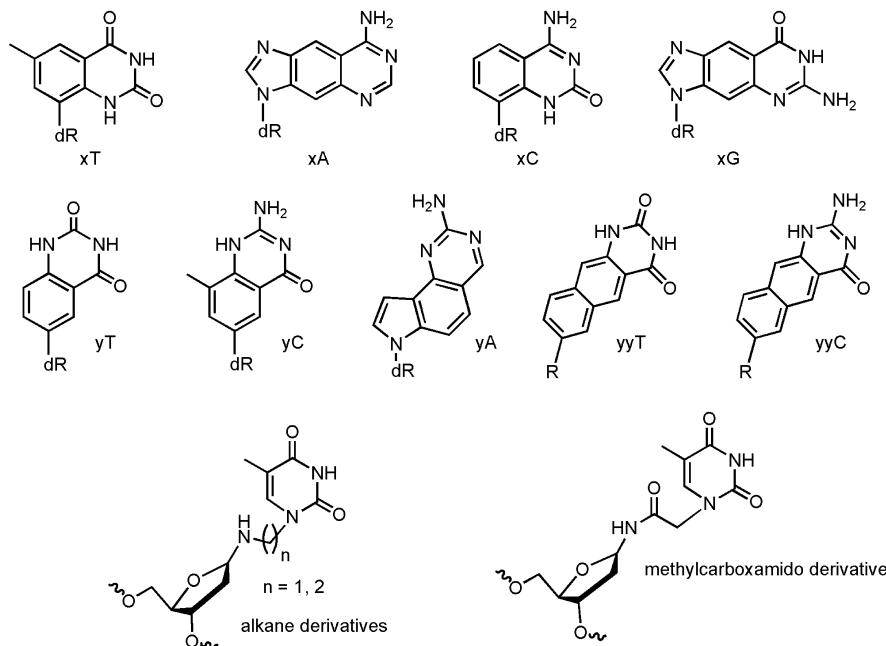


Figure 3.10: Size-expanded nucleobases.

## 3.5 Expanding the genetic alphabet—artificial DNA

Nature selected the G:C and A:T base pairs to store genetic information. The detailed knowledge of the replicative consequences of DNA lesions and mutagenic nucleobases as well as the biotechnological applications of gene replication, triggered the interest for the study of the possibility of extending the base pair schemes found in nature to other base pairs with a different structure (xenonucleic acids, XNA [299]). If novel base pairs were found and these were replicated by DNA polymerases, it will be possible to create novel artificial DNA with potential enhanced properties. Efforts in this research area have been concentrated in two directions: in the design and synthesis of novel pairs of nucleobases that are compatible and different to natural bases (orthogonal nucleobases) and in the discovery of novel DNA polymerases that could be able to replicate the unnatural bases with high efficiency.

In addition to this meaning, the term artificial DNA has also been used to describe modified backbones that can hybridize with natural nucleic acids such as; peptide nucleic acids, hexitol nucleic acids and so on used in the field of therapeutic oligonucleotides.

### 3.5.1 Orthogonal nucleobases

The first nonnatural base pairs that were evaluated for their capacity of expand the genetic alphabet were the 5-methyl-isocytidine/isoguanine [300–302], 5-(2,4-diamino)pyrimidine/2'-deoxyxanthosine [303] base pairs and 5-methylpyrimidone/6-thioguanine [304] (Figure 3.11). Some of these base pairs were replicated by DNA polymerases but selectivity and efficiency were in some cases low due to several factors [305, 306]. These results triggered the development of a large number of nonnatural base pairs including base pairs without hydrogen-bonds and extended nucleobases described above [307, 308]. In addition to the above mentioned base pairs, complex nonnatural base pairs such as the imidazopyrido-pyrimidine: naphthyridine [309, 310] unnatural base pairs and C-nucleosides carrying acetylene bonds [311] as well as purine/purine base pairs such as 2,6-diaminopurine/7-deazaxanthine (<sup>C7</sup>X) or guanine/isoguanine base pairs [312, 313] (Figure 3.11) were studied.

A large development was made by the group of Romesberg with nucleotides bearing hydrophobic base pairs (d5SICS/dNaM, Figure 3.11) that were efficiently amplified by PCR, and introduced in *E. coli* bacteria that were able to propagate the DNA information with the help of nucleotide triphosphate transporter from algae, creating a semisynthetic organism capable to replicate artificial plasmid DNA [314–317]. Similarly, Hirao's group described another set of hydrophobic base pairs (Ds/Px, Figure 3.11) that were amplified by PCR opening the possibility of generation of new high-affinity aptamers by SELEX [318–320]. Finally, the group of Benner was able to develop a DNA and RNA-like system built with eight nucleotides (named as “hachimoji”) that

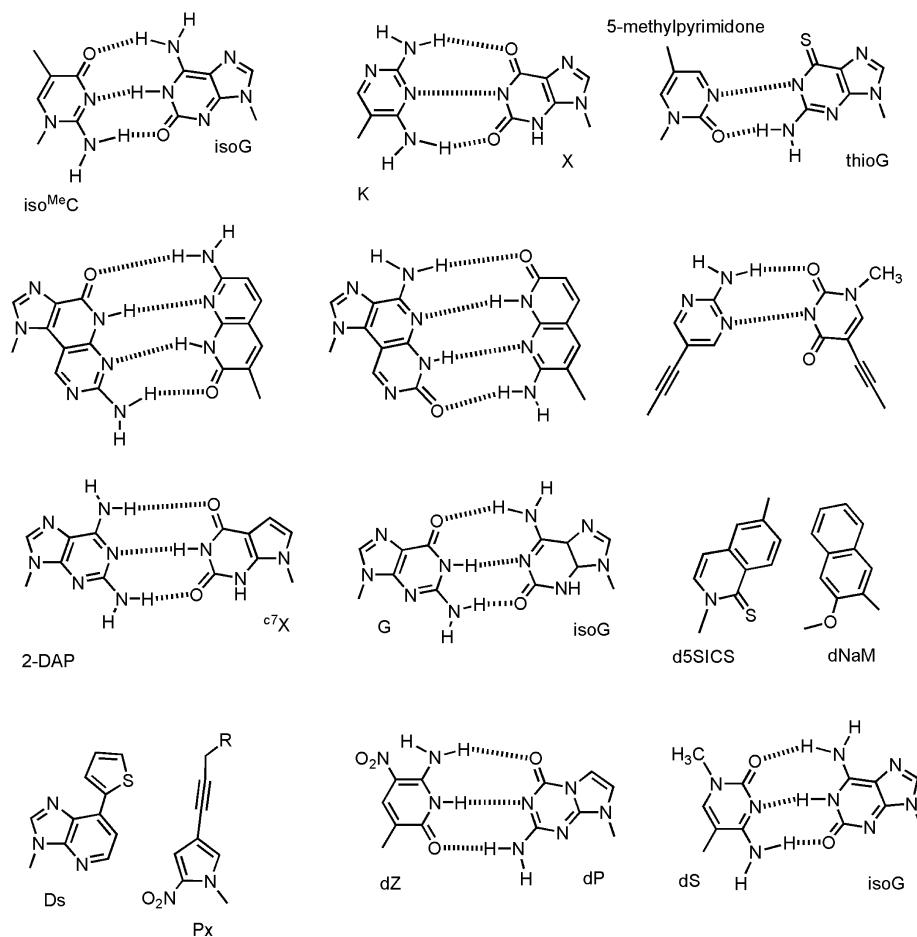


Figure 3.11: Orthogonal nucleobases.

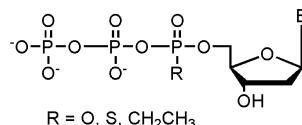
were able to form four orthogonal base pairs (dZ/dP and dS/isoG base pairs in addition to the natural bases, Figure 3.11) [321, 322]. The transcription of hachimoji DNA to hachimoji RNA was confirmed by the preparation of an active fluorescent RNA aptamer carrying the eight nucleotides [322].

### 3.5.2 Enzymatic synthesis of modified DNA—novel DNA polymerases

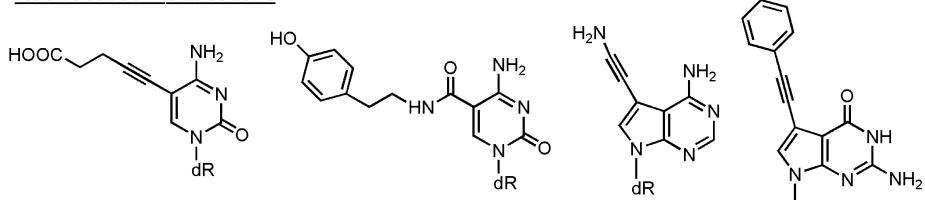
In recent years, a large number of modified oligonucleotides have been produced for biotechnology, therapeutic and diagnostic applications. An important development was the discovery of nucleic acid aptamers, obtained by in vitro selection using the

Systematic Evolution of Ligands by Exponential enrichment (SELEX) methodology. These nucleic acid derivatives are excellent alternatives to antibodies both in therapeutic and diagnostic purposes [323]. An important step in the SELEX methodology is the enzymatic synthesis of nucleic acids in order to increase the number of molecules selected in each selection step. The incorporation of modified nucleic acids in this step is desirable in order to increase the diversity and selectivity of aptamers [324]. Also, interest in the preparation of long modified nucleic acids by enzymatic synthesis will benefit the development of novel genetic polymers with applications as ligands, catalysts or materials [325]. DNA polymerases are extremely specialized enzymes with high specificity, fidelity and velocity. The extraordinary properties of DNA polymerases come from evolution resulting in DNA polymerase optimal for replicating natural nucleotides. In addition, genetic engineering and selection of novel DNA polymerases optimal for the replication of modified nucleotides are indeed possible yielding DNA polymerases specialized in the replication of nonnatural nucleotides that in turn are not able to replicate natural bases. This can generate new replication systems independent of the natural systems. The most important efforts in these directions were made by the group of Holliger that developed a large number of novel DNA polymerases capable to replicate hydrophobic base analogs described above [326] as well as numerous backbone and phosphate modifications such as nonionic ethylphosphonate bonds [327] (Figure 3.12). Most of this important research can be found in recent excellent reviews [328, 329].

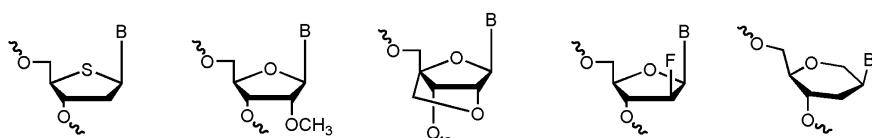
#### Phosphate modifications



#### Nucleobases modifications



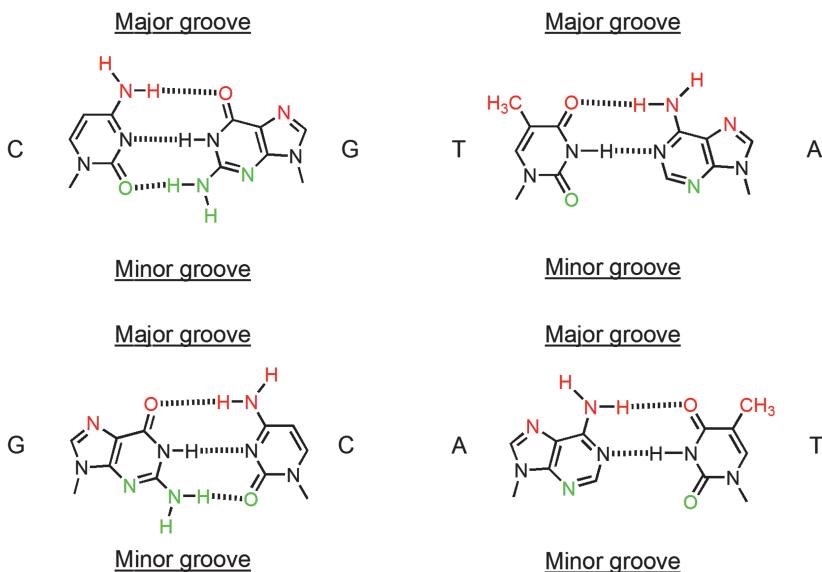
#### Sugar modifications



**Figure 3.12:** Structures of compounds used in enzymatic synthesis of modified DNA.

## 3.6 DNA-protein contacts

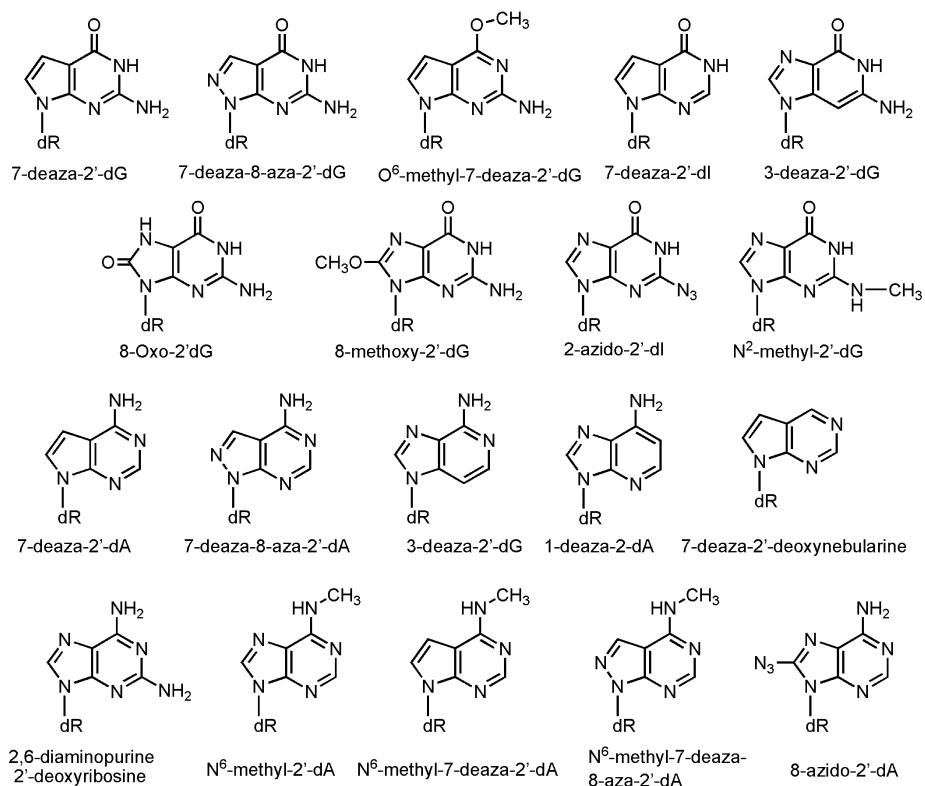
An important issue in the development of modified nucleobases has been the structural elucidation of the exact interactions between the amino acids of the proteins that bind DNA to the nucleobases of DNA. Most of these interactions occur on the major groove of DNA as it is the most spacious area for these interactions and there are clear differences between the four natural base pairs (Figure 3.13). There are one hydrogen bond donor and two acceptor groups on the major groove surface of all four dinucleotide pairs (A•T, T•A, G•C, C•G; Figure 3.13). In addition, there is a methyl group at the C-5 position of thymine that can participate in van der Waals interactions. Within the minor groove, there are two hydrogen bond acceptor sites in all four dinucleotide pairs; in the C•G and G•C dinucleotide pairs, the  $N^2$  position of G provides a hydrogen bond donor at the center of the minor groove.



**Figure 3.13:** Natural base pairs showing the atoms which may be involved in the DNA-protein interactions.

### 3.6.1 Study of DNA-protein contacts. Restriction endonucleases and bacterial DNA methylation

There are thousands of DNA-binding proteins such as transcription factors, helicases, topoisomerases, DNA-methylases and so on. However, most of the early studies on the impact of nucleobases on DNA-protein interactions were performed with the



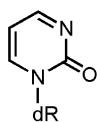
**Figure 3.14:** Structures of the modified purines designed for the elucidation of DNA-protein interactions.

bacterial methylation/restriction endonucleases systems. Figure 3.14 shows some of the purine nucleotides used in the determination of DNA-protein interactions in restriction endonucleases and bacterial DNA methylases. The role of the nitrogen atom in position 7 of purines was investigated with the use of nucleotides without the N<sup>7</sup>: 7-deaza-2'-deoxyguanosine [330–332] and 7-deaza-2'-deoxyadenosine or 2'-deoxytubercidin [332–337]. Other guanine, adenine and hypoxanthine analogs without nitrogen in position 7 used for the study of DNA-protein interactions were 7-deaza-8-aza-2'-deoxyguanosine [338, 339], 7-deaza-8-aza-2'-deoxyadenosine [340–342], O<sup>6</sup>-methyl-7-deaza-2'-deoxyguanosine [343], and 7-deaza-2'-deoxyinosine [344]. Even more other deazanucleoside purine derivatives like 3-deaza-2'-deoxyguanosine [337], 3-deaza-2'-deoxyadenosine [345] and 1-deaza-2'-deoxyadenosine, 7-deaza-2'-deoxynebularine were also used to elucidate DNA-Protein interactions [344, 346, 347]. 8-Oxo-2'-deoxyguanosine and 8-methoxy-2'-deoxyguanosine [337, 348] as well as 2,6-diaminopurine 2'-deoxyribosine [349, 350] have also been used in these type of studies. There are some bacterial DNA methylases capable to introduce methyl

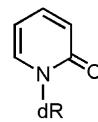
groups at the exocyclic amino groups of purines. To study these methylation processes, several *N*-methylated purines have been developed such as; *N*<sup>2</sup>-methyl-2'-deoxyguanosine [351], *N*<sup>6</sup>-methyl-2'-deoxyadenosine [352], *N*<sup>6</sup>-methyl-7-deaza-2'-deoxyadenosine and *N*<sup>6</sup>-methyl-7-deaza-8-aza-dA [341]. Azido purine nucleosides such as 2-azido-2'-deoxyinosine and 8-azido-2'-deoxyadenine [353, 354] have been used for DNA-protein crosslinking experiments.

Some of the pyrimidine derivatives used for the study of protein-DNA interactions are shown in Figure 3.15. These include several nucleosides generated by removal of the exocyclic groups on pyrimidine nucleosides or the nitrogen 3 such as; 2-pyrimidinone-2'-deoxyriboside [355–357], 2-pyridinone-2'-deoxyriboside [357], 3-deaza-thymidine [357, 358] and 5-methyl-4-pyrimidinone-2'-deoxyriboside or 2-dehydro-thymidine [359]. *N*<sup>4</sup>-Methyl-2'-deoxycytidine and 5-methyl-2'-deoxycytidine, which were used in bacterial methylases studies [352, 360, 361]. As mentioned above, the methyl group at the C5 position of thymine participates in van der Waals interactions, for this reason, 5-substituted uracil such as; 5-ethyl-dU, 5-cyano-dU, 5-halo-dU derivatives have been used for studying these interactions [349, 362–365]. In addition, 5-bromo-dU has been used in protein photocrosslinking experiments [364, 365].

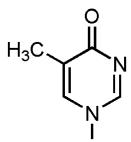
#### Pyrimidine derivatives



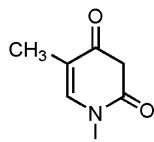
2-pyrimidinone-2'-deoxyriboside



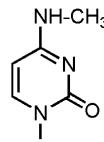
2-pyridinone-2'-deoxyriboside



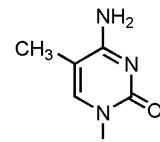
2-dehydro-T



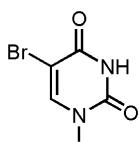
3-deaza-T



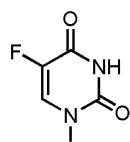
*N*<sup>4</sup>-Methyl-2'-dC



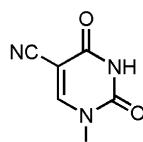
5-methyl-2'-dC



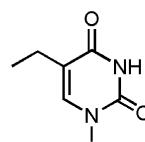
5-Br-dU



5-F-dU



5-cyano-dU



5-ethyl-dU

**Figure 3.15:** Structures of the modified pyrimidines designed for the elucidation of DNA-protein interactions.

### 3.6.2 Inhibitors of cytosine DNA methyltransferases

DNA cytosine 5-methyltransferase (DNMT) catalyzes the introduction of a methyl group at the C5 position of cytosine residues in the CpG sequence. This reaction is especially important in gene regulation of eukaryotic cells as the methylation of the CpG sequences is an epigenetic signal that triggers gene inhibition. Tumor cells have aberrant DNA methylation patterns and some genes involved in tumor suppression are hypermethylated. For this reason, DNMT is an important target for cancer treatment. Some nucleoside derivatives such as; 5-fluoro-2'-deoxycytidine, 5-aza-2'-deoxycytidine or decitabine, 5-azacytidine and zebularine are DNMT inhibitors [366]. In order to be incorporated into DNA, these nucleoside inhibitors have to be phosphorylated to the corresponding 5'-triphosphates. Sequence-selective incorporation of these nucleosides is an alternative way to generate the 5'-monophosphate derivatives by nuclease degradation or to inhibit directly DNMT by forming covalent bonds with the active site of the protein. The dinucleotide S110 (Figure 3.16) which con-

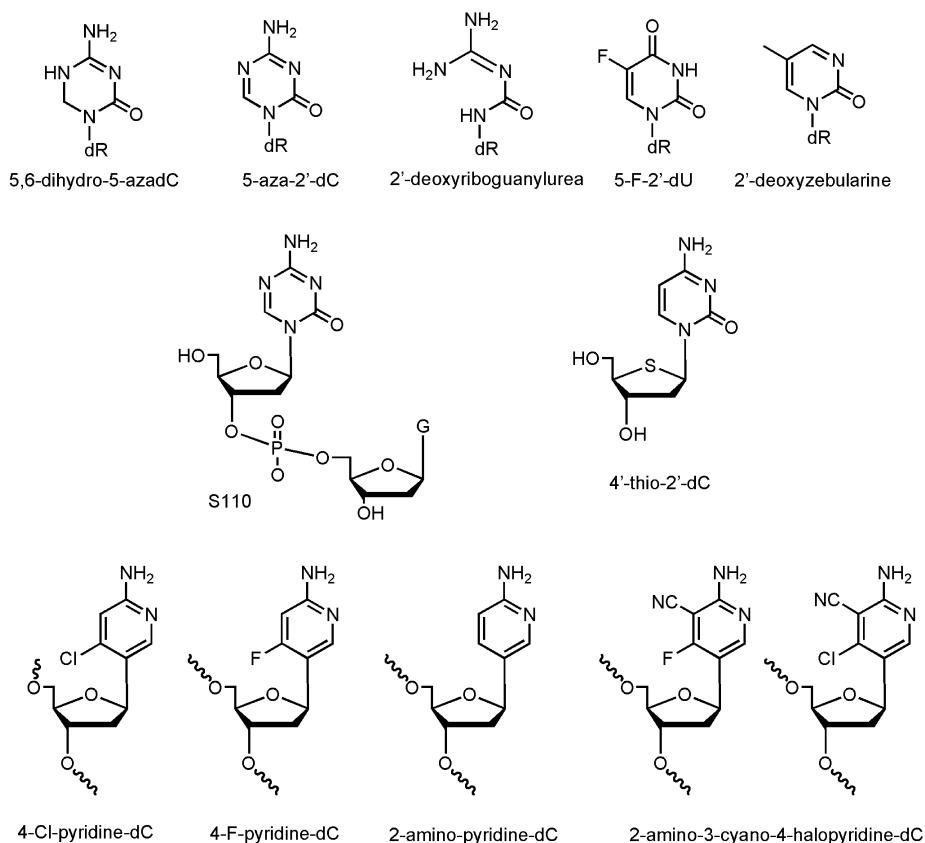


Figure 3.16: Structures of the inhibitors of cytosine DNA methyltransferases.

sists of 5-aza-2'-deoxycytidine and 2'-deoxyguanosine linked by a 5'-3' phosphate is the first FDA-approved oligonucleotide carrying DNMT nucleoside inhibitors that has been approved by FDA for cancer treatment [367–369]. Oligonucleotides carrying 5-fluoro-2'-deoxycytidine [370, 371], 5,6-dihydro-5-azacytosine [372, 373], 5-aza-2'-deoxycytidine [374–377] and 2'-deoxyzebularine [378–381] have been prepared and have been used to study the formation of covalent intermediates during the inhibition of DNMT. The synthesis of oligonucleotides carrying 5-aza-2'-deoxycytidine is complex due to the extreme lability of 5-aza-2'-deoxycytidine to aqueous acidic and basic solutions. It has been described that 5-aza-2'-deoxycytidine is degraded to 2'-deoxyriboguanylurea and this compound is easier than 5-aza-2'-deoxycytidine to be incorporated into synthetic DNA. 2'-Deoxyriboguanylurea is also an inhibitor of DNMTs [382].

Other nucleosides that have been shown to inhibit bacterial *MHhaI* methyltransferase are 4'-thio-2'-deoxycytidine (Figure 3.16) [383] and ribitol and bicyclo[3.1.0]hexane abasic sites [384] in the *HhaI* recognition sequence.

Recently, a series of oligonucleotides carrying 2-amino-4-halopyridine-C-nucleosides [385] and 2-amino-3-cyano-4-halopyridine-C-nucleosides [386] (Figure 3.16) were developed as DNMT inhibitors based on a novel aromatic nucleophilic substitution mechanism to generate protein complexes with covalent bonds to the target DNA.

## Bibliography

- [1] Watson JD, Crick FHC. Molecular structure for nucleic acids. A structure for deoxyribose nucleic acid. *Nature*. 1953;171:737–8.
- [2] Watson JD, Crick FHC. Genetical implications of the structure of deoxynucleic acid. *Nature*. 1953;171:964–7.
- [3] Topal MD, Fresco JR. Complementary base pairing and the origin of substitution mutations. *Nature*. 1976;263:285–9.
- [4] Sowers LC, Shaw BR, Veigl ML, Sedwick WD. DNA base modification: ionized base pairs and mutagenesis. *Mutat Res*. 1987;177:201–18.
- [5] Fagan PA, Fàbrega C, Eritja R, Goodman MF, Wemmer DE. NMR study of the conformation of the 2-aminopurine:cytosine mismatch in DNA. *Biochemistry*. 1996;35:4026–33.
- [6] Goodman MF, Fygenson KD. DNA polymerase fidelity: from genetics toward a biochemical understanding. *Genetics*. 1998;148:1475–82.
- [7] Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer*. 2003;3:330–8.
- [8] Ghoshal K, Jacob ST. Specific inhibition of pre-ribosomal RNA processing in extracts from the lymphosarcoma cells treated with 5-fluorouracil. *Cancer Res*. 1994;54:632–6.
- [9] Sowers LC, Eritja R, Kaplan BE, Goodman MF, Fazakerley GV. Structural and dynamic properties of a fluorouracil-adenine base pair in DNA studied by proton NMR. *J Biol Chem*. 1987;262:15436–42.
- [10] Sowers LC, Eritja R, Kaplan BE, Goodman MF, Fazakerley GV. Equilibrium between a wobble and ionized base pair formed between fluorouracil and guanine in DNA as studied by proton and fluorine NMR. *J Biol Chem*. 1988;263:14794–801.

- [11] Yu H, Bloom L, Eritja R, Goodman MF. Ionization of bromouracil and fluorouracil stimulates base pairing frequencies with guanine. *J Biol Chem.* 1993;268:15935–43.
- [12] Metzler WJ, Arndt K, Tecza E, Wasilewski J, Lu P. Lambda phage cro repressor interaction with its operator DNA: 2'-deoxy-5-fluorouracil OR3 analogues. *Biochemistry.* 1985;24:1418–24.
- [13] Habener JF, Vo CD, Le DB, Gryan GP, Ercolani L, Wang AHJ. 5-fluorodeoxyuridine as an alternative to the synthesis of mixed hybridization probes for the detection of specific gene sequences. *Proc Natl Acad Sci USA.* 1988;85:1735–9.
- [14] Liu J, Skradis A, Kolar C, Kolath J, Anderson J, Lawson T, Talmadge J, Gmeiner WH. Increased cytotoxicity and decreased in vivo toxicity of FdUMP[10] relative to 5-FU. *Nucleosides Nucleotides.* 1999;18:1789–802.
- [15] Gmeiner WH, Debinski W, Milligan C, Caudell D, Pardee TS. The applications of the novel polymeric fluoropyrimidine F10 in cancer treatment: current evidence. *Future Oncol.* 2016;12:2009–20.
- [16] Brandon ML, Mi LJ, Chaung W, Teebor G, Boorstein RJ. 5-chloro-2'-deoxyuridine cytotoxicity results from base excision repair of uracil subsequent to thymidylate synthase inhibition. *Mutat Res.* 2000;459:161–9.
- [17] Baker D, Liu P, Burdzy A, Sowers LC. Characterization of the substrate specificity of a human 5-hydroxymethyluracil glycosylase activity. *Chem Res Toxicol.* 2002;15:33–9.
- [18] Valinluck V, Liu P, Kang JI Jr, Burdzy A, Sowers LC. 5-halogenated pyrimidine lesions within a CpG sequence context mimic 5-methylcytosine by enhancing the binding of the methyl-CpG-binding domain of methyl-CpG-binding protein 2 (MeCP2). *Nucleic Acids Res.* 2005;33:3057–64.
- [19] Van Aerschot A, Everaert D, Balzarini J, Augustyns K, Jie L, Janssen G, Peeters O, Blaton N, de Ranter C, De Clercq E, Herdewijn P. Synthesis and anti-HIV evaluation of 2',3'-dideoxyribo-5-chloropyrimidine analogues: reduced toxicity of 5-chlorinated 2',3'-dideoxynucleosides. *J Med Chem.* 1990;33:1833–9.
- [20] Kumar R, Wiebe LI, Knaus EE. A mild and efficient methodology for the synthesis of 5-halogen uracil nucleosides that occurs via a 5-halogeno-6-azido-5,6-dihydro intermediate. *Can J Chem.* 1994;72:2005–10.
- [21] Theruvathu JA, Kim CH, Rogstad DK, Neidigh JW, Sowers LC. Base pairing configuration and stability of an oligonucleotide duplex containing a 5-chlorouracil-adenine base pair. *Biochemistry.* 2009;48:7539–46.
- [22] Theruvathu JA, Kim CH, Darwanto A, Neidigh JW, Sowers LC. pH-Dependent configuration of a 5-chlorouracil-guanine base pair. *Biochemistry.* 2009;48:11312–8.
- [23] Doucet J, Benoit JP, Cruse WB, Prange T, Kennard O. Coexistence of A- and B-form DNA in a single crystal lattice. *Nature.* 1989;337:190–2.
- [24] Willis MC, Hicke BJ, Uhlenbeck OC, Cech TR, Koch TH. Photocrosslinking of 5-iodouracil-substituted RNA and DNA to proteins. *Science.* 1993;262:1255–7.
- [25] Rosenthal A, Cech D, Veiko VP, Orezkaya TS, Romanova EA, Elov AA, Metelev VG, Gromova ES, Shabarova ZA. Chemische synthese von nonadesoxyribonucleotiden mit den abgewandelten basen uracil, 5-bromouracil und 5-methylcytosin nach dem triester-verfahren. *Tetrahedron Lett.* 1984;25:4353–6.
- [26] Ferrer E, Fabrega C, Güimil Garcia R, Azorín F, Eritja R. Preparation of oligonucleotides containing 5-bromouracil and 5-methylcytidine. *Nucleosides Nucleotides.* 1996;15:907–21.
- [27] Sheardy RD, Seeman NC. The synthesis of a deoxyoligonucleotide incorporating 5-iododeoxyuridine. *J Org Chem.* 1986;51:4301–3.
- [28] Ferrer E, Wiersma M, Kazimierczak B, Müller CW, Eritja R. Preparation and properties of oligodeoxynucleotides containing 5-iodouracil and 5-bromo and 5-iodocytosine. *Bioconjug Chem.* 1997;8:757–61.

- [29] Fazakerley GV, Sowers LC, Eritja R, Kaplan BE, Goodman MF. Structural and dynamic properties of a bromouracil-adenine base pair in DNA studied by proton NMR. *J Biomol Struct Dyn.* 1987;5:639–50.
- [30] Sowers LC, Goodman MF, Eritja R, Kaplan B, Fazakerley GV. Ionized and wobble base-pairing for bromouracil-guanine in equilibrium under physiological conditions. *J Mol Biol.* 1989;205:437–47.
- [31] Osterman DG, DePillis GD, Wu JC, Matsuda A, Santi DV. 5-fluorocytosine in DNA is a mechanism-based inhibitor of Hhal methylase. *Biochemistry.* 1988;27:5204–10.
- [32] Friedman S, Ansari N. Binding of the EcoRII methyltransferase to 5-fluorocytosine-containing DNA. Isolation of a bound peptide. *Nucleic Acids Res.* 1992;20:3241–8.
- [33] Schmidt S, Pein CD, Fritz HJ, Cech D. Chemical synthesis of 2'-deoxyoligonucleotides containing 5-fluoro-2'-deoxycytidine. *Nucleic Acids Res.* 1992;20:2421–6.
- [34] MacMillan AM, Chen L, Verdine GL. Synthesis of an oligonucleotide suicide substrate for DNA methyltransferase. *J Org Chem.* 1992;57:2989–91.
- [35] Marasco CJ, Sufrin JR. A convenient method for the direct incorporation of 5-fluoro-2'-deoxycytidine into oligodeoxynucleotides. *J Org Chem.* 1992;57:6363–5.
- [36] Sowers LC. <sup>15</sup>N-Enriched 5-fluorocytosine as a probe for examining unusual DNA structures. *J Biomol Struct Dyn.* 2000;17:713–23.
- [37] Evilia C, Zhang X, Kanyo J, Lu P. The synthesis of oligonucleotides containing fluoro-2'-deoxycytidine for secondary structure determination of tandem tetraloop DNA analogs. *Nucleosides Nucleotides.* 1997;16:1809–20.
- [38] Hanck T, Schmidt S, Fritz HJ. Sequence-specific and mechanism-based crosslinking of Dcm DNA cytosine-C<sup>5</sup> methyltransferase of *E.coli* K-12 to synthetic oligonucleotides containing 5-fluoro-2'-deoxycytidine. *Nucleic Acids Res.* 1993;21:303–9.
- [39] Kang JL, Burdzy A, Liu P, Sowers LC. Synthesis and characterization of oligonucleotides containing 5-chlorocytosine. *Chem Res Toxicol.* 2004;17:1236–44.
- [40] Valinluck V, Wu W, Liu P, Neidigh JW, Sowers LC. Impact of cytosine 5-halogens on the interaction of DNA with restriction endonucleases and methyltransferase. *Chem Res Toxicol.* 2006;19:556–62.
- [41] Zeng Y, Wang Y. Facile formation of an intrastand cross-link lesion between cytosine and guanine upon Pyrex-filtered UV light irradiation of d(<sup>Br</sup>CG) and duplex DNA containing 5-bromocytosine. *J Am Chem Soc.* 2004;126:6552–3.
- [42] Liu J, Verdine GL. Synthesis of photoactive DNA: incorporation of 5-Bromo-2'-deoxyadenosine into synthetic oligodeoxynucleotides. *Tetrahedron Lett.* 1992;33:4265–8.
- [43] Liu J, Fan QR, Sodeoka M, Lane WS, Verdine GL. DNA binding by an amino acid residue in the C-terminal half of the Rel homology region. *Chem Biol.* 1994;1:47–55.
- [44] Fàbrega C, Güimil García R, Díaz AR, Eritja R. Studies on the synthesis of oligonucleotides containing photoreactive nucleosides: 2-azido-2'-deoxyinosine and 8-azido-2'-deoxyadenosine. *Biol Chem.* 1998;379:527–33.
- [45] Fàbrega C, Macías MJ, Eritja R. Synthesis and properties of oligonucleotides containing 8-bromo-2'-deoxyguanosine. *Nucleosides Nucleotides Nucleic Acids.* 2001;20:251–60.
- [46] Dias E, Battiste JL, Williamson JR. Chemical probe for glycosidic conformation in telomeric DNAs. *J Am Chem Soc.* 1994;116:4479–80.
- [47] Kimura T, Kawai H, Tojo S, Majima T. One-electron attachment reaction of B- and Z-DNA modified by 8-bromo-2'-deoxyguanosine. *J Org Chem.* 2004;69:1169–73.
- [48] Nadler A, Diederichsen U. Guanosine analog with respect to Z-DNA stabilization: nucleotide with combined C8-bromo and C2'-ethynyl modifications. *Eur J Org Chem.* 2008;1544–9.
- [49] Kapdi AR, Maiti D, Sanghvi YS. Palladium-catalyzed modification of nucleosides, nucleotides and oligonucleotides. Amsterdam: Elsevier; 2018.

- [50] Hamm ML, Rajguru S, Downs AM, Cholera R. Base pair stability of 8-chloro-and 8-ido-2'-deoxyguanosine opposite 2'-deoxycytidine: implications regarding the bioactivity of 8-oxo-2'-deoxyguanosine. *J Am Chem Soc.* 2005;127:12220–1.
- [51] Sowers LC, Fazakerley GV, Eritja R, Kaplan BE, Goodman MF. Base pairing and mutagenesis: observation of a protonated base pair between 2-aminopurine and cytosine in an oligonucleotide by proton NMR. *Proc Natl Acad Sci USA.* 1986;83:5434–8.
- [52] Petrauskene OV, Schmidt S, Karyagina AS, Nikolskaya II, Gromova ES, Cech D. The interaction of DNA duplexes containing 2-aminopurine with restriction endonucleases EcoRII and Ssoll. *Nucleic Acids Res.* 1995;23:2192–7.
- [53] Diekmann S, von Kitzing E, McLaughlin L, Ott J, Eckstein F. The influence of exocyclic substituents of purine bases on DNA curvature. *Proc Natl Acad Sci USA.* 1987;84:8257–61.
- [54] Fazakerley GV, Sowers LC, Eritja R, Kaplan BE, Goodman MF. NMR studies on oligo-deoxynucleotides containing 2-aminopurine opposite adenine. *Biochemistry.* 1987;26:5641–6.
- [55] Sowers LS, Eritja R, Chen FCh, Khwaja T, Kaplan BE, Goodman MF, Fazakerley GV. Characterization of the high pH wobble structure of the 2-aminopurine.cytosine mismatch by N-15 NMR spectroscopy. *Biochem Biophys Res Commun.* 1989;165:89–92.
- [56] Eritja R, Kaplan BE, Mhaskar D, Sowers LC, Petruska J, Goodman MF. Synthesis and properties of defined DNA oligomers containing base mispairs involving 2-aminopurine. *Nucleic Acids Res.* 1986;14:5869–84.
- [57] Watanabe SM, Goodman MF. On the molecular basis of transition mutations: frequencies of forming 2-aminopurine-cytosine and adenine-cytosine base mispairs in vitro. *Proc Natl Acad Sci USA.* 1981;78:2864–8.
- [58] Sowers LC, Boulard Y, Fazakerley GV. Multiple structures for the 2-aminopurine-cytosine mispair. *Biochemistry.* 2000;39:7613–20.
- [59] Fagan PA, Fàbrega C, Eritja R, Goodman MF, Wemmer DE. NMR study of the 2-aminopurine:cytosine mismatch in DNA. *Biochemistry.* 1996;35:4026–33.
- [60] Gargallo R, Vives M, Tauler R, Eritja R. Protonation studies and multivariate curve resolution on oligodeoxynucleotides carrying the mutagenic base 2-aminopurine. *Biophys J.* 2001;81:2886–96.
- [61] Law SM, Eritja R, Goodman MF, Breslauer KJ. Spectroscopy and calorimetric characterizations of DNA duplexes containing 2-aminopurine. *Biochemistry.* 1996;35:12329–37.
- [62] Sowers LC, Mhaskar DN, Khwaja TA, Goodman MF. Preparation of imino and amino N-15 enriched 2-aminopurine deoxynucleoside. *Nucleosides Nucleotides.* 1989;8:23–34.
- [63] McLaughlin LW, Leong T, Benseler F, Piel N. A new approach to the synthesis of a protected 2-aminopurine derivative and its incorporation into oligodeoxynucleotides containing the Eco RI and Bam HI recognition sites. *Nucleic Acids Res.* 1988;16:5631–44.
- [64] Connolly BA. Synthetic oligodeoxynucleotides containing modified bases. *Methods Enzymol.* 1992;211:36–53.
- [65] Connolly BA. In: Eckstein F, editor. *Oligonucleotides and analogs. A practical approach.* New York: IRL Press; 1991. p. 155–83.
- [66] Doudna JA, Szostak JW, Rich A, Usman N. Chemical synthesis of oligoribonucleotides containing 2-aminopurine: substrates for the investigation of ribozyme function. *J Org Chem.* 1990;55:5547–9.
- [67] Schmidt S, Cech D. A new approach to the synthesis of 2-aminopurine-2'-deoxyriboside via tri-*n*-butyltin hydride reduction. *Nucleosides Nucleotides.* 1995;14:1445–52.
- [68] Fujimoto J, Nuesca Z, Mazurek M, Sowers LC. Synthesis and hydrolysis of oligodeoxyribonucleotides containing 2-aminopurine. *Nucleic Acids Res.* 1996;24:754–9.

- [69] Zhou Y, Ts'o POP. Synthesis of oligodeoxyribonucleoside methylphosphonates containing 2-aminopurine. *Nucleosides Nucleotides*. 1996;15:1635–48.
- [70] Acedo M, Fàbrega C, Aviñó A, Goodman MF, Fagan P, Wemmer D, Eritja R. A simple method for N-15 labelling of exocyclic amino groups in synthetic oligodeoxynucleotides. *Nucleic Acids Res.* 1994;22:2982–9.
- [71] Fàbrega C, Grijalvo S, Eritja R. Synthesis and properties of oligodeoxynucleotides carrying 2-aminopurine. *The Open Org Chem J.* 2011;5:1–8.
- [72] Jones AC, Neely RK. 2-aminopurine as fluorescent probe of DNA conformation and the DNA-enzyme interface. *Q Rev Biophys.* 2015;48:244–79.
- [73] Bloom L, Otto MR, Eritja R, Reha-Krantz L, Goodman MF, Beechem JM. Pre-steady-state kinetic analysis of sequence-dependent nucleotide excision by the 3'-exonuclease activity of bacteriophage T4 DNA polymerase. *Biochemistry*. 1994;33:7576–86.
- [74] Beechem JM, Otto MR, Bloom LB, Eritja R, Reha-Krantz LJ, Goodman MF. Exonuclease-polymerase active site partitioning of primer-template DNA strands and equilibrium Mg<sup>2+</sup> binding properties of bacteriophage T4 polymerase. *Biochemistry*. 1998;37:10144–55.
- [75] Raney KD, Sowers LC, Millar DP, Benkovic SJ. A fluorescence-based assay for monitoring helicase activity. *Proc Natl Acad Sci USA*. 1994;91:6644–8.
- [76] Pues H, Bleimling N, Holz B, Wölcke J, Weinhold E. Functional roles of the conserved aromatic amino acid residues at position 108 (motif IV) and position 196 (motif VIII) in base flipping and catalysis by the N<sup>6</sup>-adenine DNA methyl transferase from *Thermus aquaticus*. *Biochemistry*. 1999;26:1076–83.
- [77] Negishi K, Harada C, Ohara Y, Oohara K, Nitta N, Hayatsu H. N<sup>4</sup>-aminocytidine, a nucleoside analog that has an exceptionally high mutagenic activity. *Nucleic Acids Res.* 1983;11:5223–33.
- [78] Negishi K, Takahashi M, Yamashita Y, Nishizawa M, Hayatsu H. Mutagenesis by N<sup>4</sup>-aminocytidine: induction of AT to GC transitions and its molecular mechanism. *Biochemistry*. 1985;24:7273–8.
- [79] Takahashi M, Negishi K, Hayatsu H. Proofreading of a mutagenic nucleotide, N<sup>4</sup>-aminodeoxycytidyl acid, by *Escherichia coli* DNA polymerase I. *Biochem Biophys Res Commun.* 1987;143:104–9.
- [80] Takahashi M, Nishizawa M, Negishi K, Hanaoka F, Yamada MA, Hayatsu H. Induction of mutation in mouse FM3A cells by N<sup>4</sup>-aminocytidine-mediated replicational errors. *Mol Cell Biol.* 1988;8:347–52.
- [81] Fazakerley GV, Gdaniec Z, Sowers LC. Base-pair induced shifts in the tautomeric equilibrium of a modified DNA base. *J Mol Biol.* 1993;230:6–10.
- [82] Gdaniec Z, Ban B, Sowers LC, Fazakerley GV. Methoxyamine-induced mutagenesis of nucleic acids. A proton NMR study of oligonucleotides containing N<sup>4</sup>-methoxycytosine paired with adenine or guanine. *Eur J Biochem.* 1996;242:271–9.
- [83] Nishio H, Ono A, Matsuda A, Ueda T. The synthesis and properties of oligodeoxyribonucleotides containing N<sup>6</sup>-methoxyadenine. *Nucleic Acids Res.* 1992;20:777–82.
- [84] Nishio H, Ono A, Matsuda A, Ueda T. Nucleosides and nucleotides 111. Thermal stability of oligodeoxyribonucleotide duplexes containing N<sup>6</sup>-methoxyadenine in substitution for adenine. *Chem Pharm Bull.* 1992;40:1355–7.
- [85] Chambert S, Décout JL. Recent developments in the synthesis, chemical modifications and biological applications of sulfur modified nucleosides, nucleotides and oligonucleotides. *Org Prep Proced Int.* 2002;34:27–85.

- [86] Rao TVS, Haber MT, Sayer JM, Jerina DM. Incorporation of 4-thiothymidine into DNA by the Klenow fragment and HIV reversed transcriptase. *Bioorg Med Chem Lett.* 2000;10:907–10.
- [87] Favre A, Saintomé C, Fourrey JL, Clivio P, Laugâa P. Thionucleobases as intrinsic photoaffinity probes of nucleic acid structure and nucleic acid-protein interactions. *J Photochem Photobiol B, Biol.* 1998;42:109–24.
- [88] Connolly BA, Newmann PC. Synthesis and properties of oligonucleotides containing 4-thiothymidine, 5-methyl-2-pyrimidinone-1- $\beta$ -D-(2'-deoxyriboside) and 2-thiothymidine. *Nucleic Acids Res.* 1989;17:4957–74.
- [89] Ishihara T, Yoneda F, Tanaka K, Fuji K. Synthesis and properties of oligothymidylylate containing sulfur-modified thymidine: effect of the thiation of pyrimidine ring on the thermostability and conformation of the duplex. *Bioorg Med Chem Lett.* 1991;1:523–6.
- [90] Rajur SB, McLaughlin LW. The synthesis of oligodeoxynucleotides containing 2-thiothymidine and 5-methyl-4-pyrimidinone base analogues. *Tetrahedron Lett.* 1992;33:6081–4.
- [91] Nikiforov TT, Connolly BA. The synthesis of oligodeoxynucleotides containing 4-thiothymidine residues. *Tetrahedron Lett.* 1991;32:3851–4.
- [92] Nikiforov TT, Connolly BA. Straightforward preparation and use in oligodeoxynucleotide synthesis of 5'-O-(4,4'-dimethoxytrityl)-4-[S-(2-cyanoethyl)]-thiothymidine. *Tetrahedron Lett.* 1992;33:2379–82.
- [93] Coleman RS, Siedlecki JM. Synthesis of a 4-thio-2'-deoxyuridine-containing oligonucleotide. Development of the thiocarbonyl group as a linker element. *J Am Chem Soc.* 1992;114:9229–30.
- [94] Coleman RS, Kesicki EA. Synthesis and postsynthetic modification of oligodeoxynucleotides containing 4-thio-2'-deoxyuridine ( $d^{34}U$ ). *J Am Chem Soc.* 1994;116:11636–42.
- [95] Clivio P, Fourrey JL, Gasche J, Audic A, Favre A, Perrin C, Woisard A. Synthesis and purification of oligonucleotides containing sulfur substituted nucleobases: 4-thiouracil, 4-thiothymine and 6-mercaptopurine. *Tetrahedron Lett.* 1992;33:65–8.
- [96] Clivio P, Fourrey JL, Gasche J, Favre A. Synthesis of dinucleoside phosphates containing sulfur substituted nucleobases: 4-thiouracil, 4-thiothymine and 6-mercaptopurine. *Tetrahedron Lett.* 1992;33:69–72.
- [97] Clivio P, Fourrey JL, Gasche J, Favre A. Synthesis of dinucleoside phosphates containing 4-thio substituted nucleobases. *J Chem Soc, Perkin Trans I.* 1992;2383–8.
- [98] Kuimelis RG, Nambiar KP. Synthesis of oligodeoxynucleotides containing 2-thiopyrimidine residues – a new protection scheme. *Nucleic Acids Res.* 1994;22:1429–36.
- [99] Sintim HO, Kool ET. Enhanced base pairing and replication efficiency of thiothymidines, expanded-size variants of thymidine. *J Am Chem Soc.* 2006;128:396–7.
- [100] Faustino I, Aviñó A, Marchán I, Luque FJ, Eritja R, Orozco M. Unique tautomeric and reconstitution properties of thioketothymines? *J Am Chem Soc.* 2009;131:12845–53.
- [101] Kutyavin IV, Rhinehart RL, Lukhtanov EA, Gorn VV, Meyer RB, Gamper HB. Oligonucleotides containing 2-aminoadenine and 2-thiothymine act as selectively binding complementary agents. *Biochemistry.* 1996;35:11170–6.
- [102] Waters TR, Swann PF. Cytotoxic mechanism of 6-thioguanine: hMutS $\alpha$ , the human mismatch binding heterodimer, binds to DNA containing  $S^6$ -methylthioguanine. *Biochemistry.* 1997;36:2501–6.
- [103] Rapaport HP. The 6-thioguanine/5-methyl-2-pyrimidinone base pair. *Nucleic Acids Res.* 1988;16:7253–67.
- [104] Christopherson MS, Broom AD. Synthesis of oligonucleotides containing 2'-deoxy-6-thioguanosine at a predetermined site. *Nucleic Acids Res.* 1991;19:5719–24.
- [105] Waters TR, Connolly BA. Straightforward synthesis of 6-thiodeoxyguanosine and its incorporation into oligodeoxynucleotides. *Nucleosides Nucleotides.* 1992;11:985–98.

- [106] Nikiforov TT, Connolly BA. Oligodeoxynucleotides containing 4-thiothymidine and 6-thiodeoxyguanosine as affinity labels for the Eco RV restriction endonuclease and modification methylase. *Nucleic Acids Res.* 1992;20:1209–14.
- [107] Waters TR, Connolly BA. Interaction of the restriction endonuclease Eco RV with the deoxyguanosine and deoxycytidine bases in its recognition sequence. *Biochemistry*. 1994;33:1812–9.
- [108] Rao TS, Jayaraman K, Durland RH, Revankar GR. A novel synthesis of  $S^6$ -cyanoethyl-2'-deoxy-6-thioguanosine and its incorporation into triple helix forming oligonucleotides. *Tetrahedron Lett.* 1992;33:7651–4.
- [109] Rao TS, Durland RH, Seth DM, Myrick MA, Bodepudi V, Revankar GR. Incorporation of 2'-deoxy-6-thioguanosine into G-rich oligodeoxyribonucleotides inhibits G-tetrad formation and facilitates triplex formation. *Biochemistry*. 1995;34:765–72.
- [110] Clivio P, Fourrey JL, Favre A. Synthesis of deoxydinucleoside phosphates containing 6-thio-substituted purine nucleosides. *J Chem Soc, Perkin Trans I*. 1993;2585–90.
- [111] Xu YZ, Zheng Q, Swann PF. Synthesis by post-synthetic substitution of oligomers containing guanine modified at the 6-positions with *S*-, *N*-, *O*-derivatives. *Tetrahedron*. 1992;48:1729–40.
- [112] Milton J, Connolly BA, Nikiforov TT, Cosstick R. Site-specific disulfide bridges in oligodeoxynucleotide duplexes containing 6-mercaptopurine and 4-thiothymine bases. *J Chem Soc, Chem Commun*. 1993;779–80.
- [113] Coleman RS, Arthur JC, McCary JL. 6-Thio-2'-deoxyinosine: Synthesis, incorporation, and evaluation as a postsynthetically modifiable base in oligonucleotides. *Tetrahedron*. 1997;11191–202.
- [114] Xu YZ, Zheng Q, Swann PF. Preparation of oligodeoxynucleotides containing 6-methylthiopurine residues by chemical synthesis or specific methylation. *Nucleosides Nucleotides*. 1995;14:929–33.
- [115] Massey A, Xu YZ, Karra P. Ambiguous coding is required for the lethal interaction between methylated DNA bases and DNA mismatch repair. *DNA Repair*. 2002;1:275–86.
- [116] Hamm ML, Cholera R, Hoey CL, Gill TJ. Oligonucleotide incorporation of 8-thio-2'-deoxyguanosine. *Org Lett*. 2004;6:3817–20.
- [117] Glick GD. Methods for cross-linking nucleic acids. *Curr Prot Nucleic Acids Chem*. 2003;5.7.1–13.
- [118] Hou X, Wang G, Gaffney BL, Jones RA. Preparation of DNA and RNA fragments containing guanine N<sup>2</sup>-thioalkyl tethers. *Curr Prot Nucleic Acids Chem*. 2010;5.8.1–23.
- [119] Glick GD. Synthesis of a conformationally restricted DNA hairpin. *J Org Chem*. 1991;56:6747–7.
- [120] Wang H, Osborne SE, Zuiderweg ERP, Glick GD. Three-dimensional structure of a sulfide-stabilized non-ground-state DNA hairpin. *J Am Chem Soc*. 1994;116:5021–2.
- [121] Wang H, Zuiderweg ERP, Glick GD. Solution structure of a disulfide cross-linked DNA hairpin. *J Am Chem Soc*. 1995;117:2981–91.
- [122] Glick GD, Osborne SE, Knitt DS, Marino JP. Trapping and isolation of an alternate DNA conformation. *J Am Chem Soc*. 1992;114:5447–8.
- [123] Osborne SE, Völker J, Stevens SY, Breslauer KJ, Glick GD. Design, synthesis, and analysis of disulfide cross-linked DNA duplexes. *J Am Chem Soc*. 1996;118:11993–2003.
- [124] Goodwin JT, Glick GD. Incorporation of alkylthiol chains at C-5 of deoxyuridine. *Tetrahedron Lett*. 1993;34:5549–52.
- [125] Goodwin JT, Osborne SE, Swanson PC, Glick GD. Synthesis of a disulfide cross-linked DNA triple helix. *Tetrahedron Lett*. 1994;35:4527–30.
- [126] Osborne SE, Ellington AD. Incorporating disulfide cross-links at the terminus of oligonucleotides via solid-phase nucleic acid synthesis. *Bioorg Med Chem*. 1996;6:2339–42.

- [127] Bradley DH, Hanna MM. Synthesis and utility of 5-thiocyanato deoxyuridine and uridine phosphoramidites as masked synthons. *Tetrahedron Lett.* 1992;33:6223–6.
- [128] Hou X, Wang G, Gaffney BL, Jones RA. Synthesis of guanosine and deoxyguanosine phosphoramidites with cross-linkable thioalkyl tethers for direct incorporation into RNA and DNA. *Nucleosides Nucleotides Nucleic Acids.* 2009;28:1076–94.
- [129] Das K, Bandwar RP, White KL, Feng JY, Sarafianos SG, Tuske S, Tu X, Clark AD, Boyer PL, Hou X, Gaffney BL, Jones RA, Miller MD, Hughes SH, Arnold E. Structural basis for the role of the K65R mutation in HIV-1 reverse transcriptase polymerization, excision antagonism, and tenofovir resistance. *J Biol Chem.* 2009;284:35092–100.
- [130] Coleman RS, Kesicki EA. Template-directed cross-linking of oligonucleotides: site-specific covalent modification of dG-N7 within duplex DNA. *J Org Chem.* 1995;60:6252–3.
- [131] Coleman RS, Pires RM. Covalent cross-linking of duplex DNA using 4-thio-2'-deoxyuridine as a readily modifiable platform for introduction of reactive functionality into oligonucleotides. *Nucleic Acids Res.* 1997;25:4771–7.
- [132] Ferentz AE, Verdine GL. Disulfide cross-linked oligonucleotides. *J Am Chem Soc.* 1991;113:4000–2.
- [133] Ferentz AE, Keating TA, Verdine GL. Synthesis and characterization of disulfide cross-linked oligonucleotides. *J Am Chem Soc.* 1993;115:9006–14.
- [134] Erlanson DA, Wolfe SA, Chen L, Verdine GL. Selective base-pair destabilization enhances binding of a DNA methyltransferase. *Tetrahedron.* 1997;53:12041–56.
- [135] Mullis K, Falonna F, Scharf S, Saiki R, Horn G, Erlich H. Specific-enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symp Quant Biol.* 1986;51:263–73.
- [136] Millican TA, Mock GA, Chauncey MA, Patel TP, Eaton MAW, Gunning J, Cutbush SD, Neidle S, Mann J. Synthesis and biophysical studies of short oligodeoxynucleotides with novel modifications: a possible approach to the problem of mixed bases oligodeoxynucleotide synthesis. *Nucleic Acids Res.* 1984;12:7435–53.
- [137] François P, Perilleux D, Kempener Y, Sonveaux E. Flexible aglycone residues in duplex DNA. *Tetrahedron Lett.* 1990;31:6347–50.
- [138] Eritja R, Horowitz DM, Walker PA, Ziehler-Martin JP, Boosalis MS, Goodman MF, Itakura K, Kaplan BE. Synthesis and properties of oligonucleotides containing 2'-deoxynebularine and 2'-deoxyxanthosine. *Nucleic Acids Res.* 1986;14:8135–53.
- [139] Ikebara M, Inaoka T. Synthesis of 1-(2-deoxy- $\beta$ -D-ribofuranosyl) benzimidazole via cyclonucleosides. *Nucleosides Nucleotides.* 1985;4:515–21.
- [140] Ohsuka E, Matsuki S, Ikebara M, Takahashi Y, Matsubara K. An alternative approach to deoxyoligonucleotides as hybridization probes by insertion of deoxyinosine at ambiguous codon positions. *J Biol Chem.* 1985;260:2605–8.
- [141] Martin FH, Castro MM. Base pairing involving deoxyinosine: implications for probe design. *Nucleic Acids Res.* 1985;13:8927–38.
- [142] Kawase Y, Iwai S, Inoue H, Miura OE. Studies on nucleic acid interactions I. Stabilities of mini-duplexes ( $dG_2A_4X A_4G_2.dC_2T_4Y T_4C_2$ ) and self-complementary  $d(GGGAAXYTTCCC)$  containing deoxyinosine and other mismatched bases. *Nucleic Acids Res.* 1986;14:7727–36.
- [143] Case-Green SC, Southern EM. Studies on the base pairing properties of deoxyinosine by solid phase hybridisation to oligonucleotides. *Nucleic Acids Res.* 1994;22:131–6.
- [144] Seela F, Kaiser K. Phosphoramidites of base-modified 2'-deoxyinosine isosteres and solid-phase synthesis of  $d(GCl^*CGC)$  oligomers containing an ambiguous base. *Nucleic Acids Res.* 1986;14:1825–44.

- [145] Cubero E, Güimil García R, Luque FJ, Eritja R, Orozco M. The effect of amino groups on the stability of DNA duplexes and triplexes based on purines derived from inosine. *Nucleic Acids Res.* 2001;29:2522–34.
- [146] Acedo M, De Clercq E, Eritja R. Synthesis and biophysical and biological properties of oligonucleotides containing 2-aza-2'-deoxyinosine. *J Org Chem.* 1995;60:6262–9.
- [147] Van Aerschot A, Mag M, Herdewijn P, Vanderhaeghe H. Double protection of the heterocyclic base xanthosine and 2'-deoxyxanthosine. *Nucleoside Nucleotides.* 1989;8:159–78.
- [148] Jurczyk SC, Horlacher J, Devined KG, Benner SA, Battersby TR. Synthesis and characterization of oligonucleotides containing 2'-deoxyxanthosine using phosphoramidite chemistry. *Helv Chim Acta.* 2000;83:1517–24.
- [149] Wuenschell GE, O'Connor TR, Termini J. Stability, miscoding potential, and repair of 2'-deoxyxanthosine in DNA: implications for nitric oxide-induced mutagenesis. *Biochemistry.* 2003;42:3608–16.
- [150] Pochet S, D'Ari R. Synthesis and enzymatic polymerization of 5-amino-1-(2'-deoxy- $\beta$ -D-ribofuranosyl)imidazole-4-carboxamide-5'-triphosphate. *Nucleic Acids Res.* 1990;18:7127–31.
- [151] Pochet S, Dugué L. Oligodeoxynucleotides embodying the ambiguous base Z, 5-amino-imidazole-4-carboxamide. *Nucleosides Nucleotides.* 1995;14:1195–210.
- [152] Fernandez-Forner D, Eritja R, Bardella F, Ruiz-Perez C, Solans X, Giralt E, Pedroso E. Preparation of oligonucleotides containing dAICA using an unexpected side-reaction observed on a protected derivative of 2-aza-2'-deoxyinosine. *Tetrahedron.* 1991;47:8917–30.
- [153] Pochet S, Dugué L. Imidazole-4-carboxamide and 1,2,4-triazole-3-carboxamide deoxynucleotides as simplified DNA building blocks with ambiguous pairing capacity. *Nucleosides Nucleotides.* 1998;17:2003–9.
- [154] Lin PKT, Brown DM. Synthesis and duplex stability of oligonucleotides containing cytosine-thymine analogues. *Nucleic Acids Res.* 1989;17:10373–83.
- [155] Anand NN, Brown DM, Salisbury SA. The stability of oligodeoxyribonucleotide duplexes containing degenerate bases. *Nucleic Acids Res.* 1987;15:8167–76.
- [156] Brown DM, Lin PKT. Synthesis and duplex stability of oligonucleotides containing adenine-guanine analogues. *Carbohydr Res.* 1991;216:129–39.
- [157] Lin PKT, Brown DM. Synthesis of oligodeoxyribonucleotides containing degenerate bases and their use as primers in the polymerase chain reaction. *Nucleic Acids Res.* 1992;20:5149–52.
- [158] Kamiya H, Murata-Kamiya N, Lin PKT, Brown DM, Ohtsuka E. Nucleotide incorporation opposite degenerate bases by Taq DNA polymerase. *Nucleosides Nucleotides.* 1994;13:1483–92.
- [159] Hill F, Loakes D, Brown DM. Polymerase recognition of synthetic oligodeoxyribonucleotides incorporating degenerate pyrimidine and purine bases. *Proc Natl Acad Sci USA.* 1998;95:4258–63.
- [160] Loakes D, Brown DM. Synthesis of bicyclic N<sup>4</sup>-oxycytidine derivatives. *Nucleosides Nucleotides.* 1994;13:679–706.
- [161] Woo J, Meyer RB, Damper HB. G/C-modified oligodeoxynucleotides with selective complementarity: synthesis and hybridization properties. *Nucleic Acids Res.* 1996;24:2470–5.
- [162] Wang J, Lin KY, Matteucci MD. Synthesis and binding property of an oligonucleotide containing tetrafluorophenoxazine. *Tetrahedron Lett.* 1998;39:8385–8.
- [163] Nichols R, Andrews PC, Zhang P, Bergstrom DE. A universal nucleoside for use at ambiguous sites in DNA primers. *Nature.* 1994;369:492–3.
- [164] Loakes D, Brown DM. 5-Nitroindole as an universal base analogue. *Nucleic Acids Res.* 1994;22:4039–43.

- [165] Loakes D, Brown DM, Linde S, Hill F. 3-Nitropyrrole and 5-nitroindole as universal bases in primers for DNA sequencing and PCR. *Nucleic Acids Res.* 1995;23:2361–6.
- [166] Loakes D, Hill F, Brown DM, Salisbury A. Stability and structure of DNA oligonucleotides containing Bob-specific base analogues. *J Mol Biol.* 1997;270:426–35.
- [167] Seela F, Jawalekar A. 4-Nitroindazole: its ambiguous nature in oligonucleotides base pairing and the influence of the glycosylation position on the duplex stability. *Helv Chim Acta.* 2002;85:1857–68.
- [168] Seela F, Bourgeois W, Rosemeyer H, Wenzel T. Synthesis of 4-substituted 1H-benzimidazole 2'-deoxyribonucleosides and utility of the 4-nitro compound as universal base. *Helv Chim Acta.* 1996;79:488–98.
- [169] Van Aerschot A, Rozenski J, Loakes D, Pillet N, Schepers G, Herdewijn P. An acyclic 5-nitroindazole nucleoside analogue as ambiguous nucleoside. *Nucleic Acids Res.* 1995;23:4363–70.
- [170] Vandendriessche F, Augustynus K, Van Aerschot A, Busson R, Hoogmartens J, Herdewijn P. Acyclic oligonucleotides: possibilities and limitations. *Tetrahedron.* 1993;49:7223–38.
- [171] Seela F, Becher G. Oligonucleotides containing pyrazolo[3,4-*d*]pyrimidines: the influence of 7-substituted 8-aza-7-deaza-2'-deoxyguanosines on the duplex structure and stability. *Helv Chim Acta.* 1999;82:1640–55.
- [172] Seela F, Zulauf M. Synthesis of oligonucleotides containing [3,4-*d*]pyrimidines: the influence of 7-substituted 8-aza-7-deazaadenines on the duplex structure and stability. *J Chem Soc, Perkin Trans I.* 1999;479–88.
- [173] Seela F, Becher G. Pyrazolo[3,4-*d*]pyrimidine nucleic acids: adjustment of dA-dT to dG-dC base pair stability. *Nucleic Acids Res.* 2001;29:2069.
- [174] Becher G, He J, Seela F. Major-Groove-Halogenated DNA: the effects of bromo and iodo substituents replacing H-C(7) of 8-aza-7-deazapurine-2,6-diamine or H-C(5) of uracil residues. *Helv Chim Acta.* 2001;84:1048–65.
- [175] Seela F, Debelak H. The *N*<sup>8</sup>-(2'-deoxyribofuranoside) of 8-aza-7-deazaadenine: a universal nucleoside forming specific hydrogen bonds with the four canonical DNA constituents. *Nucleic Acids Res.* 2000;28:3224–32.
- [176] Seela F, Zulauf M, Debelak H. Base-pairing properties of 8-aza-7-deazaadenine linked via the 8-position to the DNA backbone. *Helv Chim Acta.* 2000;83:1437–53.
- [177] Sangvi Y. Heterocyclic base modifications in nucleic acids and their applications in antisense oligonucleotides. In: Crooke ST, Lebleu B, editors. *Antisense research and applications.* Boca Raton, Florida: CRC Press Inc.; 2000. p. 273–301.
- [178] Herdewijn P. Heterocyclic modifications of oligonucleotides and antisense technology. *Antisense Nucleic Acid Drug Dev.* 2000;19:297–310.
- [179] Freier SM, Altmann KH. The ups and downs of nucleic acid duplex stability: structure-stability studies on chemically-modified DNA: RNA duplexes. *Nucleic Acids Res.* 1997;25:4429–43.
- [180] Scheit KH, Rackwitz HR. Synthesis and physicochemical properties of two analogs of poly(dA): poly(2-aminopurine-9-β-D-deoxyribonucleotide) and poly 2-amino-deoxyadenylic acid. *Nucleic Acids Res.* 1982;10:4059–69.
- [181] Howard FB, Miles HT. 2NH<sub>2</sub>-T helices in the ribo- and deoxypolynucleotide series. Structural and energetic consequences of 2NH<sub>2</sub>A substitution. *Biochemistry.* 1984;23:6723–32.
- [182] Gaffney BL, Marky LA, Jones RA. The influence of the purine 2-amino group on DNA conformation and stability. II. Synthesis and physical characterization of d[CGT(2-NH<sub>2</sub>)ACG], d[CGU(2-NH<sub>2</sub>)ACG], and d[CGT(2-NH<sub>2</sub>)AT(2-NH<sub>2</sub>)ACG]. *Tetrahedron.* 1984;40:3–13.
- [183] Chollet A, Chollet-Damerius A, Kawashima EH. Synthesis of oligodeoxyribonucleotides containing the base 2-aminoadenine. *Chem Scr.* 1986;26:37–40.

- [184] Brown T, Booth ED, Craig AG. The incorporation of 2,6-diaminopurine into oligodeoxyribonucleotides by the phosphoramidite method. *Nucleosides Nucleotides*. 1989;8:1051.
- [185] Gryaznov S, Schultz RG. Stabilization of DNA: DNA and DNA: RNA duplexes by substitution of 2'-deoxyadenosine with 2'-deoxy-2-aminoadenosine. *Tetrahedron Lett*. 1994;35:2489–92.
- [186] Cano A, Goodman MF, Eritja R. Synthesis of oligodeoxyribonucleotides containing 2,6-diaminopurine. *Nucleosides Nucleotides*. 1994;13:501–9.
- [187] Luyten I, Van Aerschot A, Rozenski J, Busson R, Herdewijn P. Protection of 2,6-diaminopurine 2'-deoxyriboside. *Nucleosides Nucleotides*. 1997;16:1649–52.
- [188] Sproat B, Beijer B, Iribarren A. New synthetic routes to protected 2'-O-methylriboside-3'-O-phosphoramidites using a novel alkylation procedure. *Nucleic Acids Res*. 1990;18:41–9.
- [189] Sproat B, Iribarren A, Gümil-García R, Beijer B. New synthetic routes to synthons suitable for 2'-O-allyl oligoribonucleotide assembly. *Nucleic Acids Res*. 1991;19:733–8.
- [190] Chollet A, Kawashima EH. DNA containing the base analogue 2-aminoadenine: preparation, use as hybridization probes and cleavage by restriction endonucleases. *Nucleic Acids Res*. 1988;16:305–17.
- [191] Cheong C, Tinoco I, Chollet A. Thermodynamic studies of base pairing involving 2,6-diaminopurine. *Nucleic Acids Res*. 1988;16:5115–22.
- [192] Chazin WJ, Rance M, Chollet A, Leupin W. Comparative NMR analysis of the decadeoxynucleotide d-(GCATTAATGC)<sub>2</sub> and analogues containing 2-aminoadenine. *Nucleic Acids Res*. 1991;19:5507–13.
- [193] Wang G, Bergstrom DE. Synthesis of oligonucleotides containing  $N^2$ -[2-(imidazol-4-ylacetamido)ethyl]-2'-deoxyguanosine. *Tetrahedron Lett*. 1993;34:6725–8.
- [194] Wang G, Bergstrom DE. Synthesis of oligonucleotides containing  $N^2$ -(5-carboxypentyl)-2'-deoxyguanosine and 5-[2-(4'-methyl-2,2'-dipyrid-4-yl-carboxamido)ethylthio]-2'-deoxyuridine. *Tetrahedron Lett*. 1993;34:6721–4.
- [195] Heeb NV, Benner SA. Guanosine derivatives bearing  $N^2$ -3-imidazolepropionic acid. *Tetrahedron Lett*. 1994;35:3045–8.
- [196] Ramasamy KS, Zounes M, Gonzalez C, Freier S, Lesnik EA, Cummins LL, Griffey RH, Monia BP, Dan Cook D. Remarkable enhancement of binding affinity of heterocycle-modified DNA to DNA and RNA. Synthesis, characterization and biophysical evaluation of  $N^2$ -imidazolylpropylguanine and  $N^2$ -imidazolylpropyl-2-aminoadenine modified oligonucleotides. *Tetrahedron Lett*. 1994;35:215–8.
- [197] Manoharan M, Ramasamy KS, Mohan V, Dan Cook D. Oligonucleotides bearing cationic groups:  $N^2$ -(3-imidazolepropyl)deoxyguanosine. Synthesis, enhanced binding properties and conjugation chemistry. *Tetrahedron Lett*. 1996;37:7675–8.
- [198] Diaz AR, Eritja R, Gümil García R. Synthesis of oligodeoxynucleotides containing 2-substituted guanine derivatives using 2-fluoro-2'-deoxyinosine as common nucleoside precursor. *Nucleosides Nucleotides*. 1997;16:2035–51.
- [199] Eritja R, Diaz AR, Saison-Behmoaras E. Duplex-stabilization properties of oligodeoxynucleotides containing  $N^2$ -substituted guanine derivatives. *Helv Chim Acta*. 2000;83:1417–23.
- [200] Schmid N, Behr JP. Recognition of DNA sequences by strand replacement with polyamino-oligonucleotides. *Tetrahedron Lett*. 1995;36:1447–50.
- [201] Adib A, Potier PF, Doromina S, Huc I, Behr JP. A high-yield synthesis of deoxy-2-fluoroinosine and its incorporation into oligonucleotides. *Tetrahedron Lett*. 1997;38:2989–92.
- [202] Potier PF, Abdennaji A, Behr JP. Synthesis and hybridization properties of oligonucleotides containing polyamines at the C-2 position of purines: a pre-synthetic approach for the incorporation of spermine into oligodeoxynucleotides containing 2-(4,9,13-triazatridecyl)-2'-deoxyguanosine. *Chem Eur J*. 2000;6:4188–94.

- [203] Potier PF, Behr JP. Recognition of DNA by strand invasion with oligonucleotide-spermine conjugates. *Nucleosides Nucleotides Nucleic Acids*. 2001;20:809–13.
- [204] Seela F, Thomas H. Duplex stabilization of DNA: oligonucleotides containing 7-substituted 7-deazaadenines. *Helv Chim Acta*. 1995;78:94–108.
- [205] Seela F, Methylated CY. DNA: the influence of 7-deaza-7-methylguanine on the structure and stability of oligonucleotides. *Helv Chim Acta*. 1997;80:1073–86.
- [206] Ramzaeva N, Seela F. Duplex stabilization of 7-deazapurine DNA: oligonucleotides containing 7-bromo- or 7-iodo-7-deazaguanine. *Helv Chim Acta*. 1996;79:1549–58.
- [207] Lever C, Li X, Cosstick R, Ebel S, Brown T. Thermodynamic stability and drug binding properties of oligodeoxyribonucleotide duplexes containing 3-deazaadenine:thymine base pairs. *Nucleic Acids Res*. 1993;21:1743–6.
- [208] Seela F, Wenzel T. Oligodeoxyribonucleotides containing 4-aminobenzimidazole in place of adenine: solid-phase synthesis and base-pairing. *Helv Chim Acta*. 1995;78:833–46.
- [209] Buhr CA, Matteucci MD, Froehler BC. Synthesis of a tetracyclic 2'-deoxyadenosine analog. *Tetrahedron Lett*. 1999;40:8969–70.
- [210] Sangvi YS, Hole GD, Freier SM, Zounes MC, Gonzalez C, Cummins L, Sasmor H, Dan Cook P. Antisense oligonucleotides: synthesis, biophysical and biological evaluation of oligonucleotides containing modified pyrimidines. *Nucleic Acids Res*. 1993;21:3197–203.
- [211] Nara H, Ono A, Matsuda A. Nucleosides and nucleotides. 135. DNA duplex and triplex formation and resistance to nucleolytic degradation of oligodeoxynucleotides containing syn-norspermidine at the 5-position of 2'-deoxyuridine. *Bioconjug Chem*. 1995;6:54–61.
- [212] Ueno Y, Kumagai I, Haginiwa N, Matsuda A. Effects of 5-(N-aminoethyl)carbamoyl-2'-deoxyuridine on endonuclease stability and the ability of oligodeoxynucleotide to activate RNase H. *Nucleic Acids Res*. 1997;25:3777–82.
- [213] Ueno Y, Mikawa M, Matsuda A. Nucleosides and nucleotides. 170. Synthesis and properties of oligodeoxynucleotides containing 5-[*N*-[2-[*N,N*-bis(2-aminoethyl)amino]ethyl] carbamoyl]-2'-deoxyuridine and 5-[*N*-[3-[*N,N*-bis(3-aminopropyl)amino]propyl] carbamoyl]-2'-deoxyuridine. *Bioconjug Chem*. 1998;9:33–9.
- [214] Xodo LE, Manzini G, Quadrifolio F, van der Marel G, van Boom J. DNA hairpin loops in solution. Correlation between primary structure, thermostability and reactivity with single-strandspecific nuclease from mung bean. *Nucleic Acids Res*. 1991;19:1505–11.
- [215] Sagi J, Szemzo A, Ebinger K, Szabolcz A, Sagi G, Ruff E, Otvos L. Based-modified oligonucleotides. I Effect of 5-alkyl, 5-(1-alkenyl), 5-(1-alkynyl) substitution of the pyrimidines on duplex stability and hydrobobicity. *Tetrahedron Lett*. 1993;34:2191–4.
- [216] Froehler BC, Wadwani S, Terhorst TJ, Gerrard SR. Oligonucleotides containing C-5 propyne analogs of 2'-deoxyuridine and 2'-deoxycytidine. *Tetrahedron Lett*. 1992;33:5307–10.
- [217] Froehler BC, Jones RJ, Cao X, Terhorst TJ. Oligonucleotides derived from 5-(1-propynyl)-2'-O-allyl-uridine and 5-(1-propynyl)-2'-O-allyl-cytidine: synthesis and RNA duplex formation. *Tetrahedron Lett*. 1993;34:1003–6.
- [218] Wagner RW, Matteucci MD, Lewis JG, Gutierrez AJ, Moulds C, Froehler BC. Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. *Science*. 1993;260:1510–3.
- [219] Wagner RW, Matteucci MD, Grant D, Huang T, Froehler BC. Potent and selective inhibition of gene expression by an antisense heptanucleotide. *Nat Biotechnol*. 1996;8:40–4.
- [220] Flanagan WM, Kothavale A, Wagner RW. Effects of oligonucleotide length, mismatches and mRNA levels on C-5 propyne-modified antisense potency. *Nucleic Acids Res*. 1996;24:2936–41.

- [221] Ahmadian M, Zhang P, Bergstrom DE. A comparative study of the thermal stability of oligodeoxyribonucleotides containing 5-substituted 2'-deoxyuridines. *Nucleic Acids Res.* 1998;26:3127–35.
- [222] Gutierrez AJ, Terhorst TJ, Matteucci MD, Froehler BC. 5-Heteroaryl-2'-deoxyuridine analogs. Synthesis and incorporation into high-affinity oligonucleotides. *J Am Chem Soc.* 1994;116:5540–4.
- [223] Gutierrez AJ, Matteucci MD, Grant D, Matsumura S, Wagner RW, Froehler BC. Antisense gene inhibition by C-5-substituted deoxyuridine-containing oligodeoxyribonucleotides. *Biochemistry.* 1997;36:743–8.
- [224] Inoue H, Imura A, Ohtsuka E. Synthesis and hybridization of dodecadoxyribonucleotides containing a fluorescent pyridopyrimidine deoxynucleotide. *Nucleic Acids Res.* 1985;13:7119–28.
- [225] Matteucci MD, von Krosigk U. Hybridization properties of oligonucleotides bearing a tricyclic 2'-deoxycytidine analog based on a carbazole ring system. *Tetrahedron Lett.* 1996;37:5057–60.
- [226] Lin KY, Jones RJ, Matteucci MD. Tricyclic 2'-deoxycytidine analogs: syntheses and incorporation into oligodeoxynucleotides which have enhanced binding to complementary RNA. *J Am Chem Soc.* 1995;117:3873–4.
- [227] Kurchavov NA, Stetsenko DA, Skaptsova NV, Potapov VK, Sverdlov ED. A new phosphoramidite reagent for the incorporation of diazaphenoxazinone nucleoside with enhanced base-pairing properties into oligodeoxynucleotides. *Nucleosides Nucleotides.* 1997;16:1837–46.
- [228] Lin KY, Matteucci MD. A cytosine analogue capable of clamp-like binding to a guanine in helical nucleic acids. *J Am Chem Soc.* 1998;120:8531–2.
- [229] Ali OM, Franch T, Gerdes K, Pedersen EB. Targeting of nucleic acid junctions: addressing to a branch point an oligodeoxynucleotide conjugated with an intercalator. *Nucleic Acids Res.* 1998;26:4919–24.
- [230] Abdel-Rahman AAH, Ali OM, Pedersen EB. Insertion of 5-methyl-N<sup>4</sup>-(1-pyrenylmethyl)cytidine into DNA. Duplex, three-way junction and triplex stabilities. *Tetrahedron.* 1996;52:15311–24.
- [231] Ahlborn C, Siegmund K, Richert C. Isostable DNA. *J Am Chem Soc.* 2007;129:15218–32.
- [232] Ozaki H, Ogawa Y, Mine M, Sawai H. Effect of acridine with various linker arms attached to C5 position of 2'-deoxyuridine on the stability of DNA/DNA and DNA/RNA duplexes. *Nucleosides Nucleotides.* 1998;17:911–23.
- [233] Englisch U, Gauss DH. Chemically modified oligonucleotides as probes and inhibitors. *Angew Chem, Int Ed Engl.* 1991;30:613–29.
- [234] Lönnberg H. Solid-phase synthesis of oligonucleotide conjugates useful for delivery and targeting of potential nucleic acid therapeutics. *Bioconjug Chem.* 2009;20:1065–94.
- [235] Gooding M, Malhotra M, Evans JC, Darcy R, O'Driscoll CM. Oligonucleotide conjugates: candidates for gene silencing therapeutics. *Eur J Pharm Biopharm.* 2016;107:321–40.
- [236] Grijalvo S, Alagia A, Jorge AF, Eritja R. Covalent strategies for targeting messenger and non-coding RNAs. An updated review on siRNA, miRNA and antimiR conjugates. *Genes.* 2018;9:74.
- [237] Benizri S, Gissot A, Martin A, Vialet B, Grinstaff MW, Barthélémy P. Bioconjugated oligonucleotides: recent developments and therapeutic applications. *Bioconjug Chem.* 2019;30:366–83.
- [238] Sun JS, François JC, Montenay-Garestier T, Saison-Behmoaras T, Roig V, Thuong NT, Hélène C. Sequence-specific intercalating agents: intercalation at specific sequences on duplex DNA major groove recognition by oligonucleotide-intercalator conjugates. *Proc Natl Acad Sci USA.* 1989;86:9198–202.

- [239] Lee BL, Blake KR, Miller PS. Interaction of psolarene-derivatized oligodeoxynucleoside methylphosphonates with synthetic DNA containing a promoter for T7 RNA polymerase. *Nucleic Acids Res.* 1988;16:10681–97.
- [240] Piles U, Englisch U. Psolarene covalently linked to oligodeoxyribonucleotides: synthesis, sequence specific recognition of DNA and photo-cross-linking to pyrimidine residues of DNA. *Nucleic Acids Res.* 1989;17:285–99.
- [241] Lukhtanov EA, Kutyavin IV, Gamper HB, Meyer RB. Oligodeoxyribonucleotides with conjugated dihydropyrroloindole oligopeptides: preparation and hybridization properties. *Bioconjug Chem.* 1995;6:418–26.
- [242] Lukhtanov EA, Kutyavin IV, Meyer RB. Direct, solid phase assembly of dihydropyrroloindole peptides with conjugated oligonucleotides. *Bioconjug Chem.* 1996;7:564–7.
- [243] Afonina I, Kutyavin I, Lukhtanov E, Meyer RB, Gamper H. Sequence-specific arrest of primer extension on single-stranded DNA by an oligonucleotide-minor groove binder conjugate. *Proc Natl Acad Sci USA.* 1996;93:3199–204.
- [244] Lukhtanov EA, Lokhov SG, Gorn VV, Podyminogin MA, Mahoney W. Novel DNA probes with low background and high hybridization-triggered fluorescence. *Nucleic Acids Res.* 2007;35:e30.
- [245] Bleczinski CF, Richert C. Steroid-DNA interactions increasing stability, sequence-selectivity, DNA/RNA discrimination, and hypochromicity of oligonucleotide duplexes. *J Am Chem Soc.* 1999;121:10889–94.
- [246] Narayanan S, Gall J, Richert C. Clamping down on weak terminal base pairs: oligonucleotides with molecular caps as fidelity enhancing elements at the 5'- and 3'-terminal residues. *Nucleic Acids Res.* 2004;32:2901–11.
- [247] Dogan Z, Paulini R, Rojas Stütz JA, Narayanan S, Richert C. 5-Tethered stilbene as fidelity- and affinity-enhancing modulators of DNA duplex stability. *J Am Chem Soc.* 2002;126:4762–3.
- [248] Printz M, Richert C. Optimizing the stacking moiety and linker of 2'-acylamido caps of DNA duplexes with 3'-terminal adenine residues. *J Comb Chem.* 2007;9:306–20.
- [249] Printz M, Richert C. Pyrenylmethyldeoxyadenosine: a 3'-cap for universal DNA hybridization probes. *Chem Eur J.* 2009;15:3390–402.
- [250] Patra A, Richert C. High fidelity base pairing at the 3'-terminus. *J Am Chem Soc.* 2009;131:12671–81.
- [251] Pérez-Rentero S, Gállego I, Somoza A, Ferreira R, Janousek J, Belohradsky M, Stara I, Stary I, Eritja R. Interstrand interactions on DNA duplexes modified by TTF units at the 3' or 5'-ends. *RSC Adv.* 2012;2:4069–71.
- [252] Pons B, Kotera M, Zuber G, Behr JP. Online synthesis of diblock cationic oligonucleotides for enhanced hybridization to their complementary sequence. *ChemBioChem.* 2006;7:1173–6.
- [253] Moreau V, Voirin E, Paris C, Kotera M, Nothisen M, Rémy JS, Behr JP, Erbacher P, Lenne-Samuel N. Zip nucleic acids: new high affinity oligonucleotides as potent primers for PCR and reversed transcription. *Nucleic Acids Res.* 2009;37:e130.
- [254] Noir R, Kotera M, Pons B, Rémy JS, Behr JP. Oligonucleotide-oligospermine conjugates (Zip nucleic acids): a convenient means of finely tuning hybridization temperatures. *J Am Chem Soc.* 2008;130:13500–5.
- [255] Voirin E, Behr JP, Kotera M. Versatile synthesis of oligodeoxyribonucleotide-oligospermine conjugates. *Nat Protoc.* 2007;2:1360–7.
- [256] Nothisen M, Kotera M, Voirin E, Rémy JS, Behr JP. Cationic siRNAs provide carrier-free gene silencing in animal cells. *J Am Chem Soc.* 2009;131:11730–1.
- [257] Schweitzer BA, Kool ET. Hydrophobic, non-hydrogen-bonding bases and base pairs in DNA. *J Am Chem Soc.* 1995;117:1863–72.
- [258] Kool ET, Morales JC, Guckian KM. Mimicking the structure and function of DNA: insights into DNA stability and replication. *Angew Chem, Int Ed Engl.* 2000;39:990–1009.

- [259] Kool ET. Hydrogen bonding, base stacking, and steric effects in DNA replication. *Annu Rev Biophys Biomol Struct.* 2001;30:1–22.
- [260] Guckian KM, Schweitzer BA, Ren RXF, Shiels CJ, Paris PL, Tahmassebi DC, Kool ET. Experimental measurement of aromatic stacking affinities in the context of duplex DNA. *J Am Chem Soc.* 1996;118:8182–3.
- [261] Guckian KM, Schweitzer BA, Ren RXF, Shiels CJ, Tahmassebi DC, Kool ET. Factors contributing to aromatic stacking in water: evaluation in the context of DNA. *J Am Chem Soc.* 2000;122:2213–22.
- [262] Moran S, Ren RXF, Shiels CJ, Rummey S IV, Kool ET. Non-hydrogen bonding ‘terminator’ nucleosides increase the 3'-end homogeneity of enzymatic RNA and DNA synthesis. *Nucleic Acids Res.* 1996;24:2044–52.
- [263] Guckian KM, Kool ET. Highly precise shape mimicry by a difluorotoluene deoxynucleoside, a replication-competent substitute for thymidine. *Angew Chem, Int Ed Engl.* 1997;36:2825–8.
- [264] Morales JC, Kool ET. Efficient replication between non-hydrogen bonded nucleoside shape analogs. *Nat Struct Biol.* 1998;5:950–4.
- [265] Moran S, Ren RXF, Rummey S IV, Kool ET. Difluorotoluene, a nonpolar isostere for thymine, codes specifically and efficiently for adenine in DNA replication. *J Am Chem Soc.* 1997;119:2056–7.
- [266] Liu D, Moran S, Kool ET. Bi-stranded, multisite replication of a base pair between difluorotoluene and adenine: confirmation by ‘inverse’ sequencing. *Chem Biol.* 1997;4:919–26.
- [267] Guckian KM, Krugh TR, Kool ET. Solution structure of a DNA duplex containing a replicable difluorotoluene–adenine pair. *Nat Struct Biol.* 1998;5:954–9.
- [268] Ren RXF, Schweitzer BA, Shiels CJ, Kool ET. Formation of stable DNA loops by incorporation of nonpolar, non-hydrogen-bonding nucleoside isosteres. *Angew Chem, Int Ed Engl.* 1996;35:743–6.
- [269] Guckian KM, Morales JC, Kool ET. Structure and base pairing properties of a replicable nonpolar isostere for deoxyadenosine. *J Org Chem.* 1998;63:9652–6.
- [270] Guckian KM, Krugh TR, Kool ET. Solution structure of a nonpolar, non-hydrogen-bonded base pair surrogate in DNA. *J Am Chem Soc.* 2000;122:6841–7.
- [271] Morales JC, Kool ET. Minor groove interactions between polymerase and DNA: more essential to replication than Watson–Crick hydrogen bonds? *J Am Chem Soc.* 1999;121:2323–4.
- [272] Morales JC, Kool ET. Functional hydrogen-bonding map of the minor groove binding tracks of six DNA polymerases. *Biochemistry.* 2000;39:12979–88.
- [273] Matray TJ, Kool ET. Selective and stable DNA base pairing without hydrogen bonds. *J Am Chem Soc.* 1998;120:6191–2.
- [274] Matray TJ, Kool ET. A specific partner for abasic damage in DNA. *Nature.* 1999;399:704–8.
- [275] Morales-Rojas H, Kool ET. A porphyrin C-nucleoside incorporated into DNA. *Org Lett.* 2002;4:4377–80.
- [276] Kim TW, Delaney JC, Essigmann JM, Kool ET. Probing the active site tightness of DNA polymerase in subangstrom increments. *Proc Natl Acad Sci USA.* 2005;102:15803–8.
- [277] Lee HR, Helquist SA, Kool ET, Johnson KA. Base pair hydrogen bonds are essential for proofreading selectivity by the human mitochondrial DNA polymerase. *J Biol Chem.* 2008;283:14411–6.
- [278] Lai JS, Kool ET. Selective pairing of polyfluorinated DNA bases. *J Am Chem Soc.* 2004;126:3040–1.
- [279] Lai JS, Kool ET. Fluorous base-pairing effects in a DNA polymerase active site. *Chem Eur J.* 2005;11:2966–71.

- [280] McMinn DL, Ogawa AK, Wu Y, Liu J, Schultz PG, Romesberg FE. Efforts toward expansion of the gene alphabet: DNA polymerase recognition of a highly stable, self-pairing hydrophobic base. *J Am Chem Soc.* 1999;121:11585–6.
- [281] Henry AA, Romesberg FE. Beyond A, C, G and T: augmenting nature's alphabet. *Curr Opin Chem Biol.* 2003;7:727–33.
- [282] Yu C, Henry AA, Romesberg FE, Schultz PG. Polymerase recognition of unnatural base pairs. *Angew Chem, Int Ed Engl.* 2002;41:3841–4.
- [283] Leconte AM, Matsuda S, Romesberg FE. An efficiently extended class of unnatural base pairs. *J Am Chem Soc.* 2006;128:6780–1.
- [284] Liu H, Gao J, Lynch SR, Saito D, Maynard L, Kool ET. A four-base paired genetic helix with expanded size. *Science.* 2003;302:868–71.
- [285] Liu H, Gao J, Maynard L, Saito D, Kool ET. Toward a new genetic system with expanded dimensions: size-expanded analogues of deoxyadenosine and thymidine. *J Am Chem Soc.* 2004;126:1102–9.
- [286] Gao J, Liu H, Kool ET. Expanded-size bases in naturally sized DNA: evaluation of steric effects in Watson–Crick pairing. *J Am Chem Soc.* 2004;126:11826–31.
- [287] Liu H, Lynch SR, Kool ET. Solution structure of xDNA: a paired genetic helix with increased diameter. *J Am Chem Soc.* 2004;126:6900–5.
- [288] Liu H, Gao J, Kool ET. Size-expanded analogues of dG and dC: synthesis and pairing properties in DNA. *J Org Chem.* 2005;70:639–47.
- [289] Liu H, Gao J, Kool ET. Helix-forming properties of size-expanded DNA, an alternative four-base genetic form. *J Am Chem Soc.* 2005;127:1396–402.
- [290] Lynch SR, Liu H, Gao J, Kool ET. Toward a designed, functioning genetic system with expanded-size base pairs: solution structure of the eight-base xDNA double helix. *J Am Chem Soc.* 2006;128:14704–11.
- [291] Lu H, Krueger AT, Gao J, Liu H, Kool ET. Toward a designed genetic system with biochemical function: polymerase synthesis of single and multiple size-expanded DNA base pairs. *Org Biomol Chem.* 2010;8:2704–10.
- [292] Liu H, He K, Kool ET. yDNA: a new geometry for size-expanded base pairs. *Angew Chem, Int Ed Engl.* 2004;43:5834–6.
- [293] Lee AHF, Kool ET. Novel benzopyrimidines as widened analogues of DNA bases. *J Org Chem.* 2005;70:132–40.
- [294] Lee AHF, Kool ET. A new four-base genetic helix, yDNA, composed of widened benzopyrimidine-purine pairs. *J Am Chem Soc.* 2005;127:3332–8.
- [295] Lee AHF, Kool ET. Exploring the limits of DNA size: Naphtho-homologated DNA bases and pairs. *J Am Chem Soc.* 2006;128:9219–30.
- [296] Scremen CL, Boal JH, Wilk A, Phillips LR, Beaucage SL. [(2-Deoxy- $\alpha$ - and  $\beta$ -D-erythro-pentofuranosyl)thymin-1-yl] methane derivatives as potential conformational probes for altDNA oligonucleotides. *Bioorg Med Chem Lett.* 1996;6:207–12.
- [297] Boal JH, Wilk A, Scremen CL, Gray GN, Phillips LR, Beaucage SL. Synthesis of (2-deoxy- $\alpha$ - and  $\beta$ -D-erythro-pentofuranosyl) (thymin-1-yl)alkanes and their incorporation into oligodeoxyribonucleotides. Effect of nucleobase–sugar linker flexibility on the formation of DNA–DNA and DNA–RNA hybrids. *J Org Chem.* 1996;61:8617–26.
- [298] Carnero A, Pérez-Rentero S, Alagia A, Aviñó A, Sanghvi YS, Fernández S, Ferrero M, Eritja R. The impact of extended nucleobase-2'-deoxyribose linkers in the biophysical and biological properties of oligonucleotides. *RSC Adv.* 2017;7:9579–86.
- [299] Herdewijn P, Marlriere P. Towards safe genetically modified organism through the chemical diversification of nucleic acids. *Chem Biodivers.* 2009;6:791–808.

- [300] Horn T, Chang CA, Collins ML. Hybridization properties of the 5-methylisocytidine/isoguanosine base pair in synthetic oligonucleotides. *Tetrahedron Lett.* 1995;36:2033–6.
- [301] Roberts C, Bandaru R, Switzer C. Synthesis of oligonucleotides bearing the non standard bases iso-C and iso-G. Comparison of iso-C-iso-G, C-G and U-A base-pair stabilities in RNA/DNA duplexes. *Tetrahedron Lett.* 1995;36:3601–4.
- [302] Switzer CY, Moroney SE, Benner SA. Enzymatic recognition of the base pair between isocytidine and isoguanine. *Biochemistry.* 1993;32:10489–96.
- [303] Piccirilli JA, Krauch, Moroney SE, Benner SA. Enzymatic incorporation of a new base pair into DNA and RNA extends the genetic alphabet. *Nature.* 1990;343:33–7.
- [304] Rapaport HP. The 6-thioguanine/5-methyl-2'-pyrimidinone base pair. *Nucleic Acids Res.* 1988;16:7253–67.
- [305] Benner SA. Understanding nucleic acids using synthetic chemistry. *Acc Chem Res.* 2004;37:784–97.
- [306] Henry AA, Romesberg FE. Beyond A, C, G and T: augmenting nature's alphabet. *Curr Opin Chem Biol.* 2003;7:727–33.
- [307] Krueger AT, Kool ET. Redesigning the architecture of the base pair: towards biochemical and biological function of new genetic sets. *Chem Biol.* 2009;16:242–8.
- [308] Shivalingam A, Brown T. Synthesis of chemically modified DNA. *Biochem Soc Trans.* 2016;44:709–15.
- [309] Minakawa N, Ogata S, Takahashi M, Matsuda A. Selective recognition of unnatural imidazopyridopyrimidine: naphthyridine base pairs consisting of four hydrogen bonds by the Klenow fragment. *J Am Chem Soc.* 2009;131:1644–5.
- [310] Hikishima S, Minakawa N, Kuramoto K, Fujisawa Y, Ogawa M, Matsuda A. Synthesis of 1,8-naphthyridine C-nucleosides and their base-pairing properties in oligodeoxynucleotides: thermally stable naphthyridine: imidazopyridopyrimidine base-pairing motifs. *Angew Chem, Int Ed Engl.* 2005;44:596–8.
- [311] Doi Y, Chiba J, Morikawa T, Inouye, Artificial M. DNA made exclusively of non-natural C-nucleosides with four types of non-natural bases. *J Am Chem Soc.* 2008;130:8762–8.
- [312] Heuberger BD, Switzer C. An alternative nucleobase code: characterization of purine–purine DNA double helices bearing guanine–isoguanine and diaminopurine 7-deazaxanthine base pairs. *ChemBioChem.* 2008;9:2779–83.
- [313] Battersby TR, Albalos M, Friesenhahn MJ. An unusual mode of DNA duplex association: Watson–Crick interaction of all-purine deoxyribonucleic acids. *Chem Biol.* 2007;14:525–31.
- [314] Malyshev DA, Dhami K, Lavergne T, Chen T, Dai N, Foster JM, Correa IR, Romesberg FE. A semi-synthetic organism with an expanded genetic alphabet. *Nature.* 2014;509:385–8.
- [315] Li L, Degardin M, Lavergne T, Malyshev DA, Dhami K, Ordoukhalian P, Romesberg FE. Natural-like replication of an unnatural base pair for the expansion of the genetic alphabet and biotechnology applications. *J Am Chem Soc.* 2014;136:826–9.
- [316] Malyshev DA, Romesberg FE. The expanded genetic alphabet. *Angew Chem, Int Ed Engl.* 2015;54:11930–44.
- [317] Dien VT, Holcomb M, Feldman AW, Fischer EC, Dwyer TJ, Romesberg FE. Progress toward a semi-synthetic organism with an unrestricted expanded genetic alphabet. *J Am Chem Soc.* 2018;140:16115–23.
- [318] Kimoto M, Kawai R, Mitsui Y, Yokoyama S, Hirao I. An unnatural base pair system for efficient PCR amplification and functionalization of DNA molecules. *Nucleic Acids Res.* 2009;37:e14.
- [319] Betz K, Kimoto M, Diederichs K, Hirao I, Marx A. Structural basis forexpansion of the genetic alphabet with an artificial nucleobase pair. *Angew Chem, Int Ed Engl.* 2017;56:12000–3.
- [320] Hamashima K, Kimoto M, Hirao I. Creation of unnatural base pairs for genetic alphabet expansion toward synthetic xenobiology. *Curr Opin Chem Biol.* 2018;46:108–14.

- [321] Zhang L, Yang Z, Sefah K, Bradley KM, Hoshika S, Kim MJ, Kim HJ, Zhu G, Jiménez E, Cansiz S, Teng IT, Champanhac C, McLendon C, Liu C, Zhang W, Gerloff DL, Huang Z, Tan W, Benner SA. Evolution of functional six-nucleotide DNA. *J Am Chem Soc.* 2015;137:6734–7.
- [322] Hoshika S, Leal NA, Kim MJ, Kim MS, Karalkar NB, Kim HJ, Bates AM, Watkins NE, SantaLucia HA, Meyer AJ, DasGupta S, Piccirilli JA, Ellington AD, SantaLucia J, Georgiadis MM, Hachimoji BSA. DNA and RNA: a genetic system with eight building blocks. *Science.* 2019;363:884–7.
- [323] Wang T, Chen C, Larcher LM, Barrero RA, Veedu RN. Three decades of nucleic acid aptamer technologies: lessons learned, progress and opportunities on aptamer development. *Biotechnol Adv.* 2019;37:28–50.
- [324] Meek KN, Rangel AE, Heemstra JM. Enhancing aptamer function and stability via in vitro selection using modified nucleic acids. *Methods.* 2016;106:29–36.
- [325] Pinheiro VB, Holliger P. The XNA world: progress towards replication and evolution of synthetic genetic polymers. *Curr Opin Chem Biol.* 2012;16:245–52.
- [326] Loakes D, Gallego J, Pinheiro VB, Kool ET, Holliger P. Evolving a polymerase for hydrophobic base analogues. *J Am Chem Soc.* 2009;131:14827–37.
- [327] Arangundy-Franklin S, Taylor Al, Porebski BT, Genna V, Peak-Chew S, Viasman A, Woodgate R, Orozco M, Holliger P. A synthetic genetic polymer with an uncharged backbone chemistry based on alkyl phosphonate nucleic acids. *Nat Chem.* 2019;11:533–42.
- [328] Eremeeva E, Herdewijn P. Enzymatic synthesis using polymerases of modified nucleic acids and genes. In: Fernández-Lucas J, Camarasa Rius MJ, editors. *Enzymatic and chemical synthesis of nucleic acids derivatives.* Wiley-VCH Verlag GmbH & co, KGaA; 2019. p. 159–94. Chapter 7.
- [329] Houlihan G, Arangundy-Franklin S, Holliger P. Exploring the chemistry of genetic information storage and propagation through polymerase engineering. *Acc Chem Res.* 2017;50:1079–87.
- [330] Seela F, Driller H. Solid-phase synthesis of self-complementary hexamer d(c7GpCpc7GpCpc7GpCp) via O-3'-phosphoramidite of 7-deaza-2'-deoxyguanosine. *Nucleic Acids Res.* 1985;13:911–26.
- [331] Seela F, Driller H. Palindromic oligonucleotides containing 7-deaza-2'-deoxyguanosine: solid-phase synthesis of d(p)GG-AATTCC octamers and recognition by the endodeoxyribonuclease EcoRI. *Nucleic Acids Res.* 1986;14:2319–32.
- [332] Seela F, Driller H, Kehne A, Kaiser K. Self-complementary oligomers containing 7-deaza-2'-deoxyguanosine or 2'-deoxytubercidin. *Chem Scr.* 1986;26:173–8.
- [333] Seela F, Grein T. 7-Deaza-2'-deoxyadenosine and 3-deaza-2'-deoxyadenosine replacing dA with d(A<sub>6</sub>)-tracts: differential bending at 3' and 5'-junctions of d(A<sub>6</sub>). d(T<sub>6</sub>) and B-DNA. *Nucleic Acids Res.* 1992;20:2297–306.
- [334] Ono A, Sato M, Ohtani Y, Ueda T. Synthesis of deoxynucleotides containing 7-deazaadenine: recognition and cleavage by restriction endonuclease Bgl II and Sau 3AI. *Nucleic Acids Res.* 1984;12:8939–49.
- [335] Seela F, Kehne A. Palindromic octa- and dodecanucleotides containing 2'-deoxytubercidin: synthesis, hairpin formation, and recognition by the endodeoxyribonuclease EcoRI. *Biochem.* 1987;26:2232–8.
- [336] Seela F, Kehne A. 2'-Desoxytubercidin: synthese des O-3'-phosphoramidites und kondensation zu 2'-desoxytubercidylyl(3'→5')-2'-desoxytubercidin. *Tetrahedron.* 1985;41:5387–92.
- [337] Grein T, Lampe S, Mersmann K, Rosemeyer H, Thomas H, Seela F. 3-Deaza and 7-deazapurines: duplex stability of oligonucleotides containing modified adenine or guanine bases. *Biorg Med Chem Lett.* 1994;4:971–6.

- [338] Seela F, Driller H. Alternating d(G-C)<sub>3</sub> and d(C-G)<sub>3</sub> hexanucleotides containing 7-deaza-2'-deoxyguanosine or 8-aza-7-deaza-2'-deoxyguanosine in place of dG. *Nucleic Acids Res.* 1989;17:901–10.
- [339] Seela F, Driller H. 8-Aza-7-deaza-2'-deoxyguanosine: phosphoramidite synthesis and properties of octanucleotides. *Helv Chim Acta.* 1988;71:1191–8.
- [340] Seela F, Kaiser K. 8-Aza-7-deazaadenine  $N^8$ - and  $N^8$ - $(\beta$ -D-2'-Deoxyribofuranosides): building blocks for automated DNA synthesis and properties of oligodeoxyribonucleotides. *Helv Chim Acta.* 1988;71:1813–23.
- [341] Seela F, Kaiser K, Bindig U. 2'-Deoxy- $\beta$ -D-ribofuranosides of  $N^6$ -methylated-7-deazaadenine and 8-aza-7-deazaadenine: solid-phase synthesis of oligodeoxyribonucleotides and properties of self-complementary duplexes. *Helv Chim Acta.* 1989;72:868–81.
- [342] Seela F, Bindig U, Driller HJ, Herdering W, Kaiser K, Kehne A, Rosemeyer H, Steker H. Synthesis and application of isosteric purine 2'-deoxyribofuranosides. *Nucleosides Nucleotides.* 1987;6:11–23.
- [343] Seela F, Driller H. 7-Deaza-2'-deoxy- $O^6$ -methylguanosine: selective  $N^2$ -formylation via a formamidine, phosphoramidite synthesis and properties of oligonucleotides. *Nucleosides Nucleotides.* 1989;8:1–21.
- [344] Jiricny J, Wood SG, Martin D, Ubasawa A. Oligonucleotide duplexes containing inosine, 7-deazainosine, nebularine and 7-deazanebularine as substrates for restriction endonucleases HindII, Sall and Taq 1. *Nucleic Acids Res.* 1986;14:6579–90.
- [345] Cosstick R, Li X, Tuli DK, Williams DM, Connolly BA, Newman PC. Molecular recognition in the minor groove of the DNA helix. Studies on the synthesis of oligonucleotides and polynucleotides containing 3-deaza-2'-deoxyadenosine. Interaction of the oligonucleotides with the restriction endonuclease EcoRV. *Nucleic Acids Res.* 1990;18:4771–8.
- [346] Newman PC, Nwosu VU, Williams DM, Cosstick R, Seela F, Connolly BA. Incorporation of a complete set of deoxyadenosine and thymidine analogues suitable for the study of protein nucleic acid interactions into oligodeoxynucleotides. Application to the EcoRV restriction endonuclease and modification methylase. *Biochemistry.* 1990;29:9891–901.
- [347] Newman PC, Williams DM, Cosstick R, Seela F, Connolly BA. Interaction of the EcoRV restriction endonuclease with the deoxyadenosine and thymidine bases in its recognition hexamer d(GATATC). *Biochemistry.* 1990;29:9902–10.
- [348] Komatsu H, Kim SG, Sakabe I, Ichikawa T, Nakai M, Takaku H. Purine 8-substitution modulates the recognition endodeoxyribonucleoside EcoT1 of octadeoxyribonucleotides (dGGAATTCC). *Bioorg Med Chem Lett.* 1992;2:565–70.
- [349] Marzabal S, DuBois S, Thielking V, Cano A, Eritja R, Guschlauer W. Dam methylase from *Escherichia coli*: kinetic studies using modified DNA oligomers: Hemimethylated substrates. *Nucleic Acids Res.* 1995;23:3648–55.
- [350] Thielking V, Du Bois S, Eritja R, Guschlauer W. Dam methyltransferase from *Escherichia coli*: kinetic studies using modified DNA oligomers: Nonmethylated substrates. *Biol Chem.* 1997;378:407–15.
- [351] Ono A, Ueda T. Minor-groove-modified oligonucleotides; synthesis of decadeoxynucleotides containing hypoxanthine,  $N^2$ -methyl-guanine and 3-deazaadenine, and their interactions with restriction endonucleases BgIII, Sau 3AI and MboI. *Nucleic Acids Res.* 1987;15:3059–72.
- [352] Ono A, Ueda T. Synthesis of decadeoxyribonucleotides containing  $N^6$ -methyladenine,  $N^4$ -methylcytosine, and 5-methylcytosine: recognition and cleavage by restriction endonucleases. *Nucleic Acids Res.* 1987;15:219–32.
- [353] Fàbrega C, Güimil García R, Díaz AR, Eritja R. Studies on the synthesis of oligonucleotides containing photoreactive nucleosides: 2-azido-2'-deoxyinosine and 8-azido-2'-deoxyadenosine. *Biol Chem.* 1998;379:527–33.

- [354] Liu J, Fan QR, Sodeoka M, Lane WS, Verdine GL. DNA binding by an amino acid residue in the C-terminal half of the Rel homology region. *Chem Biol.* 1994;1:47–55.
- [355] Gildea B, McLaughlin LW. The synthesis of 2-pyrimidinone nucleosides and their incorporation into oligodeoxynucleotides. *Nucleic Acids Res.* 1989;17:2261–81.
- [356] Zhou Y, Ts'o POP. Solid-phase synthesis of oligo-2-pyrimidinone-2'-deoxyribonucleotides and oligo-2-pyrimidinone-2'-deoxyriboside methylphosphonates. *Nucleic Acids Res.* 1996;24:2652–9.
- [357] Charczuk R, Tamm C, Suri B, Bickle TA. An unusual base pairing between pyrimidine and pyridine nucleosides. *Nucleic Acids Res.* 1986;14:9530.
- [358] Eschenhof H, Strazewski P, Tamm C. A new synthesis of 3-deazathymidine and of a related phosphoramidite synthon. *Tetrahedron.* 1992;48:6225–30.
- [359] Rajur SB, McLaughlin LW. The synthesis of oligodeoxynucleotides containing 2-thiothymine and 5-methyl-4-pyrimidinone base analogues. *Tetrahedron Lett.* 1992;33:6081–4.
- [360] Butkus V, Klimašaukas S, Petrauskiené L, Manelienė Z, Janulaitis A, Minchenkova LE, Schoylkina AK. Synthesis and physical characterization of DNA fragments containing N<sup>4</sup>-methylcytosine and 5-methylcytosine. *Nucleic Acids Res.* 1987;15:8467–78.
- [361] Gromova ES, Oretskaya SL, Eritja R, Guschlauer W. Kinetic studies of Mval DNA methyltransferase interaction with modified oligonucleotide duplexes. *Biochem Mol Biol Intl.* 1995;36:247–55.
- [362] Fließ A, Wolfes H, Rosenthal A, Schwellnus K, Blöcker H, Frank R, Pingoud A. Role of the thymidine residues in DNA recognition by the EcoRI and EcoRV restriction endonucleases. *Nucleic Acids Res.* 1986;14:3463–74.
- [363] Hayakawa T, Ono A, Ueda T. Synthesis of decadeoxyribonucleotides containing 5-modified uracils and their interactions with restriction endonucleases Bgl II, Sau 3AI and Mbo I. *Nucleic Acids Res.* 1988;16:4761–76.
- [364] Babkina OV, Chutko CA, Shashkov AA, Dzhiddzhoev MS, Eritja R, Gromova ES. Iodouracil-mediated photo-cross-linking of DNA to Eco RII restriction endonuclease in catalytic conditions. *Photochem Photobiol Sci.* 2002;636–40.
- [365] Koudan EV, Subach OM, Korshunova GA, Romanova EA, Eritja R, Gromova ES. DNA duplexes containing photoreactive derivatives of 2'-deoxyuridine as photocrosslinking probes for EcoRII DNA Methyltransferase-substrate interaction. *J Biomol Struct Dyn.* 2002;20:421–8.
- [366] Yu J, Xie T, Wang Z, Wang X, Zeng S, Kang Y, Hou T. DNA methyltransferases: emerging targets for the discovery of inhibitors as potent anticancer drugs. *Drug Discov Today.* 2019;24:2323–31.
- [367] Chuang JC, Warner SL, Vollmer, Vinkayalapati H, Redkar S, Bearss DJ, Qiu X, Yoo CB, Jones PA. S110, a 5-aza-2'-deoxycytidine-containing dinucleotide, is an effective DNA methylation inhibitor in vivo and can reduce tumor growth. *Mol Cancer Ther.* 2010;9:1443–50.
- [368] Lavelle D, Sauntharajah Y, Vitkus K, Singh M, Banzon V, Phasivongsa P, Rekar S, Kanekal S, Bearss S, Shi C, Inloes R, DeSimone J. S110, a novel decitabine dinucleotide, increases fetal hemoglobin levels in baboons (*P. anubis*). *J Trans Med.* 2010;8:92.
- [369] Yoo CB, Jeong S, Egger G, Liang G, Phasivongsa P, Tang C, Redkar S, Jones PA. Delivery of 5-aza-2'-deoxycytidine to cells using oligodeoxynucleotides. *Cancer Res.* 2007;1(67):6400–8.
- [370] Chen L, MacMillan AM, Chang K, Ezaz-Nikpay K, Lane WS, Verdine GL. Direct identification of the active-site nucleophile in a DNA (cytosine-5)-methyltransferase. *Biochemistry.* 1991;30:11018–25.
- [371] Osterman GD, DePillis DG, Wu JC, Matsueda A, Santi DV. 5-Fluorocytosine in DNA is a mechanism-based inhibitor of Hhal methylase. *Biochemistry.* 1988;27:5024–10.

- [372] Sheikhnejad G, Brank A, Christman JK, Goddard A, Alvarez E, Ford H Jr, Marquez VE, Marasco CJ, Sufrin JR, O'Gara M, Cheng X. Mechanism of inhibition of DNA (cytosine C5)-methyltransferase by oligodeoxyribonucleotides containing 5,6-dihydro-5-azacytosine. *J Mol Biol.* 1999;285:2021–34.
- [373] Goddard AJ, Marquez VE. Synthesis of a phosphoramidite of 2'-deoxy-5,6-dihydro-5-azacytidine. Its potential application in the synthesis of DNA containing dihydro-5-aza- and 5-azacytosine bases. *Tetrahedron Lett.* 1988;29:1767–70.
- [374] Aviño A, Güimil-García R, Marquez VE, Eritja R. Preparation and properties of oligodeoxynucleotides containing 4-O-butylthymine, 2-fluorohypoxanthine and 5-azacytosine. *Bioorg Med Chem Lett.* 1995;5:2331–6.
- [375] Eritja R, Marquez VE, Güimil García R. Synthesis and properties of oligonucleotides containing 5-aza-2'-deoxycytidine. *Nucleosides Nucleotides.* 1997;16:1111–4.
- [376] Güimil García R, Brank AS, Marquez VE, Christman JK, Eritja R. Synthesis of oligonucleotide inhibitors of DNA (Cytosine-C5) methyltransferase containing 5-azacytosine residues at specific sites. *Antisense Nucleic Acid Drug Dev.* 2001;11:369–78.
- [377] Brank AS, Eritja R, Güimil García R, Marquez VE, Christman JK. Inhibition of Hhal DNA (Cytosine-C5) methyltransferase by oligodeoxyribonucleotides containing 5-aza-2-deoxycytidine: examination of the intertwined roles of co-factor, target transition state structure and enzyme conformation. *J Mol Biol.* 2002;323:53–67.
- [378] Marquez VE, Eritja R, Kelley JA, Vanbemmel D, Christman JK. Potent inhibition of Hhal DNA methylase by the aglycon of 2-(1*H*)-pyrimidinone riboside (zebularine) at the GCGC recognition domain. *Ann NY Acad Sci.* 2003;1002:154–64.
- [379] Dowd CL, Sutch BT, Haworth IS, Eritja R, Marquez VE, Yang AS. Incorporation of zebularine from its 2'-deoxyribonucleoside triphosphate derivative and activity as a template-coding nucleobase. *Nucleosides Nucleotides Nucleic Acids.* 2008;27:131–45.
- [380] van Bemmel DM, Brank AS, Eritja R, Marquez VE, Christman JK. DNA (Cytosine-C5) methyltransferase inhibition by oligodeoxyribonucleotides containing 2-(1*H*)-pyrimidinone (zebularine aglycon) at the enzymatic target site. *Biochem Pharmacol.* 2009;78:633–41.
- [381] Evdokimov AA, Zinov'ev VV, Kuznetsov VV, Netesova NA, Malygin EG. Design of oligonucleotide inhibitors of the human DNA-methyltransferase 1. *Mol Biol (Mosk).* 2009;43:455–63.
- [382] Lamparska K, Clark J, Babilonia G, Bedell V, Yip W, Smith SS. 2'-deoxyriboguanylurea, the primary breakdown product of 5-aza-2'-deoxycytidine, is a mutagen, an epimutagen, an inhibitor of DNA methyltransferases and an inducer of 5-azacytidine-type fragile sites. *Nucleic Acids Res.* 2012;40:9788–801.
- [383] Kumar S, Horton JR, Jones GD, Walker RT, Roberts RJ, Cheng X. DNA containing 4'-thio-2'-deoxycytidine inhibits methylation by Hhal methyltransferase. *Nucleic Acids Res.* 1997;25:2773–83.
- [384] Marquez VE, Wang P, Nicklaus MC, Maier M, Manoharan M, Christman JK, Banavali NK, Mackerell AD Jr. Inhibitors of (cytosine C5)-methyltransferase by oligonucleotides containing flexible (cyclopentane) and conformationally constrained [bicyclo3.1.0] abasic sites. *Nucleosides Nucleotides Nucleic Acids.* 2001;20:451–9.
- [385] Sato K, Kunitomo Y, Kasai Y, Utsumi S, Suetake I, Tajima S, Ichikawa S, Matsuda A. Mechanism-based inhibitor of DNA cytosine-5-methyltransferase by a  $S_NAr$  reaction with an oligodeoxyribonucleotide containing a 2-amino-4-halopyridine-C-nucleoside. *ChemBioChem.* 2018;19:865–72.
- [386] Kasai Y, Sato K, Utsumi S, Ichikawa S. Improvement of  $S_NAr$  reaction rate by an electron-withdrawing group in the crosslinking of DNA cytosine-5 methyltransferase by a covalent oligodeoxyribonucleotide inhibitor. *ChemBioChem.* 2018;19:1866–72.



Anna Aviñó, Carme Fàbrega, and Ramon Eritja

## 4 Nonradioactive labeling of oligonucleotides and postsynthetic modification of oligonucleotides

### Contents

4.1	Introduction —	143
4.2	Postsynthetic conjugation —	144
4.2.1	Amino-oligonucleotides —	144
4.2.2	Thiol- and maleimide-oligonucleotides —	148
4.2.3	Carboxyl-oligonucleotides —	149
4.2.4	Aldehyde- and hydrazide-oligonucleotides —	152
4.2.5	Alkene- and alkynyl-oligonucleotides —	154
4.2.6	Other conjugation protocols —	160
4.3	The “convertible” nucleoside —	161
4.3.1	Pyrimidine convertible nucleosides —	162
4.3.2	Purine convertible nucleosides —	163
4.4	Reagents for fluorescent labeling —	164
4.5	Fluorescent nucleobases —	168
	Bibliography —	171

### 4.1 Introduction

Recent advances in DNA detection techniques have led to the widespread use of specific nucleic acid hybridization probes as an alternative methodology for the detection of human infectious and genetic diseases. For instance, the Polymerase Chain Reaction (PCR) has increased the clinical uses of DNA-based diagnostic tests by producing an enough amount of the target sequence that simple methods can identify the pathogens from crude DNA samples [1]. In parallel, a large effort has been made to develop nonradioactive DNA labeling systems and immobilization systems for handling a large number of samples. In the oligonucleotide field, these applications have triggered the design of nucleosidic and nonnucleosidic phosphoramidite derivatives for the functionalization of oligonucleotides that have permitted the efficient preparation of oligonucleotides carrying fluorescent compounds, biotin, enzymes as well as intercalating, and DNA cleaving groups [2, 3]. More recently, the introduction of peptides, lipid, carbohydrates and receptor-mediated substrates to therapeutic oligo-

---

**Anna Aviñó, Carme Fàbrega, Ramon Eritja,** Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Networking Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Jordi Girona 18-26, E-08034 Barcelona, Spain, e-mails: aaagma@cid.csic.es, carme.fabrega@iqac.csic.es, recgma@cid.csic.es

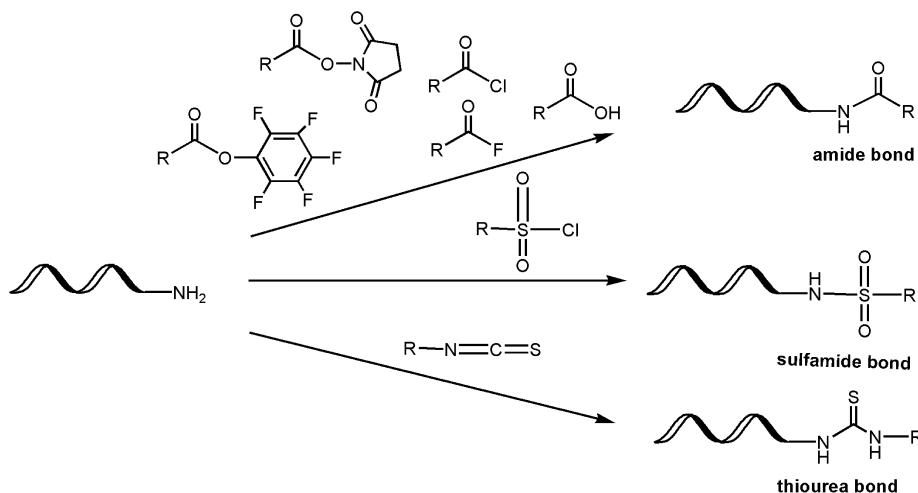
cleotides has been shown to be a critical step for the development of efficient nucleic acid-based drugs [4]. The introduction of these ligands is accomplished by two main strategies. In the first approach, a reactive group is added to the oligonucleotide at specific sites and the ligand is attached to the oligonucleotide using the specific reactivity of this group [5]. Oligonucleotides carrying reactive groups are also employed for the production of surfaces functionalized by nucleic acids needed for the preparation of DNA biosensors [6] and DNA microarrays [7]. A second approach involves the preparation of a special ligand derivative that contains the phosphoramidite function, so it can be incorporated directly to the oligonucleotide by using a cycle similar to that used for the incorporation of the nucleoside phosphoramidites. The functionalization of the oligonucleotides could be achieved at the 3' and 5' ends, at the nucleobases and carbohydrate moieties and at the phosphate groups [8–11]. In addition, there is a large interest in fluorescent nucleobases as specialized substrates for the analysis of biochemical functions. In this chapter, we will describe the advances in the chemical protocols that have allowed the extensive application of nucleic acids in the development of novel diagnostic and therapeutic tools.

## 4.2 Postsynthetic conjugation

In the postsynthetic conjugation protocols, a reactive group is added during the assembly of the oligonucleotides. Then this group reacted with the labeling molecule either before or after the final ammonia deprotection. If the labeling molecule is not stable to this condition, it is mandatory to do the conjugation with the unprotected oligonucleotide carrying the reactive group. In order to obtain the desired oligonucleotides carrying reactive groups, those have to be stable to synthesis conditions, and usually they require to be protected to avoid cross-reactions with the oligonucleotide synthesis protocols. The reactive groups that have been used in oligonucleotide field for the preparation of conjugates are: (1) amino, (2) thiol, (3) carboxyl, (4) azido, alkene and alkynyl, (5) aldehyde and (6) phosphate reactive groups such as 5'-monophosphate and phosphorothioate linkages.

### 4.2.1 Amino-oligonucleotides

Amino moieties are one of the most frequently used reactive groups in the oligonucleotide field. Although nucleobases have also amino groups, these groups are aromatic amines and have low reactivity. For this reason, primary alkyl amino groups are introduced in oligonucleotides for the selective reaction with ligands or fluorescent labels. These ligands contained amino-reactive groups such as active esters, acid chlorides, sulfonyl chlorides, isothiocyanates (Figure 4.1). Most of these compounds

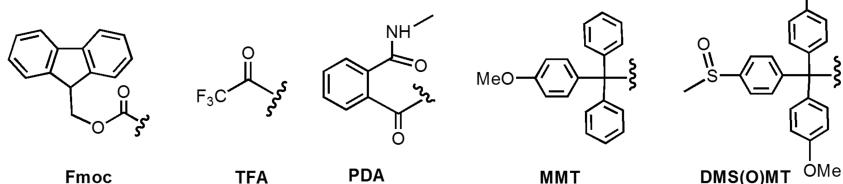
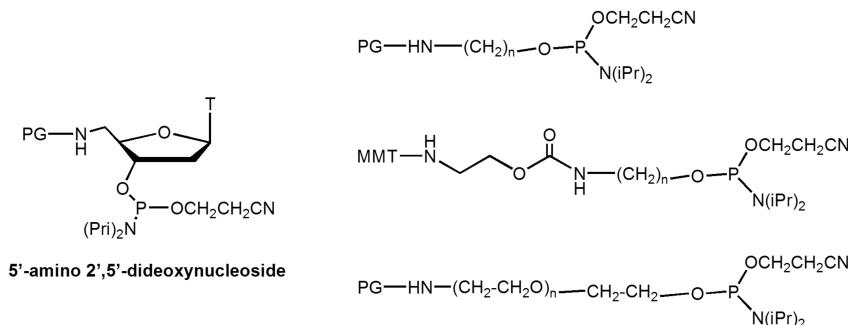
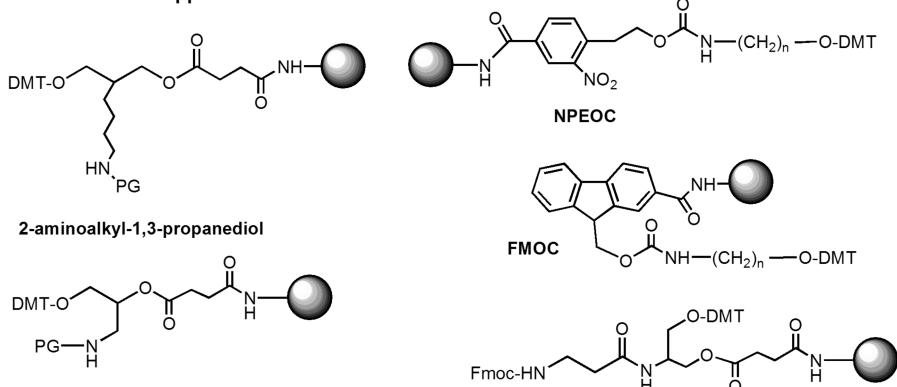
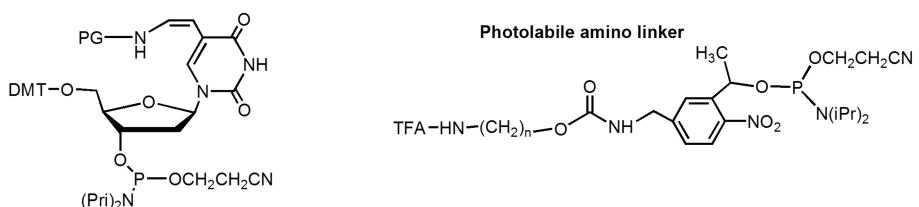


**Figure 4.1:** Reactivity of amino-oligonucleotides with carboxylic acid derivatives, sulfonyl chlorides and isothiocyanates to generate oligonucleotide conjugates.

have been established for the functionalization of proteins through the amino groups of lysines. *N*-hydroxysuccinimidyl [12, 13], halogenated phenyl esters [14] and acid fluorides [15] generate amide bonds while sulfonyl chlorides [16] generate sulfamide bonds and isothiocyanates [17] generate thioureas. Solid-phase assemblies of conjugates are done in organic solvent with a tertiary amine as a catalyst. The conjugation reactions in solutions are usually done at pH 8–9 in mixtures of aqueous buffer and a water miscible cosolvent such as ethanol, acetonitrile or dimethylformamide [18]. Sometimes the carboxylic acids are used by preactivation of the carboxylic acids with carbodimides or other condensing agents developed for peptide synthesis [19, 20]. Recently, the use of pentafluorophenyl esters has been reported for the efficient conjugation of fatty acids to oligonucleotides [21, 22].

One of the first reagents to introduce an aliphatic amino group at the 5' position of the oligonucleotides were 5'-amino 2',5'-dideoxynucleoside derivatives [23–25] (Figure 4.2). These derivatives were prepared from the corresponding 2'-deoxynucleosides and were protected with fluorenylmethoxycarbonyl (Fmoc) [23, 24] or trifluoroacetyl (TFA) moieties [25] groups that were labile to ammonia. The resulting 5'-amino-oligonucleotides were labeled with fluorescent compounds and were used as sequencing primers in the development of DNA sequencing methods [26].

Afterwards, the 5'-amino 2',5'-dideoxynucleoside derivatives were replaced by more accessible aminoalkylalcohol derivatives (Figure 4.2) such as 2-aminoethanol, 3-aminopropanol, 6-aminohexanol and 12-aminododecanol [27–31]. In addition to Fmoc and TFA groups, the amino group was also protected with the acid labile monomethoxytrityl (MMT) [27] or trityl [31] or 2-(4-biphenyl)-propyl-2-oxycarbonyl (Bpoc) [32] groups that are acid labile and, for this reason, they can be deprotected

**Amino-protecting groups (PG)****5'-amino modifiers****3'-amino-Solid Supports****Nucleoside phosphoramidites with amino groups**

**Figure 4.2:** Chemical structures of the reagents used for the introduction of reactive amino groups in oligonucleotides including phosphoramidites and solid supports as well as the protecting groups for the amino function.

independently from the nucleobase and phosphate protecting groups. Another improvement was the introduction of aminoethoxy carbamate group between the amino-hexanol and the terminal group (Figure 4.2) [33, 34]. The presence of the ether function at beta position to the amino group increases the nucleophilicity of the reactive amino and allows more efficient conjugation reactions [33, 34]. Similarly, amino-ethyleneglycol and amino-tetraethyleneglycol phosphoramidite derivatives have also been described [35]. In addition, the use of a new trityl group that carries a methyl-sulfinyl group [33] has been described. This new trityl group can be removed in milder conditions than the MMT group. Acetylation of the amino groups has been described when MMT group is used for the protection of the 5'-amino modifier [36, 37]. In order to avoid this side reaction, the capping reaction after the addition of the amino phosphoramidite is not performed. In addition, the use of the phthaloyl [38] and *N*-methyl-phthalic acid diamide (PDA) [39] protecting groups have been described and the PDA-6-aminohexyl phosphoramidite has been commercialized.

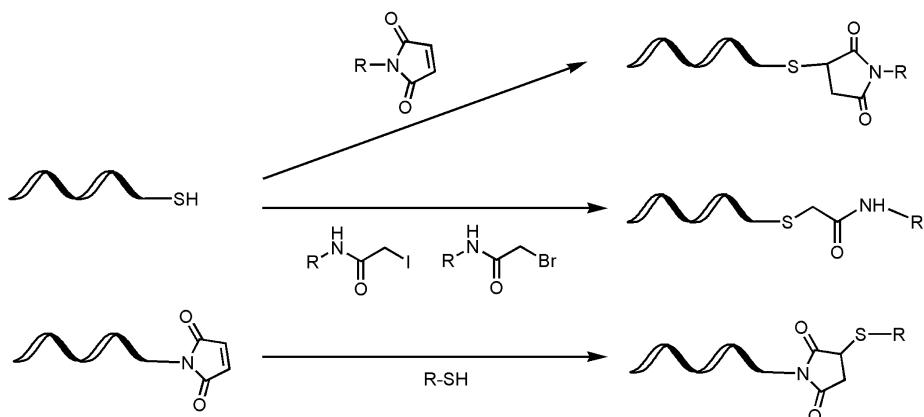
The introduction of amino groups at the 3'-end or at internal positions of oligonucleotides can be done using *N*-Fmoc-2-amino-1,3-propanediol derivatives (Figure 4.2) [40]. One of the alcohol functions is protected with the DMT group and the second alcohol function carries either the succinate linkage to connect with the solid phase or the phosphoramidite function. The *N*-Fmoc-2-amino-1,3-propanediol derivatives can be used for the addition of multiple amino groups [40]. Afterwards, the *N*-Fmoc-2-aminobutyl-1,3-propanediol derivatives were reported (Figure 4.2) [41]. The separation of the amino group from the branching point increased the reactivity of the amino group as steric effects are smaller. Furthermore, it has been described that the Fmoc group from *N*-Fmoc-2-amino-1,3-propanediol derivatives is prematurely removed and becomes acetylated during the capping step generating acetamino-oligonucleotides as side products [42]. The use of phthaloyl linkers has been suggested [42]. Moreover, the use of *p*-nitrophenylethyoxycarbonyl (NPPEC) and fluorenyloxycarbonyl (Fmoc) linkers (Figure 4.2) have been described for the introduction of amino groups at the 3'-end of oligonucleotides [43]. Other clever nonnucleoside reagents have been reported for the introduction of amino groups as well as for the direct introduction of labels [16, 44].

The introduction of reactive amino groups at the nucleobases is also possible. This is usually achieved by synthesizing 5-substituted uracil nucleosides carrying amino group [45, 46] (Figure 4.2). The introduction of the aminolinker is usually done by palladium catalyzed Heck reactions [46] although it has also been described the total synthesis of the modified 5-substituted uracil derivative [45]. Amino groups can also be introduced at the 2'-position of ribonucleosides [17]. In this case, the *N*-(5-bromopentyl)phthalimide can be used for the direct alkylation of the 2'-OH group followed by the incorporation of the appropriate protecting groups and phosphoramidite function [17]. An interesting option for the introduction of reactive amino groups into oligonucleotides is the photocleavable aminotag phosphoramidite (Figure 4.2) [47]. These phosphoramidites contain a photolabile 2-nitrobenzyl function between the

amino group and the phosphoramidite function. The addition of this phosphoramidate at the 5'-position of an oligonucleotide produced a 5'-amino-oligonucleotide that can be reacted with biotin or fluorescent compounds. The resulting conjugates can be used for affinity capture experiments with the appropriate solid supports with the advantage that the captured biomolecules can be released by photolysis for further analysis. The photocleavable oligonucleotide conjugates are useful in a variety of applications such as nonradioactive probing of DNA or RNA blots, affinity isolation, PCR and diagnostic assays [47].

#### 4.2.2 Thiol- and maleimide-oligonucleotides

Thiol groups have an extraordinary reactivity with maleimide and haloacetamido derivatives to form thioethers (Figure 4.3). A large number of maleimide and haloacetamido derivatives of fluorescent compounds are available for protein labeling through cysteine residues. For these reasons, oligonucleotides carrying thiol groups are important intermediates for oligonucleotide labeling as well as for the production of oligonucleotide-peptide [48] or oligonucleotide-protein conjugates [49]. Moreover, methods for the preparation of maleimide-oligonucleotides have been described. These modified oligonucleotides react with thiol groups to form thioethers (Figure 4.3).



**Figure 4.3:** Reactivity of thiol-oligonucleotides with maleimido and haloacetamido compounds to generate oligonucleotide conjugates.

The improvement of methods for the introduction of thiol groups at the 5'-end of oligonucleotides followed a similar path than amino-oligonucleotides. First, S-trityl-5'-mercaptop-2'-5'-dideoxy nucleosides [50] were developed as well as mercapto-alcohols [31, 51–53] such as 3-mercaptop-propanol and 6-mercaptop-hexanol (Figure 4.4). The thiol group was usually protected with the trityl group [31, 51, 52] that

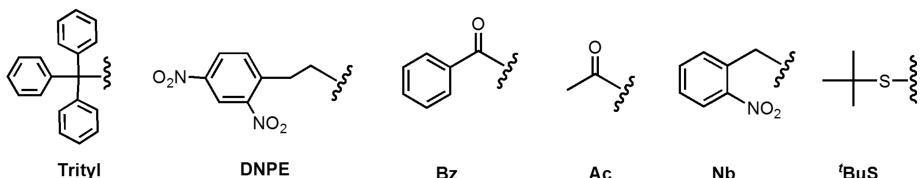
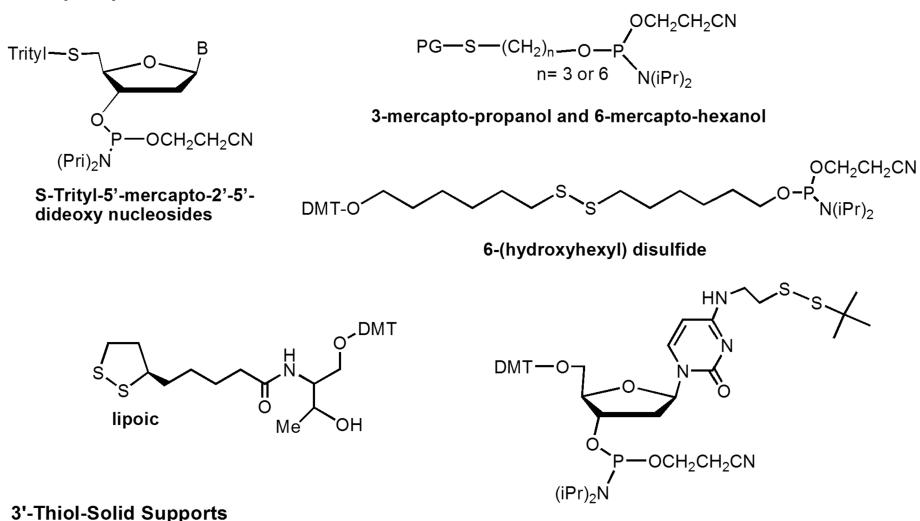
was removed with acetic acid in the presence of silver nitrate. During the scaling-up of these protocols, an important loss of the desired oligonucleotide-thiol was described [53, 54]. Small changes in the work-up protocols after deprotection have been described [54]. In a different approach, the development of the base-labile, 2,4-dinitrophenylethyl (Dnpe), group (Figure 4.4) for the protection of the thiol groups was suggested [53]. In addition, acetyl [55] and benzoyl [56] and the photolabile 2-nitrobenzyl [57] groups have also been described for the protection of the thiol groups. However, the most dramatic progress was the use of disulfide derivatives such as *bis*(3-hydroxypropyl)disulfide and *bis*(6-hydroxyhexyl) disulfide (Figure 4.4) [58, 59]. These groups are removed by the addition of thiols such as mercaptoethanol or dithiothreitol (DTT) during the ammonia deprotection step. Alternatively, the ammonia deprotection can be run as usual (without thiols) and the oligonucleotide carrying the disulfide bonds can be isolated and characterized. The free thiol oligonucleotide can be generated by a short treatment with phosphine such as tris(carboxyethyl)phosphine (TCEP) [60]. Similarly, the *tert*-butylthio group (Figure 4.4) has been described for the development of nucleoside [61–64] and nonnucleoside [65] phosphoramidites.

During the development of DNA nanobiotechnology, there has been a large demand for thiol oligonucleotides for the preparation of gold nanoparticles functionalized with oligonucleotides [66–71] and in general for the functionalization of gold surfaces with oligonucleotide probes. Thiol groups and disulfide groups can be used for the immobilization of oligonucleotides to gold surfaces. For this reason, methods for the preparation of oligonucleotides carrying disulfides such as lipoic or thioctic acid (Figure 4.4) and a steroid disulfide were described [71–76]. This implied the development of special phosphoramidites and solid supports, some of them are shown in Figure 4.4.

In addition to oligonucleotide carrying thiol group, the synthesis of oligonucleotide carrying maleimide groups has been described [77, 78]. The key point of this methodology is the use of a maleimide protecting group that remains unaltered during the ammonia-promoted deprotection of oligonucleotide functional groups, and which can be subsequently eliminated through a retro-Diels–Alder reaction. Maleimide-oligonucleotides reacted with a variety of thiol-containing compounds including fluorescent compounds, peptides oligonucleotides with phosphorothioate linkages and thiol-oligonucleotides yielding the desired oligonucleotide conjugates [79–81].

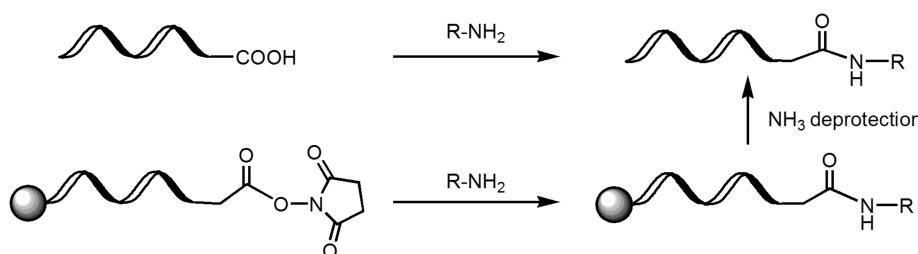
### 4.2.3 Carboxyl-oligonucleotides

Oligonucleotides carrying carboxylic acid derivatives are useful intermediates for the preparation of conjugates with compounds carrying amino groups and for the immobilization of oligonucleotides into surfaces functionalized with amino groups (Fig-

**Thiol-protecting groups (PG)****Thiol phosphoramidites**

**Figure 4.4:** Chemical structures of the reagents used for the introduction of reactive thiol groups and lipoic acid in oligonucleotides including phosphoramidites and solid supports as well as the protecting groups for the thiol function.

ure 4.5) [82, 83]. Hydroxalkylcarboxylates have been used for the preparation of phosphoramidites that allow the introduction of carboxylic acids in the 5'-end of oligonu-



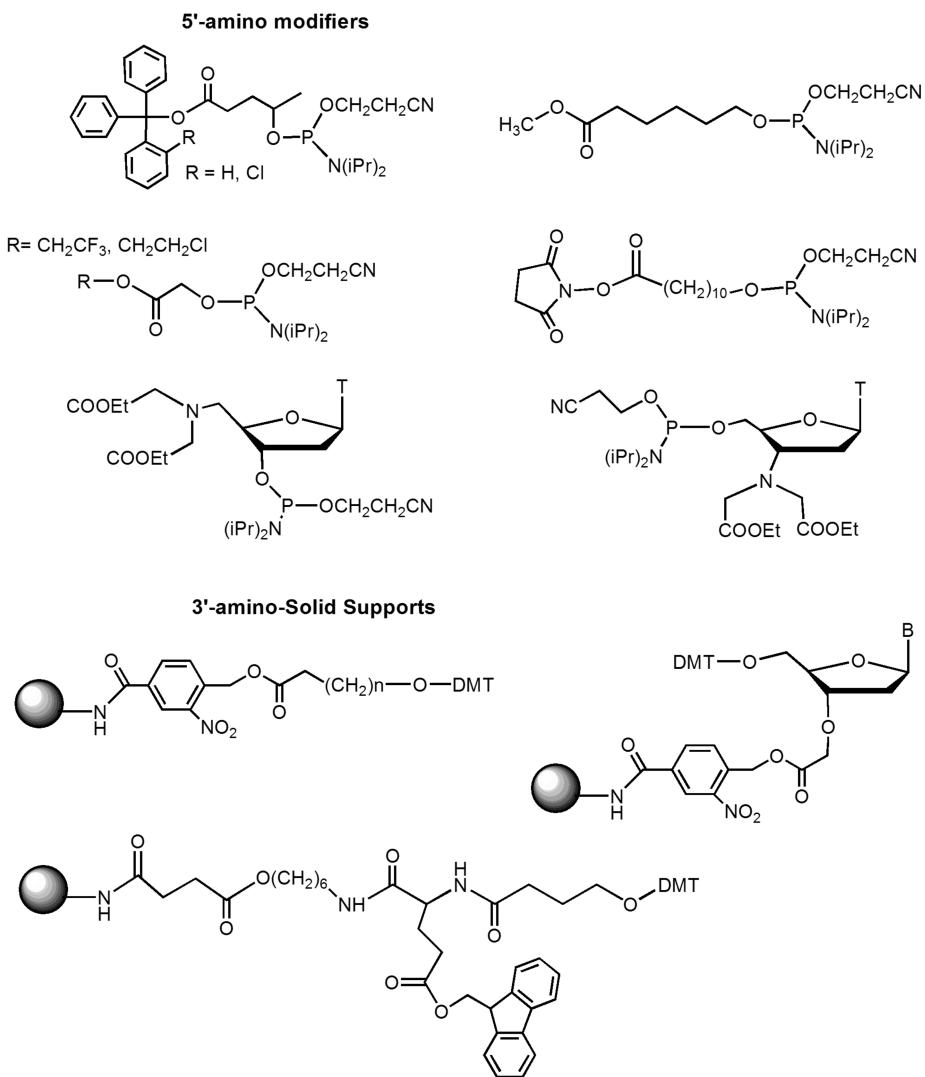
**Figure 4.5:** Reactivity of carboxyl-oligonucleotides with amino derivatives to generate oligonucleotide conjugates. The formation of the amide bond can be done either on the solid support or by postsynthetic reaction.

cleotides [82–84]. The methyl [82], chloroethyl or trifluoroethyl [84] esters that are hydrolyzed with bases have been described (Figure 4.6) for the protection of the carboxylic acid function. Additionally, the acid-labile trityl and chlorotriyl groups (Figure 4.6) have been reported [83].

The phosphoramidite of the 10-hydroxydecanic acid *N*-hydroxysuccinimidyl ester has been developed (Figure 4.6) [85]. Once incorporated to an oligonucleotide at the 5'-position, the active ester can be used to perform the reaction with amines on the support. The oligonucleotide carrying an amide bond is usually stable to ammonia deprotection allowing the efficient introduction of amino containing compounds to the 5'-end of oligonucleotides. Phosphoramidites of thymidine derivatives carrying iminodiacetic acid functions have been described [86]. Oligonucleotides carrying iminodiacetic acid moieties provide specific metal binding sites.

An interesting development from the group of Dr. Greenberg is the preparation of photolabile linkers for the introduction of carboxylic functions at the 3' position [87, 88]. The method is based on the introduction of 2-nitrobenzyl ester linkages between the solid support and the oligonucleotide (Figure 4.6). The photolysis of the 2-nitrobenzyl ester is produced with a black light lamp irradiating at 340 nm in very mild conditions. In this way, oligonucleotides carrying sensitive functions can be obtained for the production of interesting conjugates. An alternative method is the introduction of a glutamic acid protected with the fluorenylmethyl (Fm) ester between the solid support and the oligonucleotide (Figure 4.6) [71]. In this case, ammonia deprotection will generate oligonucleotides carrying carboxylic acid functions at the 3'-end [71]. An interesting multipurpose linker allows the preparation of oligonucleotides carrying several reactive groups such as amino, thiol and carboxyl groups depending on the chemical treatment performed after the assembly of the oligonucleotide [89].

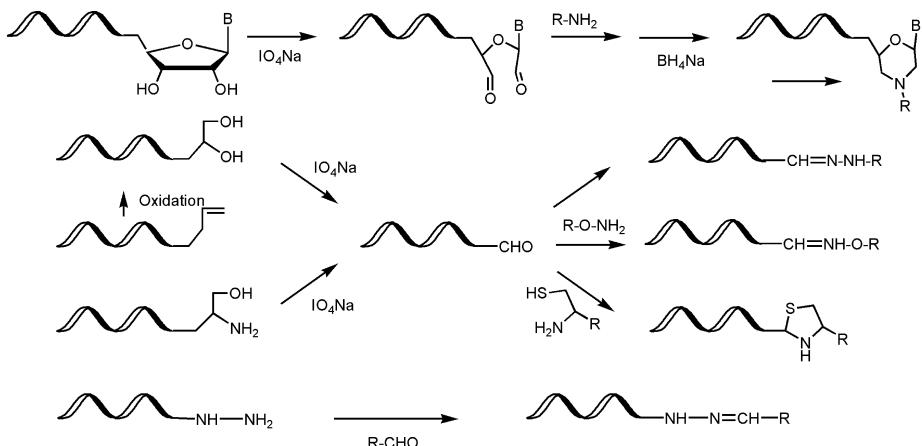
The carboxylic acid function can also be incorporated in the middle of oligonucleotides using a nucleoside derivatized in the nucleobase such as the so-called carboxy-dT phosphoramidite that contains a carboxyethyl group linked at position 5 of 2'-deoxyuridine [90].



**Figure 4.6:** Chemical structures of the reagents used for the introduction of reactive carboxyl groups in oligonucleotides.

#### 4.2.4 Aldehyde- and hydrazide-oligonucleotides

Aldehydes react with hydrazine, hydrazide and hydroxylamino derivatives with high reactivity and selectivity providing an excellent route for the preparation of oligonucleotide conjugates as well as for the immobilization of oligonucleotides to solid supports or the functionalization of proteins and polymers with oligonucleotides (Figure 4.7). Aldehydes are very reactive and they are usually generated by periodate ox-



**Figure 4.7:** Reactions involved in the generation of oligonucleotides carrying aldehyde and hydrazine oligonucleotides as well as conjugation reactions involving aldehydes, hydrazines and hydroxylamino groups.

dation of *cis*-diol groups. One of the first methods used for the preparation of oligonucleotide carrying aldehydes is the addition of a ribonucleotide at the 3'-end of the oligonucleotide [91, 92]. The 3'-terminal ribonucleotide bears a *cis*-diol function that can be oxidized with periodate generating a dialdehyde. The dialdehyde is then reacted with amines, or hydrazines generating a cyclic Schiff base that can be stabilized by reduction with sodium borohydride yielding a cyclic imine (Figure 4.7).

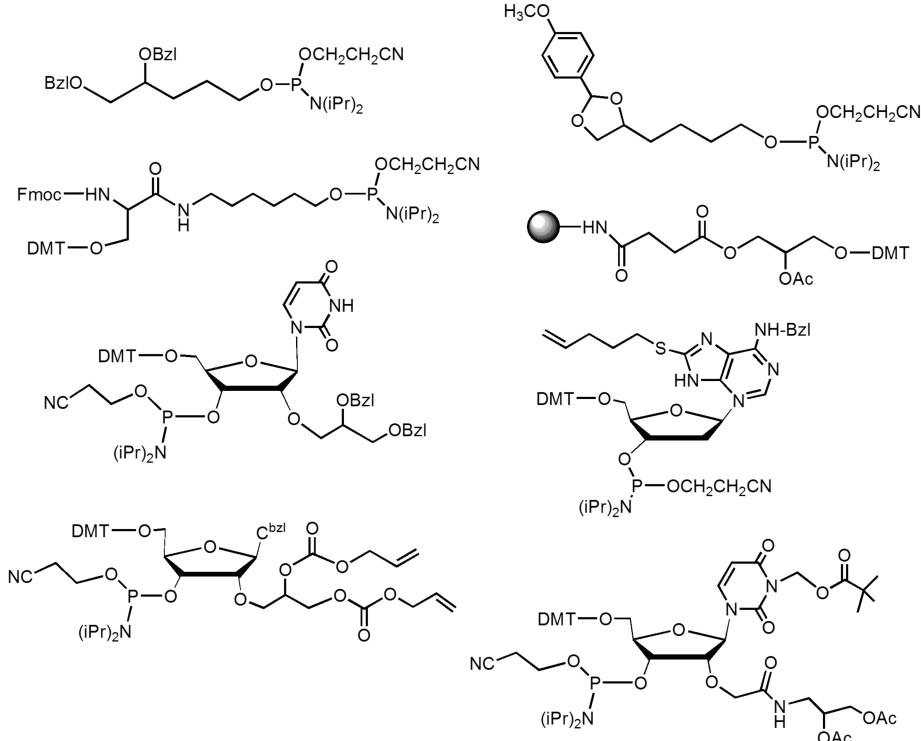
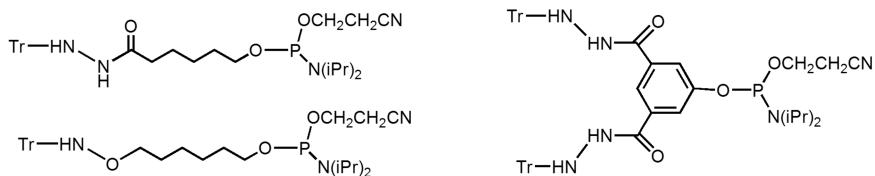
Alternatively, carboxyl-oligonucleotides have been reacted with 1-amino-2,3-propanol generating oligonucleotides bearing the desired *cis*-diol function [82]. Next, several linear triols carrying a *cis*-diol function such as 1,2,5-pentanetriol and 1,2,6-hexanetriol derivatives (Figure 4.8) have been functionalized for the incorporation at the 5'-end of oligonucleotide [92, 93]. In these protocols, the *cis*-diol function is protected either with benzoyl groups [92] or with an acid labile acetal group [93]. This last protocol has provided excellent methods for the introduction of phenanthroline derivatives [94], peptides [93] and carbohydrates [95]. Oligonucleotides carrying *cis*-aminoalcohol functions can also be employed for the generation of oligonucleotides carrying aldehydes [96, 97]. Other interesting methods for the introduction of aldehyde functions in oligonucleotides use aromatic aldehydes that are stable to oligonucleotide synthesis conditions. These include formylindole [98] and benzaldehyde [99] phosphoramidites. Another reported strategy applies amino-oligonucleotides to generate either aldehydes or hydrazino derivatives using bifunctional reagents having an *N*-hydroxysuccinimide ester on one end and either a hydrazine (SANH) or benzaldehyde (SFB) moiety at the other. Both reagents react with free amines of amino-oligonucleotides allow for the introduction of reactive aldehyde or hydrazine groups that have been used for conjugation of oligonucleotides to proteins [100].

Nucleoside phosphoramidites have also been used for the introduction of *cis*-diol functions to oligonucleotides and subsequent generation of aldehyde functions [101–107]. One of the first methods was based on the alkylation of 8-thio-2'-deoxyadenosine with 1-bromo-4-pentene for the introduction of a terminal alkene in the nucleobase at position 8 of adenine (Figure 4.8) [101, 102]. This alkene was oxidized to generate a *cis*-diol that will be used for the generation of the aldehyde function. Then several phosphoramidites (Figure 4.8) carrying protected glycerol moieties attached to the 2'-position of a ribonucleotide were reported [103–107]. This allows the introduction of an aldehyde function at any position of the oligonucleotides without the loss of the hybridization properties.

The introduction of hydrazide groups in oligonucleotides have been described (Figure 4.8) [108, 109]. These include nonnucleosidic linkers and nucleosides functionalized at the 2'-position [108]. The hydrazide function is protected either with the trityl [108] or the Fmoc [109] groups. For the introduction of an hydroxylamino group, a nonnucleoside linker protected with the trityl group (Figure 4.8) was developed [93]. The condensation of hydroxylamino groups with carbonyl groups is known as oxime ligation [93]. These reagents allow the coupling of peptides carrying trifunctional amino acids to oligonucleotides with high specificity. Moreover, phosphoramidites carrying a phthaloyl-protected aminoxy function were prepared and applied in automated oligonucleotide synthesis for the production of aminoxy-oligonucleotides [110], which were used for the attachment of oligonucleotides to microscopic polymer particles by reaction of the aminoxy function with the particle bound aldehyde or epoxide groups. The immobilized oligonucleotides were utilized as probes in hybridization assays [110].

#### 4.2.5 Alkene- and alkynyl-oligonucleotides

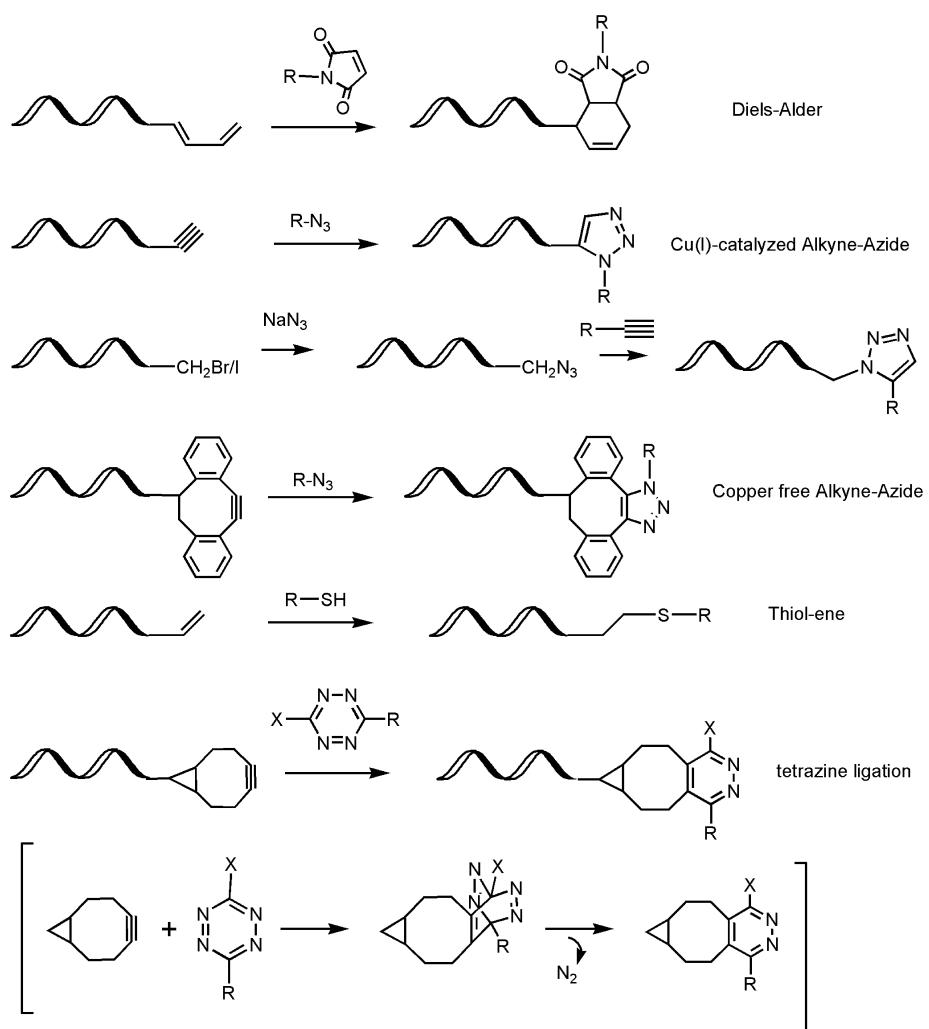
The introduction of alkene or alkyne derivatives in oligonucleotides has generated a large interest in the last years because of the need for novel protein, peptide, DNA and RNA conjugates. The description of novel bioconjugation reactions that could be performed selectively in biological samples including living cells has triggered this large interest [111]. Importantly, the standard oxidation step in the solid-phase phosphoramidite protocols is considered safe for the preparation of oligonucleotides carrying alkene and alkyne groups [112, 113]. There are several conjugation reactions that imply the use of alkene- or alkynyl-oligonucleotides (Figure 4.9). One of the first cycloaddition reactions to be applied in the oligonucleotide field was the Diels–Alder cycloaddition. This reaction is performed between a diene and an electron-deficient alkene or dienophile to obtain a cyclohexene derivative. Usually, dienes such as 1,3-hexadiene [114, 115] or furane derivatives [116, 117] are introduced into oligonucleotides (Figure 4.10) and the ligand is functionalized with maleimide groups acting as dienophiles [114–117]. This reaction has been used for the preparation of oligonucleotides.

**Phosphoramidites generating cis-diol groups****Phosphoramidites generating hydrazido or hydroxylamino groups**

**Figure 4.8:** Chemical structures of the reagents used for the generation of oligonucleotides carrying cis-diol, hydrazide or hydroxylamino groups.

cleotides carrying fluorescent compounds [115, 116], biotine [115] and peptides [114] as well as for the chemical ligation of oligonucleotides [117].

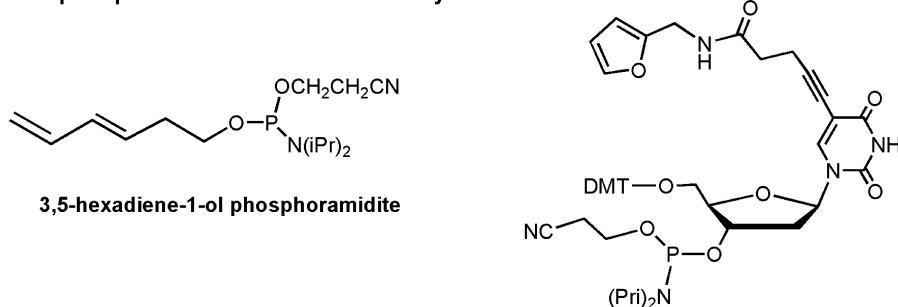
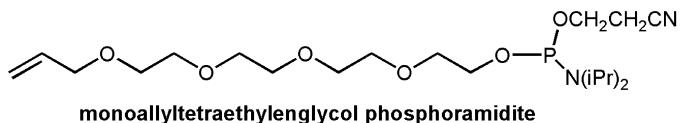
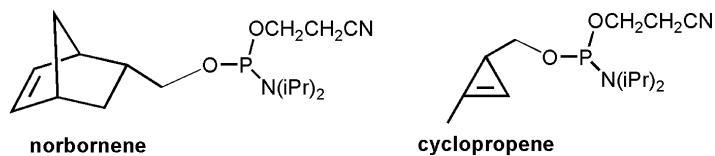
One of the most popular postsynthetic reactions on oligonucleotides is the 1,3-dipolar cycloaddition between azides and alkynes or Huisgen reaction [118] known as “click chemistry.” This reaction captured the interest of the researchers after the report of the catalytic effect of copper (I) cations [119]. The development of the click chemistry in the oligonucleotides was slower than in other fields because of the degradation observed in DNA by the presence of copper ions and ascorbic acid used for the



**Figure 4.9:** Some of the cycloaddition reactions used in the preparation of oligonucleotide conjugates.

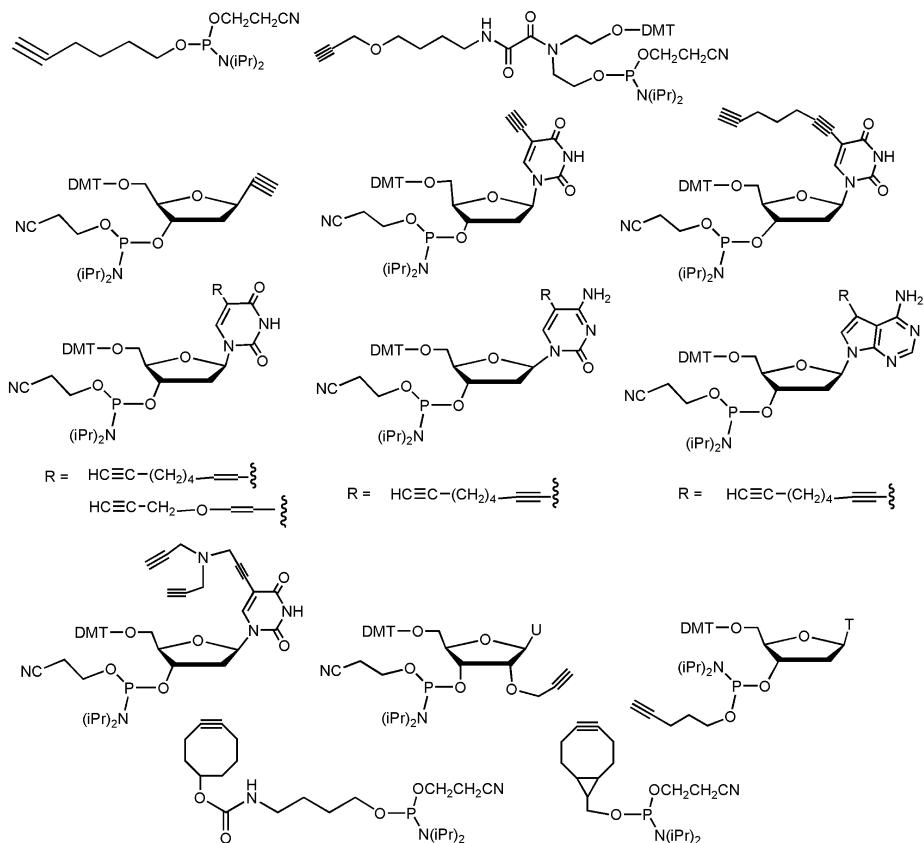
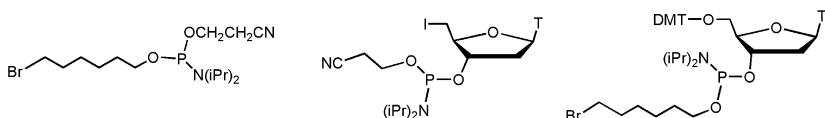
reduction of copper (II) to copper (I) [120, 121]. These problems were overcome by the use of the copper ligand tris(benzyltriazolylmethyl) amine (TBTA) [122, 123]. The important applications generated by the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) in the nucleic acids field has been covered by excellent reviews [112, 113].

Alkynes can be introduced into oligonucleotides either by direct incorporation of alkyne phosphoramidites [112, 113] or by the reaction of alkynes functionalized with reactive groups such as active esters, or amino groups with oligonucleotides carrying the corresponding reactive groups such amino or carboxylates [121]. Also,

**Diene phosphoramidite for Diels-Alder cyclization****Alkene phosphoramidite for Tiol-ene reaction****Alkene phosphoramidites for tetrazine-alkene cycloaddition**

**Figure 4.10:** Chemical structures of the phosphoramidite reagents carrying alkene and diene for the functionalization of oligonucleotides.

nucleoside triphosphates carrying alkynes can be enzymatically incorporated into DNA by DNA polymerases [124]. One of the simplest phosphoramidites used for the introduction of alkynes was the hex-5-yn-1-ol phosphoramidite (Figure 4.11) [121, 125]. In order to enhance the reactivity, linkers with an activated triple bond donor such as propynoylamino-*p*-toluic acid (PATA) and others (Figure 4.11) have been described [126, 127]. Alkynyl groups have also been introduced in nucleoside phosphoramidites. The position 5 of pyrimidines has been selected for the introduction of alkynyl groups (Figure 4.11). Alkynyl-dU [128, 129] and dC [130] have been developed by the Carell and Seela groups. The introduction of alkynyl groups to purine nucleosides has also been described using 7-deazapurines (Figure 4.11) [131, 132]. A nucleotide modification at the 2'-position of uridine has also been described [133]. Alkynyl groups have also been introduced in the phosphate linkages by the replacement of the 2-cyanoethyl group for the 4-pentynyl group (Figure 4.11) [134]. The synthesis of oligonucleotides containing 1-ethynyl-2-deoxy- $\beta$ -D-ribofuranose (Figure 4.11) has also been described [135]. The efficient conversion of the ethynyl group into several 1,2,3-triazoles is also

**Alkyne Phosphoramidites****Phosphoramidites for the generation of azido-oligonucleotides**

**Figure 4.11:** Chemical structures of the reagents described for the introduction of alkyne groups into oligonucleotides as well as phosphoramidites carrying haloalkyl groups used in the generation of azido-oligonucleotides.

reported producing novel nucleobase analogs. In another study, the synthesis of 5-tripropargylamine-dU phosphoramidite (Figure 4.11) has been reported [136]. This bifunctional derivative is able to direct two consecutive click reactions increasing the number of potential derivatives obtained by click chemistry.

The preparation of oligonucleotides carrying azido groups cannot be done directly as azido groups are not compatible with phosphoramidites as they may re-

act by the Arbusov reaction generating phosphoramidates [137]. For these reasons, azido groups are incorporated either by postsynthetic coupling such as succinimidyl 5-azidovalerate with amino-oligonucleotides [138] or by the incorporation of bromo- or iodo-alkyl derivatives (Figure 4.9) followed by displacement of the halogen by lithium or sodium azide [121]. Bromoalkyl groups have also been introduced in the phosphate linkages by replacement of the 2-cyanoethyl group for the 6-bromohexyl group (Figure 4.11) [134]. Although azido and phosphoramidite groups are generally not compatible, recently, an azido phosphoramidite was developed for the preparation of azido-oligonucleotides [139]. The phosphoramidite was stable as long as it was stored in solution but it undergoes to decomposition if the solution was evaporated to dryness.

The use of copper ions as catalysts prevents the use of the click reaction in biological systems due to the toxicity of copper ions. An interesting development from the carbohydrate field is the use of especially reactive ring-strained alkynes such as dibenzocyclooctyne (DIBO) and cyclooctyne (NSCO) that allow copper free azido-alkyne cycloaddition [140] opening the possibility of *in vivo* labeling of specific biomolecules. This method is known as ring strain-promoted azide–alkyne [3+2] cycloaddition reaction (SPAAC). Oligonucleotides carrying the cyclooctyne can be synthesized by the reaction of the appropriate active ester with amino-oligonucleotides or using dedicated phosphoramidites carrying cyclooctyne moieties (Figure 4.11) [140–142],

In addition to oligonucleotide fluorescent labeling [124, 134], CuAAC and SPAAC opened new avenues in the functionalization of surfaces with oligonucleotides [123, 143–145], chemical ligation of oligonucleotides and DNA cyclization [125, 135, 146], preparation of peptide- and protein-oligonucleotide conjugates [126, 147, 148] and the synthesis of artificial DNA nucleobases [136, 149].

Functionalization of surfaces can also be achieved by the thiol-ene chemistry [150]. Thiol-ene reaction takes place with UV radiation and forms a thioether bond. This reaction is compatible with aqueous media, which is crucial to work with biomolecules. Thiol-ene reactions proceed under mild conditions in the presence of oxygen, are regioselective, tolerate many functional groups and can be performed on aqueous solutions providing good yields. Usually, oligonucleotide carrying thiol groups are used in the thiol-ene coupling reaction [150–153] but also, the monoallyltriethoxylglycol phosphoramidite (Figure 4.10) has been reported for the preparation of oligonucleotides carrying reactive alkene groups [151].

A recent addition to the bioconjugate arsenal is the tetrazine ligation with alkene or alkynes. This cycloaddition reaction is performed between a tetrazine and a constrained alkyne or alkene such as norbornene or cyclopropene derivative (Figures 4.9 and 4.10). The Diels–Alder intermediate adduct decomposes losing a nitrogen molecule making the reaction irreversible. As tetrazines are not stable to oligonucleotide synthesis conditions, the preparation of oligonucleotides carrying tetrazine moieties is done by the reaction of tetrazines functionalized with carboxylic acid with amino-oligonucleotides [154, 155]. Cyclopropene [155] and vinyl derivatives [156]

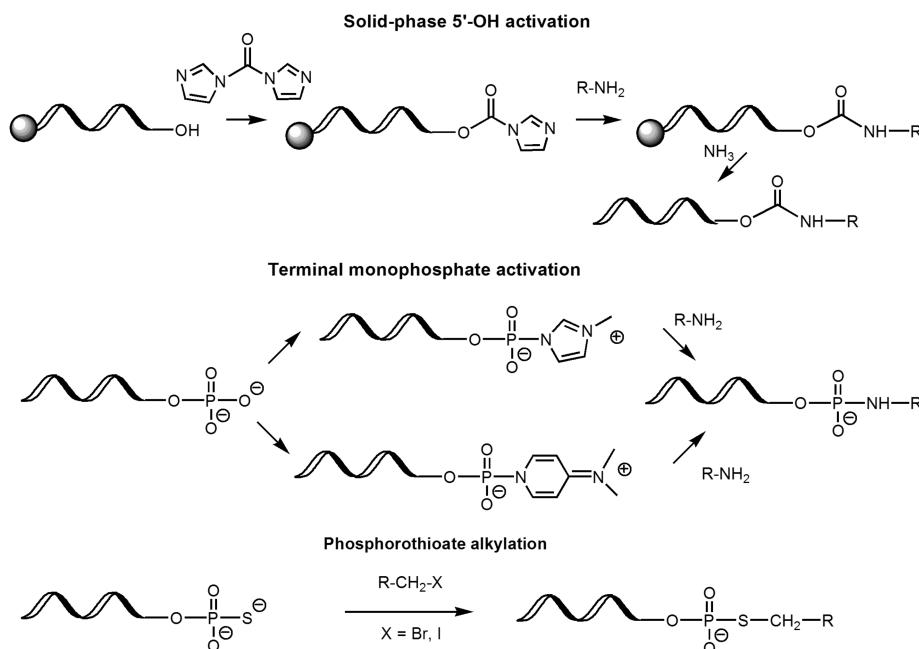
have been also introduced by enzymatic incorporation of nucleoside triphosphates. Subsequent reaction with tetrazine-fluorescent compounds allows the preparation of highly functionalized DNA molecules. An interesting enzymatic approach for the preparation of norbornene-modified nucleic acids is the use of promiscuous DNA and RNA methyltransferases using AdoMet analogs carrying a benzylic linker with norbornene derivatives as alkyl donors [157].

#### 4.2.6 Other conjugation protocols

Besides the large development of bioconjugation protocols involving oligonucleotides, there were several methods that provide simple routes to generate a wide variety of oligonucleotide conjugates. For instance, one of the first protocols for the incorporation of amino derivatives was the use of carbonyl-imidazole to activate the 5'-alcohol function at the end of the assembly of the oligonucleotide before the final deprotection. In these conditions, it is possible to activate selectively the 5'-OH and subsequent treatment with diamines [158] and amino acids [159] generate the corresponding urethane (Figure 4.12) that is stable to ammonia deprotection conditions generating the corresponding amino- or carboxyl-oligonucleotides.

Oligonucleotides carrying a terminal 5'- or 3'-monophosphate can be prepared using special phosphoramidites or solid supports. In addition, oligonucleotides can be enzymatically phosphorylated at the 5'-end using T4 oligonucleotide kinase and ATP. Oligonucleotide 3'- or 5'-monophosphates can be activated selectively without the interference of the phosphodiester bonds of the oligonucleotides. The activation protocols start with the generation of the oligonucleotide cetyltrimethylammonium salt that precipitates from aqueous solutions after the addition cetyltrimethylammonium bromide (CTAB). The resulting hydrophobic salt is activated with triphenylphosphine and dipyridyl-2,2'-disulfide (Mukaiyama's reagents [160] in the presence of either *N*-methylimidazole [161, 162] or *N,N*-dimethylaminopyridine [163–165] in dimethylsulfoxide generating an activated phosphate (Figure 4.12) that is reacted with the appropriate amino compound to generate a phosphoramidite. The isolation of the desired conjugate is usually done by precipitation with lithium perchlorate to eliminate the cetyltrimethylammonium cation [166]. The use of terminal monophosphates have provided interesting oligonucleotide conjugates carrying intercalating agents [167], fullerene derivatives and dyes for photodynamic therapy [161, 163, 164], radiolabels [162] and tags for fluorescence energy transfer [168].

Another interesting possibility is the introduction of a single phosphorothioate function especially at the terminal positions [169] as phosphorothioates are nucleophilic and they can react with many electrophiles such as bromoalkyl and bromoacetamido derivatives [168, 169], thiopyridyl [169] and tosyl [170] groups. Inter-nucleotidic phosphorothioate diester linkages have also been used for the introduction of fluorescent compounds [171, 172]. The addition of an ethylthio phosphate



**Figure 4.12:** Other less common reactions described for the preparation of oligonucleotide conjugates: (1) Activation of 5'-OH by carbonylimidazole and subsequent urea formation by reaction of primary amines. (2) Activation of 5'-monophosphate function. (3) Alkylation of phosphorothioate linkages.

group using *N*-triphenylacetyl cystamine as backbone tether facilitates the conjugation of minor groove binding molecules to oligonucleotides [173, 174]. An interesting method for the selective labeling of oligonucleotides with two different reporter groups was described. The oligonucleotide was synthesized using a stable 2-(4-methoxybenzamido)ethyl protection for a selected internucleosidic thiophosphate (PS) and a labile 2-(*N*-isopropyl-4-methoxybenzamido)-ethyl for the 3'-terminal PS or an internucleosidic phosphates. The latter group and the base protection are removed, and the 3'-terminal PS is labeled. The former protection is then cleaved by a prolonged ammonolysis, and the second reporter is introduced at the internucleosidic PS bond [175].

### 4.3 The “convertible” nucleoside

In the previous sections of this chapter, we have described the development of linker molecules needed for the introduction of reactive groups into oligonucleotides that are based on the development of nucleosidic or nonnucleosidic linker phosphoramidites

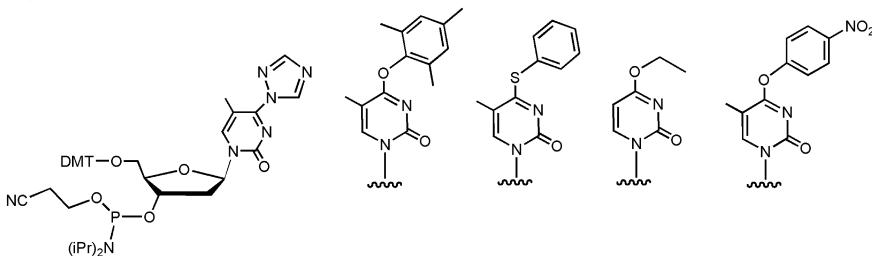
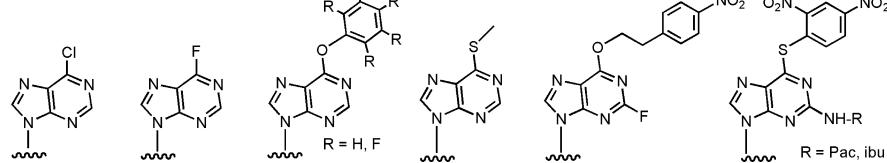
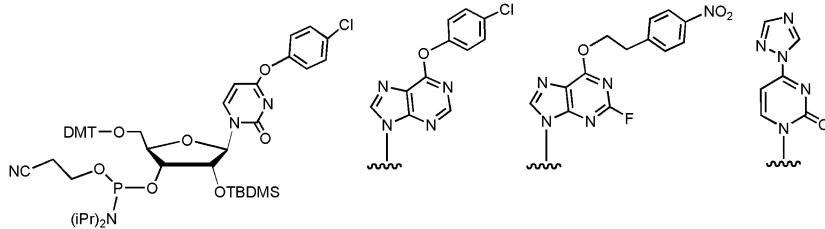
carrying the reactive groups properly protected for the oligonucleotide synthesis. An interesting alternative is the use of a modified nucleoside derivative containing a leaving group or convertible nucleoside that will be displaced by nucleophiles. This strategy allows the generation of a series of nucleoside derivatives at the end of the assembly of the sequences using a single phosphoramidite [176]. This strategy was developed by the group of Dr. Verdine for the introduction of different tethers to synthetic oligonucleotides [176, 177]. Previously, modified nucleoside phosphoramidites were used as precursors for nucleosides labile to oligonucleotide synthesis conditions such as aziridine alkylating agents [178–180]. Nowadays, this strategy is widely used for sensitive nucleobases involved in DNA repair [181, 182], for the introduction of  $N^{15}$  [183] and  $C^{13}$  [184] labels at the exocyclic positions of nucleobases and duplex-stabilization moieties [185]. The convertible nucleoside approach has been extended to RNA [186, 187] and peptide nucleic acids (PNA) [188] molecules.

#### 4.3.1 Pyrimidine convertible nucleosides

One of the first convertible nucleoside was the 4(1,2,4-triazolyl)thymidine [179, 189]. The phosphoramidite derivative (Figure 4.13) can be easily obtained by reaction of the thymidine phosphoramidite with a solution of phosphoryl tris(1,2,4-triazolide) that is generated by the mixture of phosphoryl trichloride, 1,2,4-triazol and trimethylamine [179]. The highly fluorescent triazolyl-thymidine derivative can be incorporated into oligonucleotides and, after the assembly of the oligonucleotide, it can react with amines, thiol and even alcohols to yield 5-methylcytidine, 4-thioalkylthymidine and 4-O-alkylthymidine derivatives [181, 190]. The 4(1,2,4-triazolyl)thymidine phosphoramidite is relatively stable but slowly is hydrolyzed to thymidine. For this reason, the  $O^4$ -(2,4,6-trimethylphenyl) thymidine phosphoramidite (Figure 4.13) was developed [176]. This steric hindered phenyl derivative was even stable to mild deprotection conditions generating oligonucleotides carrying  $O^4$ -(2,4,6-trimethylphenyl) thymidine residues that were reacted with amino tethers to generate site-specific modified nucleobases for further derivatization [176].

The corresponding 4-(1,2,4-triazolyl)-2'-deoxyuridine phosphoramidite derivative can also be prepared but the absence of the 5-methyl group makes this compound more susceptible to hydrolysis and, for this reason,  $O^4$ -ethyl-dU [183] and  $O^4$ -(4-nitrophenyl)-dU [191] were developed. The  $O^4$ -ethyl-dU phosphoramidite was found to generate  $N^{15}$ -labelled cytidine at the exocyclic amino group with better conversion yields than the triazolyl-dU derivative [183]. Moreover,  $O^4$ -(4-chlorophenyl)-uridine [186] and 4-(1,2,4-triazolyl)-uridine [187] have been developed as convertible nucleosides for the synthesis of RNA oligonucleotides.

An important development of the convertible nucleoside approach was the synthesis of oligodeoxynucleotides carrying 5-fluoro-2'-deoxycytidine (Figure 3.1) [192–

**Pyrimidine convertible nucleosides****Purine convertible nucleosides****RNA convertible nucleosides**

**Figure 4.13:** Chemical structures of convertible nucleosides.

195] and 5-cloro-2'-deoxycytidine [196] explained in Chapter 3. These derivatives are important suicidal inhibitors of methyltransferases and the convertible nucleoside approach was successfully used for the incorporation of these modified nucleobases into oligonucleotides.

### 4.3.2 Purine convertible nucleosides

A large variety of convertible nucleosides have been described for the generation of adenine derivatives. These include 6-methylmercaptopurine, 6-chloro- and 6-fluoropurine-2'-deoxyribosides as well as  $O^6$ -phenyl- and  $O^6$ -pentafluorophenyl-hypoxanthine 2'-deoxyribosides (Figure 4.13). A comparison between some of these derivatives for the introduction of  $^{15}N$ -labels at the exocyclic amino group of 2'-deoxyadenosine selected the  $O^6$ -pentafluorophenyl-2'-deoxyinosine because of being less prone to hydrolysis during  $^{15}N$ -ammonia treatment [183]. This derivative was also used for the introduction of  $^{13}C$ -methylamine to generate  $^{13}C$ -labeled methyladenine [184].  $O^6$ -Phenyl-2'-deoxyinosine phosphoramidite has been used for the generation of

disulfide crosslinked oligonucleotides [197, 198] and the preparation of oligonucleotides carrying 6-hystaminylpurine for metal affinity chromatography [199]. Xu et al. used 6-methylmercaptopurine phosphoramidite [178, 200] for the generation of  $^{15}\text{N}$ -labeled adenine oligodeoxynucleotides [201]. As described in Chapter 2, 6-chloro- and 6-fluoropurine-2'-deoxyribosides have been used in the preparation of oligonucleotides carrying bulky carcinogenic compounds linked to adenine using postsynthetic methods [202–206].  $O^4$ -(4-chlorophenyl)-inosine phosphoramidite (Figure 4.13) [186] was developed as convertible nucleoside for the synthesis of RNA oligonucleotides.

The introduction of modifications in position 2 of guanine is done by the use of 2-fluorohypoxanthine derivatives (Figure 4.13). The preparation of 2-fluorohypoxanthine derivatives needs the protection of position 6 of guanine. The most common 2-fluorohypoxanthine convertible nucleosides are the 2-fluoro- $O^6$ -(4-nitrophenylethyl(NPE))-2'-deoxyinosine (NPE-FdI) [183, 184] and the RNA equivalent [186] for RNA oligonucleotides (Figure 4.13). A large number of modified oligodeoxynucleotides have been generated using NPE-FdI including the selective labeling of guanine residues at the exocyclic amino group with  $^{15}\text{N}$  [183] and  $^{13}\text{C}$  [184] labels for NMR studies, the addition of duplex stabilization groups [185, 207–209], and photoreactive azido groups [210], and the preparation of guanine  $N^2$ -bulky adducts for DNA repair studies [211–213]. A similar synthon for the preparation of oligonucleotides carrying adducts at the  $N^2$ -position of guanine is 2-fluoro- $O^6$ -(trimethylsilylethyl)-2'-deoxyinosine [214]. This nucleoside is stable to synthesis conditions, but the protection of position  $O^6$  is removed under very mild conditions after displacement of the 2-fluoro group. 2-Fluoropurine 2'-deoxyribosine phosphoramidite has been also used for the preparation of oligonucleotides carrying  $^{15}\text{N}$ -labeled 2-aminopurine [183] as mentioned in Chapter 3. Finally,  $N^2$ -trifluoroacetyl- $O^6$ -(pentafluorophenyl)-2'-deoxyguanosine has been reported as a valuable synthon for the generation of 2,6-diaminopurine derivatives both for nucleosides and for the post-synthetic modification of oligonucleotides [215].

The use of the  $O^6$ -(2,4-dinitrophenyl) derivative of guanine [182] allows the generation of guanine modified nucleobases at position 6 including 6-thioguanine, 2,6-diaminopurine,  $N^6$ -alkyl-2,6-diaminopurine,  $O^6$ -alkylguanines [216] and  $S^6$ -alkylthioguanine [182].

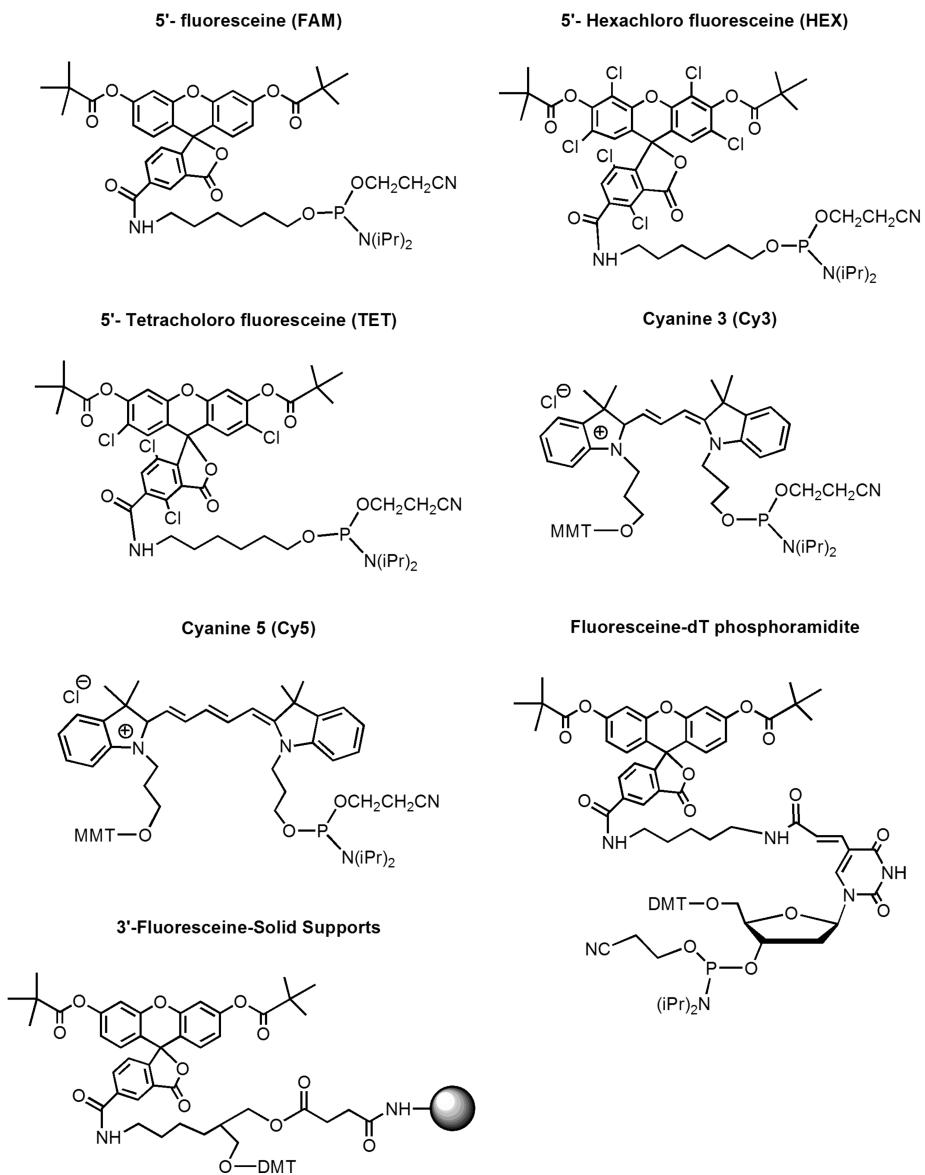
## 4.4 Reagents for fluorescent labeling

The large effort in DNA sequencing required in the Human Genome Project triggered a large interest in fluorescently-labeled oligonucleotides [11]. At the beginning DNA sequencing required a large number of sequencing primers that were fluorescently-labeled at the 5'-end and they were usually coded with four different fluorescent dyes

in order to distinguish each one of the four dideoxynucleotide enzymatic reactions. The first 5'-fluorescently labeled primers were obtained by reaction of the amino-oligonucleotides with fluorescent compounds functionalized with amino-reactive groups such as active esters, acid chlorides, acid sulfonyl chlorides or isothiocyanates [23]. Soon dedicated phosphoramidites for the direct incorporation of the fluorescent labels at the 5'-end of oligonucleotides were developed (Figure 4.14) [217–220]. The selected fluorescent dyes and their linker derivatives were selected for their emission wavelength as well as for their stability to the ammonia solution used during deprotection conditions. These derivatives were enormously useful in the DNA sequencing projects until the DNA sequencing methods changed to new methodologies in where fluorescent labels were added to the nucleobases of the dideoxynucleotides [220] eliminating the need for 5'-fluorescently labeled sequencing primers. Nevertheless, the availability of fluorescent phosphoramidites increased the number of applications for fluorescently labeled oligonucleotides including the determination of the cellular uptake of therapeutic oligonucleotides [221], the detection of mutations [222] and the development of exquisite sensors to measure the enzymatic activity of important proteins [223].

The data provided from the human genome sequencing project and the development of PCR triggered a large development of new types of fluorescently-labeled oligonucleotide probes for the detection and quantification of nucleic acids. Real-time PCR [224] appeared as one of the most efficient ways to quantify gene expression [225], and for the detection of pathogens [226]. In addition to the regular PCR primers, real-time PCR developed a new type of fluorescently-labeled oligonucleotide known as Taqman probes. These probes contain one fluorophore at the 5'-end and a quencher at the 3'-end that quenches the fluorescence of the 5'-fluorescent dye if the probe is intact. During PCR amplification, the exonuclease activity of the DNA polymerases digests the Taqman probe separating fluorophore and quencher with the subsequent increase of the sample fluorescence. Figure 4.15 shows some examples of the most common reagents used as quenchers such as; dabsyl, dabcyl, black hole, blueberry quenchers [227, 228] for the preparation of Taqman probes. Similar probes can also be used for the detection of DNA hybridization and point mutations as well as for the detection of conformational changes [229] or enzymatic reactions on nucleic acids such as DNA demethylation [230]. These probes that are not digested by the exonuclease activity of DNA polymerase are known as molecular beacons or scorpion probes [231] and they have a tremendous use in the biomedical field [223].

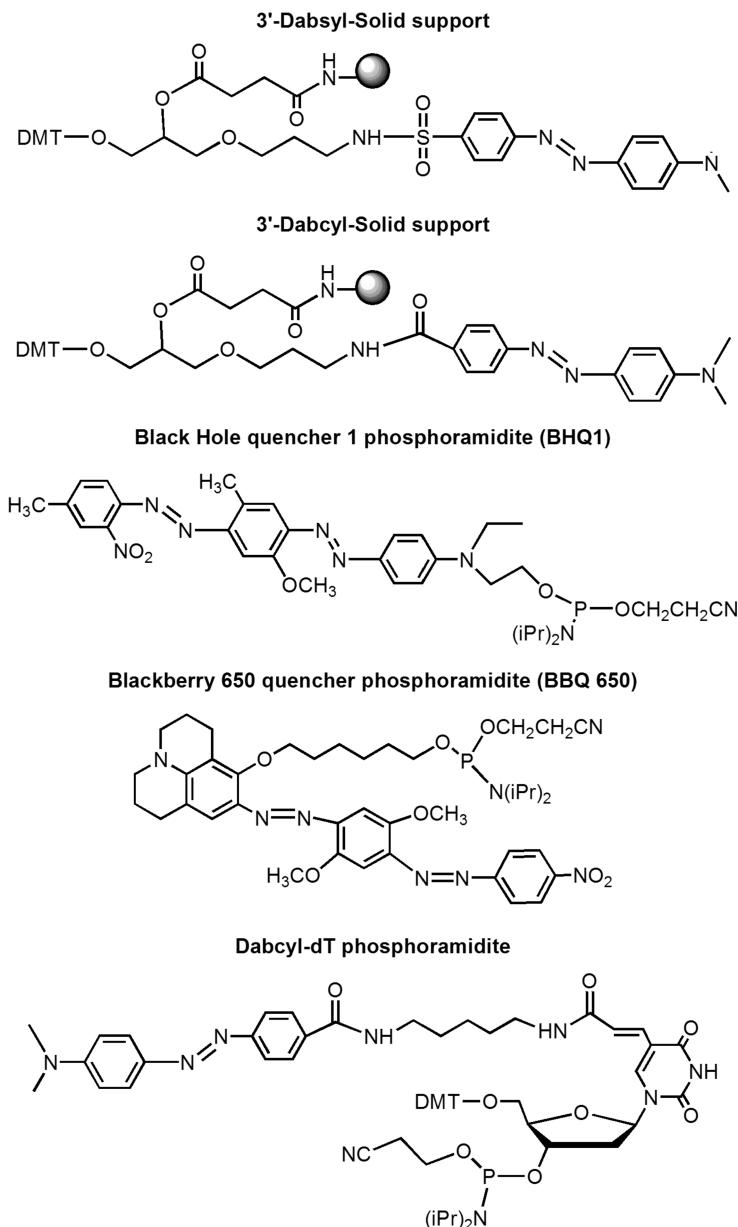
In addition to the reagents for fluorescent-labeling using nonnucleosidic linkers, there has been a large development of nucleoside phosphoramidites carrying fluorescent labels at the nucleotide residue including the nucleobase or the 2'-OH position of ribonucleotides. Figure 4.16 shows some examples described in the bibliography. Most of the labels are introduced at position 5 of 2'-deoxyuridine because this position is not involved in base pairing and the precursor nucleosides 5-bromo- or 5-iodo-2'-deoxyuridine are available [219, 232, 233]. Cytidine derivatives are usually functional-



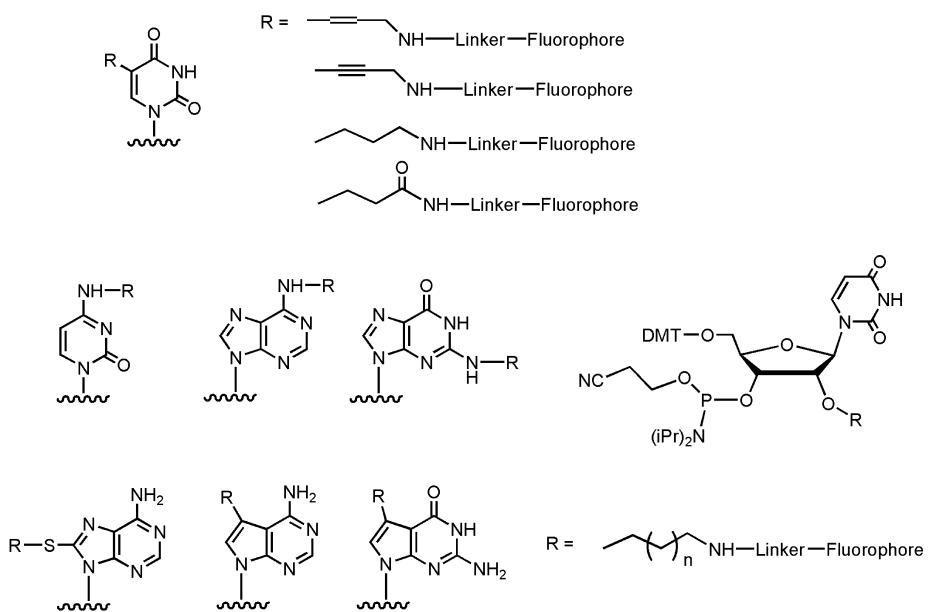
**Figure 4.14:** Chemical structures of some of the reagents described for the preparation of fluorescently labeled oligonucleotides.

ized at the exocyclic amino group [219, 234, 235]. In the same way, purine nucleosides can be functionalized through the exocyclic amino group [219, 236, 237] but also they were functionalized at position 8 by the reaction of the 8-bromopurine derivatives with thiols [238], or by palladium catalyzed reactions [239, 240]. The use of 7-deazapurine

derivatives has also been described [241, 242]. The introduction of the labels at this position is preferred as it does not disturb the natural base pairs. Another alternative anchoring site is the 2'-OH of a ribonucleotide [243].



**Figure 4.15:** Chemical structures of some of the reagents described for the preparation of oligonucleotides carrying quenchers.



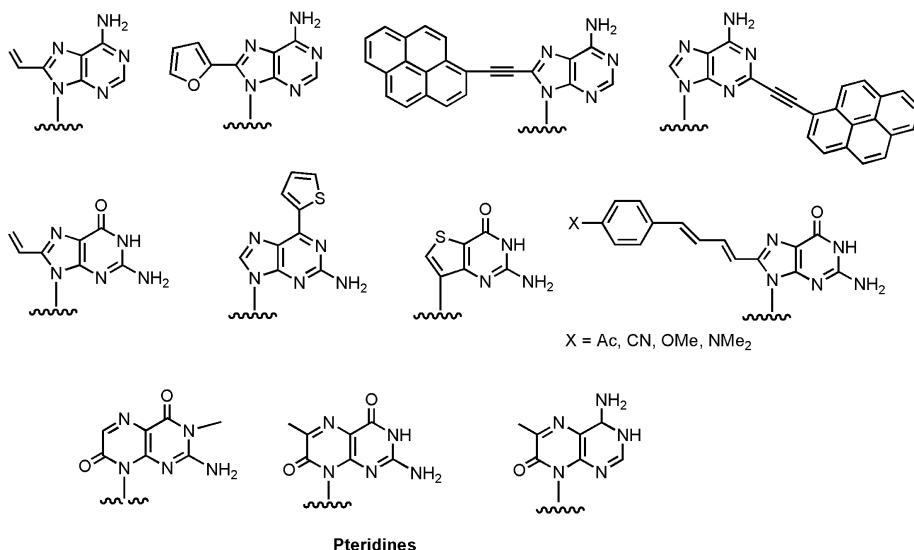
**Figure 4.16:** Chemical structures of some examples of fluorescently-labeled nucleobases.

## 4.5 Fluorescent nucleobases

An important problem in the nucleic acid field is the development of biochemical tools for the study of conformational changes on DNA, RNA as well as nucleic acids-protein complexes. Structural information on these large biomolecules can be obtained using chemically modified nucleobases that are fluorescent acting as local probes. Conformational changes can be monitored by analyzing changes in their fluorescent properties associated with the conformational changes. During the last 50 years, hundreds of fluorescent nucleobases have been developed; some of these have been described in the previous chapter. Here, we will describe some of these specialized reagents together with some of the most relevant applications. Several specialized reviews have been described [244–252].

2-Aminopurine (Chapter 3, Section 3.1.2) is one of the oldest [253] and most widely used fluorescent probes for DNA structural studies [254] as well as a local probe for DNA-binding enzymes [255]. This nucleobase is isomeric to adenine and has the ability to form Watson–Crick base pairs. Other isomeric fluorescent nucleobases are 5-methyl-2-pyrimidinone and 2-pyrimidinone described in Section 3.6.1.

Pteridines are naturally occurring fluorescent compounds similar to purines but they contain two six-member rings [256, 257] (Figure 4.17). They are excited at 300 nm and they have good quantum yields. The incorporation into oligonucleotides can in-



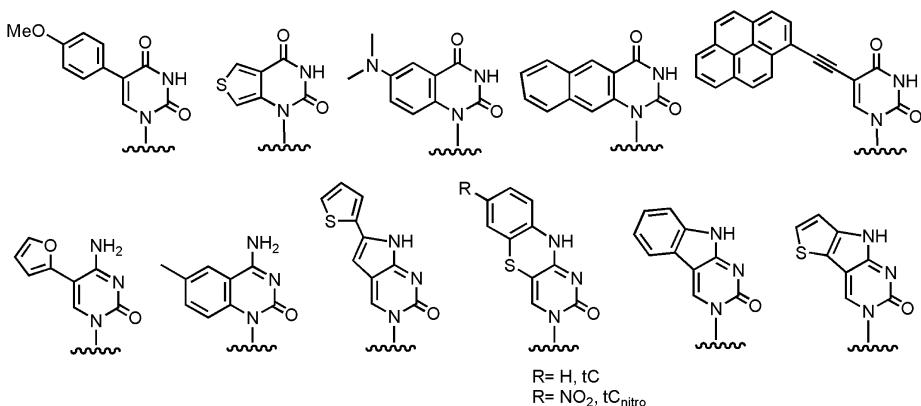
**Figure 4.17:** Chemical structures of some of fluorescent nucleobases derived from purine nucleosides.

duce either a destabilization [258] or a slight increase of the duplex structure [259, 260].

The addition of aromatic hydrocarbons such as; phenyl, furane, thiophene or pyrene to the positions not involved in Watson–Crick base pairing generates highly fluorescent nucleotides with the capacity to maintain stable duplex structures. Figure 4.17 shows some examples of purine nucleosides. Pyrimidine derivatives are shown in Figure 4.18. Pyrenylethynyl groups have been introduced at position 5 of uracil (Figure 4.18) as well as at positions 2 and 8 of adenine (Figure 4.17) [261, 262]. These derivatives present strong stacking interaction and exhibit different emission intensities when they form duplexes in where both nucleobases are in opposite sites. In addition, oligonucleotides carrying a 5-pyrenylethynyl-dU-T base pair present strong fluorescent properties in the presence of mercury ions that can be used for sensing mercury ions in water [263]. 1-Ethynylpyrene-modified guanine (Figure 4.17) and cytosine have also been described and used as optical labels for detecting DNA hybridization [264]. An important number of fluorescent nucleobases are guanine derivatives carrying conjugated aromatic systems at position 8 such as C-linked 8-aryl guanine (Figure 4.17) [265] and arylethynyl guanine derivatives [266]. Thiophene groups at position 6 of guanine have been also described as nucleoside triphosphates for the enzymatic incorporation of fluorescent tags [267]. Furan decorated nucleosides have been demonstrated to be excellent fluorescent probes as they form stable duplexes with complementary sequences and have powerful fluorescent properties [268, 269]. In an interesting work, an oligonucleotide that can form an intramolecular

quadruplex is labeled at the 3' and 5'-ends with two distinct 8-arylguanine derivatives one having a furan group and the other a thiophene group at position 8. In this combination one guanine derivative acts as a chromophore and the other acts as a quencher providing excellent sensing of quadruplex formation [270]. The addition of a simple vinyl group to the 8 position of guanine and adenine (Figure 4.17) generates fluorescent nucleosides with excellent quantum yields [271, 272]. The replacement of the carbon 8 with a nitrogen generates the fluorescent 8-aza-2'-deoxyguanosine nucleoside that has been used for mismatch discrimination [273]. The isomer 8-aza-2'-deoxyisoguanosine is also fluorescent and it can be successfully used to replace a protonated C in the Hoogsteen position of a parallel triplex [274].

Another group of fluorescent chromophores are the nucleobases formed by the addition of several fused rings. Some of these extended nucleobases have been described in Chapter 3, Sections 3.4.2 and 3.5.1. A thiophene ring fused in the position 5 and 6 of pyrimidine generates a highly emissive nucleoside thieno[3,4-*d*]pyrimidine (Figure 4.18) that have been enzymatically incorporated to RNA [275] as well as an isomeric C-nucleoside derivative [276]. The fusion of an aminophenyl group at the same position generates 7-aminoquinazoline-2,4-(1H,3H)-dione (Figure 4.18) that has the particularity of having a two-fold enhancement of fluorescence emission in duplexes in front of guanine [277]. Several fused cytosine derivatives such as; naphtopyridopyrimidine [278], indole-fused cytosine [279], 6-phenylpyrrolo-dC and 5,6-benzopyrrol-dC (Figure 4.18) [280] have been studied. Although the most used fluorescent analog is the tricycloC (tC) or 1,3-diaza-2-oxophenothiazine [281] and its nitro derivative tC<sub>nitro</sub> (Figure 4.18) [282–284]. Oligonucleotides carrying naphthodeazaadenine were found to be highly fluorescent when paired with cytosine but not fluorescent at all when paired with other nucleobases [285]. Another extended adenine derivative with high fluorescent properties was prepared by Petra et al. [286].



**Figure 4.18:** Chemical structures of some of fluorescent nucleobases derived from pyrimidine nucleosides.

Finally, an important strategy to incorporate fluorescence properties to the oligonucleotides is the addition of known fluorophores such as chromone and polycyclic aromatic hydrocarbons (pyrene, perylene, anthracene) as synthetic fluorescent nucleosides [287]. These compounds, described in Chapter 3, Section 3.4.1, do not participate in Watson–Crick base pairing but they have excellent quantum yields and they have been employed in fluorescence emission [288], fluorescence quenching [289] and detection of abasic sites [290] and  $O^6$ -alkylated guanines [291].

## Bibliography

- [1] Mullis K, Falona F, Scharf S, Saiki R, Horn G, Erlich H. Specific-enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symp Quant Biol.* 1986;51:263–73.
- [2] Goodchild J. Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. *Bioconjug Chem.* 1990;1:165–8.
- [3] Winkler J. Oligonucleotide conjugates for therapeutic applications. *Ther Deliv.* 2013;4:791–809.
- [4] Grijalvo S, Alagia A, Jorge AF, Eritja R. Covalent strategies for targeting messenger and non-coding RNAs. An updated review on siRNA, miRNA and antimiR conjugates. *Genes.* 2018;9:74.
- [5] Lönnberg H. Solid-phase synthesis of oligonucleotide conjugates useful for delivery and targeting of potential nucleic acid therapeutics. *Bioconjug Chem.* 2009;20:1065–94.
- [6] Šípová H, Homola J. Surface plasmon resonance sensing of nucleic acids: a review. *Anal Chim Acta.* 2013;773:9–23.
- [7] Pirlung MC. How to make a DNA chip. *Angew Chem, Int Ed Engl.* 2002;41:1276–89.
- [8] Agrawal S. Protocols for oligonucleotide conjugates. In: *Synthesis and analytical techniques.* Meth Mol Biol. vol. 26. Totowa, NJ: Humana Press Inc.; 1994.
- [9] Beaucage SL, Iyer RP. The functionalization of oligonucleotides via phosphoramidite derivatives. *Tetrahedron.* 1993;49:1925–63.
- [10] Eckstein F. Oligonucleotides and analogues. A practical approach. Oxford: IRL Press; 1991.
- [11] Davies MJ, Shah A, Bruce IJ. Synthesis of fluorescently labelled oligonucleotides and nucleic acids. *Chem Soc Rev.* 2000;29:97–107.
- [12] Cook AF, Vuocolo E, Brakel CL. Synthesis and hybridization of a series of biotinylated oligonucleotides. *Nucleic Acids Res.* 1988;16:4077–95.
- [13] Manoharan M, Johnson LK, Bennett CF, Vickers TA, Ecker DJ, Cowser LM, Freier SM, Can Cook P. Cholic acid-oligonucleotide conjugates for antisense applications. *Bioorg Med Chem Lett.* 1994;4:1053–60.
- [14] Balakin KV, Korshun VA, Mikhalev II, Maleev GV, Malakhov AD, Prokhorenko IA, Berlin YA. Conjugates of oligonucleotides with polyaromatic fluorophores as promising DNA probes. *Biosens Bioelectron.* 1998;13:771–8.
- [15] Pérez-Rentero S, Kielland N, Terrazas M, Lavilla R, Eritja R. Synthesis and properties of oligonucleotides carrying isoquinoline imidazo[1,2-a]azine fluorescent units. *Bioconjug Chem.* 2010;21:1622–8.
- [16] Guzaev A, Salo H, Azhayev A, Lönnberg H. Novel non-nucleosidic building blocks for the preparation of multilabeled oligonucleotides. *Bioconjug Chem.* 1996;7:240–8.

- [17] Manoharan M, Guinoss CJ, Dan Cook PD. Novel functionalization of the sugar moiety of nucleic acids for multiple labeling in the minor groove. *Tetrahedron Lett.* 1991;32:7171–7.
- [18] Eritja R. Synthesis and properties of oligonucleotides carrying cryptolepine derivatives. *Chem Biodivers.* 2004;1:289–95.
- [19] Tort N, Salvador JP, Aviñó A, Eritja R, Comelles J, Martínez E, Samitier J, Marco MP. Synthesis of steroid-oligonucleotide conjugates for a site-encoded SPR immunosensor. *Bioconjug Chem.* 2012;23:2183–91.
- [20] Vinayak R. A convenient, solid-phase coupling of rhodamine dye acids to 5' amino-oligonucleotides. *Tetrahedron Lett.* 1999;40:7611–3.
- [21] Prakash TP, Mullick AE, Lee RG, Yu J, Yeh ST, Low A, Chappell AE, Østergaard ME, Murray S, Gaus HJ, Swayze EE, Seth PP. Fatty acid conjugation enhances potency of antisense oligonucleotides in muscle. *Nucleic Acids Res.* 2019;47:6029–44.
- [22] Østergaard ME, Jackson M, Low A, Chappell AE, Lee RG, Peralta RQ, Yu J, Kinberger GA, Dan A, Carty R, Tanowitz M, Anderson P, Kim TW, Fradkin L, Mullick AE, Murray S, Rigo F, Prakash TP, Bennett CF, Swayze EE, Gaus HJ, Seth PP. Conjugation of hydrophobic moieties enhances potency of antisense oligonucleotides in the muscle of rodents and non-human primates. *Nucleic Acids Res.* 2019;47:6045–58.
- [23] Smith LM, Fung S, Hunkapiller MW, Hunkapiller TJ, Hood LE. The synthesis of oligonucleotides containing an aliphatic amino group at the 5' terminus: synthesis of fluorescent DNA primers for use in DNA sequence analysis. *Nucleic Acids Res.* 1985;13:2399–412.
- [24] Smith LM, Kaiser TJ, Sanders JZ, Hood LE. The synthesis and use of fluorescent oligonucleotides in DNA sequence analysis. *Methods Enzymol.* 1987;155:260–301.
- [25] Sproat BS, Beijer B, Rider P. The synthesis of protected 5'-amino-2',5'-dideoxyribonucleoside-3'-O-phosphoramidites; applications of 5'-amino-oligodeoxy-ribonucleotides. *Nucleic Acids Res.* 1987;15:6181–96.
- [26] Landegren U, Kaiser R, Caskey CT, Hood L. DNA diagnostics—molecular techniques and automation. *Science.* 1988;242:229–37.
- [27] Connolly BA. The synthesis of oligonucleotides containing a primary amino group at the 5'-terminus. *Nucleic Acids Res.* 1987;15:3131–9.
- [28] Kaiser RJ, MacKellar SL, Vinayak RS, Sanders JZ, Saavedra RA, Specific-primer-directed HLH. DNA sequencing using automated fluorescence detection. *Nucleic Acids Res.* 1989;17:6087–102.
- [29] Eritja R, Johnson D, Ziehler-Martin P, Walker PA, Kaplan BE. Estudio de la detección de oligonucleótidos marcados con compuestos fluorescentes utilizando la quimioluminiscencia de los ésteres del ácido oxálico. *An Quím.* 1989;85C:80–4.
- [30] Agrawal S, Christodoulou C, Gait MJ. Efficient methods for attaching non-radioactive labels to the 5' ends of synthetic oligodeoxyribonucleotides. *Nucleic Acids Res.* 1986;14:6227–45.
- [31] Sinha ND, Cook RM. The preparation and application of functionalised synthetic oligonucleotides: III. Use of H-phosphonate derivatives of protected amino-hexanol and mercapto-propanol or-hexanol. *Nucleic Acids Res.* 1988;16:2659–69.
- [32] De Vos MJ, Cravador A, Lenders JP, Houard S, Bollen A. Solid-phase non isotopic labelling of oligodeoxynucleotides using 5'-protected aminoalkyl phosphoramidites: application to the specific detection of human papilloma virus DNA. *Nucleosides Nucleotides.* 1990;9:259–73.
- [33] Kojima N, Takebayashi T, Mikami A, Ohtsuka E, Komatsu Y. Efficient synthesis of oligonucleotide conjugates on solid-support using an (aminoethoxycarbonyl)aminohexyl group for 5'-terminal modification. *Bioorg Med Chem Lett.* 2009;19:2144–7.
- [34] Komatsu Y, Kojima N, Sugino M, Mikami A, Nonaka K, Fujinawa Y, Sugimoto T, Sato K, Matsubara K, Ohtsuka E. Novel amino linkers enabling efficient labeling and convenient purification of amino-modified oligonucleotides. *Bioorg Med Chem.* 2008;16:941–9.

- [35] Coull JM, Weith HL, Bischoff R. A novel method for the introduction of an aliphatic primary amino group at the 5' terminus of synthetic oligonucleotides. *Tetrahedron Lett.* 1986;27:3991–4.
- [36] Zaramella S, Yeheskiely E, Strömberg R. A method for solid-phase synthesis of oligonucleotide 5'-peptide-conjugates using acid-labile  $\alpha$ -amino protections. *J Am Chem Soc.* 2004;126:14029–35.
- [37] Ocampo SM, Albericio F, Fernández I, Vilaseca M, Eritja R. A straightforward synthesis of 5'-peptide oligonucleotide conjugates using N $^{\alpha}$ -Fmoc-protected amino acids. *Org Lett.* 2005;7:4349–52.
- [38] Kansal VK, Huynh-Dinh T, Igolen J. Synthesis of oligodeoxyribonucleotides containing 5'-aminoalkylphosphonates. *Tetrahedron Lett.* 1988;29:5537–40.
- [39] Pitsch S, Berger S. Protected linker compounds. US patent 9.534.003, Jan 3, 2017.
- [40] Nelson PS, Sherman-Gold R, Leon R. A new and versatile reagent for incorporating multiple primary aliphatic amines into synthetic oligonucleotides. *Nucleic Acids Res.* 1989;17:7179–86.
- [41] Nelson PS, Kent M, Muthini S. Oligonucleotide labeling methods 3. Direct labeling of oligonucleotides employing a novel, non-nucleosidic, 2-aminobutyl-1,3-propanediol backbone. *Nucleic Acids Res.* 1992;20:6253–9.
- [42] Vu H, Joyce N, Rieger M, Walker D, Goldknopf I, Hill TS, Jayaraman K, Dennis Mulvey D. Use of phthaloyl protecting group for the automated synthesis of 3'-[(hydroxypropyl) amino] and 3'-[(hydroxypropyltriglycyl) oligonucleotide conjugates. *Bioconjug Chem.* 1995;6:599–607.
- [43] Aviñó A, Güimil-Garcia R, Albericio F, Mann M, Wilm M, Neubauer G, Eritja R. New carbamate supports for the preparation of 3'-amino-modified oligonucleotides. *Bioorg Med Chem.* 1996;4:1649–58.
- [44] Behrens C, Dahl O. Synthesis of achiral linker reagents for direct labelling of oligonucleotides on solid supports. *Nucleosides Nucleotides.* 1999;18:291–305.
- [45] Sawai H, Nakamura A, Sekiguchi S, Yumoto K, Endoh M, Ozaki H. Efficient synthesis of the new 5-substituted uracil nucleosides useful for linker arm incorporation. *J Chem Soc, Chem Commun.* 1994;1997–8.
- [46] Bergstrom DE, Ruth JL. Synthesis of C-5 substituted pyrimidine nucleosides via organopalladium intermediates. *J Am Chem Soc.* 1976;98:1587–9.
- [47] Olejnik J, Krzymanska-Olejnik E, Rothschild KJ. Photocleavage aminotag phosphoramidites for 5'-termini DNA/RNA labeling. *Nucleic Acids Res.* 1998;26:3572–6.
- [48] Eritja R, Pons A, Escarceller M, Giralt E, Albericio F. Synthesis of defined peptide-oligonucleotide hybrids containing a nuclear transport signal sequence. *Tetrahedron.* 1991;47:4113–20.
- [49] Aviñó A, Unzueta U, Cespedes MV, Casanova I, Vázquez E, Villaverde A, Mangues R, Eritja R. Efficient bioactive oligonucleotide-protein conjugation for cell-targeted cancer therapy. *ChemistryOpen.* 2019;8:382–7.
- [50] Sproat BS, Beijer B, Rider P, Neuner P. The synthesis of protected 5'-mercapto-2',5'-dideoxyribonucleoside-3'-O phosphoramidites; uses of 5'-mercapto-oligodeoxyribonucleotides. *Nucleic Acids Res.* 1987;15:4837–48.
- [51] Connolly BA, Rider P. Chemical synthesis of oligonucleotides containing a free sulphhydryl group and subsequent attachment of thiol specific probes. *Nucleic Acids Res.* 1985;13:4485–502.
- [52] Ansorge W, Rosenthal A, Sproat B, Schwager C, Stegemann J, Voss H. Non-radioactive automated sequencing of oligonucleotides by chemical degradation. *Nucleic Acids Res.* 1988;16:2203–6.

- [53] De la Torre B, Aviñó A, Escarceller M, Royo M, Albericio F, Eritja R. Use of a base labile protected derivative of 6-mercaptophexanol for the preparation of oligonucleotides containing a thiol group at the 5'-end. *Nucleosides Nucleotides*. 1993;12:993–1005.
- [54] Ede NJ, Tregear GN, Haralambidis J. Routine preparation of thiol oligonucleotides: application to the synthesis of oligonucleotide-peptide hybrids. *Bioconjug Chem*. 1994;5:373–8.
- [55] Kiupers WHA, van Boeckel CAA. A new strategy for the solid-phase synthesis of 5'-thiolated oligodeoxynucleotides. *Tetrahedron*. 1993;49:10931–44.
- [56] Glick GD. Synthesis of a conformationally restricted DNA hairpin. *J Org Chem*. 1991;56:6746–7.
- [57] Takada T, Kawano Y, Nakamura M, Tamana K. Photo-triggered generation of a free thiol group on DNA: application to DNA conjugation. *Tetrahedron Lett*. 2012;53:78–81.
- [58] Salo H, Guzaev A, Lönnberg H. Disulfide-tethered solid supports for synthesis of photoluminescent oligonucleotide conjugates: hydrolytic stability and labeling on the support. *Bioconjug Chem*. 1998;9:365–71.
- [59] Dubey IY, Fedoryak DM. Synthesis and use of disulfide-based H-phosphonate reagent for 3'- and/or 5'-oligonucleotide labelling via mercaptoalkyl linker. *Biopolym Chem*. 1998;14:163–72.
- [60] Zimmermann JL, Nicolaus T, Neuert G, Blank K. Thiol-based, site-specific and covalent immobilization of biomolecules for single-molecule experiments. *Nat Protoc*. 2010;5:975–85.
- [61] Gottschling D, Seliger H, Tarrasón G, Piulats J, Eritja R. Synthesis of oligodeoxynucleotides containing *N*<sup>4</sup>-mercaptoethylcytosine and their use in the preparation of oligonucleotide-peptide conjugates carrying c-myc tag sequence. *Bioconjug Chem*. 1998;9:831–7.
- [62] Manning B, Pérez-Rentero S, Garibotti AV, Ramos R, Eritja R. Modified oligonucleotides for biosensing applications. *Sens Lett*. 2009;7:774–81.
- [63] Pérez-Rentero S, Garibotti AV, Eritja R. Solid-phase synthesis of oligodeoxynucleotides carrying *N*<sup>4</sup>-[2-(*t*-butyldisulfanyl)ethyl]-5-methylcytosine. *Molecules*. 2010;15:5692–707.
- [64] Garibotti AV, Pérez-Rentero S, Eritja R. Functionalization and self-assembly of DNA bidimensional arrays. *Int J Mol Sci*. 2011;12:5641–51.
- [65] Pérez-Rentero S, Grijalvo S, Ferreira R, Eritja R. Synthesis of oligonucleotides carrying thiol groups using a simple reagent derived from threoninol. *Molecules*. 2012;17:10026–45.
- [66] Alivisatos AP, Johnsson KP, Peng X, Wilson TE, Loweth CJ, Bruchez MP Jr, Schultz PG. Organization of ‘nanocrystal molecules using DNA. *Nature*. 1996;382:609–11.
- [67] Mirkin CA, Letsinger RL, Mucic RC, Storhoff JJ. A DNA-based method for rationally assembling nanoparticles into macroscopic materials. *Nature*. 1996;1996(382):607–9.
- [68] Elghanian R, Storhoff JJ, Mucic RC, Letsinger RL, Mirkin CA. Selective colorimetric detection of polynucleotides based on the distance-dependent optical properties of gold nanoparticles. *Science*. 1997;277:1078–81.
- [69] Rana S, Bajaj A, Mout R, Rotello VM. Monolayer coated gold nanoparticles for delivery applications. *Adv Drug Deliv Rev*. 2012;64:200–16.
- [70] Hu R, Zhang X-B, Kong R-M, Zhao X-H, Jiang J, Tan W. Nucleic acid-functionalized nanomaterials for bioimaging applications. *J Mater Chem*. 2011;21:16323–34.
- [71] De la Torre BG, Morales JC, Aviñó A, Iacopino D, Ongaro A, Fitzmaurice D, Murphy D, Doyle H, Redmond G, Eritja R. Synthesis of oligonucleotides carrying anchoring groups and their use in the preparation of oligonucleotide-gold conjugates. *Helv Chim Acta*. 2002;85:2594–607.
- [72] Dougan JA, Reid AK, Graham D. Thiocctic acid modification of oligonucleotides using an H-phosphonate. *Tetrahedron Lett*. 2010;51:5787–90.

- [73] Dougan JA, Karlsson C, Smith WE, Graham D. Enhanced oligonucleotide-nanoparticle conjugate stability using thioctic acid modified oligonucleotides. *Nucleic Acids Res.* 2007;35:3668–75.
- [74] Pérez-Rentero S, Grijalvo S, Peñuelas G, Fàbrega C, Eritja R. Thioctic acid derivatives as building blocks to incorporate DNA oligonucleotides onto gold nanoparticles. *Molecules.* 2014;19:10495–523.
- [75] Tatulchenkov MY, Prokhorenko IA, Kvach MV, Navakouski ME, Stepanova IA, Pilchenko NV, Gontarev SV, Sharko OL, Korshun VA, Shmanai VV. Phosphoramidite reagents and solid-phase supports based on hydroxyprolinol for the synthesis of modified oligonucleotides. *Russ J Bioorg Chem.* 2017;43:386–96.
- [76] Letsinger RL, Elghanian R, Viswanadham G, Mirkin CA. Use of a steroid cyclic disulphide anchor in constructing gold nanoparticle-oligonucleotide conjugates. *Bioconjug Chem.* 2000;11:289–91.
- [77] Sánchez A, Pedroso E, Grandas A. Maleimide-dimethylfuran exo adducts: effective maleimide protection in the synthesis of oligonucleotide conjugates. *Org Lett.* 2011;13:4364–7.
- [78] Sánchez A, Pedroso E, Grandas A. Easy introduction of maleimides at different positions of oligonucleotide chains for conjugation purposes. *Org Biomol Chem.* 2012;10:8478–83.
- [79] Sánchez A, Pedroso E, Grandas A. Oligonucleotide cyclization: the thiol-maleimide reaction revisited. *Chem Commun.* 2013;49:309–11.
- [80] Sánchez A, Pedroso E, Grandas A. Conjugation reactions involving maleimides and phosphorothioate oligonucleotides. *Bioconjug Chem.* 2012;23:300–7.
- [81] Paris C, Brun O, Pedroso E, Grandas A. Exploiting protected maleimides to modify oligonucleotides, peptides and peptide nucleic acids. *Molecules.* 2015;20:6389–408.
- [82] Kremsky JN, Wooters JL, Dougherty JP, Meyers RE, Collins M, Brown EL. Immobilization of DNA via oligonucleotides containing an aldehyde or carboxylic acid group at the 5' terminus. *Nucleic Acids Res.* 1987;15:2891–909.
- [83] Kachalova AV, Stetsenko DA, Romanova EA, Tashlitsky VN, Gait MJ, Oretskaya TS. A new and efficient method for synthesis of 5'-conjugates of oligonucleotides through amide-bond formation on solid phase. *Helv Chim Acta.* 2002;85:2409–16.
- [84] Hovinen J, Guzaev A, Azhayev A, Lönnberg H. Novel non-nucleosidic phosphoramidite building blocks for versatile functionalization of oligonucleotides at primary hydroxy groups. *J Chem Soc, Perkin Trans I.* 1994;2745.
- [85] Lebedev AV, Combs D, Hogrefe RI. Preactivated carboxyl linker for the rapid conjugation of alkylamines to oligonucleotides on solid support. *Bioconjug Chem.* 2007;18:1530–6.
- [86] Jonklaas MD, Kane RR. The synthesis of 3'- and 5'-iminodiacetic acid derivatives of thymidine and their incorporation into synthetic oligonucleotides. *Tetrahedron Lett.* 2000;41:4035–7.
- [87] Yoo DJ, Greenberg MM. Synthesis of oligonucleotides containing 3'-alkyl carboxylic acids using universal, photolabile solid phase synthesis supports. *J Org Chem.* 1995;60:3358–64.
- [88] Kahl JD, Greenberg MM. Solution-phase bioconjugate synthesis using protected oligonucleotides containing 3'-alkyl carboxylic acids. *Bioconjug Chem.* 1999;64:507–10.
- [89] Hovinen J, Guzaev A, Azhayev A, Lönnberg H. Novel solid supports for the preparation of 3'-derivatized oligonucleotides: introduction of 3'-alkylphosphate tether groups bearing amino, carboxy, carboxamido, and mercapto functionalities. *Tetrahedron.* 1994;50:7203–18.
- [90] Allabush F, Mendes PM, Tucker JHR. Acrylamide-dT: a polymerisable nucleoside for DNA incorporation. *RSC Adv.* 2019;9:31511–6.
- [91] Timofeev EN, Kochetkova SV, Mirzabekov AD, Florentiev VL. Regioselective immobilization of short oligonucleotides to acrylic copolymer gels. *Nucleic Acids Res.* 1996;24:3142–8.

- [92] Jones DS, Hachmann JP, Osgood SA, Hayag MS, Barstad PA, Iverson GM, Coutts SM. Conjugates of double-stranded oligonucleotides with poly(ethyleneglycol) and keyhole limpet hemocyanin: a model for treating systemic lupus erythematosus. *Bioconjug Chem*. 1994;5:390–9.
- [93] Forget D, Boturyn D, Defrancq E, Lhomme J, Dumy P. Highly efficient synthesis of peptide–oligonucleotide conjugates: chemoselective oxime and thiazolidine formation. *Chem Eur J*. 2001;7:3976–84.
- [94] Deroo S, Defrancq E, Moucheron C, Kirsch-De Mesmaeker A, Dumy P. Synthesis of an oxyamino-containing phenanthroline derivative for the efficient preparation of phenanthroline oligonucleotide oxime conjugates. *Tetrahedron Lett*. 2003;44:8379–82.
- [95] Forget D, Renaudet O, Defrancq E, Dumy P. Efficient preparation of carbohydrate–oligonucleotide conjugates (COCs) using oxime bond formation. *Tetrahedron Lett*. 2001;42:7829–32.
- [96] Forget D, Renaudet O, Boturyn D, Defrancq E, Dumy P. 3'-Oligonucleotides conjugation via chemoselective oxime bond formation. *Tetrahedron Lett*. 2001;42:9171–4.
- [97] Singh Y, Defrancq E, Dumy P. New method to prepare peptide–oligonucleotide conjugates through glyoxylic oxime formation. *J Org Chem*. 2004;69:8544–6.
- [98] Okamoto A, Tainaka K, Saito I. A facile incorporation of the aldehyde function into DNA: 3-formylindole nucleoside as an aldehyde-containing universal nucleoside. *Tetrahedron Lett*. 2002;43:4581–83.
- [99] Achilles K, Kiedrowski GV. Kinetic model studies on the chemical ligation of oligonucleotides via hydrazone formation. *Bioorg Med Chem Lett*. 2005;15:1229–33.
- [100] Kozlov IA, Melnyk PC, Stromsborg KE, Chee MS, Barker DL, Zhao C. Efficient strategies for the conjugation of oligonucleotides to antibodies enabling highly sensitive protein detection. *Biopolymers*. 2004;73:621–30.
- [101] Trévisiol E, Renard A, Defrancq E, Lhomme J. The oxyamino-aldehyde coupling reaction: an efficient method for the derivatization of oligonucleotides. *Tetrahedron Lett*. 1997;38:8687–90.
- [102] Trévisiol E, Renard A, Defrancq E, Lhomme J. Fluorescent labelling of oligodeoxyribonucleotides by the oxyamino-aldehyde coupling reaction. *Nucleosides Nucleotides Nucleic Acids*. 2000;19:1427–39.
- [103] Kachalova AV, Stetsenko DA, Gait MJ, Oretskaya TS. Synthesis of oligonucleotide 2'-conjugates via amide bond formation in solution. *Bioorg Med Chem Lett*. 2004;14:8801–4.
- [104] Zubin EM, Stetsenko DA, Zatsepina TS, Gait MJ, Oretskaya TS. Oligonucleotides containing 2'-O-[2-(2, 3-dihydroxypropyl) amino-2-oxoethyl] uridine as suitable precursors of 2'-aldehyde oligonucleotides for chemoselective ligation. *Bioorg Med Chem*. 2005;13:4912–20.
- [105] Zatsepina TS, Stetsenko DA, Arzumanov AA, Romanova EA, Gait MJ, Oretskaya TS. Synthesis of peptide–oligonucleotide conjugates with single and multiple peptides attached to 2'-aldehydes through thiazolidine, oxime, and hydrazine linkages. *Bioconjug Chem*. 2002;13:822–30.
- [106] Zatsepina TS, Stetsenko DA, Gait MJ, Oretskaya TS. Synthesis of DNA conjugates by solid-phase fragment condensation via aldehyde–nucleophile coupling. *Tetrahedron Lett*. 2005;46:3191–5.
- [107] Tilquin JM, Dechamps M, Sonveaux E. Incorporation of an aldehyde function in oligonucleotides. *Bioconjug Chem*. 2001;12:451–7.
- [108] Zatsepina TS, Gait MJ, Oretskaya TS, Stetsenko DA. Synthesis of 2'-hydrazine oligonucleotides and their efficient conjugation with aldehydes and 1,3-diketones. *Tetrahedron Lett*. 2006;47:5515–8.

- [109] Raddatz S, Mueller-Ibeler J, Kluge J, Wass L, Burdinski G, Havens JR, Onofrey TJ, Wang D, Schweitzer M. Hydrazide oligonucleotides: new chemical modification for chip array attachment and conjugation. *Nucleic Acids Res.* 2002;30:4793–802.
- [110] Salo H, Virta P, Hakala H, Prakash TP, Kawasaki AM, Manoharan M, Lönnberg H. Aminoxy functionalized oligonucleotides: preparation, on-support derivatization, and postsynthetic attachment to polymer support. *Bioconjug Chem.* 1999;10:815–23.
- [111] Kolb HC, Finn MG, Sharpless KB. Click chemistry: diverse chemical function from a few good reactions. *Angew Chem, Int Ed Engl.* 2001;40:2004–21.
- [112] Gramlich PME, Wirges CT, Manetto A, Postsynthetic CT. DNA modification through the copper-catalyzed azide–alkyne cycloaddition reaction. *Angew Chem, Int Ed Engl.* 2008;47:8350–8.
- [113] El-Sagheerab AH, Brown T. Click chemistry with DNA. *Chem Soc Rev.* 2010;39:1388–405.
- [114] Marchan V, Ortega S, Pulido D, Pedroso E. Diels–Alder cycloadditions in water for the straightforward preparation of peptide–oligonucleotide conjugates. *Nucleic Acids Res.* 2006;34:e24.
- [115] Hill KW, Taunton-Rigby J, Carter JD, Kropp E, Vagle K, Pieken W, McGee DPC, Husar GM, Leuck M, Anziano DJ, Sebesta DP. Diels–Alder bioconjugation of diene-modified oligonucleotides. *J Org Chem.* 2001;66:5352–8.
- [116] Graham D, Grondin A, McHugh C, Fruk L, Smith WE. Internal labeling of oligonucleotide probes by Diels–Alder cycloaddition. *Tetrahedron Lett.* 2002;43:4785–8.
- [117] El-Sagheer AH, Cheong VV, Brown T. Rapid chemical ligation of oligonucleotides by the Diels–Alder reaction. *Org Biomol Chem.* 2011;9:232–5.
- [118] Huisgen R. 1,3-Dipolar cycloadditions chemistry. vol. 1. New York: Willey; 1984. p. 1–176.
- [119] Tornoe CW, Christensen C, Meldal M. Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J Org Chem.* 2002;67:3057–64.
- [120] Kanan MW, Rozenman MM, Sakurai K, Snyder TM, Liu DR. Reaction discovery enabled by DNA-templated synthesis and in vitro selection. *Nature.* 2004;431:545.
- [121] Alvira M, Eritja R. Synthesis of oligonucleotides carrying 5'-5' linkages using copper-catalyzed cycloaddition reactions. *Chem Biodivers.* 2007;4:2798–809.
- [122] Chan TR, Hilgraf R, Sharpless KB, Fokin VV. Polytriazoles as copper(I)-stabilizing ligands in catalysis. *Org Lett.* 2004;6:2853–5.
- [123] Devaraj NK, Miller GP, Ebina W, Kakaradov B, Collman JP, Kool ET, Chidsey CED. Chemoselective covalent coupling of oligonucleotide probes to self-assembled monolayers. *J Am Chem Soc.* 2005;127:8600–1.
- [124] Gierlich J, Gutsmiedl K, Gramlich PME, Schmidt A, Burley GA, Carell T. Synthesis of highly modified DNA by a combination of PCR with alkyne-bearing triphosphates and click chemistry. *Chem Eur J.* 2007;13:9486–94.
- [125] El-Sagheer AH, Kumar R, Findlow S, Werner JN, Lane AN, Brown T. A very stable cyclic DNA miniduplex with just two base pairs. *ChemBioChem.* 2008;50–2.
- [126] Wenska M, Alvira M, Steunenberg P, Stenberg A, Murtola M, Strömberg R. An activated triple bond linker enables ‘click’ attachment of peptides to oligonucleotides on solid support. *Nucleic Acids Res.* 2011;39:9047–59.
- [127] Kupryushkin MS, Konevets DM, Vasilyeva SV, Kuznetsova AS, Stetsenko DA, Pyshnyi DV. Oligonucleotide functionalization by a novel alkyne-modified nonnucleosidic reagent obtained by versatile building block chemistry. *Nucleosides Nucleotides Nucleic Acids.* 2013;32:306–19.

- [128] Gierlich J, Burley GA, Gramlich PME, Hammond DM, Carell T. Click chemistry as a reliable method for the high-density postsynthetic functionalization of alkyne-modified DNA. *Org Lett.* 2006;8:3639–42.
- [129] Seela F, Sirivolu VR. Nucleosides and oligonucleotides with diynyl side chains: base pairing and functionalization of 2'-deoxyuridine derivatives by the copper(I)-catalyzed alkyneazide 'Click' cycloaddition. *Helv Chim Acta.* 2007;90:535–52.
- [130] Seela F, Sirivolu VR. Pyrrolo-dC oligonucleotides bearing alkynyl side chains with terminal triple bonds: synthesis, base pairing and fluorescent dye conjugates prepared by the azide–alkyne “click” reaction. *Org Biomol Chem.* 2008;6:1674–87.
- [131] Seela F, Sirivolu VR. DNA containing side chains with terminal triple bonds: base-pair stability and functionalization of alkynylated pyrimidines and 7-deazapurines. *Chem Biodivers.* 2006;3:509–14.
- [132] Seela F, Sirivolu VR, Chittepu P. Modification of DNA with octadiynyl side chains: synthesis, base pairing, and formation of fluorescent coumarin dye conjugates of four nucleobases by the alkyne-azide “click” reaction. *Bioconjug Chem.* 2008;19:211–24.
- [133] Sirivolu VR, Chittepu P, Seela F. DNA with branched internal side chains: synthesis of 5-tripropargylamine-dU and conjugation by an azide-alkyne double click reaction. *ChemBioChem.* 2008;9:2305–16.
- [134] Berndl S, Herzig N, Kele P, Lachmann D, Li X, Wolfbeis OS, Wagenknecht HA. Comparison of a nucleosidic vs non-nucleosidic postsynthetic “click” modification of DNA with base-labile fluorescent probes. *Bioconjug Chem.* 2009;20:558–64.
- [135] Lietard J, Meyer A, Vasseur JJ, Morvan F. New strategies for cyclization and bicyclization of oligonucleotides by click chemistry assisted by microwaves. *J Org Chem.* 2008;73:191–200.
- [136] Nakahara M, Kuboyama T, Izawa A, Hari Y, Imanishi T, Obika S. Synthesis and base-pairing properties of C-nucleotides having 1-substituted 1H-1,2,3-triazoles. *Bioorg Med Chem Lett.* 2009;19:3316–9.
- [137] Coppola C, Simeone L, De Napoli L, Montesarchio D. On the compatibility of azides in phosphoramidite-based couplings: Synthesis of a novel, convertible azido-functionalized CyPLOS analogue. *Eur J Org Chem.* 2011;1155–65.
- [138] Seo TS, Li Z, Ruparel H, Ju J. Click chemistry to construct fluorescent oligonucleotides for DNA sequencing. *J Org Chem.* 2003;68:609–12.
- [139] Fomich MA, Kvach MV, Navakouski MJ, Weise C, Baranovsky AV, Korshun VA, Shmanai VV. Azide phosphoramidite in direct synthesis of azide-modified oligonucleotides. *Org Lett.* 2014;16:4590–3.
- [140] Shelbourne M, Chen X, Brown T, El-Sagheer AH. Fast copper-free click DNA ligation by the ring-strain promoted alkyne-azide cycloaddition reaction. *Chem Commun.* 2011;47:6257–9. Copper-free click DNA ligation by the ring-strain promoted alkyne-azide.
- [141] Singh I, Heaney F. Solid phase strain promoted “click” modification of DNA via [3+2]-nitrile oxide–cyclooctyne cycloadditions. *Chem Commun.* 2011;47:2706–8.
- [142] Jawalekar AM, Malik S, Verkade JMM, Gibson B, Barta NS, Hodges JC, Rowan A, van Delft FL. Oligonucleotide tagging for copper-free click conjugation. *Molecules.* 2013;18:7346–63.
- [143] Oberhansl S, Hirtz M, Lagunas A, Eritja R, Martínez E, Fuchs H, Samitier J. Facile modification of silica substrates provides a platform for direct-writing surface click chemistry. *Small.* 2012;8:541–5.
- [144] Galán T, Prieto B, Alvira M, Eritja R, Götz G, Bäuerle P, Samitier J. Label-free electrochemical DNA sensor using “click”-functionalized PEDOT electrodes. Application to Hepatitis C virus detection. *Biosens Bioelectron.* 2015;74:751–6.
- [145] Nie J, Li JP, Deng H, Pan HC. Progress on click chemistry and its application in chemical sensors. *Chin J Anal Chem.* 2015;43:609–17.

- [146] Kumar R, El-Sagheer A, Tumpane J, Lincoln P, Wilhelmsson LM, Brown T. Template-directed oligonucleotide strand ligation, covalent intramolecular DNA circularization and catenation using click chemistry. *J Am Chem Soc.* 2007;129:6859–64.
- [147] Shabanpoor F, Gait MJ. Development of a general methodology for labelling peptide–morpholino oligonucleotide conjugates using alkyne–azide click chemistry. *Chem Commun.* 2013;49:10260–2.
- [148] Khatwani SL, Mullen DG, Hast MA, Beese LS, Distefano MD, Taton TA. Covalent protein–oligonucleotide conjugates by copper-free click reaction. *Bioorg Med Chem.* 2012;20:4532–9.
- [149] Chittipu P, Sirivolu VR, Seela F. Nucleosides and oligonucleotides containing 1,2,3-triazole residues with nucleobase tethers: synthesis via the azide–alkyne ‘click’ reaction. *Bioorg Med Chem.* 2008;16:8427–39.
- [150] Weinrich D, Köhn M, Jonkheijm P, Westerlind U, Dehmelt L, Engelkamp H, Christianen PCM, Kuhlmann J, Maan JC, Nüsse D, Schröder H, Wacker R, Voges E, Breinbauer R, Kunz H, Niemeyer CM, Waldmann H. Preparation of biomolecule microstructures and microarrays by thiol–ene photoimmobilization. *ChemBioChem.* 2010;11:235–47.
- [151] Escorihuela J, Bañuls MJ, Grijalvo S, Eritja R, Puchades R, Maquieira A. Direct covalent attachment of DNA microarrays by rapid thiol–ene “Click” chemistry. *Bioconjug Chem.* 2014;25:618–27.
- [152] Bañuls MJ, Jiménez-Meneses P, Meyer A, Vasseur JJ, Morvan F, Escorihuela J, Puchades R, Maquieira A. Improved performance of DNA microarray multiplex hybridization using probes anchored at several points by thiol–ene or thiol–yne coupling chemistry. *Bioconjug Chem.* 2017;28:496–506.
- [153] Clark KD, Varona M, Anderson JL. Ion-tagged oligonucleotides coupled with a magnetic liquid support for the sequence-specific capture of DNA. *Angew Chem, Int Ed Engl.* 2017;56:7630–3.
- [154] Wu H, Cisneros BT, Cole CM, Devaraj NK. Bioorthogonal tetrazine-mediated transfer reactions facilitate reaction turnover in nucleic acid-templated detection of microRNA. *J Am Chem Soc.* 2014;136:17942–5.
- [155] Seckute J, Yang J, Devaraj NK. Rapid oligonucleotide-templated fluorogenic tetrazine ligations. *Nucleic Acids Res.* 2013;41:e148.
- [156] Busskamp H, Batroff E, Niederwieser A, Abdel-Rahman OS, Winter RF, Wittmann V, Marx A. Efficient labelling of enzymatically synthesized vinyl-modified DNA by an inverse-electron-demand Diels–Alder reaction. *Chem Commun.* 2014;50:10827.
- [157] Muttah F, Muthmann N, Reichert D, Anhäuser L, Rentmeister A. A benzylic linker promotes methyltransferase catalyzed norbornene transfer for rapid bioorthogonal tetrazine ligation. *Chem Sci.* 2017;8:7947–53.
- [158] Wachter L, Jablonski JA, Ramachandran KL. A simple and efficient procedure for the synthesis of 5'-aminoalkyl oligodeoxynucleotides. *Nucleic Acids Res.* 1986;14:7985–94.
- [159] Gottikh M, Asseline U, Thuong NT. Synthesis of oligonucleotides containing a carboxyl group at either their 5' end or their 3' end and their subsequent derivatization by an intercalating agent. *Tetrahedron Lett.* 1990;31:6657–60.
- [160] Mukaiyama T, Matsueda R, Suzuki M. Peptide synthesis via the oxidation-reduction condensation by the use of 2, 2'-dipyridyldisulfide as an oxidant. *Tetrahedron Lett.* 1970;22:1901–4.
- [161] Bouteirine AS, Tokuyama H, Takasugi M, Isobe H, Nakamura E, Hélène C. Fullerene-oligonucleotide conjugates: photoinduced sequence-specific DNA cleavage. *Angew Chem, Int Ed Engl.* 1994;33:2462–5.

- [162] Boutorine AS, Le Doan T, Battioni JP, Mansuy D, Dupré D, Hélène C. Rapid routes of synthesis of chemically reactive and highly radioactively labeled  $\alpha$ - and  $\beta$ -oligonucleotide derivatives for in vivo studies. *Bioconjug Chem*. 1990;1:350–6.
- [163] Boutorine AS, Brault D, Takasugi M, Delgado O, Hélène C. Chlorin-oligonucleotide conjugates: synthesis, properties, and red light-induced photochemical sequence-specific DNA cleavage in duplexes and triplexes. *J Am Chem Soc*. 1996;118:9469–76.
- [164] Bergamin M, Da Ros T, Spalluto G, Boutorine A, Prato M. Synthesis of a hybrid fullerene-trimethoxyindole–oligonucleotide conjugate. *Chem Commun*. 2001;17–8.
- [165] Grimm GN, Boutorine AS, Hélène C. Rapid routes of synthesis of oligonucleotide conjugates from non-protected oligonucleotides and ligands possessing different nucleophilic or electrophilic functional groups. *Nucleosides Nucleotides Nucleic Acids*. 2000;19:1943–65.
- [166] Knorre DG, Alekseyev PV, Gerassimova YV, Silnikov VN, Maksakova GA, Godovikova TS. Intraduplex photo-cross-linking of *p*-azidoaniline residue and amino acid side chains linked to the complementary oligonucleotides via a new phosphorylating intermediate formed in the Mukaiyama system. *Nucleosides Nucleotides*. 1998;17:397–410.
- [167] Silver GC, Sun JS, Nguyen CH, Boutorine AS, Bisagni E, Hélène C. Stable triple-helical DNA complexes formed by benzopyridoindole- and benzopyridoquinoxaline-oligonucleotide conjugates. *J Am Chem Soc*. 1997;119:263–8.
- [168] Mergny JL, Boutorine AS, Garestier T, Belloc F, Rougée M, Bulychev NV, Koshkin AA, Bourson J, Lebedev AV, Valeur B, Thuong NT, Hélène C. Fluorescence energy transfer as a probe for nucleic acid structures and sequences. *Nucleic Acids Res*. 1994;22:920–8.
- [169] Alefelder S, Patel BK, Eckstein F. Incorporation of terminal phosphorothioates into oligonucleotides. *Nucleic Acids Res*. 1998;26:4983–8.
- [170] Herrlein MK, Nelson JS, Letsinger RL. A covalent lock for self-assembled oligonucleotide conjugates. *J Am Chem Soc*. 1995;117:10151–2.
- [171] Fidanza JA, McLaughlin LW. Introduction of reporter groups at specific sites in DNA containing phosphorothioate diesters. *J Am Chem Soc*. 1989;111:9117–9.
- [172] Fidanza JA, Ozaki H, McLaughlin LW. Site-specific labeling of DNA sequences containing phosphorothioate diesters. *J Am Chem Soc*. 1992;55:09–17.
- [173] O'Donnell MJ, Herbert N, McLaughlin LW. The stereospecific introduction of reporter groups to oligodeoxynucleotides by the labeling of individual phosphorus diastereomers. *Bioorg Med Chem Lett*. 1994;4:1001–4.
- [174] O'Donnell MJ, Rajur SB, McLaughlin LW. Synthesis and properties of a hoechst-like minor-groove binding agent tethered to an oligodeoxynucleotide. *Bioorg Med Chem*. 1995;3:743–50.
- [175] Guzaev AP, Manoharan M. Selective phosphate protection: a novel synthesis of double-labeled oligonucleotides. *Org Lett*. 2001;3:3071–4.
- [176] MacMillan AM, Verdine GL. Engineering tethered DNA molecules by the convertible nucleoside approach. *Tetrahedron*. 1991;47:2603–16.
- [177] MacMillan AM, Verdine GL. Synthesis of functionally tethered oligodeoxynucleotides by the convertible nucleoside approach. *J Org Chem*. 1990;55:5931–3.
- [178] Webb TR, Matteucci MD. Sequence-specific cross-linking of deoxyoligonucleotides via hybridization-triggered alkylation. *J Am Chem Soc*. 1986;108:2764–5.
- [179] Webb TR, Matteucci MD. Hybridization triggered cross-linking of deoxyoligonucleotides. *Nucleic Acids Res*. 1986;14:7661–74.
- [180] Webb TR, Matteucci MD. Synthesis and crosslinking properties of a deoxyoligonucleotide containing N<sup>8</sup>,N<sup>6</sup>-ethanodeoxyadenosine. *Tetrahedron Lett*. 1987;28:2469–72.

- [181] Xu YZ, Zheng Q, Swann PF. Synthesis of DNA containing modified bases by post-synthetic substitution. Synthesis of oligomers containing 4-substituted thymine: O<sup>4</sup>-alkylthymine, 5-methylcytosine, N<sup>4</sup>-(dimethylamino)-5-methylcytosine, and 4-thiothymine. *J Org Chem.* 1992;57:3839–45.
- [182] Zheng Q, Wang Y, Lattmann E. Introduction of structural diversity into oligonucleotides containing 6-thioguanine via on-column conjugation. *Tetrahedron.* 2003;59:1925–32.
- [183] Acedo M, Fàbrega C, Aviñó A, Goodman M, Fagan P, Wemmer D, Eritja R. A simple method for N-15 labelling of exocyclic amino groups in synthetic oligodeoxynucleotides. *Nucleic Acids Res.* 1994;22:2982–9.
- [184] Hofmann M, Acedo M, Fagan P, Wemmer D, Eritja R, Díaz AR. Synthesis of oligodeoxynucleotides containing N<sup>6</sup>-[<sup>13</sup>C-methyl]adenine and N<sup>2</sup>-[<sup>13</sup>C-methyl]guanine. *J Chem Soc, Perkin Trans I.* 1997;1825–blpage8.
- [185] Eritja R, Díaz AR, Saison-Behmoaras E. Duplex-stabilization properties of oligodeoxynucleotides containing N<sup>2</sup>-substituted guanine derivatives. *Helv Chim Acta.* 2000;83:1417–23.
- [186] Allerson CR, Chen SL, Verdine GL. A chemical method for site-specific modification of RNA: the convertible nucleoside approach. *J Am Chem Soc.* 1997;119:7423–33.
- [187] Aviñó A, Güimil-García R, Eritja R. Synthesis of oligoribonucleotides containing 4-thiouridine using the convertible nucleoside approach and the FpmP group. *Nucleosides Nucleotides Nucleic Acids.* 2004;23:1767–77.
- [188] De la Torre BG, Eritja R. Synthesis of labelled PNA oligomers by a post-synthetic modification approach. *Bioorg Med Chem Lett.* 2003;13:391–3.
- [189] Le Brun S, Duchange N, Namane A, Zakin MM, Huynh-Dinh T, Igolen J. Simple chemical synthesis and hybridization properties of non-radioactive DNA probes. *Biochimie.* 1989;71:319–24.
- [190] Xu YZ, Zheng Q, Urwin G, Swann PF. Site-specific introduction of functional groups onto bases in synthetic oligonucleotides for biological applications. *Nucleosides Nucleotides Nucleic Acids.* 1997;16:1559–62.
- [191] De la Torre BG, Morales JC, Aviñó A, Iacopino D, Ongaro A, Fitzmaurice D, Murphy D, Doyle H, Redmond G, Eritja R. Synthesis of oligonucleotides carrying anchoring groups and their use in the preparation of oligonucleotide-gold conjugates. *Helv Chim Acta.* 2002;85:2594–607.
- [192] Schmidt S, Pein CD, Fritz HJ, Cech D. Chemical synthesis of 2'-deoxyoligonucleotides containing 5-fluoro-2'-deoxycytidine. *Nucleic Acids Res.* 1992;20:2421–6.
- [193] MacMillan AM, Chen L, Verdine GL. Synthesis of an oligonucleotide suicide substrate for DNA methyltransferase. *J Org Chem.* 1992;57:2989–91.
- [194] Sowers LC. 15N-Enriched 5-fluorocytosine as a probe for examining unusual DNA structures. *J Biomol Struct Dyn.* 2000;17:713–23.
- [195] Evilia C, Zhang X, Kanyo J, Lu P. The synthesis of oligonucleotides containing fluoro-2'-deoxycytidine for secondary structure determination of tandem tetraloop DNA analogs. *Nucleosides Nucleotides.* 1997;16:1809–20.
- [196] Kang JI, Burdzy A, Liu P, Sowers LC. Synthesis and characterization of oligonucleotides containing 5-chlorocytosine. *Chem Res Toxicol.* 2004;17:1236–44.
- [197] Ferentz AE, Verdine GL. Disulfide cross-linked oligonucleotides. *J Am Chem Soc.* 1991;113:4000–2.
- [198] Ferentz AE, Keating TA, Verdine GL. Synthesis and characterization of disulfide cross-linked oligonucleotides. *J Am Chem Soc.* 1993;115:9006–14.
- [199] Min C, Verdine GL. Immobilized metal affinity chromatography of DNA. *Nucleic Acids Res.* 1996;24:3806–10.

- [200] Xu YZ. Post-synthetic introduction of labile functionalities onto purine residues via 6-methylthiopurines in oligodeoxyribonucleotides. *Tetrahedron*. 1996;52:10737–50.
- [201] Xu YZ, Ramesh V, Swann PF. Site-specific <sup>15</sup>N-labelling of adenine in DNA for NMR studies. *Bioorg Med Chem Lett*. 1996;6:1179–82.
- [202] Stezowski JJ, Joos-Guba G, Schönwälter KH, Straub A, Glusker JP. Preparation and characterization in solution of oligonucleotides alkylated by activated carcinogenic polycyclic aromatic hydrocarbons. *J Biomol Struct Dyn*. 1987;5:615–37.
- [203] Lee H, Hinz M, Stezowski JJ, Harvey RG. Syntheses of polycyclic aromatic hydrocarbon-nucleoside and oligonucleotide adducts specifically alkylated on the amino functions of deoxyguanosine and deoxyadenosine. *Tetrahedron Lett*. 1990;31:6773–6.
- [204] Lee H, Luna E, Hinz M, Stezowski JJ, Kiselyov AS, Harvey RG. Synthesis of oligonucleotide adducts of the bay region diol epoxide metabolites of carcinogenic polycyclic aromatic hydrocarbons. *J Org Chem*. 1995;60:5604–13.
- [205] Kim SJ, Stone MP, Harris CM, Harris TM. Postoligomerization synthesis of oligodeoxynucleotides containing polycyclic aromatic hydrocarbon adducts at the N<sup>6</sup> position of deoxyadenosine. *J Am Chem Soc*. 1992;114:5480–1.
- [206] Harris CM, Zhou L, Strand EA, Harris TM. New strategy for the synthesis of oligodeoxynucleotides bearing adducts at exocyclic amino sites of purine nucleosides. *J Am Chem Soc*. 1991;113:4328–9.
- [207] Díaz AR, Eritja R, Güimil García R. Synthesis of oligodeoxynucleotides containing 2-substituted guanine derivatives using 2-fluoro-2'-deoxyinosine as common nucleoside precursor. *Nucleosides Nucleotides*. 1997;16:2035–51.
- [208] Schmid N, Behr JP. Recognition of DNA sequences by strand replacement with polyamino-oligonucleotides. *Tetrahedron Lett*. 1995;36:1447–50.
- [209] Adib A, Potier PF, Doromina S, Huc I, Behr JP. A high-yield synthesis of deoxy-2-fluoroinosine and its incorporation into oligonucleotides. *Tetrahedron Lett*. 1997;38:2989–92.
- [210] Fàbrega C, Güimil García R, Díaz AR, Eritja R. Studies on the synthesis of oligonucleotides containing photoreactive nucleosides: 2-azido-2'-deoxyinosine and 8-azido-2'-deoxyadenosine. *Biol Chem*. 1998;379:527–33.
- [211] Ramesha AR, Kroth H, Jerina DM. Novel trifluoroethanol mediated synthesis of benzo[a]pyrene 7,8-diol 9,10-epoxide adducts at the N<sup>2</sup>-position of deoxyguanosine and the N<sup>6</sup>-position of deoxyadenosine. *Tetrahedron Lett*. 2001;42:1003–5.
- [212] Zajc B, Lakshman MK, Sayer JM, Jerina DM. Epoxide and diol epoxide adducts of polycyclic aromatic hydrocarbons at the exocyclic amino group of deoxyguanosine. *Tetrahedron Lett*. 1992;33:3409–12.
- [213] Laxmi YRS, Suzuki N, Dasaradhi L, Johnson F, Shibutani. Preparation of oligodeoxynucleotides containing a diastereoisomer of  $\alpha$ -(N<sup>2</sup>-2'-deoxyguanosinyl) tamoxifen by phosphoramidite chemical synthesis. *Chem Res Toxicol*. 2002;15:218–25.
- [214] DeCorte BL, Tsarouhtsis D, Kuchimanchi S, Cooper MD, Horton P, Harris CM, Harris TM. Improved strategies for post oligomerization synthesis of oligodeoxynucleotides bearing structurally defined adducts at the N<sup>2</sup> position of deoxyguanosine. *Chem Res Toxicol*. 1996;9:630–7.
- [215] Gao H, Fathi R, Gaffney BL, Goswani B, Kung PP, Rhee Y, Jin R, Jones RA. 6-O-(Pentafluorophenyl)-2'-deoxyguanosine: a versatile synthon for nucleoside and oligonucleotide synthesis. *J Org Chem*. 1992;57:6954–9.
- [216] Xu YZ, Zheng Q, Swann PF. Synthesis by post-synthetic substitution of oligomers containing guanine modified at the 6-positions with S-, N-, O-derivatives. *Tetrahedron*. 1992;48:1729–40.

- [217] Schubert F, Ahlert K, Cech D, Rosenthal A. One-step labelling of oligonucleotides with fluoresceine during automated synthesis. *Nucleic Acids Res.* 1990;18:3427.
- [218] Hall LM, Gerowska M, Brown T. A highly fluorescent DNA toolkit: synthesis and properties of oligonucleotides containing new Cy3, Cy5 and Cy3B monomers. *Nucleic Acids Res.* 1992;40:e108.
- [219] Markiewicz WT, Gröger G, Rösch R, Zebrowska A, Markiewicz M, Klotz M, Hinz M, Godzina P, Seliger H. A new method of synthesis of fluorescently labelled oligonucleotides and their application in DNA sequencing. *Nucleic Acids Res.* 1997;25:3672–80.
- [220] Prober JM, Trainor GL, Dam RJ, Hobbs FW, Robertson CW, Zagursky RJ, Cocuzza AJ, Jensen MA, Baumeister K. A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science.* 1987;238:336–41.
- [221] Jaroszewskia JW, Cohen JS. Cellular uptake of antisense oligodeoxynucleotides. *Adv Drug Deliv Rev.* 1991;6:235–50.
- [222] Thelwell N, Millington S, Solinas A, Booth J, Brown T. Mode of action and application of Scorpion primers to mutation detection. *Nucleic Acids Res.* 2000;3752–61.
- [223] Dai N, Kool ET. Fluorescent DNA-based enzyme sensors. *Chem Soc Rev.* 2011;40:5756–70.
- [224] Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res.* 1996;6:986–94.
- [225] Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. *BioTechniques.* 2005;39:75–85.
- [226] Heid CA, Stevens J, Livak KJ, Williams PM. Real-time PCR in virology. *Nucleic Acids Res.* 2002;30:1292–305.
- [227] Johansson MK, Fidder H, Dick D, Cook RM. Intramolecular dimers: a new strategy to fluorescence quenching in dual-labeled oligonucleotide probes. *J Am Chem Soc.* 2002;124:6950–6.
- [228] Crisalli P, Kool ET. Multiple pathway quenchers: efficient quenching of common fluorophores. *Bioconjug Chem.* 2011;22:2435–54.
- [229] Holzhauser C, Wagenknecht HA. “DNA traffic lights”: concept of wavelength-shifting DNA probes and application in an aptasensor. *ChemBioChem.* 2012;13:1136–8.
- [230] Tintoré M, Aviñó A, Ruiz FM, Eritja R, Fàbrega C. Development of a novel fluorescence assay based on the use of the thrombin binding aptamer for the detection of O<sup>6</sup>-alkylguanine-DNA alkyltransferase activity. *J Nucleic Acids.* 2010;2010:632041.
- [231] Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. *Nat Biotechnol.* 1996;4:303–8.
- [232] Okamoto A, Tainaka K, Unzai T, Saito I. Synthesis and fluorescence properties of dimethylaminonaphthalene–deoxyuridine conjugates as polarity-sensitive probes. *Tetrahedron.* 2007;63:3465–70.
- [233] Ruth JL. Oligodeoxynucleotides with reporter groups attached to the base. In: Eckstein F, editor. *Oligonucleotides and analogues. A practical approach.* Oxford: IRL Press; 1991. p. 255–82.
- [234] Pieles U, Sproat BS, Lamm GM. A protected biotin containing deoxycytidine building block for solid phase synthesis of biotinylated oligonucleotides. *Nucleic Acids Res.* 1990;18:4355–60.
- [235] Roget A, Bazin H, Teoule R. Synthesis and use of labelled nucleoside phosphoramidite building blocks bearing a reporter group biotinyl, dinitropenyl, pyrenyl and dansyl. *Nucleic Acids Res.* 1989;17:7643–51.
- [236] Casale R, McLaughlin LW. Synthesis and properties of an oligodeoxynucleotide containing a polycyclic aromatic hydrocarbon site specifically bound to the N<sup>2</sup> amino group of a 2'-deoxyguanosine residue. *J Am Chem Soc.* 1990;112:5264–71.

- [237] Stezowski JJ, Stigler RD, Joos-Guba G, Lösch R, Carrell HL, Peck RM, Glusker JP. Stereochemical properties of nucleosides alkylated by activated carcinogens. *Cancer Res.* 1984;44:5555–66.
- [238] Pieles U, Sproat BS, Neuner P, Cramer F. Preparation of a novel psoralen containing deoxyadenosine building block for the facile solid phase synthesis of psoralen-modified oligonucleotides for a sequence specific crosslink to a given target sequence. *Nucleic Acids Res.* 1989;17:8967–78.
- [239] Shinohara Y, Matsumoto K, Kugenuma K, Morii Y, Saito Y, Saito I. Design of environmentally sensitive fluorescent 2'-deoxyguanosine containing arylethynyl moieties: distinction of thymine base by base-discriminating fluorescent (BDF) probe. *Bioorg Med Chem Lett.* 2010;20:2817–20.
- [240] Seo YJ, Ryu JH, Kim BH. Quencher-free, end-stacking oligonucleotides for probing single-base mismatches in DNA. *Org Lett.* 2005;7:4931–3.
- [241] Saito Y, Miyauchi Y, Okamoto A, Saito I. Base-discriminating fluorescent (BDF) nucleoside: distinction of thymine by fluorescence quenching. *Chem Commun.* 2004;1704–5.
- [242] Saito Y, Suzuki A, Ishioroshi S, Saito I. Synthesis and photophysical properties of novel push–pull-type solvatochromic 7-deaza-2'-deoxypurine nucleosides. *Tetrahedron Lett.* 2011;52:4726–9.
- [243] Nakamura M, Ohtoshi Y, Yamana K. Helical pyrene-array along the outside of duplex RNA. *Chem Commun.* 2005;5163–5.
- [244] Teo YN, Kool ET. DNA-multichromophore systems. *Chem Rev.* 2012;112:4221–45.
- [245] Xu W, Chan KM, Kool ET. Fluorescent nucleobases as tools for studying DNA and RNA. *Nat Chem.* 2017;9:1043–55.
- [246] Matsika S. Modified nucleobases. *Top Curr Chem.* 2015;355:209–44.
- [247] Sinkeldam RW, Greco NJ, Tor Y. Fluorescent analogs of biomolecular building blocks: design, properties, and applications. *Chem Rev.* 2010;110:2579–619.
- [248] Wilhemsson LM. Fluorescent nucleic acid base analogs. *Q Rev Biophys.* 2010;43:159–83.
- [249] Dodd DW, Hudson RHE. Intrinsically fluorescent base-discriminating nucleoside analogs. *Mini-Rev Org Chem.* 2009;6:378–91.
- [250] Wilson JN, Fluorescent KET. DNA base replacements: reporter and sensors for biological systems. *Org Biomol Chem.* 2006;4:4265–74.
- [251] Ranasinghe RT, Brown T. Fluorescence based strategies for genetic analysis. *Chem Commun.* 2005;5487–502.
- [252] Kool ET. Replacing the nucleobases in DNA with designer molecules. *Acc Chem Res.* 2002;35:936–43.
- [253] Ward DC, Reich E, Stryer L. Fluorescence studies of nucleotides and polynucleotides. Formycin, 2-aminopurine riboside, 2,6-diaminopurine riboside, and their derivatives. *J Biol Chem.* 1969;244:1228–37.
- [254] Gargallo R, Vives M, Tauler R, Eritja R. Protonation studies and multivariate curve resolution on oligodeoxynucleotides carrying the mutagenic base 2-aminopurine. *Biophys J.* 2001;81:2886–96.
- [255] Jones AC, Neely RK. 2-aminopurine as fluorescent probe of DNA conformation and the DNA-enzyme interface. *Q Rev Biophys.* 2015;48:244–79.
- [256] Hawkins ME, Brand L, Michael LJ. Fluorescent pteridine probes for nucleic acid analysis. *Methods Enzymol.* 2008;450:201–31.
- [257] Hawkins ME. Fluorescent pteridine nucleoside analogs. A window on DNA interactions. *Cell Biochem Biophys.* 2001;34:257–81.

- [258] Hawkins ME, Pfleiderer W, Jungmann O, Balis FM. Synthesis and fluorescence characterization of pteridine adenosine nucleoside analogs for DNA incorporation. *Anal Biochem.* 2001;298:231–40.
- [259] Rösler A, Pfleiderer W. Synthesis of condensed  $N^1$ -(2'-deoxy- $\beta$ -D-ribofuranosyl)lumazines, new fluorescent building blocks in oligonucleotide synthesis. *Helv Chim Acta.* 1997;80:1869–81.
- [260] Maurinsk Y, Pfleiderer W. Synthesis of base-modified oligonucleotides containing 6- and 7-aryl lumazines. *Nucleosides Nucleotides.* 1995;14:795–8.
- [261] Hwang GT, Seo YJ, Kim BH. Pyrene-labeled deoxyuridine and deoxyadenosine: fluorescent discriminating phenomena in their oligonucleotides. *Tetrahedron Lett.* 2005;46:1475–7.
- [262] Forster U, Lommel K, Sauter D, Grünwald C, Engels JW, Wachtveitl J. 2-(1-Ethynylpyrene)-adenosine as a folding probe for RNA – pyrene in or out. *ChemBioChem.* 2010;11:664–72.
- [263] Okamoto A, Ochi Y, Saito I. Modulation of base selectivity for a base-discriminating fluorescent nucleobase by addition of Mercury ion. *Bioorg Med Chem Lett.* 2005;15:4279–81.
- [264] Wagner C, Rist M, Mayer-Enthart E, Wagenknecht HA. 1-Ethynylpyrene-modified guanine and cytosine as optical labels for DNA hybridization. *Org Biomol Chem.* 2005;3:2062–3.
- [265] Manderville RA, Wetmore SD. C-linked 8-aryl guanine nucleobase adducts: biological outcomes and utility as fluorescent probes. *Chem Sci.* 2016;7:3482–93.
- [266] Shinohara Y, Matsumoto K, Kugenuma K, Morii T, Saito Y, Saito I. Design of environmentally sensitive fluorescent 2'-deoxyguanosine containing arylethylnyl moieties: distinction of thymine base by base-discriminating fluorescent (BDF) probe. *Bioorg Med Chem Lett.* 2010;20:2817–20.
- [267] Kimoto M, Mitsui T, Yokoyama S, Hirao I. A unique fluorescent base analogue for the expansion of the genetic alphabet. *J Am Chem Soc.* 2010;132:4988–9.
- [268] Greco NJ, Tor Y. Furan decorated nucleoside analogues as fluorescent probes: synthesis, photophysical evaluation, and site-specific incorporation. *Tetrahedron.* 2007;63:3515–27.
- [269] Srivatsan SG, Tor Y. Fluorescent pyrimidine ribonucleotide: synthesis, enzymatic incorporation, and utilization. *J Am Chem Soc.* 2007;129:2044–53.
- [270] Blanchard DJM, Cserenyi TZ, Manderville RA. Dual fluorescent deoxyguanosine mimics for FRET detection of G-quadruplex folding. *Chem Commun.* 2015;51:16829–31.
- [271] Ben Gaiel N, Glasser N, Ramalanjaona N, Beltz H, Wolff P, Marquet R, Burger A, Mély Y. 8-Vinyl-deoxyadenosine, an alternative fluorescent nucleoside analog to 2'-deoxyribosyl-2-aminopurine with improved properties. *Nucleic Acids Res.* 2005;33:1031–9.
- [272] Nadler A, Strohmeier J, Diederichsen U. 8-Vinyl-2'-deoxyguanosine as a fluorescent 2'-deoxyguanosine mimic for investigating DNA hybridization and topology. *Angew Chem, Int Ed Engl.* 2011;50:5392–6.
- [273] Seela F, Jiang D, Xu K. 8-Aza-2'-deoxyguanosine: base pairing, mismatch discrimination and nucleobase anion fluorescence sensing in single-stranded and duplex DNA. *Org Biomol Chem.* 2009;7:3463–73.
- [274] Seela F, Jiang D, Budow S. Triples with 8-aza-2'-deoxyisoguanosine replacing protonated dC: probing third strand stability with a fluorescent nucleobase targeting duplex DNA. *ChemBioChem.* 2010;11:1443–50.
- [275] Srivatsan SG, Weizman H, Tor Y. A highly fluorescent nucleoside analog based on thieno[3,4-*d*]pyrimidine senses mismatched paring. *Org Biomol Chem.* 2008;6:1334–8.
- [276] Tor Y, Del valle S, Jaramillo D, Srivatsan SG, Rios A, Weizman H. Designing new isomeric fluorescent nucleobase analogues: the thieno[3,4-*d*]pyrimidine core. *Tetrahedron.* 2007;63:3608–14.

- [277] Xie Y, Maxson T, Tor Y. Fluorescent nucleoside analogue displays enhanced emission upon pairing with guanine. *Org Biomol Chem*. 2010;8:5053–5.
- [278] Okamoto A, Tainaka K, Saito I. Synthesis and properties of a novel fluorescent nucleobase, naphthopyrimidine. *Tetrahedron Lett*. 2003;44:6871–4.
- [279] Seio K, Kanamori T, Tokugawa M, Ohzeki H, Masaki Y, Tsunoda H, Ohkubo A, Sekine M. Fluorescent properties of oligonucleotides doubly modified with an indole-fused cytosine analog and 2-aminopurine. *Bioorg Med Chem*. 2013;21:3197–201.
- [280] Elmehriki AAH, Suchy M, Chicas KJ, Wojciechowski F, Hudson RHE. Syntehsis and spectral characterization of environmentally responsive fluorescent deoxycytidine analogs. *Artificial DNA PNA XNA*. 2014;5:e29174.
- [281] Lane RSK, Magennis SW. Two-photon excitation of the fluorescent nucleobase analogues 2-AP and tC. *RSC Adv*. 2012;2:11397–403.
- [282] Preus S, Kilsa K, Wilhelmsson LM, Albinsson N. Photophysical and structural properties of the fluorescent nucleobase analogues of the tricyclic cytosine (tC) family. *Phys Chem Chem Phys*. 2010;12:8881–92.
- [283] Preus S, Börjesson K, Kilsa K, Albinsson N, Wilhelmsson LM. Characterization of nucleobase analogue FRET acceptor tC<sub>nitro</sub>. *J Phys Chem*. 2010;114:1050–6.
- [284] Bood M, Sarangamath S, Wranne MS, Grotli M, Wilhelmsson LM. Fluorescent nucleobase analogues for base-base FRET in nucleic acids: synthesis, photophysics and applications. *Beilstein J Org Chem*. 2018;14:114–29.
- [285] Okamoto A, Tanaka K, Fukuta T, Saito I. Cytosine detection by a fluorescein-labeled probe containing base-discriminating fluorescent nucleobase. *ChemBioChem*. 2004;5:958–63.
- [286] Petra DGI, de Boer RF, Koomen GJ, Meewenoord NJ, Kuyl-Yeheskiely E, van der Marel GA, van Boom JH. Synthesis and properties of a fluorescent nucleotide derivative. *Recl Trav Chim Pays-Bas*. 1996;115:99–102.
- [287] Wilson JN, Gao J, Kool ET. Oligodeoxyfluorosides: strong sequence dependence of fluorescence emission. *Tetrahedron*. 2007;63:3427–33.
- [288] Spadafora M, Postupalenko VY, Shvadchak VV, Klymchenko AS, Mély Y, Burger A, Benhida R. Efficient synthesis of ratiometric fluorescent nucleosides featuring 3-hydroxychromone nucleobases. *Tetrahedron*. 2009;65:7809–16.
- [289] Wilson JN, Cho Y, Tan S, Cuppoletti A, Kool ET. Quenching of fluorescent nucleobases by neighboring DNA: the “insulator” concept. *ChemBioChem*. 2008;9:279–85.
- [290] Lee SH, Wang S, Kool ET. Templated chemistry for monitoring damage and repair directly in duplex DNA. *Chem Commun*. 2012;48:8069–71.
- [291] Dahlmann HA, Berger FD, Kung RW, Wyss LA, Gubler I, McKeague M, Wetmore SD, Sturla SJ. Fluorescent nucleobase analogues with extended pi surfaces stabilize DNA duplexes containing O6-alkylguanine adducts. *Helv Chim Acta*. 2018;101:e1800066.

Ramon Eritja

## 5 Nucleic acids triple helices

### Contents

5.1	Triplex formation by oligonucleotides —	<b>188</b>
5.1.1	Parallel triplex —	<b>189</b>
5.1.2	Antiparallel triplex —	<b>189</b>
5.2	Stabilization of parallel triplex by modified nucleobases —	<b>190</b>
5.2.1	5-Substituted pyrimidines —	<b>190</b>
5.2.2	Modified nucleobases that mimic protonated cytosine —	<b>192</b>
5.2.3	Cytosine derivatives with increased basicity —	<b>194</b>
5.3	Modified nucleobase aimed at stabilizing antiparallel triple helices —	<b>195</b>
5.4	Dealing with interruptions on the polypurine: polypyrimidine track —	<b>196</b>
5.4.1	Heterocyclic compounds aimed to the recognition of GC base pair inversion —	<b>197</b>
5.4.2	Heterocyclic compounds aimed to the recognition of AT base pair inversion —	<b>199</b>
5.4.3	Nucleoside derivatives able to stabilize triplexes in the presence of interruptions —	<b>200</b>
5.5	Formation of triple helices in alternate strands —	<b>201</b>
5.6	Modified nucleobases at the Watson–Crick positions —	<b>203</b>
5.7	Triplex stabilization by modified backbones —	<b>204</b>
5.8	Triplex stabilization by the addition of crosslinkers or intercalating agents —	<b>207</b>
5.9	Targeting single-stranded and double-stranded nucleic acids by triplex formation using clamps or hairpins. Strand-displacement —	<b>208</b>
5.9.1	Targeting the polypurine strand —	<b>209</b>
5.9.2	Targeting the polypyrimidine strand —	<b>211</b>
	Bibliography —	<b>213</b>

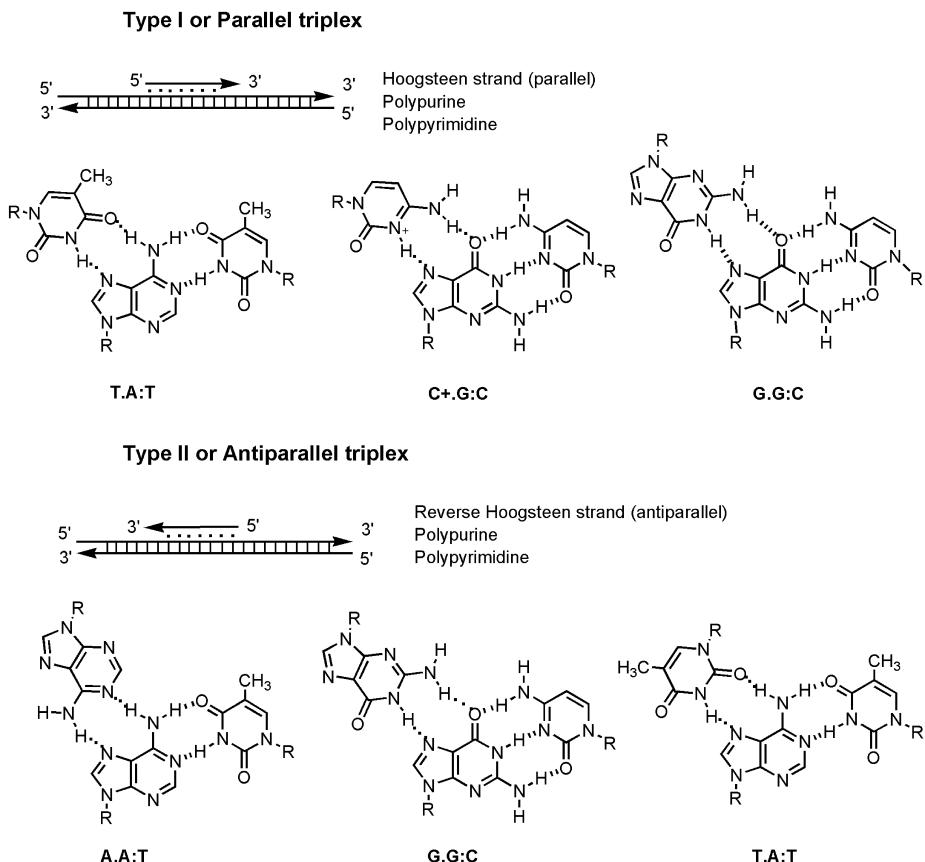
In biological systems, DNA is in the form of a double helix but depending on the sequence and ionic environment it is possible to observe the formation of other forms such as triple and quadruple helices. The formation of three-stranded nucleic acid structures were observed for the first time in the 1950s when studying the base-pairing properties of RNA homopolymers [1, 2]. Later, it was demonstrated that triple helices are formed both in DNA and RNA molecules as long as they have homopurine-homopyrimidine sequences by the formation of specific hydrogen bonds. These structures triggered the development of a large number of applications. In this chapter, we will review the development of the synthetic methodologies and potential applications of the technologies based on triple helices. Excellent reviews on this area written by several authors can be found in the bibliography [3–15].

---

Ramon Eritja, Networking Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Jordi Girona 18-26, E-08034 Barcelona, Spain, e-mail: recgma@cid.csic.es

## 5.1 Triplex formation by oligonucleotides

Oligonucleotides able to form triplexes with target nucleic acid sequences have been largely studied as DNA-binding molecules of potential interest in diagnosis or therapeutics [3–15]. Typically, triplexes are formed in homopurine-homopyrimidine sequences of duplex DNA by interaction with a single-stranded triplex-forming oligonucleotide (TFO), which binds to the major groove of Watson–Crick double-helical DNA. Depending on the orientation of the triplex-forming oligonucleotide with respect to the central oligopurine Watson–Crick strand, triplexes are classified into two main categories: (i) parallel and (ii) antiparallel (Figure 5.1).



**Figure 5.1:** Parallel and antiparallel triplexes. Strand orientation and chemical structure of the corresponding triads.

### 5.1.1 Parallel triplex

In the pyrimidine triplex I motif or parallel triplex, an oligonucleotide made of cytosine and thymidine residues (C,T-TFO) binds parallel to the homopurine strand via Hoogsteen base pairs forming T,A:T and C+,G:C triads [16–18]. Protonation of cytosine is required for the Hoogsteen base pair between protonated C and the  $N^7$  of guanine. For this reason, the stability of parallel triplex is pH-dependent being more stable in acidic pH (pH 4–5) near the pKa of cytosine [19, 20]. At physiological conditions, the stability of the parallel triplex is low especially if the G,C content is high. In the C,T-parallel triplex triads are isomeric.

It has been described that the C+,G:C triad can be replaced by G,G:C triad (Figure 5.1) [21]. In this case, the TFO is formed by G and T residues (parallel G,T-TFO). In this case, the stability of the triplex is not pH-dependent because there is no need of protonation. Unfortunately, the triads in the G,T-parallel triplex are not isomeric and every time that there is a change from a T,A:T triad to a G,G:C triad, the stability decreases so this type of triplex is not observed in mixed A,G sequences [22].

In general C,T-parallel triplets are more studied from the structural and biochemical point of view than antiparallel triplets [11]. This is because C,T-parallel triplets have a clear hypochromic change upon triplex formation that can be used to follow the dissociation of the third strand. On the contrary, the dissociation of the antiparallel triplets cannot be observed in UV melting curves. Although it is difficult to compare the relative stability of parallel and antiparallel triplets, it has been suggested that C,T-parallel triplets are more stable than antiparallel triplets [23, 24].

### 5.1.2 Antiparallel triplex

In the purine triplex II motif or antiparallel triplex, the TFO is made of guanine and adenine residues (G,A-TFO) and it binds antiparallel to the homopurine strand via reverse Hoogsteen base pairs forming A,A:T and G,G:C triads (Figure 5.1). In this case, there is no need for base protonation to establish two hydrogen bonds between the Watson–Crick purine with the reverse Hoogsteen purine so the stability of this triplex is pH-independent [25].

It has been described that the A,A:T triad can be replaced by the T,A:T triad (Figure 5.1) [25]. In this case, the TFO is formed by G and T residues (antiparallel G,T-TFO). The G,T-antiparallel is also pH-independent and, in general, is slightly less stable than the G,A-antiparallel triplet [26]. As mentioned above, the lack of hypochromicity upon antiparallel triplex formation difficult the analysis of the relative stability of triplets by UV spectrometry and for this reason, there is much less data on this type of triplets. Although antiparallel-TFO binding to duplex target can be observed and measured by gel foot-printing assays [24, 25, 27], fluorescent FRET analysis [28], reso-

lution of melting curves by multivariate curve resolution [29] and UV-VIS denaturation studies of oligonucleotide-gold conjugates [30].

## 5.2 Stabilization of parallel triplex by modified nucleobases

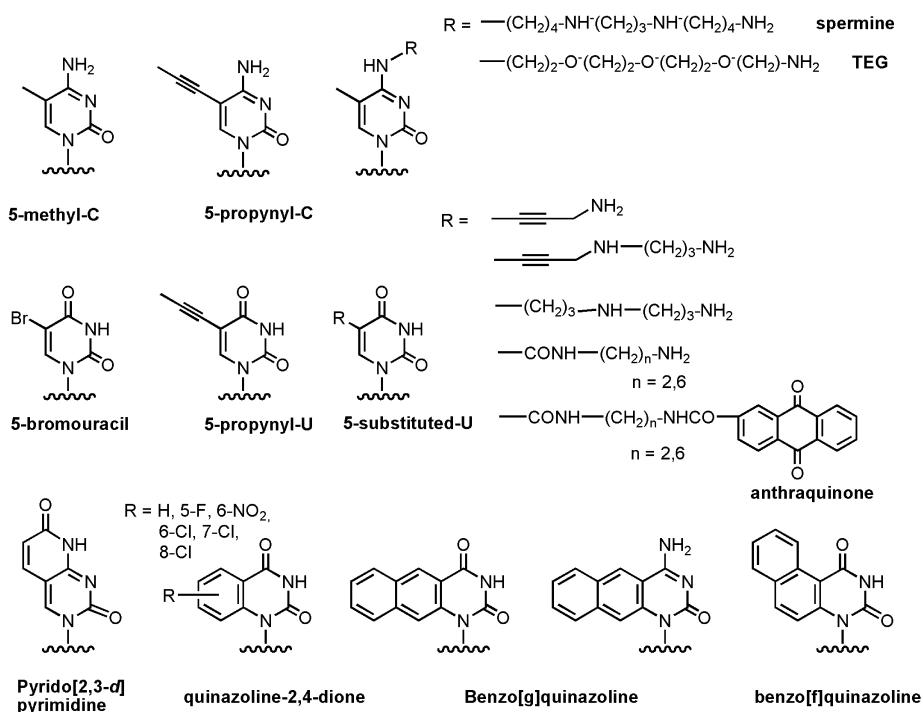
One of the main problems of the parallel triplex is the need for protonation of cytosine residues in the Hoogsteen strand. In order to increase the stability of parallel triplexes at physiological neutral conditions, a large effort in the development of modified nucleobases has been made.

### 5.2.1 5-Substituted pyrimidines

Replacement of cytosine by 5-methylcytosine (Figure 5.2) in TFO has been one of the older chemical modifications that were demonstrated to stabilize parallel triplex [31–35]. The triplex-stabilization properties of 5-methyl-C have been assigned to an increased pKa (4.4 versus 4.2 of cytosine), and an increase in entropy due to stronger stacking interactions and desolvation of the helix [33]. The substitution of thymine by 5-bromouracil has also a positive impact on the stabilization of parallel triplex [32]. The presence of 5-methylcytosine and 5-bromouracil has an additive stabilization effect and the combination of these two modifications allows the observation of parallel triplexes at neutral pH [32, 35]. On the contrary, other 5-substituted pyrimidines such as 5-iodouracil, 5-bromocytosine, 5-iodocytosine and 5-aminouracil had negative effects on the stability of parallel triplexes [36–38]. The results were in good agreement with those obtained by molecular dynamics calculations [39].

5-Methylcytosine nucleobase has also been modified at the 4 position by the addition of long, positively charged chains that may add favorable electrostatic interactions with the phosphate groups. The 4-amino group has been replaced by spermine and other polyamines (Figure 5.2) [40, 41], or *N*-alkylated with an aminotetraethyleneglycol (TEG) chain (Figure 5.2) [42]. The rationale for these modifications is that spermine is known to stabilize triple helices, even in the absence of Mg<sup>2+</sup> and at neutral pH [43]. Moreover, the covalent linkage of spermine to the 5' end of an oligonucleotide has been shown to produce more stable triplexes [44].

The addition of a propynyl group at 5 positions of both uracil and cytosine (Figure 5.2) generates an important class of triplex-stabilization nucleobases [45]. The introduction of a propynyl group brings hydrophobicity to the triplex complex, favoring both desolvation and stacking interactions. Substitution of T by 5-propynyl-U was clearly stabilizing the parallel triplex ( $\Delta T_m = 2.4\text{ }^\circ\text{C}/\text{modification}$ ); however, the replacement of C by 5-propynyl-C was not [45, 46]. The cooperative association of two



**Figure 5.2:** Chemical structures of modified nucleobases with potential triplex-stabilization properties.

short oligonucleotides with a DNA duplex, to yield a triplex, was certainly enhanced when 5-propynyl-U was substituted for T at the ends of both of the short oligonucleotides [47]. NMR Studies of a parallel intramolecular triplex in which T was replaced by 5-propynyl-U showed an A-DNA like conformation exhibiting an increased stacking due to the propynyl group [48]. Thermal denaturation experiments and gel shift assays have also been used to compare the effect of many different nucleobase and backbone modifications on oligonucleotides triplex-forming ability [49]. The most stable parallel triple helices were those in which the third strand contained 5-propynyl-U or 5-methyl-C.

The substitution of T by 5-(aminopropargyl)-uracil (Figure 5.2) at the Hoogsteen strand provokes an increase in stability of about 12 °C [50]. The contribution of the positively charged groups was demonstrated by comparison with 5-propargyl at different pHs. The uracil derivative carrying both 5-aminopropargyl and 2'-aminoethoxy modifications have been incorporated into triplex-forming oligonucleotides; the combination of the two amino groups on the same nucleoside greatly enhances triplex stability [51, 52]. Previously, nucleosides with 2'-O-(2-aminoethyl)ribose were described to stabilize triple helices [53]. Other 5-modified uracil derivatives carrying two positive

charges have also been proposed (Figure 5.2) [54]. Unfortunately, these derivatives did not increase the stability of parallel triplexes.

5-Methoxycarbonyl-2'-deoxyuridine phosphoramidite was introduced to oligonucleotides. Nucleophilic attack with diamines generated uracil derivatives carrying amino groups at the 5 position. The addition of anthraquinone systems by post-synthetic protocols allowed the preparation of TFO carrying either positively charged groups or aromatic systems able to intercalate between base pairs (Figure 5.2) [55]. Only the anthraquinone-modified bases, when placed at the 5' end of an oligonucleotide chain, showed a significant stabilizing effect on parallel triple helices.

Polycyclic nucleobase derivatives have also been studied in the parallel triplex. Some of these modified nucleobases have been described in Chapters 3 and 4 due to their biophysical and fluorescent properties. Pyrido[2,3-*d*]pyrimidine nucleoside (Figure 5.2) has an extended system that, in principle, should favor the stacking interactions with other nucleobases. The modified nucleobase recognizes with high selectivity AT base pairs within parallel triplex [56].

Different systems with fused benzene-thymine rings have been examined. The use of 2, 4-quinazolinedione (Figure 5.2) as a substitute for thymine in the canonical T:A:T has been described [57] with poor stabilizing results. The analogs with halo or nitro groups at the benzene ring (Figure 5.2) did not afford better results [58].

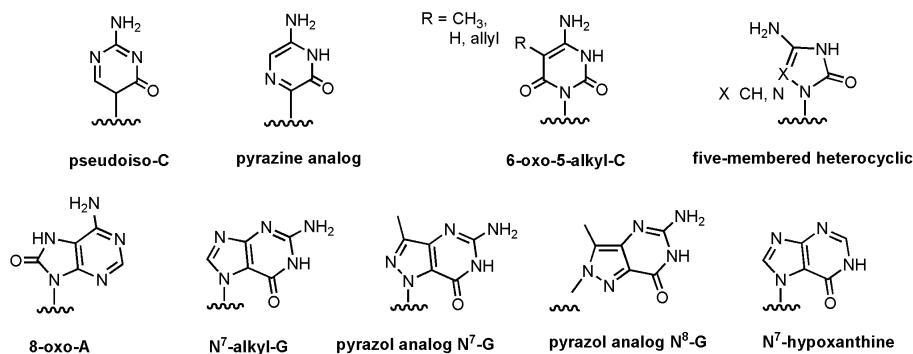
The use of benzo[g]- and benzo[f]quinazoline-2,4-dione-(1*H*,3*H*)-dione (Figure 5.2) as substitutes for thymine in the canonical T.A:T triplet has been reported [59]. Benzo[g]quinazoline and benzo[f]quinazoline formed triple-stranded structures with slightly decreased stabilities. In addition, benzo[g]quinazoline revealed strong fluorescence emission properties which can be used to monitor selectively the formation of triple-helical structures [60]. Benzo[g]quinazoline was also used to evaluate the affinity of a segment of the HIV Tat protein for a stem-loop segment of the TAR RNA [61].

The nucleoside derivative 4-amino-1*H*-benzo[g]quinazoline-2-one (Figure 5.2) is a fluorescent analog of cytosine [62]. The 2'-O-methyl ribonucleoside derivative of this heterocycle was synthesized and exhibited a fluorescence emission centered at 456 nm. When introduced in triplex-forming oligonucleotides, this modified nucleoside was used to reveal the protonation state of triplets in triple-stranded structures and the triplex formation was detected by quenching of the fluorescence emission.

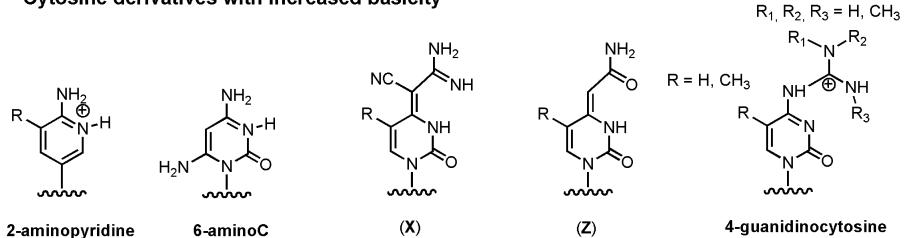
### 5.2.2 Modified nucleobases that mimic protonated cytosine

Neutral nucleoside derivatives that reproduce the double donor hydrogen bond pattern of protonated cytosine have been designed as derivatives of either the pyrimidine or the purine rings (Figure 5.3). The first group of nucleobases should, in principle, give rise to the most stable triplexes, since they cause little or no distortion in the sugar-phosphate backbone. In the second group of analogs, the presence of a purine

### Neutral nucleobases that mimic protonated cytosine



### Cytosine derivatives with increased basicity



**Figure 5.3:** Chemical structures of modified nucleobases that mimic protonated cytosines and cytosine derivatives with increased basicity.

system in the pyrimidine-rich Hoogsteen strand separates the anomeric carbon from the Watson–Crick duplex, breaking the isomorphism of the triple helix.

Pseudoisocytosine (Figure 5.3) was obtained from pseudouridine [63–65]. Several pseudoisocytosine derivatives have been prepared and introduced in oligonucleotide analogs. These include the 2'-deoxynucleoside [65], the ribo- and 2'-O-methylribonucleosides [63–65], and the PNA derivative [66]. When pseudoisocytosine is replacing cytosine in parallel tripleplexes, it is possible to observe tripleplexes at neutral pH [63, 64]. The 2'-O-methyl derivative of pseudoisocytosine formed more stable tripleplexes than the 2'-deoxy derivative [65]. The 2'-O-methylribo pyrazine analog (Figure 5.3) was also prepared [67]. When introduced in a single position of the third strand sequence, this modified nucleobase was shown to form tripleplexes with no pH-dependence and more stable than those formed by C at neutral pH. Pseudouridine and pseudoisocytidine have been also introduced into TFO with pyrrolidino backbone [68–70]. Replacing T by pyrrolidino pseudouridine was found to be destabilizing but, on the contrary, replacing C by pyrrolidino pseudoisocytidine was found to enhance triplex stability.

6-Oxocytosines either unsubstituted at the 5-position or substituted with methyl or allyl groups (Figure 5.3) were also prepared [71–75]. These modified nucleobases

have been incorporated into 2'-deoxyribo- [71, 73, 75], ribo- [75], 2'-O-methylribo-nucleosides [72, 75], and as a glycerol derivative [74]. Triplets containing the 6-oxocytosine 2'-O-methylribonucleoside were more stable than those formed by the methyl-6-oxocytosine derivative [72] suggesting that the methyl group at the 5 position might cause unfavorable steric interactions.

Other five-membered heterocyclic systems have been designed as neutral analogs of protonated cytosine (Figure 5.3) [76]. So far, only the preparation of the deoxyribonucleosides has been described.

Several groups have proposed to use of the oxidative damage product 8-oxoadenine (oxoA, Figure 5.3) [77–79] or its  $N^2$ -methyl derivative [80] to replace protonated cytosine in C<sup>+</sup>.G:C triads. The synthesis of 8-oxoadenine derivatives is described in Chapter 2. Both melting denaturation experiments [77] and footprinting techniques [80] showed that oxoA formed oxoA-G·C triplets with no pH dependence, interacting either with isolated G-C pairs or with (G-C)<sub>n</sub> tracts. Triple helices containing oxoA were more stable than those containing 5-methyl-C at pH > 7.4, however, the situation was reversed below this pH value [77].

Nucleosides having a C'<sup>1</sup>-N<sup>7</sup> guanine glycosidic bond (Figure 5.3) were prepared by glycosylation of N<sup>2</sup>-isobutyryylguanosine [81–83]. This nucleoside analog can replace protonated C in parallel triplexes. The affinity of N<sup>7</sup>-guanine for G-C base pairs was shown to be the same at pH 7.0 or 7.5, but they are sequence-dependent [82, 83].

The capacity of pyrazole analogs of N<sup>7</sup>-guanine (Figure 5.3) [84, 85] to mimic protonated C was also evaluated. Footprinting experiments [84, 85] showed that their affinity for G:C pairs was different. The affinity of the N<sup>8</sup> derivative was smaller than that of N<sup>7</sup> derivative, probably as a result of the distortion caused by the position of the sugar-nucleobase linkage. NMR studies of intramolecular triplexes containing one N<sup>7</sup>-pyrazol derivative.G:C triplet in the middle of the Hoogsteen strand showed that even though the triplet is not isomorph with T.A:T, the distortion caused by the N<sup>7</sup>-pyrazol derivative was found to be small [86].

The  $\alpha$ - and  $\beta$ -N<sup>7</sup>-hypoxantine-2'-deoxynucleosides as well as the ribo- and 2'-O-methylribonucleosides (Figure 5.3) have also been evaluated as protonated cytosine analogs able to interact with G:C pairs [87, 88]. The stability of the triplets, the affinity and selectivity of these analogs were assessed using several techniques. The  $\alpha$ -analog yielded less stable triplexes [87], but the  $\beta$ -N<sup>7</sup>-hypoxantine-2'-deoxyriboside gave more stable triple helices [88, 89]. The riboside derivatives afforded less stable triplexes.

### 5.2.3 Cytosine derivatives with increased basicity

Several C-nucleosides with the 2-aminopyridine ring (Figure 5.3) have been prepared and evaluated as cytosine analogs with a higher pKa and the same pattern

of hydrogen donor groups [90–93]: (i) 2'-deoxy-5-(2-aminopyridine) nucleosides, either in the  $\alpha$ - or  $\beta$ -configuration (pKa 6.2 and 5.9, respectively [90]), (ii) the 5-(2-amino-3-methylpyridine)  $\beta$ -2'-deoxynucleoside and (iii) the 2'-O-methylribo-5-(2-aminopyridine) derivative. At neutral pH, the affinity for duplexes with G:C base pairs was similar for both anomers [90]. At acidic pH 2-aminopyridine analogs formed the most stable triplexes. Other authors have also shown that 2-aminopyridine can suitably replace protonated cytosine in triple helices [94].

Oligonucleotides carrying 6-aminocytosine (Figure 5.3) were not able to replace cytosine in parallel triplexes [95] even that this nucleobase is more basic than cytosine (pKa = 6.8). It was suggested that the 6-amino group might change the conformation of the glycosidic bond, preventing the formation of the *anti* conformation needed for triplex formation.

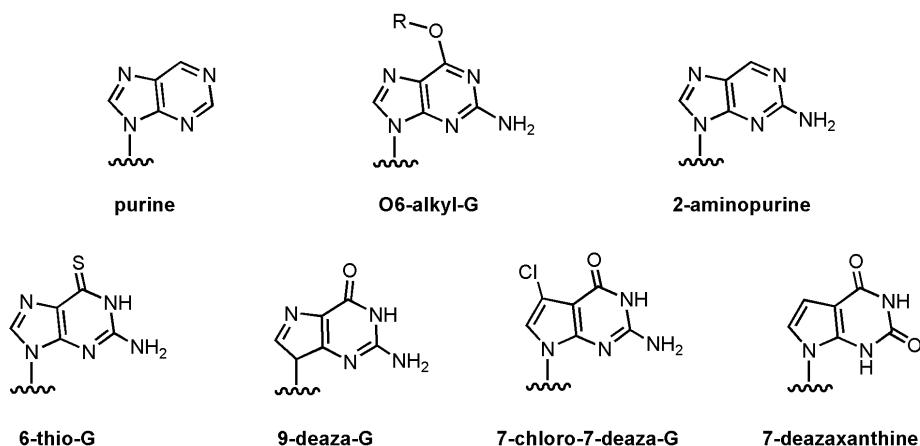
The addition of a guanidine group at position 4 of cytosine (Figure 5.3) was initially devised as a nucleobase able to recognize G:C or C:G pairs [8, 96]. 4-Guanidino-5-methylcytosine derivatives (Figure 5.3) have also been prepared [97].

Two additional neutral cytosine derivatives, resembling the 4-guanidinocytosine analogs, were designed to have similar binding properties. Nucleobases with either two hydrogen bond donor groups (**X**, Figure 5.3), or one acceptor and one donor groups (**Z**, Figure 5.3), were prepared and introduced in oligonucleotides [98, 99]. Footprinting experiments were carried out to assess whether **X**- and **Z**-containing oligonucleotides were able to recognize duplexes, with contradictory results. The nuclease S1 treatment indicated that one of these modified oligonucleotides was able to interact with a duplex and generate protection of this strand, while DNase 1 digestion suggested that no triplex was formed.

### 5.3 Modified nucleobase aimed at stabilizing antiparallel triple helices

Several base analogs have been proposed to substitute adenine in triplex-forming oligonucleotides (TFO) to form antiparallel triplexes (Figure 5.4). Purine 2'-deoxyriboside (Chapter 3) was found to bind to C:G base pairs by the formation of one H-bond to cytosine [100]. O<sup>6</sup>-Alkyl-dG derivatives linked to a psoralen and an acridine derivative [101] and 2-aminopurine (Chapter 3) [102] were proposed as adenine substitutes in antiparallel triplexes. The ability to form antiparallel triplexes of four isomeric forms (7-, 9-,  $\alpha$ - and  $\beta$ -) of 2-aminopurine 2'-deoxyriboside were analyzed [103]. Both the  $\alpha$ -9- and the  $\beta$ -7-isomers bind to A:T base pairs similar to thymine in a GT-oligonucleotide. The  $\alpha$ -9-isomer binds also to G:C base pair with a similar stability to A:T base pair [103].

Triple helix formation involving guanine-rich oligonucleotides is inhibited by physiological ions, particularly K<sup>+</sup>, most likely due to oligonucleotide aggregation



**Figure 5.4:** Chemical structures of modified nucleobases with potential stabilization properties of antiparallel tripleplexes.

such as the formation of guanine quartet structures. Several guanine analogs have been designed to prevent G-quartet structure (Figure 5.4). Oligonucleotides carrying 6-thioguanine were found to resist K<sup>+</sup> mediated inhibition but triplexes were less stable than triplexes with guanines [104–107]. The increased radius and decreased electronegativity of sulfur at the 6-position of guanine destabilize potential guanine quartets. Substitution of T by 7-deazaxanthines in G,T-rich oligonucleotides has also shown triplex stabilization [104, 108, 109]. 9-Deazaguanine and 6-thio-7-deazaguanine have been also prepared to avoid K<sup>+</sup>-inhibition of triplex however, a strong destabilization was observed [110, 111]. Recently, 7-chloro-7-deaza guanine was found to resist K<sup>+</sup>-inhibition and to form triplexes with similar or slightly better stability than triplexes having guanine [112].

## 5.4 Dealing with interruptions on the polypurine: polypyrimidine track

An important limitation of triplex formation is the presence of interruptions in the polypurine-polypyrimidine tracks. This is caused by the presence of a pyrimidine on a polypurine track or the presence of a purine on a pyrimidine track. These are known as GC base pair or AT base pair inversions. In order to find potential solutions, there has been a large development of non-natural nucleosides and one of the most successful directions was the design and use of heterocyclic compounds that interact with both base pairs in the major groove. In addition, research has been directed to search for compounds that are neutral or able to stack so they can help to cope with the presence of several interruptions.

### 5.4.1 Heterocyclic compounds aimed to the recognition of GC base pair inversion

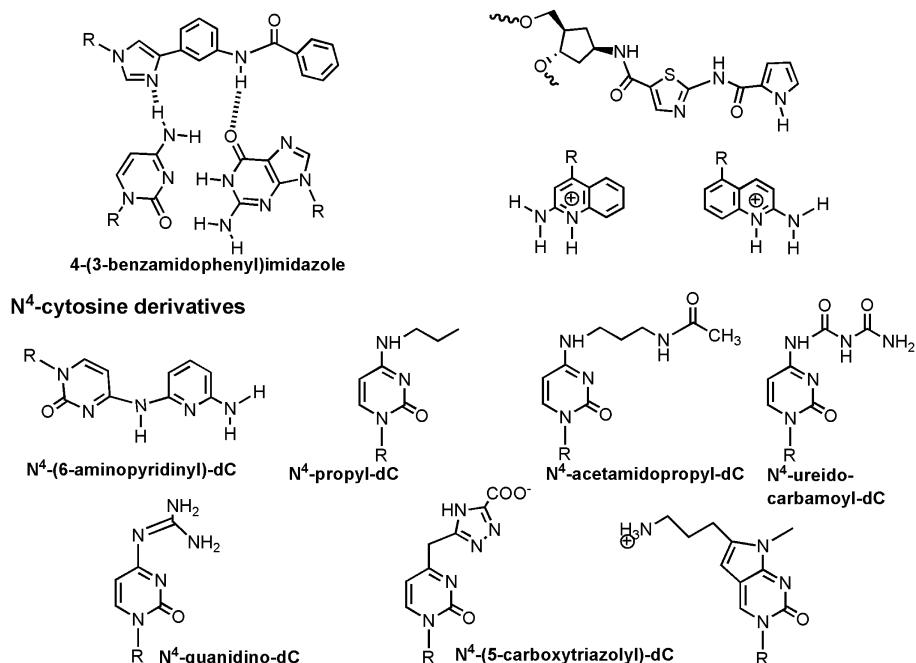
One of the first molecules of this type was a 4-(3-benzamidophenyl)imidazole derivative (Figure 5.5) described by Griffin et al. [113]. The incorporation of this compound in a parallel triplex allowed the specific binding to T:A and C:G base pairs but not to the inverse A:T and G:C base pairs. NMR experiments showed that the binding of this imidazole derivative was produced by intercalation instead of the expected hydrogen bonding [114, 115].

A series of  $N^4$ -cytosine derivatives (Figure 5.5) have been studied as potential bidentate ligands to bind Watson–Crick bases in parallel triplexes [116–120].  $N^4$ -(6-aminopyridinyl)-C (Figure 5.5) and  $N^4$ -(propyl)-dC (Figure 5.5) form stable base pairs with inverse GC base pairs [116, 118].  $N^4$ -(acetamidopropyl)-dC (Figure 5.5) form stable base pairs with direct CG base pairs.  $N^4$ -(3-carboxypropyl) and  $N^4$ -(5-carboxytriazolyl)-C (Figure 5.5) form stable base pairs with direct CG and inverse TA base pairs [117]. Oligonucleotides carrying  $N^4$ -guanidino [96, 119],  $N^4$ -anthraniloyl-,  $N^4$ -ureido- $N^4$ -ureidocarbamoyl-C [120] (Figure 5.5) derivatives have also been prepared [120]. Unfortunately, the incorporation of some of these nucleosides is troublesome and the resulting TFOs have limited binding selectivity in parallel triplexes.

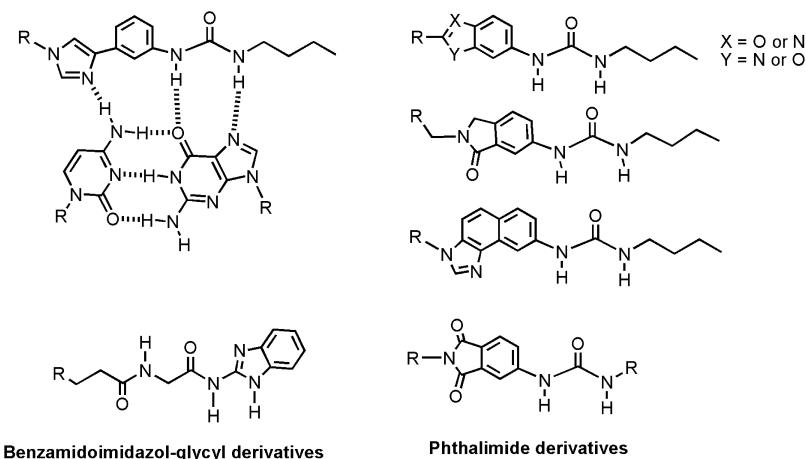
Several carbocyclic derivatives such as 5-substituted 2-aminoquinolines were developed by Li et al. [121–123]. 5-substituted 2-aminoquinolines form stable triplex with sequences having C:G inversion however, 4-substituted 2-aminoquinoline was specific for G:C base pairs [123]. Substituted 3*H*-pyrrolo[2,3-*d*]pyrimidin-2(7*H*)-one nucleoside analogs were incorporated to TFO presenting a good selectivity for C:G inversions [124–126]. The thiazol derivative (Figure 5.5) shows selective binding of inverted pyrimidine-purine base pair over direct purine-pyrimidine within a parallel triplex. However, no discrimination between CG or TA base pair was found [127].

Other heterocyclic derivatives designed to form three Hoogsteen hydrogen bonds with Watson–Crick bases are shown in Figure 5.5. For example, the benzaminoimidazole-glycyl derivative (Figure 5.5) described by Sasaki et al. [128] was demonstrated by NMR to bind a C:G base pair by the formation of three hydrogen bonds. Various phthalimide derivatives (Figure 5.5) have been prepared and incorporated in TFO [129]. However, the modified TFO carrying these derivatives did not from the expected hydrogen bonds by NMR [130]. Similar results were found with the naphthimidazole nucleoside (Figure 5.5) designed by Zimmermann et al. [131]. On the other hand, the expected hydrogen bonds were detected by NMR with the ureido benzimidazole and benzoxazole derivatives designed by Sun et al. [132]. In an attempt to improve the properties of the artificial nucleobase, the ureido isoindolin-1-one derivative (Figure 5.5) was developed by Mertz et al. [133]. Unfortunately, these derivatives did not reach the expected results [134]. Several other heterocyclic compounds such as phenylimidazole which

### Two hydrogen-bonds nucleobases for C:G recognition



### Three hydrogen-bonds nucleobases for C:G recognition

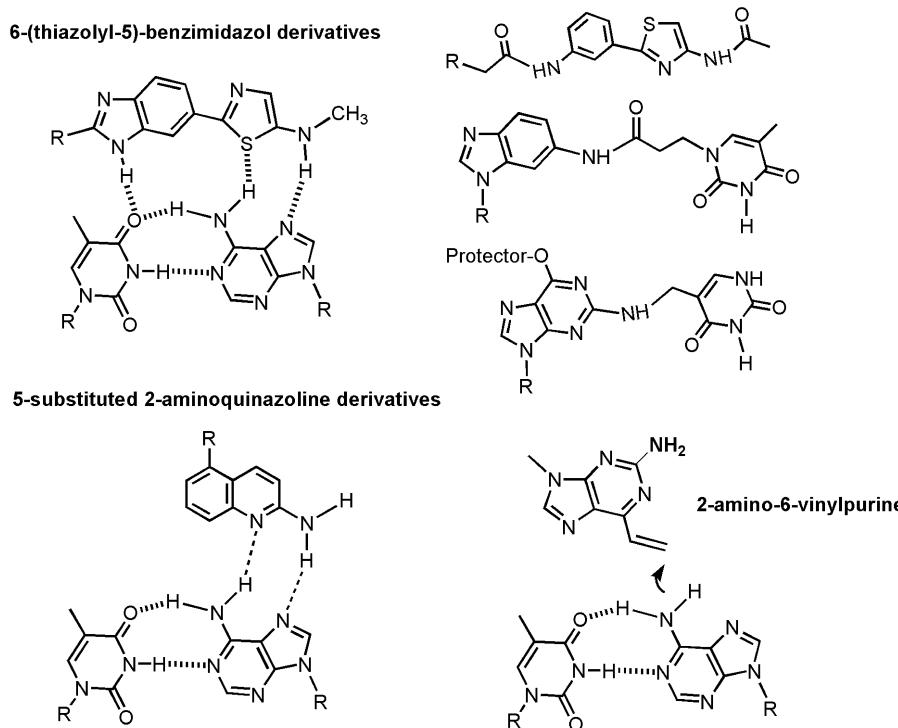


**Figure 5.5:** Chemical structures of compounds designed to compensate interruptions on the poly-purine: polypyrimidine tracks.

may interact with Watson–Crick bases through the major groove have been described and their triplex binding properties are being evaluated [135–138].

### 5.4.2 Heterocyclic compounds aimed to the recognition of AT base pair inversion

Targeting TA base pair inversion in parallel triplexes is more complex due to the presence of the methyl group of T and for this reason, is less studied. One of the most successful series of compounds for AT recognition are based on 6-(thiazolyl-5)-benzimidazole nucleobase [139, 140]. This aromatic derivative, bearing a rigid nucleobase, was designed to produce three Hoogsteen hydrogen bonds with the A:T base pair (Figure 5.6). However, this derivative did not accomplish the expected stability. Then a more successful design was obtained with an aminophenyl-thiazole derivative constructed to form three Hoogsteen hydrogen bonds [141, 142], being one of the best derivatives for the recognition of an A:T interruption.



**Figure 5.6:** Chemical structures of heterocyclic compounds for the recognition of A:T base pair inversions.

Other alternative nucleoside that has been proposed to form two hydrogen bonds with A:T base pair is a guanine analog carrying an uracil derivative linked at the  $N^2$  position (Figure 5.6). This derivative was only evaluated at the nucleoside level [143] and its incorporation into oligonucleotides was not reported. A similar derivative where the

uracil moiety was attached to an aminobenzimidazole was described by Van Craynest et al. [144] however; the properties of TFOs containing these compounds were not reported.

Some of the heterocyclic derivatives described in the previous section have also been studied with some success for the recognition of A:T base pairs including the 3*H*-pyrrolo[2,3-*d*]pyrimidin-2(7*H*)-one [126, 145] and 5-substituted 2-aminoquinazoline (Figure 5.6) [121–123].

Finally, Sasaki et al. reported the incorporation of 2-amino-6-vinylpurine into TFOs to achieve selective cross-linking with the 6-NH<sub>2</sub> of the Watson–Crick adenine of a TA base pair [146].

#### 5.4.3 Nucleoside derivatives able to stabilize triplexes in the presence of interruptions

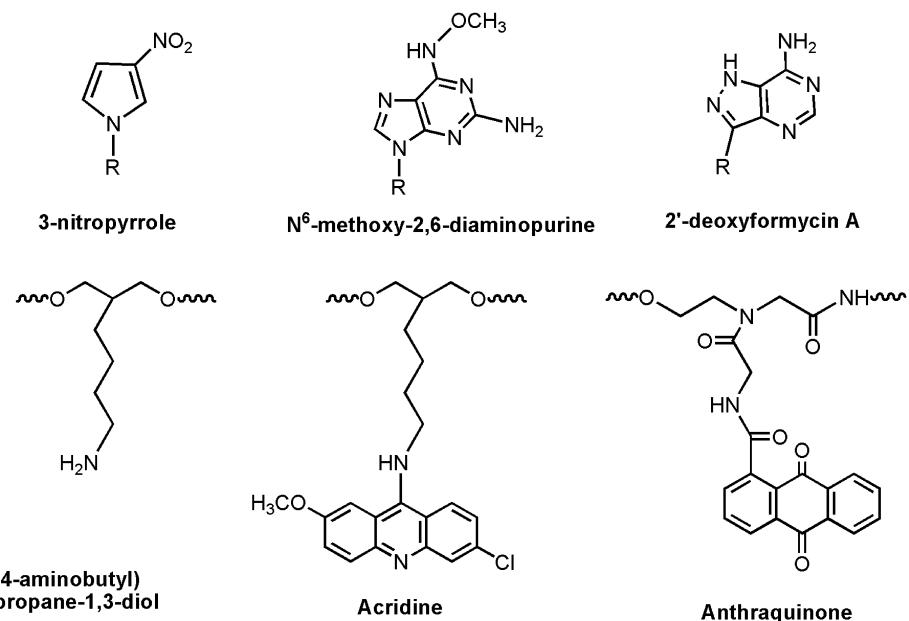
In addition to novel heterocyclic compounds designed to bind both Watson–Crick bases, interest was directed to search for compounds that are neutral to interruptions. One of the first questions to solve was the ability of the natural nucleotides to bind the pyrimidine site [147]. It was found that the best nucleobase to bind to a T:A interruption was G [147, 148]. The structural characteristics of the G:T:A triad was determined by NMR [149]. The best nucleobase to bind to a C:G interruption is T [147]. In order to avoid destabilization produced by unfavorable steric hindrance effects, several abasic site analogs (Chapter 2) were studied. Good results were found for the (4-aminobutyl) propane-1,3-diol (Figure 5.7) probably due to interactions of protonated amino group and phosphates [150].

Universal bases that were designed to bind all four natural bases in duplex structures (Chapter 3) were also used to cope with interruptions in TFOs [151–153]. Azole derivatives (Figure 5.7) were shown to bind to both T:A and C:G inversion sites but more specifically to T:A inversion sites [152]. 3-Nitropyrrole (Figure 5.7) was found to bind specifically to C:G inversion site and *N*<sup>6</sup>-methoxy-2,6-diaminopurine (Figure 5.7) binds to T:A inversion site [151, 153].

A systematic study of six uracil derivatives to bind at C:G and T:A inversion sites in a G,T-TFO to form antiparallel triplexes found thymine and 5-fluorouracil as the best binders for C:G inversion sites [154]. 5-Fluorouracil binds also to T:A inversion sites [154].

A study involving eight nucleobase analogs opposite to several interruptions of a parallel triplex was reported [155]. In this study, it was found that 5-propynyl- and 5-bromo-U (Figure 5.2) bind better than T to C:G inverted sites. Furthermore, 5-propynyl-C (Figure 5.2) and 5-bromo-C (Chapter 3) were found to have a similar affinity than C to bind G:C base pair [155].

The use of *N*-(2-hydroxyethyl)glycine residue having several small ligands was studied to bind to a T:A inverted site in a parallel triplex context [156]. Only the



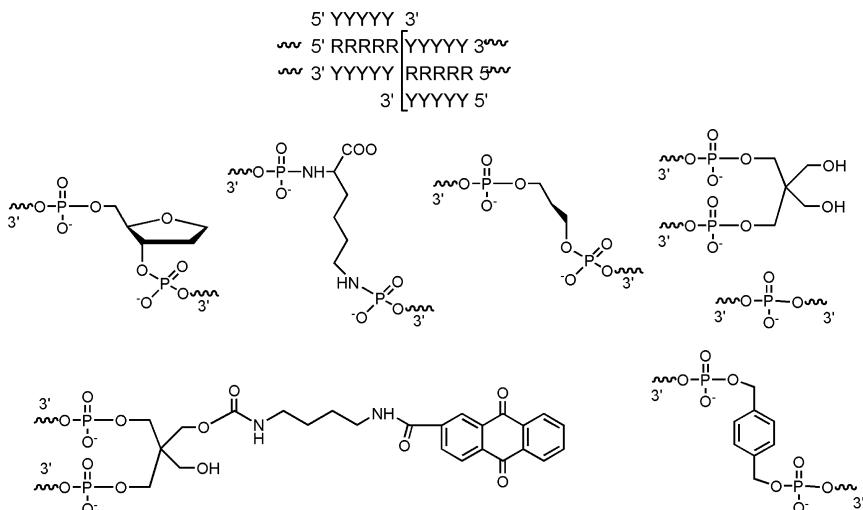
**Figure 5.7:** Chemical structures of compounds neutral to polypurine: polypyrimidine interruptions.

monomer carrying an anthraquinone molecule (Figure 5.7) gave a more stable triplex than the unmodified oligonucleotide. In addition, 2'-deoxyformycin A was found to bind with high affinity to C:G sites in antiparallel tripleplexes [157].

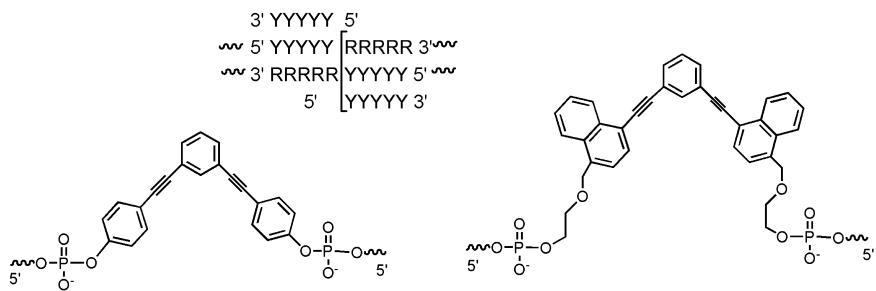
## 5.5 Formation of triple helices in alternate strands

In some cases, the DNA target is made of several adjacent oligopurine-oligopyrimidine tracks. Then it is possible to link two or more TFOs with the appropriate spacer molecules that act as spacers (Figure 5.8), and the triplex formation may be enhanced due to cooperative binding. This approach is known as alternate-strand recognition or “switched” triple helix. The most common mode of alternate-strand triple helix formation is when two parallel tripleplexes are adjacent. In this case, pyrimidine triplex-forming oligonucleotides are in opposite directions (Figure 5.8). This requires joining the TFO sequences either with 3'-3' or 5'-5' linkages to change the orientation at the junction. Several Linker molecules [158–162] have been used to introduce 3'-3' linkages including 2-deoxy-1,4-anhydroribitol [158], 1,3-propanediol, xylene [159], regular phosphodiester linkages [160, 161] and lysine [162] (Figure 5.8). In an interesting development, a pentaerythritol derivative (Figure 5.8) was designed for the synthesis of 3'-3' symmetric and asymmetric oligonucleotides [163–165]. In addition, the pentaerythritol derivative was further functionalized to produce 3'-3' symmetric oligonucleotides

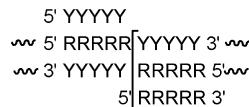
## Two adjacent parallel triplexes 3'-3' linkages



## Two adjacent parallel triplexes, 5'-5' linkages



## Parallel and antiparallel triplexes



## Two antiparallel triplexes at distant sites

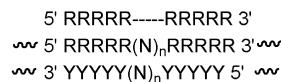


Figure 5.8: Adjacent polypurine: polypyrimidine tracks. Alternated triplexes.

carrying an anthraquinonyl group that had a higher affinity for their targets than the unmodified 3'-3' linked TFOs.

The design of the 5'-5' linked oligonucleotides was found to be more complex due to the larger distance between the 5'-ends of the third strand. This goal was achieved by using lineal molecules connected through the bases [166–168] or using aromatic compounds interconnected with triple bonds that act as intercalating agents [169–

171]. These intercalating systems provide a high stabilization of alternate triplexes enabling the formation of parallel triplexes at physiological pH [170].

The second type of alternate-strand triple helix is when the two TFO to be linked are designed to form two different types of triplexes: one parallel and the other antiparallel. Then TFO consists of both C,T sequences (to form parallel triplex) and G,A or G,T sequences (to form antiparallel triplexes). In this case, both halves have the same polarity (Figure 5.8) and TFO are easier to prepare [172–180]. In these mixed triplexes crossing the mayor groove is difficult due to the structure of the phosphate bonds. Strand recognition is easier at purine-pyrimidine junctions than at pyrimidine-purine junctions [172–180].

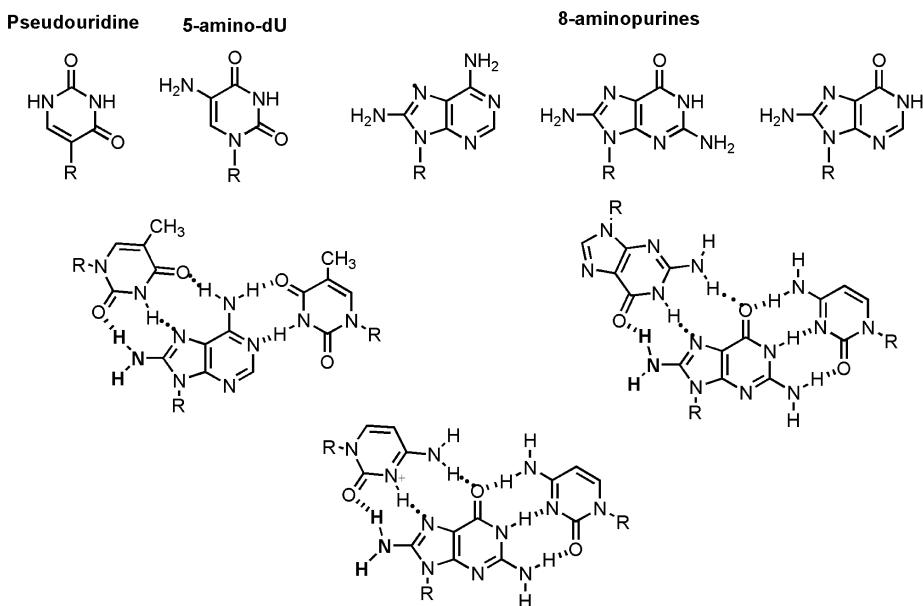
A third strategy consists in linking two TFO sequences having their triplex target sequences separated by several bases, which are incompatible with triplex formation (Figure 5.8). Two homopurine sequences of 12 bases separated by 10 bases (roughly one helix turn) have been simultaneously bound by hybrid oligonucleotides carrying a flexible linker molecule longer than 20–25 rotatable bonds [181].

## 5.6 Modified nucleobases at the Watson–Crick positions

In the search for new triads to increase the stability of triplexes, several novel nucleobases have been incorporated at the Watson–Crick positions. Although the use of these new nucleobases is limited due to the fact that the modification is located at the target site, it is possible to overcome this inconvenient by designing hairpins or clamps to target single-stranded RNA molecules as well as to target double-stranded DNA molecules by the so-called strand displacement phenomenon. In the next sections, the “clamp” strategy is discussed further as it opens a wide range of potential applications.

Both 2'-deoxyriboside and riboside derivatives of pseudouridine (Figure 5.9) are able to form two types of new triads. The T.pseudoU:T [182] and the A.pseudoU:A triad [183]. In these triads, pseudouridine is located at the Watson–Crick position at the purine strand (replacing an A) and binding of the nucleoside at the Hoogsteen strand (T or A) is made through H-bonds with pseudouridine instead of the A in the normal T.A:T or A.A:T triads. A similar result was found when 5-amino-U (Figure 5.9) is located at the central position [184, 185]. The amino group at position 5 of uracil is acting as the H-donor group for Hoogsteen pairing as it does one of the N-H groups of pseudouridine. In addition, 5-aminouracil was reported to bind to G, 2-aminopurine and T within an antiparallel context and C, T and A in parallel orientation [185].

The introduction of an amino group at position 8 of the Watson–Crick purines (A, G and hypoxanthine, Figure 5.9) produces a high stabilization of parallel triplexes [186–190]. The triplex stabilizing properties of 8-aminopurines were predicted in the



**Figure 5.9:** Chemical structures of modified nucleobases at the Watson–Crick positions with triplex stabilization properties.

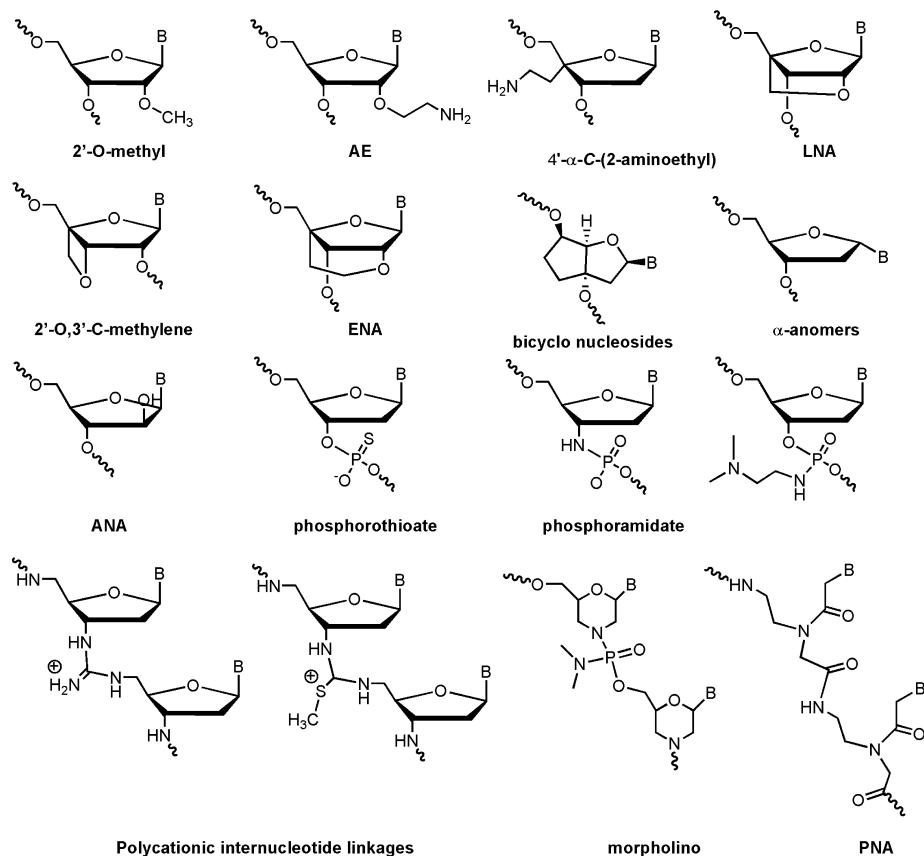
70's [191–193] but experimental data was not available due to the self-aggregation of homopolymers containing these bases. The synthesis of oligonucleotides carrying 8-aminopurines has been described and a high triplex stabilization effect both in parallel and antiparallel tripleplexes was found [186–190]. Theoretical calculations have demonstrated that the high triplex-stabilization properties of the amino group at position 8 is due to a combined effect of the gain of one Hoogsteen purine-pyrimidine H-bond (Figure 5.9) and the propensity of the amino group to be integrated into the “spine of hydration” located in the minor-major groove of the triplex [188]. In order to use the triplex-stabilization properties of 8-aminopurines, hairpins formed by a polypyrimidine part linked to a polypurine sequence carrying 8-aminopurines were prepared and used for binding polypyrimidine sequences [194, 195]. Oligonucleotides carrying 8-aminopurines produce also a high stabilization of parallel duplexes [196]. Oligonucleotides carrying 8-aminoguanine produce also more stable antiparallel duplexes and tripleplexes [197].

## 5.7 Triplex stabilization by modified backbones

The use of oligonucleotides as therapeutic agents has triggered one of the largest developments of nucleic acid analogs having modified backbones in order to protect the

integrity of the oligonucleotides to the *in vivo* nuclease degradation. Hundreds of new modified backbones have been developed and their properties have been tested [198]. During the study of their biophysical properties, most of these artificially-modified backbones were tested for potential triplex stabilization properties mostly in parallel triplexes. A detailed description of these studies is out of the scope of this chapter but the most important developments in the triplex stabilization properties of modified backbones will be the subject of this section.

One of the modifications most commonly used in the design of TFO's are 2'-O-methyl-RNA units (Figure 5.10). Parallel triplexes formed with TFOs carrying 2'-O-methyl-RNA were reported to be more thermally stable than those formed by DNA oligomers [199–202]. In addition, 2'-O-methyl RNAs are easier to handle, and are relatively nuclease resistant, especially in circular oligonucleotides [203].



**Figure 5.10:** Chemical structures of modified backbones with potential triplex stabilization properties.

Another 2'-O-alkyl-RNA derivatives that have been used for their triplex stabilization properties are the 2'-O-(2-aminoethyl)-RNA (AE) residues (Figure 5.10). The presence of a short amino alkyl group at the 2' position of the ribose of the third strand allow the protonated amino group to form intermolecular contacts with the phosphate groups of the DNA duplex [51, 53, 204–206]. TFOs carrying aminoethyl-RNA residues and photoreactive psoralen were reported to knock out the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene *in vivo* [207]. The addition of 5-(aminopropargyl)uridine residues together with the 2'-O-(2-aminoethyl)-RNA modifications in a TFO designed to make a parallel triplex induced a clear increase in triplex stabilization detected even at neutral pH [51–53, 206, 208–210]. Other amino alkyl nucleoside derivatives with triplex stabilization properties are 4'- $\alpha$ -C-(2-aminoethyl)-2'-deoxypyrimidine nucleosides [211]. In these compounds, an aminoethyl group is attached at the 4' position pointing at the minor groove in a similar way to the 2'-O-(2-aminoethyl)-RNA derivatives.

Conformationally restricted nucleosides such as 2',4'-C-methylene bridged nucleic acids (2',4'-BNA) [212–214] known as locked nucleic acids (LNAs) [215, 216] were reported to form stable parallel triplexes. Surprisingly, full-length LNA oligonucleotides were not able to form triplexes and for this reason, it is recommended that only a few residues (one every 2–3 nucleotides) are modified by LNAs to achieve optimal triplex stabilization [215]. The isomeric 2'-O,3'-C-methylene bridged nucleic acids (Figure 5.10) has also been reported but TFOs carrying this analog with 4',5'-phosphatediester bonds do form parallel triplexes [217]. The substitution of the methylene group of the LNA for an ethylene group was reported [218]. TFO's carrying 2'-O,4'-C-ethylene nucleic acids (ENA) (Figure 5.10) can form parallel triplexes with dsDNA with high stability including at physiological pH [218] as described for LNA-modified TFOs.

Other conformationally restricted nucleosides that have triplex stabilization properties are the bicyclonucleotides developed by the group of Leumann [219]. In this case, the bicyclo thymidine derivative is the most efficient derivative for the stabilization of parallel triplexes.

Oligonucleotides fully modified with the  $\alpha$ -anomers of nucleosides (Figure 5.10) of purine-rich and pyrimidine-rich have been studied for their ability to form triplexes. It has been reported that C,T-oligonucleotides made by  $\alpha$ -anomers form parallel triplexes similar to the natural nucleosides [220, 221]. G,T-oligonucleotides form triplexes in the parallel orientation but GA-oligonucleotides are not able to form antiparallel triplexes [220]. Fully modified oligonucleotides with arabino nucleosides (ANA) form parallel triplexes only with DNA duplexes [222].

Oligonucleotides carrying modified phosphates have also been reported to facilitate triplex formation. Among them, phosphoramidate oligonucleotides (Figure 5.10) are reported to be highly potent in the formation of parallel triplexes through Hoogsteen base pairing [223–225]. Oligonucleotides carrying phosphorothioate linkages have also been studied as this modification is frequently used in the development

of antisense oligonucleotides. Phosphorothioate linkages in polypyrimidine oligonucleotides have been shown to destabilize parallel triplexes but they still performed better than unmodified oligonucleotides in the *in vitro* inhibition of gene transcription by T7 RNA polymerase via triplex formation [226]. In another study pyrimidine-rich stereoregular Rp-phosphorothioate oligonucleotides were reported to be not able to form parallel triplexes but, on the contrary, G,T-oligonucleotides had comparable affinities to their targets than the unmodified oligonucleotides [227].

The negative charge of phosphodiester linkages is responsible for electrostatic repulsion as phosphates are relatively close in the minor groove of duplexes and triplexes. Replacement of phosphate bonds by neutral or positively charged internucleotide bonds can stabilize triplex formation at the same time than provides degradation-resistant properties as these bonds are not recognized by nucleases. Polycationic internucleotide linkages such as guanidino (Figure 5.10) [228–230], methylisothiouronium [231–233], and *N,N*-dimethylaminoethyl phosphoramidates [234] are the best examples of this type of modified oligonucleotides developed for triplex formation showing clear stabilization of parallel triplexes.

Uncharged modified internucleotide linkages have been also studied. The stability of triplexes formed by TFO carrying morpholino (Figure 5.10) linkages was reported [235, 236]. Pyrimidine-rich morpholino oligonucleotides bind to their corresponding duplex targets to form parallel triplexes in low salt concentration and in the absence of magnesium ions [235]. The formation of parallel triplexes by pyrimidine-rich morpholino oligonucleotides was confirmed by other authors who also studied the formation of antiparallel triplexes. Unfortunately, the formation of antiparallel triplexes by G,A- or G,T- morpholino TFO oligonucleotides was not observed [236].

Other uncharged oligonucleotide derivative with extraordinary triplex-stabilization properties are Peptide Nucleic Acids (PNA) (Figure 5.10). In this case, the ribose phosphate backbone is totally replaced by a neutral, non-quiral aminoethylglycine backbone [237]. PNA oligomers form very stable parallel triplexes being able to displace one strand to form PNA.PNA.DNA triplexes [230–241].

## 5.8 Triplex stabilization by the addition of crosslinkers or intercalating agents

The formation of triple helices offers the possibility of targeting genomic DNA by synthetic oligonucleotides but the pH-dependence of parallel triplexes difficult the potential *in vivo* applications. In the previous sections of this chapter, we have reported the results obtained with the use of modified oligonucleotides including changes in nucleobases, carbohydrate and phosphates moieties. An alternative to increase the

affinity of TFOs to their target duplex sequences is to connect oligonucleotides to ligands that are able to stabilize triplex structures such as minor groove binders, intercalating agents, and crosslinkers [9]. The protocols used for the synthesis of the oligonucleotides carrying crosslinkers or intercalating agents have been discussed in Chapter 4.

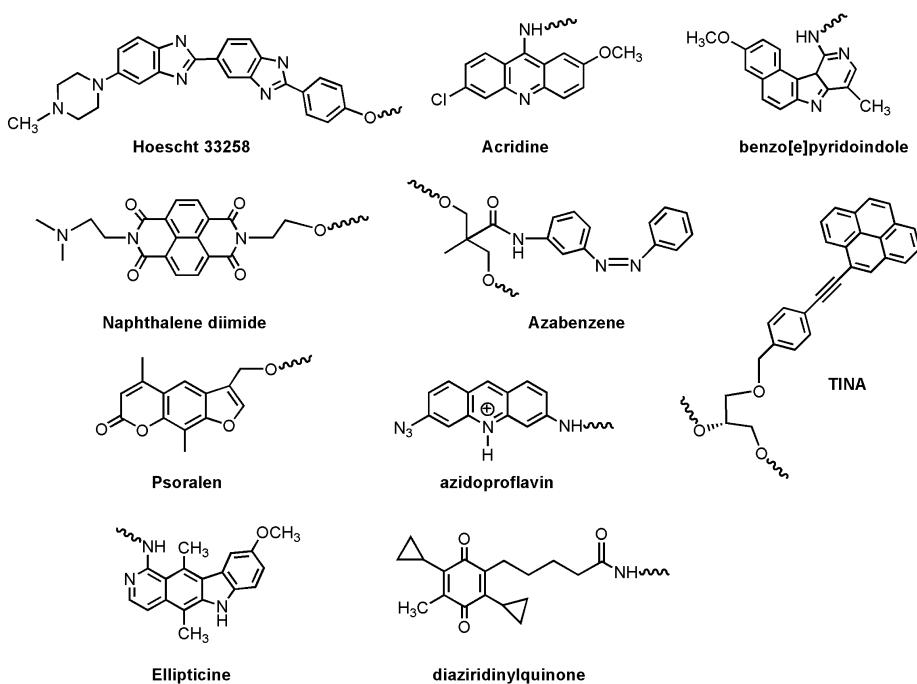
Oligonucleotides functionalized at their 3' or 5'-ends with minor groove binders such as pyrrole/imidazole polyamines [242], Hoescht 33258 (Figure 5.11) [243–245] have been reported, demonstrating that this strategy can be successfully applied for increasing the binding affinity of TFOs.

Oligonucleotides carrying intercalating agents at the 3' or 5'-ends are the most frequently used derivatives especially oligonucleotides carrying acridine derivatives (Figure 5.11) [246–249]. Naphthalene-diimide (Figure 5.11) and perylene-based intercalating agents have also been used to enhance parallel triplex formation [250–252]. Similarly, enhanced triplex stabilization properties have been reported for azobenzene [253, 254], benzopyridoindole (Figure 5.11), benzopyridoquinoxaline [255] and dipyridophenazine [256] tethered oligonucleotides. Azobenzene (Figure 5.11) allows the photoregulation of triplex formation. When the azobenzene takes the *trans* form, a stable triplex is formed. By isomerization of the *trans*-azobenzene to its *cis* form by UV light irradiation, the TFO dissociates from duplex [254]. More recently, the insertion of 1-pyrenylethynyl (TINA) derivatives (Figure 5.11) into the middle of homopyrimidine TFOs led to a high increase of the thermal stability of parallel triplexes [257].

The use of photoactivable crosslinkers such as psoralen linked to TFOs [258, 259] has been profusely used for a variety of *in vitro* experiments such as antigenic inhibition of gene expression [260, 261], and for the generation of site-specific mutations [262–266]. Other photoreactive groups such as ellipticine (Figure 5.11) [267, 268], *p*-azidophenacyl [269] quinacridine [270] or azidoproflavin (Figure 5.11) [271, 272] derivatives have been tethered to TFOs for the development of specific endonucleases and inhibit gene expression. Moreover, alkylating agents such as; bromoacetyl groups [273, 274] cyclopropanpyrroloindole [275] or diaziridinylquinone (Figure 5.11) [276] derivatives have also been linked to TFOs in order to generate site-specific interstrand crosslinks with target DNA.

## 5.9 Targeting single-stranded and double-stranded nucleic acids by triplex formation using clamps or hairpins. Strand-displacement

One of the strategies for increasing the affinity of oligonucleotides to bind nucleic acids by triplex formation is by linking the Hoogsteen-strand to one of the Watson–



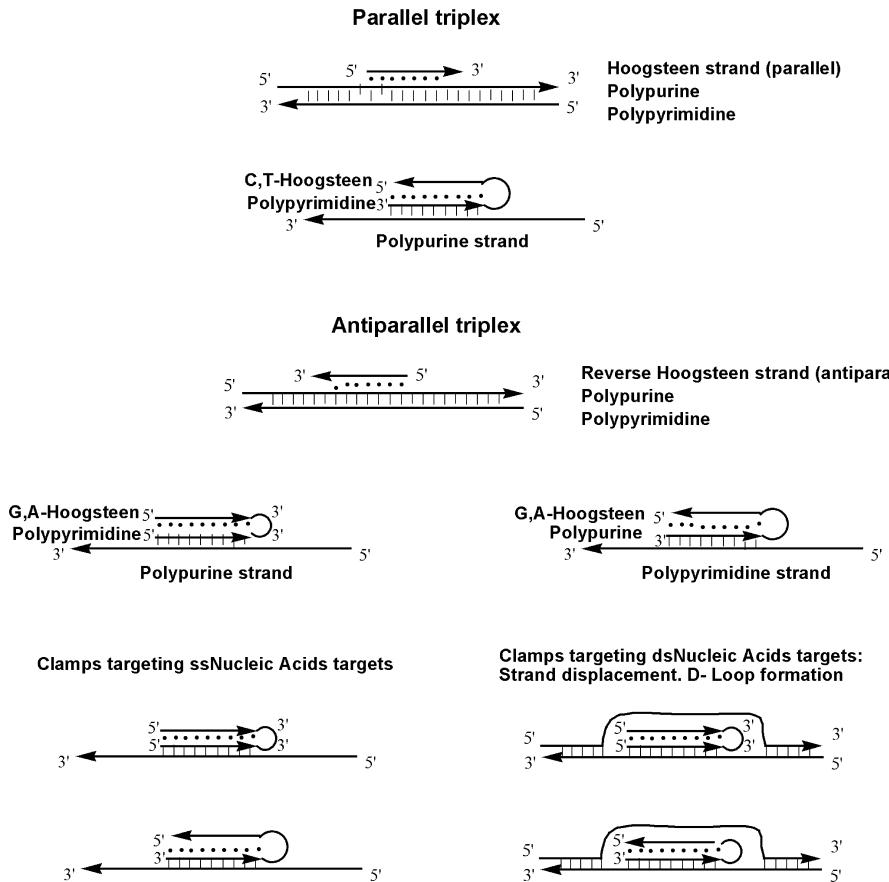
**Figure 5.11:** Chemical structures of compounds with potential triplex stabilization properties by crosslinking or intercalation.

Crick strands. This type of fold-back oligonucleotides are known as clamps or hairpins. These clamps or hairpins bind single-stranded nucleic acids with high affinity because the recognition properties of complementary oligonucleotides are complemented by the action of the TFO moiety. In some cases, it has been demonstrated that strong affinity of the clamps to their target sequence allows the binding to double-stranded nucleic acids. In this last example, the natural complementary sequence is displaced by the clamp due to the stronger binding properties of the clamp. This phenomena known as strand displacement has captured a large interest as it is one of the more effective methods to interact with double-stranded genomic DNA [14, 15].

As mentioned before, there are two types of triplexes: parallel triplexes and antiparallel triplexes (Figure 5.12) and each triplex has two strands (homopurine and homopyrimidine). For this reason, there are four potential ways of connecting the TFO and the Watson–Crick strands (Figure 5.12).

### 5.9.1 Targeting the polypurine strand

The most studied strategy for triplex formation using clamps is the one that targets the polypurine strand (Figure 5.12). The connection of the Hoogsteen polypyrimidine



**Figure 5.12:** Targeting single-stranded and double-stranded nucleic acids by triplex formation. Strand-displacement.

strand with the polypyrimidine Watson–Crick generates clamps that can be easily synthesized because the polarity of the strands allows the straight synthesis using regular phosphoramidites. For this reason, these clamps were the first to be synthesized and their properties studied [277]. In general, the connection between the Hoogsteen and the Watson–Crick polypyrimidine domains is done by adding linking molecules such as ethyleneglycol oligomers [277, 278] or 4–5 nucleotide residues [279–282]. These so-called foldback TFOs were demonstrated to bind to double-stranded DNA provoking the displacement of the complementary Watson–Crick polypyrimidine strand [283]. In addition to foldback TFOs, it is possible to connect both ends generating circular or cyclic oligonucleotides that have an extraordinary binding affinity to their target single-stranded nucleic acids [284–286]. Clamps and cyclic oligonucleotides have also been used for binding to adjacent pyrimidine/purine tracks [287].

The clamp strategy is profusely used with peptide nucleic acids (PNAs). As described in Section 5.7, homopyrimidine PNAs were found to have a strong affinity for homopurine DNA targets forming PNA.PNA.DNA triplexes that were able to displace the Watson–Crick polypyrimidine strand [238–241]. In these triplexes, two homopyrimidine PNA molecules participate in triplex formation. Then, connecting two homopyrimidine PNA molecules (*bis*-PNA), an extra entropic effect is added and the resulting *bis*-PNA acquires a strong affinity for polypurine targets [288, 289]. In order to avoid the pH dependence of the parallel triplex, the authors increase the affinity even further by replacing some of the Hoogsteen cytosines for pseudouracils [66, 289, 290]. The addition of oligomers of ethyleneglycol or positively charged linkers for the connection of the Hoogsteen and the Watson–Crick moieties has also a beneficial effect [66, 289]. The mechanism of strand displacement was confirmed with the addition of a peptide sequence GlyGlyHis that can be cleaved by nickel ions in the linker zone [291]. The study of the fragments generated after the formation of the triplexes followed by degradation with nickel ions confirmed the formation of a D-loop in which the formation of the triplex releases the Watson–Crick polypyrimidine strand [291]. The mechanism and specificity of the *bis*-PNA binding to double-stranded DNA has been studied [292, 293]. The large affinity and specificity of *bis*-PNA were found to be appropriate for specific binding at genomic levels and for *in vivo* applications [292, 294]. Short *bis*-PNA molecules in conjunction with methylases and restriction enzymes have been used for the generation of large DNA fragments of genomic DNA [295]. The use of *bis*-PNA clamps or PNA openers allows also the direct quantification of specific DNA targets [296]. The introduction of a fluorescent and a quencher derivative into a PNA probe generates PNA beacons that can be used for the direct detection of a specific DNA sequence in double-stranded DNA samples with the help of *bis*-PNAs clamps or PNA openers that facilitates hybridization by binding to the complementary strand [297]. *Bis*-PNA clamps have also been linked to peptides that are capable to recruit transcription factors. In this way, *bis*-PNAs are able to direct the deposition of transcription factors to a specific DNA area acting as artificial synthetic transcription activators [298]. Moreover, the addition of a 40 bases long donor DNA fragment to a *bis*-PNA clamp promotes the anchoring of the DNA fragment that facilitates the site-directed recombination that is used for the correction of mutations [299]. The strand displacement mechanism of *bis*-PNA clamps has been recently visualized using origami DNA and atomic force microscopy (AFM) [300]. The multiple applications of *bis*-PNA clamps have been reported in several reviews [301, 302].

### 5.9.2 Targeting the polypyrimidine strand

Targeting of the polypyrimidine has been achieved by two different families of fold-back triplex forming oligonucleotides [303]. First, the parallel clamps in which the

connection of the reversed Hoogsteen polypurine strand is made with the polypyrimidine Watson–Crick strand (Figure 5.12) [304]. This implies the formation of 3'-3' or 5'-5' internucleotide linkages [305] and the use of a combination of standard and reversed phosphoramidites [303]. Second, polypurine clamps or hairpins in where the polypurine reverse Hoogsteen is linked with the polypurine Watson–Crick strand (Figure 5.12) [306]. In this strategy, the orientation of the strands allows the synthesis using standard phosphoramidites. This last strategy known as PolyPurine Reverse Hoogsteen (PPRH) technology has been recently reviewed [307].

In addition to clamps or hairpins, other oligonucleotide DNA structures have been described in the bibliography. These include cyclic oligonucleotides [279, 287, 308], wedged [309] and tail-clamps [310, 311]. In all of these cases, a higher affinity polypyrimidine target and an increase in specificity has been reported.

The synthesis of parallel clamps needs the assembly of one of the strands in the reversed (5'-3') direction using 5'-phosphoramidites. Alternatively, it has been described the use of asymmetric branching units avoiding the need of reversed 5'-phosphoramidites [312]. An alternative method for the preparation of parallel clamps avoiding the use of 5'-phosphoramidites is the preparation of both polypurine and polypyrimidine strands in a separate support and link them using the Cu(I)-catalyzed cycloaddition reaction [313].

An important issue in the design of clamps and PPRHs is the presence of interruptions in the target polypyrimidine sequence. In parallel clamps, it has been suggested the use of the complementary base in the Watson–Crick strand and the use of C in the Hoogsteen strand for G interruptions and the use of G in A interruptions [314]. In PPRH, it has been suggested to add an A in the Watson–Crick position and another A in the Hoogsteen position [315] or maintaining the pyrimidine interruption within the PPRH sequence [309]. A concern on G-rich sequences present in PPRHs is the possibility to have competing G-quadruplex structures however, it has been reported that PPRHs can form tripleplexes even under conditions that facilitates G-quadruplex formation [316].

Parallel clamps have been used in the isolation of polypyrimidine RNA sequences of biological interest such as bacterial RNA [317] and miRNA [318]. Applications of the PolyPurine Reverse Hoogsteen (PPRH) technology include target validation in cancer research [319], inhibition of genes involved in cancer [320–325], correction of genes in mammalian cells [326] as well as for the capture of miRNA [327] and double-stranded DNA for the analysis of DNA methylation status [328] and for the detection of *Pneumocystis* [329].

## Bibliography

- [1] Felsenfeld G, Davies DR, Rich A. Formation of a three-stranded polynucleotide molecule. *J Am Chem Soc.* 1957;79:2023.
- [2] Rich A. Formation of two- and three-stranded helical molecules by polyinosinic and polyadenylic acid. *Nature.* 1958;181:521–5.
- [3] Thuong NT, Hélène C. Sequence-specific recognition and modification of double-helical DNA by oligonucleotides. *Angew Chem, Int Ed Engl.* 1993;32:666–90.
- [4] Chan PP, Triplex GPM. DNA: fundamentals, advances, and potential applications for gene therapy. *J Mol Med.* 1997;75:267–82.
- [5] Vasquez KM, Wilson JH. Triplex-directed modification of genes and gene activity. *Trends Biochem Sci.* 1998;23:4–9.
- [6] Hélène C. Sequence-selective recognition and cleavage of double-helical DNA. *Curr Opin Biotechnol.* 1993;4:29–36.
- [7] Gowers DM, Fox KR. Towards mixed sequence recognition by triple helix formation. *Nucleic Acids Res.* 1999;27:1569–77.
- [8] Doronina SO, Behr JP. Towards a general triple helix mediated DNA recognition scheme. *Chem Soc Rev.* 1997;26:63–71.
- [9] Fox KR. Targeting DNA with triplexes. *Curr Med Chem.* 2000;7:17–37.
- [10] Luyten I, Herdewijn P. Hybridization properties of base-modified oligonucleotides within the double and triple helix motif. *Eur J Med Chem.* 1998;33:515–76.
- [11] Robles J, Grandas A, Pedroso E, Luque FJ, Eritja R, Nucleic OM. Acid triple helices: stability effects of nucleobase modifications. *Curr Org Chem.* 2002;6:1333–68.
- [12] Malnuit V, Duca M, Targeting BR. DNA base pair mismatch with artificial nucleobases. Advances and perspectives in triple helix strategy. *Org Biomol Chem.* 2011;9:326–36.
- [13] Soyfer VN, Potaman VN. Triple-helical nucleic acids. New York: Springer; 1996.
- [14] Jain A, Wang G, Vasquez KM. DNA triple helices: biological consequences and therapeutic potential. *Biochimie.* 2008;90:1117–30.
- [15] Vasquez KM, Glazer PM. Triplex-forming oligonucleotides: principles and applications. *Q Rev Biophys.* 2002;35:89–107.
- [16] Mills M, Arimondo PB, Lacroix L, Garestier T, Hélène C, Klump H, Mergny JL. Energetics of strand-displacement reactions in triple helices: a spectroscopic study. *J Mol Biol.* 1999;291:1035–54.
- [17] Soliva R, Laughton CA, Luque FJ, Orozco M. Molecular dynamic simulations of d(G.C.C) triple helix in aqueous solution. *J Am Chem Soc.* 1998;120:11226–33.
- [18] Shields G, Laughton C, Orozco M. Molecular dynamics simulations of the d(T.A.T) triple helix. *J Am Chem Soc.* 1997;119:7463–9.
- [19] Singleton SF, Dervan PB. Influence of pH on the equilibrium association constants for oligodeoxyribonucleotide-directed triple helix formation at single DNA sites. *Biochemistry.* 1992;31:10995–1003.
- [20] Lee JS, Johnson DA, Morgan AR. Complexes formed by (pyrimidine) $n$  (purine) $n$  DNAs on lowering the pH are three-stranded. *Nucleic Acids Res.* 1979;6:3073–91.
- [21] Khomyakova EB, Gousset H, Liquier J, Huynh-Dinh T, Gouyette C, Takahashi M, Florentiev VL, Taillandier E. Parallel intramolecular DNA triple helix with G and T bases in the third strand stabilized by Zn (2+) ions. *Nucleic Acids Res.* 2000;28:3511–6.
- [22] Aviñó A, Cubero E, Gargallo R, González C, Orozco M, Eritja R. Structural properties of G,T-parallel duplexes. *J Nucleic Acids.* 2010;2010:763658.

- [23] Scaria PV, Shafer RH. Calorimetric analysis of triple helices targeted to the d(G<sub>3</sub>A<sub>4</sub>G<sub>3</sub>) d(C<sub>3</sub>T<sub>4</sub>C<sub>3</sub>) duplex. *Biochemistry*. 1996;35:10985–94.
- [24] Chandler SP, Fox KR. Specificity of antiparallel DNA triple helix formation. *Biochemistry*. 1996;35:15038–48.
- [25] Beal PA, Dervan PB. Second structural motif for recognition of DNA by oligonucleotide-directed triple helix formation. *Science*. 1991;251:1360–3.
- [26] Aviñó A, Cubero E, González C, Eritja R, Orozco M. Antiparallel triple helices. Structural characteristics and stabilization by 8-amino derivatives. *J Am Chem Soc*. 2003;125:16127–38.
- [27] Keppler MD, Neidle S, Fox KR. Stabilization of TG- and AG-containing antiparallel DNA triplexes by triplex-binding ligands. *Nucleic Acids Res*. 2001;29:1935–42.
- [28] Yang M, Ghosh SS, Millar DP. Direct measurement of thermodynamic and kinetic parameters of DNA triple helix formation by fluorescence spectroscopy. *Biochemistry*. 1994;33:15329–37.
- [29] Jaumot J, Eritja R, Aviñó A, Tauler R, Gargallo R. Analytical resolution of parallel and antiparallel oligonucleotide triple helices formation and melting processes by means of multivariate curve resolution. *J Biomol Struct Dyn*. 2003;21:267–78.
- [30] Murphy D, Eritja R, Redmond G. Monitoring denaturation behaviour and comparative stability of DNA triple helices using oligonucleotide-gold nanoparticle conjugates. *Nucleic Acids Res*. 2004;32:e65.
- [31] Lee JS, Woodsworth ML, Latimer LJ, Morgan AR. Poly (pyrimidine) poly (purine) synthetic DNAs containing 5-methylcytosine form stable triplexes at neutral pH. *Nucleic Acids Res*. 1984;12:6603–14.
- [32] Povsic TJ, Dervan PB. Triple helix formation by oligonucleotides on DNA extended to the physiological pH range. *J Am Chem Soc*. 1989;111:3059–61.
- [33] Xodo LE, Manzini G, Quadrifoglio F, van der Marel GA, van Boom JH. Effect pf 5-methylcytosine on the stability of triple-stranded DNA-a thermodynamic study. *Nucleic Acids Res*. 1991;19:5625–31.
- [34] Lee JS, Woodsworth ML, Latimer LJP. Monoclonal antibodies specific for poly (dG) Poly (dC) and poly (dG) Poly (dm5C). *Biochemistry*. 1984;23:3277–81.
- [35] Ferrer E, Fàbrega C, Güimil-García R, Azorín F, Eritja R. Preparation of oligonucleotides containing 5-bromouracil and 5-methylcytidine. *Nucleosides Nucleotides*. 1996;15:907–21.
- [36] Ferrer E, Wiersma M, Kazimierczak B, Müller CW, Eritja R. Preparation and properties of oligodeoxynucleotides containing 5-ido-uracil, 5-bromo- and 5-ido-cytosine. *Bioconjug Chem*. 1997;8:757–61.
- [37] Ferrer E, Neubauer G, Mann M, Eritja R. Synthesis and biophysical properties of oligonucleotides containing 5-amino-2'-deoxyuracil and 5-N-acetamido-2'-deoxyuracil. *J Chem Soc, Perkin Trans I*. 1997;2051–8.
- [38] Eritja R, Adam V, Aviñó A, Díaz AR, Fàbrega C, Ferrer E, Grøtli M, Güimil García R, Hofmann M, Márquez VE, Wiersma M. Preparation of oligonucleotides containing non-natural base analogs. *Nucleosides Nucleotides*. 1997;16:697–702.
- [39] Yang L, Liu M, Deng W, Wang C, Bai C, Kan L-S. Influence of 5-bromodeoxycytosine substitution on triplex DNA stability and conformation. *Biophys Chem*. 1999;76:25–34.
- [40] Barawkar DA, Kumar VA, Ganesh KN. Triplex formation at physiological pH by oligonucleotides incorporating 5-Me-dC-(N<sup>4</sup>-spermine). *Biochem Biophys Res Commun*. 1994;205:1665–70.
- [41] Barawkar DA, Rajeev KG, Kumar VA, Ganesh KN. Triplex formation at physiological pH by 5-Me-dC-N<sup>4</sup>-(spermine) [X] oligodeoxynucleotides: non protonation of N<sup>3</sup> in X of X\*G:C triad and effect of base mismatch/ionic strength on triplex stabilities. *Nucleic Acids Res*. 1996;24:1229–37.

- [42] Rajeev KG, Jadhav VR, Ganesh KN. Triplex formation at physiological pH: comparative studies on DNA triplexes containing 5-Me-dC tethered at N<sup>4</sup> with spermine and tetraethyleneoxyamine. *Nucleic Acids Res.* 1997;25:4187–93.
- [43] Hampel KJ, Crosson P, Lee JS. Polyamines favor DNA triplex formation at neutral pH. *Biochemistry.* 1991;30:4455–9.
- [44] Tung CH, Breslauer KJ, Stein S. Polyamine-linked oligonucleotides for DNA triple helix formation. *Nucleic Acids Res.* 1993;21:5489–94.
- [45] Froehler BC, Wadwani S, Terhorst TJ, Gerrard SR. Oligodeoxynucleotides containing C-5 propyne analogs of 2'-deoxyuridine and 2'-deoxycytidine. *Tetrahedron Lett.* 1992;33:5307–10.
- [46] Eritja R, Ferrer E, Güimil-García R, Orozco M. Modified oligonucleotides with triple-helix stabilization properties. *Nucleosides Nucleotides.* 1999;18:1619–21.
- [47] Colocci N, Dervan PB. Cooperative binding of 8-mer oligonucleotides containing 5-(1-propynyl)-2'-deoxyuridine to adjacent DNA sites by triple-helix formation. *J Am Chem Soc.* 1994;116:785–6.
- [48] Phipps AK, Tarköy M, Schultze P, Feigón J. Solution structure of an intramolecular DNA triplex containing 5-(1-propynyl)-2'-deoxyuridine residues in the third strand. *Biochemistry.* 1998;37:5820–30.
- [49] Mills M, Arimondo PB, Lacroix L, Garestier T, Klump H, Mergny JL. Chemical modification of the third strand: differential effects on purine and pyrimidine triple helix formation. *Biochemistry.* 2002;41:357–66.
- [50] Bijapur J, Keppler MD, Berqvist S, Brown T, Fox KR. 5-(1-propargylamino)-2'-deoxyuridine (U<sup>P</sup>): a novel thymidine analogue for generating DNA triplexes with increased stability. *Nucleic Acids Res.* 1999;27:1802–9.
- [51] Sollogoub M, Domínguez B, Fox KR, Brown T. Synthesis of a novel bis-amino-modified thymidine monomer for use in DNA triplex stabilization. *Chem Commun.* 2000;2315–6.
- [52] Sollogoub M, Darby RAJ, Cuenoud B, Brown T, Stable FKR. DNA triple helix formation using oligonucleotides containing 2'-aminoethoxy, 5-propargylamino-U. *Biochemistry.* 2002;41:7224–31.
- [53] Cuenoud B, Casset F, Husken D, Natt F, Wolf RM, Altmann K-H, Marti P, Moser HE. Dual recognition of double-stranded DNA by 2'-aminoethoxy-modified oligonucleotides. *Angew Chem, Int Ed Engl.* 1998;37:1288–91.
- [54] Michel J, Moreau S. Use of C5-diamino-substituted-pyridine in triple helix forming oligonucleotides. *Nucleosides Nucleotides.* 1999;18:1633–7.
- [55] Ono A, Haginoya N, Kiyokawa M, Minakawa N, Matsuda A. A novel and convenient post-synthetic modification method for the synthesis of oligodeoxyribonucleotides carrying amino linkers at the 5 position of 2'-deoxyuridine. *Bioorg Med Chem Lett.* 1994;4:361–6.
- [56] Staubli AB, Dervan PB. Sequence specificity of the non-natural pyrido[2,3-d]pyrimidine nucleoside in triple helix formation. *Nucleic Acids Res.* 1994;22:2637–42.
- [57] Michel J, Toulmé J-J, Vercauteren J, Moreau S. Quinazoline-2,4(1H,3H)-dione as a substitute for thymine in triple-helix forming oligonucleotides: a reassessment. *Nucleic Acids Res.* 1996;24:1127–35.
- [58] Michel J, Gueguen G, Vercauteren J, Moreau S. Triplex stability of oligodeoxynucleotides containing substituted quinazoline-2, 4-(1H, 3H)-dione. *Tetrahedron.* 1997;53:8457–78.
- [59] Godde F, Toulmé J-J, Moreau S. Benzoquinazoline derivatives as substitutes for thymine in nucleic acid complexes. Use of fluorescence emission of benzo[g]quinazoline-2,4-(1H,3H)-dione in probing duplex and triplex formation. *Biochemistry.* 1998;37:13765–75.

- [60] Godde F, Aupeix K, Moreau S, Tolumé J-J. A fluorescent base analog for probing triple helix formation. *Antisense Nucleic Acid Drug Dev.* 1998;8:469–76.
- [61] Arzumanov A, Godde F, Moreau S, Tolumé J-J, Weeds A, Gait MJ. Use of the fluorescent nucleoside analogue benzo[g]quinazoline 2'-O-methyl- $\beta$ -D-ribofuranoside to monitor the binding of the HIV-1 Tat protein or of antisense oligonucleotides to the TAR RNA stem loop. *Helv Chim Acta.* 2000;83:1424–36.
- [62] Godde F, Toulmé J-J, Moreau S. 4-amino-1H-benzo[g]quinazoline-2-one: a fluorescent analog of cytosine to probe protonation sites in triplex forming oligonucleotides. *Nucleic Acids Res.* 2000;28:2977–85.
- [63] Ono A, Ts'o POP, Kan LS. Triplex formation of oligonucleotides containing 2'-O-methylpseudoisocytidine in substitution for 2'-deoxycytidine. *J Am Chem Soc.* 1991;113:4132–3.
- [64] Ono A, Ts'o POP, Kan LS. Triplex formation of an oligonucleotide containing 2'-O-methylpseudoisocytidine with a DNA duplex at neutral pH. *J Org Chem.* 1992;57:3225–30.
- [65] Chin T-M, Lin S-B, Lee S-Y, Chang M-L, Cheng AY-Y, Chang F-C, Pasternack L, Huang D-H, Kan LS. "Paper-Clip" type triple helix formation by 5'-d-(TC)<sub>3</sub>Ta(CT)<sub>3</sub>Cb(AG)<sub>3</sub> ( $a$  and  $b$  = 0–4) as a function of loop size with and without the pseudoisocytosine base in the Hoogsteen strand. *Biochemistry.* 2000;39:12457–64.
- [66] Egholm M, Christensen L, Dueholm KL, Buchardt O, Coull J, Nielsen PE. Efficient pH-independent sequence-specific DNA binding by pseudoisocytosine-containing bis-PNA. *Nucleic Acids Res.* 1995;23:217–22.
- [67] von Krosigk U, Benner SA. pH-independent triple helix formation by an oligonucleotide containing a pyrazine donor-donor-acceptor base. *J Am Chem Soc.* 1995;117:5361–2.
- [68] Häberli A, Leumann CJ. Synthesis of pyrrolidine C-nucleosides via Heck reaction. *Org Lett.* 2001;3:489–92.
- [69] Häberli A, Leumann CJ. DNA binding properties of oligodeoxynucleotides containing pyrrolidino C-nucleosides. *Org Lett.* 2002;4:3275–8.
- [70] Mayer A, Häberli A, Leumann CJ. Synthesis and triplex forming properties of pyrrolidino pseudoisocytidine containing oligodeoxynucleotides. *Org Biomol Chem.* 2005;3:1653–8.
- [71] Xiang G, Soussou W, McLaughlin LW. A new pyrimidine nucleoside (m5oxC) for the pH-independent recognition of GC base pairs by oligonucleotide-directed triplex formation. *J Am Chem Soc.* 1994;116:11155–6.
- [72] Berressem R, Engels JW. 6-Oxocytidine a novel protonated C-base analogue for stable triple helix formation. *Nucleic Acids Res.* 1995;23:3465–72.
- [73] Xiang G, Bogacki R, McLaughlin LW. Use of a pyrimidine nucleoside that functions as a bidentate hydrogen bond donor for the recognition of isolated or contiguous GC base pairs by oligonucleotide-directed triplex formation. *Nucleic Acids Res.* 1996;24:1963–70.
- [74] Xiang G, McLaughlin LW. A cytosine analogue containing a conformationally flexible acyclic linker for triplex formation at sites with contiguous GC base pairs. *Tetrahedron.* 1998;54:375–92.
- [75] Parsch U, Engels JW. pH-Independent triple-helix formation with 6-oxocytidine as cytidine analogue. *Chem Eur J.* 2000;6:2409–24.
- [76] Bédu E, Benhida R, Devys M, Novel FJ-L. 2'-deoxycytidine analogues as pH independent substitutes of protonated cytosines in triple helix forming oligonucleotides. *Tetrahedron Lett.* 1999;40:835–8.
- [77] Miller PS, Bhan P, Cushman CD, Trapane TL. Recognition of a guanine-cytosine base pair by 8-oxoadenine. *Biochemistry.* 1992;31:6788.

- [78] Jetter MC, Hobbs FW. 7, 8-Dihydro-8-oxoadenine as a replacement for cytosine in the third strand of triple helixes. Triplex formation without hypochromicity. *Biochemistry*. 1993;32:3249–54.
- [79] Miller PS, Bi G, Kipp SA, Fok V, Delong RK. Triplex formation by a psoralen-conjugated oligodeoxyribonucleotide containing the base analog 8-oxo-adenine. *Nucleic Acids Res.* 1996;24:730–6.
- [80] Krawczyk SH, Milligan JF, Wadwani S, Moulds C, Froehler BC, Matteucci MD. Oligonucleotide-mediated triple helix formation using an N<sup>3</sup>-protonated deoxycytidine analog exhibiting pH-independent binding within the physiological range. *Proc Natl Acad Sci USA*. 1992;89:3761–4.
- [81] Rao TS, Durland RH, Revankar GR. Synthesis of oligonucleotides containing 7-(2-deoxy- $\beta$ -D-erythro-pentofuranosyl)guanine and 8-amino-2'-deoxyguanosine. *J Heterocycl Chem.* 1994;31:935–40.
- [82] Hunziker J, Priestley ES, Brunar H, Dervan PB. Design of an N<sup>7</sup>-glycosylated purine nucleoside for recognition of GC base pairs by triple helix formation. *J Am Chem Soc.* 1995;117:2661–2.
- [83] Brunar H, Dervan PB. Sequence composition effects on the stabilities of triple helix formation by oligonucleotides containing N<sup>7</sup>-deoxyguanosine. *Nucleic Acids Res.* 1996;24:1987–91.
- [84] Koh JS, Dervan PB. Design of a nonnatural deoxyribonucleoside for recognition of GC base pairs by oligonucleotide-directed triple helix formation. *J Am Chem Soc.* 1992;114:1470–8.
- [85] Priestley ES, Dervan PB. Sequence composition effects on the energetics of triple helix formation by oligonucleotides containing a designed mimic of protonated cytosine. *J Am Chem Soc.* 1995;117:4761–5.
- [86] Radhakrishnan J, Patel DJ, Priestley ES, Nash HM, Dervan PB. NMR structural studies on a nonnatural deoxyribonucleoside which mediates recognition of GC base pairs in pyrimidine-purine-pyrimidine DNA tripleplexes. *Biochemistry*. 1993;32:11228–34.
- [87] Marfurt J, Hunziker J, Leumann C. Recognition of a GC base pair by  $\alpha$ -N<sup>7</sup>-deoxyinosine within the pyrimidine-purine-pyrimidine DNA triple helical motif. *Bioorg Med Chem Lett.* 1996;6:3021–4.
- [88] Marfurt J, Parel SP, Leumann C. Strong, specific, monodentate GC base pair recognition by N<sup>7</sup>-inosine derivatives in the pyrimidine-purine-pyrimidine triple-helical binding motif. *Nucleic Acids Res.* 1997;25:1875–82.
- [89] Marfurt J, Leumann C. Evidence for C–H···O hydrogen bond assisted recognition of a pyrimidine base in the parallel DNA triple-helical motif. *Angew Chem, Int Ed Engl.* 1998;37:175–7.
- [90] Bates PJ, Laughton CA, Jenkins TC, Capaldi DC, Roselt PD, Reese CB, Neidle S. Efficient triple helix formation by oligodeoxyribonucleotides containing  $\alpha$ -or  $\beta$ -2-amino-5-(2-deoxy-D-ribofuranosyl) pyridine residues. *Nucleic Acids Res.* 1996;24:4176–84.
- [91] Cassidy SA, Slickers P, Trent JO, Capaldi DC, Roselt PD, Reese CB, Neidle S. Recognition of GC base pairs by triplex forming oligonucleotides containing nucleosides derived from 2-aminopyridine. *Nucleic Acids Res.* 1997;25:4891–8.
- [92] Hildbrand S, Blaser A, Parel SP, Leumann CJ. 5-Substituted 2-aminopyridine C-nucleosides as protonated cytidine equivalents: increasing efficiency and selectivity in DNA triple-helix formation. *J Am Chem Soc.* 1997;119:5499–511.
- [93] Hildbrand S, Enhancing LCJ. DNA triple helix stability at neutral pH by the use of oligonucleotides containing a more basic deoxycytidine analog. *Angew Chem, Int Ed Engl.* 1996;35:1968–70.
- [94] Chen DL, McLaughlin LW. Use of pKa differences to enhance the formation of base triplets involving C–G and G–C base pairs. *J Org Chem.* 2000;65:7468–74.

- [95] Pudlo JS, Wadwani S, Milligan JF, Matteucci MD. Synthesis of 6-amino-2'-O-methylcytidine, a protonated cytidine analog for triple helix binding studies. *Bioorg Med Chem Lett.* 1994;4:1025–8.
- [96] Doronina SO, Behr J-P. Synthesis of 4-guanidinopyrimidine nucleosides for triple helix-mediated guanine and cytosine recognition. *Tetrahedron Lett.* 1998;39:547–50.
- [97] Robles J, Grandas A, Pedroso E. Synthesis of modified oligonucleotides containing 4-guanidino-2-pyrimidinone nucleobases. *Tetrahedron.* 2001;57:179–94.
- [98] Blanalt-Feidt S, Doronina SO, Behr J-P. Synthesis of non-natural pyrimidine nucleosides. *Nucleosides Nucleotides.* 1999;18:605–6.
- [99] Blanalt-Feidt S, Doronina SO, Behr J-P. Synthesis of C-4 substituted pyrimidines exhibiting various H-bonding patterns. *Tetrahedron Lett.* 1999;40:6229–32.
- [100] Stilz HU, Dervan PB. Specific recognition of CG base pairs by 2'-deoxynebularine within the purine-purine-pyrimidine triple-helix motif. *Biochemistry.* 1993;32:2177–85.
- [101] Raynaud F, Asseline U, Roig V, Thuong NT. Synthesis and characterization of O<sup>6</sup>-modified deoxyguanosine-containing oligodeoxyribonucleotides for triple-helix formation. *Tetrahedron.* 1996;52:2047–64.
- [102] Roig V, Kurfust R, Thuong NT. Oligo-β-and-α-deoxyribonucleotides involving 2-aminopurine and guanine for triple-helix formation. *Tetrahedron Lett.* 1993;34:1601–4.
- [103] Parel SP, Leumann CJ. Triple-helix formation in the antiparallel binding motif of oligodeoxynucleotides containing N9- and N7-2-aminopurine deoxynucleosides. *Nucleic Acids Res.* 2001;29:2260–7.
- [104] Olivas WM, Maher-III LJ. Overcoming potassium-mediated triplex inhibition. *Nucleic Acids Res.* 1995;23:1936–41.
- [105] Marathias VM, Sawicki MJ, Bolton PH. 6-Thioguanine alters the structure and stability of duplex DNA and inhibits quadruplex DNA formation. *Nucleic Acids Res.* 1999;27:2860–7.
- [106] Rao TS, Durland RH, Seth DM, Myrick MA, Bodepudi V, Revankar GR. Incorporation of 2'-deoxy-6-thioguanosine into G-rich oligodeoxyribonucleotides inhibits G-tetrad formation and facilitates triplex formation. *Biochemistry.* 1995;34:765–72.
- [107] Gee JE, Revankar GR, Rao TS, Hogan ME. Triplex formation at the rat neu gene utilizing imidazole and 2'-deoxy-6-thioguanosine base substitutions. *Biochemistry.* 1995;34:2042–8.
- [108] Milligan JF, Krawczyk SH, Wadwani S, Matteucci MD. An anti-parallel triple helix motif with oligodeoxynucleotides containing 2'-deoxyguanosine and 7-deaza-2'-deoxy xanthosine. *Nucleic Acids Res.* 1993;21:327–33.
- [109] Faraji AF, Krawczyk SH, Matteucci MD, Glazer PM. Potassium-resistant triple helix formation and improved intracellular gene targeting by oligodeoxyribonucleotides containing 7-deazaxanthine. *Nucleic Acids Res.* 1997;25:633–40.
- [110] Rao TS, Lewis AF, Durland RH, Revankar GR. A total synthesis of 2'-deoxy-9-deazaguanosine (9-deaza-dG) and its incorporation into triple helix forming oligodeoxyribonucleotides with antiparallel motif. *Tetrahedron Lett.* 1993;34:6709–12.
- [111] Rao TS, Lewis AF, Hill TS, Revankar GR. Incorporation of 2'-deoxy-9-deazaguanosine and 2'-deoxy-7-deaza-6-thioguanosine into G-rich oligodeoxyribonucleotides. *Nucleosides Nucleotides.* 1995;14:1–12.
- [112] Aubert Y, Perrouault L, Hélène C, Giovannangeli C, Asseline U. Synthesis and properties of triple helix-forming oligodeoxyribonucleotides containing 7-chloro-7-deaza-2'-deoxyguanosine. *Bioorg Med Chem.* 2001;9:1617–24.
- [113] Griffin LC, Kiessling LL, Beal PA, Gillespie P, Dervan PB. Recognition of all four base pairs of double-helical DNA by triple-helix formation: design of non-natural deoxyribonucleosides for pyrimidine-purine base pair. *J Am Chem Soc.* 1992;114:7976–82.

- [114] Koshlap KM, Gillespie P, Dervan PB, Feigon J. Nonnatural deoxyribonucleoside D3 incorporated in an intramolecular DNA triplex binds sequence-specifically by intercalation. *J Am Chem Soc.* 1993;115.
- [115] Wang E, Koshlap KM, Gillespie P, Dervan PB, Feigon J. Solution structure of a pyrimidine-purine-pyrimidine triplex containing the sequence-specific intercalating non-natural base D3. *J Mol Biol.* 1996;257:1052–69.
- [116] Huang CY, Miller PS. Triplex formation by an oligodeoxyribonucleotide containing N<sup>4</sup>-(6-aminopyridinyl)-2'-deoxycytidine. *J Am Chem Soc.* 1993;115:10456–7.
- [117] Verma S, Miller PS. Interactions of cytosine derivatives with T-A interruptions in pyrimidine-purine-pyrimidine DNA triplexes. *Bioconjug Chem.* 1996;7:600–5.
- [118] Huang CY, Bi G, Miller PS. Triplex formation by oligonucleotides containing novel deoxycytidine derivatives. *Nucleic Acids Res.* 1996;24:2606.
- [119] Robles J, Grandas A, Pedroso E. 4-Guanidino-2-pyrimidinone nucleobases: synthesis and hybridization properties. *Nucleosides Nucleotides Nucleic Acids.* 2003;22:1085–7.
- [120] Guzzo-Pernell N, Tregebar GW, Haralambidis J, Lawlor JM. The design and synthesis of N<sup>4</sup>-anthraniloyl-2'-dC, the improved syntheses of N<sup>4</sup>-carbamoyl-and N<sup>4</sup>-ureidocarbamoyl-2'-dC, incorporation into oligonucleotides and triplex formation testing. *Nucleosides Nucleotides.* 1998;17:1191–207.
- [121] Li J-S, Fan YH, Zhang Y, Marky LA, Gold B. Design of triple helix forming C-glycoside molecules. *J Am Chem Soc.* 2003;125:2084–93.
- [122] Li J-S, Shikiya R, Marky LA, Gold B. Triple helix forming TRIPside molecules that target mixed purine/pyrimidine DNA sequences. *Biochemistry.* 2004;43:1440–8.
- [123] Li J-S, Chen F-X, Shikiya R, Marky LA, Gold B. Molecular recognition via triplex formation of mixed purine/pyrimidine DNA sequences using oligoTRIPs. *J Am Chem Soc.* 2005;127:12657–65.
- [124] Ranasinghe RT, Rusling DA, Powers VEC, Fox KR, Brown T. Recognition of CG inversions in DNA triple helices by methylated 3 H-pyrrolo [2,3-d] pyrimidin-2 (7 H)-one nucleoside analogues. *Chem Commun.* 2005;2555–7.
- [125] Rusling DA, Powers VEC, Ranasinghe RT, Wang Y, Osborne SD, Fox KR, Brown T. Four base recognition by triplex-forming oligonucleotides at physiological pH. *Nucleic Acids Res.* 2005;33:3025–32.
- [126] Gerrard SR, Srinivasan N, Fox KR, Brown T. CG Base pair recognition within Dna triple helices using N-methyl-3H-pyrrolo[2,3-d]pyrimidin-2(7H)-one nucleoside analogues. *Nucleosides Nucleotides Nucleic Acids.* 2007;26:1363–7.
- [127] Lehmann TE, Greenberg WA, Liberles DA, Wada CK, Dervan PB. Triple-helix formation by pyrimidine oligonucleotides containing non-natural nucleosides with extended aromatic nucleobases: intercalation from the major groove as a method for recognizing C-G and T-A base pairs. *Helv Chim Acta.* 1997;80:2002–22.
- [128] Sasaki S, Nakashima S, Nagatsugi F, Tanaka Y, Hisatome M, Maeda M. Design of a novel artificial nucleobase for the selective formation of a triple-complex with a cytosine-guanine base pair. *Tetrahedron Lett.* 1995;36:9521–4.
- [129] Lengeler D, Weisz K. Novel phthalimide nucleosides for the specific recognition of a CG Watson–Crick base pair. *Tetrahedron Lett.* 2001;42:1479–81.
- [130] Xiao Z, Weisz K. Substituent effects of phthalimide-based nucleoside analogs on binding a CG Watson–Crick base pair. *J Phys Org Chem.* 2007;20:771–7.
- [131] Zimmermann SC, Schmitt P. Model studies directed toward a general triplex DNA recognition scheme: a novel DNA base that binds a CG base-pair in an organic solvent. *J Am Chem Soc.* 1995;117:10769–70.

- [132] Lecubin F, Benhida R, Fourrey J-L, Sun J-S. NMR recognition studies of C·G base pairs by new easily accessible heterobicyclic systems. *Tetrahedron Lett.* 1999;40:8085–8.
- [133] Mertz E, Mattei S, Zimmerman SC. Synthetic receptors for CG base pairs. *Org Lett.* 2000;19:2931–4.
- [134] Mertz E, Mattei S, Zimmerman SC. Synthesis and duplex DNA recognition studies of oligonucleotides containing a ureido isoindolin-1-one homo-N-nucleoside. A comparison of host–guest and DNA recognition studies. *Bioorg Med Chem.* 2004;12:1517–26.
- [135] Wang W, Purwanto MGM, Weisz K. CG base pair recognition by substituted phenylimidazole nucleosides. *Org Biomol Chem.* 2004;2:1194–8.
- [136] Purwanto MGM, Weisz K. Binding of imidazole-derived nucleosides to a CG base pair. *J Org Chem.* 2004;69:195–7.
- [137] Purwanto MGM, Weisz K. Non-natural nucleosides for the specific recognition of Watson–Crick base pairs. *Curr Org Chem.* 2003;7:427–46.
- [138] Purwanto MGM, Lengeler D, Weisz K. Nucleosides derived from urocanic acid: potential ligands for CG base pairs. *Tetrahedron Lett.* 2002;43:61–4.
- [139] Guianvarc'h D, Fourrey JL, Maurisse R, Sun JS, Synthesis BR. Incorporation into triplex-forming oligonucleotide, and binding properties of a novel 2'-deoxy-C-nucleoside featuring a 6-(thiazolyl-5) benzimidazole nucleobase. *Org Lett.* 2002;4:4209–12.
- [140] Jazouli M, Guianvarc'h D, Bougrin K, Soufiaoui M, Vierling P, Benhida R. Synthesis and studies of modified oligonucleotides-directed triple helix formation at the purine-pyrimidine interrupted site. *Nucleosides Nucleotides Nucleic Acids.* 2003;22:1277–80.
- [141] Guianvarc'h D, Benhida R, Fourrey JL, Maurisse R, Sun JS. Incorporation of a novel nucleobase allows stable oligonucleotide-directed triple helix formation at the target sequence containing a purine · pyrimidine interruption. *Chem Comm.* 2001;1814–5.
- [142] Guianvarc'h D, Fourrey J-L, Maurisse R, Sun J-S, Benhida R. Design of artificial nucleobases for the recognition of the AT inversion by triple-helix forming oligonucleotides: a structure–stability relationship study and neighbor bases effect. *Bioorg Med Chem.* 2003;11:2751–9.
- [143] Saito A, Kuroda R. Design and synthesis of a new type of modified nucleoside for triple helix-mediated adenine-thymine base pair recognition: formation of hydrogen bonds to the far-side adenine. *Tetrahedron Lett.* 1999;40:4837–40.
- [144] Van Craynest N, Guianvarc'h D, Peyron C, Benhida R. Efficient synthesis of extended guanine analogues designed for recognition of an A.T inverted base pair in triple helix based-strategy. *Tetrahedron Lett.* 2004;45:6243–7.
- [145] Rusling DA, Brown T, Fox KR. DNA triple-helix formation at target sites containing duplex mismatches. *Biophys Chem.* 2006;123:134–40.
- [146] Nagatsugi F, Matsuyama Y, Maeda M, Sasaki S. Selective cross-linking to the adenine of the TA interrupting site within the triple helix. *Bioorg Med Chem Lett.* 2002;12:487–9.
- [147] Beal PA, Dervan PB. The influence of single base triplet changes on the stability of a Pur·Pur-Pyr triple helix determined by affinity cleaving. *Nucleic Acids Res.* 1992;20:2773–6.
- [148] Griffin LC, Dervan PB. Recognition of thymine adenine. Base pairs by guanine in a pyrimidine triple helix motif. *Science.* 1989;245:967–71.
- [149] Jiang L, Russu IM. Proton exchange and local stability in a DNA triple helix containing a G·TA triad. *Nucleic Acids Res.* 2001;29:4231–7.
- [150] Kandimalla ER, Manning AN, Venkataraman G, Sasisekharan V, Agrawal S. Single strand targeted triplex formation: targeting purine-pyrimidine mixed sequences using abasic linkers. *Nucleic Acids Res.* 1995;23:4510–7.

- [151] Orson FM, Klysik J, Bergstrom DE, Ward B, Glass GA, Hua P, Kinsey BM. Triple helix formation: binding avidity of acridine-conjugated AG motif third strands containing natural, modified and surrogate bases opposed to pyrimidine interruptions in a polypurine target. *Nucleic Acids Res.* 1999;27:810–6.
- [152] Durland RH, Rao TS, Bodepudi V, Seth DM, Jayaraman K, Revankar GR. Azole substituted oligonucleotides promote antiparallel triplex formation at non-homopurine duplex targets. *Nucleic Acids Res.* 1995;23:647–53.
- [153] Kukreti S, Sun JS, Loakes D, Brown D, Nguyen CH, Bisagni E, Garestier T, Hélène C. Triple helices formed at oligopyrimidine-oligopurine sequences with base pair inversions: effect of a triplex-specific ligand on stability and selectivity. *Nucleic Acids Res.* 1998;26:2179–83.
- [154] Durland RH, Rao TS, Revankar GR, Tinsley JH, Myrick MA, Seth DM, Rayford J, Singh P, Jayaraman K. Binding of T and T analogs to CG base pairs in antiparallel triplexes. *Nucleic Acids Res.* 1994;22:3233–40.
- [155] Amasova OA, Fresco JR. A search for base analogs to enhance third-strand binding to 'inverted' target base pairs of triplexes in the pyrimidine/parallel motif. *Nucleic Acids Res.* 1999;27:4632–5.
- [156] Mokhir AA, Connors WH, Richert C. Synthesis and monitored selection of nucleotide surrogates for binding T: a base pairs in homopurine–homopyrimidine DNA triple helices. *Nucleic Acids Res.* 2001;29:3674–84.
- [157] Rao TS, Hogan ME, Revankar GR. Synthesis of triple helix forming oligonucleotides containing 2'-deoxyformycin A. *Nucleosides Nucleotides.* 1994;13:95–107.
- [158] Horne DA, Dervan PB. Recognition of mixed-sequence duplex DNA by alternate-strand triple-helix formation. *J Am Chem Soc.* 1990;112:2435–7.
- [159] McCurdy S, Moulds C, Froehler B. Deoxyoligonucleotides with inverted polarity synthesis and use in triple-helix formation. *Nucleosides Nucleotides.* 1991;10:287–90.
- [160] De Napoli L, Messere A, Montesarchio D, Pepe A, Piccialli G, Varra M. Synthesis and triple helix formation by alternate strand recognition of oligonucleotides containing 3'-3' phosphodiester bonds. *J Org Chem.* 1997;62:9024–30.
- [161] De Napoli L, Di Fabio G, Messere A, Montesarchio D, Piccialli G, Varra M. A Facile solid-phase synthesis of oligonucleotides containing a 3'-3' phosphodiester bond for alternate strand triple-helix formation. *Eur J Org Chem.* 1998;2119–25.
- [162] Barone G, De Napoli L, Di Fabio G, Giancola C, Messere A, Montesarchio D, Petraccone L, Piccialli G. Oligonucleotides containing a lysine residue as 3'-3' junction for alternate strand triple helix formation. *Bioorg Med Chem.* 2001;9:2895–900.
- [163] Hoshika S, Ueno Y, Matsuda A. Nucleosides and nucleotides. 218. Alternate-strand triple-helix formation by the 3'-3'-linked oligodeoxynucleotides using a purine motif. *Bioconjug Chem.* 2003;14:607–13.
- [164] Hoshika S, Ueno Y, Kamiya H, Matsuda A. Nucleosides and nucleotides. Part 226: Alternate-strand triple-helix formation by 3'-3'-linked oligodeoxynucleotides composed of asymmetrical sequences. *Bioorg Med Chem Lett.* 2004;14:3333–6.
- [165] Ueno Y, Mikawa M, Hoshika S. Nucleosides and Nucleotides. 208. Alternate-strand triple-helix formation by the 3'-3'-linked oligodeoxynucleotides with the anthraquinonyl group at the junction point. *Bioconjug Chem.* 2001;12:635–42.
- [166] Asseline U, Thuong NT. 5'-5' Tethered oligonucleotides via nucleic bases: a potential new set of compounds for alternate strand triple-helix formation. *Tetrahedron Lett.* 1994;35:5221–4.
- [167] Zhou BW, Marchand C, Asseline U, Thuong NT, Sun JS, Garestier T, Hélène C. Recognition of alternating oligopurine/oligopyrimidine tracts of DNA by oligonucleotides with base-to-base linkages. *Bioconjug Chem.* 1995;6:516–23.

- [168] Ueno Y, Ogawa A, Nakagawa A, Matsuda A. Nucleosides and nucleotides. 162. Facile synthesis of 5'-5'-linked oligodeoxyribonucleotides with the potential for triple-helix formation. *Bioorg Med Chem Lett.* 1997;6:2817–22.
- [169] Jessen CH, Pedersen EB. Design of an intercalating linker leading to the first efficiently 5',5'-linked alternate-strand Hoogsteen triplex with high stability and specificity. *Helv Chim Acta.* 2004;87:2465–71.
- [170] Filichev VV, Nielsen MC, Bomholt N, Jessen CH, Pedersen EB. High thermal stability of 5'-5'-linked alternate Hoogsteen triplexes at physiological pH. *Angew Chem, Int Ed Engl.* 2006;45:5311–5.
- [171] Bomholt N, Filichev VV, Pedersen EB. The first postsynthetic 5'-5' intercalators in triplex DNA – solid-phase postsynthetic Sonogashira reaction and homocouplings on arylacetylenes. *Helv Chim Acta.* 2009;92:716–30.
- [172] Jayasena SD, Johnston BH. Intramolecular triple-helix formation at  $(Pu_nPy_n)_n$ .  $(Pu_nPy_n)_n$  tracts: recognition of alternate strands via Pu.PuPy and Py.PuPy base triplets. *Biochemistry.* 1992;31:320–7.
- [173] Jayasena SD, Johnston BH. Sequence limitations of triple helix formation by alternate-strand recognition. *Biochemistry.* 1993;32:2800.
- [174] Jayasena SD, Johnston BH. Oligonucleotide-directed triple helix formation at adjacent oligopurine and oligopyrimidine DNA tracts by alternate strand recognition. *Nucleic Acids Res.* 1992;20:5279–88.
- [175] Balatskaya SV, Belotserkovskii BP, Johnston BH. Alternate-strand triplex formation: modulation of binding to matched and mismatched duplexes by sequence choice in the Pu-Pu-Py block. *Biochemistry.* 1996;35:13328–37.
- [176] de Bizemont T, Duval-Valentin G, Sun J-S, Bisagni E, Garestier T, Hélène C. Alternate strand recognition of double-helical DNA by (T,G)-containing oligonucleotides in the presence of a triple helix-specific ligand. *Nucleic Acids Res.* 1996;24:1136–43.
- [177] de Bizemont T, Sun J-S, Garestier T, Hélène C. New junction models for alternate-strand triple-helix formation. *Chem Biol.* 1998.
- [178] Washbrook E, Alternate-strand FKR. DNA triple-helix formation using short acridine-linked oligonucleotides. *Biochem J.* 1994;301:569–75.
- [179] Washbrook E, Fox KR. Comparison of antiparallel A-AT and T-AT triplets within an alternate strand DNA triple helix. *Nucleic Acids Res.* 1994;22:3977–82.
- [180] Brodin P, Sun JS, Mouscadet JF, Auclair C. Optimization of alternate-strand triple helix formation at the 5'-TpA-3' and 5'-ApT-3' junctions. *Nucleic Acids Res.* 1999;27:3029–34.
- [181] Kessler DJ, Pettitt BG, Cheng YK, Smith SR, Jayaraman K, Vu HM, Hogan ME. Triple helix formation at distant sites: hybrid oligonucleotides containing a polymeric linker. *Nucleic Acids Res.* 1993;21:4810–5.
- [182] Trapane TL, Christopherson MS, Roby CD, Ts'o POP, Wang D. DNA triple helices with C-nucleosides (deoxypseudouridine) in the second strand. *J Am Chem Soc.* 1994;116:8412–3.
- [183] Bandaru R, Hashimoto H, Switzer C. Inverted motif for oligonucleotide triplexes: adenosine-pseudouridine-adenosine. *J Am Chem Soc.* 1995;60:786–7.
- [184] Rana VS, Barawkar DA, Ganesh KN. Molecular recognition of 5-amino-dU in the central strand of a DNA triplex: formation of triads A\*U#:A in parallel and G\*U#:A in antiparallel motif. *J Org Chem.* 1996;61:3578–9.
- [185] Rana VS, Ganesh KN. Recognition of 5-aminouracil (U#) in the central strand of a DNA triplex: orientation selective binding of different third strand bases. *Nucleic Acids Res.* 2000;28:1162–9.

- [186] Güimil-García R, Bachi A, Eritja R, Luque FJ, Orozco M. Triple helix stabilization properties of oligonucleotides containing 8-amino-2'-deoxyguanosine. *Bioorg Med Chem Lett.* 1998;8:3011–6.
- [187] Kawai K, Saito I, Sugiyama H. Stabilization of Hoogsteen base pairing by introduction of NH<sub>2</sub> group at the C8 position of adenine. *Tetrahedron Lett.* 1998;39:5221–4.
- [188] Güimil-García R, Ferrer E, Macías MJ, Eritja R, Orozco M. Theoretical calculations, synthesis and base-pairing properties of oligonucleotides containing 8-amino-2'-deoxyadenosine. *Nucleic Acids Res.* 1999;27:1991–8.
- [189] Soliva R, Güimil García R, Blas JR, Eritja R, Asensio JL, González C, Luque FJ, Orozco M. DNA-triplex stabilizing properties of 8-aminoguanine. *Nucleic Acids Res.* 2000;28:4531–9.
- [190] Cubero E, Güimil-García R, Luque FJ, Eritja R, Orozco M. The effect of amino groups on the stability of DNA duplexes and triplexes based on purines derived from inosine. *Nucleic Acids Res.* 2001;29:2522–34.
- [191] Hattori M, Frazier J, Milles HT. Poly (8-aminoguanylic acid). Formation of ordered self-structures and interaction with poly (cytidylic acid). *Biochemistry.* 1975;18:5033–45.
- [192] Hattori M, Frazier J, Milles HT. The structure of triple-stranded G.2C polynucleotide helices. *Biopolymers.* 1976;15:523–31.
- [193] Kumar RK, Gunjal AD, Ganesh KN. 8-Amino-2'-deoxyadenosine: 2'-deoxythymidine base pairing: identification of novel reverse Hoogsteen mode in solution. *Biochem Biophys Res Commun.* 1994;204:788–93.
- [194] Aviñó A, Morales JC, Frieden M, de la Torre BG, Güimil-García R, Cubero E, Luque FJ, Orozco M, Azorín F, Eritja R. Parallel-stranded hairpins containing 8-aminopurines. Novel efficient probes for triple-helix formation. *Bioorg Med Chem Lett.* 2001;11:1761–3.
- [195] Cubero E, Aviñó A, de la Torre BG, Frieden M, Eritja R, Luque FJ, González C, Orozco M. Hoogsteen-based parallel-stranded duplexes of DNA. The effect of 8-amino derivatives. *J Am Chem Soc.* 2002;124:3133–42.
- [196] Aviñó A, Frieden M, Morales JC, de la Torre BG, Güimil-García R, Azorín F, Gelpí JL, Orozco M, González C, Eritja R. Properties of triple helices formed by parallel-stranded hairpins containing 8-aminopurines. *Nucleic Acids Res.* 2002;30:2609–19.
- [197] Aviñó A, Cubero E, González C, Eritja R, Orozco M. Antiparallel triple helices. Structural characteristics and stabilization by 8-amino derivatives. *J Am Chem Soc.* 2003;125:16127–38.
- [198] Bell NM, Micklefield J. Chemical modification of oligonucleotides for therapeutic, bioanalytical and other applications. *ChemBioChem.* 2009;10:2691–703.
- [199] Shimizu M, Konishi A, Shimada Y, Inoue H, Ohtsuka E. Oligo(2'-O-methyl)ribonucleotides effective probes for duplex DNA. *FEBS Lett.* 1992;302:155–8.
- [200] Shimizu M, Koizumi T, Inoue H, Ohtsuka E. Effects of 5-methyl substitution in 2'-O-methyloligo-(pyrimidine)nucleotides on triple-helix formation. *Bioorg Med Chem Lett.* 1994;4:1029–32.
- [201] Escudé C, Sun JS, Rougée M, Garestier T, Hélène C. Stable triple helices are formed upon binding of RNA oligonucleotides and their 2'-O-methyl derivatives to double-helical DNA. *C R Acad Sci Paris (Ser III).* 1992;315:521–5.
- [202] Asensio JL, Carr R, Brown T, Lane AN. Conformational and thermodynamic properties of parallel intramolecular triple helices containing a DNA, RNA, or 2'-OMeDNA third strand. *J Am Chem Soc.* 1999;121:11063–70.
- [203] Wang S, Kool ET. Relative stabilities of triple helices composed of combinations of DNA, RNA and 2'-O-methyl-RNA backbones: chimeric circular oligonucleotides as probes. *Nucleic Acids Res.* 1995;23:1157–64.

- [204] Puri N, Majumdar A, Cuenoud B, Miller PS, Seidman MM. Importance of clustered 2'-O-(2-aminoethyl) residues for the gene targeting activity of triple helix-forming oligonucleotides. *Biochemistry*. 2004;43:1343–51.
- [205] Blommers MJJ, Natt F, Jahnke W, Cuenoud B. Dual recognition of double-stranded DNA by 2'-aminoethoxy-modified oligonucleotides: the solution structure of an intramolecular triplex obtained by NMR spectroscopy. *Biochemistry*. 1998;37:17714–25.
- [206] Buchini S, Leumann CJ. 2'-O-Aminoethyl oligoribonucleotides containing novel base analogues: Synthesis and triple-helix formation at pyrimidine/purine inversion site. *Eur J Org Chem*. 2006;3152–68.
- [207] Puri N, Majumdar A, Cuenoud B, Natt F, Martin P, Boyd A, Miller PS, Seidman MM. Targeted gene knockout by 2'-O-aminoethyl modified triplex forming oligonucleotides. *J Biol Chem*. 2001;276:28991–8.
- [208] Puri N, Majumdar A, Cuenoud B, Natt F, Martin P, Boyd A, Miller PS, Seidman MM. Minimum number of 2'-O-(2-aminoethyl) residues required for gene knockout activity by triple helix forming oligonucleotides. *Biochemistry*. 2002;41:7716–24.
- [209] Cardew AS, Brown T, Fox KR. Secondary binding sites for heavily modified triplex forming oligonucleotides. *Nucleic Acids Res*. 2012;40:3753–62.
- [210] Osborne SD, Powers VEC, Rusling DA, Lack O, Fox KR, Brown T. Selectivity and affinity of triplex-forming oligonucleotides containing 2'-aminoethoxy-5-(3-aminoprop-1-ynyl)uridine for recognizing AT base pairs in duplex DNA. *Nucleic Acids Res*. 2004;32:4439–47.
- [211] Atsumi N, Ueno Y, Kanazaki M, Shuto S, Matsuda A. Nucleosides and nucleotides. Part 214: thermal stability of triplexes containing 4' α-C-Aminoalkyl-2'-deoxynucleosides. *Bioorg Med Chem*. 2002;10:2933–60.
- [212] Obika S, Hari Y, Sugimoto T, Sekiguchi M, Imanishi T. Triplex-forming enhancement with high sequence selectivity by single 2'-O,4'-C-methylene bridged nucleic acid (2',4'-BNA) modification. *Tetrahedron Lett*. 2000;41:8923–7.
- [213] Obika S, Uneda T, Sugimoto T, Nanbu D, Minami T, Doi T, Imanishi T. 2'-O,4'-C-methylene bridged nucleic acid (2',4'-BNA): synthesis and triplex-forming properties. *Bioorg Med Chem*. 2001;9:1001–11.
- [214] Torigoe H, Hari Y, Sekiguchi M, Obika S, 2'-O IT. 4'-C-Methylene bridged nucleic acid modification promotes pyrimidine motif triplex DNA formation at physiological pH. *J Biol Chem*. 2001;276:2354–60.
- [215] Sun BW, Babu R, Sorensen MD, Zakrzewska K, Wengel J, Sun JS. Sequence and pH effects of LNA-containing triple helix-forming oligonucleotides: physical chemistry, biochemistry, and modeling studies. *Biochemistry*. 2004;43:4160–9.
- [216] Vester B, Wengel J. LNA (locked nucleic acid): high-affinity targeting of complementary RNA and DNA. *Biochemistry*. 2004;43:13233–40.
- [217] Savy P, Benhida R, Fourrey JL, Maurisse R, Sun JS. DNA triplex structures are stabilized by the incorporation of 3'-endo blocked pyrimidine nucleosides in the Hoogsteen strand. *Bioorg Med Chem Lett*. 2000;10:2287–9.
- [218] Koizumi M, Morita K, Daigo M, Tsutsumi S, Abe K, Obika S, Imanishi T. Triplex formation with 2'-O,4'-C-ethylene-bridged nucleic acids (ENA) having C3'-endo conformation at physiological pH. *Nucleic Acids Res*. 2003;31:3267–73.
- [219] Bolli M, Leumann C. Triple-helix formation of oligodeoxynucleotides containing [(3'S,5'R)-2'-deoxy-3',5'-ethano-β-D-ribofuranosyl]nucleosides (“bicyclo-deoxynucleosides”). *Angew Chem, Int Ed Engl*. 1995;34:694–6.
- [220] Noonberg SB, François JC, Praseuth D, Guieysse-Peugeot AL, Lacoste J, Garestier T, Hélène C. Triplex formation with α anomers of purine-rich and pyrimidine-rich oligodeoxynucleotides. *Nucleic Acids Res*. 1995;23:4042–9.

- [221] Morvan F, Chaix C, Zeissler A, Rayner B, Imbach JL. Triple helix forming  $\alpha$ -oligonucleotides containing 5-methylcytosine and/ or 5-bromouracil. *Nucleosides Nucleotides*. 1995;14:975–7.
- [222] Noronha A, Damha MJ. Triple hélices containing arabinonucleotides in the third (Hoogsteen) strand: effects of inverted stereochemistry at the 2'-position of the sugar moiety. *Nucleic Acids Res.* 1998;26:2665–71.
- [223] Escudé C, Giovannangeli C, Sun JS, Lloyd DH, Chen JK, Gryaznov SM, Garestier T, Hélène C. Stable triple helices formed by oligonucleotide N3' → P5' phosphoramidates inhibit transcription elongation. *Proc Natl Acad Sci USA*. 1996;93:4365–9.
- [224] Zhou-Sun BW, Sun JS, Gryaznov SM, Liquier J, Garestier T, Hélène C. A physico-chemical study of triple helix formation by an oligodeoxythymidylate with N3' → P5' phosphoramidate linkages. *Nucleic Acids Res.* 1997;25:1782.
- [225] Mondragón-Sánchez JA, Liquier J, Gryaznov SM, Taillandier E. A pyrimidine motif DNA triplex with a third N3' → P5' phosphoramidate dC, T strand studied by FTIR and UV spectroscopy. *J Mol Struct.* 2003;661–662:183–93.
- [226] Xodo L, Alunni-Fabbroni M, Manzini G, Quadrifoglio F. Pyrimidine phosphorothioate oligonucleotides from triple-stranded hélices and promote transcription inhibition. *Nucleic Acids Res.* 1994;22:3322–30.
- [227] Hacia JG, Wold BJ, Dervan PB. Phosphorothioate oligonucleotide-directed triple helix formation. *Biochemistry*. 1994;33:5367–9.
- [228] Dempcy RO, Browne K, Bruice TC. Synthesis of the polycation thymidyl DNG, its fidelity in binding polyanionic DNA/RNA, and the stability and nature of the hybrid complexes. *J Am Chem Soc.* 1995;117:6140–1.
- [229] Blasko A, Dempcy RO, Minyat EE, Bruice TC. Association of short-strand DNA oligomers with guanidinium-linked nucleosides. A kinetic and thermodynamic study. *J Am Chem Soc.* 1996;118:7892–9.
- [230] Blasko A, Dempcy RO, Minyat EE, Bruice TC. Fidelity of binding of the guanidinium nucleic acid (DNG) d(Tg)<sub>4</sub>-T-azido with short strand DNA oligomers (A<sub>5</sub>G<sub>3</sub>A<sub>5</sub>, GA<sub>4</sub>G<sub>3</sub>A<sub>4</sub>G, G<sub>2</sub>A<sub>3</sub>G<sub>3</sub>A<sub>3</sub>G<sub>2</sub>, G<sub>2</sub>A<sub>2</sub>G<sub>5</sub>A<sub>2</sub>G<sub>2</sub>). A kinetic and thermodynamic study. *Biochemistry*. 1997;36:7821–31.
- [231] Arya DP, Bruice TC. Positively charged deoxynucleic methylthioureas: synthesis and binding properties of pentameric thymidyl methylthiourea. *J Am Chem Soc.* 1998;120:12419–27.
- [232] Arya DP, Bruice TC. Fidelity of deoxynucleic S-methylthiourea (DNmt) binding to DNA oligomers: influence of C mismatches. *J Am Chem Soc.* 1999;121:10680–4.
- [233] Arya DP, Bruice TC. Triplex-helix of DNA oligomers with methylthiourea-linked nucleosides (DNmt): a kinetic and thermodynamic analysis. *Proc Natl Acad Sci USA*. 1999;96:4384–9.
- [234] Robles J, Ibáñez V, Grandas A, Pedroso E. Synthesis and triple helix-forming ability of oligonucleotides with N,N-dimethylaminoethyl phosphoramidate linkages. *Tetrahedron Lett.* 1999;40:7131–4.
- [235] Lacroix L, Arimondo PB, Takasugi M, Hélène C, Mergny JL. Pyrimidine morpholino oligonucleotides form a stable triple helix in the absence of magnesium ions. *Biochem Biophys Res Commun.* 2000;270:363–9.
- [236] Basye J, Trent JO, Gao D, Ebbinghaus SW. Triplex formation by morpholino oligodeoxyribonucleotides in the HER-2/neu promoter requires the pyrimidine motif. *Nucleic Acids Res.* 2001;29:4873–80.
- [237] Nielsen PE, Egholm M, Berg RH, Buchardt O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science*. 1991;254:1497–500.
- [238] Knudsen H, Nielsen PE. Antisense properties of duplex- and triplex-forming PNAs. *Nucleic Acids Res.* 1996;24:494–500.

- [239] Wittung P, Nielsen P, Nordén B. Extended DNA-recognition repertoire of peptide nucleic acid (PNA): PNA – dsDNA triplex formed with cytosine-rich homopyrimidine PNA. *Biochemistry*. 1997;36:7973–9.
- [240] Wittung P, Nielsen P, Nordén B. Observation of a PNA – PNA – PNA triplex. *J Am Chem Soc*. 1997;119:3189–90.
- [241] Nielsen PE, Egholm M, Buchardt O. Evidence for (PNA)<sub>2</sub>/DNA triplex structure upon binding of PNA to dsDNA by strand displacement. *J Mol Recognit*. 1994;7:165–70.
- [242] Szewczyk JW, Baird EE, Dervan PB. Sequence-specific recognition of DNA by a major and minor groove binding ligand. *Angew Chem, Int Ed Engl*. 1996;35:1487–9.
- [243] Robles J, Rajur SB, McLaughlin LW. A parallel-stranded DNA triplex tethering a Hoechst 33258 analogue results in complex stabilization by simultaneous major groove and minor groove binding. *J Am Chem Soc*. 1996;118:5820–1.
- [244] Robles J, McLaughlin LW. DNA triplex stabilization using a tethered minor groove binding Hoechst 33258 analogue. *J Am Chem Soc*. 1997;119:6014–21.
- [245] Rajur SB, Robles J, Wiederolt K, Kuimelis RG, McLaughlin LW. Hoechst 33258 tethered by a hexa (ethylene glycol) linker to the 5'-termini of oligodeoxynucleotide 15-mers: duplex stabilization and fluorescence properties. *J Org Chem*. 1997;62:523–9.
- [246] Fox KR. Formation of DNA triple helices incorporating blocks of G.GC and T.AT triplets using short acridine-linked oligonucleotides. *Nucleic Acids Res*. 1994;22:2016–21.
- [247] Orson FM, Kinsey BM, McShan WM. Linkage structures strongly influence the binding cooperativity of DNA intercalators conjugated to triplex forming oligonucleotides. *Nucleic Acids Res*. 1994;22:479–84.
- [248] Sun JC, Giovannangeli C, François JC, Kurfust M, Monteny-Garestier T, Asseline U, Saison-Behmoaras T, Thuong NT, Hélène C. Triple-helix formation by alpha oligodeoxynucleotides and alpha oligodeoxynucleotide-intercalator conjugates. *Proc Natl Acad Sci USA*. 1991;88:6023–7.
- [249] Shchyolkina AK, Timofeev EN, Lysov YP, Florentiev VL, Jovin TM, Arndt-Jovin DJ. Protein-free parallel triple-stranded DNA complex formation. *Nucleic Acids Res*. 2001;29:986–95.
- [250] Gianolio DA, Segismundo JM, McLaughlin LW. Tethered naphthalene diimide-based intercalators for DNA triplex stabilization. *Nucleic Acids Res*. 2000;28:2128–34.
- [251] Bevers S, Schutte S, McLaughlin LW. Naphthalene- and perylene-based linkers for the stabilization of hairpin tripleplexes. *J Am Chem Soc*. 2000;122:5906–15.
- [252] Gianolio DA, McLaughlin LW. Tethered naphthalene diimide intercalators enhance DNA triplex stability. *Bioorg Med Chem*. 2001;9:2329–34.
- [253] Asanuma H, Yoshida T, Liang X, Komiyama M. Azobenzene-appended oligonucleotides form unexpectedly stable triple-helices. *Chem Lett*. 2000;108–9.
- [254] Liang X, Asanuma H, Komiyama M. Photoregulation of DNA triplex formation by azobenzene. *J Am Chem Soc*. 2002;124:1877–83.
- [255] Silver GC, Nguyen CH, Bourtchine AS, Bisagni E, Garestier T, Hélène C. Conjugates of oligonucleotides with triplex-specific intercalating agents. Stabilization of triple-helical DNA in the promoter region of the gene for the  $\alpha$ -subunit of Interleukin 2 (IL-2R $\alpha$ ). *Bioconjug Chem*. 1997;8:15–22.
- [256] Ossipov D, Zamaratski E, Chattopadhyaya J. Dipyrido[3,2- $a$ :2',3']phenazine-tethered oligo-DNA: synthesis and thermal stability of their DNA.DNA and DNA.RNA duplexes and DNA.DNA.DNA tripleplexes. *Helv Chim Acta*. 1999;82:2186–200.
- [257] Filichev VV, Pedersen EB. Stable and selective formation of Hoogsteen-type tripleplexes and duplexes using twisted intercalating nucleic acids (TINA) prepared via postsynthetic Sonogashira solid-phase coupling reactions. *J Am Chem Soc*. 2005;127:14849–58.

- [258] Bates P, Macaulay VM, McLean MJ, Jenkins TC, Reszka AP, Laughton CA, Neidle S. Characteristics of triplex-directed photoadduct formation by psoralen-linked oligodeoxynucleotides. *Nucleic Acids Res.* 1995;23:4283–9.
- [259] Gasparro FP, Havre PA, Olack GA, Gunther EJ, Glazer PM. Site-specific targeting of psoralen photoadducts with a triplex helix-forming oligonucleotide: characterization of psoralen monoadduct and crosslink formation. *Nucleic Acids Res.* 1994;22:2845–52.
- [260] Takasugi M, Guendouz A, Chassignol M, Decout JL, Lhomme J, Thuong NT, Hélène C. Sequence-specific photo-induced cross-linking of the two strands of double-helical DNA by a psoralen covalently linked to a triple helix-forming oligonucleotide. *Proc Natl Acad Sci USA.* 1991;88:5602–6.
- [261] Giovannangeli C, Thuong NT, Hélène C. Oligodeoxynucleotide-directed photo-induced cross-linking of HIV proviral DNA via triple-helix formation. *Nucleic Acids Res.* 1992;20:4275–81.
- [262] Wang G, Levy DD, Seidman MM, Glazer PM. Targeted mutagenesis in mammalian cells mediated by intracellular triple helix formation. *Mol Cell Biol.* 1995;15:1759–68.
- [263] Wang G, Seidman MM, Glazer PM. Mutagenesis in mammalian cells induced by triple helix formation and transcription-coupled repair. *Science.* 1996;271:802–5.
- [264] Havre PA, Gunther EJ, Gasparro FP, Glazer PM. Targeted mutagenesis of DNA using triple helix-forming oligonucleotides linked to psoralen. *Proc Natl Acad Sci USA.* 1993;90:7879–83.
- [265] Wang G, Glazer PM. Altered repair of targeted psoralen photoadducts in the context of an oligonucleotide-mediated triple helix. *J Biol Chem.* 1995;270:22595–601.
- [266] Raha M, Wang G, Seidman MM, Glazer PM. Mutagenesis by third-strand-directed psoralen adducts in repair-deficient human cells: high frequency and altered spectrum in a xeroderma pigmentosum variant. *Proc Natl Acad Sci USA.* 1996;93:2941–6.
- [267] Perrouault L, Asseline U, Rivalle C, Thuong NT, Bisagni E, Giovannangeli C, LeDoan T, Hélène C. Sequence-specific artificial photo-induced endonucleases based on triple helix-forming oligonucleotides. *Nature.* 1990;344:358–60.
- [268] LeDoan T, Perrouault L, Asseline U, Thuong NT, Rivalle C, Bisagni E, Hélène C. Recognition and photo-induced cleavage and cross-linking of nucleic acids by oligonucleotides covalently linked to ellipticine. *Antisense Res Dev.* 1991;1:43–54.
- [269] Praseuth D, Perrouault L, LeDoan T, Chassignol M, Thuong N, Hélène C. Sequence-specific binding and photocrosslinking of alpha and beta oligodeoxynucleotides to the major groove of DNA via triple-helix formation. *Proc Natl Acad Sci USA.* 1991;88:5602–6.
- [270] Teulade-Fichou MP, Perrin D, Boutorine A, Polverari D, Vigneron JP, Lehn JM, Su JS, Garestier T, Hélène C. Direct photocleavage of HIV-DNA by quinacridine derivatives triggered by triplex formation. *J Am Chem Soc.* 2001;123:9283–92.
- [271] Praseuth D, Le Doan T, Chassignol M, Decout JL, Habhou N, Lhomme J, Thuong NT, Hélène C. Sequence-targeted photosensitized reactions in nucleic acids by oligo-alpha.-deoxynucleotides and oligo-beta-deoxynucleotides covalently linked to proflavin. *Biochemistry.* 1988;27:3031–8.
- [272] LeDoan T, Perrouault L, Praseuth D, Habhou N, Decout JL, Thuong NT, Lhomme J, Hélène C. Sequence-specific recognition, photocrosslinking and cleavage of the DNA double helix by an oligo-( $\alpha$ -thymidylate covalently linked to an azidoproflavine derivative. *Nucleic Acids Res.* 1987;15:7749–60.
- [273] Grant KB, Dervan PB. Sequence-specific alkylation and cleavage of DNA mediated by purine motif triple helix formation. *Biochemistry.* 1996;35:12313–9.
- [274] Povsic TJ, Strobel SA, Dervan PB. Sequence-specific double-strand alkylation and cleavage of DNA mediated by triple-helix formation. *J Am Chem Chem Soc.* 1992;114:5934–41.

- [275] Dempcy RO, Kutyavin IV, Mills AG, Lukhtanov EA, Meyer RB. Linkers designed to intercalate the double helix greatly facilitate DNA alkylation by triplex-forming oligonucleotides carrying a cyclopropapyrroloindole reactive moiety. *Nucleic Acids Res.* 1999;27:2931–7.
- [276] Reed MW, Wald A, Meyer RB. Triplex-directed interstrand DNA cross-linking by diazirinidylquinone-oligonucleotide conjugates. *J Am Chem Soc.* 1998;120:9730–4.
- [277] Giovannangeli C, Montonay-Garestier T, Rougée M, Chassignol M, Thuong NT, Hélène C. Single-stranded DNA as a target for triple-helix formation. *J Am Chem Soc.* 1991;113:7775–7.
- [278] Rumney S IV, Kool ET. Structural optimization of non-nucleotide loop replacements for duplex and triplex DNAs. *J Am Chem Soc.* 1995;117:5635–46.
- [279] Wang S, Kool ET. Recognition of single-stranded nucleic acids by triplex formation: the binding of pyrimidine-rich sequences. *J Am Chem Soc.* 1994;116:8857–8.
- [280] Wang S, Booher MA, Kool ET. Stabilities of nucleotide loops bridging the pyrimidine strands in DNA pyrimidine.purine-pyrimidine triplexes: special stability of the CTTG loops. *Biochemistry.* 1994;33:4639–44.
- [281] Kandimalla ER, Agrawal S. Single-strand triplex formation: stability, specificity and RNase H activation properties. *Gene.* 1994;149:115–21.
- [282] Xodo LE, Manzini G, Quadrifoglio F. Spectroscopic and calorimetric investigation on the DNA triplex formed by d(CTCTTCTTCTTCTTCT) and d(GAGAAGAAAGA) at acidic pH. *Nucleic Acids Res.* 1990;18:3557–64.
- [283] Kandimalla ER, Manning AN, Agrawal S. Single strand targeted triplex formation: strand displacement of duplex DNA by foldback triplex-forming oligonucleotides. *J Biomol Struct Dyn.* 1995;13:483–91.
- [284] Kool ET. Circular oligonucleotides: new concepts in oligonucleotide design. *Annu Rev Biophys Struct.* 1996;25:1–28.
- [285] Kool ET. Molecular recognition by circular oligonucleotides: increasing the selectivity of DNA binding. *J Am Chem Soc.* 1991;113:6265–6.
- [286] Prakash G, Kool ET. Structural effects in the recognition of DNA by circular oligonucleotides. *J Am Chem Soc.* 1992;114:3523–7.
- [287] Maksimenko AV, Volkov EM, Bertrand JR, Porumb H, Malvy C, Shabarova ZA, Gottikh MB. Targeting of single-stranded DNA and RNA containing adjacent pyrimidine and purine tracts by triple helix formation with circular and clamp oligonucleotides. *Eur J Biochem.* 2000;267:3592–603.
- [288] Griffith MC, Risen LM, Greig MJ, Lesnik EA, Sprangle KG, Griffey R, Kiely JS, Freier SM. Single and bis peptide nucleic acids as triplexing agents: binding and stoichiometry. *J Am Chem Soc.* 1995;117:831–2.
- [289] Kurakin A, Larsen HJ, Nielsen PE. Cooperative strand displacement by peptide nucleic acid (PNA). *Chem Biol.* 1998;5:81–9.
- [290] Neuner P, New MP. Fmoc pseudoisocytosine monomer for the synthesis of a bis-PNA molecule by automated solid-phase Fmoc chemistry. *Bioconjug Chem.* 2002;13:676–8.
- [291] Footer M, Egholm M, Kron S, Coull JM, Matsudaira P. Biochemical evidence that a D-loop is part of a four-stranded PNA-DNA bundle. Nickel-mediated cleavage of duplex DNA by a Gly-Gly-His bis-PNA. *Biochemistry.* 1996;35:10673–9.
- [292] Kuhn H, Demidov VV, Nielsen P, Frank-Kamenetskii MD. An experimental study of mechanism and specificity of peptide nucleic acids (PNA) binding to duplex DNA. *J Mol Biol.* 1999;286:1337–45.
- [293] Hansen GI, Bentin T, Larsen HJ, Nielsen PE. Structural isomers of bis-PNA bound to a target in duplex DNA. *J Mol Biol.* 2001;307:67–74.
- [294] Demidov VV, Bukanov NO, Duplex DV. DNA capture. In: Nielsen PE, Egholm M, editors. Peptide nucleic acids. Protocols and applications. Horizon Scientific Press; 1999. p. 175–84.

- [295] Veselkov AG, Demidov VV, Frank-Kamenetskii MD, Nielsen P. PNA as a rare genome-cutter. *Nature*. 1996;379:214.
- [296] Broude NE, Demidov VV, Kuhn H, Gorenstein J, Pulyaeva H, Volkovitsky P, Drukier AK, Frank-Kamenetskii MD. PNA openers as a tool for direct quantification of specific targets in duplex DNA. *J Biomol Struct Dyn*. 1999;17:237–44.
- [297] Kuhn H, Demidov VV, Gildea BD, Fiandaca MJ, Coull JCC PNA beacons for duplex DNA. *Antisense Nucleic Acid Drug Dev*. 2001;11:265–70.
- [298] Liu B, Han Y, Corey DR, Kodadek T. Towards synthetic transcription factors to DNA by a PNA-peptide chimera. *J Am Chem Soc*. 2002;124:1838–9.
- [299] Rogers FA, Vasquez KM, Egholm M, Glazer PM. Site-directed recombination via bifunctional PNA-DNA conjugates. *Proc Natl Acad Sci*. 2002;99:16695–700.
- [300] Yamazaki T, Aiba Y, Yasuda K, Sakai Y, Tamanaka Y, Kuzuya A, Ohya Y, Komiyama M. Clear-cut observation of PNA invasion using nanomechanical DNA origami devices. *Chem Commun*. 2012;48:11361–3.
- [301] Nielsen PE. Peptide nucleic acids (PNA) in chemical biology and drug discovery. *Chem Biodivers*. 2010;7:786–804.
- [302] Nielsen PE. Sequence-selective targeting of duplex DNA by peptide nucleic acids. *Curr Opin Mol Ther*. 2010;12:184–91.
- [303] Aviñó A, Eritja R, Ciudad CJ, Noé V. Parallel clamps and polypurine hairpins (PPRH) for gene silencing and triplex-affinity capture: design, synthesis and use. *Curr Protoc Nucleic Acids Chem*. 2019;e78.
- [304] Kandimalla ER, Agrawal S. Single strand targeted triplex formation: parallel-stranded DNA hairpin duplexes for targeting pyrimidine strands. *J Am Chem Soc*. 1995;117:6416–7.
- [305] Kandimalla ER, Hoogsteen AS. DNA duplexes of 3'-3' and 5'-5' linked oligonucleotides and triplex formation with RNA and DNA pyrimidine single strands: experimental and molecular modeling studies. *Biochemistry*. 1996;35:1532–9.
- [306] Coma S, Noé V, Eritja R, Ciudad CJ. Strand displacement of double-stranded DNA by triplex-forming antiparallel purine-hairpins. *Oligonucleotides*. 2005;15:269–83.
- [307] Ciudad CJ, Rodríguez L, Villalobos X, Félix AJ, Noé V. Polypurine reverse Hoogsteen hairpins as a gene silencing tool for cancer. *Curr Med Chem*. 2017;24:2809–26.
- [308] Grima MG, Gargallo R, Aviñó A, Eritja R. Synthesis and triplex-forming properties of cyclic oligonucleotides with G,A-antiparallel strands. *Chem Biodivers*. 2005;2:275–85.
- [309] Rodriguez L, Villalobos X, Solé A, Lliberós C, Ciudad CJ, Noé V. Improved design of PPRHs for gene silencing. *Mol Pharm*. 2015;12:867–77.
- [310] Nadal A, Eritja R, Esteve T, Plà M. Parallel- and antiparallel-tail-clamps hairpins increase the efficiency of triplex formation with structured DNA and RNA targets. *ChemBioChem*. 2005;6:1034–42.
- [311] Nadal A, Coll A, Aviñó A, Esteve T, Eritja R, Plà M. Efficient sequence-specific purification of *Listeria innocua* mRNA species by triplex affinity capture with parallel tail-clamps. *ChemBioChem*. 2006;7:1039–47.
- [312] Aviñó A, Grima MG, Frieden M, Eritja R. Synthesis and triple-helix stabilization properties of branched oligonucleotides carrying 8-aminoadenine. *Helv Chim Acta*. 2004;87:303–16.
- [313] Alvira M, Eritja R. Synthesis of oligonucleotides carrying 5'-5' linkages using copper-catalyzed cycloaddition reactions. *Chem Biodivers*. 2007;4:2798–809.
- [314] Aviñó A, Frieden M, Morales JC, de la Torre BG, Güimil García R, Azorín F, Gelpí JL, Orozco M, González C, Eritja R. Properties of triple helices formed by parallel-stranded hairpins containing 8-aminopurines. *Nucleic Acids Res*. 2002;30:2609–19.

- [315] de Almagro MC, Coma S, Noé V, Ciudad CJ. Polypurine hairpins directed against the template strand of DNA knock down the expression of mammalian genes. *J Biol Chem.* 2009;284:11579–89.
- [316] Solé A, Delagoutte E, Ciudad CJ, Noé V, Alberti P. Polypurine reverse-Hoogsteen (PPRH) oligonucleotides can form triplexes with their target sequences even under conditions where they fold into G-quadruplexes. *Sci Rep.* 2017;7:39898.
- [317] Carrascosa LG, Gómez-Montes S, Aviñó A, Nadal A, Pla M, Eritja R, Lechuga LM. Sensitive and label-free biosensing of RNA with predicted secondary structures by a triplex affinity capture method. *Nucleic Acids Res.* 2012;40:e56.
- [318] Aviñó A, Huertas CS, Lechuga LM, Eritja R. Sensitive and label-free detection of miRNA-145 by triplex formation. *Anal Bioanal Chem.* 2016;408:885–93.
- [319] Villalobos X, Rodríguez L, Solé A, Lliberós C, Mencia N, Ciudad CJ, Noé V. Effect of polypurine reverse Hoogsteen hairpins on relevant cancer target genes in different human cell lines. *Nucleic Acid Ther.* 2015;25:198–208.
- [320] Rodríguez L, Villalobos X, Dakhel S, Padilla L, Hervas R, Hernández JL, Ciudad CJ, Noé V. Polypurine reverse Hoogsteen hairpins as a gene therapy tool against survivin in human prostate cancer PC3 cells in vitro and in vivo. *Biochem Pharmacol.* 2013;86:1541–54.
- [321] de Almagro MC, Mencia N, Noé V, Ciudad CJ. Coding polypurine hairpins cause target-induced cell death in breast cancer cells. *Hum Gene Ther.* 2011;22:451–63.
- [322] Félix AJ, Ciudad CJ, Noé V. Functional pharmacogenomics and toxicity of PolyPurine reverse Hoogsteen hairpins directed against survivin in human cells. *Biochem Pharmacol.* 2018;155:8–20.
- [323] Medina Enríquez MM, Félix AJ, Ciudad CJ, Noé V. Cancer immunotherapy using PolyPurine reverse Hoogsteen hairpins targeting the PD-1/PD-L1 pathway in human tumor cells. *PLoS ONE.* 2018;13:e0206818.
- [324] Bener G, Félix AJ, Sánchez de Diego C, Pascual Fabregat I, Ciudad CJ, Noé V. Silencing of CD47 and SIRP $\alpha$  by polypurine reverse Hoogsteen hairpins to promote MCF-7 breast cancer cells death by PMA-differentiated THP-1 cells. *BMC Immunol.* 2016;17:32.
- [325] Aubets E, Noé V, Ciudad CJ. Targeting replication stress response using polypurine reverse Hoogsteen hairpins directed against WEE1 and CHK1 genes in human cáncer cells. *Biochem Pharmacol.* 2020;175:113911.
- [326] Félix AJ, Ciudad CJ, Noé V. Correction of the aprt gene using repair-polypurine reverse Hoogsteen hairpins in mammalian cells. *Mol Ther Nucleic Acids.* 2019;19:683–95.
- [327] Huertas CS, Aviñó A, Kurachi C, Piqué A, Sandoval J, Eritja R, Esteller M, Lechuga LM. Label-free DNA-methylation detection by direct ds-DNA fragment screening using poly-purine hairpins. *Biosens Bioelectron.* 2018;120:47–54.
- [328] Ribes A, Santiago-Felipe S, Aviñó A, Candela-Noguera V, Eritja R, Sancenón F, Martínez-Máñez R, Aznar E. Design of oligonucleotide-capped mesoporous silica nanoparticles for the detection of miRNA-145 by duplex and triplex formation. *Sens Actuators B, Chem.* 2018;277:598–603.
- [329] Calvo-Lozano O, Aviñó A, Friaza V, Medina-Escuela A, Huertas CS, Calderón El, Eritja R, Lechuga LM. Fast and accurate pneumocystis pneumonia diagnosis in human samples using a label-free plasmonic biosensor. *Nanomaterials.* 2020;10:1246.

Raimundo Gargallo, Carlos González, Carme Fàbrega, Anna Aviñó,  
and Ramon Eritja

## 6 Nucleic acids quadruplex

### Contents

6.1	Oligonucleotides forming G-quadruplex —	232
6.1.1	Guanine modifications on the tetrads —	234
6.1.2	Modifications of the 2'-deoxyribose phosphate backbone of the guanine tetrads —	237
6.1.3	Modifications at the loops —	241
6.1.4	Applications of aptamers based in G-quadruplex. Functionalization at the 3' or 5'-ends —	244
6.2	Oligonucleotides forming i-motif —	248
6.2.1	Cytosine modifications —	251
6.2.2	Modifications of the 2'-deoxyribose phosphate backbone in the i-motif core and at the loop positions —	253
6.2.3	i-motif structures functionalized at the 3' or 5'-ends —	258
	Bibliography —	259

DNA is mostly in the form of a double helix. However, in G-rich regions DNA can adopt other three-dimensional structures such as the G-quadruplex (G4), a structure formed by guanine tetrads stabilized by hydrogen bonds [1]. G4 is a very stable structure *in vitro* and it is found *in vivo* especially in promoter regions of oncogenes and in telomeres, having a relevant role in different biological processes. The complementary C-rich regions have also the ability to form a folded structure known as i-motif or i-tetraplex or i-DNA [2]. This structure consists of parallel-stranded duplexes held together through intercalated base pairs. The formation of i-motif structures requires protonation to form the C:C<sup>+</sup> base pair, so their stability is low at neutral pH values. Initially, these structures were considered *in vitro* model structures with no particular biological relevance. However, this vision has dramatically changed after the visualization of the presence of these structures using monoclonal antibodies in living cells [3, 4]. Nowadays, these structures receive special attention due to their biological functions and implications in gene regulation as well as other important biological

---

**Raimundo Gargallo**, Dept. of Chemical Engineering and Analytical Chemistry, University of Barcelona, Martí i Franquès 1, E-08028 Barcelona, Spain, e-mail: raimon\_gargallo@ub.edu

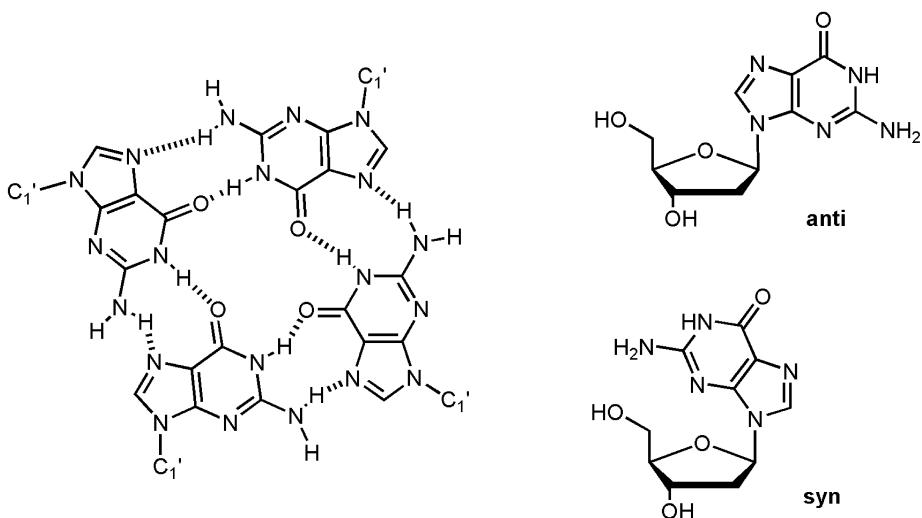
**Carlos González**, Instituto de Química Física “Rocasolano”, CSIC, Serrano 119, E-28006 Madrid, Spain, e-mail: cgonzalez@iqfr.csic.es

**Carme Fàbrega, Anna Aviñó, Ramon Eritja**, Networking Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Jordi Girona 18-26, E-08034 Barcelona, Spain, e-mails: carme.fabrega@iqac.csic.es, aaagma@cid.csic.es, recgma@cid.csic.es

processes [5]. Synthetic oligonucleotides are key elements for the structural elucidation of these structures. In addition, the discovery of specific nucleic acids sequences or aptamers [6] that fold in G-quadruplex structures have triggered an increasing interest in the development of modified oligonucleotides in order to generate potential drugs by specific interaction with target proteins. In this chapter, we will describe the development of modified oligonucleotides to generate G-quadruplex and i-motif structures with improved properties.

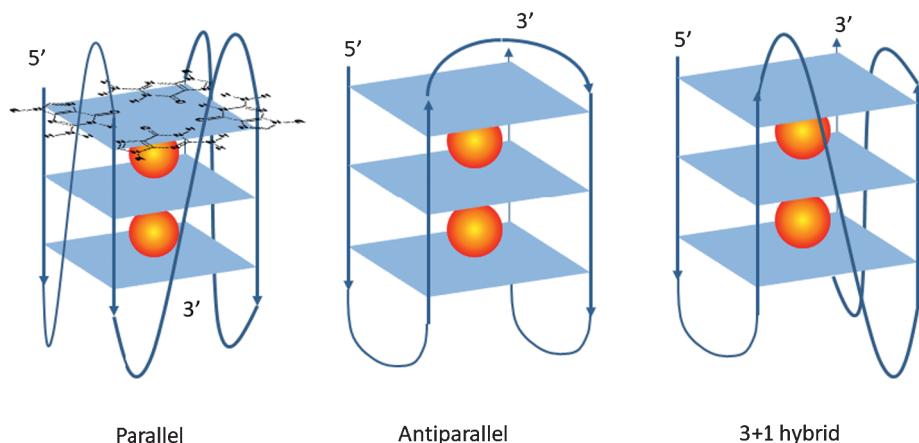
## 6.1 Oligonucleotides forming G-quadruplex

G-quadruplexes are formed by stacked of successive G-tetrads (Figure 6.1). In order to build these tetrads a series of at least 2-3 consecutive guanines are needed. Optimally, four groups of consecutive guanines are needed for an intramolecular G-quadruplex. These structures can be classified by the number of strands as tetrameric, dimeric or monomeric (intramolecular) [7]. Another classification is based on the relative orientation of the strands being (1) all strands in the same direction (parallel quadruplex), (2) three strands in one direction and one in the opposite direction (hybrid quadruplex or 3 + 1), (3) two consecutive strands in one direction and the other two in opposite direction (antiparallel quadruplex) and (4) two opposite strands in one direction and the other two in opposite direction (antiparallel quadruplex) (Figure 6.2).



**Figure 6.1:** (a) Chemical structure of G tetrads; (b) *syn*, *anti* conformations of 2'-deoxyguanosine.

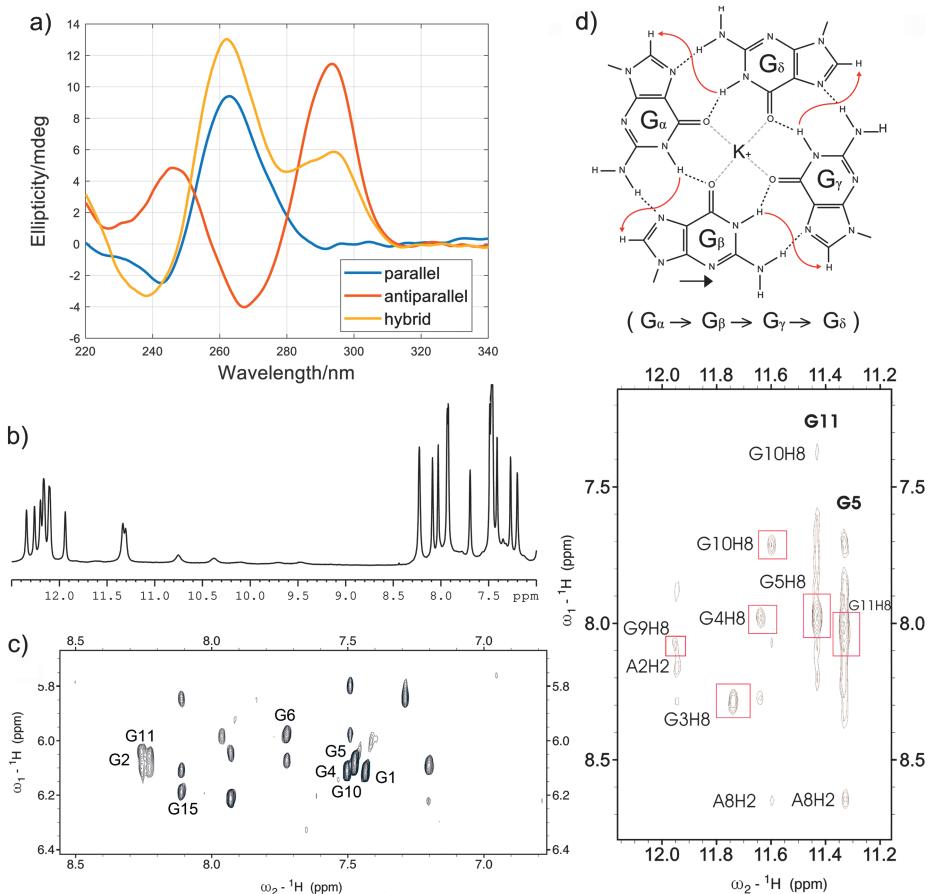
The relative orientation of the strands dictates the conformation of the guanine nucleosides of the tetrads that can be either *syn* or *anti* (Figure 6.1). In an intramolecu-



**Figure 6.2:** Scheme of folding topologies of G-rich sequences into parallel, antiparallel and 3 + 1 hybrid G-quadruplex structures. Orange spheres indicate the counterion. Blue squares indicate the G-tetrads, as in Figure 6.1a [81].

lar quadruplex, several types of loops can be found. Lateral loops are when the loop connects two adjacent antiparallel strands. Diagonal loops are when the loop connects two opposing antiparallel strands. Propeller loops connect adjacent parallel strands [7]. The determination of the structure and the biological properties of oligonucleotides forming G-quadruplex use a plethora of biophysical and molecular biology methods that can be found in excellent reviews [7–9]. Several bioinformatics methods to identify potential G-quadruplex sites have been reported [10, 11].

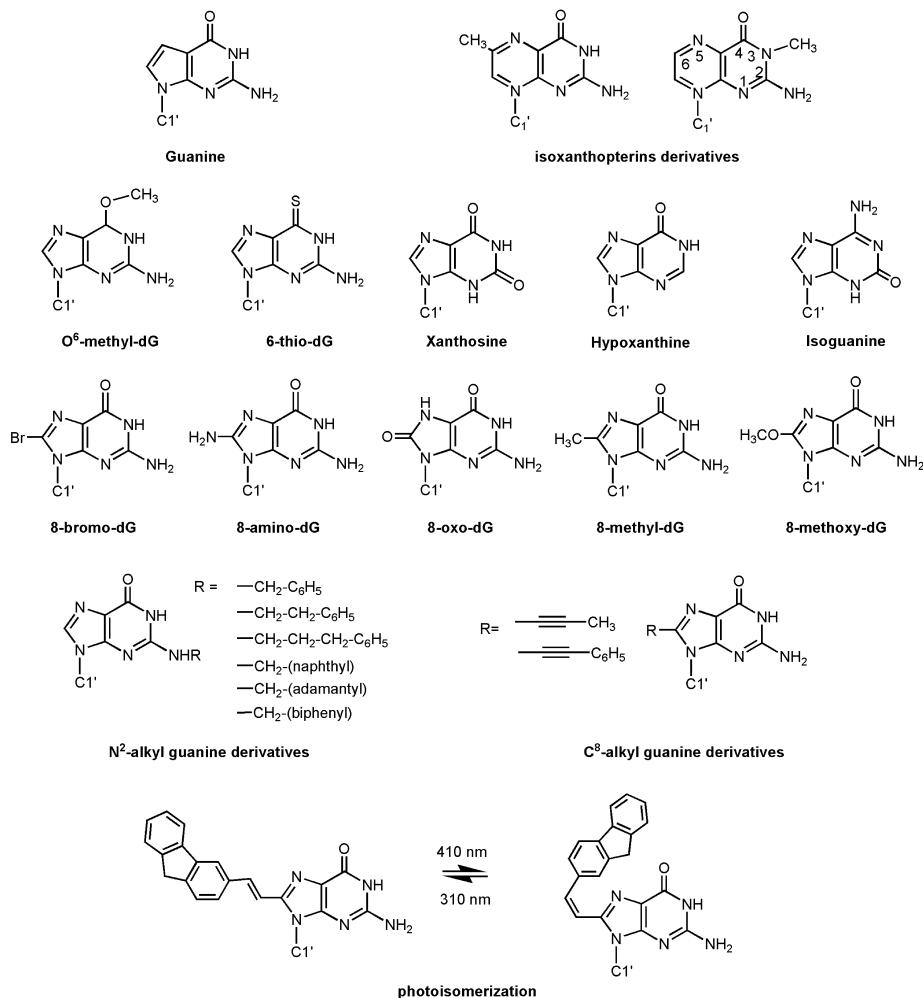
Circular dichroism (CD) and Nuclear Magnetic Resonance (NMR) are probably the most used techniques among those devoted to study G-quadruplex structures. CD spectra of G-quadruplex structures are strongly dependent on the folding topology. Parallel G-quadruplex show characteristic positive and negative signals at 260 and 240 nm, respectively. On the contrary, antiparallel G-quadruplex show positive signals at 245 and 290 nm, and a negative signal at 265 nm, approximately. Hybrid structures show a mixture of both signatures (Figure 6.3). NMR is also a very useful technique for characterizing noncanonical DNA structures. In the case of G-quadruplex, the imino protons of the guanines involved in the G-tetrad resonate around 11–12 ppm, which allows distinguishing G-tetrad from Watson–Crick (12–13 ppm) base pair formation very easily. G-quadruplex formation can be confirmed by characteristic imino-amino cross-peaks in 2D NOESY experiments. The presence of guanines in *syn* conformation, typical of antiparallel G-quadruplex, can be detected by their strong intra-residual H1'-H8 NOE cross-peaks [12, 13]. Although detection of G-quadruplex by NMR is usually straightforward from simple  $^1\text{H}$  experiments, the complete assignment of their NMR spectra normally requires site-directed isotopically labeled samples [13].



**Figure 6.3:** (a) CD spectra of three G-rich sequences forming parallel ( $5'$ -TGGGGG- $3'$ ), antiparallel ( $5'$ -GGTTGGTGTTGG- $3'$ ) and hybrid ( $5'$ -CGGGCACGGGAGGAAGGGGGCGGG- $3'$ ) structures. (b) NMR spectra of the TBA. (c)  $^1\text{H}$ -aromatic region of a 2D NOESY spectrum of TBA. Intra residual  $^1\text{H}$ - $^1\text{H}$  cross-peaks of guanines are labeled. *Syn* guanines (1,5,10,14) exhibit more intense NOEs than anti guanines. (d) Example of identification of G-tetrad alignment identified from NOESY data on the basis of the characteristic imino-H8 connectivity indicated by red squares [12].

### 6.1.1 Guanine modifications on the tetrads

In a comparative study, several guanine and isoanthopterin derivatives (Figure 6.4) have been used to replace guanine at specific sites of the tetrameric parallel quadruplex TG<sub>4</sub>T. Authors conclude that most nonguanine derivatives are deleterious for G-quadruplex formation except for 8-bromo-dG and 6-methyl isoanthopterin which were found to accelerate quadruplex formation [14]. This work was extended to TG<sub>4</sub>T quadruplexes carrying 8-aminoguanines (8-amino-dG) confirming the acceleration of the association of tetrameric G-quadruplex by this 8-substituted guanine



**Figure 6.4:** Chemical structures of guanine analogs introduced in G-quadruplexes.

derivative [15]. On the other hand, guanine substitution by 8-aminoguanine in an intramolecular antiparallel G-quadruplex was found to be destabilizing [16]. The effect of *O*<sup>6</sup>-methyl-dG in telomeric G-quadruplex sequence was analyzed in order to determine the potential effect of alkylating agents used in chemotherapy [17]. As expected methylation of guanines induces a clear destabilization of G-quadruplex especially when methylation occurs at the guanines present in the middle of the G-quadruplex stack. This destabilization has been used for the development of a novel fluorescence assays for the detection of the repair activity of *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase [18]. This method based on the conformational change from methylated random coil to unmethylated G-quadruplex with affinity to thrombin was

further developed to nanosensors, one based on the observation of the aggregation of nanoparticles [19] and another at the single molecule level using DNA origami and atomic force microscopy (AFM) [20]. The substitution of 6-thioguanine (6-thio-dG) for guanine stabilize duplex DNA however inhibits G-quadruplex formation [21, 22]. As 6-thioguanine is used in chemotherapy, authors suggest that the cytotoxic properties of 6-thioguanine may imply the disruption of the quadruplex structures present at the telomeres and other sites of the genome [21].

The effect of the presence of the oxidative DNA lesion 8-oxo-dG in telomeric G-quadruplex sequences was also analyzed [23]. Similarly, to what has been found with *O*<sup>6</sup>-methyl-dG, the presence of 8-oxo-dG destabilized G-quadruplex especially in the middle of the GGG sequence. When the lesion was at the 5'-end the stability of G-quadruplex was similar to the natural oligonucleotide [23]. In a recent development, it has been shown that the double substitution of two base-modified guanine analogs, xanthosine and 8-oxo-dG, within a G-tetrad can drive the reversal of G-tetrad polarity without producing changes in the overall G-quadruplex structure [24–27].

The synthesis of oligonucleotides carrying 8-methyl-dG has been reported [28–30]. This 8-substituted derivative of guanine has a preference for the *syn* conformation because of the steric hindrance of the methyl group. The presence of this nucleobase in duplex DNA maintains the hybridization properties of the modified oligonucleotides but in primer extension experiments this compound is capable of generating transversions and deletions [28]. The incorporation of 8-methyl-dG in the tetramolecular TG<sub>4</sub>T quadruplex gave unexpected complex results [29]. Depending on the position of the substitution mixtures of parallel and antiparallel quadruplex were observed and the preference for the *syn* conformation was not observed in all the cases, as in some of the quadruplex 8-methyl-dG was found in the *anti* conformation [29]. On the other hand, the study of an intramolecular antiparallel G-quadruplex in the 5' end of the retinoblastoma (Rb) gene reported the stabilization of the G-quadruplex due to the preference for the *syn* conformation of 8-methyl-dG [30].

Similarly, the incorporation of 8-bromo-dG in tetramolecular TG<sub>3</sub>T quadruplex had different effects depending on the position of the 8-bromo-dG substitution. The d(TG<sup>Br</sup>GGT) and d(TGG<sup>Br</sup>GT) tetramers were more stable than the unmodified sequence, however the d(TGGG<sup>Br</sup>T) tetramer was much less stable [31]. On the other hand, the substitution of G by 8-bromo-dG in the intramolecular telomeric sequence gave a clear improvement of the NMR signals as it was able to avoid the presence of minor conformers [32]. These authors recommended the use of 8-bromo-dG to overcome the difficulty of nucleic acid structure determination arising from conformational heterogeneity [32, 33]. In a study done with the intramolecular antiparallel thrombin binding aptamer (TBA), it was observed that the presence of single 8-bromo-dG substitutions in the tetrads has the preference to adopt a *syn* glycosidic conformation, producing the correct tetrad and enhanced quadruplex stability. However, when two 8-bromo-dG substitutions are introduced in *anti* positions of different tetrads it was observed a conformational flip from *syn* to *anti* conformation of 8-bromo-G

to favor loop-tetrad interaction and it preserves the overall TBA stability [34]. In a comparative study, the substitution of guanine by 8-bromo-guanine, 8-methoxy-guanine, 8-amino-guanine, and 8-oxo-guanine in a well-defined human telomeric G-quadruplex was studied. In this model quadruplex the highest stabilizing effect was found when 8-bromo-dG was replacing a single *syn* guanine followed by the substitution of 8-methoxy-guanine and 8-aminoguanine [35]. Substitutions of 8-oxo-guanine lead to drastic changes in NMR imino proton spectra and a large decrease in the stability [35].

One of the most studied G-quadruplex is the thrombin binding aptamer (TBA) [36]. This 15mer oligonucleotide folds in an intramolecular antiparallel quadruplex with a chair-like conformation and a core of two G-tetrads connected by three loops [37, 38]. The anticoagulant properties of the thrombin binding aptamer [39] triggered the search for modified TBA derivatives with enhanced stability to nucleases and increased anticoagulant properties. One of the approaches is the preparation of combinatorial libraries. A library of all possible substitutions of guanine by isoguanine (Figure 6.4) in the TBA was prepared by split and mix synthesis [40]. Some of the modified aptamers had enhanced binding activity which was attributed to the serum stability rather than thermodynamic stability. In another study, a series of *N*<sup>2</sup> and *C*<sup>8</sup>-alkyl guanine derivatives (Figure 6.4) were introduced in the G-tetrads [41]. These positions are not forming the H-bonding of the tetrads and are available for attaching one or more groups pointing away from the chair-like structure. The increased activities for the substitutions on *C*<sup>8</sup> positions may be explained by the stabilization of *syn* conformation of the G residues, while the increased activities for the substitutions on *N*<sup>2</sup> positions may be due to the interaction with thrombin.

A method for the photoregulation of G-quadruplex formation using *cis-trans* photoisomerization of a double bond was described [42]. In the *trans* form, the quadruplex is folded but upon irradiation, at 410 nm the quadruplex is unfolded. This isomerization is reversible. The efficacy of the photoregulation was analyzed in the TBA as a model quadruplex. In a separate study, the use of a guanine derivative carrying a photolabile group in the TBA was analyzed. The photocaged TBA derivative can be irreversibly unmasked and allow quadruplex formation as well as thrombin binding [43].

### 6.1.2 Modifications of the 2'-deoxyribose phosphate backbone of the guanine tetrads

The search for aptamers with enhanced stability to nucleases for potential *in vivo* use lead to the introduction of 3'-3' and 5'-5' inversions and to the study of the subsequent changes in the structure of G-quadruplex especially in the TBA (3'-G<sub>1</sub>G<sub>2</sub>T<sub>3</sub>T<sub>4</sub>G<sub>5</sub>G<sub>6</sub>T<sub>7</sub>G<sub>8</sub>T<sub>9</sub>G<sub>10</sub>G<sub>11</sub>T<sub>12</sub>T<sub>13</sub>G<sub>14</sub>G<sub>15</sub>-5') [44, 45]. A small library of TBA derivatives containing a 3'-3' and 5'-5' inversion polarity site was prepared [45] and analyzed. From this library, one

modified TBA molecule ( $3'$ -GGT- $5'$ - $5'$ -TGGTGTGGTTGG- $3'$ ) presented higher thermal stability and display a smaller but interesting anticoagulant activity [46]. The structure of this modified aptamer was characterized by an unusual folding with three parallel strands and one antiparallel strand with an unusual *syn anti* distribution of the guanines [46]. The binding to the thrombin of this modified TBA derivative was investigated using several biophysical methods [47, 48]. TBA analogs containing one or two inversions of polarity sites at the ends of the TBA sequence, with the aim to increase resistance to exonucleases were prepared [49]. A clear improvement of affinity to thrombin was found when a purine nucleotide was introduced at the  $3'$ -end with unnatural  $3'$ - $3'$  phosphate bonds [49]. The effect of the presence of one or two  $3'$ - $3'$  or  $5'$ - $5'$  inversion sites was also studied in the tetrameric TG<sub>3</sub>T [50] and TG<sub>4</sub>T [51] quadruplexes. All the modified tetrameric quadruplex were formed with unexpected changes in the glycosidic conformation of guanosines and base stacking.

The replacement of some deoxynucleotides by ribonucleotides induces interesting changes in the topology of G-quadruplexes as guanosine has a preference for the *syn* conformation. For this reason, the replacement of some dG residues by guanosines in TBA and in a telomeric sequence induces a large conformational change and monomeric antiparallel topologies are converted to dimeric parallel structures [52, 53]. From the stability point of view, replacement of the  $2'$ -deoxynucleotides for ribonucleotides or  $2'$ -O-methyl-RNA derivatives (Figure 6.5) in several quadruplexes were found to be destabilizing in short intramolecular G-quadruplexes but they are stabilizing in tetrameric TG<sub>4</sub>T oligonucleotides [54].

The impact of  $2'$ -deoxy- $2'$ -fluoro-D arabino (F-ANA) (Figure 6.5) units in TBA has been well studied. This analog confers an excellent resistance to degradation by nucleases. When F-ANA-G derivatives are substituting Gs that are in *syn* conformation the G-quadruplex structure is highly modified and multiple conformations are observed. On the contrary, the replacement of Gs in *anti* conformation by F-ANA-G produces stable antiparallel quadruplex with an increase in binding affinity to thrombin [55]. This work has been extended recently to the analysis of a microarray containing all 32768 possible DNAs-carrying F-ANA/DNA chimeric sequences to fully map the binding affinity landscape of the thrombin-binding G-quadruplex aptamer [56]. In this work, the authors confirmed that the substitution of *syn*-oriented guanines by F-ANA in TBA does not tolerate the presence of F-ANA residues in these positions. Moreover, the modification of thymidines T3 and T12 residues induce an increased binding and stability as these residues directly participate in thrombin binding. In conclusion, a high F-ANA content translated into a considerable increase in quadruplex stability [56].

Conformationally restricted nucleosides such as Locked Nucleic Acids (LNA) (Figure 6.5) have been profusely used to control or modulate the folding of G-quadruplexes. In a study involving the *Oxytrichia trifalax* telomeric sequence, the incorporation of LNA residues can induce the change of antiparallel to parallel G-quadruplex structures [57]. The tetrameric TG<sub>4</sub>T and TG<sub>3</sub>T quadruplexes carrying several LNA

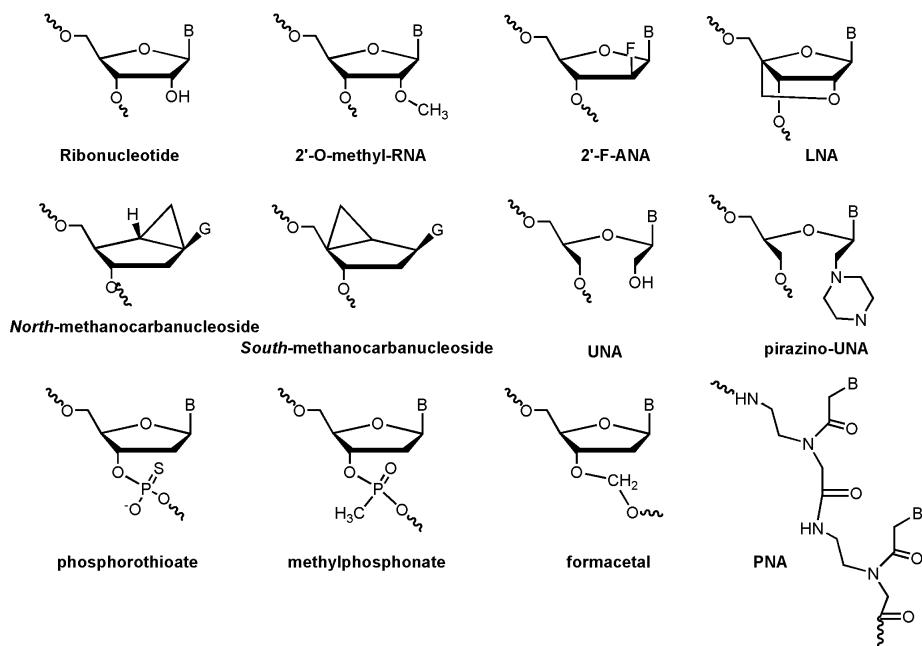


Figure 6.5: Chemical structures of backbone modifications introduced in G-quadruplexes.

residues have been studied by NMR [58, 59]. The molecule adopts a parallel stranded conformation, showing a right-handed helicity and the guanine residues in an almost planar conformation. The thermal stability of quadruplexes carrying LNA is higher than the DNA quadruplex but similar to the RNA [58, 59]. The thrombin binding aptamer modified with LNA has been also studied by NMR [60–62]. A small number of LNA substitutions maintain the antiparallel structure of modified TBAs and the subsequent binding to thrombin although a decrease in the affinity to thrombin is usually observed [60–62]. In a comparative study, the effects of sugar-modified guanosines on the structure and stability of a (4 + 0) parallel and a (3 + 1) hybrid G-quadruplex was evaluated. To this end, over 60 modified sequences containing a single substitution of LNA-G, F-ANA-G and 2'-deoxy-2'-fluoro-riboguanosine (FG) were prepared [63]. LNA-G substitutions into *anti* position guanines within a guanine-tetrad lead to a more stable G-quadruplex, while substitutions into *syn* positions disrupt the native G-quadruplex conformation.

*North* and *South* bicyclo[3.1.0]hexane pseudonucleosides or methanocarbanucleosides (Figure 6.5) are other conformationally restricted nucleosides that have been used to study the influence of *North/anti* and *South/syn* conformers in the stability of the TBA quadruplex [64]. An advantage of these modified pseudonucleosides over LNAs is that both *North-* and *South-*locked platforms can be prepared by shifting the position of the fused cyclopropane ring. In addition, the *North*-pseudosugar confor-

mation favors the *anti* glycosyl orientation, whereas the *South*-pseudosugar conformation favors the *syn* disposition of the base. The introduction of methanocarbanucleosides at selected positions indicated that the *syn/anti* glycosyl conformation is a more restrictive factor for the TBA stability than the sugar puckering.

The use of guanine flexible nucleosides replacing the guanines of the tetrads has also been reported. Unlocked Nucleic Acids (UNA) (Figure 6.5) are acyclic RNA derivatives that lack the C-C bond between positions 2' and 3'. The systematic replacement of each of the nucleosides in TBA by UNA units has been described. The substitution by uridine derivatives at the loops maintains or slightly increased the stability of the antiparallel TBA structure [65]. On the contrary, the replacement of the guanines of the tetrads is unfavorable. The anticoagulant properties of the modified TBA are maintained or even increased in some cases [65]. Another flexible nucleoside modification that has been evaluated in the loop positions of the TBA quadruplex is the 2'-C-piperazino-UNA monomer [66]. This monomer is characterized by a more efficient stabilization of quadruplexes structures in comparison to the regular UNA and increases the thermodynamic stability of TBA in a position depending manner retaining the quadruplex topology.

Peptide Nucleic Acids (PNA, Figure 6.5) and PNA/DNA hybrid molecules have been also used to investigate G-quadruplex structural features, especially in tetramolecular tetraplexes. One of the first studies on PNA quadruplex was the observation of a dimeric G-quadruplex formed by the *Oxytricha nova* DNA telomeric sequence d(G<sub>4</sub>T<sub>4</sub>G<sub>4</sub>) with a peptide nucleic acid (PNA) carrying the same sequence (H-G<sub>4</sub>T<sub>4</sub>G<sub>4</sub>-Lys-NH<sub>2</sub>, G<sub>4</sub>-PNA) [67]. The G<sub>4</sub>-PNA is also able to self-pair giving full-PNA G-quadruplexes that can be dimeric or tetrameric, although the dimeric form is preferred [68]. Several PNA-DNA chimeras having the TG<sub>3</sub>T sequences were prepared using a special solid-phase protocol that combines the formation of amide, phosphodiester and phosphoramidate linkages. The resulting PNA-DNA chimeras carrying one or two PNA units were able to form parallel tetrameric structures with similar stability than the unmodified DNA TG<sub>3</sub>T sequence [69]. A detailed report on the thermodynamic properties of the TG<sub>4</sub>T sequence carrying one PNA modification was described [70]. The study confirms that the incorporation of a PNA unit into TG<sub>4</sub>T oligonucleotides results in the formation of tetrameric parallel quadruplexes and the modification changes their folding and biophysical properties. Thermal denaturation experiments showed an increase in quadruplex stability for chimeric quadruplexes. Singular value decomposition analysis suggested the presence of kinetically stable intermediate species in the quadruplex formation process [70]. The self-assembly of the PNA sequence TG<sub>3</sub> comprising three tandem guanine residues and a lysine residue at the C-terminus to enhance solubility in water was reported [71]. The all-PNA tetramer was characterized by electrospray-mass spectrometry. A bimolecular homo-PNA quadruplex was obtained by self-assembly of a special bis-PNA prepared using a bifunctional linker that was functionalized with the G<sub>3</sub>T PNA sequence [72]. PNA oligomers targeting quadruplex forming oligoribonucleotides can also form hybrid

PNA-RNA quadruplexes [73]. In an interesting development, a short PNA molecule carrying three PNA-G units and an acridone molecule was able to form a hybrid 3 + 1 PNA-DNA bimolecular complex with a three-repeat fragment of the human telomere [74]. The combined effects of tetrad formation and stabilization of quartet by the “capping” effect of the intercalating agent trigger quadruplex formation.

Oligonucleotides containing phosphorothioate (Figure 6.5) substitutions at different internucleotide sites were studied. It was found that these linkages do not disrupt the antiparallel intramolecular quadruplex [75]. The substitutions placed between planes of G-quartets led to a drop in the formation free energy, and the stability decreases linearly with the number of these modifications. The effect of the phosphorothioate bonds was also analyzed in parallel tetrameric quadruplexes. In this case the phosphorothioate is present in all the four G of the tetrads. The resulting modified TBA was less stable than the unmodified TBA [52]. The effect of several chemical modifications including phosphorothioate linkages, 2'-O-methyl-RNA, L-nucleotides and LNA units on a G-quadruplex based DNAzymes with peroxidase activity was described. 2'-OMe-RNA modification was found to yield the best results by stabilizing parallel G-quadruplexes which increased peroxidase activity about 3-fold higher than the unmodified DNA-form [76].

Oligonucleotides carrying methylphosphonate linkages were studied in tetrameric G-quadruplex. The modified TBA variants suffered a strong destabilization [54]. A series of TBA analogs were synthesized containing one or more phosphodiester linkages replaced by a natural formacetal group [77]. The results obtained show no loss of anticoagulant activity by a single formacetal substitution; however, in oligodeoxynucleotides carrying two noncontiguous formacetal groups the result was the sum of the effects obtained from the corresponding monosubstituted TBA derivatives. The *in vivo* anticoagulant properties of an oligodeoxynucleotide containing four formacetal groups showed an increase of the anticoagulant effect and an extended half-life compared to the unmodified TBA [77].

### 6.1.3 Modifications at the loops

G-quadruplex can be formed by one, two or four oligonucleotides strands. When the G-quadruplex is formed by four strands all the 2'-deoxyguanosines are in the *anti* conformation. In the other two cases, the 2'-deoxyguanosine residues can be in *syn* or *anti* conformation and the connecting loops can exert some type of control on the final structures. In a detailed study, the impact of the length and composition of the loops and the number of tetrads in the structure of the intramolecular TBA quadruplex was reported [78]. Modifications of the loops maintained the main structure of the antiparallel quadruplex but induce relatively large changes in its stability. In a separate report, the substitution of T by A in the loops of the TBA quadruplex was found to have a strong impact on the stability of the quadruplex and in the interaction

with thrombin [79]. Moreover, the presence of guanines in the loops of TBA provoked a large topology alteration and the monomeric antiparallel structure was changed to a dimeric parallel structure with additional G.G.T.T tetrads [80]. In a recent work, a loop that contains a potential C-C<sup>+</sup> base pair was found to exert a pH-dependent stabilization of the G-quadruplex structure present within the SMARCA4 gene promoter region [81].

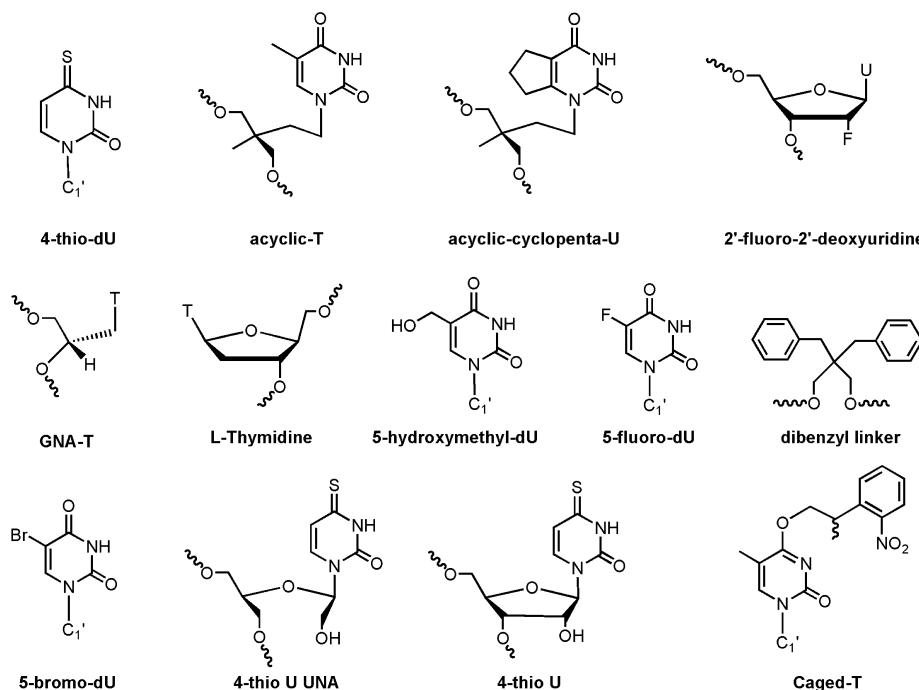
The introduction of modified nucleosides in the loops also modulates the structural and biological properties of the quadruplexes. These effects have been studied in the thrombin binding aptamer that has two lateral TT loops in the interaction with thrombin. Substitution of thymidines of the loops by 4-thio-2'-deoxyuridine (Figure 6.6) residues in the TBA increased its anticoagulant properties [82]. The replacement of the T residues of the loops by an acyclic thymine derivative (Figure 6.6) generated changes in the stability of TBA and one modified derivative had an increased anticoagulant activity [83]. Oligonucleotides carrying a similar acyclic derivative but containing a cyclopenta uracil derivative (Figure 6.6) instead of a thymine has also been reported for the modification of TBA loops. In this case the introduction of the acyclic derivative improves the stability of the quadruplex structure especially at position 7. The anticoagulant activity of the modified TBAs was also enhanced [84]. Moreover, its folding ability was more affected by modification in the thymidines 4 and 13 than in positions 3 and 12 of TBA. Similar results were found in the systematic replacement of the TBA residues by F-ANA (Figure 6.5) [55, 56], LNA (Figure 6.5) [62] and UNA (Figure 6.5) [65].

Modified thrombin-binding aptamers (TBAs) carrying uridine (U), 2'-deoxy-2'-fluorouridine (FU) (Figure 6.6) and *North*-methanocarbathymidine (Figure 6.5) residues in the loop regions were synthesized. The replacement of T in the TGT loop by U and FU resulted in an increased stability of the antiparallel quadruplex structure while the presence of methanocarbathymidine in the same position destabilizes this structure. The substitution of the Ts in the TT loops by these nucleoside derivatives induced destabilization of the antiparallel quadruplex [85].

The effects of site-specific substitutions such as thymine glycol nucleic acid (GNA, Figure 6.6) and L-thymidine in the loops of model G-quadruplexes were studied [86]. The substitutions of the Ts in the loop positions with GNA-T decrease TBA stability except for a single modification in T7 position. L-thymidine substitutions in the loops were not well tolerated.

The substitution of thymidines in the loops of TBA by the naturally occurring oxidation product hydroxymethyl-dU (Figure 6.6) was also reported [87]. Modified TBA derivatives retain the ability to fold into the antiparallel intramolecular quadruplex structure as well as maintain the thermal stability compared to the unmodified TBA. Although TBA analogs presented lower affinities to the thrombin, it was found that modified TBAs at positions 3, 7 and 9 have improved anticoagulant properties [87].

Oligonucleotides carrying 5-fluoro-2'-deoxyuridine residues instead of T have been also prepared [88]. Single substitutions maintain the chair-like conformation



**Figure 6.6:** Chemical structures of compounds introduced at the loop positions of G-quadruplexes.

observed for unmodified TBA. TBAs carrying FdU at positions 4 and 13 had a remarkable improvement both in the melting temperature and in the anticoagulant activity in comparison with unmodified TBA [88].

In addition to anticoagulant properties, it has been described that TBA has mild antiproliferative properties [8]. This situation can be reversed by the substitution of the nucleotides in the loops by a dibenzyl linker (Figure 6.6) obtaining TBA derivatives with higher antiproliferative activity and lower anticoagulant properties [89].

Literature data suggests that the antiproliferative activity of the single-stranded guanosine-rich oligodeoxyribonucleotides (GROs) depends on the binding to biological targets such as a specific cellular protein (GRO-binding protein) [8] but also in the contribution of the guanine-based degradation products [90]. Modified TBA analogs containing L-residues and inversion of polarity sites lose the anticoagulant activity however gain antiproliferative properties against two cancer cell lines [91]. As these TBA derivatives are more resistant to degradation by nucleases, these data support the important role in the antiproliferative activity of the binding of aptamers to GRO-binding proteins. The introduction of several inversions of the polarity near the loops can reproduce the folding to the chair-like structure but the resulting TBA derivatives lose the anticoagulant activity increasing the antiproliferative and antimotility activities in Calu-6 cells [92].

The substitution of thymidines at positions 4 and 13 of the TBA by dU, 5-bromo-dU and 5-hydroxymethyl-dU (Figure 6.6) maintained the antiparallel quadruplex of unmodified TBA but it can dissociate the anticoagulant and antiproliferative activities [93]. In this work, the authors describe that all the modified TBAs have antiproliferative activity but only TBA modified with dU, 5-bromo-dU maintain the anticoagulant properties similar to unmodified TBA.

The effect of single incorporation of all four canonical UNAs (Figure 6.5) and a novel 4-thiouracil UNA derivative (Figure 6.6), as well as single incorporation of 4-thiouridine (Figure 6.6) was reported [94]. TBA variants containing these modified nucleotide residues maintain the TBA folding topology. Certain UNA residues may improve G-quadruplex anticoagulant properties. Interestingly, some TBA variants with low anticoagulant properties have significant antiproliferative properties in HeLa cells [94].

The introduction of alkyl phosphorothioate linkages to TBA weakens the affinity of modified TBAs to thrombin; however, the antiproliferative activity in A549 cells was maintained [95]. Unfortunately, no antiproliferative activity was found in MC7 cells.

Recently, dimer TBA sequences were prepared by the connection of two molecules of TBA and a nucleotide or 1,3-propandiol linker [96]. The antiproliferative activities of the bis-TBA oligonucleotides for eight human cancer cell lines were reported. The most pronounced antiproliferative effect of the bis-TBA molecules on the cell viability was found for lung cancer cells RL-67 and central nervous system cancer cells U87 [96].

The thrombin binding properties and the anticoagulant activity of the TBA can be regulated by the introduction of a caged thymidine in the TT loops [97]. In this work, TBA derivatives carrying a photosensitive thymidine (Figure 6.6) in the TT loops were prepared. The resulting TBA derivatives did not bind to thrombin and subsequently, they did not present anticoagulant properties. Upon irradiation quantitative formation of the uncaged TBA was observed and both the thrombin binding and anticoagulant properties were fully recovered [97].

#### **6.1.4 Applications of aptamers based in G-quadruplex. Functionalization at the 3' or 5'-ends**

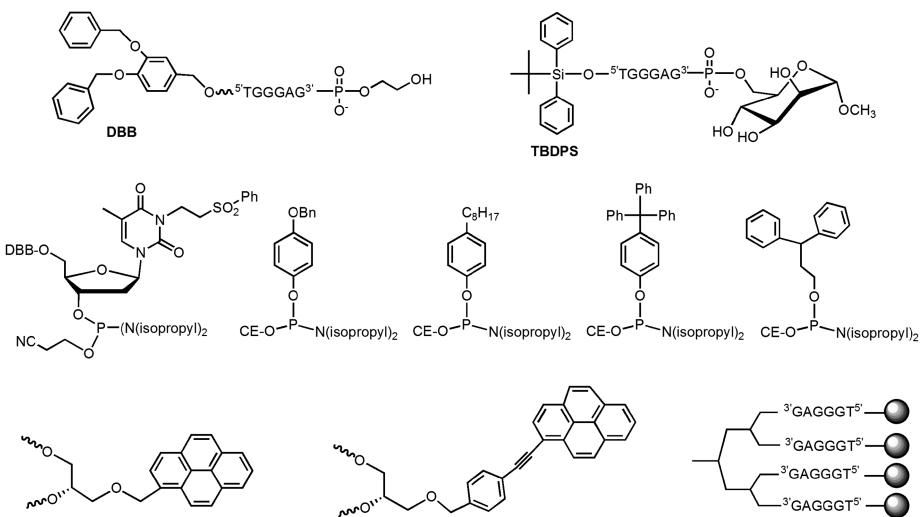
A wide range of pathologies such as cancer, viral infections as well as blood coagulation have been targeted using aptamers that fold into G-quadruplex [1, 98]. The main advantages found for aptamers with a G-quadruplex structure are higher stability and increased cellular uptake [99–103].

Several G-rich oligonucleotides have been described to possess antiviral properties specially anti-HIV-1 inhibition properties [104]. In a first approach G-quadruplex based aptamers have been successfully designed to target a number of several HIV proteins such as HIV-1 reverse transcriptase [105, 106], HIV-1 RNase H [107] and HIV-1

integrase [108–111]. Especially relevant is the 93del aptamer that adopts a dimeric quadruplex folding topology inhibiting HIV-1 integrase at nanomolar concentration [111]. Afterwards, these efforts generated the aptamer T30177 (also known as AR177 or Zintevir) with higher nuclease stability and integrase inhibition capacity [112]. Later it was found that the primary target for the antiviral action of AR177 (Zintevir) is the glycoprotein gp120 [113–115]. The cationic V3 loop region of the viral envelope glycoprotein gp120 was found to be the primary target of the phosphorothioate 8mer 5'-TTGGGGTT-3' [116, 117] as well as a series of G-rich oligonucleotides carrying the DMT group at the 5'-end [118–120]. From these oligonucleotides, the 6mer 5'-DMT-d(TGGGAG)-3' known as Hotoda sequence was found to retain most of the antiviral activity by binding to HIV-1 gp120 protein [121].

As the DMT group is relatively labile a large number of hydrophobic moieties including aromatic compounds and silyl groups were tested for the functionalization of the 5'-end of the Hotoda 6mer [122–126]. The most potent activity and the least cytotoxicity was the 6-mer bearing a 3,4-dibenzylxyloxybenzyl (Figure 6.7, DBB) group at the 5'-end [123]. In addition different modified phosphate analogs were added at the 3'-end including 2-hydroxyethylphosphate, 2-hydroxyethylthiophosphate, or methylphosphate group [123]. Among various 3'- and 5'-end-modified 6-mers that were tested, the 6-mer bearing a DBB group at the 5'-end and a 2-hydroxyethylphosphate group at the 3'-end was found the most appropriate as antiviral [123]. The 6-mer bearing a DBB group at the 5'-end enhanced the formation of a parallel G-quadruplex. The presence of the parallel G-quadruplex structure and the hydrophobic nature of the group at the 5'-ends were found to be the requirements for the anti-HIV-1 activity [124]. The substitution of guanines for *N*<sup>2</sup>-methyl-2'-deoxyguanosines showed an increase of the antiviral activity as expected for the observed stabilization of the G-quadruplex structure [126]. In order to simplify the synthesis of the DBB-oligonucleotides a novel T phosphoramidite protected at the nucleobase (Figure 6.7) and bearing the DBB group at the 5'-position was described [127]. A detailed thermodynamic and kinetic analysis of the association and dissociation of the DBB-modified parallel quadruplex demonstrated that the hydrophobic group at the 5'-end increased the rate of formation of the quadruplex that is responsible for the antiviral activity [127].

The addition of carbohydrates such as glucose, sucrose or mannose at the 5'- and 3'-end of the Hotoda 6mer was reported [128]. The combination of a mannose residue at the 3'-end and the *tert*-butyldiphenylsilyl (TBDPS) at the 5-end (Figure 6.7) was found to be the best combination for quadruplex formation resulting in a 3-fold greater antiviral activity [128]. Novel anti-HIV G-quadruplex-forming oligonucleotides carrying aromatic groups at the 5'-end (Figure 6.7) were studied [129]. Several of these oligonucleotide derivatives showed pronounced activity preferentially against HIV-1, especially the oligonucleotide carrying the 4-benzylxyloxy phenyl derivative. These oligonucleotides also showed high binding affinities for the HIV-1 envelope gp120 and gp41 proteins [129].



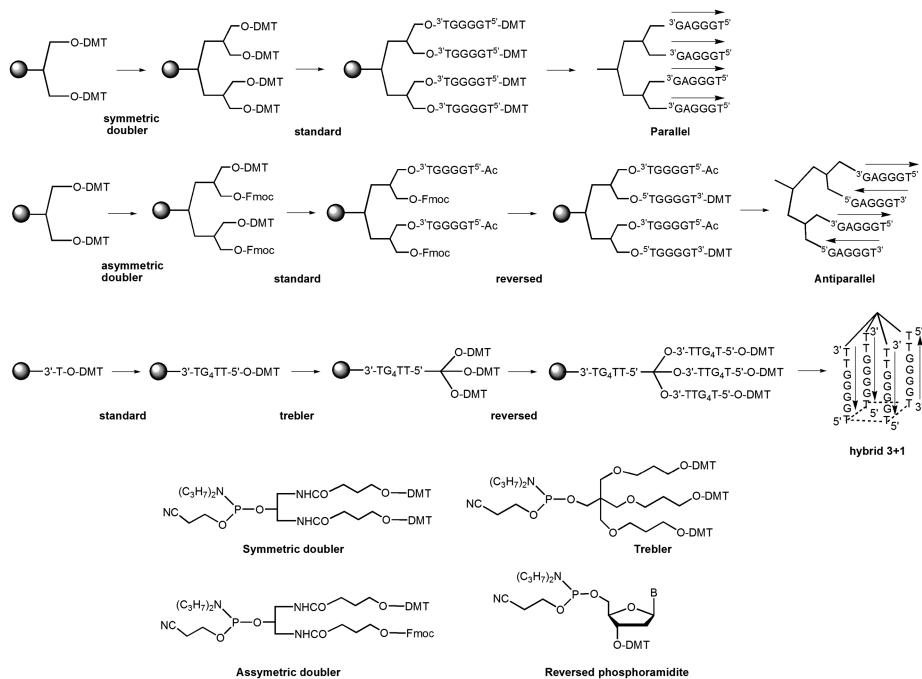
**Figure 6.7:** Chemical structures introduced at the terminal positions of G-quadruplexes.

Enhanced anti-HIV-1 activity and stabilization of the G-quadruplex of Hotoda 6mer and T30177 aptamers was also achieved by the incorporation of LNA residues (Figure 6.5) and intercalating compounds derived from pyrenylmethylglycerol (Figure 6.7) [130]. In the modified T30177 aptamers, the presence of the DMT group produces more active aptamers that stimulate the formation of aggregates. The increased activity seems to correlate with the presence of the high-order G-quadruplex structures [130].

A dramatic improvement of the stability of the G-quadruplex parallel structures was reported by linking the four 6mer strands (Figure 6.7) [131]. The tetra-end-linked oligonucleotides have an enhanced thermal stability because the intramolecular structure of the quadruplex improved the association kinetics. This stability produced a significant increase of affinity to viral gp120 protein and the subsequent increase of anti-HIV-1 activity. This is especially relevant if the oligonucleotides are capped at the 5'-end that improves their resistance to serum nucleases degradation.

The synthesis of tetra-end-linked oligonucleotides is performed using branched phosphoramidites [132–136] (Figure 6.8). These branched phosphoramidites can be symmetric or asymmetric doublers or treblers. The combination of branched phosphoramidites, regular nucleoside phosphoramidites and reversed nucleoside phosphoramidites allow the preparation of a large variety of tetra-end-linked oligonucleotides with several strand orientations (Figure 6.8).

The incorporation of lipid residues at the 3' or 5'-ends of G-rich antiviral oligonucleotides has been also reported [137–139]. The addition of a cholesterol molecule to the G-rich 20mer phosphorothioate oligonucleotide 5'-d(TGGGGCTTACCTTGCG-AACA-3') potentiates the formation of a parallel G-quadruplex improving the phar-



**Figure 6.8:** Synthesis of tetra-end linked G-quadruplex using branched phosphoramidites. Synthetic strategies for the preparation of parallel, antiparallel and hybrid 3 + 1 tetra-end linked G-quadruplexes. Bottom: chemical structures of symmetric and asymmetric doubler and trebler phosphoramidites as well as reversed phosphoramidites used in these protocols.

macokinetic properties of the known antisense inhibitor of human cytomegalovirus [136]. The synthesis and antiviral activity of lipid–oligonucleotide conjugates (Lipoquads) forming a highly stable tetramolecular parallel G-quadruplex were recently described [139, 140]. These molecules block HIV-1 and HIV-2 entry with submicromolar activities. Because the behavior of envelope proteins is similar in several other enveloped viruses, Lipoquads have broader activities against enveloped viruses such as hepatitis virus C [141].

G-rich oligonucleotide conjugates containing acridine and quindoline derivatives linked through a threoninol molecule have been reported [142]. These modified TBA conjugates maintained the intramolecular antiparallel quadruplex structure with higher thermal stability than the unmodified TBA oligonucleotide [142].

The synthesis of carbohydrate–DNA conjugates containing the oligonucleotide sequences of G-quadruplexes such as TBA and human telomere sequence were reported and analyzed by NMR [143]. The solution structure of the fucose–TBA conjugate showed stacking interactions between the carbohydrate and the DNA G-tetrad in addition to hydrogen bonding and hydrophobic contacts. Moreover, the introduction of carbohydrates at the 5'-end of the quadruplex telomeric sequence was shown to alter

its folding topology suggesting the possibility of the modulation of the folding of the G-quadruplex by the introduction of modifications at the ends [143].

Recently, the possibility of linking peptides to TBA to modulate the interaction between  $\alpha$ -thrombin and the TBA aptamer was reported [144]. Short peptide fragments present in the acidic region of the human factor V, known to interact directly with exosite I of thrombin, were added to the 5'-end of TBA. Surface Plasmon Resonance (SPR) showed enhanced detection of thrombin by one of the peptide-TBA conjugates [144].

The affinity of TBA for metal ions has been used to build sensors for potassium ions. In this case, the conformational change of the TBA during the folding process is used for the detection of metal ions. For this purpose, TBA functionalized with 5'-fluoresceine and a quencher [145] at the 3'-end as well as 5'-functionalized with pyrene [146] was developed for the detection of potassium ion. The area of biosensors using aptamers is very active and it has been summarized in recent reviews [147–149].

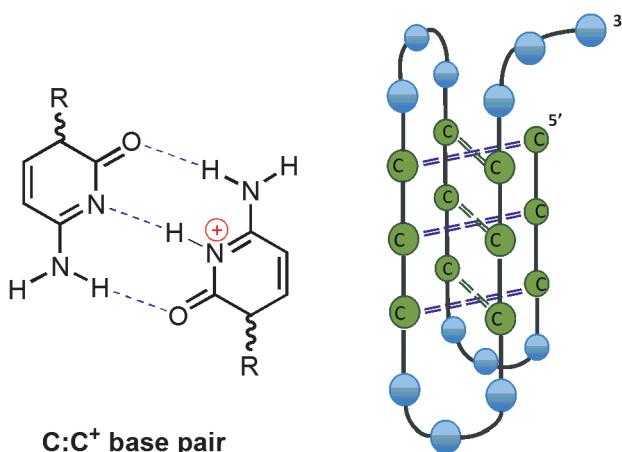
The G-quadruplex aptamer AS1411 also known as AGRO100 has been shown to reduce cell proliferation by binding to nucleolin that is overexpressed in cancer cells [150]. A large number of nanomaterials and small molecules such as fluorescent probes and, drugs have been functionalized with aptamer AS1411 in order to facilitate *in vivo* targeting by using the nucleolin binding properties [151]. Recently, AS1411 has been found to have anti-HIV-1 activity acting as a cell entry inhibitor [152, 153].

## 6.2 Oligonucleotides forming i-motif

C-rich oligonucleotides can fold into a singular structure known as i-motif. These are composed by two parallel parallel duplexes arranged in an antiparallel way. Each one of the parallel duplexes are maintained by the formation of intercalated C:C<sup>+</sup> base pairs (Figure 6.9) [154]. Because the formation of this base pair requires the protonation of the cytosine bases at N<sup>3</sup> position, its maximal stability against pH is near the pK<sub>a</sub> of free cytosine, around 4.5. As other nucleic acid structures, the stability is also temperature dependent. Hence, the highest stability of i-motif structure *in vitro* is found at low temperature and pH values near 4.5.

I-Motif structures can be intramolecular or monomeric or intermolecular both dimeric or tetrameric (Figure 6.9). Monomeric i-motif structures are those formed by a C-rich sequence that folds showing intramolecularly yielding the formation of three loops. It has been demonstrated that both, the length and nature of bases present at the loops may affect dramatically to the stability of the resulting i-motif structure [2, 154].

The biophysical study of the stability of i-motif structures is usually carried out by means of spectroscopic techniques, such as circular dichroism or nuclear mag-

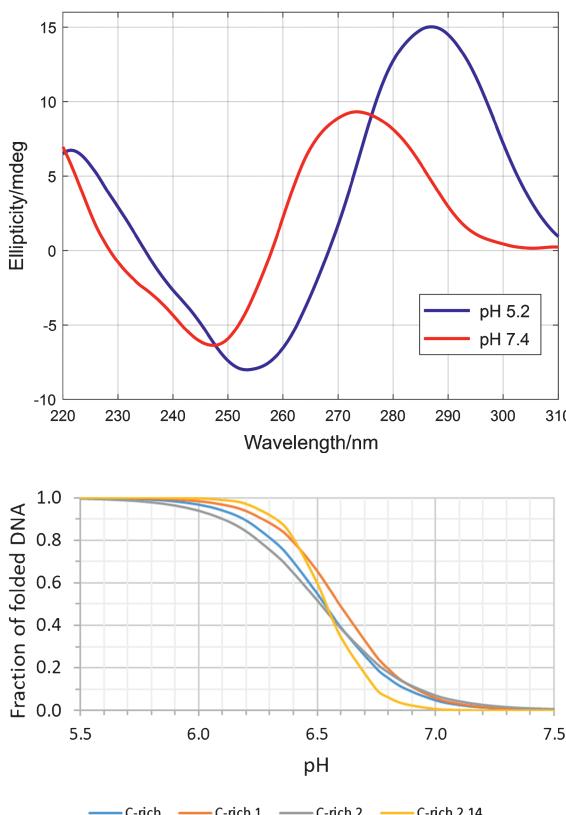


**Figure 6.9:** (a) C:C<sup>+</sup> base pair showing the protonation of the N<sup>3</sup> position of one of the C bases- (b) Scheme of a tetrameric i-motif structure showing the antiparallel disposition of two parallel duplexes maintained by the C:C<sup>+</sup> base pairs.

netic resonance. Figure 6.10 shows the typical CD spectrum of an i-motif structure, which features are an intense positive signal around 288 nm and a less intense negative band around 255 nm. At neutral pH, the CD spectrum corresponding to the nucleic acid sequence is clearly different from that of the i-motif structure. By using CD spectroscopy, or another spectroscopic technique such as molecular absorption, it is possible to monitor the folding of a C-rich sequence into an i-motif structure induced by pH or temperature changes. In the case of acid-base titrations, the midpoint of the transition from the folded to the unfolded sequence is called the pH<sub>1/2</sub> and gives an estimate of the stability of the i-motif structure in front of pH changes. As example, Figure 6.8b shows the folding transitions of a C-rich sequence induced by pH. Small variations in both, position of pH<sub>1/2</sub> and cooperativity are observed when chemical modifications are introduced into the backbone.

The NMR spectra of hemi-protonated C:C<sup>+</sup> base pairs are very characteristic, exhibiting imino signals around 15.0–16.0 ppm, and amino protons signals around 9.0–10.0 ppm, with their corresponding NOE cross-peaks (Figure 6.11). Although observation of these signals is usually considered an evidence of i-motif formation, it must be taken into account that C:C<sup>+</sup> base pairs may occur in other DNA secondary structures, such as parallel duplexes. Definitive evidence of i-motif formation arises from peculiar NOE patterns in the NOESY spectra. In particular, the presence of multiple long distance NOEs between sugar H1' protons, and between C:C<sup>+</sup> amino protons and H2'/H2'' sugar protons (Figure 6.11). The former ones occur through the minor groove and the latter through the major groove of the i-motif.

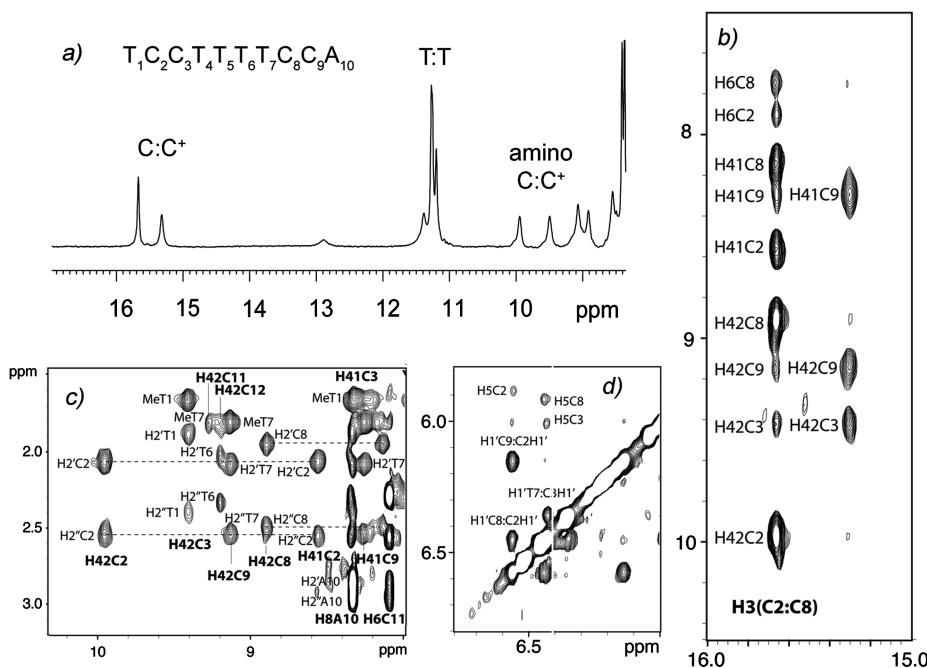
NMR is also an excellent technique to detect capping interaction in i-motif structures. For example, T:T base pairs, occurring very frequently at the sides of the i-motif



**Figure 6.10:** (a) CD spectra of the  $(C_3TA_2)_3C_3T$  sequence measured in 10 mM acetate or phosphate buffer and 15 °C. (b) Fraction of folded DNA into i-motif structure calculated from acid-base titrations of several C-rich oligonucleotides carrying 2'-deoxycytidine modifications by means of CD spectroscopy.

C-stack, can be detected by characteristic sharp signals between 10.0 and 11.5 ppm (Figure 6.11).

Because they are complementary to G-rich oligonucleotides the natural occurrence of i-motif forming sequences follows the same trend than G-quadruplex forming sequences [154, 155]. For these reasons, i-motif sequences can be found in telomeric, centromeric and promoter regions supporting their role in biologically relevant functions as modulators of gene expression [2, 155]. Additionally, these structures are interesting in the field of nanotechnology, since they have been used to control the assembly of supramolecular structures or in the design of molecular motors and sensing systems such as nanoswitches driven by pH changes to monitor biological processes [156–161]. The large number of potential applications for i-motif forming oligonucleotides has triggered the interest for nuclease resistant oligonucleotide derivatives

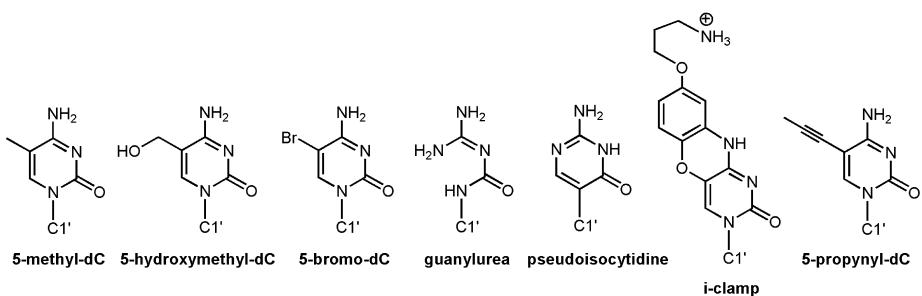


**Figure 6.11:** Example of an NMR characterization of an i-motif structure. (a) NMR spectra of the oligonucleotide sequence shown in the panel with the usual signals found in i-motifs indicated. (b) typical imino-amino cross-peaks for C:C<sup>+</sup> base pairs. (c) inter-strand amino – H<sub>2</sub>'/H<sub>2</sub>'' and (d) H<sub>1</sub>'-H<sub>1</sub>' cross-peaks.

and the generation of novel oligonucleotides as potential pH-sensors able to form i-motif structures at different pHs [158–160].

### 6.2.1 Cytosine modifications

Cytosines present at CpG positions can be modified in living cells to several derivatives including 5-methyl-dC and 5-hydroxymethyl-dC (Figure 6.12). These epigenetic modifications that were described in Section 2.3.3 have been studied in the context of i-motif formation [162–164]. The modification of C to 5-methyl-dC induces a small increase of the stability of i-motif structures that is in agreement with the increased pK<sub>a</sub> of 5-methyl-dC (the pK<sub>a</sub> of isolated 5-methyl-dC is 4.5, the pK<sub>a</sub> of isolated dC is 4.4 [165]). On the contrary, the substitution of C by 5-hydroxymethyl-dC decreases the stability of the i-motif [163]. The addition of several crowding agents eliminates the stabilizing or destabilizing properties due to the presence of the epigenetic modifications [163]. In another study, the effect of multiple modifications in telomeric i-motif suggested that DNA i-motifs were stabilized when they are modified with one or two 5-methyl-dC residues. On the other side, the introduction of 5-hydroxymethyl-



**Figure 6.12:** Chemical structures of cytidine analogs introduced in i-motif structures.

dC residues caused the destabilization of the i-motif structure [162]. The impact of 5-methyl-dC and 5-bromo-dC (Figure 6.12) in i-motif sequences was also analyzed. In this case, 5-bromo-dC has a lower pK<sub>a</sub> than dC (the pK<sub>a</sub> of isolated 5-bromo-C nucleobase is 2.45 [166]). The relative values of the pK<sub>a</sub>'s of isolated nucleosides are reproduced in the pH mid-transitions of the modified i-motif [5.9, 6.3 and 6.4 for i-motif carrying 5-bromo-dC (pK<sub>a</sub> 2.45), dC (pK<sub>a</sub> 4.4), 5-methyl-dC (pK<sub>a</sub> 4.5)] [167]. Moreover, the stability of the i-motifs at pH 5 follows the same order (45.5, 5-bromo-dC; 55.7, dC; 59.1, 5-methyl-dC) [167]. The changes found in the pH structural mid-transitions of 5-bromo-dC and 5-methyl-dC were used to generate fluorescent DNA nanodevices expanding the pH sensing regime from 5.3 to 7.5 [160].

5-Azacytosine derivatives are chemotherapeutic compounds acting as DNA methyltransferase inhibitors [168] after incorporation in DNA as described in Section 3.6.2. The genome of patients treated with 5-azacytosine derivatives may contain 5-azacytosine replacing cytosine. Due to the metabolic instability of 5-azacytosine, guanylurea residues are generated at 5-azacytosine positions [169]. In order to analyze the impact of this chemical modification in the stability of i-motif Wright et al. [170] prepared oligonucleotides carrying guanylurea (Figure 6.12) residues. The substitution of C by guanylurea decreased the thermal stability and the pH mid-transition of the i-motif structures [171].

Pseudoisocytidine (Figure 6.8) was described in Section 5.2.2 as a neutral analog of protonated cytidine allowing the observation of parallel triplexes at neutral pH. Stabilization of the i-motif was observed at neutral pH when C's located at the external C:C<sup>+</sup> base pairs were replaced by pseudoisocytidine [172]. Curiously, when pseudoisocytidine is located in the central C:C<sup>+</sup> base pairs the resulting i-motifs are only observed at acidic pH. Mir et al. suggest that protonated base pairs are needed to stabilize the i-motif structure [171].

The phenoxazine 2'-deoxynucleoside (Figure 6.12) was described in Section 3.3.3 as duplex stabilizing pyrimidine derivative because the phenoxazine ring provides a rigid scaffold for the introduction of appending amino group designed to interact with the N<sup>7</sup> Hoogsteen position of the complementary guanine (G-clamp effect). Tsvetkov

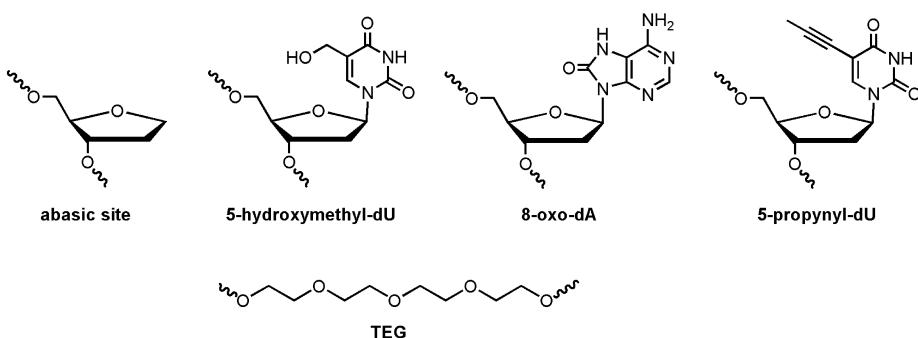
et al. reported the use of a similar phenoxazine nucleobase, named as i-clamp (Figure 6.12), for the stabilization of i-motif structures [172]. The i-clamp modification generated an increase of the stability on both intramolecular and intermolecular i-motifs that was reported to be due to the interaction of the protonated amino pendant group with the phosphate groups within the neighboring DNA backbone. The optimal location for the introduction of i-clamp modifications was found to be two i-clamp residues on opposite strands providing a thermal stabilization of around 10–11 °C at pH 5.8 [172].

Branched oligonucleotides carrying 5-propynyl-dC (Figure 6.12) residues were reported [173]. The low  $pK_a$  value of this nucleoside ( $pK_a$  3.3) compared to dC ( $pK_a$  4.5) preclude the use of this analog at neutral pH. Strong stabilization of the modified i-motif structures at pH 3.5 was observed when branching residues linking two oligonucleotide strands in a parallel orientation were introduced. The immobilization of oligonucleotides carrying 5-propynyl-dC on gold nanoparticles generated DNA-gold nanoparticles aggregates at pH 5.0 indicating the formation of stable i-motif structures [173].

### **6.2.2 Modifications of the 2'-deoxyribose phosphate backbone in the i-motif core and at the loop positions**

The first studies on the effect of modified backbones in the stability of i-motif were performed at the same time than the development of modified oligonucleotides for triplex formation. In the case of triplex forming oligonucleotides designed to form parallel triplex, the C,T-TFO can be C-rich if the target ds-DNA is G:C rich. In this case, C,T-TFO may form i-motif structures that compete with triplex formation. So in the search for new modifications for triplex formation attention should be made to avoid i-motif formation. Lacroix et al. [174] studied the triplex versus i-motif formation of several modifications in the 16 base oligonucleotide designed to bind human neurotrophin 4/5 gene (5'-TCCTCCTTTCCCT-3'). They found that 2'-O-methyl-RNA, unmodified RNA, 5-propynyl-dC, methylphosphonates, and phosphoramidates were good modifications for triplex formation because they were unable to form i-motif. Phosphorothioate linkages were neutral but 5-methyl-dC (Figure 6.12) and 5-propynyl-dU (Figure 6.13) were stabilizing i-motif. Special emphasis was made with the substitution of the T's of the loops for 5-propynyl-dU residues that were considered inappropriate for TFO due to the strong self-assembly properties yielding competing i-motif structures [174]. An important outcome of this study is the confirmation of a previous study that showed the inability of RNA and 2'-O-methyl-RNA to form i-motif [175] indicating that RNA polypyrimidine oligonucleotides are an attractive choice for triplex formation [175].

A more detailed study on the effect of phosphorothioate linkages on the stability of i-motif was reported [176]. Different oligonucleotides carrying phosphorothioate link-



**Figure 6.13:** Chemical structures of compounds introduced at the loop positions of i-motif structures.

ages (Figure 6.5) were analyzed. In all cases, the melting temperatures of the phosphorothioate oligomers were equal or slightly inferior to the melting temperature of the natural phosphodiester oligonucleotides. For long oligonucleotides, a small change of pH leads to a completely different melting profile: the curves are reversible at pH 6.4 or lower, and a hysteresis is obtained at pH 6.8.

In a different study, the role of the chirality of the phosphorothioate linkages in the stability of i-motif was studied [177], Kanehara et al. reported that the stability of the i-motif of the  $C_n$  carrying phosphorothioate linkages was dependent on the P-chirality of the molecule: the  $S_p$  conformation produced a slightly more stable structure than  $R_p$  [177]. However, in a second study carried out with the sequence TCCCC the melting temperatures were in disagreement with the previous study. In this case, the all Rp-phosphorothioate oligonucleotide had the same stability than the unmodified oligonucleotide while the all Sp-phosphorothioate was decreased by 11 °C.

A possible explanation provided by the authors was that the i-motif structure of  $d(C_4)$  was different from all of the other i-motif structures in terms of a small twist angle and  $C4'$ -exo deoxyribose conformation [178].

The effect of one or two phosphorothiolate linkages into the  $d(TCCCC)$  sequence was also described [179, 180]. The phosphorothiolate linkage shifts the conformation of the sugar where the sulfur is attached at the 3'-position to the  $C3'$ -endo (North) pucker. The study demonstrates that the incorporation of this linkage stabilizes the i-motif with no perturbation of the overall structure [179].

In a detailed study, the effect of DNA lesions (Chapter 2) in the stability of the human telomeric sequence 5'-(CCCTAA)<sub>3</sub>CCCT-3' was analyzed [181]. Specifically, Dvorakova et al. studied the replacement of the adenines of the loops by 8-oxo-dA and by the abasic site (Figure 6.13) as well as the substitution of thymines of the loop by 5-hydroxymethyl-dU (Figure 6.13) and the substitution of dC's by the deamination product dU. The results demonstrate that the substitution of adenines for abasic sites and 8-oxoadenine in the TAA loops and the substitution of T by 5-hydroxymethyluracil were neutral modifications provoking only small changes in the stability of the i-motif.

On the contrary, the substitution of cytosine by uracil reduced the stability of the i-motif as well as provoked a shift on the pH mid transition toward more acidic pH values [181].

The substitution of the nucleotides in the loops of an intramolecular i-motif for a tetra(ethyleneglycol) (TEG, Figure 6.13) linker maintain the i-motif structure although the stability is slightly decreased [182]. When the replacement of the loops by TEG is done in several positions the formation of different topologies is observed indicating that the nucleotide loops are contributing to the topology of the i-motif.

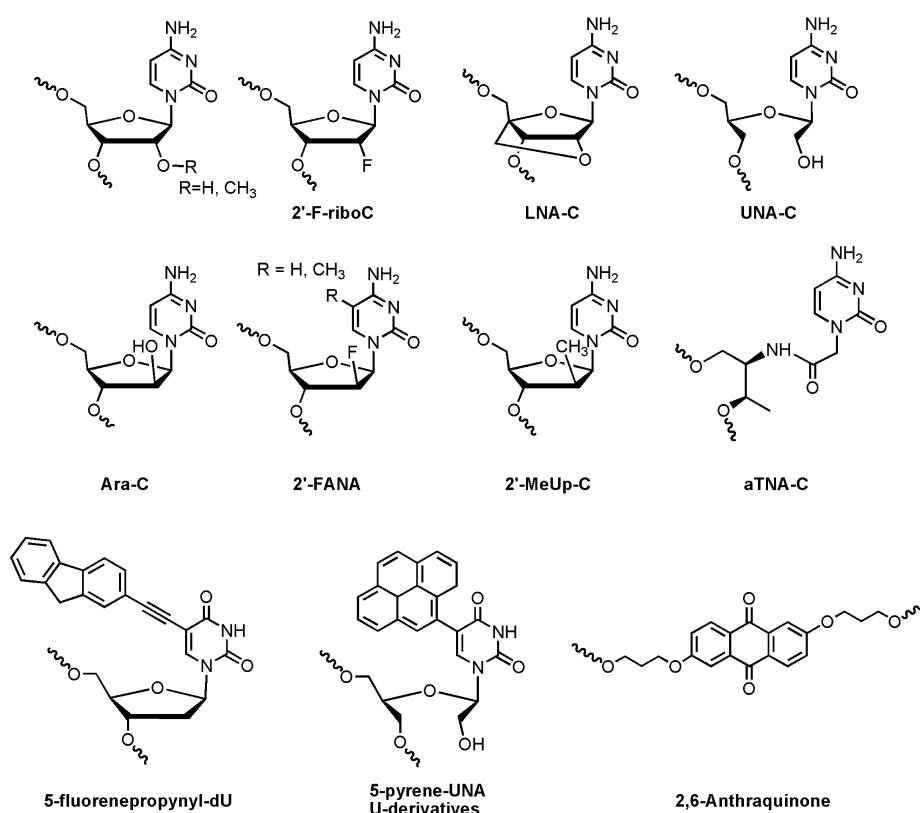
The addition of a dC oligomer at the 2' and 3' positions of a ribonucleotide generates a branched oligonucleotide with two parallel dC oligomer strands [183]. This branched oligonucleotide is able to form an i-motif that was characterized by gel electrophoresis, CD and NMR [183]. Bonnet et al. reported the use of a cyclic peptide as scaffold for the preparation of parallel and antiparallel tetrameric i-motifs [184]. Each C-rich strand is attached to the  $\epsilon$ -amino group of a lysine by using click chemistry. A combination of orthogonal copper catalyzed alkyne-azide cycloaddition with the oxime condensation allows the immobilization of two different types of strands. The resulting end-linked tetrameric molecules have a dramatic improvement in the stability resulting in i-motif that can be visualized at neutral pH [184].

An interesting topology for i-motif structures can be obtained by linking the 3' and 5'-ends of the i-motif generating circular oligonucleotides [185–188]. Li et al. prepared the first i-motif circular oligonucleotide by chemical ligation at acidic pH of the vertebrate telomeric 28mer sequence carrying a phosphate at the 5'-end that was located in the central C:C base pairs. In this way, the formation of the i-motif triggered the circularization of the oligonucleotide while the presence of mismatches inhibited the circularization [185]. In a second approach, smaller circular oligonucleotides (9-, 12-bases) were made by the use of dimeric structures as circularization templates [186]. On the other hand, DNA nanocircles consisting of 6 to 10 repeats of the hexameric telomeric sequence (resulting in circle sizes of 36, 42, 48, 54 and 60 nt) were prepared by cyclization/ligation with T4 DNA ligase [187]. Using the methodology described by Li et al. a fluorescein-tagged circular structure of i-motif was prepared [188]. The authors suggested that these fluorescein-labeled circular i-motifs could be used for the identification of proteins with affinity to i-motifs to elucidate the biological role of i-motifs.

A comparison of the effect of the 2'-OH group on the stability of the tetrameric i-motif formed by d(TCCCC) was reported [189]. The incorporation of ribonucleotides and 2'-O-methyl-RNA (Figure 6.14) residues destabilized the tetrameric i-motif. On the contrary, the incorporation of arabinonucleotides maintains the stability of the tetrameric i-motif [189]. A detailed NMR study of RNA i-motifs has been reported [190]. Four C-rich RNAs ( $r(UC_5)$ ,  $r(C_5)$ ,  $r(C_5U)$  and  $r(UC_3)$ ) were studied showing the presence of multiple intercalated structures at acidic pH. The RNA i-motifs are less stable than DNA i-motifs [190]. Interestingly, by mixing equivalent amount of C-rich RNA and C-rich DNA molecules a hybrid DNA-RNA i-motif containing two RNA and two DNA

strands was kinetically formed [191]. The stability of this hybrid i-motif allowed its characterization by CD spectra and UV melting experiments. This hybrid was slowly converted into the most stable all-DNA i-motif and ssRNA [191].

The replacement of the 2'-OH function of RNA for a fluoride atom generates 2'-fluoro-2'-deoxycytidine (Figure 6.14) that stabilizes the i-motif structure [192]. Fenna et al. reported that the introduction of a single 2'-fluoro-2'-deoxycytidine residue in the d(TCCCCC) sequence is enhancing the stability while the introduction of an RNA residue provokes an important decrease of the stability of the i-motif [189]. The authors suggest that the instability of the RNA i-motif may be due to the solvation of the hydroxyl groups, a process that is not so efficient for the fluorine substitution [192].



**Figure 6.14:** Chemical structures of backbone modifications introduced in the i-motif structures.

The introduction of 2'-deoxy-2'-fluoro arabinocytosine (F-ANA-C, Figure 6.14) [193] in the d(TCCCCC) sequence provoke a large stabilization of the i-motif as demonstrated by an increase of the melting temperature and pH mid transition that passes from 6.1 (unmodified) to 6.8–7.6 [194]. NMR studies shows that 2'-deoxy-2'-fluoroarabinose

ring adopts a *C2'-endo* conformation, instead of the *C3'-endo* conformation found in unmodified i-motifs. This substitution does not alter the overall i-motif structure but leads to a number of favorable sequential and interstrand electrostatic interactions that stabilize i-motif structure [194] and slow down the i-motif unfolding dynamics [193]. The stabilization of i-motif by 2'-deoxy-2'-fluoroarabinose can be further increased by the introduction of the 5-methyl group at the cytosine base [195]. The introduction of 2'-deoxy-2'-fluoro-5-methyl-arabinocytidine (Figure 6.14) residues in the telomeric i-motif allows the NMR observation of the i-motif structure at pH 7.0 [195].

The presence of a methyl group at the arabino position generates the (2'S)-2'-deoxy-2'-methyl-cytidine (CMeup, Figure 6.14). The effect of this modification in the telomeric i-motif induced a small stabilization with the CMeUp:CMeUp base pair more stable than the CMeUp:dC base pair [196].

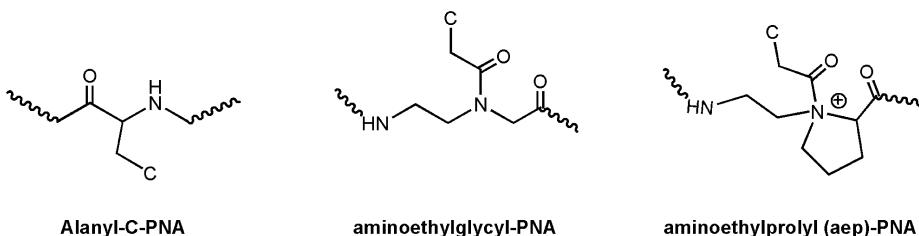
Locked nucleic acid (LNA; Figure 6.3 and 6.14) is a conformationally constrained nucleotide with a 2'-O-4'-C methylene bridge that locks the nucleotide analog in a *C3'-endo* sugar. As the *C3'-endo* conformation is found in unmodified i-motifs, the introduction of this modification was analyzed [197]. As expected the introduction of one or two LNA residues in the intramolecular i-motif formed by d(TCCCCC) produces more stable i-motifs [197]. The same stabilization was also observed in the c-myc C-rich sequence, an intramolecular i-motif present in the promoter region of c-myc proto-oncogene [198].

In a systematic study, the 22 bases fragment of the human telomeric C-rich strand was modified with unlocked nucleic acids (Figure 6.3 and Figure 6.14, UNA) [199]. When UNA residues were replacing dC residues of the i-motif, a significant loss of the thermodynamic stability of the i-motif structure was observed. In contrast any UNA modification of the TAA internal loops was found stabilizing especially at the A's of the central TAA loop without significant changes in i-motif topology [199].

The effect of acyclic threoninol nucleic acid (aTNA, Figure 6.14) residues on the stability of the intramolecular telomeric i-motifs was described [200]. In general, the substitution of dC by acyclic C modification reduced the stability of the resulting structures. The effect is dependent on the position of the modification and the type of pair implicated, being the pairing dC–acyclic-C less favorable than the acyclic-C – acyclic-C base pair. When a single substitution was introduced, the positions near the loops generate the most stable i-motifs [200].

Fluorene and pyrene-modified uracil derivatives including acyclic derivatives (Figure 6.14) have been described [201, 202]. These compounds introduced in the loop regions of the i-motif were sensitive to the formation and melting of i-motif structures. Anthraquinones (Figure 6.14) [203], acyclic pyrenes (TINA, Figure 6.7, [204]) and porphyrins [205] have been introduced in the loop positions of i-motifs. The insertion of anthraquinone and acyclic pyrene at the central TAA loop of the telomeric C-rich sequence was found to stabilize the i-motif structure [203, 204].

Alanyl-PNA (Figure 6.15) is a regular peptide backbone with alanyl amino acids which have nucleobases connected to the  $\beta$ -position of alanine. The oligomers H-(Gly-



**Figure 6.15:** Chemical structures of modified peptide nucleic acid (PNA) backbones introduced in the i-motif structures.

AlaC<sub>4</sub>-Lys containing a glycine unit as every second amino acid were able to form stable complexes at pH 4.5 including C:C<sup>+</sup> base pairs [206].

The formation of i-motif structures on regular 2-aminoethylglycine PNA backbones was also reported by the analysis of the self-assembly properties of PNA-TC<sub>8</sub>. The formation of a C-C<sup>+</sup> tetrameric i-motif in acidic pH was observed [207]. Interestingly, the PNA i-motif was more stable than unmodified dTC<sub>8</sub> at pH 3 but less stable at pH higher than 4.5 [207]. Using nanoelectrospray ionization spectroscopy coupled with H/D exchange the formation of i-motif of the C-rich PNA oligomer C<sub>5</sub>T was also described [208]. This methodology confirmed that the thermal stability of the PNA oligomer is similar to the unmodified DNA oligonucleotide however, the PNA i-motif structure was observed only at a very narrow pH around pH 4.0–4.5 [208].

Modi et al. reported that the equimolar mixture of C-rich DNA and PNA oligonucleotides yielded a hybrid i-motif complex composed of two DNA strands and two PNA strands. DNA and PNA strands were arranged antiparallel to each other. The hybrid i-motif has enhanced stability and was observed in a wider pH range than the PNA one [209]. Similarly, Chakraborty et al. described the formation of hybrid RNA-PNA i-motif composed of two RNA strands and two PNA [210].

The PNA derivative, aminoethylprolyl-PNA (aep, Figure 6.15) has a special protonated backbone with interesting hybridization properties. Gade et al. reported the synthesis of aep-PNA-C<sub>5</sub>. The C-rich sequence forms hybrid DNA:aep-PNA (1:1) i-motif with enhanced stability probably due to additional strong ion-ion attractive interactions between tertiary amine backbone of aep-PNA and the phosphate backbone of DNA [211]. The formation of i-motif by only aep-PNA was not observed.

### 6.2.3 i-motif structures functionalized at the 3' or 5'-ends

The incorporation of lipophilic modifications at the 3'-terminal position of a C-rich oligonucleotide was reported by Sun et al. [212]. These authors demonstrate that alkyl chains containing more than 14 carbon atoms increased the stability of short tetrameric i-motifs [212].

C-rich oligonucleotides carrying the human telomeric intramolecular i-motif were combined with carboxyl-modified single-walled carbon nanotubes (SWNTs). The contact with SWNTs induced i-motif formation by shifting the pKa of C:C<sup>+</sup> base pairs [213]. The addition of dehydrating agents such as polyethyleneglycol as cell mimic crowding agents to single-walled carbon nanotubes (SWNTs) can drive i-motif causing more water to be released and consequently stabilizing i-motif structures [214]. Human telomeric intramolecular i-motif were also treated with graphene quantum dots (GQDs) [215]. GQDs promote the formation of the i-motif structures through end-stacking of the bases at the loop regions, thus reducing its solvent-accessible area.

The covalent attachment of C<sub>60</sub> fullarene to the human telomeric intramolecular i-motif shifted the pH-induced conformational transition of the i-motif possibly due to the hydrophobic interactions between the terminal fullerenes and the internal TAA loop in the DNA strand [216].

The covalently attachment of a donor (fluorescein) and an acceptor (tetramethylrhodamine) to the 5' and 3' ends of the DNA was described in Chapter 4. The molecular beacons have been profusely used for monitoring the intramolecular folding of an oligonucleotide forming intramolecular i-DNA motifs [217]. Two amphiphilic dendrons were covalently attached to the 3' and 5' ends of an intramolecular i-motif sequence [218]. The resulting DNA-dendron conjugates behave as a DNA molecular motor sensing pH changes. The reversible folding / unfolding was controlled by the opening and closing of the i-motif structure following pH changes [218]. Based on the changes of fluorescence during the folding and unfolding of an intramolecular i-motif Liu et al. described a proton-fueled DNA nanomachine [159]. This principle has been extensively exploited in the development of pH-sensitive devices for intra-cellular sensing and bioimaging [158] as well as smart surfaces, intelligent nanopores triggered by pH changes, molecular logic gates and DNA nanosprings [159].

## Bibliography

- [1] Collie GW, Parkinson GN. The application of DNA and RNA G-quadruplexes to therapeutic medicines. *Chem Soc Rev.* 2011;40:5867–92.
- [2] Benabou S, Aviñó A, Eritja R, González C, Gargallo R. Fundamental aspects of the nucleic acid I-motif structures. *RSC Adv.* 2014;4:26956–80.
- [3] Biffi G, Tannahill D, McCafferty J, Balasubramanian S. Quantitative visualization of DNA G-quadruplex structures in human cells. *Nat Chem.* 2013;5:182–6.
- [4] Zeraati M, Langley DB, Schofield P, Moye AL, Rouet R, Hughes WE, Bryan TM, Dinger ME, Christ D. I-motif DNA structures are formed in the nuclei of human cells. *Nat Chem.* 2018;10:631–7.
- [5] Hänsel-Hertsch R, Di Antonio M, Balasubramanian S. DNA G-quadruplexes in the human genome: detection, functions and therapeutic potential. *Nat Rev Mol Cell Biol.* 2017;18:279–84.

- [6] Mallikaratchy P. Evolution of complex target SELEX to identify aptamers against mammalian cell-surface antigens. *Molecules*. 2017;22:215.
- [7] Sanohe Y, Sugiyama H. Overview of formation of G-quadruplex structures. *Current Protocols Nucleic Acids Chem*. 2010;17.2.1–17.
- [8] Dapic V, Abdomerovic V, Marrington R, Peberdy J, Rodger A, Trent JO, Bates PJ. Biophysical and biological properties of quadruplex oligodeoxyribonucleotides. *Nucleic Acids Res*. 2003;31:2097–107.
- [9] Lane AN, Chaires JB, Gray RD, Trent JO. Stability and kinetics of G-quadruplex structures. *Nucleic Acids Res*. 2008;36:5482–515.
- [10] Huppert JL, Balasubramanian S. Prevalence of quadruplex in the human genome. *Nucleic Acids Res*. 2005;33:2908–16.
- [11] Todd AK. Bioinformatics approaches to quadruplex sequence location. *Methods*. 2007;43:246–51.
- [12] Martín-Pintado N, Yahyaee-Anzahae M, Deleavy GF, Portella G, Orozco M, Damha MJ, González C. Dramatic effect of furanose C2' substitution on structure and stability: directing the folding of the human telomeric quadruplex with a single fluorine atom. *J Am Chem Soc*. 2013;135:5344–7.
- [13] Adrian M, Heddi B, Phan AT. NMR spectroscopy of G-quadruplexes. *Methods*. 2012;57:11–24.
- [14] Gros J, Rosu F, Amrane S, Cian AD, Gabelica V, Lacroix L, Mergny JL. Guanines are a quartet's best friend: impact of base substitutions on the kinetics and stability of tetramolecular quadruplexes. *Nucleic Acids Res*. 2007;35:3064–75.
- [15] Gros J, Aviñó A, López de la Osa J, González C, Lacroix L, Pérez A, Orozco M, Eritja R, Mergny JL. 8-Aminoguanine accelerates tetramolecular G-quadruplex formation. *Chem Commun*. 2008;2926–8.
- [16] Lopez de la Osa J, Gonzalez C, Gargallo R, Rueda M, Cubero E, Orozco M, Aviñó A, Eritja R. Destabilization of quadruplex DNA by 8-aminoguanine. *ChemBioChem*. 2006;7:46–8.
- [17] Mekmaysy CS, Petraccone L, Garbett NC, Ragazzon PA, Gray R, Trent JO, Chaires JB. Effect of O<sup>6</sup>-methylguanine on the stability of G-quadruplex DNA. *J Am Chem Soc*. 2008;130:67100–1.
- [18] Tintoré M, Aviñó A, Ruiz FM, Eritja R, Fàbrega C. Development of a novel fluorescence assay based on the use of the thrombin binding aptamer for the detection of O<sup>6</sup>-alkylguanine-DNA alkyltransferase activity. *J Nucleic Acids*. 2010;2010:632041.
- [19] Tintoré M, Mazzini S, Polito L, Marelli M, Latorre A, Somoza A, Aviñó A, Fàbreg C, Eritja R. Gold-coated superparamagnetic nanoparticles for single methyl discrimination in DNA aptamers. *Int J Mol Sci*. 2015;16:27625–39.
- [20] Tintoré M, Gállego I, Manning B, Eritja R, DNA FC. Origami as DNA repair nanosensor at the single-molecule level. *Angew Chem, Int Ed Engl*. 2013;52:7747–50.
- [21] Marathias VM, Sawicki MJ, Bolton PH. 6-Thioguanine alters the structure and stability of duplex DNA and inhibits quadruplex DNA formation. *Nucleic Acids Res*. 1999;27:2860–7.
- [22] Spacova N, Cubero E, Sponer J, Orozco M. Theoretical study of the guanine to 6-thioguanine substitution in duplexes, triplexes, and tetraplexes. *J Am Chem Soc*. 2004;126:14642–50.
- [23] Szalai VA, Singer MJ, Thorp HH. Site-specific probing of oxidative reactivity and telomerase function using 7,8-dihydro-8-oxoguanine in telomeric DNA. *J Am Chem Soc*. 2002;124:1625–31.
- [24] Cheong VV, Lech CJ, Heddi B, Phan AT. Inverting the G-tetrad polarity of a G-quadruplex by using xanthine and 8-oxoguanine. *Angew Chem, Int Ed Engl*. 2016;55:160–3.
- [25] Benz A, Hartig JS. Redesigned tetrads with altered hydrogen bonding patterns enable programming of quadruplex topologies. *Chem Commun*. 2008;4010–2.
- [26] Singh V, Benz A, Hartig JS. G quadruplexes stabilised by 8-oxo-2'-deoxyguanosine. *Chem Eur J*. 2011;17:10838–43.

- [27] Cheong VV, Hedd B, Lech CJ, Phan AT. Xanthine and 8-oxoguanine in G-quadruplexes: formation of a G-G-X-O tetrad. *Nucleic Acids Res.* 2015;43:10506–14.
- [28] Kohda K, Tsunomoto H, Minoura Y, Tanabe K, Shibutani S. Synthesis, miscoding specificity, and thermodynamic stability of oligodeoxynucleotide containing 8-methyl-2'-deoxyguanosine. *Chem Res Toxicol.* 1996;9:1278–84.
- [29] Virgilio A, Esposito V, Randazzo A, Mayol L, Galeone A. 8-Methyl-2'-deoxyguanosine incorporation into parallel DNA quadruplex structures. *Nucleic Acids Res.* 2005;33:6188–95.
- [30] Xu Y, Sugiyama H. Formation of the G-quadruplex and i-motif structures in retinoblastoma susceptibility genes (Rb). *Nucleic Acids Res.* 2006;34:949–54.
- [31] Esposito V, Randazzo A, Piccialli G, Petraccone L, Giancola C, Mayol L. Effects of an 8-bromodeoxyguanosine incorporation on the parallel quadruplex [d(TGGGT)]<sub>4</sub>. *Org Biomol Chem.* 2004;2:313–8.
- [32] Matsugami A, Xu Y, Noguchi Y, Sugiyama H, Katahira M. Structure of a human telomeric DNA sequence stabilized by 8-bromoguanosine substitutions, as determined by NMR in a K<sup>+</sup> solution. *FEBS J.* 2007;274:3545–56.
- [33] Xu Y, Noguchi Y, Sugiyama H. The new models of the human telomere d[AGGG(TTAGGG)3] in K<sup>+</sup> solution. *Bioorg Med Chem.* 2006;14:5584–91.
- [34] Aviñó A, Mazzini S, Fàbrega C, Peñalver P, Gargallo R, Morales JC, Eritja R. The effect of L-thymidine, acyclic thymine and 8-bromoguanine on the stability of model G-quadruplex structures. *Biochim Biophys Acta.* 2017;1861:1205–12.
- [35] Lech CJ, Lim JKC, Lim JW, Amrane S, Hedd B, Phan AT. Effects of site-specific guanine C<sup>8</sup>-modifications on an intramolecular DNA G-quadruplex. *Biophys J.* 2011;101:1987–98.
- [36] Aviñó A, Fàbrega C, Tintoré M, Eritja R. Thrombin binding aptamer, more than a simple aptamer. Chemically modified derivatives and biomedical applications. *Curr Pharm Des.* 2012;18:2036–47.
- [37] Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ. Selection of single stranded-DNA molecules that bind and inhibit human thrombin. *Nature.* 1992;355:564–6.
- [38] Macaya RF, Schultze P, Smith FW, Roe JA, Feigón J. Thrombin-binding DNA aptamer forms a unimolecular quadruplex structure in solution. *Proc Natl Acad Sci USA.* 1993;90:3745–9.
- [39] Griffin LC, Tidmarsh GF, Bock LC, Toole JJ, Leung LL. In vivo anticoagulant properties of a novel nucleotide-based thrombin inhibitor and demonstration of regional anticoagulation in extracorporeal circuits. *Blood.* 1993;81:3271–6.
- [40] Nallagatla SR, Heuberger B, Haque A, Switzer C. Combinatorial synthesis of thrombin-binding aptamers containing iso-guanine. *J Comb Chem.* 2009;11:364–9.
- [41] He GX, Krawczyk SH, Swaminathan S, Shea RG, Dougherty JP, Terhorst T, Law VS, Griffin LC, Coutre S, N<sup>2</sup> BN. C<sup>8</sup>-substituted oligodeoxynucleotides with enhanced thrombin inhibitory activity in vitro and in vivo. *J Med Chem.* 1998;41:2234–42.
- [42] Ogasawara S, Maeda M. Reversible photoswitching of a G-quadruplex. *Angew Chem, Int Ed Engl.* 2009;48:6671–4.
- [43] Jayapal P, Mayer G, Heckel A, Wenmohs F. Structure-activity relationship of a caged thrombin binding DNA aptamer: insight gained from molecular dynamics simulation studies. *J Struct Biol.* 2009;166:241–50.
- [44] Esposito V, Virgilio A, Randazzo A, Galeone A, Mayol L. A new class of DNA quadruplexes formed by oligodeoxynucleotides containing a 3'-3' or 5'-5' inversion of polarity. *Chem Commun.* 2005;31:3953–5.
- [45] Esposito V, Galeone A, Mayol L, Randazzo A, Virgilio A, Virno A. A mini-library of TBA analogues containing 3'-3' and 5'-5' inversion of polarity sites. *Nucleosides Nucleotides Nucleic Acids.* 2007;26:1145–9.

- [46] Martino L, Virno A, Randazzo A, Virgilio A, Esposito V, Giancola C, Bucci M, Cirino G, Mayol L. A new modified thrombin binding aptamer containing a 5'-5' inversion of polarity site. *Nucleic Acids Res.* 2006;34:6653–62.
- [47] Russo Krauss I, Merlino A, Randazzo A, Mazzarella L, Sica F. Crystallization and preliminary X-ray analysis of the complex of human a-thrombin with a modified thrombin-binding aptamer. *Acta Crystallogr.* 2010;F66:961–3.
- [48] Pagano B, Martino L, Randazzo A, Giancola C. Stability and binding properties of a modified thrombin binding aptamer. *Biophys J.* 2008;94:562–9.
- [49] Esposito V, Scuotto M, Capuozzo A, Santamaria R, Varra M, Mayol L, Virgilio A, Galeone A. A straightforward modification in the thrombin aptamer improving stability, affinity to thrombin and nuclease resistance. *Org Biomol Chem.* 2014;12:8840–3.
- [50] Esposito V, Virgilio A, Pepe A, Oliveira G, Mayol L, Galeone A. Effects of the introduction of inversion of polarity sites in the quadruplex forming oligonucleotide TGGGT. *Bioorg Med Chem.* 2009;17:1997–2001.
- [51] Virgilio A, Esposito V, Mayol L, Galeone A. More than one non-canonical phosphodiester bond in the G-tract: formation of unusual parallel G-quadruplex structures. *Org Biomol Chem.* 2014;12:534–40.
- [52] Tang CF, Shafer RH. Engineering the quadruplex fold: nucleoside conformation determines both folding topology and molecularly in guanine quadruplexes. *J Am Chem Soc.* 2006;128:5966–73.
- [53] Qi J, Shafer RH. Human telomere quadruplex: refolding and selection of individual conformers via RNA/DNA chimeric editing. *Biochemistry.* 2007;46:7599–606.
- [54] Saccà B, Lacroix L, Mergny JL. The effect of chemical modifications on the thermal stability of different G-quadruplex-forming oligonucleotides. *Nucleic Acids Res.* 2005;33:1182–92.
- [55] Peng CG, Damha MJ. G-quadruplex induced stabilization by 2'-deoxy-2'-fluoro-D-arabinonucleic acids (2'F-ANA). *Nucleic Acids Res.* 2007;35:4977–88.
- [56] Lietard J, Assi HA, Gómez-Pinto I, González C, Somoza MM, Damha MJ. Mapping the affinity landscape of thrombin-binding aptamers on 2'-F-ANA/DNA chimeric G-quadruplex microarrays. *Nucleic Acids Res.* 2017;45:1619–32.
- [57] Dominick PK, Jarstfer MB. A conformationally constrained nucleotide analogue controls the folding topology of a DNA G-quadruplex. *J Am Chem Soc.* 2004;126:5050–1.
- [58] Nielsen JT, Arar K, Petersen M. NMR solution structures of LNA (locked nucleic acid) modified quadruplexes. *Nucleic Acids Res.* 2006;34:2006–14.
- [59] Randazzo A, Esposito V, Ohlenschläger O, Ramachandran R, Mayol L. NMR structure of a parallel LNA quadruplex. *Nucleic Acids Res.* 2004;32:3083–92.
- [60] Randazzo A, Esposito V, Ohlenschläger O, Ramachandran R, Virgilio A, Mayol L. Structural studies on LNA quadruplexes. *Nucleosides Nucleotides Nucleic Acids.* 2005;24:795–800.
- [61] Virno A, Randazzo A, Giancola C, Bucci M, Cirino G, Mayol L. A novel thrombin binding aptamer containing a G-LNA residue. *Bioorg Med Chem.* 2007;15:5710–8.
- [62] Bonifacio L, Church FC, Jarstfer MB. Effect of locked-nucleic acid on a biologically active G-quadruplex. A structure-activity relationship of the thrombin aptamer. *Int J Mol Sci.* 2008;9:422–33.
- [63] Li Z, Lech CJ, Phan AT. Sugar-modified G-quadruplexes: effects of LNA-, 2'F-RNA- and 2'F-ANA-guanosine chemistries on G-quadruplex structure and stability. *Nucleic Acids Res.* 2014;42:4068–79.
- [64] Saneyoshi H, Mazzini S, Aviñó A, Portella G, González C, Orozco M, Marquez V, Eritja R. Conformationally rigid nucleoside probes help understand the role of sugar pucker and nucleobase orientation in the thrombin binding aptamer. *Nucleic Acids Res.* 2009;37:5589–601.

- [65] Pasternak A, Hernandez FJ, Rasmussen LM, Vester B, Wengel J. Improved thrombin binding aptamer by incorporation of a single unlocked nucleic acid monomer. *Nucleic Acids Res.* 2011;39:1155–64.
- [66] Jensen TB, Henriksen JR, Rasmussen BE, Rasmussen LM, Andersen TL, Wengel J, Pasternak A. Thermodynamics and biological evaluation of a thrombin binding aptamer modified with several unlocked nucleic acids (UNA) monomers and a 2'-C-piperazino-UNA-monomer. *Bioorg Med Chem.* 2011;19:4739–45.
- [67] Datta B, Schmitt C, Armitage BA. Formation of a PNA<sub>2</sub>-DNA<sub>2</sub> hybrid quadruplex. *J Am Chem Soc.* 2003;125:4111–8.
- [68] Datta B, Bier M, Roy S, Armitage BA. Quadruplex formation by a guanine-rich PNA oligomer. *J Am Chem Soc.* 2005;127:4199–207.
- [69] Esposito V, Randazzo A, Messere A, Galeone A, Petraccone L, Giancola C, Piccialli G, Mayol L. Synthesis and structural characterization of PNA-DNA quadruplex-forming chimeras. *Eur J Org Chem.* 2003;3364–71.
- [70] Petraccone L, Pagano B, Esposito V, Randazzo A, Piccialli G, Barone G, Mattia CA, Giancola C. Thermodynamics and kinetics of PNA-DNA quadruplex-forming chimeras. *J Am Chem Soc.* 2005;127:16215–23.
- [71] Krishnan-Ghosh Y, Stephens E, Balasubramanian S. A PNA<sub>4</sub> quadruplex. *J Am Chem Soc.* 2004;126:5944–5.
- [72] Pinto B, Rusciano G, D'Errico S, Borbone N, Sasso A, Piccialli V, Mayol L, Oliviero G, Piccialli G. Synthesis and label free characterization of a bimolecular PNA homo quadruplex. *Biochim Biophys Acta.* 2017;1861:1222–8.
- [73] Marin VL, Armitage BA. Hybridization of complementary and homologous peptide nucleic acid oligomers to a guanine quadruplex-forming RNA. *Biochemistry.* 2006;45:1745–54.
- [74] Paul A, Sengupta P, Krishnan Y, Combining LS. G-quadruplex targeting motifs on a single peptide nucleic acid scaffold: a hybrid (3 + 1) PNA-DNA bimolecular quadruplex. *Chem Eur J.* 2008;14:8682–9.
- [75] Zaitseva M, Kaluzhny D, Shchyolkina A, Borisova O, Smirnov I, Pozmogova G. Conformation and thermostability of oligonucleotide d(GGTTGGTGTGGTTGG) containing thiophosphoryl internucleotide bonds at different positions. *Biophys Chem.* 2010;146:1–6.
- [76] Li C, Zhu L, Zhu Z, Fu H, Jenkins G, Wang C, Zou Y, Lua X, Yang CJ. Backbone modification promotes peroxidase activity of G-quadruplex-based DNAzyme. *Chem Commun.* 2012;48:8347–9.
- [77] He GX, Williams JP, Postich MJ, Swaminathan S, Shea RG, Terhorst T, Law VS, Mao CT, Sueoka C, Coutre S, Bischofberger N. In vitro and in vivo activities of oligodeoxynucleotide-based thrombin inhibitors containing neutral formacetal linkages. *J Med Chem.* 1998;41:4224–31.
- [78] Smirnov I, Shafer RH. Effect of the sequence and size on DNA aptamer stability. *Biochemistry.* 2000;39:1462–8.
- [79] Nagatoishi S, Isono N, Tsumoto K, Sugimoto N. Loop residues of thrombin-binding DNA aptamer impact G-quadruplex stability and thrombin binding. *Biochimie.* 2011;93:1231–8.
- [80] Aviñó A, Portella G, Ferreira R, Gargallo R, Mazzini S, Gabélica V, Orozco M, Eritja R. Specific loop modifications of the thrombin binding aptamer trigger the formation of parallel structures. *FEBS J.* 2014;281:1085–99.
- [81] Benabou S, Mazzini S, Aviñó A, Eritja R, Gargallo R. A pH-dependent bolt involving cytosine bases located in the lateral loops of antiparallel G-quadruplex structures within the SMARCA4 gene promotor. *Sci Rep.* 2019;9:15807.
- [82] Mendelboum Raviv S, Horváth A, Aradi J, Bagoly Z, Fazakas F, Batta Z, Muszbek L, Hársfalvi J. 4-thio-deoxyuridylate-modified thrombin aptamer and its inhibitory effect on fibrin clot formation, platelet aggregation and thrombus growth on subendothelial matrix. *J Thromb Haemost.* 2008;6:1764–71.

- [83] Coppola T, Varra M, Oliveira G, Galeone A, D'Isa G, Mayol L, Morelli E, Bucci MR, Vellecco V, Cirino G, Borbone N. Synthesis, structural studies and biological properties of new TBA analogues containing an acyclic nucleotide. *Bioorg Med Chem*. 2008;16:8244–53.
- [84] Borbone N, Bucci MR, Oliviero G, Morelli E, Amato J, D'Atri V, D'Errico S, Valentina Vellecco V, Cirino G, Piccialli G, Fattorusso C, Varra M, Mayol L, Persico M, Scuotto M. Investigating the role of T7 and T12 residues on the biological properties of thrombin-binding aptamer: enhancement of anticoagulant activity by a single nucleobase modification. *J Med Chem*. 2012;55:10716–28.
- [85] Aviñó A, Mazzini S, Ferreira R, Gargallo R, Marquez VE, Eritja R. The effect on quadruplex stability of North-nucleoside derivatives in the loops of the thrombin-binding aptamer. *Bioorg Med Chem*. 2012;20:4186–93.
- [86] Aviñó A, Mazzini S, Fàbrega C, Peñalver P, Gargallo R, Morales JC, Eritja R. The effect of L-thymidine, acyclic thymine and 8-bromoguanine on the stability of model G-quadruplex structures. *Biochim Biophys Acta*. 2017;1861:1205–12.
- [87] Virgilio A, Petraccone L, Scuotto M, Vellecco V, Bucci MR, Mayol L, Varra M, Esposito G V. 5-Hydroxymethyl-2'-deoxyuridine residues in the thrombin binding aptamer: investigating anticoagulant activity by making a tiny chemical modification. *ChemBioChem*. 2014;15:2427–34.
- [88] Virgilio A, Petraccone L, Vellecco V, Bucci MR, Varra M, Irace C, Santamaria R, Pepe A, Mayol L, Esposito V, Galeone A. Site-specific replacement of the thymine methyl group by fluorine in thrombin binding aptamer significantly improves structural stability and anticoagulant activity. *Nucleic Acids Res*. 2015;43:10602–11.
- [89] Scuotto M, Rivieccio E, Varone A, Corda D, Bucci MR, Vellecco V, Cirino G, Virgilio A, Esposito V, Galeone A, Borbone N, Varra M, Mayo L. Site specific replacements of a single loop nucleoside with a dibenzyl linker may switch the activity of TBA from anticoagulant to antiproliferative. *Nucleic Acids Res*. 2015;43:7702–16.
- [90] Zhang N, Bing T, Liu X, Qi C, Shen L, Wang L, Shangguan D. Cytotoxicity of guanine-based degradation products contributes to the antiproliferative activity of guanine-rich oligonucleotides. *Chem Sci*. 2015;6:3831–8.
- [91] Esposito V, Russo A, Amato T, Varra M, Vellecco V, Bucci MR, Russo G, Virgilio A, Galeone A. Backbone modified TBA analogues endowed with antiproliferative activity. *Biochim Biophys Acta*. 2017;1861:1213–21.
- [92] Esposito V, Russo A, Vellecco V, Bucci MR, Russo G, Mayol L, Virgilio A, Galeone A. Thrombin binding aptamer analogues containing inversion of polarity sites endowed with antiproliferative and anti-motility properties against Calu-6 cells. *Biochim Biophys Acta*. 2018;1862:2645–50.
- [93] Esposito V, Russo A, Amato T, Vellecco V, Bucci MR, Mayol L, Russo G, Virgilio A, Galeone A. The “Janus face” of the thrombin binding aptamer: Investigating the anticoagulant and antiproliferative properties through straightforward chemical modifications. *Bioorg Chem*. 2018;76:202–9.
- [94] Kotkowiak W, Lisowiec-Wachnicka J, Grynda J, Kierzek R, Wengel J, Pasternak A. Thermodynamic, anticoagulant, and antiproliferative properties of thrombin binding aptamer containing novel UNA derivative. *Mol Ther Nucleic Acids*. 2017;10:304–16.
- [95] Yang X, Zhu Y, Wang C, Guan Z, Zhang L, Yang Z. Alkylation of phosphorothioated thrombin binding aptamers improves the selectivity of inhibition of tumor cell proliferation upon anticoagulation. *Biochim Biophys Acta*. 2017;1861:1864–9.
- [96] Antipova O, Samoylenkova N, Savchenko E, Zavyalova E, Revischin A, Pavlova G, Kopylov A. Bimodular antiparallel G-quadruplex nanoconstruct with antiproliferative activity. *Molecules*. 2019;24:3625.

- [97] Heckel A, Mayer G. Light regulation of aptamer activity: an anti-thrombin aptamer with caged thymidine nucleobases. *J Am Chem Soc.* 2005;127:822–3.
- [98] Choi EW, Nayak LV, Bates PJ. Cancer-selective antiproliferative activity is a general property of some G-rich sequence oligodeoxynucleotides. *Nucleic Acids Res.* 2010;38:1623–35.
- [99] Chang T, Qi C, Meng J, Zhang N, Bing T, Yang X, Cao Z, Shangguan D. General cell-binding activity of intramolecular G-quadruplex with parallel structure. *PLoS ONE.* 2013;8:e62348.
- [100] Gatto B, Palumbo M, Sissi C. Nucleic acid aptamers based on the G-quadruplex structure: therapeutic and diagnostic potential. *Curr Med Chem.* 2009;16:1248–65.
- [101] Do NQ, Chung WJ, Truong THA, Hedi B, Phan T. G-quadruplex structure of an anti-proliferative DNA sequence. *Nucleic Acids Res.* 2017;45:7487–93.
- [102] Petraccone L, Barone C, Giancola C. Quadruplex-forming oligonucleotides as tools in anticancer therapy and aptamers design: energetic aspect. *Curr Med Chem Anticancer Agents.* 2005;5:463–75.
- [103] Grijalvo S, Alagia A, Gargallo R, Eritja R. Cellular uptake studies of antisense oligonucleotides using g-quadruplex-nanostructures: the effect of cationic residue in the biophysical and biological properties. *RSC Adv.* 2016;6:76099–109.
- [104] Musumeci D, Riccardi C, Montesarchio D. G-Quadruplex forming oligonucleotides as anti-HIV agents. *Molecules.* 2015;20:17511–32.
- [105] Schneider DJ, Feigon J, Hostomsky S, Gold L. High-affinity ssDNA inhibitors of the reverse transcriptase of type 1 human immunodeficiency virus. *Biochemistry.* 1995;34:9599–610.
- [106] Michalowski D, Chitima-Matsiga R, Held DM, Burke DH. Novel bimodular DNA aptamers with guanosine quadruplexes inhibit phylogenetically diverse HIV-1 reverse transcriptases. *Nucleic Acids Res.* 2008;36:7124–35.
- [107] Andreola ML, Pileur F, Calmels C, Ventura M, Tarrago-Litvak L, Toulmé JJ, Litvak S. DNA aptamers selected against the HIV-1 RNase H display in vitro antiviral activity. *Biochemistry.* 2001;40:10087–94.
- [108] Rando RF, Ojwang J, Elbaggari A, Reyes GR, Tinder R, McGrath MS, Hogan ME. Suppression of human immunodeficiency virus type 1 activity in vitro by oligonucleotides which form intramolecular tetrads. *J Biol Chem.* 1995;270:1754–60.
- [109] Mazumder A, Neamati N, Ojwang J, Sunder S, Rando RF, Pommier Y. Inhibition of the human immunodeficiency virus type 1 integrase by guanosine quartet structures. *Biochemistry.* 1996;35:13762–71.
- [110] de Soulttrait VR, Lozach PY, Altmeyer R, Tarrago-Litvak L, Litvak S, Andreóla ML. DNA aptamers derived from HIV-1 RNase H inhibitors are strong anti-integrase agents. *J Mol Biol.* 2002;324:195–203.
- [111] Chou SH, Chin KH, Wang AHJ. DNA aptamers as potential anti-HIV agents. *Trends Biochem Sci.* 2005;30:231–4.
- [112] Jing N, Rando RF, Pommier Y, Hogan ME. Ion selective folding of loop domains in a potent anti-HIV oligonucleotide. *Biochemistry.* 1997;36:12498–505.
- [113] Esté JA, Cabrera C, Schols D, Cherepanov P, Gutierrez A, Witvrouw M, Pannecoque C, Debysier Z, Rando RF, Clotet B, Desmyter J, De Clercq E. Human immunodeficiency virus glycoprotein gp120 as the primary target for the antiviral action of AR177 (Zintevir). *Mol Pharmacol.* 1998;53:340–5.
- [114] Urata H, Kumashiro T, Kawahata T, Otake T, Akagi M. Anti-HIV-1 activity and mode of action of mirror image oligodeoxynucleotide analogue of Zintevir. *Biochem Biophys Res Comm.* 2004;313:55–61.
- [115] Cherepanov P, Esté JA, Rando RF, Ojwang JO, Reekmans G, Steinfeld R, David G, De Clercq E, Debysier Z. Mode of interaction of G-quartets with the integrase of human immunodeficiency virus type 1. *Mol Pharmacol.* 1997;52:771–80.

- [116] Wyatt JR, Vickers TA, Roberson JL, Buckheit RW, Klimkait T, DeBaets E, Davis PW, Rayner B, Imbach JL, Ecker DJ. Combinatorially selected guanosine-quartet structure is a potent inhibitor of human immunodeficiency virus envelope-mediated cell fusion. *Proc Natl Acad Sci USA*. 1994;91:1356–60.
- [117] Wyatt JR, Davis PW, Freier SM. Kinetics of G-quartet-mediated tetramer formation. *Biochemistry*. 1996;35:8002–8.
- [118] Furukawa H, Momota K, Agatsuma T, Yamamoto I, Kimura S, Shimada K. Mechanism of inhibition of HIV-1 Infection in vitro by guanine-rich oligonucleotides modified at the 5'-terminal by dimethoxytrityl residue. *Nucleic Acids Res*. 1994;22:5621–7.
- [119] Hotoda H, Momota K, Furukawa H, Nakamura T, Kaneko M, Kimura S, Shimada K. Biologically active oligodeoxyribonucleotides. 2: Structure activity relationships of anti-HIV-1 pentadecadeoxyribonucleotides bearing 5'-end-modifications. *Nucleosides Nucleotides*. 1994;13:1375–95.
- [120] Agatsuma T, Yamamoto I, Furukawa H, Nishigaki T. Guanine-rich oligonucleotide modified at the 5' terminal by dimethoxytrityl residue inhibits HIV-1 replication by specific interaction with the envelope glycoprotein. *Antivir Res*. 1996;31:137–48.
- [121] Furukawa H, Momota K, Agatsuma T, Yamamoto I, Kimura S, Shimada K. Identification of a phosphodiester hexanucleotide that inhibits HIV-1 infection in vitro on covalent linkage of its 5'-end with a dimethoxytrityl residue. *Antisense Nucleic Acid Drug Dev*. 1997;7:167–75.
- [122] Hotoda H, Koizumi M, Koga R, Momota K, Ohmine T, Furukawa H, Nishigaki T, Kinoshita T, Kaneko M, Kimura S, Shimada K. Biologically active oligodeoxyribonucleotides-IV: anti-HIV-1 activity of TGGGAG having hydrophobic substituent at its 5'-end via phosphodiester linkage. *Nucleosides Nucleotides Nucleic Acids*. 1996;15:531–8.
- [123] Koizumi M, Koga H, Hotoda K, Momota T, Ohmine H, Furukawa T, Agatsuma T, Nishigaki K, Abe T, Kosaka S, Tsutsumi J, Sone J, Kaneko M, Kimura S, Shimada K. Biologically active oligodeoxyribonucleotides—IX. Synthesis and anti-HIV-1 activity of hexadeoxyribonucleotides, TGGGAG, bearing 3'-and 5'-end-modification. *Bioorg Med Chem*. 1997;5:2235–43.
- [124] Hotoda H, Koizumi M, Koga R, Kaneko M, Momota K, Ohmine T, Furukawa H, Agatsuma T, Nishigaki T, Sone J, Tsutsumi S, Kosaka T, Abe K, Kimura S, Shimada K. Biologically active oligodeoxyribonucleotides. 5. 5'-End-substituted d(TGGGAG) possesses anti-human immunodeficiency virus type 1 activity by forming a G-quadruplex structure. *J Med Chem*. 1998;41:3655–63.
- [125] Koizumi M, Koga H, Hotoda K, Ohmine T, Furukawa H, Agatsuma T, Nishigaki T, Abe K, Kosaka T, Tsutsumi S, Sone J, Kaneko M, Kimura S, Shimada K. Biologically active oligodeoxyribonucleotides. Part 11: The least phosphate-modification of quadruplex-forming hexadeoxyribonucleotide TGGGAG, bearing 3'- and 5'-end-modification, with anti-HIV-1 activity. *Bioorg Med Chem*. 1998;6:2469–75.
- [126] Koizumi M, Akahori K, Ohmine T, Tsutsumi S, Sone J, Kosaka T, Kaneko M, Kimura S, Shimada K. Biologically active oligodeoxyribonucleotides. Part 12: N2-Methylation of 2'-deoxyguanosines enhances stability of parallel G-quadruplex and anti-HIV-1 activity. *Bioorg Med Chem*. 2000;8:2213–16.
- [127] D'Onofrio J, Petraccone L, Erra E, Martino L, Di Dabio G, De Napoli L, Giancola C, Montesarchio D. 5'-Modified G-quadruplex forming oligonucleotides endowed with anti-HIV activity: synthesis and biophysical properties. *Bioconjug Chem*. 2007;18:1194–204.
- [128] D'Onofrio J, Petraccone L, Martino L, Di Dabio G, Iadonisi A, Balzarini J, Giancola C, Montesarchio D. Synthesis, biophysical characterization, and anti-HIV activity of glyco-conjugate G-quadruplex-forming oligonucleotide. *Bioconjug Chem*. 2008;19:607–16.

- [129] Di Fabio G, D'Onofrio J, Chiapparelli M, Hoorelbeke B, Montesarchio D, Balzarini J, De Napoli L. Discovery of novel anti-HIV active G-quadruplex-forming oligonucleotide. *Chem Commun.* 2011;47:2363–5.
- [130] Pedersen EB, Nielsen JT, Nielsen C, Filichev VV. Enhanced anti HIV-1 activity of G-quadruplexes comprising locked nucleic acids and intercalating nucleic acids. *Nucleic Acids Res.* 2011;39:2470–81.
- [131] Oliveira G, Amato J, Borbone N, D'Errico S, Galeone A, Mayol L, Haider S, Olubiyi O, Hoorelbeke B, Balzarini J, Piccialli G. Tetra-end-linked oligonucleotides forming DNA G-quadruplex: a new class of aptamers showing anti-HIV activity. *Chem Commun.* 2010;46:8971–3.
- [132] Oliveira G, Amato J, Borbone N, Galeone A, Varra M, Piccialli G, Mayol L. Unusual monomolecular DNA quadruplex structures using bunch-oligonucleotides. *Nucleosides Nucleotides Nucleic Acids.* 2005;24:739–41.
- [133] Oliveira G, Amato J, Borbone N, D'Errico S, Galeone A, Petraccone L, Varra M, Piccialli G, Mayol L. Synthesis and characterization of monomolecular DNA G-quadruplex formed by tetra-end-linked oligonucleotides. *Bioconjug Chem.* 2006;17:889–98.
- [134] Petraccone L, Martino L, Duro I, Oliveira G, Borbone N, Piccialli G, Giancola C. Physico-chemical analysis of G-quadruplex containing bunch-oligonucleotides. *Int J Biol Macromol.* 2007;40:242–7.
- [135] Borbone N, Oliveira G, Amato J, D'Errico S, Galeone A, Piccialli G, Mayol L. Synthesis and characterization of tetra-end linked oligonucleotides capable of forming monomolecular G-quadruplex. *Nucleosides Nucleotides Nucleic Acids.* 2007;26:1231–6.
- [136] Ferreira R, Alvira M, Aviñó A, Gómez-Pinto I, González C, Gabelica V, Eritja R. Synthesis and structural characterization of stable branched DNA G-quadruplexes using the trebler phosphoramidite. *ChemistryOpen.* 2012;1:106–14.
- [137] Musumeci D, Montesarchio D. Synthesis of a cholesterol-HEG phosphoramidite derivative and its application to lipid-conjugates of the anti-HIV 5'TGGGAG3' Hotoda's sequence. *Molecules.* 2012;17:12378–92.
- [138] Wolfe JL, Goodchild J. Modulation of tetraplex formation by chemical modifications of a G4-containing phosphorothioate oligonucleotide. *J Am Chem Soc.* 1996;118:6301–2.
- [139] Lyonnais S, Grijalvo S, Alvarez-Fernández C, Fleta E, Martínez J, Meyerhans A, Sánchez-Palomino S, Mirambeau G, Eritja R. Lipid-oligonucleotide conjugates forming G-Quadruplex (lipoquads) as potent inhibitors of HIV entry. *Proceedings.* 2017;1:670.
- [140] Lyonnais S, Eritja R, Grijalvo S, Sanchez-Palomino S, Alvarez C, Meyerhans A, Martínez Vesga J, Fleta E, Díez Antón HM, Koutsoudakis G, Mirambeau G. Antiviral agents comprising an oligonucleotide-lipid conjugate forming G-quadruplexes. European patent, application number 15382470.1 filed on 28.09.2015 published EP3 147 364 A1, 29.03.2017.
- [141] Koutsoudakis G, Paris de León A, Herrera C, Dorner M, Pérez-Vilaró G, Lyonnais S, Grijalvo S, Eritja R, Meyerhans A, Mirambeau G, Díez J. Oligonucleotide-lipid conjugates forming G-quadruplex structures are potent and pangenotypic hepatitis C virus entry inhibitors in vitro and ex vivo. *Antimicrob Agents Chemother.* 2017;61:e02354–16.
- [142] Aviñó A, Mazzini S, Ferreira R, Eritja R. Synthesis and structural properties of oligonucleotides covalently linked to acridine and quindoline derivatives through a threoninol linker. *Bioorg Med Chem.* 2010;18:7348–56.
- [143] Gómez-Pinto I, Vengut-Climent E, Lucas R, Aviñó A, Eritja R, González C, Carbohydrate MJC. DNA interaction at G-quadruplexes: folding and stability changes by attaching sugars at the 5'-end. *Chem Eur J.* 2013;19:1920–7.
- [144] Aviñó A, Jorge AF, Huertas CS, Cova TFGG, Pais A, Lechuga LM, Eritja R, Fàbrega C. Aptamer-peptide conjugates as a new strategy to modulate human  $\alpha$ -thrombin binding affinity. *Biochim Biophys Acta.* 2019;1863:1610–30.

- [145] Nagatoishi S, Nojima T, Galezowska E, Juskowiak B, Takenaka, G-quadruplex-based S. FRET probes with thrombin-binding aptamer (TBA) sequence designed for the efficient fluorimetric detection of the potassium ion. *ChemBioChem*. 2006;7:1730–7.
- [146] Nagatoishi S, Nojima T, Juskowiak B, Takenaka S. A pyrene-labeled G-quadruplex oligonucleotide as a fluorescent probe for potassium ion detection in biological applications. *Angew Chem, Int Ed Engl*. 2005;44:5067–70.
- [147] Roxo C, Kotkowiak W, Pasternak AG. Quadruplex-forming aptamers-characteristics, applications, and perspectives. *Molecules*. 2019;24:3781.
- [148] Krashenina OA, Novopashina DS, Apartsin EK, Venyaminova AG. Recent advances in nucleic acid targeting probes and supramolecular constructs based on pyrene-modified oligonucleotides. *Molecules*. 2017;22:2108.
- [149] Liu J, Cao Z, Lu Y. Functional nucleic acids sensors. *Chem Rev*. 2009;109:1948–98.
- [150] Bates PJ, Laber DA, Miller DM, Thomas SD, Trent JO. Discovery and development of the G-rich oligonucleotide AS1411 as a novel treatment for cancer. *Exp Mol Pathol*. 2009;86:151–64.
- [151] Bates PJ, Reyes-Reyes EM, Malik MT, Murphy EM, O'Toole MG, Trent JO. G-quadruplex oligonucleotide AS1411 as a cancer-targeting agent: uses and mechanisms. *Biochim Biophys Acta*. 2017;2017(1861):1414–28.
- [152] Perrone R, Butovskaya E, Lago S, Garzino-Demo A, Pannecouque C, Palù G, Richter SN. The G-quadruplex-forming aptamer AS1411 potently inhibits HIV-1 attachment to the host cell. *Int J Antimicrob Agents*. 2016;47:311–6.
- [153] Métifiot M, Amrane S, Mergny JL, Andreola ML. Anticancer molecule AS1411 exhibits low nanomolar antiviral activity against HIV-1. *Biochimie*. 2015;118:173–5.
- [154] Guéron M, Leroy JL. The i-motif in nucleic acids. *Curr Opin Struct Biol*. 2000;10:326–31.
- [155] Abou Assi H, Garaví M, González C, Damha MJ. i-Motif DNA: structural features and significance to cell biology. *Nucleic Acids Res*. 2018;46:8038–56.
- [156] Mergny JL, Sen D. DNA quadruple helices in nanotechnology. *Chem Rev*. 2019;119:6290–325.
- [157] Dong Y, Yang Z, Liu D. DNA nanotechnology based on i-motif structures. *Acc Chem Res*. 2014;47:1853–60.
- [158] Dembska A, Bielecka P, Juskowiak B. pH-Sensing fluorescence oligonucleotide probes based on an i-motif scaffold: a review. *Anal Methods*. 2017;9:6092–106.
- [159] Liu D, Balasubramanian S. A proton-fuelled DNA nanomachine. *Angew Chem, Int Ed Engl*. 2003;42:5734–6.
- [160] Halder S, Krishnan Y. Design of ultrasensitive DNA-based fluorescent pH sensitive nanodevices. *Nanoscale*. 2015;7:10008–12.
- [161] Alba JJ, Sadurní A, Gargallo R. Nucleic acid i-motif structures in analytical chemistry. *Crit Rev Anal Chem*. 2016;46:443–54.
- [162] Xu B, Devi G, Shao F. Regulation of telomeric I-motif stability by 5-methylcytosine and 5-hydroxymethylcytosine modification. *Org Biomol Chem*. 2015;13:5646–51.
- [163] Bhavsar-Jog YP, Van Dornshuld E, Brooks TA, Tschumper GS, Wadkins RM. Epigenetic modification, dehydration, and molecular crowding effects on the thermodynamics of i motif structure formation from C-rich DNA. *Biochemistry*. 2014;53:1586–94.
- [164] Dvořáková Z, Renčík D, Kejnovská I, Školáková-Bednářová K, Sagi J, Vorlíčková M. I-Motif of cytosine-rich human telomere DNA fragments containing natural base lesions. *Nucleic Acids Res*. 2018;46:1624–34.
- [165] Karino N, Ueno Y, Matsuda A. Synthesis and properties of oligonucleotides containing 5-formyl-2'-deoxycytidine: in vitro DNA polymerase reactions on DNA templates containing 5-formyl-2'-deoxycytidine. *Nucleic Acids Res*. 2001;29:2456–63.
- [166] Kulikowski T, Shugar D. Methylation and tautomerism of 1-substituted 5-fluorocytosines. *Acta Biochim Pol*. 1979;26:145–60.

- [167] Lannes L, Halder S, Krishnan Y, Schwalbe H. Tuning the pH response of i-motif DNA oligonucleotides. *ChemBioChem*. 2015;16:1647–56.
- [168] Christman JK. 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene*. 2002;21:5483–95.
- [169] Lamparska K, Clark J, Babilonia G, Bedell V, Yip W, Smith SS. 2'-Deoxyriboguanylurea, the primary breakdown product of 5-aza-2'-deoxyribocytidine, is a mutagen, an epimutagen, an inhibitor of DNA methyltransferases and an inducer of 5-azacytidine type fragile sites. *Nucleic Acids Res.* 2012;2012(40):9788–801.
- [170] Wright EP, Lamparska K, Smith SS, Waller ZAE. Substitution of cytosine with guanylurea decreases the stability of i-motif DNA. *Biochemistry*. 2017;66:4879–83.
- [171] Mir B, Solés X, González C, Escaya N. The effect of the neutral cytidine protonated analogue pseudoisocytidine on the stability of i-motif structures. *Sci Rep.* 2017;7:2772.
- [172] Tsvetkov VB, Zatsepin TS, Belyaev ES, Kostyukevich YI, Shpakovski GV, Podgorsky VV, Pozmogova GE, Varizhuk AM, Aralov AV. I-clamp phenoxazine for the fine tuning of DNA i-motif stability. *Nucleic Acids Res.* 2018;46:2751–64.
- [173] Seela F, Budow S, Leonard P. Oligonucleotides forming an i-motif: the pH-dependent assembly of individual strands and branched structures containing 2'-deoxy-5-propynylcytidine. *Org Biomol Chem*. 2007;5:1858–72.
- [174] Lacroix L, Mergny JL. Chemical modification of pyrimidine TFOs: effect on i-motif and triple helix formation. *Arch Biochem Biophys*. 2000;381:153–63.
- [175] Lacroix L, Mergny JL, Leroy JL, Helene C. Inability of RNA to form the i-motif: implications for triplex formation. *Biochemistry*. 1996;35:8715–22.
- [176] Mergny JL, Lacroix L. Kinetics and thermodynamics of i-DNA formation: phosphodiester versus modified oligodeoxynucleotides. *Nucleic Acids Res.* 1998;26:4797–803.
- [177] Kanehara H, Mizuguchi M, Tajima K, Kanaori K, Makino K. Spectroscopic evidence for the formation of four-stranded solution structure of oligodeoxycytidine phosphorothioate. *Biochemistry*. 1997;36:1790–7.
- [178] Kanaori K, Sakamoto S, Yoshida H, Guga P, Stec W, Tajima K, Makino K. Effect of phosphorothioate chirality on i-motif structure and stability. *Biochemistry*. 2004;43:5672–9.
- [179] Brazier JA, Fisher J, Cosstick R. Stabilization of the DNA I-motif structure by incorporation of 3'-S-phosphorothiolate linkages. *Angew Chem, Int Ed Engl*. 2006;45:114–7.
- [180] Cosstick R, Buckingham J, Brazier J, Fisher J. Stabilization of multi-stranded nucleic acid structures using 3'-S-phosphorothiolate linkages. *Nucleosides Nucleotides Nucleic Acids*. 2007;26:555–8.
- [181] Dvorakova Z, Renciuk D, Kejnovska I, Skolakova P, Bednarova K, Sagi J, Vorlickova M. i-Motif of cytosine-rich human telomere DNA fragments containing natural base lesions. *Nucleic Acids Res.* 2018;46:1624–34.
- [182] Yang Y, Sun Y, Xing Y, Zhang T, Wang Z, Yang Z, Liu D. Influence of tetra(ethylene glycol) (EG<sub>4</sub>) substitution at the loop region on the intramolecular DNA i-motif. *Macromolecules*. 2012;45:2643–7.
- [183] Robidoux S, Klinck S, Gehring RK, Damha MJ. Association of branched oligonucleotides into the i-motif. *J Biomol Struct Dyn*. 1997;15:517–27.
- [184] Bonnet R, Murat P, Spinelli N, Defrancq E. Click-click chemistry on a peptidic scaffold for easy access to tetrameric DNA structures. *Chem Commun*. 2012;48:5992–4.
- [185] Li T, Liu D, Chen J, Lee AHF, Qi J, Chan ASC. Construction of circular oligodeoxyribonucleotides on the new structural basis of I-motif. *J Am Chem Soc*. 2001;123:12901–2.
- [186] Liu D, Chen J, Lee AHF, Chow LMC, Chan ASC, Li T. Small circular oligodeoxynucleotides achieved from self-assembling entities. *Angew Chem, Int Ed Engl*. 2003;42:797–9.

- [187] Hartig JS, Kool ET. Small circular DNAs for synthesis of the human telomere repeat: varied sizes, structures and telomere-encoding activities. *Nucleic Acids Res.* 2004;32:e152.
- [188] Zhou T, Li X, Ng MTT, Wang Y, Quek NM, Luo J, Yuan W, Tan CH, Zeng H, Li T. Synthesis and characterization of circular structures of i-motif tagged with fluoresceins. *Bioconjug Chem.* 2009;20:644–7.
- [189] Collin D, Gehring K. Stability of chimeric DNA/RNA cytosine tetrads: implications for i-motif formation by RNA. *J Am Chem Soc.* 1998;120:4069–72.
- [190] Snoussi K, Nonin-Lecomte S, Leroy JL. The RNA i-motif. *J Mol Biol.* 2001;309:139–53.
- [191] Chakraborty S, Krishnan Y. Kinetic hybrid i-motifs: intercepting DNA with RNA to form a  $\text{DNA}_2\text{-RNA}_2$  i-motif. *Biochimie.* 2008;90:1088–95.
- [192] Fenna CP, Wilkinson CP, Arnold JRP, Cosstick R, Fisher J. The effect of 2'-fluorine substitutions on DNA i-motif conformation and stability. *Chem Commun.* 2008;3567–9.
- [193] Abou Assi H, Harkness RW, Martin-Pintado N, Wilds CJ, Campos-Olivas R, Mittermaier AK, González C, Damha MJ. Stabilization of I-motif structures by 2'- $\beta$ -fluorination of DNA. *Nucleic Acids Res.* 2016;44:4998–5009.
- [194] Abou Assi H, El-Khoury R, González C, Damha MJ. 2-Fluoroarabinonucleic acid modification traps G-quadruplex and i-motif structures in human telomeric DNA. *Nucleic Acids Res.* 2017;45:11535–46.
- [195] Abou Assi H, Lin YC, Serrano I, González C, Damha MJ. Probing synergistic effects of DNA methylation and 2'- $\beta$ -fluorination on i-motif stability. *Chem Eur J.* 2018;24:471–7.
- [196] Aviñó A, Dellafiore M, Gargallo R, González C, Iribarren AM, Montserrat J, Eritja R. Stabilization of telomeric i-motif structures by (2'S)-2'-deoxy-2'-methyl-cytidine residues. *ChemBioChem.* 2017;18:1123–8.
- [197] Kumar N, Petersen M, Maiti S. Tunable c-MYC LNA i-motif. *Chem Commun.* 2009;1532–4.
- [198] Kumar N, Nielsen JT, Maiti S, Petersen M. I-motif formation with locked nucleic acids (LNA). *Angew Chem, Int Ed Engl.* 2007;46:9220–22.
- [199] Pasternak A, Wengel J. Modulation of i-motif thermodynamic stability by the introduction of UNA (unlocked nucleic acid) monomers. *Bioorg Med Chem Lett.* 2010;21:752–5.
- [200] Pérez-Rentero S, Gargallo R, González C, Eritja R. Modulation of the stability of i-motif structures using an acyclic threoninol cytidine derivative. *RSC Adv.* 2015;5:63278–81.
- [201] Lee IJ, Park M, Joo T, Kim BH. Using fluorescence changes of F1U units at terminal and mid-loop positions to probe i-motif structures. *Mol BioSyst.* 2012;8:486–90.
- [202] Perlikova P, Karlsen KK, Pedersen EB, Wengel J. Unlocked nucleic acids with a pyrene-modified uracil: synthesis, hybridization studies, fluorescent properties and i-motif stability. *ChemBioChem.* 2014;15:146–56.
- [203] Gouda AS, Amine MS, Pedersen EB. Improved i-motif thermal stability by insertion of anthraquinone monomers. *Org Biomol Chem.* 2017;15:6613–21.
- [204] El-Sayed AA, Pedersen EB, Khaireldin NA. Studying the influence of the pyrene intercalator TINA on the stability of DNA i-motifs. *Nucleosides Nucleotides Nucleic Acids.* 2012;31:872–9.
- [205] Stephenson AWI, Partridge AC, Filichev VV. Synthesis of  $\beta$ -pyrrolid-modified porphyrins and their incorporation into DNA. *Chem Eur J.* 2011;17:6227–38.
- [206] Diederichsen U. Oligomers with intercalating cytosine-cytosine+ base pairs and peptide backbone: DNA i-motif analogues. *Angew Chem, Int Ed Engl.* 1998;37:2273–6.
- [207] Sharma NK, Ganesh KN. PNA C-C+ i-motif: superior stability of PNA TC8 tetraplexes compared to DNA TC8 tetraplexes at low pH. *Chem Commun.* 2005;4330–2.
- [208] Krishnan-Ghosh Y, Stephens E, Balasubramanian S. PNA forms an i-motif. *Chem Commun.* 2005;5278–80.
- [209] Modi S, Wani AH, Krishnan Y. The PNA-DNA hybrid I-motif: implications for sugar-sugar contacts in i-motif tetramerization. *Nucleic Acids Res.* 2006;34:4354–63.

- [210] Chakraborty S, Modi S, Krishnan Y. The RNA 2-PNA 2 hybrid i-motif-a novel RNA-based building block. *Chem Commun.* 2008;70–2.
- [211] Gade CR, Sharma NK. Hybrid DNA i-motif: aminoethylprolyl-PNA (pC5) enhance the stability of DNA (dC5) i-motif structure. *Bioorg Med Chem Lett.* 2017;27:5424–8.
- [212] Sun Y, Ji Y, Wang D, Wang J, Liu D. Stabilization of an intermolecular i-motif by lipid modification of cytosine-oligodeoxynucleotides. *Org Biomol Chem.* 2018;16:4857–63.
- [213] Li X, Peng Y, Ren J, Qu X. Carboxyl-modified single-walled carbon nanotubes selectively induce human telomeric i-motif formation. *Proc Natl Acad Sci.* 2006;103:19658–63.
- [214] Zhao C, Ren J, Qu X. Single-walled carbon nanotubes binding to human telomeric i-motif DNA under molecular-crowding conditions: more water molecules released. *Chem Eur J.* 2008;14:5435–9.
- [215] Chen X, Zhou X, Han T, Wu J, Zhang J, Guo S. Stabilization and induction of oligonucleotide i-motif structure via graphene quantum dots. *ACS Nano.* 2013;7:531–7.
- [216] Jin KS, Shin SR, Ahn B, Jin A, Rho Y, Kim H, Kim SJ, Ree M. Effect of C60 fullerene on the duplex formation of i-motif DNA with complementary DNA in solution. *J Phys Chem B.* 2010;114:4783–8.
- [217] Mergny JL. Fluorescence energy transfer as a probe for tetraplex formation: the i-motif. *Biochemistry.* 1999;38:1573–81.
- [218] Sun YW, Liu HJ, Xu LJ, Wang LY, Fan QH, Liu DS. DNA-molecular-motor-controlled dendron association. *Langmuir.* 2010;26:12496–9.



Andreia F. Jorge, Santiago Grijalvo, Anna Aviñó, Carme Fàbrega,  
and Ramon Eritja

## 7 Advances in therapeutic oligonucleotide chemistry

### Contents

7.1	Introduction to therapeutic oligonucleotides —	274
7.1.1	Antisense oligonucleotides (ASOs) —	274
7.1.1.1	Gapmers and steric blockers —	276
7.1.1.2	Exon-skipping —	277
7.1.2	RNA interference (RNAi) —	278
7.1.2.1	Short interfering RNA (siRNA) —	278
7.1.2.2	Anti-miRNA oligonucleotides and miRNA mimics —	279
7.1.2.3	Anti-lncRNA —	280
7.1.3	Aptamers —	280
7.1.4	Immunostimulation and CpG oligonucleotides —	281
7.1.5	DNA- and RNA-decoys —	281
7.1.6	Triplex forming oligonucleotides —	282
7.1.7	Ribozymes and DNAzymes —	282
7.1.8	Anti-proliferative oligonucleotides —	283
7.2	Phosphate modified backbones —	283
7.2.1	Oligonucleoside phosphorothioates and phosphorodithioates —	284
7.2.2	Neutral phosphate backbones: Oligonucleoside <i>O</i> -alkylphosphates and methylphosphonates —	286
7.2.3	Oligonucleoside phosphoramidates and thiophosphoramidates —	288
7.2.4	Oligonucleoside phosphonoacetates and thiophosphonoacetates —	290
7.2.5	Oligonucleoside boranophosphates or borane phosphonates —	291
7.3	Oligonucleotide carrying 2'- <i>O</i> -alkyl-RNAs —	294
7.4	Oligonucleotides carrying modified carbohydrates —	295
7.5	Oligonucleotides carrying conformationally restricted sugars —	297
7.6	Oligonucleotides carrying acyclic derivatives —	299
7.7	Phosphorodiamidate morpholino oligomers (PMO) —	300
7.8	Peptide nucleic acids (PNA) —	301
7.9	Nonphosphorus internucleoside linkages —	303
7.10	Modification of the 5'-position of siRNA —	305
	Bibliography —	306

---

**Andreia F. Jorge, Santiago Grijalvo, Anna Aviñó, Carme Fàbrega, Ramon Eritja**, Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Networking Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Jordi Girona 18-26, E-08034 Barcelona, Spain, e-mails:  
andreiajorge09@gmail.com, santiago.grijalvo@iqac.csic.es, aaagma@cid.csic.es,  
carme.fabrega@iqac.csic.es, recgma@cid.csic.es

## 7.1 Introduction to therapeutic oligonucleotides

Short oligonucleotides are being used as therapeutic agents exerting their inhibitory action of by blocking translation or transcription of a specific gene or by stimulating the degradation of a particular messenger RNA [1–10] (Figure 7.1). Table 7.1 shows the key features of the oligonucleotides already approved for human use and those currently under assessment in late phases of clinical research. In order to achieve the desired inhibition, oligonucleotides should penetrate inside the cells and bind readily to their complementary sequence before exonuclease activity degrades them. A large research activity has been made in order to design modified oligonucleotides with enhanced nuclease resistance and cellular uptake, as well as higher selectivity to target tissues. The research made in this topic is too vast to be covered in this book, however, it has been extensively discussed elsewhere [11–29]. Herein, a comprehensive description of the most interesting oligonucleotide derivatives, their synthesis and properties and the main mechanisms of action will be described.

### 7.1.1 Antisense oligonucleotides (ASOs)

The most studied nucleic acid-based therapeutics used in the downregulation of gene expression are the antisense oligonucleotides (ASOs) (Figure 7.1). The use of synthetic oligonucleotides for the inhibition of the binding of tRNA to ribosomes was first mentioned in the 1970s during the development of dinucleotides linked by ester or carbamoyl linkages [30]. Moreover, gene regulation by antisense RNA was observed in bacteria [31]. However, the first demonstration that synthetic ASOs could be applied therapeutically to inhibit protein translation was described in 1978 by Zamecnick and Stephenson [32, 33]. At this time, oligonucleotides were prepared by the phosphotriester method. The development of the solid-phase phosphoramidite methods in the 1980s facilitated the production of antisense oligonucleotides with several modifications revolutionizing one decade later the synthesis of these molecules at a large scale.

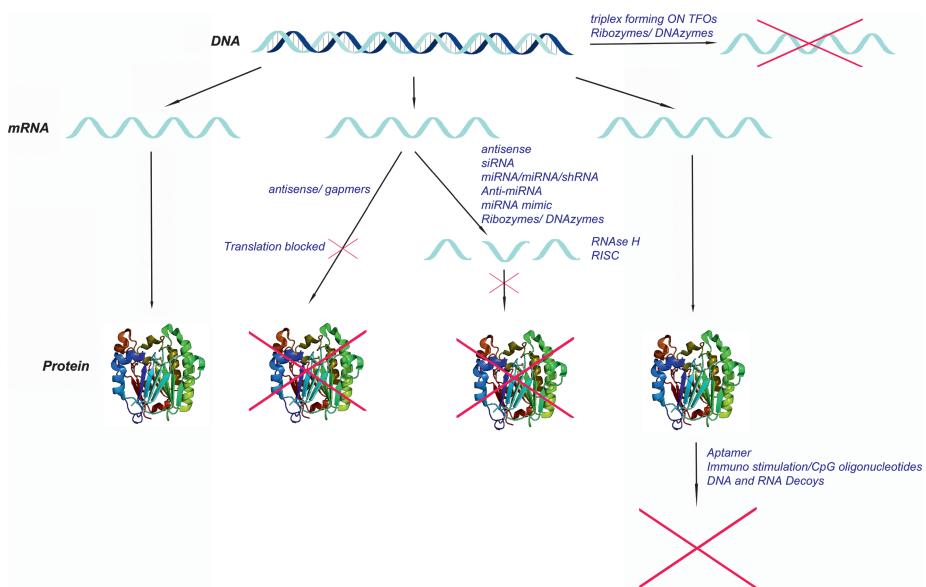
ASOs are chemically modified single-stranded DNA molecules, typically 15–30 nucleotides in length, which specifically bind to endogenous target messenger RNA (mRNA) by Watson–Crick base pairing rules [1, 34]. Following these rules, complementary base pairs are held together via specific hydrogen-bonding interactions and strengthened by the hydrophobic interactions arisen from purine-pyrimidine shape complementarity and base stacking. The chemical modification of ASOs is a requisite to guarantee stability in physiological conditions and to increase their potency and specificity to silence the mRNA target [12, 35]. Oligonucleoside phosphorothioates [36] were the first backbone modifications employed in the protection of ASOs and contributed to advance ASOs as therapeutic agents. This modification is easy to introduce via phosphoramidite chemistry and extends the half-lives of ASOs in serum. In addition, it binds to serum proteins facilitating cellular uptake [21, 23, 37]. The breakdown

**Table 7.1:** Oligonucleotides approved for human use or in advanced clinical evaluation.

<b>Drug</b>	<b>Type</b>	<b>Modif.</b>	<b>Conditions</b>	<b>Target</b>	<b>Year</b>	<b>Status</b>
Vitravene Formivirsen	ASO	PS	Citomegalovirus Retinitis	IE2	1998	Discont.
Macugen Pegaptanib	Aptamer	PS, 2'-F, 2'-OMe	Aged-related macular degeneration	VEGF 165	2004	Active
Kynamro Mipomersen	ASO, Gapmer	PS, MOE	Homozygous familial hypercholesterolemia	Apo B-100	2013	Active
Exondys 51 Eteplirsen	ASO	PMO	Duchene muscular dystrophy	Exon 51 dystrophine	2016	Active
Spinraza Nusinersen	ASO	PS, MOE	Spinal muscular dystrophy	SMN2	2016	Active
Tegsedi Inotersen	ASO	PS, MOE, gapmer	hATTR	Transthyretin	2018	Active
Onpattro Patisiran	siRNA	2'-OMe	Familial amyloid polyneuropathy	Transthyretin	2018	Active
Givosiran Givlaari	siRNA	GalNAc, 2'-OMe 2'-F	Acute hepatic porphyria	Aminolevulinate synthase	2018	Active
Waylivra Volanesorsen	ASO	MOE	Familial chylomicronemia syndrome	apolipoprotein C-III	2019	Active
Vyondys 53 Golodirsen	ASO	PMO	Duchene muscular dystrophy	Exon 53 dystrophin	2019	Active
Viltepso Viltolarsen	ASO	PMO	Duchene muscular dystrophy	Exon 53 dystrophin	2020	Active
Inclisiran	siRNA	GalNAc 2'-OMe 2'-F	Hypercholesterolemia	PCSK9	On going	Phase 3
Miravirsen	anti-miRNA	LNA, PS	Hepatitis C virus	miRNA-122	On going	Phase 2

ASO: antisense oligonucleotide; PS: phosphorothioate; 2'-OMe: 2'-O-methyl-RNA; 2'-F: 2'-F-RNA; LNA: locked nucleic acids, MOE: methoxyethyl; GalNAc: N-acetylgalactosamine; PMO: phosphorodi-amidate morpholino; hATTR: human transthyretin amyloidosis, PCSK9: proprotein convertase subtilisin kesin type 9.

of the HIV-1 pandemic was also an important factor for the development of the antisense technology in the 1990s. For this reason, the first antisense oligonucleotide approved for human treatment was Vitravene (Table 7.1), an antiviral phosphorothioate derivative designed to treat retinitis caused by cytomegalovirus, a disease that appeared in some AIDS patients causing blindness if untreated [38]. Equally relevant was the introduction of 2'-O-methyl-RNA modified oligonucleotides that dramatically



**Figure 7.1:** Schematic representation of the central dogma of molecular biology showing the potential sites of action of therapeutic oligonucleotides.

increased the resistance of ASOs to nucleases [39]. Later on, several 2'-O-alkyl-RNA derivatives were developed (Section 7.4).

Hitherto, it is generally accepted that the inhibitory activity of ASOs can be processed through two main mechanisms: (i) Ribonuclease H (RNase H)-dependent mechanism [40] and (ii) non-degradative steric blocking mechanisms [41] (Figure 7.1). In the degradative mechanism, RNase H enzyme binds to the heteroduplex mRNA-ASO through a binding domain inserted on the N terminus of the protein and catalyzes the cleavage of the mRNA strand. In humans, the specific enzyme recruited is RNase H1 and it is ubiquitously found in the nucleus, mitochondria and cytoplasm [40]. The cleaved mRNA is then processed by the typical cellular degradation pathways while the intact ASO is again available to hybridize with an additional target mRNA [42]. In the non-degradative steric blocking mechanisms, the binding of ASOs to pre-mRNA and mRNA creates steric hindrance that blocks the access of the ribosomal machinery and, therefore, inhibits translation, without mRNA direct degradation. This strategy is being used in the modulation of the splicing by exon-skipping described below.

#### 7.1.1.1 Gapmers and steric blockers

The number of modifications on the ASOs backbone may also modulate their activity. The triggered mechanisms by which ASOs exerts their antisense activity are thus

dependent on ASO modifications and design. RNase H-mediated oligonucleotides often include internucleotide linkage modifications such as phosphorothioate (PS) and other phosphate modifications (Section 7.2) [43]. Sugar modifications (Section 7.4) are generally considered incompatible with RNase-H-mediated gene silencing. However, they can be implemented in a “gapmer” design, where the unmodified central DNA core guarantees high affinity to RNase-H while their overhangs can be successfully protected with 2'-O-alkyl-RNA modifications [44–46]. Gapmers are special ASOs made of a central window carrying PS linkages which directs the action of RNase H, flanked by modified O-alkyl-RNA nucleotides fragments that increase the binding affinity of the gapmer for the mRNA target. Mipomersen (Table 7.1) is a 20-bases antisense gapmer designed to inhibit apolipoprotein B approved for human use in 2013 to treat hyperlipidemia by reducing the amount of low-density lipoprotein [47, 48]. Recently, Volanesorsen (Table 7.1) was approved for the treatment of familial chylomicronemia syndrome. This oligonucleotide is an antisense oligonucleotide gapmer carrying phosphorothioate and 2'-methoxyethyl (MOE) modifications which selectively binds the 3' untranslated region of apolipoprotein CIII mRNA. This interaction prevents the translation of the apolipoprotein CIII mRNA and allows ribonuclease H1-mediated mRNA degradation, thereby lowering the plasma triglyceride levels [49].

### 7.1.1.2 Exon-skipping

ASOs can selectively interfere in the intermediary processing steps of RNA metabolism, to redirect splicing or prevent translation by modifying 5' cap or polyadenylation site [41]. The design of ASOs complementary to splice regions [50–52] has received much attention due to their ability to originate a steric block to the binding of splicing factors to the pre-mRNA (e. g., RNA-binding proteins or components of the spliceosome) and block RNA sequences required for splicing, as small nuclear RNA [53]. This strategy beneficially suits the treatment of diseases with a known splicing defect, since rationally designed ASOs permit either to induce exon exclusion or exon inclusion, as it is the mechanism of eteplirsen [54], nusinersen [55], Vyondys 53 and Viltepso [56] respectively, all of these approved ASOs for neurological disorders. In this particular strategy, modification of ASOs with phosphorodiamidate morpholino oligomers (PMO) (Section 7.8) activates splice-switching and steric blocking mechanisms while enhances cell uptake due to their neutral charge [57]. Three PMO derivatives, Vyondys 53, Golodirsen and Viltepso have been recently approved for the treatment of Duchene Muscular Dystrophy [58], acting on exons 51 and 53 of dystrophin (Table 7.1). Nusinersen is a phosphorothioate oligonucleotide made of 2'-methoxyethyl (MOE)-RNA nucleotides (Section 7.4). The action of Nusinersen is based on the restoration of survival motor neuron 2 (SMN2) by exon skipping compensating the loss of function of an analogous SMN1 protein that has been mutated [55]. A recent conformationally

constrained DNA known as tricyclo-DNA (tc-DNA, Section 7.5) was found to have a remarkable ability to promote exon-skipping [59, 60].

An important breakthrough was the development of one drug tailored to one patient with a genetic brain disease. This patient had a mutation in the CLN 7 gene causing neuronal ceroid lipofuscinosis (Batten's disease). In 2017, it was developed a personalized antisense oligonucleotide (22 bases) named as Milasen that was similarly modified as nusinersen and it was approved for human use for intrathecal administration being the first drug developed for one single patient [61].

### 7.1.2 RNA interference (RNAi)

RNA interference (RNAi) pathway was firstly identified by Mello and Fire [62] and it is a natural regulatory process whereby small-sized double-stranded RNAs (dsRNA) downregulate gene expression. These endogenous noncoding RNAs are active regulators of gene expression acting at a post-transcriptional level and are commonly classified in three main subclasses in animals: (i) small interfering RNA (siRNA), (ii) microRNA (miRNA) and (iii) Piwi-interacting RNA (piRNA). Endogenously, siRNA are diced from longer RNA transcripts by an enzyme known as Dicer to generate an oligonucleotide duplex with 21–23 nt in length [63], while miRNA are processed from longer hairpin transcripts by the consecutive action of two RNase III proteins, Drosha and Dicer [64], to end up with approx. 22 nt in length. After the breakdown, siRNA and miRNA are loaded into RNA-Induced Silencing Complex (RISC) in a double-stranded conformation. This complex is composed by Dicer, Argonaute2 (Ago2) and transactivation response RNA-binding (TRBP) proteins [65–67]. In the case of siRNA, a process deemed RISC maturation triggers the release of the sense strand whereas the antisense strand is paired to a complementary mRNA. The perfect match between antisense siRNA and mRNA results in endonucleolytic cleavage of the target mRNA promoted by the slicing effector Ago2 of the RNAi process [68]. Conversely, complementarity between the seed region of one strand of the hairpin duplex of miRNA (positions 2–8 from the 5' miRNA) with the 3' untranslated region (3' UTR) of the target mRNA is the only prerequisite to silence the expression of the target genes through mechanisms of translation repression and mRNA destabilization [69]. Fully complementarity between miRNA-mRNA activates a process of degradation of the mRNA, while a partial complementarity induces translational inhibition.

#### 7.1.2.1 Short interfering RNA (siRNA)

RNAi-based therapeutics involve the delivery of synthetic small RNA duplexes, for instance, siRNA, artificial miRNA and small hairpin RNA (shRNA) into a determined

target cell to produce gene silencing effect. Ago2-mediated mechanisms, as RNase-H-dependent ones, may be modulated by the design of chemically modified oligonucleotides [70–75]. The first siRNA-based approved drug, Patisiran, was developed to treat patients with polyneuropathy caused by hereditary transthyretin-mediated amyloidosis (hATTR) in adults, by exploiting the use of lipid nanoparticles to deliver the drug directly into the liver [76, 77]. Patisiran (Alnylam Pharmaceuticals) was developed in parallel with Inotersen (Ionix Pharmaceuticals), a new antisense gapmer designed to bind the same transthyretin RNA target [78]. The approval for human use of Givosiran (Table 7.1) for the treatment of hepatic porphyria was an important milestone because it was the first time to use *N*-acetylgalactosamine (GalNAc) trimer for active transport to the liver [79]. Subcutaneously administered siRNA molecule accumulates in target hepatocytes inhibiting aminolevulinate acid synthase 1. The excellent results found in this formulation triggered the development of Inclisiran (Table 7.1) taking advantage from the same GalNAc targeting system allowing only one subcutaneous administration every 6 months [80, 81]. At present, Inclisiran is in advanced clinical phase 3 for the treatment of familial hypercholesterolemia targeting proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 is a serine protease that binds to the LDL receptor to stimulate the lysosomal degradation of the LDL receptor. If there is an excess of PCSK9, there is a decrease in LDL receptors that causes elevation of LDL-C levels as they are not taken up by the receptors. Inclisiran was designed to reduce the levels of PCSK9, thus ensuring the maintenance of the level of free LDL receptors and healthy LDL-C levels in the blood [80, 81].

#### 7.1.2.2 Anti-miRNA oligonucleotides and miRNA mimics

The misregulation of miRNAs has proved to be associated with several human conditions, including cancer, neurodegenerative and autoimmune diseases [82, 83]. The use of synthetic miRNA emerged as an attractive tool to regulate the expression of endogenous miRNA. miRNA-based therapeutics may be divided into two strategies; (i) miRNA mimics (agonist, dsRNA), used to restore the suppressed miRNA level, and (ii) inhibitors of endogenous miRNA (antagonist, ssRNA), known as antimiRNAs or antagonists, with potential to down-regulate overactive miRNA expression [26, 84, 85].

AntimiRNAs, as antisense oligonucleotides, are single-stranded RNAs fully complementary to mature miRNA that degrades or repress miRNA translation mediated by Argonaute-containing miRNA induced silencing complex. One of the most advanced antimiRNAs in clinical evaluation is Miravirsen, a 15-mer LNA-modified oligonucleotide carrying phosphorothioate linkages designed to bind with a high affinity to miR-122, a host factor for Hepatitis virus C (HCV) [86]. Treatment of HCV

patients with Miravirsen has been shown to reduce the viral load without side effects [87]. The development of novel and efficient nucleotide prodrugs for HCV was a serious competitor for the use of Miravirsen in the treatment of HCV patients.

Several miRNAs have tumor-suppressor activities and their absence can induce cancer formation [82]. It has been found that the tumor-suppression miR-34a is lost or repressed in several human tumors such as colon cancer, hepatocarcinoma and others [88]. The miRNA mimic MRX34 encapsulated with lipid nanoparticles was shown to recover miR-34a function in a subset of patients with advanced solid tumors, although the clinical trial was halted because of immune-related side effects [89].

#### 7.1.2.3 Anti-lncRNA

Long noncoding RNAs (lncRNAs) are a diverse group of transcripts that do not encode any protein but they may be involved in modulation of gene expression [90, 91]. Gene silencing can be used to investigate the function of a lncRNA, using either ASOs or duplex RNAs. However, the use of gene silencing for lncRNAs is less straightforward than for mRNA, because cellular localization differs among lncRNAs. ASOs may be the silencing method of choice when a target RNA is thought to function in the nucleus, whereas duplex RNAs may be a better choice when a target is thought to function in the cytoplasm. The preclinical study for the treatment of Angelman syndrome was an example of the inhibition of long noncoding RNAs by ASOs. This syndrome is caused by a defect affecting the expression of the maternal gene UBE3A, which encodes an E3 ubiquitin protein ligase. An antisense transcript is expressed at the paternal UBE3A locus and represses gene expression of that allele. Reduction of murine Ube3a antisense transcript levels was achieved in mice using phosphorothioate 2'-MOE gapmer ASOs126. Reducing transcript levels using two different ASOs led to increased expression of the paternal allele of Ube3a [92].

Following the same strategy, several studies have exploited the use of ASOs for targeting long noncoding RNAs as lncRNA ASBEL considered to be involved in Wnt/β-catenin-associated tumorigenesis in breast cancer [93] and the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) a nuclear localized RNA that is upregulated in cancer cells [94].

#### 7.1.3 Aptamers

Aptamers are nontoxic and nonimmunogenic single-stranded DNA or RNA oligonucleotides, having a length of 40 to 100 nucleotides, which specifically bind with high affinity to target molecules including a large variety of compounds such as small organic molecules, amino acids, proteins, antibodies, cells, bacteria and tissues [95–100]. These synthetic molecules have similar binding affinities as antibodies and may

be synthesized at a larger scale with longer stability and storage life. Aptamers are identified from DNA libraries by the systematic evolution of ligands by exponential enrichment (SELEX) [95].

Pegaptanib (Macugen, Table 7.1) was the first aptamer approved for human therapy. This aptamer is a phosphorothioate oligonucleotide with a high affinity to the vascular endothelial growth factor (VEGF). It is formulated by conjugation to polyethylene glycol (PEG) and it is used for the treatment of age-related macular degeneration by intravitreal injection [101, 102].

In order to increase the stability of aptamers to nucleases, the synthesis of oligonucleotides carrying the mirror image L-nucleotides (Spiegelmers) have been proposed [103]. Some of these aptamers commercialized by NOXXON Pharma reached clinical trials for cancer treatment [104].

Aptamers are frequently used in the nanoformulations for drug delivery including anticancer compounds [105–107] as well as siRNA drugs [108]. Several aptamers used in drug delivery are able to form G-quadruplex [106, 109] as explained in Chapter 6.

#### 7.1.4 Immunostimulation and CpG oligonucleotides

One of the potential side effects of therapeutic oligonucleotides can be the stimulation of the innate immune system. Several reports have demonstrated that the stimulation of the immune system by siRNA molecules depends on different factors: (i) chemical structure; (ii) length of siRNA; (iii) specific nucleotide sequence; (iv) relative concentration of siRNA at the time of transfection and (v) target cell type [110–112]. During the development of therapeutic siRNAs it has been found that some modifications can reduce these undesired side effects [113–115].

A totally different approach is to exploit the innate stimulation properties of oligonucleotides to boost tumor immunity to enhance the efficacy of cancer treatments [116]. Oligonucleotides carrying unmethylated CpG (CpG oligonucleotides) are recognized by Toll-like receptor-9 (TLR-9) generating an increased stimulation of the innate response that is used for cancer vaccine adjuvants [117–119]. Other oligonucleotides applied for innate response stimulation are double-stranded RNA carrying 5'-diphosphate [120] or 5'-triphosphate [121] groups that interact with the retinoic acid-inducible gene I (RIG-I).

#### 7.1.5 DNA- and RNA-decoys

Transcription factors have become attractive targets for potential therapeutic development, because bind specific sequences found in the promoter regions which are 6–10 base pairs. As transcription factors can recognize relatively short DNA sequences,

short oligodeoxynucleotides carrying the consensus binding sites can be used for therapeutic purposes. This strategy involves the intracellular delivery of such oligonucleotides named as DNA-decoy oligonucleotides [122–124]. Similarly, RNA-decoys can be employed to capture RNA-binding proteins or other RNA molecules such as miRNAs through RNA-RNA interactions [125].

### 7.1.6 Triplex forming oligonucleotides

Nucleic acid triplets are formed when a DNA or RNA oligonucleotide binds to a polypurine-polypyrimidine rich sequence. Single-stranded polypurine or polypyrimidine oligonucleotides or triplex-forming oligonucleotides (TFOs) can form triplets with target nucleic acid sequences (see Chapter 5). TFOs were well studied in the 1990s for their potential therapeutic applications such as gene silencing known as antigene strategy [126, 127] or site-specific mutagenesis [128]. The possibility of targeting double-stranded DNA and the limited amount of genomic DNA (two copies per cell) triggered the interest for TFOs. Unfortunately, the lower stability of triplets compared to duplexes as well as the protection of genomic DNA by nucleosomes and the nuclear membrane were important factors to decrease the interest for the antigene strategy. More recently, the development of oligonucleotides that connect triplex-forming strands with Watson–Crick duplex strands such as *bis*-PNA [129], *bis*-LNA [130] and polypurine hairpins [131] demonstrated that higher affinity oligonucleotides can be prepared to induce strand displacement, putting the focus back on the potential applications of oligonucleotides based on triplex formation.

### 7.1.7 Ribozymes and DNAzymes

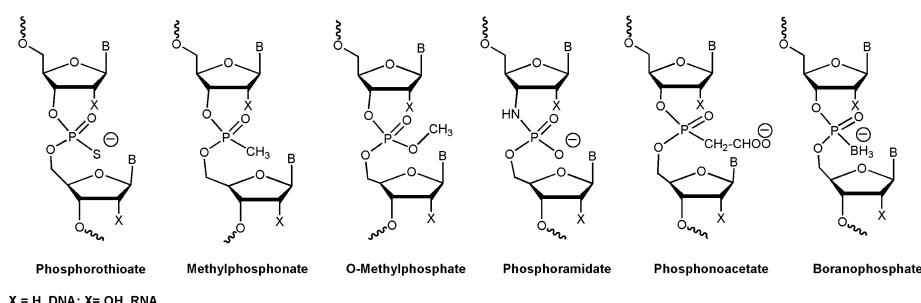
Ribozymes and DNAzymes are molecules that can bind specific DNA or RNA sequences and slice the target sequence in a specific site. Ribozymes were discovered in the 1980s when studying RNA splicing in *Tetrahymena* observing that the self-catalyzed splicing of an mRNA was possible without any enzyme [132]. Later, other RNA molecules were found to be able to catalyze a large variety of chemical reactions generating the hypothesis of an origin of life involving self-replicating RNA [133]. DNA oligonucleotides named as DNAzymes can also act as reaction catalysts and generate enzymatic activity such as hydrolysis of RNA molecules [134]. DNAzymes were generated through in vitro selection techniques [135]. Ribozymes and DNAzymes can be redesigned to cut a specific mRNA, and thus increasing the inhibitory effect as they do not need the action of Ribonuclease H [136]. Moreover, a large number of biosensors are based on the use of DNAzymes for signal amplification [137].

### 7.1.8 Anti-proliferative oligonucleotides

Several synthetic nucleosides such as 5-fluoro-2'-deoxyuridine, cytarabine and gemcitabine known as antimetabolites are being used in cancer chemotherapy as well as antivirals. The mechanism of action of these nucleosides involves the cellular phosphorylation of nucleosides to yield 5'-monophosphates. The modified nucleotides can be further phosphorylated to nucleoside 5'-triphosphates and incorporated into DNA or RNA. In addition, modified nucleosides could directly interfere with the biosynthesis of the natural nucleoside 5'-phosphates such as the inhibition of the biosynthesis of thymidylic acid. An interesting alternative to generate the modified nucleoside 5'-monophosphate is by fragmentation in monomers of oligonucleotides by degradation of cellular exonucleases [138]. The antitumoral action of the decamer FdU<sub>10</sub> composed by ten monomers of 5-fluoro-2'-deoxyuridine monophosphate (FdU) has been demonstrated in preclinical models of acute myeloid leukemia [139], acute lymphocytic leukemia [140], prostate cancer [141] and glioblastoma multiform (GBM) [142]. Recently, DNA nanoassemblies were designed to carrying in the scaffold polymeric fluoropyrimidine strands representing a promising approach for cancer therapy [143, 144].

## 7.2 Phosphate modified backbones

Modification at the phosphodiester bond generates oligonucleotides with enhanced nuclease resistance while maintaining the capacity of binding to their target mRNA. In addition, the presence or absence of the negative charge at the phosphate positions has a profound influence on the biophysical, pharmacokinetic and biochemical properties of the oligonucleotides. Figure 7.2 shows some of the phosphate modifications that have been proposed for potential therapeutic applications [13, 145, 146].

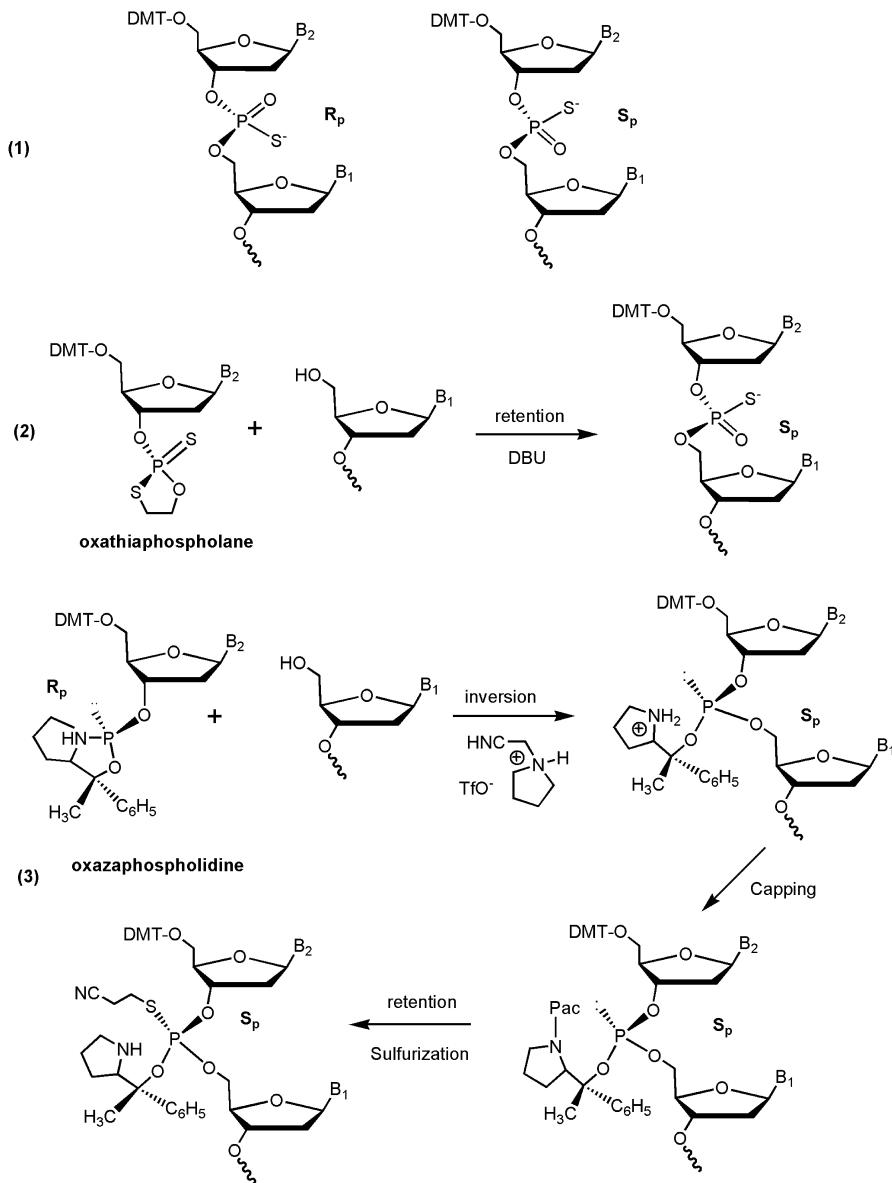


**Figure 7.2:** Main classes of phosphate modified backbones developed for therapeutic oligonucleotides.

### 7.2.1 Oligonucleoside phosphorothioates and phosphorodithioates

The replacement of oxygen for sulfur generates phosphorothioate linkages that are the most common modifications. The large majority of the therapeutic oligonucleotides carry this modification mainly because it can be easily introduced by replacing the oxidation reagent for a sulfurizing as explained in Chapter 1. This simple modification prevents oligonucleotide degradation catalyzed by nucleases and activates the degradation of target mRNA by RNase H [36, 37]. Moreover, phosphorothioates bind to serum proteins increasing the pharmacokinetic properties of oligonucleotides favoring their distribution in the different tissues, especially in the liver and avoiding kidney elimination [21, 147, 148]. Some authors have expressed concerns regarding their potential toxicity that may arise from protein binding [149] and the impairment of hybridization properties [150]. The introduction of the sulfur generates a chiral center and this implies the formation of two diastereoisomers in each phosphorothioate linkage designated  $R_p$  and  $S_p$  (Figure 7.3) [151]. A therapeutic oligonucleotide carrying several phosphorothioate linkages contains a mixture of  $2^n$  diastereoisomers (being  $n$  the number of phosphorothioate linkages). As the biophysical and biological properties of each diastereoisomer may be different, a large effort in developing methods for the control of the stereoselectivity of phosphorothioate linkages has been done [150]. The enzymatic synthesis of phosphorothioate-oligonucleotides can be used for the generation of oligonucleotides carrying  $R_p$ -phosphorothioate bonds [152].

The first chemical method for the stereocontrolled synthesis of phosphorothioate linkages was based on the use of enantiomerically pure DMT-nucleoside oxathiaphospholane (Figure 7.3) derivatives [150, 153–155]. The oxathiaphospholane monomers react with the 5'-OH of the growing oligonucleotide catalyzed by 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) to generate the enantiomerically pure phosphorothioate bonds. The reaction proceeds with retention of the configuration with a coupling yield of around 92–94 % [150]. Phosphoramidite [156–162] and *H*-phosphonate [163] chemistries have been also described for the generation of stereodefined phosphorothioate linkages. Especially relevant is the use of nucleoside 3'-oxazaphospholidine derivatives (Figure 7.3) as monomers known as SOSICS (stereocontrolled oligonucleotide synthesis with iterative capping and sulfurization) that uses the capping and sulfurization steps for the release of the chiral auxiliary reagent [162]. A phenoxyacetic (Pac) anhydride solution was utilized for the capping reaction. This solution truncates the unreacted hydroxyl group but also facilitates the removal of the chiral auxiliary group by acylation of the pyrrolidine nitrogen formed during the coupling reaction (Figure 7.3). The electrophilic reagent *S*-cyanoethyl methylthiosulfonate [164] is used as sulfurizing reagent triggering the elimination of the chiral auxiliary group. This method has allowed the synthesis of several stereocontrolled derivatives of Mipomersen (Table 7.1) that contains 2'-MOE and 19 phosphorothioate modifications. The study of the biophysical and biomedical properties of stereospecific Mipomersen

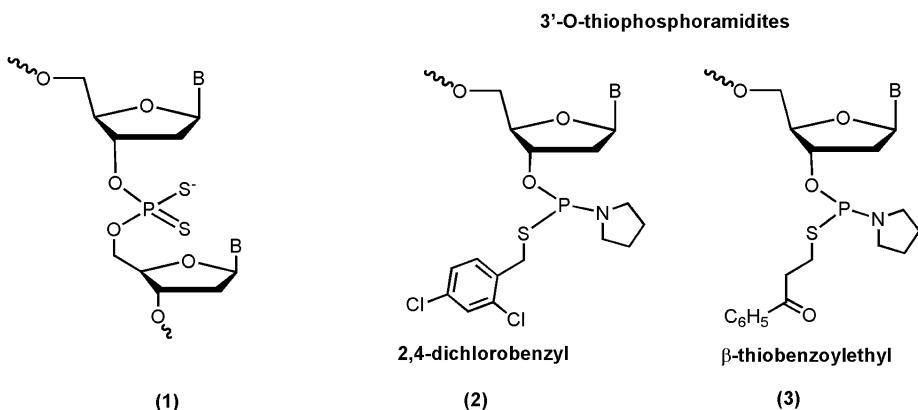


**Figure 7.3:** Stereocontrolled synthesis of phosphorothioates. Chemical structures of  $R_p$ - and  $S_p$ -phosphorothioates (1). Coupling reactions using oxathiaphospholane monomers (2). Reactions involved in the stereocontrolled oligonucleotide synthesis with iterative capping and sulfuration (SOSICS) method using oxazaphospholidine monomers (3).

derivatives demonstrates that duplexes carrying  $R_p$ -isomers have a small increased thermal stability however, ASOs carrying  $S_p$ -isomers have increased lipophilicity and increased metabolic stability. Moreover, the authors found a special configuration of

the terminal 3'-positions that promotes target mRNA cleavage by RNase H1 providing a more durable response than the stereo random ASOs in mice [162].

The substitution of the two non-bridging oxygens of phosphates by sulfur generates achiral phosphorodithioate linkages known as dithioate DNA (Figure 7.4). These linkages are isostructural and isopolar to natural phosphodiester, stable to degradation by nucleases and can react with electrophiles to generate oligonucleotide conjugates [165, 166]. Dithioate DNA can be synthesized using deoxynucleoside thiophosphoramidites [165–169], diamidites [170], *H*-phosphonodithioates [171, 172] and dithiophosphate triester [173, 174] as synthons. The more convenient and efficient protocols use deoxynucleoside 3'-*O*-thiophosphoramidites as protected monomers. Figure 7.4 shows the structure of two thiophosphoramidites showing the 2,4-dichlorobenzyl [165, 167, 168] and the  $\beta$ -thiobenzoylethyl [166, 168] protecting groups for the thiophosphate function. Dithioate DNA molecules are very resistant to nucleases, form stable duplexes although they are slightly destabilized, and they can direct RNaseH degradation of target mRNA [175, 176]. Polycytidine dithioate oligomers have shown strong anti-HIV-1 activity [177].



**Figure 7.4:** Synthesis of phosphorodithioate DNA (1). Chemical structures of 3'-*O*-thiophosphoramidites carrying 2,4-dichlorobenzyl (2) or  $\beta$ -thiobenzoylethyl (3) thiophosphate protecting groups.

## 7.2.2 Neutral phosphate backbones: Oligonucleoside *O*-alkylphosphates and methylphosphonates

The negatively charged phosphodiester backbone is considered correlated with poor pharmacokinetic properties of oligonucleotides. For this reason, the first aim in the development of novel modified oligonucleotides for therapeutic uses was the removal of the negative charge of oligonucleotides.

The first and simplest neutral modification was obtained with *O*-alkyl phosphates as these are synthetic intermediates in oligonucleotide synthesis. The synthesis of oligonucleotides carrying methyl [178], ethyl [179, 180], isopropyl [181, 182], trifluoroethyl [183, 184] and neopentyl [185] phosphotriester linkages have been reported. The phosphoramidites synthons can be easily prepared by small variations of standard methods [183]. The most important synthetic challenge is the lability of the alkylphosphates to ammonia and iodine solution, especially in the case of the methylphosphates. This problem can be overcome by replacing the ammonia solution by a solution of potassium carbonate in methanol [186]. In order to increase the efficacy of these deprotection conditions more labile linkers such as oxanyl [187] or malonyl [188] have been reported for the preparation of phosphotriester oligonucleotides. Moreover, the use of extremely labile groups such as; *N*-pent-4-enoyl [189, 190], fluorenylmethoxycarbonyl (Fmoc) [191] or isopropoxyacetyl [183] moieties have been reported. Finally, the replacement of the iodine solution for nonaqueous oxidation solutions such as *tert*-butylperoxide solutions has been recommended [187].

Oligonucleotides carrying methyl phosphotriester are DNA alkylphosphotriester derivatives. Methylphosphotriester modified oligonucleotides are nuclease resistant, form more stable duplex due to the absence of interstrand phosphate repulsions and can block DNA replication [192]. On the other hand, the complex deprotection protocols, the presence of stereoisomers and the lower gene inhibitory properties compared to phosphorothioate oligonucleotides [193] are common difficulties encountered during the preclinical evaluation of these modified oligonucleotides.

Methylphosphonate oligonucleosides stood out as neutral phosphate backbone because they were one of the first modifications that clearly demonstrated to possess selective gene inhibitory properties in cell culture [194, 195]. In these compounds, one of the nonbridged phosphate oxygens has been replaced by a methyl group (Figure 7.2). In the 1980s the method of choice for the production of methylphosphonate oligonucleotides employed polystyrene supports and DMT-nucleoside 3'-methylphosphonic imidazolides that were *in situ* generated by the reaction of DMT-nucleosides and methylphosphonic bis(imidazolide) [196, 197]. These methods showed the instability of the methylphosphonates to ammonia solution and the final deprotection was carried out using a solution of ethylenediamine in anhydrous ethanol. This treatment provoked a side reaction during the removal of the benzoyl group of cytidine that yields the ethylenediamine adduct of cytidine. These side products were produced in lower amounts and for short methylphosphonate oligonucleotides having a terminal phosphodiester nucleotide could be easily removed by chromatographic methods [197]. The development of the phosphoramidite protocols triggered the development of DMT-protected nucleoside 3'-methylphosphonamidites [198–203] which are the equivalent of P-III reagents for methylphosphonates synthesis. Miller's group recommended a deprotection protocol consisting of two steps. First, hydrazine hydrate in a pyridine-acetic acid buffer was used for the removal of the benzoyl groups of C and A.

Then the treatment with ethylenediamine/ethanol cleaved the oligonucleotide from the support and removed the isobutyryl group of G [198]. This two-steps protocol was optimized to a one-pot protocol by using a treatment with diluted ammonia followed by the addition of the ethylenediamine/ethanol solution [204, 205]. Further improvements came by changing the protecting groups of the nucleobases for more labile ones preventing the side reactions, such as the *tert*-butylphenoxyacetyl groups [206] and the *N*-pent-4-enoyl groups [207].

The presence of the methyl group in the methylphosphonate function generated a chiral center with the subsequent generation of diastereoisomeric mixtures. Stereoselective methods for the generation of chirally pure methylphosphonate linkages have been developed [208–211]. The  $R_p$  isomer binds the target RNA with a higher affinity than the racemic mixture [208]. The synthesis of RNA-modified with single enantiomerically pure methylphosphonate linkages has been described [211]. These modified RNAs are valuable tools for studying the interaction of RNA with proteins or small molecules [212].

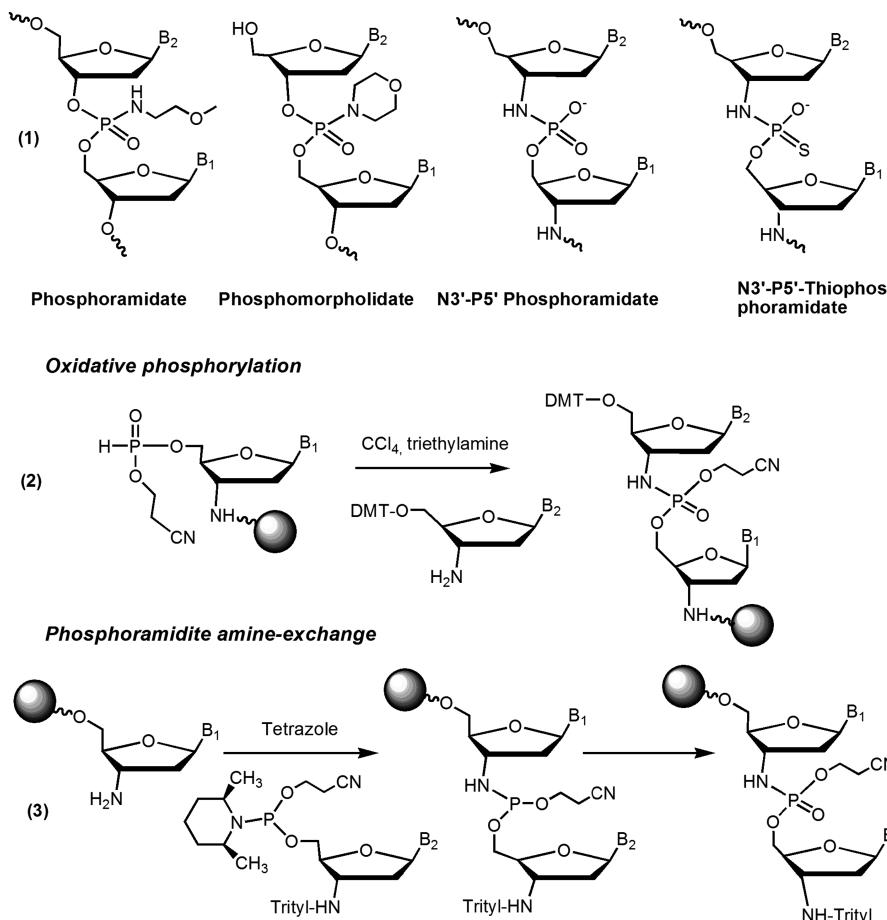
The use of sulfurizing reagents instead of the standard iodine oxidation solution after the coupling of phosphonamidites generates oligodeoxynucleoside methylphosphonothioates [213, 214]. These derivatives have high stability to nucleases; however, their presence provokes a small decrease in duplex stability [214].

Methylphosphonate oligonucleotides are readily taken up by mammalian cells in culture [215]. The cellular uptake pathway seems to involve fluid-phase endocytosis or adsorptive endocytosis, which is different from what is observed for negatively charged oligonucleotides [216]. Methylphosphonate oligonucleotides are not substrates for RNaseH [217] but chimeric oligonucleotides carrying mixed backbones can direct degradation of the target RNA by RNase H becoming interesting alternatives for antisense experiments [218, 219].

### 7.2.3 Oligonucleoside phosphoramidates and thiophosphoramidates

The substitution of nonbridged phosphate oxygens by primary or secondary amines generates chiral phosphoramidites as shown in Figure 7.5. These compounds can be prepared by oxidation of *H*-phosphonate diesters in the presence of primary or secondary amines catalyzed by iodine or carbon tetrachloride [220]. These derivatives have been shown to specifically degrade maternal messages in *Xenopus* oocytes and embryos [221] and are highly resistant to exonucleases [222].

Oligonucleoside  $N3' \rightarrow P5'$  phosphoramidates (Figure 7.5) are the most studied oligonucleotides carrying phosphoramidate linkages [223]. In this case, the  $3'-OH$  is replaced by a  $3'$ -amine group generating an achiral phosphate derivative. Two solid-phase methods have been reported for the synthesis of these compounds that are outlined in Figure 7.5. In the first method, the coupling reaction between the amino-



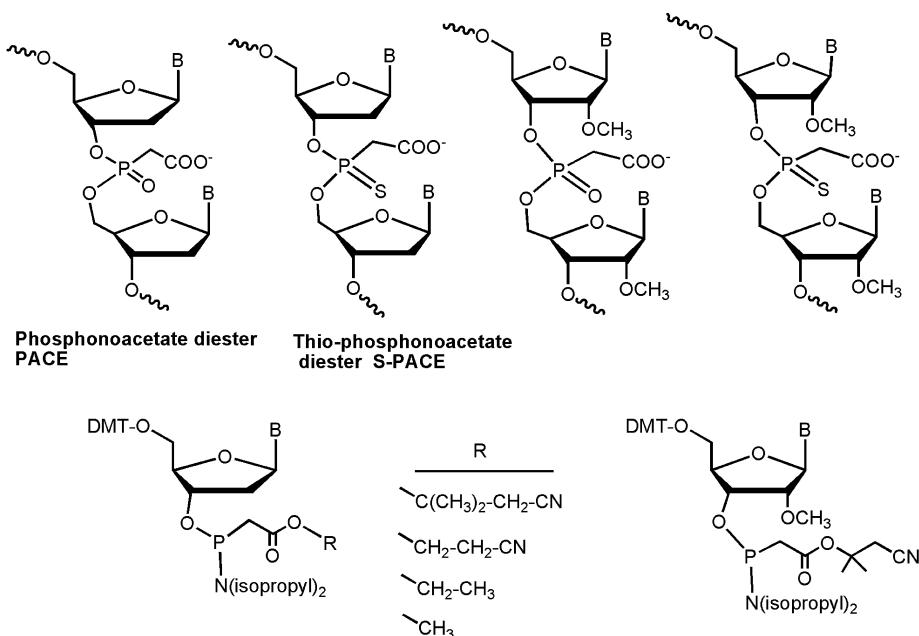
**Figure 7.5:** Chemical structures of several oligonucleotide phosphoramidate backbones (1). Synthesis of N3'-P5' phosphoramidate using oxidative phosphorylation (2) or phosphoramidite amino-exchange (3).

nucleoside and the following nucleoside is an oxidative phosphorylation coupling of 5'-O-DMT-protected 3'-aminonucleoside with a *H*-phosphonate diester linked to the solid support [224–226]. The *H*-phosphonate diester is generated in each step with the formation of the 5'-phosphoramidite and hydrolysis with tetrazole and water [226]. This method has been adapted for the synthesis of oligonucleotide  $N3' \rightarrow P5'$  thiophosphoramidates [227]. The second approach is based on a phosphoramidite-amine exchange [228]. The elongation of the oligonucleotide chain is done in the  $5' \rightarrow 3'$  direction using 3'-*N*-trityl-protected nucleosides 5'-O-[*(cis*-2,6-dimethylpiperidino)(2-cyanoethyl) phosphoramidite] [228–231]. The last method is claimed to be more efficient and compatible with other chemistries suitable for the generation of important oligonucleotide conjugates and chimeras [228–231].

The biophysical and biological properties of oligonucleotide  $N3' \rightarrow P5'$  phosphoramidates and thio-phosphoramidates have been reviewed [223]. These include powerful antisense inhibitory properties [232]; improved triplex-forming properties [233] explained in Chapter 5, Section 5.7; RNA mimetics [234]; anticancer properties as potent telomerase template antagonists [235–237]; and powerful probes for fluorescence *in situ* hybridization (FISH) techniques [238].

### 7.2.4 Oligonucleoside phosphonoacetates and thiophosphonoacetates

The substitution of one of the nonbridged phosphate oxygens by an acetate group (Figure 7.6) generates a phosphonoacetate (PACE) diester linkage [239]. At neutral pH the acetate group is ionized generating a negatively charged backbone and formed heteroduplexes with complementary sequences with similar stability than phosphorothioates [239, 240].



**Figure 7.6:** Chemical structures of several oligonucleoside phosphonoacetates (PACE) and thiophosphonoacetates. Phosphoramidite monomers used in the synthesis of PACE-DNA.

The synthesis of these modified oligonucleotides has been done by solid-phase protocols using the appropriate DMT-nucleoside 3'-phosphinoamidites (Figure 7.6)

[239, 241]. The carboxylic group can be protected in the form of methyl, ethyl, 2-cyanoethyl or 2-cyano-1,1-dimethylethyl ester (Figure 7.6). The tetrazole-catalyzed coupling generates phosphinoacetate intermediates that can be oxidized with (1*S*)-(+)-(10-camphorsulfonyl)oxaaziridine to generate PACE linkages or with 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage's reagent, Section 1.8.2) to generate thiophosphonoacetates (thio-PACE, Figure 7.6). As described for methylphosphonates [204], PACE and thio-PACE linkages are sensitive to basic aqueous media. For this reason, it is essential to reduce the time for the final deprotection as much as possible. In addition, the removal of the protecting group of the ester function has to be done avoiding aqueous basic conditions. The methyl and ethyl ester used in the first protocols [241] were changed to 2-cyanoethyl or 2-cyano-1,1-dimethylethyl esters which can be removed by  $\beta$ -elimination with a strong nonnucleophilic base such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in anhydrous conditions [239].

Both PACE and thio-PACE oligonucleotides stimulate RNase H and are resistant to degradation by exonucleases [240]. The neutral esterified forms of these derivatives are taken up by cells in culture and can be used for cell transfection in the absence of transfecting lipids [240, 242]. Chimeric 2'-*O*-methyl oligoribonucleotides containing PACE and thio-PACE linkages have been prepared using the appropriate DMT-protected 2'-*O*-methyl-nucleoside 3'-phosphinoamidites (Figure 7.6) [243, 244]. When siRNA was modified at the passenger strand with PACE and thio-PACE modification the resulting siRNA maintained gene silencing activity [243]. Single-stranded PACE and thio-PACE oligomers have good cellular permeability and can be used as microRNA inhibitors [243] or antisense inhibition of huntingtin in patient-derived cells [244].

### 7.2.5 Oligonucleoside boranophosphates or borane phosphonates

Boranophosphates or borane phosphonates (Figure 7.2) can be considered as a hybrid between phosphodiester and methylphosphonate linkages. The borane moiety (-BH<sub>3</sub>) is isoelectronic to oxygen in phosphodiesters and phosphorothioates and isoelectronic and isosteric with the methyl group in methylphosphonates [245, 246]. Boranophosphates are negatively charged activating RNase H degradation of complementary RNA [247] but exhibit some of the properties of methylphosphonates such as nuclease resistance and enhanced cell penetration by passive transport [248–250]. In addition, boron can absorb thermal neutrons so oligonucleotides carrying boranophosphates can be used to treat tumor cells by boron neutron capture therapy (BNCT) [245].

Oligonucleoside boranophosphates can be prepared either by enzymatic synthesis using DNA polymerases and nucleoside  $\alpha$ -boranotriphosphates [251] or by chemical methods [248, 249]. Several chemical methods have been reported for the synthesis of oligonucleoside boranophosphates (Figure 7.7). The first reported

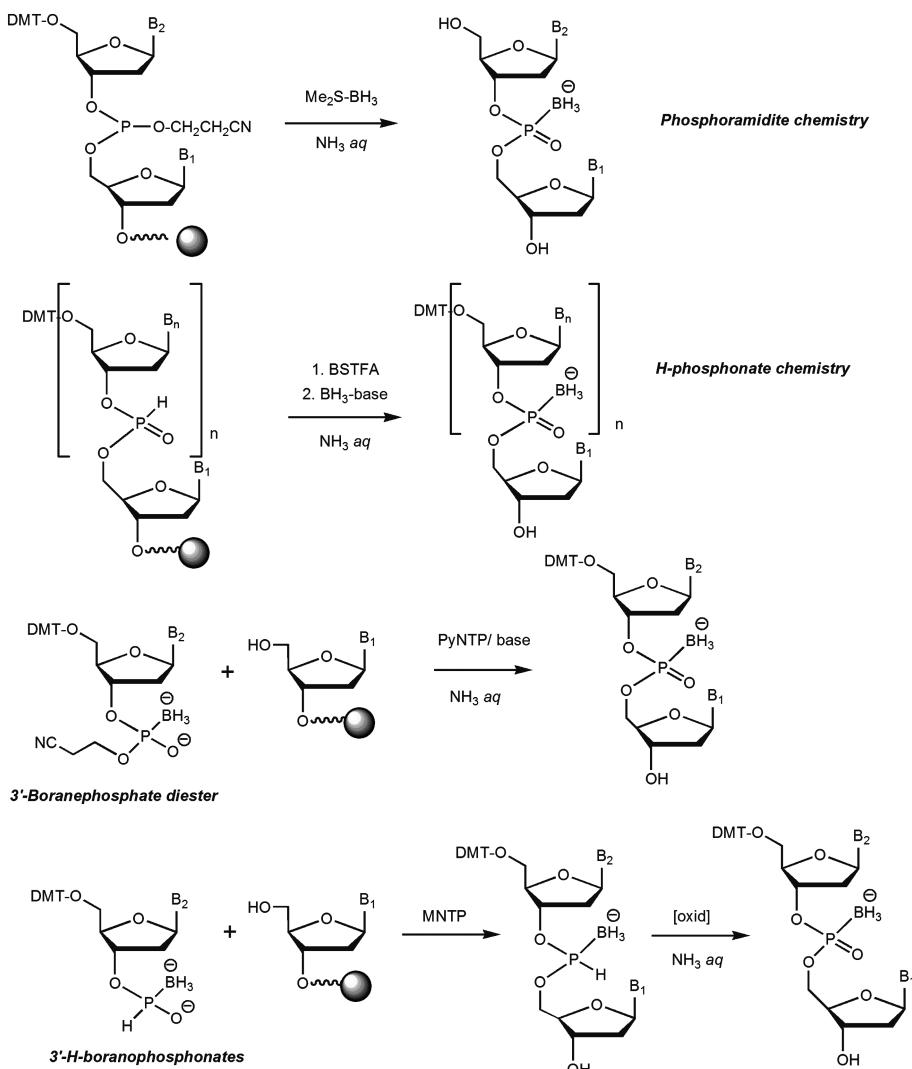


Figure 7.7: Synthesis of oligonucleoside boranophosphates and borane phosphonates.

protocols used standard phosphoramidites and the iodine oxidation step was replaced by oxidation of the phosphite-triester with  $\text{Me}_2\text{S}-\text{BH}_3$  [252]. This method was used to generate short oligonucleotides because the DMT group was not compatible with the borane reagent employed in the oxidation step. The solution to this problem was solved by the use of benzhydroxybis(trimethylsilyloxy)silyl group for the protection of the 5'-hydroxyl [253, 254]. The exocyclic amines of adenine and cytosine were protected with dimethoxytrityl and trimethoxytrityl, respectively, whereas  $N^2$ -(9-fluorenylmethoxycarbonyl) or  $N^2$ -trimethoxytrityl groups were used for gua-

nine protection. Thymine was protected with the  $N^3$ -anisoyl group. Oxidation was performed with THF • BH<sub>3</sub>. The method was extended to the synthesis of methylborane phosphine-DNA chimeras which had a good cellular permeability [255].

A second more efficient protocol was achieved by the solid-phase H-phosphonate method followed by the oxidation with borane that was done in one single step after the assembly of the oligonucleotide sequence [256, 257]. The third method was based on the use of boranophosphodiester monomers (Figure 7.7) that were activated with special condensing reagents similar to the phosphotriester approach (Section 1.2.2). In this case, pre-boronated monomer units are used to introduce the borane function avoiding the side reactions that can be caused by the borane reagents [258, 259]. The protecting group of the boranophosphodiester monomer units could be either methyl [258] or 2-cyanoethyl [259] groups. The recommended condensing agents for methyl boranophosphodiester units were *N,N'*-bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP-Cl) [258], or 3-nitro-1,2,4-triazol-1-yl-tris(pyrrolidin-1-yl)phosphonium hexafluorophosphate (PyNTP) or a new reagent, 1,3-dimethyl-2-(3-nitro-1,2,4-triazol-1-yl)-2-pyrrolidin-1-yl-1,3,2-diazaphopholidinium hexafluoro-phosphate (MNTP) that was developed for the coupling of 2-cyanoethyl boranophosphodiester units [259]. In addition, thymine and guanine were protected in their 4 and 6 positions with benzoyl and diphenylcarbamoyl groups (Chapter 1, Section 1.5). In a variation of the method the exocyclic amino groups were protected with 4-azidobenzyl group that was removed with methyldiphenylphosphine [260].

The fourth method uses nucleoside *H*-boranephosphonate as monomer units [261, 262] and, BOP-Cl [261] or MNTP [262] as condensing agents. The *H*-boranephosphonate diester intermediate can be oxidized to boranophosphate linkages [262] but also to boranophosphorothioate and boranophosphoramidite linkages [263]. This method was also extended to the synthesis of locked nucleic acid (LNA)-modified boranephosphate oligonucleotides [263].

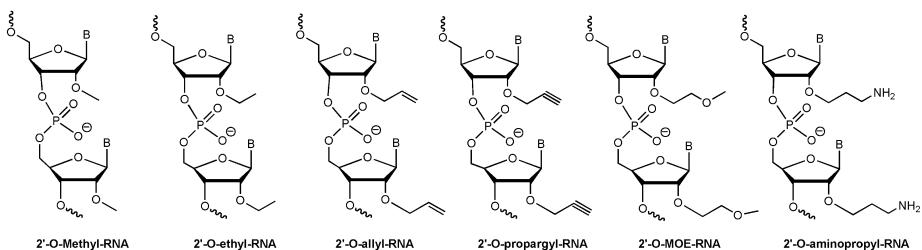
Oligonucleoside boranophosphates are chiral centers so each of these modified linkages produced a mixture of diastereoisomers. Some authors have reported methods for the stereocontrolled synthesis of small oligonucleoside boranophosphates [264–266].

Boranophosphates-modified siRNA duplexes [267] and single-stranded siRNA [268] have been synthesized and evaluated in model cellular systems. Both siRNA duplexes and single-stranded molecules were able to induce the desired inhibition of gene expression with higher potency than unmodified siRNA [267, 268].

An interesting property of oligonucleoside boranophosphates is the capacity of reducing metallic ions to generate silver, gold and platinum nanoparticles [269, 270]. The boranophosphate DNA-mediated metallization process has been demonstrated on single-walled carbon nanotubes generating novel hybrid nanomaterials with interesting applications in nanoelectronics and catalysis [270].

### 7.3 Oligonucleotide carrying 2'-O-alkyl-RNAs

The introduction of alkyl groups at the 2'-hydroxyl group of oligoribonucleotides (Figure 7.8) yields RNA analogues that are resistant to cleavage by alkali and ribonucleases. Most of the therapeutic oligonucleotides carry 2'-O-alkyl-RNAs units specially 2'-O-methyl and 2'-O-methoxyethyl (MOE) residues (Table 7.1). 2'-O-Methyl-RNA modifications are naturally occurring [271] however; their synthesis was first reported by Inoue *et al.* using the phosphotriester methodology [272]. Later, the synthesis of the corresponding 2'-O-methyl-RNA phosphoramidites was reported [273–276]. These reagents can be used for the assembly of oligonucleotides following phosphoramidite protocols similar to those employed for DNA synthesis. Sometimes longer coupling times and more efficient activators are used [276] to compensate for the slower coupling rate due to the bulkiness of the groups at 2' position. Other 2'-O-alkyl-RNA derivatives such as ethyl [277, 278], allyl [279, 280], propargyl [281] and methoxyethyl (MOE) [282, 283] and aminopropyl [284] have been developed for antisense studies being MOE derivatives the most popular due to the stronger stability to nuclease degradation and the beneficial pharmacokinetic properties [285]. The approved drugs Macugen, Onpatro, Givlaari and the drug in phase 3 Inclisiran contain 2'-O-methyl-RNA modifications and the 2'-MOE are present in the approved drugs Kynamro, Spinraza, Tegsedi and Waylivra (Table 7.1).



**Figure 7.8:** Chemical structures of oligonucleotides carrying 2'-O-alkyl-RNAs.

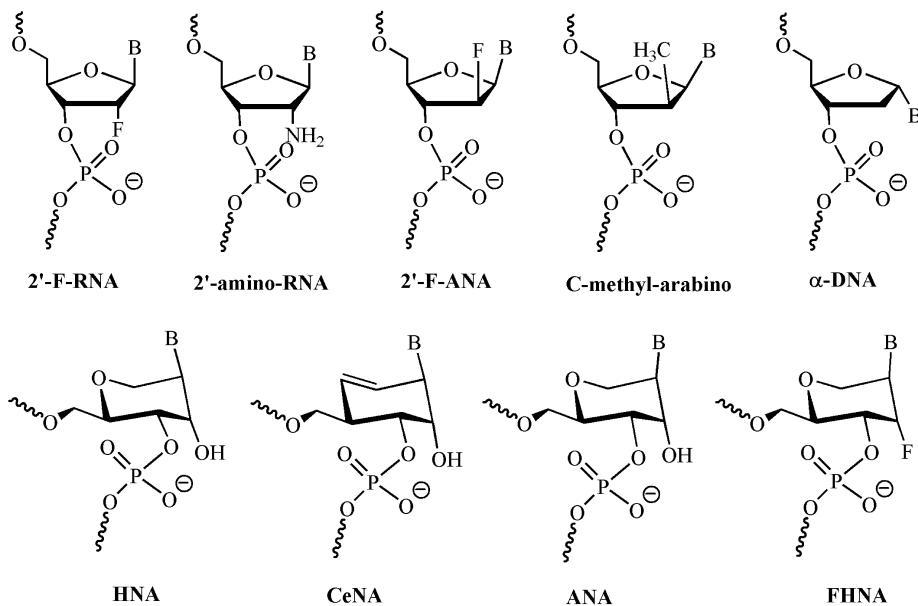
Most of the 2'-O-alkyl-RNA molecules forms more stable duplexes with complementary RNA than natural RNA [286]. This is especially relevant in the alkyl derivatives that have electron withdrawing groups at the  $\beta$  or  $\gamma$  position (allyl, propargyl and MOE) [286]. Longer alkyl groups such as; butyl, pentyl or octyl increase the stability to nucleases but induce unfavorable homoduplex interactions [281]. A special case is the presence of protonable moieties at the  $\gamma$ -position such as the 3-(*N,N*-dimethylamino)propyl group which maintained a good stability towards nucleases and interesting duplex stabilization properties [287].

Most importantly, 2'-O-alkyl-RNA:RNA duplexes are strongly stabilized while 2'-O-alkyl-RNA:DNA are not. This unique combination of chemical and biological

stability and efficient hybridization makes the 2'-O-alkyl RNA good candidates for antisense studies and for some biochemical applications such as purification or depletion of RNA-protein complexes [273, 288]. 2'-O-Alkyl RNA analogues were also used for the preparation of more stable ribozymes [289] and aptamers [290]. 2-O-alkyl-RNAs are frequently used in the gapmer technology (Section 7.1.1.1) [291]. Recently, two novel 2'-O-RNA modifications, 2'-O-benzyl, and 2'-O-methyl-4-pyridine have been introduced to the guide strand of siRNA showing increased inhibition potency and duration compared to unmodified siRNA [70, 292].

## 7.4 Oligonucleotides carrying modified carbohydrates

A large research effort has been devoted to the search for modified oligonucleotides carrying chemically-modified carbohydrates able to hybridize with complementary RNA but being nuclease-resistant. From the large number of reported modified carbohydrates, we selected for their relevance the following derivatives: 2'-amino-RNA nucleosides, 2'-fluoro-RNA nucleosides (F-RNA), 2'-fluoroarabino nucleosides (2'F-ANA), 2'-methylarabino nucleosides, hexitols such as hexitol nucleosides (HNA), cyclohexene nucleosides (CeNA), altritol nucleosides (ANA) and 3'-fluoro hexitol nucleosides (FHNA), as well as  $\alpha$ -nucleosides (Figure 7.9).



**Figure 7.9:** Chemical structures of oligonucleotides carrying modified carbohydrates.

2'-amino- and 2'-fluoro-RNA nucleosides are analogues in which the 2'-hydroxyl functions were replaced by amino or fluoro groups [44, 293]. These derivatives have been extensively used for the modification of aptamers [294] and in the elucidation of the mechanism of action of ribozymes [295, 296] as well as to generate nuclease resistant antisense oligonucleotides and ribozymes [297]. The clinical drugs Macugen, Givlaari and Inclisiran contain 2'-fluoro-RNA modifications (Table 7.1). In a detailed study comparing 24 substitutions at the 2' position, the presence of a 2'-fluoro substituent was the most duplex stabilizing modification [286]. Some of these modified nucleosides can be converted to 5-triphosphate nucleoside derivatives which are recognized by T7 RNA polymerases and be used for enzymatic synthesis of modified RNA with enhanced nuclease resistance [298].

Arabino (ANA) and 2'-fluoroarabino nucleosides (2'F-ANA) were considered interesting alternatives after investigation that the oxygen or fluoro group at the 2' *arabino* position will drive the *N*-to *S*- equilibrium of the sugar to the *S*- form enforcing a topology similar to the one present in native DNA/RNA duplexes [299, 300]. This hypothesis was confirmed as ANA and 2'F-ANA modified oligonucleotides elicit RNase H-mediated hydrolysis of target RNA, maintaining adequate hydrolytic stability [299, 300]. The synthesis of the ANA and 2'F-ANA phosphoramidites has been described [300, 301]. The solid-phase assembly of the modified oligonucleotides proceeds in similar yields than standard phosphoramidites. Sulfurization of phosphite triester intermediates can be performed with Beaucage's reagent (Section 1.8.2) generating 2'F-ANA-oligonucleoside phosphorothioates [300]. 2'F-ANA modified oligonucleotides are extensively analyzed in preclinical studies especially as antisense [302–304], and antimiRNA [305] oligonucleotides.

The synthesis and silencing properties of siRNA duplexes carrying 2'-C-methyl-ribo and arabino nucleosides were reported [306]. Best silencing activities were obtained when the modifications were located in the antisense 3'-overhang region. Modifications in positions 5 and 6 of the seed region had a positive effect on the selectivity avoiding potential off-target effects [306].

One of the most studied carbohydrate modifications is the substitution of the five member ring of ribose for a six member ring carbohydrates. Pyranose nucleic acids were first prepared to elucidate why pentoses instead of hexoses were selected for building DNA and RNA [307]. After 30 years of studies on the structural and biochemical properties of hexose nucleic acids, several of them have been developed as convenient modifications for antisense and siRNA oligonucleotides [308, 309]. The most successful hexoses modifications were 2,3-dideoxy-D-*arabino*-hexitol nucleic acids (HNA) [310], cyclohexene nucleic acids (CeNA) [311], D-altritol nucleosides (ANA) [312, 313] and 3'-fluoro hexitol nucleic acid (FHNA) [314]. Most of these modifications are being used to enhance nuclease resistance as well as to improve gene inhibitory activity and decrease toxicity [315, 316]. Recently, the siRNA hexopyranose ANA modification has been introduced in the Patisiran (Table 7.1) siRNA sequence to silence the transthyretin gene in conjunction with the trivalent *N*-acetylgalactosamine (GalNAc)

ligand for targeted delivery to hepatocytes [317]. Although, ANA residues were detrimental at the 5' end of the antisense strand, the siRNAs with ANA at position 6 or 7 in the seed region recover the activity found for Patisiran. The siRNA with ANA at position 7 in the seed region had an interesting activity in a mouse model [317].

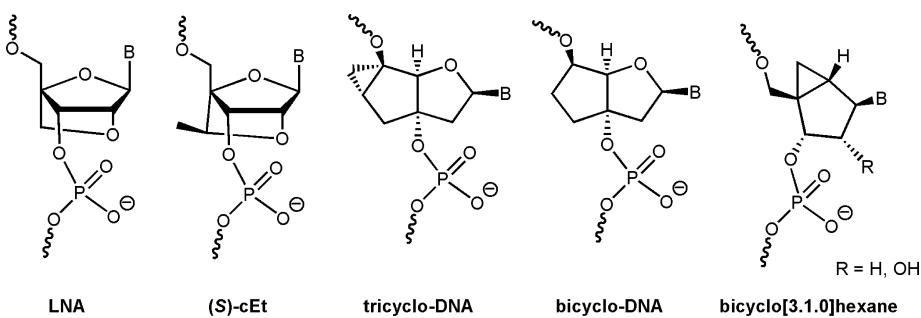
Nucleotide units in DNA and RNA are characterized by a  $\beta$  configuration at the anomeric center of the sugar moiety. The anomeric  $\alpha$ -nucleosides can be synthesized and the appropriate phosphoramidite has been reported [318, 319]. Oligodeoxyribonucleotides [318, 319] and oligoribonucleotides [320] can be prepared by phosphoramidite chemistry similar to standard oligonucleotides showing enhanced nuclease stability relative to the natural DNA and RNA. They bind tightly to complementary RNA to form parallel-stranded hybrids that were not substrates for RNase H [321, 322]. The inhibition of the translation initiation via RNase-H independent mechanism has been demonstrated [323]. Oligodeoxyribonucleotides carrying  $\alpha$ -nucleotides have interesting triplex-stabilizing properties that have been described in Chapter 5, Section 5.7.

As mentioned in Section 7.1.3 oligonucleotides carrying the mirror image L-nucleotides (Spiegelmers) are being developed to obtain nuclease resistant aptamers with a longer active life in serum [103, 104].

## 7.5 Oligonucleotides carrying conformationally restricted sugars

The sugar-phosphate backbone of DNA is highly flexible. The two most stable conformations of the furanose ring are the *C3'-endo (North)* and the *C2'-endo (South)* forms. These conformations have similar stability and the energy barrier separating them is low. Novel nucleosides have been developed that stabilize the *North* conformation as these compounds form more stable RNA:DNA duplexes. Some of the hexitol derivatives and *arabino* derivatives mentioned in the previous section have restricted conformation in the desired *North* form. The most relevant conformationally restricted nucleoside derivatives are shown in Figure 7.10 including 2'-*O*, 4'-C-methylene- $\beta$ -D-ribofuranosyl nucleosides (locked nucleic acids, LNA) [324, 325]; constrained ethyl (CEt) [326], *bicyclo*-DNA [327], *tricyclo*-DNA [328] and *North bicyclo[3.1.0]hexane pseudonucleosides* [329, 330].

LNA (Figure 7.10) is the most studied derivative of this group. It can be considered an RNA derivative in which the ribose moiety is blocked by an oxymethylene bridge connecting the 2' and 4' positions, locking the conformation of the sugar to the northern conformation [324]. Several properties such as extremely high binding affinity to target RNA, improved mismatch discrimination, and high stability in serum make these derivatives very important as diagnostic probes and for therapeutic applications [331]. LNAs have been profusely employed for miRNA detection [332] but also



**Figure 7.10:** Chemical structures of oligonucleotides carrying conformationally restricted sugars.

they are very important nucleoside derivatives for the modification of antisense [333] and siRNA oligonucleotides [334] due to their high binding affinity. Fully substituted LNAs have a trend to form homoduplexes due to the large stability of LNA:LNA duplexes and for this reason most of the oligonucleotides carrying LNA modifications contain one LNA for every 2–3 nucleotides [335]. LNAs have also been used in the design of improved DNAzymes (LNAzymes, [336]), aptamers (LNA aptamers, [337]) and triplex forming oligonucleotides [338] as described in Chapter 5, Section 5.7. However, the LNAs hold strong therapeutic potential in the development of locked nucleic acids-based anti-miRs [84] with several compounds in clinical trials such as Maravirsen (Table 7.1) and the recently described antimiR-92a for cardiovascular diseases [339].

Constrained 2'-*O*-ethyl (cEt) (Figure 7.10) modified oligonucleotides [326] have similar enhanced binding affinity than LNAs [340, 341] however, the oligonucleotides having cEt showed enhanced stability to exonuclease digestion [340] and improved potency *in vivo* [342]. These modifications and some variations of them have been used to modify antisense oligonucleotides in preclinical studies with interesting results in animal models [343–345].

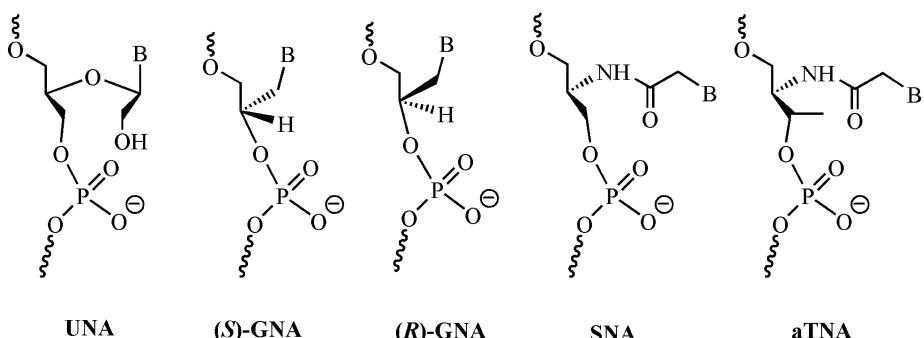
*Bicyclo-DNA* [327, 346] and *tricyclo-DNA* [328, 347] oligonucleotides (Figure 7.10) are two important nucleoside derivatives developed by the group of Leumann. These conformationally restricted nucleosides bind to their complementary DNA and RNA forming double and triple helices with slightly higher affinity than natural oligonucleotides. Moreover, bicyclo- and tricyclo-oligonucleotides are around one order of magnitude more stable to degradation by exonucleases than natural oligonucleotides. Tricyclo-DNA carrying phosphorothioate linkages can be transfected with lipofectamine but they are also active in the absence of lipofectamine in cell culture experiments seeking the correction of aberrant splicing by exon-skipping [348]. These interesting pharmacological properties as well as the unprecedented cellular uptake properties by many tissues after systemic administration triggered the development of tricyclo-DNA oligonucleotide for the treatment of Duchenne muscular dystrophy using exon-skipping [59, 60].

The effect of bicyclo-[3.1.0]hexane (methanocarba) 2'-deoxyribo- [329] and ribo [330]-pseudosugars (Figure 7.10) on RNA interference activity has been reported. The incorporation of these modifications into siRNA duplexes increased their thermal stabilities, substantially enhanced serum stabilities, and decreased innate immunostimulation. Gene-silencing activities of methanocarba-modified siRNAs were comparable to those obtained with the LNA modification [329]. In addition, *North* and *South* bicyclo-[3.1.0]hexane 2'-deoxyribo-pseudonucleosides were described as interesting tools for structural analysis of G-quadruplex as explained in Chapter 6, Section 6.1.2 [349].

## 7.6 Oligonucleotides carrying acyclic derivatives

Unlocked nucleic acid or 2',3'-seco-RNA is an acyclic RNA analogue in which the bond between the C2' and C3' atoms of the ribose ring was removed (UNA, Figure 7.11) [350]. These derivatives, instead of having a frozen conformation, are very flexible providing a distinct structural feature to modulate structural parameters on therapeutic oligonucleotides such as siRNA [351] and aptamers [352]. This flexibility can be beneficial at a certain position of siRNA as found during the screening of the impact of modified nucleosides in the off-target effects within the seed region of siRNA [353]. In this study, UNA incorporation in position 7 of the siRNA was found to be the most potent modification to reduce off-target effects without affecting the inhibitory properties of the UNA-modified siRNA [354]. Thermodynamic analysis of the RNA duplexes carrying single UNA-modifications shows a clear decrease in the stability when the UNA modification is placed at the central positions but only a small decrease when UNA modification is near the 3' or 5'-ends [354]. Moreover, UNA modified-siRNAs showed a dramatic increase in serum stability in mice inducing potent gene inhibition in xenograft model human pancreatic cancer [355]. UNA is also able to modulate the thermodynamic properties of several DNA structures including quadruplexes, triplexes or i-motif as explained in Chapter 6, Sections 6.1.2 and 6.2.2 [356, 357].

Glycerol nucleic acids (GNA, Figure 7.11) are the simplest acyclic nucleosides that form stable Watson–Crick antiparallel duplexes [358–360]. Both (R)- and (S)- enantiomers can be prepared and both form stable duplexes with good thermodynamic properties [361, 362]. The synthesis of the corresponding phosphoramidites has been described [363–365]. GNA has been used for the preparation of nucleic acid nanostructures. A 4-helix junction (4HJ) [366] mimicking a DNA Holliday junction was prepared [367]. Because the GNA backbone contains only one stereocenter per repeating unit, it was possible to synthesize two mirror-image nanostructures using S- and R-GNA that were more stable than the corresponding unmodified DNA nanostructure. The flexible GNA modifications have also been used as flexible linkers in the study of novel glucose-DNA base pairs [368, 369] and in the loops of model G-quadruplex es (Chapter 6, Section 6.1.3). Recently, a GNA nucleotide located in the seed region was demon-



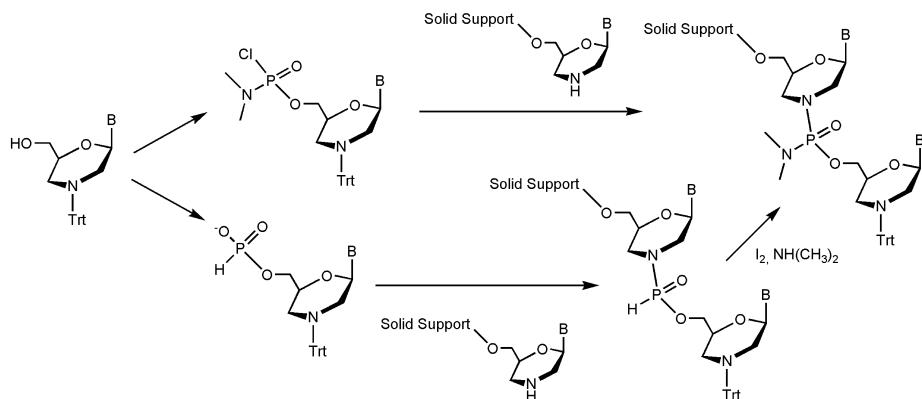
**Figure 7.11:** Chemical structures of oligonucleotides carrying acyclic derivatives.

strated to mitigate hepatotoxicity while maintaining comparable siRNA levels in total liver [370]. In addition, a siRNA with (S)-GNA in the seed region resulted in greater *in vitro* potencies over identical sequences containing (R)-GNA [371].

Acyclic Threoninol Nucleic Acid (aTNA, Figure 7.11) and Serinol Nucleic Acids (SNA, Figure 7.11) are acyclic nucleic acids bearing threoninol (2-amino-1,3-butanediol) or serinol (2-amino-1,3-propanediol) backbones tethered to one of the natural nucleobases (Figure 7.11). These oligomers are characterized by a more flexible scaffold than the natural DNA/RNA, and they form stable homoduplex in an antiparallel manner and right-handed structure as well as DNA/RNA heteroduplex [372–374]. Serinol has a chiral center generating two stereoisomers (D-, L-serinol) and threoninol has two chiral centers generating four stereoisomers (D-, L-threoninol and *allo*-D- and *allo*-L-threoninol). Each one of them is available in optically pure form. siRNA duplexes carrying L-aTNA [375, 376] and (R)-SNA [377] have demonstrated that these modifications are compatible with the RNA interference mechanisms increasing the stability of the siRNA duplexes toward degradation by nucleases. Furthermore, flexibility may be important at certain positions of siRNA to improve biological activity especially at the 3'-terminal position because of favorable interactions with the PAZ domain of the RNA-interference silencing complex (RISC) [378]. Moreover, antisense oligonucleotide modified with serinol backbones were shown to elicit exon skipping activity in *in vitro* cellular model of Duchenne muscular dystrophy [379].

## 7.7 Phosphorodiamidate morpholino oligomers (PMO)

Phosphorodiamidate morpholino oligomers or morpholino antisense oligomers (PMO, Figure 7.12) are unique synthetic antisense oligonucleotides that have a morpholino ring instead of the ribose sugar and a neutral phosphorodiamidate backbone

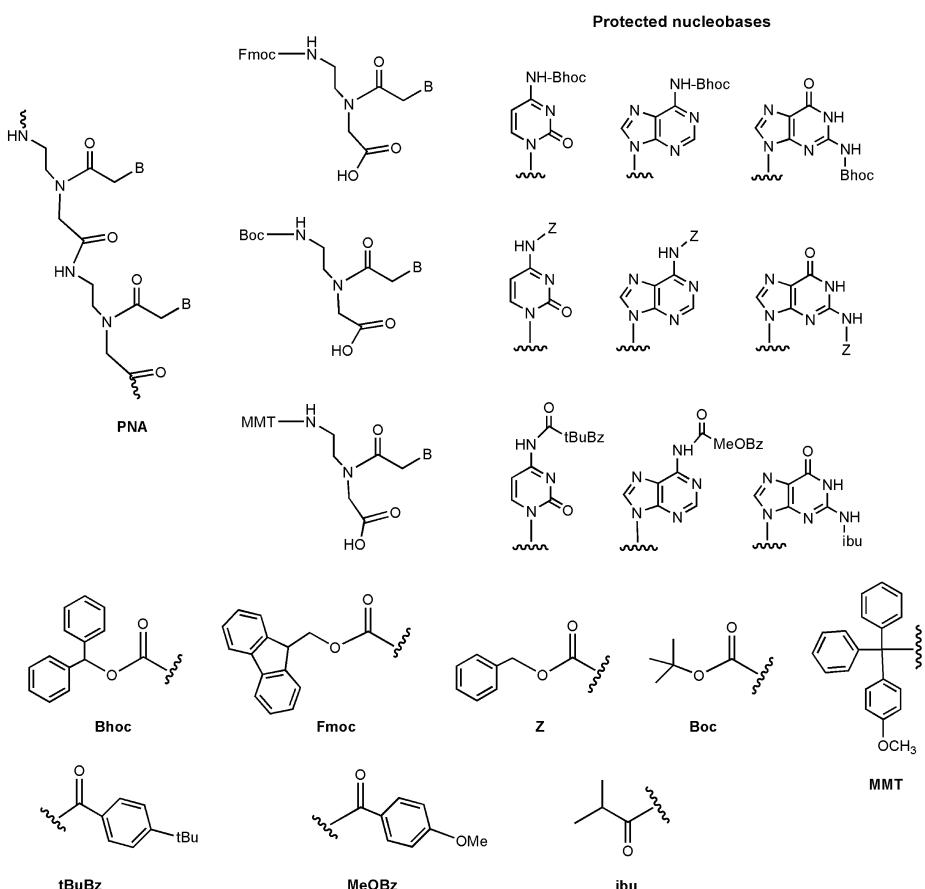


**Figure 7.12:** Synthesis of phosphorodiamidate oligomers (PMO).

[57, 380–382]. The synthesis of these oligomers can be done by solid-phase synthesis protocols either by the use of chlorophosphoramidite activated monomers [383] or using the appropriate H-phosphonate intermediates [384] as shown in Figure 7.12. Several derivatives of PMO including morpholino derivatives with an anionic backbone [385] or nucleobase pending cationic groups [386] have been reported. The presence of a chiral center in the phosphorodiamidite linkage generates a mixture of diastereoisomers. The mechanism of action of PMOs for gene silencing is significantly different than antisense DNA or siRNA agents. Rather than degrading the target mRNA enzymatically by RNase H, PMOs act as steric blockers, which bind to the target mRNA sequence and hinders the access to the protein translation machinery [387]. PMOs have been widely used in developmental biology to unravel the specific role of each gene in early embryonic development by loss-of-function analysis [388]. Moreover, PMOs are very effective to modulate pre-mRNA splicing by exon-skipping mechanism and inhibiting miRNA action [389]. PMOs are safe and effective therapeutic drugs for a broad range of diseases including Duchenne muscular dystrophy. Three drugs based in PMO chemistry have been approved for human use: Eteplirsen [54], Golodirsen [58] and Viltepso [56] (Table 7.1). Pyrimidine PMOs can form stable parallel triplets as described in Chapter 5, Section 5.7.

## 7.8 Peptide nucleic acids (PNA)

Peptide Nucleic Acids (Figure 7.13) are DNA analogs in which the natural ribosephosphodiester backbone is replaced by a neutral, nonchiral aminoethylglycine backbone and the nucleobases are linked to the backbone by methylene carbonyl groups [390–392]. PNAs bind tightly to DNA and RNA although the hybrid formed is not a substrate for RNase H. PNA oligomers form also very stable parallel triplets being



**Figure 7.13:** Synthesis of Peptide Nucleic Acids (PNA) showing the most common protection schemes.

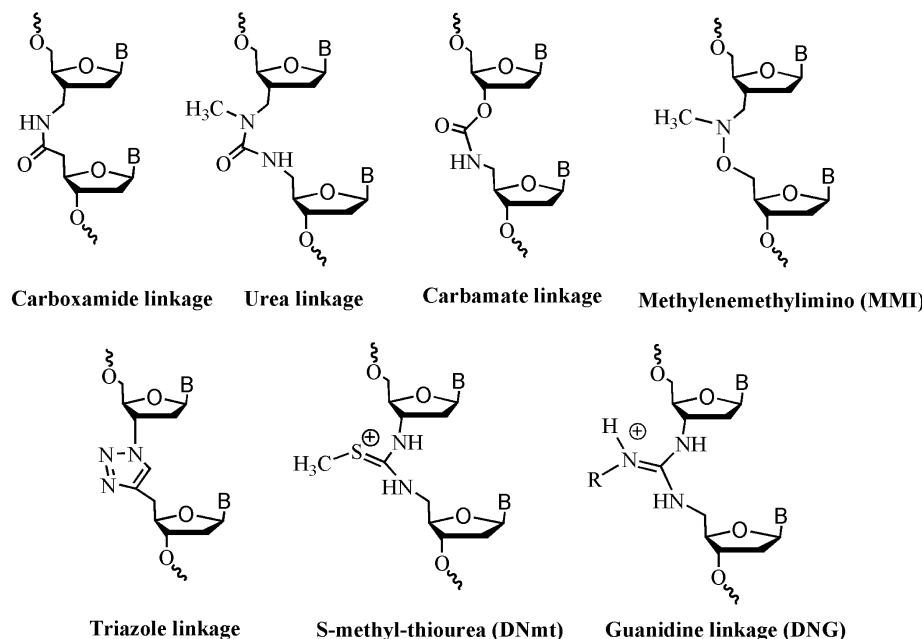
able to displace one duplex strand to form PNA.PNA.DNA triplexes [393] especially in the form of foldback *bis*-PNA probes [394] as described in Chapter 5, Sections 5.7 and 5.9.1.

PNA can be prepared in relatively large amounts using solid-phase peptide synthesis protocols [391, 392, 395]. Two main types of monomers can be used as standard peptide synthesis: the *tert*-butoxycarbonyl (Boc)/benzyloxycarbonyl (Z) strategy [396] or the fluorenylmethoxycarbonyl (Fmoc)/benzhydryloxycarbonyl (Bhoc) [397]. Figure 7.13 shows the chemical structures of the monomers and the protecting groups. A third strategy for PNA synthesis relies on the ammonia-labile protecting groups and the acid labile monomethoxytrityl (MMT) for the amino group [398, 399]. This strategy is compatible with oligonucleotide synthesis protocols and it can be used for the synthesis of DNA-PNA hybrid molecules [400].

PNA have great potential as therapeutic agents, diagnostic tools and probes in molecular biology. There are a large number of applications which use PNA oligomers and a plethora of derivatives that have been designed for specific applications. This activity has been well covered by excellent reviews [391, 392] and it cannot be covered in this chapter. Especially relevant as therapeutic agents are the use of antisense PNA-peptide conjugates as antimicrobial agents to treat multidrug-resistant bacterial strains [401–404].

## 7.9 Nonphosphorus internucleoside linkages

In addition to PNA oligomers several backbones carrying nonphosphorus internucleoside linkages have been reported [405] (Figure 7.14). Replacement of phosphate bonds by neutral or positively charged internucleotide bonds provides important resistance to degradation properties as these bonds are not recognized by nucleases. Additionally, these modified backbones may provide enhanced cellular uptake or increased affinity to complementary nucleic acids targets due to the removal of the negatively charged phosphate backbone. Some of these modified backbones have been explained in Chapter 5, Section 5.7 as they can stabilize triplex structures.



**Figure 7.14:** Chemical structures of oligonucleotides carrying non-phosphorus internucleoside linkages.

Short oligonucleotides carrying carboxamido linkages such as carboxymethyl bonds were between the first modified oligonucleotides to be prepared [404] and to show some degree of interference with the nucleic acids functions [30]. These compounds were prepared mostly at the dinucleotide level [30, 406, 407]. Dimeric phosphoramidite building blocks carrying carboxamido linkages were reported for the synthesis mixed phosphodiester-carboxamido backbone oligonucleotides [408, 409]. Carbamate linkages were also between the first modified oligonucleotides reported in the bibliography [30]. However, their synthesis was further optimized [410–412]. Additionally, dimeric phosphoramidite building blocks carrying carbamate and urea (Figure 7.14) linkages have also been reported [413, 414] including dimers carrying LNA units in order to increase their binding properties [415].

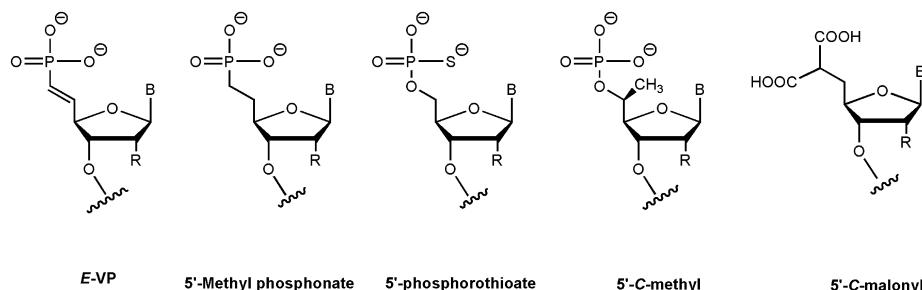
Modified backbones including oxime functions such as methylenemethylimino (MMI) [416] or methyleneoxy(methylimino) (MOMI) [417] linkages were reported. These linkages are achiral, neutral and can be incorporated in phosphorothioate oligonucleotides with an improvement of their pharmacological properties [417].

The replacement of the phosphodiester bond by neutral heterocyclic compounds such as imidazole and triazole was also reported [418]. Especially relevant was the use of triazole linkages that can be prepared by the azide-alkyne cycloaddition (click chemistry) [418–421]. Triazolyl-functionalized oligonucleotides were prepared by using triazolyl-dinucleoside phosphoramidites [419] or by click reaction between azido- and alkynyl-oligonucleotides [420]. Triazolyl-oligonucleotides can achieve DNA binding affinities similar to those of unmodified oligonucleotides and they can be used as nuclease resistant PCR-primers [421].

The incorporation of positive charges in oligonucleotides may increase their DNA/RNA-binding properties and their cell-permeation properties. For these reasons there is a large interest in the positively-charged linkages [422]. This interest has crystallized in the development of S-methylthiourea (DNmt) linkages [423–425] and the deoxynucleic Guanidine (DNG) linkages [426–430] (Figure 7.14). Short oligonucleotides carrying several DNmt linkages [424, 425] and DNmt/DNA chimeras [423] have been prepared demonstrating excellent DNA-binding and DNA-discrimination properties as well as nuclease resistance. The first synthesis of a pentathymidine oligomer carrying DNG linkages was done in solution by condensation of 3'-aminonucleosides with 5'-isothiocyanatenucleosides resulting in thiourea formation that was oxidized with peracetic acid at the end of the assembly process [426, 427]. Next, building blocks for solid-phase synthesis were developed including all natural nucleobases [428–430]. Positively charged DNGs were demonstrated to bind telomeric RNA sequences with very high affinity [431]. The combination of positively charged DNGs and neutral PNA chimeras provided an excellent combination of modified backbones demonstrating sequence specific and faster strand invasion properties. The DNG/PNA chimeras may extend the potential utility of PNA in diagnostics, biomolecular probes, and anti-sense/antigene therapeutics [432].

## 7.10 Modification of the 5'-position of siRNA

Recent advances in siRNA field paid special attention to the development of RNA modifications to improve potency and selectivity avoiding undesired off-target effects [73, 75]. The first nucleotide at the 5'-end of the guide strand needs to be phosphorylated for strand loading and proper Ago2-mediated cleavage [433]. Chemical phosphorylation of the 5'-end of oligonucleotides can be efficiently achieved using specially designed phosphoramidites [434–436]. Blocking phosphorylation of the 5'-hydroxyl by chemical modification of the 5'-ribose of the guide strand can interfere with intracellular phosphorylation; however, the activity of these 5'-modified guide strands can be restored if a 5'-phosphate is introduced chemically [292]. Unfortunately, the 5'-phosphate can be removed by cellular phosphatases, resulting in an accumulation of biologically inactive siRNAs. Phosphatase-resistant analogues of the 5'-phosphate such as; 5'-(E)-vinylphosphate [437–440], 5'-methyl phosphonate [441], 5'-C-methyl analog [441] and 5'-phosphorothioate [441] (Figure 7.15) have been reported. Chemically introduced, metabolically stable 5'-phosphate mimics lead to higher metabolic stability, increased RISC loading, and higher gene silencing activities of chemically modified siRNAs [437–441]. Moreover, 5'-(E)-vinylphosphate improves tissue accumulation and efficacy of lipid-conjugated siRNAs *in vivo* [442, 443].



**Figure 7.15:** Chemical structures of the modifications described for the protection of the 5'-position of siRNA.

Recently, a 5'-C-malonyl-modified nucleotide was incorporated at the 5'-terminus of chemically modified RNA oligonucleotides [444]. The 5'-C-malonyl siRNAs showed improved *in vitro* gene silencing and high levels of Ago2 loading providing a dramatically improved metabolic stability to the antisense strand of the siRNA duplexes [444].

## Bibliography

- [1] Murray JAH. Antisense RNA and DNA. *Modern Cell Biology Series*. vol. 11. New York: John Wiley & Sons Inc Publ; 1992.
- [2] Crooke ST, Lebleu B. Antisense research and applications. Boca Raton, Florida: CRC Press Inc.; 1993.
- [3] Agrawal S. Protocols for oligonucleotides and analogs. *Synthesis and properties. Methods in Mol Biol.* vol. 20. Totowa, NJ: Humana Press Inc.; 1993.
- [4] Agrawal S. Protocols for oligonucleotide conjugates. *Synthesis and analytical techniques. Methods in Mol Biol.* vol. 26. Totowa, NJ: Humana Press Inc.; 1994.
- [5] Kurreck J. Therapeutic oligonucleotides. Oxford: RSC Publishing; 2008.
- [6] Goodchild J. Therapeutic oligonucleotides. *Methods and protocols. Meth Mol Biol Series.* vol. 764. New York: Springer; 2011.
- [7] Nimesh S, Chandra R, Gupta N. *Advances in Nanomedicine for the Delivery of Therapeutic Nucleic Acids*. Woodhead Publishing; 2017.
- [8] Agrawal S, Gait MJ. *Advances in nucleic acid therapeutics. Drug Discovery Series.* vol. 68. Oxford: RSC Publishing; 2019.
- [9] Obika S, Sekine M. *Synthesis of therapeutic oligonucleotides*. Singapore: Springer; 2018.
- [10] Gissberg O, Zain R, Lundin KE. *Oligonucleotide-based therapies. Methods and protocols. Meth Mol Biol.* vol. 2036. Springer; 2019.
- [11] Goodchild J. Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. *Bioconjug Chem.* 1990;1:165–86.
- [12] Uhlmann E, Peymann A. Antisense oligonucleotides: a new therapeutic principle. *Chem Rev.* 1990;90:544–84.
- [13] Beaucage SL, Iyer RP. The synthesis of modified oligonucleotides by the phosphoramidite approach and their applications. *Tetrahedron.* 1993;49:6123–94.
- [14] Braasch DA, Corey DR. Novel antisense and peptide nucleic acid strategies for controlling gene expression. *Biochemistry.* 2002;41:4503–10.
- [15] Urban E, Noe CR. Structural modifications of antisense oligonucleotides. *Il Farmaco.* 2003;58:243–58.
- [16] Corey DC. Chemical modification: the key to clinical application of RNA interference? *J Clin Invest.* 2007;117:3165–22.
- [17] Oh YK, Park TG. siRNA delivery systems for cancer treatment. *Adv Drug Deliv Rev.* 2009;61:850–62.
- [18] Winkler J. Oligonucleotide conjugates for therapeutic applications. *Ther Deliv.* 2013;4:791–809.
- [19] Sharma VK, Sharma RK, Singh SK. Antisense oligonucleotides: modifications and clinical trials. *Med Chem Commun.* 2014;5:1454–71.
- [20] Lundin KE, Gissberg O, Smith CIE. Oligonucleotide therapies: the past and the present. *Hum Gene Ther.* 2015;26:475–85.
- [21] Geary RS, Norris D, Yu R, Bennett CF. Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides. *Adv Drug Deliv Rev.* 2015;87:46–51.
- [22] Juliano RL. The delivery of therapeutic oligonucleotides. *Nucleic Acids Res.* 2016;44:6518–48.
- [23] Crooke ST. Molecular mechanism of antisense oligonucleotides. *Nucleic Acids Ther.* 2017;27:70–7.
- [24] Crooke ST, Wang S, Vickers TA, Shen W, Liang XH. Cellular uptake and trafficking of antisense oligonucleotides. *Nat Biotechnol.* 2017;35:230–7.

- [25] Khvorova A, Watts JK. The chemical evolution of oligonucleotide therapies of clinical utility. *Nat Biotechnol.* 2017;35:238–48.
- [26] Chakraborty C, Sharma AR, Sharma G, Doss GP, Lee SS. Therapeutic miRNA and siRNA moving from bench to clinic as next generation on medicine. *Mol Ther Nucleic Acids.* 2017;8:132–43.
- [27] Grijalvo S, Alagia A, Jorge AF, Eritja R. Covalent strategies for targeting messenger and non-coding RNAs. An updated review on siRNA, miRNA and antimiRNA conjugates. *Genes.* 2018;9:74.
- [28] Yin W, Rogge M. Targeting RNA: a transformative therapeutic strategy. *Clin Transl Sci.* 2019;12:98–112.
- [29] Smith CIE, Zain R. Therapeutic oligonucleotides: state of the art. *Annu Rev Pharmacol Toxicol.* 2019;59:605–30.
- [30] Gait MJ, Jones AS, Walker RT. Synthetic analogues of polynucleotides. Part XII. Synthesis of thymidine derivatives containing an oxyacetamido- or an oxyformamidino-linkage instead of a phosphodiester group. *J Chem Soc Perkin I.* 1974;1684–6.
- [31] Green PJ, Pines O, Inouye M. The role of antisense RNA in gene regulation. *Annu Rev Biochem.* 1986;55:569–97.
- [32] Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci.* 1978;75:280–4.
- [33] Stephenson ML, Zamecnik PC. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci.* 1978;75:285–8.
- [34] Dias N, Stein C. Antisense oligonucleotides: basic concepts and mechanisms. *Mol Cancer Ther.* 2002;1:347–55.
- [35] Kurreck J. Antisense technologies: improvement through novel chemical modifications. *Eur J Biochem.* 2003;270:1628–44.
- [36] Eckstein F. Nucleoside phosphorothioates. *J Am Chem Soc.* 1970;92:4718–23.
- [37] Eckstein F. Phosphorothioates, essential components of therapeutic oligonucleotides. *Nucleic Acids Ther.* 2014;24:374–87.
- [38] de Smet MD, Meenken C, Van Den Horn GJ. Fomivirsen – a phosphorothioate oligonucleotide for the treatment of CMV retinitis. *Ocular Immunol Inflamm.* 1999;7:189–98.
- [39] Shibahara S, Mukai S, Morisawa H, Nakashima H, Kobayashi S, Yamamoto N. Inhibition of human immunodeficiency virus (HIV-1) replication by synthetic oligo-RNA derivatives. *Nucleic Acids Res.* 1989;17:239–52.
- [40] Wu H, Lima WF, Zhang H, Fan A, Sun H, Crooke ST. Determination of the role of the human RNase H1 in the pharmacology of DNA-like antisense drugs. *J Biol Chem.* 2004;279:17181–9.
- [41] Bennett CF, Swayze EE. RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu Rev Pharmacol Toxicol.* 2010;50:259–93.
- [42] Cerritelli SM, Crouch RJ. Ribonuclease H: the enzymes in eukaryotes. *FASEB J.* 2009;276:1494–505.
- [43] Agrawal S, Kandimalla ER. Antisense therapeutics: is it as simple as complementary base recognition? *Mol Med Today.* 2000;6:72–81.
- [44] Manoharan M. 2'-Carbohydrate modifications in antisense oligonucleotide therapy: importance of conformation, configuration and conjugation. *Biochim Biophys Acta.* 1999;1489:117–30.
- [45] Agrawal S, Gewirtz AM. Oligonucleotide therapeutics for hematologic disorders. *Biochim Biophys Acta.* 1999;1489:85–96.
- [46] Aboul-Fadl T. Antisense oligonucleotides: the state of the art. *Curr Med Chem.* 2005;12:763–71.

- [47] Raal FJ, Santos RD, Bloom DJ, Marais AD, Charng MJ, Cromwell WC, Lachmann RH, Gaudet D, Tan JL, Chasan-Taber S, Tribble DL, Flaim JAD, Crooke ST. Mipomersen, an apolipoprotein B synthesis inhibitor, for lowering of LDL cholesterol concentration in patients with homozygous familial hypercholesterolemia: a randomized, double-bind, placebo-controlled trial. *Lancet*. 2010;375:998–1006.
- [48] Ajero E, Rader DJ. New therapeutic approaches for familial hypercholesterolemia. *Annu Rev Med*. 2018;69:113–31.
- [49] Witztum JL, Gaudet D, Freedman SD, Alexander VJ, Digenio A, Williams KR, Yang Q, Hughes SG, Geary RS, Arca M, Stroes ESG, Bergeron J, Soran H, Civeira F, Hemphill L, Tsimikas S, Blom DJ, O’Dea L, Bruckert E. Volanesorsen and triglyceride levels in familial chylomicronemia syndrome. *N Engl J Med*. 2019;381:531–42.
- [50] Zamecnik P, Goodchild J, Taguchi Y, Sarin PS. Inhibition of replication and expression of human T-cell lymphotropic virus type III in culture cells by exogenous synthetic oligo-deoxynucleotides complementary to viral RNA. *Proc Natl Acad Sci USA*. 1986;83:4143–6.
- [51] Zaia JA, Rossi JJ, Murakawa GJ, Spallone PA, Stephens DA, Kaplan B, Eritja R, Wallace RB, Cantin EM. Inhibition of human immunodeficiency virus (HIV) using an oligonucleoside-methylphosphonate targeted to the TAT-3 gene. *J Virol*. 1988;62:3914–7.
- [52] Järver P, O’Donovan L, Gait MJ. A chemical view of oligonucleotides for exon skipping and related drug applications. *Nucleic Acids Ther*. 2014;24:37–47.
- [53] Havens MA, Hastings ML. Splice-switching antisense oligonucleotides as therapeutic drugs. *Nucleic Acids Res*. 2016;44:6549–63.
- [54] Charleston JS, Schnell FJ, Dworzak J, Donoghue C, Lewis S, Chen L, Young GD, Milici AJ, Voss J, DeAlwis U, Wentworth B, Rodino-Klapac LR, Sahenk Z, Frank D, Mendell JR. Eteplirsen treatment for Duchenne muscular dystrophy: exon skipping and dystrophin production. *Neurology*. 2018;90:e2146–54.
- [55] Finkel RS, Mercuri E, Darras BT, Connolly AM, Kuntz NL, Kirschner J, Chiriboga CA, Saito K, Servais L, Tizzano E, Topaloglu H, Tulinius M. Nusinersen versus Sham control in infantile-onset spinal muscular atrophy. *N Engl J Med*. 2017;377:1723–32.
- [56] Dhillon, Viltolarsen S. First approval. *Drugs*. 2020;80:1027–31.
- [57] Summerton J. Morpholino antisense oligomers; the case for an RNAase H-independent structural type. *Biochim Biophys Acta*. 1999;1489:141–58.
- [58] Aartsma-Rus A, Corey DR. The 10th oligonucleotide therapy approved: Golodirsen for Duchenne Muscular Dystrophy. *Nucleic Acids Ther*. 2020;30:67–70.
- [59] Goyenvalle A, Griffith G, Babbs A, El Andaloussi S, Ezzat K, Avril A, Dugovic B, Chaussenot R, Ferry A, Voit T, Amthor H, Bühr C, Schürch S, Wood MJA, Davies KE, Vaillend C, Leumann C, García L. Functional correction in mouse models of muscular dystrophy using exon-skipping tricyclo-DNA oligomers. *Nat Med*. 2015;21:270–5.
- [60] Relizani K, Griffith G, Echevarría L, Zarrouki F, Facchinetto P, Vaillend C, Leumann C, García L, Goyenvalle A. Efficacy and safety profile of tricyclo-DNA antisense oligonucleotides in Duchenne muscular dystrophy mouse model. *Mol Ther Nucleic Acids*. 2017;8:144–57.
- [61] Kim J, Hu C, El Achkar CM, Black LE, Douville J, Larson A, Pendergast MK, Goldkind SF, Lee EA, Kuniholm A, Soucy A, Vaze J et al. Patient-customized oligonucleotide therapy for a rare genetic disease. *N Engl J Med*. 2019;381:1644–52.
- [62] Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998;391:806–11.
- [63] Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*. 2001;411:494–8.

- [64] Ha M, Kim VN. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol.* 2014;15:509–24.
- [65] Wilson RC, Tambe A, Kidwell MA, Noland CL, Schneider CP, Doudna JA. TRBP-complex formation ensures accurate mammalian microRNA biogenesis. *Mol Cell.* 2015;57:397–407.
- [66] Schirle NT, MacRae IJ. The crystal structure of human Argonaute2. *Science.* 2012;336:1037–40.
- [67] Alagia A, Eritja R. Biochemistry of RNA silencing in eLS. Chichester: John Wiley & Sons, Ltd; 2017. a0021019.
- [68] Wilson RC, Doudna JA. Molecular mechanism of RNA interference. *Annu Rev Biophys.* 2013;42:217–39.
- [69] Huntzinger E, Izaurralde E. Gene silencing by micro-RNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet.* 2011;12:99–110.
- [70] Kenski DM, Butora G, Willingham AT, Cooper AJ, Fu W, Qi N, Soriano F, Davies IW, Flanagan WM. *Mol Ther Nucleic Acids.* 2012;1:e5.
- [71] Davis S, Lollo B, Freier S, Esau C. Improved targeting of miRNA with antisense oligonucleotides. *Nucleic Acids Res.* 2006;34:2294–304.
- [72] Lam JK, Chow MYT, Zhang Y, Leung SWS. siRNA versus miRNA as therapeutics for gene silencing. *Mol Ther Nucleic Acids.* 2015;4:e252.
- [73] Alagia A, Eritja R. siRNA and RNAi optimization. *WIREs RNA.* 2016;7:316–29.
- [74] Hu B, Zhong L, Weng Y, Peng L, Huang Y, Zhao Y, Liang XJ. Therapeutic siRNA: state of the art. *Signal Transduct Target Ther.* 2020;5:101.
- [75] Egli M, Re-engineering MM. RNA molecules into therapeutic agents. *Acc Chem Res.* 2019;52:1036–47.
- [76] Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV, Tournev I, Schmidt HH, Coelho T, Berk JL, Lin K-P, Vita G et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N Engl J Med.* 2018;379:11–21.
- [77] Suhr OB, Coelho T, Buades J, Pouget J, Conceicao I, Berk J, Schmidt H, Waddington-Cruz M, Campistol JM, Bettencourt BR, Vaishnav A, Gollob J, Adams D. Efficacy and safety of patisiran. *N Engl J Med.* 2015;10:109.
- [78] Benson MD, Waddington-Cruz M, Berk JL, Polydefkis M, Dyck PJ, Wang AK, Planté-Bordeneuve V, Barroso FA, Merlini G, Obici L, Scheinberg M, Brannagan TH III et al. Inotersen treatment for patients with hereditary transthyretin amyloidosis. *N Engl J Med.* 2018;379:22–31.
- [79] Balwani M, Sardh E, Ventura P, Aguilera Peiró P, Rees DC, Stölzel U, Bissell DM, Bonkovsky HL, Windyga J, Anderson KE, Parker C, Silver SM, Keel SB, Wang JD, Stein PE, Harper P, Vassiliou D, Wang B, Phillips J, Ivanova A, Langendonk JG, Kauppinen R, Minder E, Horie Y, Penz C, Chen J, Liu S, Ko JJ, Sweetser MT, Garg P, Vaishnav A, Kim JB, Simon AR, Gouya L. Phase 3 trial of RNAi therapeutic Givosiran for acute intermittent porphyria. *N Engl J Med.* 2020;382:2289–301.
- [80] Kosmas CE, Muñoz Estrella A, Sourlas A, Delia Silverio D, Hilario E, Montan PD, Inclisiran GE. A new promising agent in the management of hypercholesterolemia. *Diseases.* 2018;6:63.
- [81] Ray KK, Wright RS, Kallend D, Koenig W, Leiter LA, Raal FJ, Bisch JA, Richardson T, Jaros M, Wijngaard PLJ, Kastelein JJP. Two phase 3 trials of inclisiran in patients with elevated LDL cholesterol. *N Engl J Med.* 2020;382:1507–19.
- [82] Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. *Dev Biol.* 2007;302:1–12.
- [83] Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat Genet.* 2007;39:673–7.
- [84] Van Rooij E, Kauppinen S. Development of microRNA therapeutics is coming of age. *EMBO Mol Med.* 2014;6:851–64.

- [85] Li Z, Rana TM. Therapeutic targeting of microRNAs: current status and future challenges. *Nat Rev Drug Discov*. 2014;13:622–38.
- [86] Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, van der Meer AJ, Patick AK, Chen A, Zhou Y, Persson R, King BD, Kauppinen S, Levin AA, Hodges MR. Treatment of HCV infection by targeting microRNA. *N Engl J Med*. 2013;368:1685–94.
- [87] van der Ree MH, Van der Meer AJ, de Bruijne J, Maan R, van Vliet A, Welzel TM, Zeuzem S, Lawitz EJ, Rodriguez-Torres M, Kupcova V, Wiercinska-Drapalo A, Hodges MR, Janssen HLA, Reesink HW. Long term safety and efficacy of microRNA-targeted therapy in chronic hepatitis C patients. *Antivir Res*. 2014;111:53–9.
- [88] Okada N, Lin C-P, Ribeiro MC, Biton A, Lai G, He X, Bu P, Vogel H, Jablons DM, Keller AC, Wilkinson JE, He B, Speed TP, He L. A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression. *Genes Dev*. 2014;28:438–50.
- [89] Beg MS, Brenner AJ, Sachdev J, Borad M, Kang YK, Stoudemire J, Smith S, Bader AG, Kim S, Hong DS. Phase I study of MRX34, a liposomal miR-34a mimic, administered twice weekly in patients with advanced solid tumors. *Invest New Drugs*. 2017;35:180–8.
- [90] Matsui M, Corey DR. Non-coding RNAs as drug targets. *Nat Rev Drug Discov*. 2017;16:167–79.
- [91] Wahlestedt C. Targeting long non-coding RNA to therapeutically upregulate gene expression. *Nat Rev Drug Discov*. 2013;12:433–46.
- [92] Meng L, Ward AJ, Chun S, Bennett CF, Beaudet AL, Rigo F. Towards a therapy for Angelman syndrome by targeting a long non-coding RNA. *Nature*. 2015;518:409–12.
- [93] Xia Y, Xiao X, Deng X, Zhang F, Zhang X, Hu Q, Sheng W. Targeting long non-coding RNA ASBEL with oligonucleotide antagonist for breast cancer therapy. *Biochem Biophys Res Commun*. 2017;489:386–92.
- [94] Mendell JT. Targeting a long noncoding RNA in breast cancer. *N Engl J Med*. 2016;374:2287–9.
- [95] Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science*. 1990;249:505–10.
- [96] Colas P, Cohen B, Jenssen T, Grishina I, McCoy J, Brent R. Genetic selection of peptide-aptamers that recognize and inhibit cyclic-dependent kinase 2. *Nature*. 1996;380:548–50.
- [97] Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. *Nature*. 1990;346:818–22.
- [98] Shaltiel-Kyrylo R, Frenkel-Pinter M, Egoz-Matia N, Frydman-Marom A, Shalev DE, Segal D, Gazit E. Inhibiting  $\alpha$ -synucleic oligomerization by stable cell-penetrating  $\beta$ -synucleic fragments recovers phenotype of Parkinson’s disease model flies. *PLoS ONE*. 2010;5:e13863.
- [99] Shigdar S, Qiao L, Zhou SF, Xiang D, Wang T, Li Y, Lim LY, Kong L, Li L, Duan W. RNA aptamers targeting cancer stem cell marker CD133. *Cancer Lett*. 2013;330:84–95.
- [100] Vuyisich M, Beal PA. Controlling protein activity with ligand regulated RNA aptamers. *Chem Biol*. 2002;9:907–13.
- [101] Gragoudas ES, Adamis AP, Cunningham ET, Feinsod M, Guyer DR. Pegaptanib for neovascular age-related macular degeneration. *N Engl J Med*. 2004;351:2805–16.
- [102] Bunka DHJ, Platonova O, Stockley PG. Development of aptamer therapeutics. *Curr Opin Pharmacol*. 2010;10:557–62.
- [103] Denekas T, Tröltzsch M, Vater A, Klussmann S, Messlinger K. Inhibition of stimulated meningeal blood flow by a calcitonin gene-related peptide binding mirror-image RNA oligonucleotide. *Br J Pharmacol*. 2006;148:536–43.
- [104] Ludwig H, Weisel K, Petrucci MT, Leleu X, Cafro AM, Garderet L, Leitgeb C, Foa R, Greil R, Yakoub-Agha I, Zboralski D, Dümmeler T, Beyer D, Kruschinski A, Riecke K, Baumann M, Engelhardt M. Olaptesed pegol, an anti-CXCL12/SDF-1 Spiegelmer, alone and with bortezomib-dexamethasone in relapsed/refractory multiple myeloma: a phase IIa study. *Leukemia*. 2017;31:997–1000.

- [105] Bates PJ, Reyes-Reyes EM, Malik MT, Murphy EM, Toole MGO, Trent JO. G-quadruplex oligonucleotide AS1411 as a cancer-targeting agent: uses and mechanisms. *Biochim Biophys Acta.* 2017;1861:1414–28.
- [106] Nabavinia MS, Ghooobi A, Chargoo F, Nabavina M, Ramezani M, Abnous K. Anti-MUC1 aptamer: a potential opportunity for cancer treatment. *Med Res Rev.* 2017;37:1518–39.
- [107] Riccardi C, Fàbrega C, Grijalvo S, Vitiello G, D'Errico G, Eritja R, Montesarchio D. AS1411-decorated niosomes as effective nanocarriers for Ru(III)-based drugs in anticancer strategies. *J Mat Chem B.* 2018;6:5368–84.
- [108] Ano Bom APD, da Costa Neves PC, Bonacossa de Almeida CE, Silva D, Missailidis S. Aptamers as delivery agents of siRNA and chimeric formulations for the treatment of cancer. *Pharmaceutics.* 2019;11:684.
- [109] Platella C, Riccardi C, Montesarchio D, Roviello GN, Musumeci D. G-quadruplex-based aptamers against protein targets in therapy and diagnostics. *Biochim Biophys Acta.* 2017;1861:1429–47.
- [110] Whitehead KA, Dahlman JE, Langer RS, Anderson DG. Silencing or stimulation? siRNA delivery and the immune system. *Annu Rev Chem Biomol Eng.* 2011;2:77–96.
- [111] Robbins M, Judge A, MacLachlan I. SiRNA and innate immunity. *Oligonucleotides.* 2009;19:89–102.
- [112] Sioud M. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. *J Mol Biol.* 2005;348:1079–90.
- [113] Eberle F, Giessler K, Deck C, Heeg K, Peter M, Richert C, Dalpke AH. Modifications in small interfering RNA that separate immunostimulation from RNA interference. *J Immunol.* 2008;180:3229–37.
- [114] Alagia A, Terrazas M, Eritja R. RNA/aTNA chimeras: RNAi effects and nuclease resistance of single and double stranded RNAs. *Molecules.* 2014;19:17872–96.
- [115] Reyes-Darias JA, Sánchez-Luque FJ, Morales JC, Pérez-Rentero S, Eritja R, Berzal-Herranz A. Glucose conjugation of anti-HIV-1 oligonucleotides containing unmethylated CpG motifs reduces their immunostimulatory activity. *ChemBioChem.* 2015;16:584–91.
- [116] Krieg AM. CpG still rocks! Update of an accidental drug. *Nucleic Acids Ther.* 2012;22:77–89.
- [117] Shirota H, Tross D, Klinman DM. CpG oligonucleotides as cancer vaccine adjuvants. *Vaccines.* 2015;3:390–407.
- [118] Klinman DM, Klaschik S, Sato T, Tross D. CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases. *Adv Drug Deliv Rev.* 2009;61:248–55.
- [119] Kandimalla ER, Agrawal S. Chemistry of CpG DNA. *Curr Prot Nucleic Acids Chem.* 2003;4.16.1–14.
- [120] Goubau D, Schlee M, Deddouche S, Pruijssers AJ, Zillingter T et al. Antiviral immunity via RIG-I-mediated recognition of RNA bearing 5'-diphosphates. *Nature.* 2014;514:372–5.
- [121] Hornung V, Ellegast J, Kim S, Brzozka K, Jung A et al. 5'-Triphosphate RNA as the ligand for RIG-I. *Science.* 2006;314:994–7.
- [122] Hecker M, Wagner AH. Transcription factor decoy technology: a technology update. *Biochem Pharmacol.* 2017;144:29–34.
- [123] Mann MJ, Dzau VJ. Therapeutic applications of transcription factor decoy oligonucleotides. *J Clin Invest.* 2000;106:1071–5.
- [124] Brennan P, Donev R, Hewamana S. Targeting transcription factors for therapeutic benefit. *Mol BioSyst.* 2008;4:909–19.
- [125] Almeida MI, Reis RM, Calin GA. Decoy activity through microRNAs: the therapeutic implications. *Expert Opin Biol Ther.* 2012;12:1153–9.

- [126] Rogers FA, Lloyd JA, Glazer PM. Triplex-forming oligonucleotides as potential tools for modulation of gene expression. *Curr Med Chem*. 2005;5:319–26.
- [127] Thuong NY, Hélène C. Sequence-specific recognition and modification of double-helical DNA by oligonucleotides. *Angew Chem, Int Ed Engl*. 1993;32:666–90.
- [128] Knauert MP, Glazer PM. Triplex forming oligonucleotides: sequence-specific tools for gene targeting. *Hum Mol Genet*. 2001;10:2243–51.
- [129] Hansen GI, Bentin T, Larsen HJ, Nielsen PE. Structural isomers of bis-PNA bound to a target in duplex DNA. *J Mol Biol*. 2001;307:67–74.
- [130] Geny S, Moreno PM, Krzywkowski T, Gissberg O, Andersen NK, Isse AJ, El-Madani AM, Lou C, Pabon YV, Anderson BA, Zaghoul EM, Zain R, Hrdlicka PJ, Jørgensen PT, Nilsson M, Lundin KE, Pedersen EB, Wengel J, Smith CIE. Next-generation bis-locked nucleic acids with stacking linker and 2'-glycylamino-LNA show enhanced DNA invasion into supercoiled duplexes. *Nucleic Acids Res*. 2016;44:2007–19.
- [131] Aviñó A, Eritja R, Ciudad CJ, Noé V. Parallel clamps and polypurine hairpins (PPRH) for gene silencing and triplex-affinity capture: design, synthesis and use. *Curr Prot Nucleic Acids Chem*. 2019;e78.
- [132] Zaug AJ, Been MD, Cech TR. The tetrahymena ribozyme acts like an RNA restriction endonuclease. *Nature*. 1986;324:429–33.
- [133] Cech TR. The efficiency and versatility of catalytic RNA: implications for an RNA world. *Genes*. 1993;135:33–6.
- [134] Morrison D, Rothenbroker M, Li Y. DNAzymes: selected for applications. *Small Methods*. 2018;2:1700319.
- [135] Santoro SW, Joyce GF. A general purpose RNA-cleaving DNA enzyme. *Proc Natl Acad Sci USA*. 1997;94:4262–6.
- [136] Sioud M, Iversen PO. Ribozymes, DNAzymes and small interfering RNAs as therapeutics. *Curr Drug Targets*. 2005;6:647–53.
- [137] Zhou W, Ding J, Liu J. Theranostic DNAzymes. *Theranostics*. 2017;7:1010–25.
- [138] Gmeiner WH, Debinski W, Milligan C, Caudell D, Pardee TS. The applications of the novel polymeric fluoropyrimidine F10 in cancer treatment: current evidence. *Future Oncol*. 2016;12:2009–20.
- [139] Pardee TS, Gomes E, Jennings-Gee J, Caudell D, Gmeiner WH. Unique dual targeting of thymidylate synthase and topoisomerase 1 by FdUMP[10] results in high efficacy against AML and low toxicity. *Blood*. 2012;119:3561–70.
- [140] Pardee TS, Stadelman K, Jennings-Gee J, Caudell DL, Gmeiner WH. The poison oligonucleotide F10 is highly active against acute lymphoblastic leukemia while sparing normal hematopoietic cells. *Oncotarget*. 2014;5:4170–9.
- [141] Gmeiner WH, Willingham MC, Bourland JD, Hatcher HC, Smith TL, D'Agostino RB Jr., Blackstock W. F10 inhibits growth of PC3 xenografts and enhances the effects of radiation therapy. *J Clin Oncol Res*. 2014;2:1028.
- [142] Gmeiner WH, Lema-Tome C, Gibo D, Jennings-Gee J, Milligan C, Debinski W. Selective antitumor activity of the npvel fluoropyrimidine polymer F10 towards G48a orthotopic GBM tumours. *J Neuro-Oncol*. 2014;116:447–54.
- [143] Mou Q, Ma Y, Pan G, Xue B, Yan D, Zhang C, Zhu X. DNA trojan horses: self-assembled floxuridine containing DNA polyhedral for cancer therapy. *Angew Chem*. 2017;129:12702–6.
- [144] Jorge AF, Aviñó A, Pais A, Eritja R, Fàbrega C. DNA-based nanoscaffolds as vehicles for 5-fluoro-2'-deoxyuridine oligomers in colorectal cancer therapy. *Nanoscale*. 2018;10:7238–49.
- [145] Sproat B. Chemistry and applications of oligonucleotide analogues. *J Biotechnol*. 1995;41:221–38.

- [146] Bell NM, Micklefield J. Chemical modification of oligonucleotides for therapeutic, bioanalytical and other applications. *ChemBioChem*. 2009;10:2691–703.
- [147] Srinivasan SK, Iversen P. Review of *in vivo* pharmacokinetics and toxicology of phosphorothioate oligonucleotides. *J Clin Labor Anal*. 1995;9:129–37.
- [148] Koller E, Vincent TM, Chappell A, De S, Manoharan M, Bennett CF. Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes. *Nucleic Acids Res*. 2011;39:4795–807.
- [149] Henry SP, Beattie G, Yeh G, Chappel A, Giclas P, Mortari A, Jagels MA, Kornbrust DJ, Levin AA. Complement activation is responsible for acute toxicities in rhesus monkeys treated with a phosphorothioate oligodeoxynucleotide. *Int Immunopharm*. 2002;2:1657–66.
- [150] Guga P, Stec WJ. Synthesis of phosphorothioate oligonucleotides with stereodefined phosphorothioate linkages. *Curr Prot Nucleic Acid Chem*. 2003;4.17.1–28.
- [151] Wilk A, Stec WJ. Analysis of oligo(deoxynucleoside phosphorothioate)s and their diastereomeric composition. *Nucleic Acids Res*. 1995;23:530–4.
- [152] Lackey DB, Patel J. Biochemical synthesis of chirally pure  $R_p$  oligonucleotide phosphorothioates. *Biotechnol Lett*. 1997;19:475–8.
- [153] Stec WJ, Grajkowski A, Koziolkiewicz M, Uznanski B. Novel route to oligo(deoxyribonucleoside phosphorothioate): stereocontrolled synthesis of P-chiral oligo(deoxyribonucleoside phosphorothioates). *Nucleic Acids Res*. 1991;19:5883–8.
- [154] Stec WJ, Grajkowski A, Karwowski B, Kobylanska A, Koziolkiewicz M, Misiura K, Okruszek A, Wilk A, Guga P, Boczkowska M. Diastereoisomers of nucleoside 3'-O-(2-thio-1,3,2-oxathia(selena)phospholanes): building blocks for stereocontrolled synthesis of oligo(deoxyribonucleoside phosphorothioate)s. *J Am Chem Soc*. 1995;117:12019–29.
- [155] Stec WJ, Grajkowski A, Boczkowska M, Guga P, Koziolkiewicz M, Sochacki M, Wieczorek M, Blaszczyk J. Deoxyribonucleoside 3'-O-(2-thio- and 2-oxo-spiro-4,4-pentamethylene-1,3,2-oxathiaphospholane)s: monomers for stereocontrolled synthesis of oligo(deoxyribonucleoside phosphorothioate)s and chimeric PS/PO oligonucleotides. *J Am Chem Soc*. 1998;120:7156–67.
- [156] Iyer RP, Guo MJ, Yu D, Agrawal S. Solid-phase stereoselective synthesis of oligonucleotide phosphorothioates: the nucleoside bicyclic oxazaphospholidines as novel synthons. *Tetrahedron Lett*. 1998;39:2491–4.
- [157] Wilk A, Grajkowski A, Phillips LR, Beauchage SL. Deoxyribonucleoside cyclic *N*-acylphosphoramidites as a new class of monomers for the stereocontrolled synthesis of oligothymidyl- and oligodeoxycytidyl-phosphorothioates. *J Am Chem Soc*. 2000;122:2149–56.
- [158] Oka N, Yamamoto M, Sato T, Wada T. Solid-phase synthesis of stereoregular oligodeoxyribonucleoside phosphorothioates using bicyclic oxazaphospholidine derivatives as monomer units. *J Am Chem Soc*. 2008;130:16031–7.
- [159] Nukuga Y, Yamada K, Ogata T, Oka N, Wada T. Stereocontrolled solid-phase synthesis of phosphorothioate oligoribonucleotides using 2'-O-(2-cyanoethoxymethyl)-nucleoside 3'-Oxazaphospholidine monomers. *J Org Chem*. 2012;77:7913–22.
- [160] Oka N, Kondo T, Fujiwara S, Maizuru Y, Wada T. Stereocontrolled synthesis of oligoribonucleoside phosphorothioates by an oxazaphospholidine approach. *Org Lett*. 2009;11:967–70.
- [161] Li M, Lightfoot HL, Halloy F, Malinowska AL, Berk C, Behera A, Schümperli D, Hall J. Synthesis and cellular activity of stereochemically-pure 2'-O-(2-methoxyethyl)-phosphorothioate oligonucleotides. *Chem Commun*. 2017;53:541–4.

- [162] Iwamoto N, Butler DCD, Svrzikapa N, Mohapatra S, Zlatev I, Sah DWY, Meena, Standley SM, Lu G, Apponi LH, Frank-Kamenetsky M, Zhang JJ, Vargesse C, Verdine GL. Control of phosphorothioate stereochemistry substantially increases the efficacy of antisense oligonucleotides. *Nat Biotechnol.* 2017;35:845–51.
- [163] Iwamoto N, Oka N, Sato T, Wada T. Stereocontrolled solid-phase synthesis of oligonucleoside H-phosphonate by an oxazaphospholidine approach. *Angew Chem, Int Ed Engl.* 2009;48:496–9.
- [164] Brill WKD. Thioalkylation of nucleoside H-phosphonates and its application to solid phase synthesis of oligonucleotides. *Tetrahedron Lett.* 1995;36:703–6.
- [165] Beaton G, Dellinger D, Marshall WS, Caruthers MH. Synthesis of oligonucleotide phosphorodithioates. In: Eckstein F, editor. *Oligonucleotides and analogues. A practical approach.* Oxford: IRL Press; 1991. p. 109–35.
- [166] Wiesler WT, Marshall WS, Caruthers MH. Synthesis and purification of phosphorodithioate DNA. In: Agrawal S, editor. *Protocols for nucleotides and analogs.* Meth Mol Biol. vol. 20. Totowa, NJ: Humana Press Inc; 1993. p. 191–224.
- [167] Brill WKD, Tang JY, Ma YX, Caruthers MH. Synthesis of oligodeoxynucleoside phosphorodithioates via thioamidites. *J Am Chem Soc.* 1989;111:2321–2.
- [168] Wiesler WT, Caruthers MH. Synthesis of phosphorodithioate DNA via sulfur-linked base-labile protecting group. *J Org Chem.* 1996;61:4272–81.
- [169] Bjergård K, Dahl O. Solid phase synthesis of oligodeoxyribonucleoside phosphorodithioates from thiophosphoramidites. *Nucleic Acids Res.* 1991;19:5843–50.
- [170] Grandas A, Marshall WS, Nielsen J, Caruthers MH. Synthesis of deoxycytidine oligomers containing phosphorodithioate linkages. *Tetrahedron Lett.* 1989;30:543–6.
- [171] Brill WKD, Yau EK, Caruthers MH. Oxidative and nonoxidative formation of internucleotide linkages. *Tetrahedron Lett.* 1989;30:6621–4.
- [172] Beaton G, Brill WKD, Grandas A, Ma YX, Nielsen J, Yau E, Caruthers MH. Synthesis of oligonucleotide phosphorodithioates. *Tetrahedron.* 1991;47:2377–88.
- [173] Porritt GM, Reese CB. Use of the 2,4-dinitrobenzyl protecting group in the synthesis of phosphorodithioate analogues of oligodeoxyribonucleotides. *Tetrahedron Lett.* 1990;31:1319–22.
- [174] Eldrup AB, Bjergård K, Felding J, Kehler J, Dahl O. Preparation of oligodeoxyribonucleoside phosphorodithioates by a triester method. *Nucleic Acids Res.* 1994;22:1797–804.
- [175] Cummins L, Graff D, Beaton G, Marshall WS, Caruthers MH. Biochemical and physicochemical properties of phosphorodithioate DNA. *Biochemistry.* 1996;35:8734–41.
- [176] Ghosh MK, Ghosh K, Dahl O, Cohen JS. Evaluation of some properties of a phosphorodithioate oligodeoxyribonucleotide for antisense applications. *Nucleic Acids Res.* 1993;21:5761–6.
- [177] Marshall WS, Beaton G, Stein CA, Matsukura M, Caruthers MH. Inhibition of human immunodeficiency virus activity by phosphorodithioate oligodeoxycytidine. *Proc Natl Acad Sci USA.* 1992;89:6265–9.
- [178] Miller PS, Fang KN, Kondo NS, Ts'o POP. Syntheses and properties of adenine and thymine nucleoside alkyl phosphotriesters, the neutral analogs of dinucleoside monophosphates. *J Am Chem Soc.* 1971;93:6657–65.
- [179] Miller PS, Barrett JC, Ts'o POP. Synthesis of oligodeoxyribonucleotide ethyl phosphotriesters and their specific complex formation with transfer ribonucleic acid. *Biochemistry.* 1974;13:4887–96.
- [180] Miller PS, Chandrasegaran S, Dow DL, Pulford SM, Kan LS. Synthesis and template properties of an ethyl phosphotriester modified decadeoxyribonucleotide. *Biochemistry.* 1982;21:5468–74.

- [181] Stec WJ, Zen G, Gallo KA, Byrd RA, Uznanski B, Guga P. Synthesis and absolute configuration of P-chiral O-isopropyl oligonucleotide triesters. *Tetrahedron Lett.* 1985;26:2191–4.
- [182] Yamana K, Nishijima Y, Negishi K, Yashiki T, Nishio K, Nakano H, Sangen O. Deoxyribonucleoside 3'-phosphorobisamidites in the synthesis of isopropyl phosphotriester oligodeoxyribonucleotide analogues. *Tetrahedron Lett.* 1991;32:4721–4.
- [183] Koziolkiewicz M, Wilk A. Oligodeoxyribonucleotide phosphotriesters. In: Agrawal S, editor. *Protocols for nucleotides and analogs*. Meth Mol Biol. vol. 20. Totowa, NJ: Humana Press Inc; 1993. p. 207–24.
- [184] Uznanski B, Grajkowski A, Wilk A. Isopropoxyacetic group for convenient base protection during solid-support synthesis of oligodeoxyribonucleotides and their triester analogs. *Nucleic Acids Res.* 1989;17:4863–71.
- [185] Hau JF, Asseline U, Thuong NT. Octathymidylates involving alternating neopentylphosphothionotriester-phosphodiester linkages with controlled stereochemistry at the modified P-center. *Tetrahedron Lett.* 1991;32:2497–8.
- [186] Kuijpers WHA, Huskens J, Koole LH, van Boeckel CAA. Synthesis of well-defined phosphate-methylated DNA fragments: the application of potassium carbonate in methanol as deprotecting agent. *Nucleic Acids Res.* 1990;18:5197–52.
- [187] Alul RH, Singman CN, Zhang G, Letsinger RL. Oxalyl-CPG: a labile support for synthesis of sensitive oligonucleotide derivatives. *Nucleic Acids Res.* 1991;19:1527–32.
- [188] Guzaev A, Lönnberg H. A novel solid support for synthesis of 3'-phosphorylated chimeric oligonucleotides containing internucleosidic methyl phosphotriester and methylphosphonate linkages. *Tetrahedron Lett.* 1997;38:3989.
- [189] Iyer RP, Yu D, Jiang Z, Agrawal S. Synthesis, biophysical properties, and stability studies of mixed backbone oligonucleotides containing segments of methylphosphotriester internucleotidic linkages. *Tetrahedron.* 1996;52:14419–36.
- [190] Iyer RP, Yu D, Ho NH, Devlin T, Agrawal S. Methyl phosphotriester oligonucleotides: facile synthesis using *N*-pent-4-enoyl nucleoside phosphoramidites. *J Org Chem.* 1995;60:8132–3.
- [191] Koole LH, Moody HM, Broeders NLHL, Quaedflieg PJLM, Kuijpers WHA, van Genderen MHP, Coenen AJJM, van der Wal S, Buck HM. Synthesis of phosphate-methylated DNA fragments using 9-fluorenylmethoxycarbonyl as transient base protecting group. *J Org Chem.* 1989;54:1657–64.
- [192] Moody HM, van Genderen MHP, Koole LH, Kocken HJM, Meijer EM, Buck HM. Regiospecific inhibition of DNA duplication by antisense phosphate-methylated oligodeoxynucleotides. *Nucleic Acids Res.* 1989;17:4769–82.
- [193] Marcus-Sekura CJ, Woerner AM, Shinozuka K, Zon G, Quinnan GV Jr. Comparative inhibition of chloramphenicol acetyltransferase gene expression by antisense oligonucleotide analogues having alkyl phosphotriester, methylphosphonate and phosphorothioate linkages. *Nucleic Acids Res.* 1987;15:5749–63.
- [194] Miller PS, Agris CH, Aurelian L, Blake KR, Murakami A, Reddy PM, Spitz SA, Ts'o POP. Control of ribonucleic acid function by oligonucleoside methylphosphonates. *Biochimie.* 1985;67:769–76.
- [195] Kulka M, Smith CC, Aurelian L, Fishelevich R, Meade K, Miller P, Ts'o POP. Site specificity of the inhibitory effects of oligo(nucleoside methylphosphonate)s complementary to the acceptor splice junction of herpes simplex virus type 1 immediate early mRNA 4. *Proc Natl Acad Sci USA.* 1989;86:6868–72.
- [196] Miller PS, Agris CH, Murakami A, Reddy PM, Spitz SA, Ts'o POP. Preparation of oligodeoxyribonucleoside methylphosphonates on a polystyrene support. *Nucleic Acids Res.* 1983;11:6225–42.

- [197] Miller PS, Reddy PM, Murakami A, Blake KR, Lin SB, Agris CH. Solid-phase syntheses of oligodeoxyribonucleoside methylphosphonates. *Biochemistry*. 1986;25:5092–7.
- [198] Miller PS, Cushman CD, Levis JT. Synthesis of oligo-2'-deoxyribonucleoside methylphosphonates. In: Eckstein F, editor. Oligonucleotides and analogues. A practical approach. Oxford: IRL Press; 1991. p. 137–54.
- [199] Miller PS, Agris CH, Blandin M, Murakami A, Reddy PM, Spitz SA, Ts'o POP. Use of methylphosphonic dichloride for the synthesis of oligonucleoside methylphosphonates. *Nucleic Acids Res.* 1983;11:5189–204.
- [200] Jäger A, Engels J. Synthesis of deoxynucleoside methylphosphonates via a phosphoramidite approach. *Tetrahedron Lett.* 1984;25:1437–40.
- [201] Agrawal S, Goodchild J. Oligodeoxynucleoside methylphosphonates: synthesis and enzymic degradation. *Tetrahedron Lett.* 1987;28:3539–42.
- [202] Thaden J, Miller PS. Automated synthesis of oligodeoxyribonucleoside methylphosphonates having [*N*-(3-aminoprop-1-yl)-*N*-(2-hydroxyethyl) 2-aminoethyl] phosphate or methylphosphonic acid at the 3' end using a modified controlled pore glass support. *Bioconjug Chem.* 1993;4:395–401.
- [203] Zhang Z, Nichols A, Tang JX, Tang JY. In situ generation of methylphosphonamidites for synthesis of oligonucleotide methylphosphonates. *Chem Commun.* 1997;1235–6.
- [204] Hogrefe RI, Reynolds MA, Vaghefi MM, Young KM, Riley TA, Klem RE, Arnold LJ Jr. An improved method for the synthesis and deprotection of methylphosphonate oligonucleotide. In: Agrawal S, editor. Protocols for nucleotides and analogs. Meth Mol Biol. vol. 20. Totowa, NJ: Humana Press Inc; 1993. p. 143–64.
- [205] Hogrefe RI, Vaghefi MM, Reynolds MA, Young KM, Arnold LJ Jr. Deprotection of methyiphosphonate oligonucleotides using a novel one-pot procedure. *Nucleic Acids Res.* 1993;21:2031–8.
- [206] Sinha ND, Michaud DP, Roy SK, Casale RA. Synthesis of oligodeoxynucleoside methylphosphonates utilizing the tert-butylphenoxyacetyl group for exocyclic amine protection. *Nucleic Acids Res.* 1994;22:3119–23.
- [207] Habus I, Devlin T, Iyer RP, Agrawal S. Improved synthesis of oligonucleoside methylphosphonate analogs. *Bioorg Med Chem Lett.* 1996;6:1393–8.
- [208] Reynolds MA, Hogrefe RI, Jaeger JA, Schwartz DA, Riley TA, Marvin WB, Daily WJ, Vaghefi MV, Beck TA, Knowles SK, Klem RE, Arnold LJ Jr. Synthesis and thermodynamics of oligonucleotides containing chirally pure  $R_p$  methylphosphonate linkages. *Nucleic Acids Res.* 1996;24:4584–91.
- [209] Jaworska-Maslanka MM, Kacperczyk W, Korczynski D, Lesnikowski ZJ. Studies on stereospecific formation of P-chiral internucleotide linkage: synthesis of all- $R_p$  and all- $S_p$  methylphosphonate pentanucleotide d(MMTrA<sub>pMe</sub>T<sub>pMe</sub>T<sub>pMe</sub>C<sub>pMe</sub>TAc) via Grignard activated coupling. *Antisense Nucleic Acid Drug Dev.* 1997;7:23–30.
- [210] Schell P, Engels JW. Rp-Diastereoselective synthesis of dinucleoside methylphosphonates by the phosphoramidite approach. *Tetrahedron Lett.* 1998;39:8629–32.
- [211] Rosmanitz P, Eisenhardt S, Bats JW, Engels JW. New proline derived chiral building blocks for nucleoside methylphosphonate synthesis. *Tetrahedron*. 1994;50:5719–34.
- [212] Flür S, Micura R. Chemical synthesis of RNA with site-specific methylphosphonate modifications. *Methods*. 2016;107:79–88.
- [213] Brill WKD, Caruthers MH. Synthesis of nucleoside methylphosphonothioates. *Tetrahedron Lett.* 1987;28:3205–8.
- [214] Padmapriya AA, Agrawal S. Synthesis of oligodeoxynucleoside methylphosphonothioates. *Bioorg Med Chem Lett.* 1993;3:761–4.

- [215] Miller PS, McParland KB, Jayaraman K, Ts'o POP. Biochemical and biological effects of nonionic nucleic acid methylphosphonates. *Biochemistry*. 1981;20:1874–80.
- [216] Shoji Y, Akhtar S, Periasamy A, Herman B, Juliano RL. Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages. *Nucleic Acids Res.* 1991;19:5543–50.
- [217] Furdon PJ, Dominski Z, Kole R. RNase H cleavage of RNA hybridized to oligonucleotides containing methylphosphonate, phosphorothioate and phosphodiester bonds. *Nucleic Acids Res.* 1989;17:9193–204.
- [218] Agrawal S, Mayrand SH, Zamecnik PC, Pederson T. Site-specific excision from RNA by RNase H and mixed-phosphate-backbone oligodeoxynucleotides. *Proc Natl Acad Sci USA*. 1990;87:1401–5.
- [219] Giles RV, Spiller DG, Tidd DM. Chimeric oligodeoxynucleotide analogs: chemical synthesis, purification, and molecular and cellular biology protocols. *Methods Enzymol.* 1999;313:95–135.
- [220] Froehler BC. Deoxynucleoside H-phosphonate diester intermediates in the synthesis of internucleotide phosphate analogues. *Tetrahedron Lett.* 1986;27:5575–8.
- [221] Dagle JM, Walder JA, Weeks DL. Targeted degradation of mRNA in *Xenopus* oocytes and embryos directed by modified oligonucleotides: studies of An2 and cyclin in embryogenesis. *Nucleic Acids Res.* 1990;18:4751–7.
- [222] Ozaki H, Kitamura M, Yamana K, Murakami A, Shimidzu T. Syntheses and properties of oligothymidylate analogs containing stereoregulated phosphoramorpholidate and phosphodiester linkages in an alternating manner. *Bull Chem Soc Jpn.* 1990;63:1929–36.
- [223] Gryaznov SM. Oligonucleotide N3' → P5' phosphoramidates and thio-phosphoramidates as potential therapeutic agents. *Chem Biodivers.* 2010;7:477–93.
- [224] Gryaznov SM, Chen JK. Oligodeoxyribonucleotide N3' → P5' phosphoramidates: synthesis and hybridization properties. *J Am Chem Soc.* 1994;116:3143–4.
- [225] Chen JK, Schultz RG, Lloyd DH, Gryaznov SM. Synthesis of oligodeoxyribonucleotide N3' → P5' phosphoramidates. *Nucleic Acids Res.* 1995;23:2661–8.
- [226] Gryaznov SM, Lloyd DH, Chen JK, Schultz RG, Dedionisio LA, Ratmeyert L, Wilson WD. Oligonucleotide N3' → P5' phosphoramidates. *Proc Natl Acad Sci USA.* 1995;92:5798–802.
- [227] Pongracz K, Gryaznov SM. Oligonucleotide N3' → P5' thiophosphoramidates: synthesis and properties. *Tetrahedron Lett.* 1999;40:7661–4.
- [228] Fearon KL, Nelson JS. Synthesis and purification of oligonucleotide N3' → P5' phosphoramidates and their phosphodiester and phosphorothioate chimeras. *Curr Prot Nucleic Acid Chem.* 2000;4.7.1–20.
- [229] McCurdy SN, Nelson JS, Hirschbein BL, Fearon KL. An improved method for the synthesis of N3' → P5' phosphoramidate oligonucleotides. *Tetrahedron Lett.* 1997;38:207–10.
- [230] Nelson JS, Fearon KL, Nguyen MQ, McCurdy SN, Frediani JE, Foy MF, Hirschbein BL. *J Org Chem.* 1997;62:7278–87.
- [231] Fearon KL, Nelson JS, Hirschbein BL, Foy MF, Nguyen MQ, McCurdy SN, Frediani JE, Okruszek A, DeDionisio LA, Raible AM, Boyd V. An improved synthesis of oligonucleotide N3' → P5' phosphoramidates and their chimera using hindered phosphoramidite monomers and a novel handle for reversed phase purification. *Nucleic Acids Res.* 1998;26:3813–24.
- [232] Skorski T, Perrotti D, Nieborowska-Skorska M, Gryaznov S, Calabretta B. Antileukemia effect of C-myc N3' → P5' phosphoramidate antisense oligonucleotides *in vivo*. *Proc Natl Acad Sci USA.* 1997;94:3966–71.
- [233] Escudé C, Giovannangeli C, Sun JS, Lloyd DH, Chen JK, Gryaznov SM, Garestier T, Helene C. Stable triple helices formed by oligonucleotide N3' → P5' phosphoramidates inhibit transcription elongation. *Proc Natl Acad Sci USA.* 1996;93:4365–9.

- [234] Rigl CT, Lloyd DH, Tsou DS, Gryaznov SM, Wilson WD. Structural RNA mimetics: N3' → P5' phosphoramidate DNA analogs of HIV-1 RRE and TAR RNA form A-type helices that bind specifically to Rev and Tat-related peptides. *Biochemistry*. 1997;36:650–9.
- [235] Akiyama M, Hidemitsu T, Shammas MA, Hayashi T, Hamasaki M, Tai YT, Richardson P, Gryaznov S, Munshi NC, Anderson KC. Effects of oligonucleotide N3' → P5' thio-phosphoramidate (GRN163) targeting telomerase RNA in human multiple myeloma cells. *Cancer Res.* 2003;63:6187–94.
- [236] Shea-Herbert B, Pongracz K, Shay JW, Gryaznov SM. Oligonucleotide N3' → P5' phosphoramidates as efficient telomerase inhibitors. *Oncogene*. 2002;21:638–42.
- [237] Shea-Herbert B, Gellert GC, Hochreiter A, Pongracz K, Wright WE, Zielinska D, Chin AC, Harley CB, Shay JW, Gryaznov SM. Lipid modification of GRN163, an N3' → P5' thio-phosphoramidate oligonucleotide, enhances the potency of telomerase inhibition. *Oncogene*. 2005;24:5262–8.
- [238] Zou Y, Gryaznov SM, Shay JW, Wright WE, Cornforth MN. Asynchronous replication timing of telomeres at opposite arms of mammalian chromosomes. *Proc Natl Acad Sci USA*. 2004;101:12928–33.
- [239] Dellinger DJ, Yamada CM, Caruthers MH. Oligodeoxyribonucleotide analogs functionalized analogs functionalized with phosphonoacetate and thiophosphonoacetate diesters. *Curr Prot Nucleic Acid Chem*. 2004;4.24.1–26.
- [240] Sheehan D, Lunstad B, Yamada CM, Stell BG, Caruthers MH, Dellinger DJ. Biochemical properties of phosphonoacetate and thiophosphonoacetate oligodeoxyribonucleotides. *Nucleic Acids Res*. 2003;31:4109–18.
- [241] Dellinger DJ, Sheehan D, Christensen NK, Lindberg JG, Caruthers MH. Solid-phase chemical synthesis of phosphonoacetate and thiophosphonate oligodeoxyribonucleotides. *J Am Chem Soc*. 2003;125:940–50.
- [242] Yamada CM, Dellinger DJ, Caruthers MH. Synthesis and biological activity of phosphonocarboxylate DNA. *Nucleosides Nucleotides Nucleic Acids*. 2007;26:539–46.
- [243] Threlfall RN, Torres AG, Krivenko A, Gait MJ, Caruthers MH. Synthesis and biological activity of phosphonoacetate- and thiophosphonoacetate-modified 2'-O-methyl oligoribonucleotides. *Org Biomol Chem*. 2012;10:746–54.
- [244] Matsui M, Threlfall RN, Caruthers MH, Corey DR. Effect of 2'-O-methyl/thiophosphonoacetate-modified antisense oligonucleotides on huntingtin expression in patient-derived cells. *Artificial DNA PNA XNA*. 2014;5:e1146391.
- [245] Shaw BR, Madison J, Sood A, Spielvogel BF. Oligonucleoside boranophosphate (borane phosphonate). In: Agrawal S, editor. *Protocols for nucleotides and analogs*. Meth Mol Biol. vol. 20. Totowa, NJ: Humana Press Inc; 1993. p. 225–43.
- [246] Shaw BR, Sergueev D, He K, Porter K, Summers J, Sergueeva ZA, Rait V. Boranephosphate backbone: a mimic of phosphodiesters, phosphorothioates, and methyl phosphonates. *Methods Enzymol*. 1999;313:226–57.
- [247] Rait VK, Shaw BR. Boranophosphates support the RNase H cleavage of polyribonucleotides. *Antisense Nucleic Acid Drug Dev*. 1999;9:53–60.
- [248] Kundu R. Borane phosphonate DNA: a versatile unnatural internucleotide linkage. *New J Chem*. 2019;43:4323–8.
- [249] Li P, Sergueeva ZA, Dobrikov M, Shaw BR. Nucleoside and oligonucleotide boranophosphates: chemistry and properties. *Chem Rev*. 2007;107:4746–96.
- [250] Shaw BR, Dobrikov M, Wang X, Wan J, He K, Lin JL, Li P, Rait V, Sergueeva ZA, Sergueev D. Reading, writing, and modulating genetic information with boranophosphate mimics of nucleotides, DNA, and RNA. *Ann NY Acad Sci*. 2003;1002:12–29.

- [251] Li H, Porter K, Huang F, Shaw BR. Boron-containing oligodeoxyribonucleotide 14mer duplexes: enzymatic synthesis and melting studies. *Nucleic Acids Res.* 1995;23:4495–501.
- [252] Sood A, Shaw BR, Spielvogel. Boron-containing nucleic acids. 2. Synthesis of oligodeoxynucleoside boranophosphates. *J Am Chem Soc.* 1990;112:9000–1.
- [253] McCuen NB, Noé MS, Sierzchala AB, Higson AP, Caruthers MH. Synthesis of mixed sequence borane phosphonate DNA. *J Am Chem Soc.* 2006;128:8138–9.
- [254] McCuen NB, Noé MS, Olesiak M, Sierzchala AB, Caruthers MH, Higson AP. Phosphorous Sulfur Silicon. *2009;183:349–63.*
- [255] Krishna H, Solid-Phase CMH. Synthesis, thermal denaturation studies, nuclease resistance, and cellular uptake of (oligodeoxyribonucleoside)methylborane phosphineDNA chimeras. *J Am Chem Soc.* 2011;133:9844–54.
- [256] Sergueev DS, Shaw BR. H-Phosphonate approach for solid-phase synthesis of oligodeoxyribonucleoside boranophosphates and their characterization boranophosphates. *J Am Chem Soc.* 1998;120:9417–27.
- [257] Higson AP, Sierzchala A, Brummel H, Zhao Z, Caruthers MH. Synthesis of an oligothymidylate containing boranophosphate linkages. *Tetrahedron Lett.* 1998;39:3899–902.
- [258] Wada T, Shimizu M, Oka N, Saigo K. A new boranophosphorylation reaction for the synthesis of deoxyribonucleoside boranophosphates. *Tetrahedron Lett.* 2002;43:4137–40.
- [259] Shimizu M, Saigo K, Wada T. Solid-phase synthesis of oligodeoxyribonucleoside boranophosphates by the boranophotriester method. *J Org Chem.* 2006;71:4262–9.
- [260] Kawanaka T, Shimizu M, Shintani N, Wada T. Solid-phase synthesis of backbone-modified DNA analogs by the boranophotriester method using new protecting groups for nucleobases. *Bioorg Med Chem Lett.* 2008;18:3783–6.
- [261] Higashida R, Oka N, Kawanaka T, Wada T. Nucleoside *H*-boranophosphonates: a new class of boron-containing nucleotide analogues. *Chem Commun.* 2009;2466–8.
- [262] Sato K, Imai H, Shuto T, Iwata Hara R, Wada T. Solid-phase synthesis of phosphate/boranophosphate chimeric DNAs using the *H*-phosphonate-*H*-boranophosphonate method. *J Org Chem.* 2019;84:15032–41.
- [263] Uehara S, Hiura S, Higashida R, Oka N, Wada T. Solid-phase synthesis of *P*-boronated oligonucleotides by the *H*-boranophosphonate method. *J Org Chem.* 2014;79:3465–72.
- [264] Iwamoto N, Oka N, Wada T. Stereocontrolled synthesis of oligodeoxyribonucleoside boranophosphates by an oxazaphospholidine approach using acid-labile *N*-protecting groups. *Tetrahedron Lett.* 2012;53:4361–4.
- [265] Johnson CN, Spring AM, Sergueev D, Shaw BR, Germann MW. Structural basis of the RNase H1 activity on stereo regular borano phosphonate DNA/RNA hybrids. *Biochemistry.* 2011;50:3903–12.
- [266] Sergueeva ZA, Sergueev DS, Ribeiro AA, Summers JS, Shaw BR. Individual isomers of dinucleotide boranophosphates as synthons for incorporation into oligonucleotides: synthesis and configurational assignment. *Helv Chim Acta.* 2000;83:1377–91.
- [267] Hall AHS, Wan J, Shaughnessy EE, Shaw BR, Alexander KA. RNA interference using boranophosphate siRNAs: structure–activity relationships. *Nucleic Acids Res.* 2004;32:5991–6000.
- [268] Hall AHS, Wan J, Spesock A, Sergueeva Z, Shaw BR, Alexander KA. High potency silencing by single-stranded boranophosphate siRNA. *Nucleic Acids Res.* 2006;34:2773–81.
- [269] Russell C, Roy S, Ganguly S, Qian X, Caruthers MH, Nilsson M. Formation of silver nanostructures by rolling circle amplification using boranephosphonate-modified nucleotides. *Anal Chem.* 2015;87:6660–6.
- [270] Ganguly S, Paul S, Yehezkel O, Cha J, Caruthers MH. Boranephosphonate DNA-mediated metallization of single-walled carbon nanotubes. *Chem Mater.* 2017;29:2239–45.

- [271] Poole A, Penny D, Sjöberg BJ. Methyl-RNA: an evolutionary bridge between RNA and DNA? *Chem Biol*. 2000;7:R207–16.
- [272] Inoue H, Hayase Y, Imura A, Iwai S, Miura K, Ohtsuka E. Synthesis and hybridization studies on two complementary nona(2'-O-methyl)ribonucleotides. *Nucleic Acids Res*. 1987;15:6131–48.
- [273] Sproat BS, Lamond AI. 2'-O-Methyloligonucleotides: synthesis and applications. In: Eckstein F, editor. *Oligonucleotides and analogues. A practical approach*. Oxford: IRL Press; 1991. p. 49–86.
- [274] Sproat BS. Synthesis of 2'-O-alkyloligonucleotides. In: Agrawal S, editor. *Protocols for nucleotides and analogs*. Meth Mol Biol. vol. 20. Totowa, NJ: Humana Press Inc; 1993. p. 115–41.
- [275] Sproat BS, Beijer B, Iribarren A. New synthetic routes to protected purine 2'-O-methylriboside-3'-O-phosphoramidites using a novel alkylation procedure. *Nucleic Acids Res*. 1990;18:41–9.
- [276] Sproat BS, Lamond AI, Beijer B, Neuner P, Ryder U. Highly efficient chemical synthesis of 2'-O-methyloligonucleotides and tetrabiotinylated derivatives; novel probes that are resistant to degradation by RNA or DNA specific nucleases. *Nucleic Acids Res*. 1989;1989(17):3373–86.
- [277] Cotten M, Oberhauser B, Brunar H, Holzner A, Issakides G, Noe CR, Schaffner G, Wagner E, Birnstiel ML. 2'-O-Methyl, 2'-O-ethyl oligoribonucleotides and phosphorothioate oligodeoxyribonucleotides as inhibitors of the in vitro U7 snRNP-dependent mRNA processing event. *Nucleic Acids Res*. 1991;19:2629–35.
- [278] Wagner E, Oberhauser B, Holzner A, Brunar H, Issakides G, Schaffner G, Cotten M, Knollmüller M, Noe CR. A simple procedure for the preparation of protected 2'-O-methyl or 2'-O-ethyl ribonucleoside-3'-O-phosphoramidites. *Nucleic Acids Res*. 1991;19:5965–71.
- [279] Iribarren AM, Sproat BS, Neuner P, Sulston I, Ryder U, Lamond AI. 2'-O-Alkyl oligoribonucleotides as antisense probes. *Proc Natl Acad Sci USA*. 1990;87:7747–51.
- [280] Sproat BS, Iribarren A, Guimil Garcia R, Beijer B. New synthetic routes to synthons suitable for 2'-O-allyloligonucleotide assembly. *Nucleic Acids Res*. 1991;19:733–8.
- [281] Grøtli M, Douglas M, Eritja R, Sproat BS. 2'-O-Propargyl oligoribonucleotides: synthesis and hybridisation. *Tetrahedron*. 1998;54:5899–914.
- [282] Martin P. Ein neuer Zugang zu 2'-O-Alkylribonucleosiden und Eigenschaften deren Oligonucleotide. *Helv Chim Acta*. 1995;78:486–504.
- [283] Cuenoud B, Casset F, Hüskens D, Natt F, Wolf RM, Altmann KH, Martin P, Moser HE. Dual recognition of double-stranded DNA by 2'-aminoethoxy-modified oligonucleotides. *Angew Chem*. 1998;37:1288–91.
- [284] Griffey RH, Monia BP, Cummins LL, Freier S, Greig MJ, Guinoss CJ, Lesnik E, Manalili SM, Mohan V, Owens S, Ross BR, Sasmor H, Wanczewicz E, Weiler K, Wheeler PD, Dan Cook P. 2'-O-Aminopropyl ribonucleotides: a zwitterionic modification that enhances the exonuclease resistance and biological activity of antisense oligonucleotides. *J Med Chem*. 1996;39:5100–9.
- [285] Teplova M, Minasov G, Tereshko V, Inamati GB, Dan Cook P, Manoharan M, Egli M. Crystal structure and improved antisense properties of 2'-O-(2-methoxyethyl)-RNA. *Nat Struct Biol*. 1999;6:535–9.
- [286] Freier SM, Altmann K-H. The ups and downs of nucleic acid duplex stability: structure-stability studies on chemically-modified DNA: RNA duplexes. *Nucleic Acids Res*. 1997;25:4429–43.
- [287] Prakash TP, Manoharan M, Fraser AS, Kawasaki AM, Lesnik EA, Owens SR. Zwitterionic oligonucleotides with 2'-O-[3-(*N*, *N*-dimethylamino) propyl]-RNA modification: synthesis and properties. *Tetrahedron Lett*. 2000;41:4855–9.

- [288] Lamond AI, Sproat BS. Antisense oligonucleotides made of 2'-O-alkylRNA: their properties and applications in RNA biochemistry. *FEBS Lett.* 1993;325:123–7.
- [289] Christoffersen RE, Marr JJ. Ribozymes as human therapeutic agents. *J Med Chem.* 1995;38:2023–37.
- [290] Ng EW, Shima DT, Calias P, Cunningham ET Jr, Guyer DR, Adamis AP. Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nat Rev Drug Discov.* 2006;5:123–32.
- [291] Monia BP, Lesnik EA, Gonzalez C, Lima WK, McGee D, Guinoss CJ, Kawasaki AM, Cook PD, Freier SM. Evaluation of 2'-modified oligonucleotide containing 2'-deoxy gaps as antisense inhibitors of gene expression. *J Biol Chem.* 1993;268:14514–22.
- [292] Butora G, Kenski DM, Cooper AJ, Fu W, Nqi N, Li JJ, Flanagan WM, Davies IW. Nucleoside optimization for RNAi: a high-throughput platform. *J Am Chem Soc.* 2011;133:16766–9.
- [293] Benseler F, Williams DM, Eckstein F. Synthesis of suitably-protected phosphoramidites of 2'-fluoro-2'-deoxyguanosine and 2'-amino-2'-deoxyguanosine for incorporation into oligoribonucleotides. *Nucleosides Nucleotides.* 1992;1992(11):1333–51.
- [294] London GM, Mayosi BM, Khati M. Isolation and characterization of 2'-F-RNA aptamers against whole HIV-1 subtype C envelope pseudovirus. *Biochem Biophys Chem Commun.* 2015;456:428–33.
- [295] Williams DM, Pieken WA, Eckstein F. Function of specific 2'-hydroxyl groups of guanosines in a hammerhead ribozyme probed by 2' modifications. *Proc Natl Acad Sci USA.* 1992;89:918–21.
- [296] Olsen DB, Benseler F, Aurup H, Pieken WA, Eckstein F. Study of a hammerhead ribozyme containing 2'-modified adenosine residues. *Biochemistry.* 1991;30:9735–41.
- [297] Pieken WA, Olsen DB, Benseler F, Aurup H, Eckstein F. Kinetic characterization of ribonuclease- resistant 2'-modified hammerhead ribozymes. *Science.* 1991;253:314–7.
- [298] Aurup H, Williams DM, Eckstein F. 2'-Fluoro- and 2'-amino-2'-deoxynucleoside 5'-triphosphates as substrates for T7 RNA polymerase. *Biochemistry.* 1992;31:9636–41.
- [299] Viazovkina E, Mangos MM, Elzagheid MI, Damha MJ. Solid-phase synthesis of 2'-deoxy-2'-fluoro- $\beta$ -D-oligoarabinonucleotides (2'F-ANA) and their phosphorothioate derivatives. *Curr Prot Nucleic Acids Chem.* 2002;4.15.1–22.
- [300] Noronha AM, Wilds CJ, Lok C-N, Viazovkina K, Arion D, Parniak MA, Damha MJ. Synthesis and biophysical properties of arabino nucleic acids (ANA): circular dichroic spectra, melting temperatures and ribonuclease H susceptibility of ANA: RNA hybrid duplexes. *Biochemistry.* 2000;39:7050–62.
- [301] Wilds CJ, Damha MJ. 2'-Deoxy-2'-fluoro- $\beta$ -D-oligoarabinonucleotides and oligonucleotides (2'F-ANA): synthesis and physicochemical studies. *Nucleic Acids Res.* 2000;28:3625–35.
- [302] Kalota A, Karabon L, Swider CR, Viazovkina E, Elzagheid M, Damha MJ, Gewirtz AM. 2'-deoxy-2'-fluoro-beta-D-arabinonucleic acid (2'F-ANA) modified oligonucleotides (ON) effect highly efficient, and persistent, gene silencing. *Nucleic Acids Res.* 2006;34:451–61.
- [303] Wilds CJ, Damha MJ. 2'F-Arabinonucleic acids (2'F-ANA) – History, properties, and new frontiers. *Can J Chem.* 2008;86:641–56.
- [304] Souleimanian N, Deleavy GF, Soifer H, Wang S, Tiemann K, Damha MJ, Stein CA. Antisense 2'-deoxy, 2'-fluoroarabino nucleic acid (2'F-ANA) oligonucleotides: in vitro gymnotic silencers of gene expression whose potency is enhanced by fatty acids. *Mol Ther Nucleic Acids.* 2012;1:e43.
- [305] Deleavy GF, Damha MJ. Designing chemically modified oligonucleotides for targeted gene silencing. *Chem Biol.* 2012;19:937–54.
- [306] Dellafiore M, Aviñó A, Alagia A, Montserrat J, Iribarren AM, Eritja R. siRNA modified with 2'-deoxy-2'-C-methylpyrimidine nucleosides. *ChemBioChem.* 2018;19:1409–13.

- [307] Pitsch S, Wendeborn S, Jaun B, Eschenmoser A. Why pentose- and not hexose-nucleic acids?? Part VII. Pyranosyl-RNA ('p-RNA'). Preliminary communication. *Helv Chim Acta*. 1993;76:2161–83.
- [308] Herdewijn P. Nucleic acids with a six-membered carbohydrate mimic in the backbone. *Chem Biodivers*. 2010;7:1–59.
- [309] Herdewijn P. Conformationally restricted carbohydrate-modified nucleic acids and antisense technology. *Biochim Biophys Acta*. 1999;1489:167–79.
- [310] Hendrix C, Rosemeyer H, Verheggen I, Seela F, Van Aerschot A, Herdewijn P. 1',5'-Anhydrohexitol Oligonucleotides: synthesis, base pairing and recognition by regular oligodeoxyribonucleotides and oligoribonucleotides. *Chem Eur J*. 1997;3:110–20.
- [311] Wang J, Verbeure B, Luyten I, Lescrinier E, Froeyen M, Hendrix C, Rosemeyer H, Seela F, Van Aerschot A, Herdewijn P. Cyclohexene Nucleic Acids (CeNA): Serum stable oligonucleotides that activate RNase H and increase duplex stability with complementary RNA. *J Am Chem Soc*. 2000;122:8595–602.
- [312] Allart B, Khan K, Rosemeyer H, Schepers G, Hendrix C, Rothenbacher K, Seela F, Van Aerschot A, Herdewijn P. D-Altritol nucleic acids (ANA): hybridisation properties, stability, and initial structural analysis. *Chem Eur J*. 1999;5:2424–31.
- [313] Abramov M, Schepers G, Van Aerschot A, Herdewijn P. Fmoc-protected altritol phosphoramidite building blocks and their application in the synthesis of altritol nucleic acids (ANAs). *Eur J Org Chem*. 2007;1446–56.
- [314] Egli M, Pallan PS, Allerson CR, Prakash TP, Berdeja A, Yu J, Lee S, Watt A, Gaus H, Bhat B, Swayze EE, Seth PP. Synthesis, improved antisense activity and structural rationale for the divergent RNA affinities of 3'-fluoro hexitol nucleic acid (FHNA and Ara-FHNA) modified oligonucleotides. *J Am Chem Soc*. 2011;133:16642–9.
- [315] Fisher M, Abramov M, Van Aerschot A, Rozenski J, Dixit V, Juliano RL, Herdewijn P. Biological effects of hexitol and altritol-modified siRNAs targeting B-Raf. *Eur J Pharmacol*. 2009;606:38–44.
- [316] Bramsen JB, Laursen MB, Nielsen AF, Hansen TB, Bus C, Langkjær N, Babu BR, Højland T, Abramov M, Van Aerschot A, Odadzic D, Smiclus R, Haas J, Andree C, Barman J, Wenska M, Srivastava P, Zhou C, Honcharenko D, Hess S, Müller E, Bobkov GV, Mikhailov SN, Fava E, Meyer TF, Chattopadhyaya J, Zerial M, Engels JW, Herdewijn P, Wengel J, Kjems J. A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity. *Nucleic Acids Res*. 2009;37:2867–81.
- [317] Kumar P, Degaonkar R, Guenther DC, Abramov M, Schepers G, Capobianco M, Jiang Y, Harp J, Kaittanis C, Janas MM, Castoreno A, Zlatev I, Schlegel MK, Herdewijn P, Egli M, Manoharan M. *Nucleic Acids Res*. 2020;48:4028–40.
- [318] Morvan F, Rayner B, Imbach JL.  $\alpha$ -Oligodeoxynucleotides. In: Agrawal S, editor. *Protocols for nucleotides and analogs*. Meth Mol Biol. vol. 20. Totowa, NJ: Humana Press Inc; 1993. p. 261–83.
- [319] Morvan F, Rayner B, Leonetti J-P, Imbach J-L.  $\alpha$ -DNA VII. Solid phase synthesis of  $\alpha$ -anomeric oligodeoxyribonucleotides. *Nucleic Acids Res*. 1988;16:833–47.
- [320] Debart F, Rayner B, Degols G, Imbach J-L. Synthesis and base-pairing properties of the nuclease-resistant  $\alpha$ -anomeric dodecaribonucleotide  $\alpha$ -[r(UCUUACCCACA)]. *Nucleic Acids Res*. 1992;20:1193–200.
- [321] Gagnor C, Bertrand J-R, Thenet S, Lemaitre M, Morvan F, Rayner B, Malvy C, Lebleu B, Imbach J-L, Paoletti C.  $\alpha$ -DNA VI: Comparative study of  $\alpha$ - and  $\beta$ -anomeric oligodeoxyribonucleotides in hybridization to mRNA and in cell free translation inhibition. *Nucleic Acids Res*. 1987;15:10419–36.

- [322] Cazenave C, Chevrier M, Thuong NT, Helene C. Rate of degradation of  $[\alpha]$ - and  $[\beta]$ -oligodeoxynucleotides in *Xenopus* oocytes. Implications for anti-messenger strategies. *Nucleic Acids Res.* 1987;15:10507–21.
- [323] Boiziau C, Kurfurst R, Cazenave C, Roig V, Thuong NT, Toulme J-J. Inhibition of translation initiation by antisense oligonucleotides via an RNase-H independent mechanism. *Nucleic Acids Res.* 1991;19:1113–9.
- [324] Veedu RN, Locked WJ. Nucleic acids: promising nucleic acid analogs for therapeutic applications. *Chem Biodivers.* 2010;7:536–42.
- [325] Kaur H, Babu BR, Maiti S. Perspectives on chemistry and therapeutic applications of Locked Nucleic Acid (LNA). *Chem Rev.* 2007;107:4672–97.
- [326] Pallan PS, Allerson CR, Berdeja A, Seth PP, Swayze EE, Prakash TP, Egli M. Structure and nuclease resistance of 2',4'-constrained 2'-O-methoxyethyl (cMOE) and 2'-O-ethyl (cEt) modified DNAs. *Chem Commun.* 2012;48:8195–7.
- [327] Bolli M, Trafelet HU, Leumann C. Watson–Crick base-pairing properties of bicyclo-DNA. *Nucleic Acids Res.* 1996;24:4660–7.
- [328] Renneberg D, Leumann CJ. Watson–Crick base-pairing properties of tricyclo-DNA. *J Am Chem Soc.* 2002;124:5993–6002.
- [329] Terrazas M, Ocampo SM, Perales JC, Marquez V, Eritja R. Effect of North bicyclo[3.1.0]hexane pseudosugars on RNA interference. A novel class of siRNA modification. *ChemBioChem.* 2011;12:1056–65.
- [330] Terrazas M, Aviñó A, Siddiqui MA, Marquez VE, Eritja R. A direct, efficient method for the preparation of siRNAs containing ribo-like North bicyclo[3.1.0]hexane pseudosugars. *Org Lett.* 2011;13:2888–91.
- [331] Jepsen J, Sorensen MD, Locked WJ. Nucleic acid: a potent nucleic acid analog in therapeutics and biotechnology. *Oligonucleotides.* 2004;14:130–46.
- [332] Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, Horvitz HR, Kauppinen S, Plasterk RHA. MicroRNA expression in zebrafish embryonic development. *Science.* 2005;309:310–1.
- [333] Wahlestedt C, Salmi P, Good L, Kela J, Johnsson T, Hokfelt T, Broberger C, Porreca F, Lai J, Ren K, Ossipov M, Koshkin A, Jakobsen N, Skouw J, Ørum H, Jacobsen MH, Wengel J. Potent and nontoxic antisense oligonucleotides containing Locked Nucleic Acids. *Proc Natl Acad Sci USA.* 2000;97:5633–8.
- [334] Elmen J, Thonberg H, Ljungberg K, Frieden M, Westergaard M, Xu Y, Wahren B, Liang Z, Ørum H, Koch T, Wahlestedt C. Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Res.* 2005;33:439–47.
- [335] Wengel J, Petersen M, Frieden M, Kock T. Chemistry of locked nucleic acids (LNA): design, synthesis and bio-physical properties. *Lett Pept Sci.* 2003;10:237–53.
- [336] Jadhav VM, Scaria V, Maiti S. Antagomirzymes: oligonucleotide enzymes that specifically silence microRNA function. *Angew Chem, Int Ed Engl.* 2009;48:2557–60.
- [337] Schmidt KS, Borkowski S, Kurreck J, Stephens AW, Bald R, Hecht M, Fribe M, Dinkelborg L, Erdmann VA. Application of locked nucleic acids to improve aptamer *in vivo* stability and targeting function. *Nucleic Acids Res.* 2004;32:5757–65.
- [338] Sun BW, Babu R, Sorensen MD, Zakrzewska K, Wengel J, Sun JS. Sequence and pH effects of LNA-containing triple helix-forming oligonucleotides: physical chemistry, biochemistry, and modeling studies. *Biochemistry.* 2004;43:4160–9.
- [339] Abplanalp WT, Fischer A, John D, Zeiher AM, Gosgnach W, Darville H, Montgomery R, Pestano L, Alleé G, Paty I, Fougerousse F, Dimmeler S. Efficiency and target derepression of anti-miR-92a: results of a first in human study. *Nucleic Acid Ther.* 2020, in press, DOI:10.1089/nat.2020.0871.

- [340] Seth PP, Vasquez G, Allerson CA, Berdeja A, Gaus H, Kinberger GA, Prakash TP, Migawa MT, Bhat B, Swayze EE. Synthesis and biophysical evaluation of 2',4'-constrained 2' O-methoxyethyl and 2',4'-constrained 2' O-ethyl nucleic acid analogues. *J Org Chem.* 2010;75:1569–81.
- [341] Blade H, Bradley D, Diorazio L, Evans T, Hayter BR, Howell GP. Modular synthesis of constrained ethyl (cEt) purine and pyrimidine nucleosides. *J Org Chem.* 2015;80:5337–43.
- [342] Seth PP, Siwkowski A, Allerson CA, Vasquez G, Lee S, Prakash TP, Wancewicz EV, Wirczell D, Swayze EE. Short antisense oligonucleotides with novel 2'-4' conformationally restricted nucleoside analogues show improved potency without increased toxicity in animals. *J Med Chem.* 2009;52:10–3.
- [343] Pandey SK, Wheeler TM, Justice SL, Kim A, Younis HS, Gattis D, Jauvin D, Puymirat J, Swayze EE, Freier SM, Bennett CF, Thornton CA, MacLeod AR. Identification and characterization of modified antisense oligonucleotides targeting DMPK in mice and nonhuman primates for the treatment of myotonic dystrophy type 1. *J Pharmacol Exp Ther.* 2015;355:329–40.
- [344] Burel SA, Han SR, Lee HS, Norris DA, Lee BS, Machemer T, Park SY, Zhou T, He G, Kim Y, MacLeod AR, Monia BP, Lio S, Kim TW, Henry SP. Preclinical evaluation of the toxicological effects of a novel constrained ethyl modified antisense compound targeting signal transducer and activator of transcription 3 in mice and cynomolgus monkeys. *Nucleic Acid Ther.* 2013;23:213–27.
- [345] Prakash TP, Siwkowski A, Allerson CR, Migawa MT, Lee S, Gaus HJ, Black C, Seth PP, Swayze EE, Bhat B. Antisense oligonucleotides containing conformationally constrained 2',4'-(*N*-methoxy)aminomethylene and 2',4'-aminoxyimethylene and 2'-O,4'-C-aminomethylene bridged nucleoside analogues show improved potency in animal models. *J Med Chem.* 2010;53:1636–50.
- [346] Bolli M, Litten JC, Schütz R, Leumann C. Bicyclo-DNA: a Hoogsteen-selective pairing system. *Chem Biol.* 1996;3:197–206.
- [347] Steffens R, Leumann C. Synthesis and thermodynamic and biophysical properties of tricyclo-DNA. *J Am Chem Soc.* 1999;121:3249–55.
- [348] Renneberg D, Bouliong E, Reber U, Schümperli D, Leumann CJ. Antisense properties of tricyclo-DNA. *Nucleic Acids Res.* 2002;30:2751–7.
- [349] Saneyoshi H, Mazzini S, Aviñó A, Portella G, González C, Orozco M, Marquez V, Eritja R. Conformationally rigid nucleoside probes help understand the role of sugar pucker and nucleobase orientation in the thrombin binding aptamer. *Nucleic Acids Res.* 2009;37:5589–601.
- [350] Langkjær N, Pasternak A, Wengel J. UNA (unlocked nucleic acid): a flexible RNA mimic that allows engineering of nucleic acid duplex stability. *Bioorg Med Chem.* 2009;17:5420–5.
- [351] Werk D, Wengel J, Wengel SL, Grunert HP, Zeichhardt H, Kurreck J. Application of small interfering RNAs modified by unlocked nucleic acid (UNA) to inhibit the heart-pathogenic coxsackievirus B3. *FEBS Lett.* 2010;584:591–8.
- [352] Campbell MA, Wengel J. Locked vs. unlocked nucleic acids (LNA vs. UNA): contrasting structures work towards common therapeutic goals. *Chem Soc Rev.* 2011;40:5680–9.
- [353] Bramsen JB, Pakula MM, Hansen TB, Bus C, Langkjær N, Odadzic D, Smiclus R, Wengel SL, Chattopadhyaya J, Engels JW, Herdewijn P, Wengel J, Kjems J. A screen of chemical modifications identifies position-specific modification by UNA to most potently reduce siRNA off-target effects. *Nucleic Acids Res.* 2010;38:5761–73.
- [354] Pasternak A, Wengel J. Thermodynamics of RNA duplexes modified with unlocked nucleic acid nucleotides. *Nucleic Acids Res.* 2010;38:6697–706.

- [355] Laursen MB, Pakula MM, Gao S, Fluiter K, Mook OR, Baas F, Langklaer N, Wengel SL, Wengel J, Kjems J, Bramsen JB. Utilization of unlocked nucleic acid (UNA) to enhance siRNA performance in vitro and in vivo. *Mol BioSyst.* 2010;6:862–70.
- [356] Pasternak A, Wengel J. Unlocked nucleic acid – an RNA modification with broad potential. *Org Biomol Chem.* 2011;9:3591–7.
- [357] Kotkowiak W, Kotkowiak M, Kierzek R, Pasternak A. Unlocked nucleic acids: implications of increased conformational flexibility for RNA/DNA triplex formation. *Biochem J.* 2014;464:203–11.
- [358] Zhang L, Peritz A, Meggers E. A simple glycol nucleic acid. *J Am Chem Soc.* 2005;127:4174–5.
- [359] Zhang L, Peritz AE, Carroll PJ, Meggers E. Synthesis of glycol nucleic acids. *Synthesis.* 2006;645–53.
- [360] Schlegel MK, Peritz AE, Kittigowittana K, Zhang L, Meggers E. Duplex formation of the simplified nucleic acid GNA. *ChemBioChem.* 2007;8:927–32.
- [361] Meggers E, Zhang L. Synthesis and properties of the simplified nucleic acid glycol nucleic acid. *Acc Chem Res.* 2010;43:1092–102.
- [362] Zhang S, Switzer C, Chaput JC. The resurgence of acyclic nucleic acids. *Chem Biodivers.* 2010;7:245–58.
- [363] Zhang S, Chaput JC. Synthesis of Glycerol Nucleic Acid (GNA) phosphoramidite monomers and oligonucleotide polymers. *Curr Prot Nucleic Acids Chem.* 2010;4.40.1–18.
- [364] Acevedo OL, Andrews RS. Synthesis of propane-2,3-diol combinatorial monomers. *Tetrahedron Lett.* 1996;1996(37):3913–4.
- [365] Schlegel MK, Meggers E. Improved phosphoramidite building blocks for the synthesis of the simplified nucleic acid GNA. *J Org Chem.* 2009;74:4615–8.
- [366] Zhang RS, McCullum EO, Chaput JC. Synthesis of two mirror image 4-helix junctions derived from glycerol nucleic acid. *J Am Chem Soc.* 2008;130:5846–7.
- [367] Kallenbach NR, Ma R-I, Seeman NC. An immobile nucleic acid junction constructed from oligonucleotides. *Nature.* 1983;305:829–31.
- [368] Vengut-Climent E, Gómez-Pinto I, Lucas R, Peñalver P, Aviñó A, Fonseca-Guerra C, Bickelhaupt FM, Eritja R, González C, Morales JC. Glucose-nucleobase pseudo base pairs as a new biomolecular interaction in a DNA context. *Angew Chem, Int Ed Engl.* 2016;55:8643–7.
- [369] Vengut-Climent E, Peñalver P, Lucas R, Gómez-Pinto I, Aviñó A, Muro-Pastor A, Galbis E, de Paz V, Fonseca Guerra C, Bickelhaupt M, Eritja R, González C, Morales JC. Glucose-nucleobase pairs within DNA: impact of increased hydrophobicity, wider linking unit and DNA polymerase nucleotide insertion studies. *Chem Sci.* 2018;9:3544–54.
- [370] Janas MM, Schlegel MK, Harbison CE, Yilmaz VO, Jiang Y, Parmar R, Zlatev I, Castoreno A, Xu H, Shulga-Morskaya S, Rajeev KG, Manoharan M, Keirstead ND, Maier MA, Jadhav V. Selection of GalNAc-conjugated siRNAs with limited off-target-driven rat hepatotoxicity. *Nat Commun.* 2018;9:723.
- [371] Schlegel MK, Foster DJ, Kel'in AV, Zlatev I, Bisbe A, Jayaraman M, Lackey JG, Rajeev KG, Charissé K, Harp J, Pallan PS, Maier MA, Egli M, Manoharan M. Chirality dependent potency enhancement and structural impact of glycol nucleic acid modification on siRNA. *J Am Chem Soc.* 2017;139:8537–46.
- [372] Asanuma H, Toda T, Murayama K, Liang X, Kashida H. Unexpectedly stable artificial duplex from flexible acyclic threoninol. *J Am Chem Soc.* 2010;132:14702–3.
- [373] Murayama K, Tanaka Y, Toda T, Kashida H, Asanuma H. Highly stable duplex formation by artificial nucleic acids acyclic threoninol nucleic acid (aTNA) and serinol nucleic acid (SNA) with acyclic scaffolds. *Chem Eur J.* 2013;19:14151–8.
- [374] Kashida H, Murayama K, Toda T, Asanuma H. Control of the chirality and helicity of oligomers of serinol nucleic acids (SNA) by sequence design. *Angew Chem, Int Ed Engl.* 2011;50:1285–8.

- [375] Alagia A, Terrazas M, Eritja R. RNA/aTNA chimeras: RNAi effects and nuclease resistance of single and double stranded RNAs. *Molecules*. 2014;19:17872–96.
- [376] Alagia A, Terrazas M, Eritja R. Modulation of the RNA interference activity using central mismatched siRNAs and acyclic threoninol nucleic acids (aTNA) units. *Molecules*. 2015;20:7602–19.
- [377] Kamiya Y, Takai J, Ito H, Murayama K, Kashida H, Asanuma H. Enhancement of stability and activity of siRNA by terminal substitution with serinol nucleic acid (SNA). *ChemBioChem*. 2014;15:2549–55.
- [378] Alagia A, Jorge AF, Aviñó A, Cova TFGG, Crehuet R, Grijalvo S, Pais AAC, Eritja R. Exploring PAZ/3'-overhang interaction to improve siRNA specificity. A combined experimental and modeling study. *Chem Sci*. 2018;9:2074–86.
- [379] Le BT, Murayama K, Shabanpoor F, Asanuma H, Veedu RN. Antisense oligonucleotide modified with serinol nucleic (SNA) induces exon skipping in mdx myotubes. *RSC Adv*. 2017;7:34049–52.
- [380] Summerton J, Weller D. Morpholino antisense oligomers: design, preparation and properties. *Antisense Nucleic Acid Drug Dev*. 1997;7:187–95.
- [381] Amantana A, Iversen PL. Pharmacokinetics and biodistribution of phosphorodiamidate morpholino antisense oligomers. *Curr Opin Pharmacol*. 2005;5:550–5.
- [382] Summerton JE. Morpholino, siRNA, and SDNA compared: impact of structure and mechanism of action on off-target effects and sequence specificity. *Curr Top Med Chem*. 2007;7:651–60.
- [383] Bhadra J, Pattanayak S, Sinha S. Synthesis of morpholino monomers, chlorophosphoramidate monomers, and solid-phase synthesis of short morpholino oligomers. *Curr Protoc Nucleic Acid Chem*. 2015;2015:4.65.1–4.65.26.
- [384] Bhadra J, Kundu J, Ghosh KC, Sinha S. Synthesis of phosphorodiamidate morpholino oligonucleotides by the H-phosphonate method. *Tetrahedron Lett*. 2015;56:4565–8.
- [385] Zhang N, Tan C, Cai P, Jiang Y, Zhang P, Zhao Y. Synthesis and properties of morpholino chimeric oligonucleotides. *Tetrahedron Lett*. 2008;49:3570–3.
- [386] Paul S, Pattanayak S, Sinha S. Synthesis and cell transfection properties of cationic uracil-morpholino tetramer. *Tetrahedron Lett*. 2014;55:1072–6.
- [387] Moulton JD. Using morpholinos to control gene expression. *Curr Protoc Nucleic Acid Chem*. 2006;4.30.1–26.
- [388] Heasman J. Morpholino oligos: making sense of antisense? *Dev Biol*. 2002;243:209–14.
- [389] Morcos PA. Achieving targeted and quantifiable alteration of mRNA splicing with morpholino oligos. *Biochem Biophys Res Commun*. 2007;358:521–7.
- [390] Nielsen PE, Egholm M, Berg RH, Buchardt O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science*. 1991;254:1497–500.
- [391] Nielsen PE, Peptide EM. Nucleic acids. Protocols and applications. England: Horizon Scientific Press; 1999.
- [392] Nielsen PE. Peptide nucleic acids. Methods and protocols. *Meth Mol Biol*. vol. 2105. New York: Humana Press; 2020.
- [393] Nielsen PE, Egholm M, Buchardt O. Evidence for (PNA)<sub>2</sub>/DNA triplex structure upon binding of PNA to dsDNA by strand displacement. *J Mol Recognit*. 1994;7:165–70.
- [394] Egholm M, Christensen L, Dueholm KL, Buchardt O, Coull J, Nielsen PE. Efficient pH-independent sequence-specific DNA binding by pseudoisocytosine-containing bis-PNA. *Nucleic Acids Res*. 1995;23:217–22.
- [395] Braasch DA, Nulf CJ, Corey DR. Synthesis and purification of peptide nucleic acids. *Curr Protoc Nucleic Acid Chem*. 2002;4.11.1–18.

- [396] Dueholm KL, Egholm M, Behrens C, Christensen L, Hansen HF, Vulpius T, Petersen KH, Berg RH, Nielsen PE, Buchardt O. Synthesis of peptide nucleic acid monomers containing the four natural nucleobases: thymine, cytosine, adenine and guanine and their oligomerization. *J Org Chem.* 1994;59:5767–73.
- [397] Thomson SA, Josey JA, Cadilla R, Gaul MD, Hassman CF, Luzzio MJ, Pipe AJ, Reed KL, Rica DJ, Wiethe RW, Noble SA. Fmoc mediated synthesis of peptide nucleic acids. *Tetrahedron.* 1995;51:6179–94.
- [398] Will DW, Breipohl G, Langer D, Knolle J, Uhlmann E. The synthesis of polyamide nucleic acids using a novel monomethoxytrityl protecting group strategy. *Tetrahedron.* 1995;51:12069–82.
- [399] Ferrer E, Eisenhut M, Eritja R. A convenient route for the preparation of peptide nucleic acid monomers carrying acid-labile groups for the protection of the amino function. *Lett Peptide Sci.* 1999;6:209–19.
- [400] Uhlmann E, Will DW, Breipohl G, Langner D, Ryte A. Synthesis and properties of PNA/DNA chimeras. *Angew Chem, Int Ed Engl.* 1996;35:2632–5.
- [401] Good L, Sandberg R, Larsson O, Nielsen PE, Antisense WC. PNA effects in *Escherichia coli* are limited by the outer-membrane LPS layer. *Microbiology.* 2000;148:2665–70.
- [402] Good L, Awasthi SK, Dryselius R, Larsson O, Nielsen PE. Bactericidal antisense effects of Peptide-PNA conjugates. *Nature Biotech.* 2001;19:360–4.
- [403] Hansen AM, Bonke G, Larsen CJ, Yavari N, Nielsen PE, Franzyk H. Antibacterial peptide nucleic acid-antimicrobial peptide (PNA-AMP) conjugates: antisense targeting of fatty acid biosynthesis. *Bioconjug Chem.* 2016;27:863–7.
- [404] Goltermann L, Yavari N, Zhang M, Ghosal A, Nielsen PE. PNA Length restriction of antibacterial activity of peptide-PNA conjugates in *Escherichia coli* through effects of the inner membrane. *Front Microbiol.* 2019;10:1032.
- [405] Uhlmann E, Peyman A. Oligonucleotide analogs containing dephosphointernucleoside linkages. In: Agrawal S, editor. *Protocols for nucleotides and analogs.* Meth Mol Biol. vol. 20. Totowa, NJ: Humana Press Inc; 1993. p. 355–89.
- [406] Edge MD, Hodgson A, Jones AS, MacCoss M, Walker RT. Synthetic analogs of polynucleotides. Part IX. Synthesis of 3'-O-carboxymethyl-2'-deoxyribonucleosides and their use in the synthesis of an analog of 2'-deoxyadenyl-(3'-5')thymidine 3'-phosphate. *J Chem Soc Perkin I.* 1973;290–4.
- [407] Nyilas A, Glemarec C, Chattopadhyaya J. Synthesis of [3'(O)-5'(C)]-oxyacetamido linked nucleosides. *Tetrahedron.* 1990;46:2149–64.
- [408] Idziak I, Just G, Damha MJ, Giannaris PA. Synthesis and hybridization properties of amide-linked thymidine dimers incorporated into oligodeoxynucleotides. *Tetrahedron Lett.* 1993;34:5417–20.
- [409] De Mesmaeker A, Lebrenton J, Waldner A, Lebreton J, Hoffmann P, Fritsch V, Wolf RM, Freier SM. Amides as a new type of backbone modification in oligonucleotides. *Angew Chem, Int Ed Engl.* 1994;33:226–9.
- [410] Mungall WS, Kaiser JK. Carbamate analogs of oligonucleotides. *J Org Chem.* 1977;42:703–6.
- [411] Stirchak D, Summerton JE, Weller DD. Uncharged stereoregular nucleic acid analogs. 1. Synthesis of a cytosine-containing oligomer with carbamate internucleoside linkages. *J Org Chem.* 1987;52:4202–6.
- [412] Coull JM, Carlton DV, Weith HL. Synthesis and characterization of a carbamate-linked oligonucleoside. *Tetrahedron Lett.* 1987;28:745–8.
- [413] Kutterer KMK, Just G. Synthesis and hybridization studies of urea and carbamate linked thymidine dimers incorporated into oligodeoxynucleosides. *Bioorg Med Chem Lett.* 1994;4:435–8.

- [414] Waldner A, De Mesmaeker A, Lebrenton J, Fritsch V, Wolf RM. Ureas as backbone replacements for the phosphodiester linkage in oligonucleotides. *Synlett*. 1994;57–61.
- [415] Thorpe C, Epple S, Woods B, El-Sagheer AH, Brown T. Synthesis and biophysical properties of carbamate-locked nucleic acid (LNA) oligonucleotides with potential antisense applications. *Org Biomol Chem*. 2019;17:5341–8.
- [416] Morvan F, Sanghvi YS, Perbost M, Vasseur JJ, Bellon L. Oligonucleotide mimics for antisense therapeutics: solution phase and automated solid-support synthesis of MMI linked oligomers. *J Am Chem Soc*. 1996;118:255–6.
- [417] Debart F, Vasseur JJ, Sanghvi YS, Cook PD. Synthesis and incorporation of methyleneoxy (methylimino) linked thymidine dimer into antisense oligonucleosides. *Bioorg Med Chem Lett*. 1992;2:1479–82.
- [418] von Matt P, Altmann KH. Replacement of the phosphodiester linkage in oligonucleotides by heterocycles: the effect of triazole-and imidazole-modified backbones on DNA/RNA duplex stability. *Bioorg Med Chem Lett*. 1997;7:1553–6.
- [419] El-Sagheer AH, Brown T. Synthesis and polymerase chain reaction amplification of DNA strands containing an unnatural triazole linkage. *J Am Chem Soc*. 2009;131:3958–64.
- [420] Varizhuk A, Chizhov A, Florentiev V. Synthesis and hybridization data of oligonucleotide analogs with triazole internucleotide linkages, potential antiviral and antitumor agents. *Bioorg Chem*. 2011;39:127–31.
- [421] Varizhuk AM, Kaluzhny DN, Novikov RA, Chizhov AO, Smirnov IP, Chuvilin AN, Tatarinova ON, Fisunov GY, Pozmogova GE, Florentiev VL. Synthesis of triazole-linked oligonucleotides with high affinity to DNA complements and an analysis of their compatibility with biosystems. *J Org Chem*. 2013;78:5964–9.
- [422] Jain ML, Bruice PY, Szabo IE, Bruice TC. Incorporation of positively charged linkages into DNA and RNA backbones: a novel strategy for antigene and antisense agents. *Chem Rev*. 2012;112:1284–309.
- [423] Arya DP, Bruice TC. Positively charged deoxynucleic methylthioureas: synthesis and binding properties of pentameric thymidyl methylthiourea. *J Am Chem Soc*. 1998;120:12419–27.
- [424] Arya DP, Bruice TC. Fidelity of deoxynucleic S-methylthiourea (DNmt) binding to DNA oligomers: influence of C mismatches. *J Am Chem Soc*. 1999;121:10680–4.
- [425] Challa H, Bruice TC. Incorporation of positively charged deoxynucleic S-methylthiourea linkages into oligodeoxyribonucleotides. *Bioorg Med Chem Lett*. 2001;11:2423–7.
- [426] Dempcy RO, Browne K, Bruice TC. Synthesis of the polycation thymidyl DNG, its fidelity in binding polyanionic DNA/RNA, and the stability and nature of the hybrid complexes. *J Am Chem Soc*. 1995;117:6140–1.
- [427] Blasko A, Dempcy RO, Minyat EE, Bruice TC. Association of short-strand DNA oligomers with guanidinium-linked nucleosides. A kinetic and thermodynamic study. *J Am Chem Soc*. 1996;118:7892–9.
- [428] Linkletter BA, Szabo IE, Bruice TC. Solid-phase synthesis of oligopurine deoxynucleic guanidine (DNG) and analysis of binding with DNA oligomers. *Nucleic Acids Res*. 2001;29:2370–6.
- [429] Szabo IE, Bruice TC. DNG cytidine: synthesis and binding properties of octameric guanidinium-linked deoxycytidine oligomer. *Bioorg Med Chem*. 2004;12:4233–4.
- [430] Challa H, Bruice TC. Deoxynucleic guanidine: synthesis and incorporation of purine nucleosides into positively charged DNG oligonucleotides. *Bioorg Med Chem*. 2004;12:1475–81.
- [431] Park M, Bruice TC. Development of potential anticancer agents that target the telomere sequence. *Bioorg Med Chem Lett*. 2010;20:3982–6.

- [432] Barawkar DA, Kwok Y, Bruice TW, Bruice TC. Deoxynucleic guanidine/peptide nucleic acid chimeras: synthesis, binding and invasion studies with DNA. *J Am Chem Soc.* 2000;122:5244–50.
- [433] Lima WF, Wu H, Nichols JG, Sun H, Murray HM, Crooke ST. Binding and cleavage specificities of human Argonaute2. *J Biol Chem.* 2009;284:26017–28.
- [434] Horn T, Urdea MS. A chemical 5'-phosphorylation of oligodeoxyribonucleotides that can be monitored by trityl cation release. *Tetrahedron Lett.* 1986;27:4705–8.
- [435] Celebuski JE, Chan C, Jones RA. Synthesis and utility of a DNA phosphorylating agent based on 2-(triphenylsilyl)ethanol. *J Org Chem.* 1992;57:5535–8.
- [436] Guzaev A, Salo H, Azhayaev A, Lönnberg H. A new approach for chemical phosphorylation of oligonucleotides at the 5'-terminus. *Tetrahedron.* 1995;51:9375–84.
- [437] Parmar R, Willoughby JLS, Liu J, Foster DJ, Brigham B, Theile CS, Charisse K, Akinc A, Guidry E, Pei Y, Strapps W, Cancilla M, Stanton MG, Rajeev KG, Sepp-Lorenzino L, Manoharan M, Meyers R, Maier MA, Jadhav V. 5'-(E)-Vinylphosphonate: a stable phosphate mimic can improve the RNAi activity of siRNA-GalNAc conjugates. *ChemBioChem.* 2016;17:985–9.
- [438] Prakash TP, Kinberger GA, Murray HM, Chappell A, Riney S, Graham MJ, Lima WF, Swayze EE, Seth PP. Synergistic effect of phosphorothioate, 5'-vinylphosphonate and GalNAc modifications for enhancing activity of synthetic siRNA. *Bioorg Med Chem Lett.* 2016;26:2817–20.
- [439] Lima WF, Prakash TP, Murray HM, Kinberger GA, Li W, Chappell AE, Li CS, Murray SF, Gaus H, Seth PP, Swayze EE, Crooke ST. Single-stranded siRNAs activate RNAi in animals. *Cell.* 2012;150:883–94.
- [440] Yu D, Pendergraft H, Liu J, Kordasiewicz HB, Cleveland DW, Swayze EE, Lima WF, Crooke ST, Prakash TP, Corey DR. Single-stranded RNAs use RNAi to potently and allele-selectively inhibit mutant Huntingtin expression. *Cell.* 2012;150:895–908.
- [441] Prakash TP, Lima WF, Murray HM, Li W, Kinberger GA, Chappell AE, Gaus H, Seth PP, Bhat B, Crooke ST, Swayze EE. Identification of metabolically stable 5'-phosphate analogs that support single-stranded siRNA activity. *Nucleic Acids Res.* 2015;43:2993–3011.
- [442] Haraszti RA, Roux L, Coles AH, Turanov AA, Alterman JF, Echeverria D, Godinho BMDC, Aronin N, Khvorova A. 5-Vinylphosphonate improves tissue accumulation and efficacy of conjugated siRNAs in vivo. *Nucleic Acids Res.* 2017;45:7581–92.
- [443] Biscans A, Caiazzo J, Davis S, McHugh N, Sousa J, Khvorova A. The chemical structure and phosphorothioate content of hydrophobically modified siRNAs impact extrahepatic distribution and efficacy. *Nucleic Acids Res.* 2020;48:7665–80.
- [444] Zlatev I, Foster DJ, Liu J, Charisse K, Brigham B, Parmar RG, Jadhav V, Maier MA, Rajeev KG, Egli M, Manoharan M. 5'-C-Malonyl RNA: small interfering RNAs modified with 5'-monophosphate bioisostere demonstrate gene silencing activity. *ACS Chem Biol.* 2016;11:953–60.



Andreia F. Jorge, Santiago Grijalvo, Anna Aviñó, Carme Fàbrega,  
and Ramon Eritja

## 8 Oligonucleotide conjugates and DNA nanotechnology

### Contents

8.1	Oligonucleotide conjugates —	331
8.1.1	Lipid-oligonucleotide conjugates (LOC) —	332
8.1.2	Peptide-oligonucleotide conjugates (POC) —	334
8.1.3	Proteins functionalized with oligonucleotides (PFO) —	337
8.1.4	Carbohydrate-oligonucleotide conjugates (COCs) —	338
8.1.5	Oligonucleotide functionalized with substrates for receptor-mediated recognition —	341
8.2	DNA nanotechnology —	342
8.2.1	Gold nanoparticles functionalized with oligonucleotides —	342
8.2.2	DNA nanostructures and DNA origami —	343
8.2.3	DNA nanostructures in drug delivery —	344
	Bibliography —	346

### 8.1 Oligonucleotide conjugates

Oligonucleotides are essential tools for DNA detection and manipulation. At the end of the 1980s, the discovery of the polymerase chain reaction (PCR) and the potential use of oligonucleotides as inhibitors of gene expression triggered a high demand for oligonucleotides and conjugates with new, tailored properties. These new properties were obtained as a result of the addition of special molecules to oligonucleotides, resulting in oligonucleotide conjugates or chimeras. In Chapter 4, we have described the methods used for the preparation of oligonucleotides carrying fluorescent compounds as well as lipid and other ligands. Here, we summarize some of the most commonly used oligonucleotide conjugates for potential therapeutic applications with special emphasis on the results from our group. A large number of excellent reviews can be found in the bibliography for further reading [1–11].

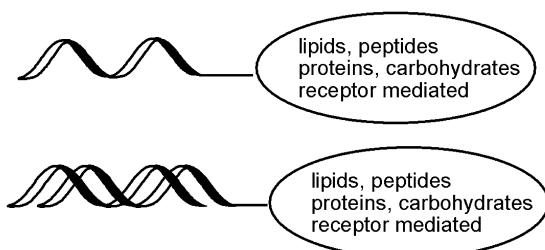
Generally, the production of oligonucleotide conjugates for therapeutics aims to develop novel compounds with enhanced stability to nucleases and favorable pharmacokinetics. Most of the modifications are located at the 3' or 5'-ends of the oligonucleotides but other internal positions within the nucleotide phosphate backbone are also available. Certain tethered ligands have been found to improve the

---

**Andreia F. Jorge, Santiago Grijalvo, Anna Aviñó, Carme Fàbrega, Ramon Eritja**, Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Networking Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Jordi Girona 18-26, E-08034 Barcelona, Spain, e-mails:  
andreiajorge09@gmail.com, santiago.grijalvo@iqac.csic.es, aaagma@cid.csic.es,  
carme.fabrega@iqac.csic.es, recgma@cid.csic.es

cellular delivery of oligonucleotides and increase their affinity for the target gene. Other modifications may play the role of synthetic nucleases. Other modifications can modulate the extent of protein binding either to enhance biodistribution or to lessen side effects due to nonspecific protein binding [1]. This field has become especially relevant for the design of novel therapeutic oligonucleotide for *in vivo* targeting after the excellent results obtained in liver targeting of siRNA using the triantennary N-acetylgalactosamine (GalNAc) discussed in the previous Chapter 7.

We have classified the conjugates depending on the nature of the organic ligand in lipid-oligonucleotide conjugates (LOC), peptide-oligonucleotide conjugates (POC), oligonucleotide functionalized proteins (OFP), carbohydrate-oligonucleotide conjugate (COC) and oligonucleotides carrying substrates for receptor binding (Figure 8.1).

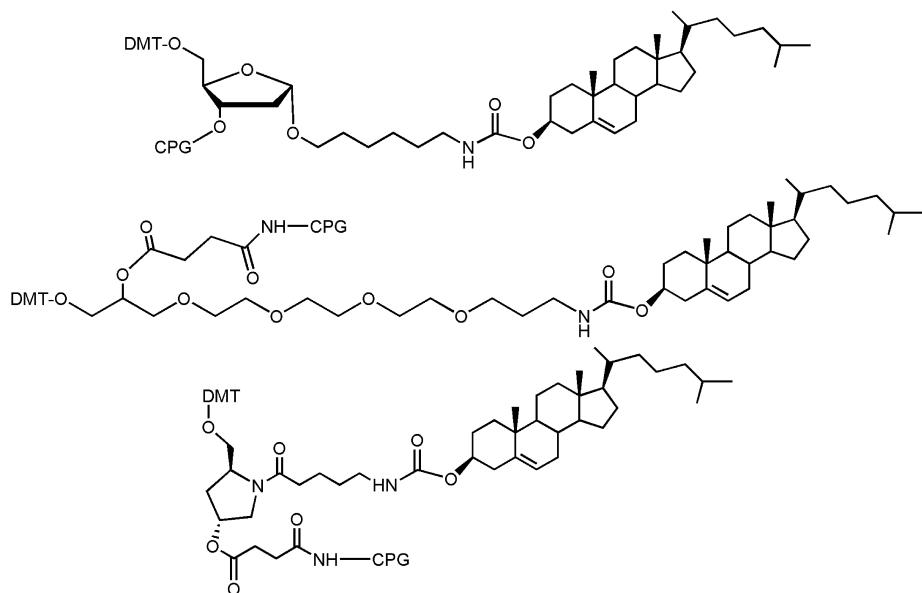


**Figure 8.1:** General scheme of oligonucleotide conjugates.

### 8.1.1 Lipid-oligonucleotide conjugates (LOC)

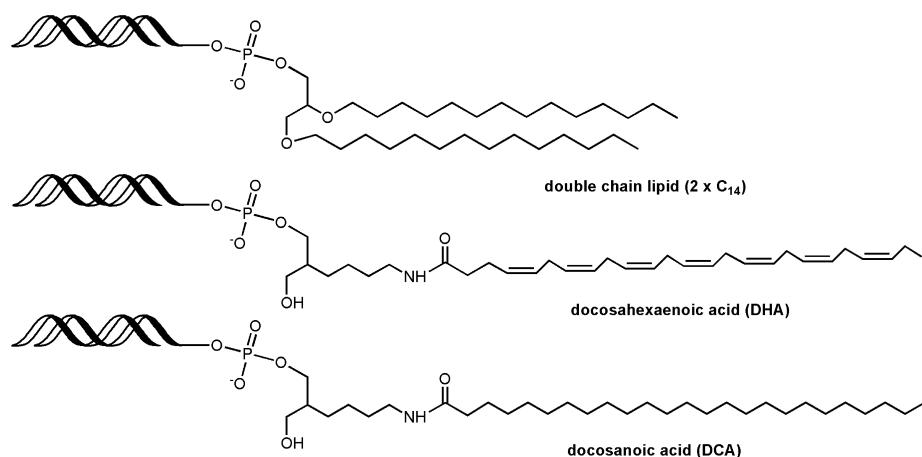
The conjugation of oligonucleotides with lipids affords molecules with improved interesting properties such as better cellular uptake, binding to serum protein and nuclease resistance [12–14]. This includes oligonucleotides carrying cholesterol [15], fatty acids [16, 17], cholic acid [18], steroids [19, 20] and others such as tocopherol [21], dexamethasone [22] or acridines [23].

The synthesis of lipid-oligonucleotide conjugates (LOCs) can be done by postsynthetic conjugation usually by reacting active ester of lipid carboxyl derivatives with amino-oligonucleotides (Section 4.2.1) [24, 25]. Other postsynthetic conjugation protocols include azide-alkyne cycloadditions (click reaction, Section 4.2.5) [26]. Dedicated phosphoramidite and functionalized solid support are employed for the conjugation of most common lipids such as cholesterol [15], or fatty acids [16]. Figure 8.2 shows the chemical structures of the most commonly used reagents for the introduction of cholesterol at the 3'-end. Similar phosphoramidite derivatives are available for the introduction of cholesterol at the 5'-end. The connecting bonds between cholesterol and the oligonucleotides are usually urethane or amide or triazole bonds that are stable to ammonia deprotection. An alternative solution is the use of ether bonds as reported [27, 28].



**Figure 8.2:** Chemical structures of the most commonly used reagents for the synthesis of oligonucleotide-cholesterol conjugates.

Oligonucleotides carrying a double-tail lipid modification (Figure 8.3) showed better incorporation in lipid model membranes and cell systems. The  $\beta 2$  integrin (CR3) receptor was directly involved in the enhanced internalization of this compound [29, 30]. Double-lipid modified oligonucleotides have been shown to be useful reagents for the assembly and fusion of liposomes [31].



**Figure 8.3:** Chemical structures of lipid-siRNA conjugates.

It has been reported that the introduction of docosahexaenoic acid (DCH, Figure 8.3) into siRNA improved the siRNA activity in the brain [32, 33]. Surprisingly, the saturated form of DCH, docosanoic acid (DCA, Figure 8.3), conjugated siRNA supports extrahepatic delivery to a wide range of tissues including muscle, heart, lung, fat and adrenal glands [34–38]. Other factors involved in enhancing the inhibitory activity in extrahepatic tissues include the hydrophobicity of the lipid [38], the phosphorothioate content [35, 39], the linker chemistry (cleavable versus stable) [35] and the valency of fatty acid conjugates [37].

Finally, it was found that the addition of lipids to oligonucleotides that form non-canonical DNA structures stabilized these structures and may enhance some properties such as antiviral activity (Section 6.1.4) [40–42].

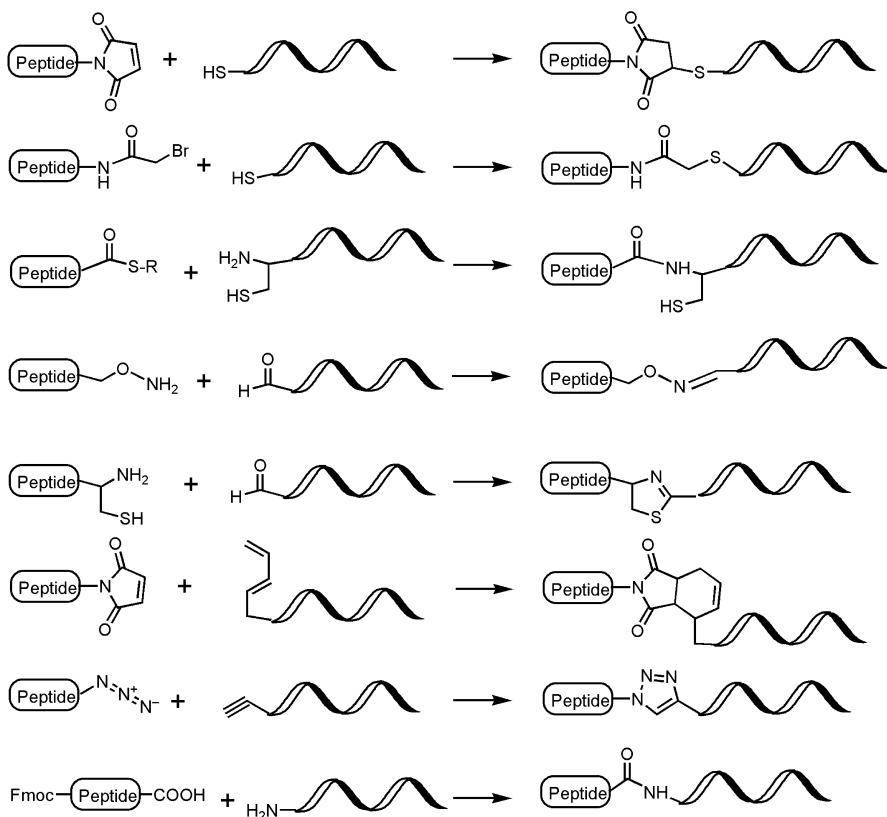
### 8.1.2 Peptide-oligonucleotide conjugates (POC)

Covalent attachment of peptides to oligonucleotides has been extensively explored by improving cellular uptake of inhibitory oligonucleotides. For this reason, several comprehensive reviews can be found [43–49].

The preparation of oligonucleotide-peptide conjugates (POCs) can be an interesting challenge since the conventional protection schemes are not compatible. For example, amide-type protecting groups are used to protect nucleobases. These protecting groups are removed by ammonia under conditions that could hydrolyze peptide bonds or cause some undesired side reactions. However, all standard protection schemes in solid-phase peptide synthesis use acid treatments which could cause partial depurination of DNA. Despite this inconvenient, two different strategies that overcome these problems have been described: (1) the post-synthetic conjugation approach [50]; and (2) the stepwise solid-phase synthesis approach [44, 51].

In the post-synthetic conjugation approach, although the oligonucleotide and the peptide are built in a separate support using standard protocols, they are conveniently functionalized for the production of a covalent bond after their synthesis and purification. In the stepwise solid-phase approach, the oligonucleotide-peptide conjugate is prepared in a single support using special protecting groups and modified protocols that minimize undesired side-reactions [52]. In a comparative study, both methodologies yielded similar results during the preparation of oligonucleotides carrying nuclear localization sequences [52].

Several coupling reactions have been reported in the post-synthetic conjugation approach (Figure 8.4) including maleimide-thiol [50, 53], bromoacetyl-thiol [54], oxime and thiazolidine formation [55, 56], native ligation [57], Diels–Alder [58, 59] and copper-catalyzed azide-alkyne 2 + 2 cycloaddition [60]. Some of these reactions were introduced in Chapter 4. Moreover, the use of protected peptide fragments have also been reported [61].



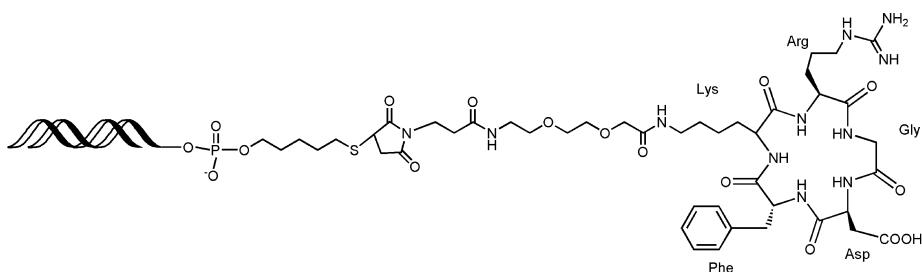
**Figure 8.4:** Postsynthetic conjugation reactions used in the preparation of oligonucleotide-peptide conjugates.

In the stepwise approach, POCs are prepared by stepwise addition of amino acids followed by successive additions of nucleoside phosphoramidites on the same solid support. This is usually performed by first assembling the peptide using *t*-butoxycarbonyl (Boc)-protected amino acids with base labile groups such as 9-fluorenylmethyl (Fm), 9-fluorenylmethoxycarbonyl (Fmoc) and trifluoroacetyl (TFA) groups to protect side chains. Although these protocols generate oligonucleotide-3'-peptide, they could not be used to incorporate peptides at the 5' end or in the middle of the oligonucleotide sequence. A method for the solid-phase synthesis of oligonucleotide-5'-peptide conjugates has been described. It is based on the use of the 2-(biphenyl-4-yl)propan-2-yloxycarbonyl (Bpoc) group for the protection of the  $\alpha$ -amino group [62]. These groups can be removed under mildly acidic conditions suitable for DNA. In addition, we have developed an efficient method to synthesize short oligonucleotide-5'-peptide conjugates via stepwise synthesis using commercially available Fmoc-protected amino acids [63, 64]. Several linkers and solid supports have been developed to facilitate the stepwise synthesis protocols [65].

The peptide-oligonucleotide conjugates containing the so-called cell penetration peptide (CPP) are one of the most studied POCs. Cell penetration peptides include penetratin, transportan [66–70], Tat-peptide [66, 68, 69], membrane translocation motifs [71], nuclear localization sequences [52, 72–74], guanidine peptides [75], amphipathic peptides [76] and protamines [71]. Most of these POCs showed improved cellular uptake arriving to have equivalent delivery efficacy than cationic liposomes [77]. CPP conjugation had very good success in increasing the permeability of single-stranded oligonucleotides particularly morpholino oligomers (PMO) and peptide nucleic acids (PNA) involved in exon skipping [78–80].

Another strategy for increasing permeability of oligonucleotides has been the conjugation of oligonucleotides with peptides that have an affinity for cellular receptors. Oligonucleotides carrying the arginine-glycine-aspartic acid tripeptide motif (RGD), which is known to be powerful and selective ligand of the  $\alpha_V\beta_3$  integrin receptor (Figure 8.5) have been reported [55, 60, 81]. In addition, oligonucleotides carrying the cyclic RGD peptide have been used for covering titanium implants and the improvement of osteoblast adhesion [82]. Histidine-rich peptides derived from bombesin were covalently linked to a splice switching antisense oligonucleotide for endosomal disruption. The conjugates were tested for their ability to correct splicing in prostate cancer cells that express the bombesin receptors. Trivalent conjugates that included both the targeting sequence and several histidine residues were substantially more effective than conjugates containing only the bombesin or histidine moieties [83]. Oligonucleotides carrying the somatostatin analog, Tyr3-octreotate, were prepared [84]. Octreotide is a cyclic octapeptide derived from somatostatin. Somatostatin receptors are overexpressed by a variety of neoplastic tissues, especially endocrine tumors. The introduction of octreotate into oligonucleotides was designed to increase the uptake of anticancer oligonucleotides by tumor cells. Oligonucleotide phosphorothioates carrying octreotate were prepared by post-synthetic conjugation. The resulting conjugates exhibited a high binding affinity to somatostatin receptors [84]. *In vivo* cellular uptake proved to be better using the peptide nucleic acid-octreotate conjugate [85]. In a comparative study of the enhancement of cell permeability of siRNAs in tumor cells induced by peptides, a peptide carrier based on a cyclic anti-HER2 peptide (AHNP) linked to a Tat cell-penetrating peptide (Tat-AHNP) has been reported to enter HER2<sup>+</sup> breast cancer cells with better efficiency than cyclic RGD peptide and octreotide [60].

The introduction of peptide sequences into oligonucleotides as a nonradioactive labels have been also pursued. The great diversity of antibodies raised against peptides triggered the synthesis of POCs as alternative labeling systems. Oligonucleotides carrying *c-myc* tag-sequence have been reported [86–88]. The conjugates were recognized by an anti-*c-myc* monoclonal antibody while detection was achieved through interaction with a peroxidase-conjugated antibody and a chemiluminescent substrate, as well as with gold-labeled antibody and inductively coupled plasma-mass spectrometry (ICPMS) [89]. Oligonucleotides carrying fibrin/filaggrin citrullinated pep-



**Figure 8.5:** Chemical structure of a siRNA linked to the cyclic RGD (ArgGlyAsp) peptide, a powerful ligand for integrin receptors.

tides have been reported [90]. These oligonucleotide-citrullinated peptide conjugates have been used to develop ELISA-based tests for the detection of anticitrullinated protein/peptide antibodies in human serum from rheumatoid arthritis patients [90].

The preparation of double-stranded DNAs (dsDNAs) conjugated to peptide fragments corresponding to transcription factors (TFs) was reported [90]. These POCs offer unique opportunities for the specific recognition of naturally occurring transcription factors given by the bivalent interaction surface provided by the peptide and the ds-DNA [91].

### 8.1.3 Proteins functionalized with oligonucleotides (PFO)

Proteins functionalized with oligonucleotides (PFO) are versatile molecular tools for biotechnology, biomedicine and material science [92–94]. PFOs have been used to fabricate arrangements of proteins using the specificity of base-pairing of nucleic acids or to immobilize proteins to surfaces in the fabrication of protein arrays or other supramolecular devices [95]. Additionally, several immunological bioassays are based on the use of these chimeras [96]. The attachment of oligonucleotides to proteins or other polymers have shown to enhance nucleic acids delivery in gene inhibition strategies [97, 98].

Several methods have been used for the preparation of POFs including (a) disulfide formation between thiolated-oligonucleotide and cysteine containing proteins [99], (b) thiol-maleimide reaction between thiolated-oligonucleotide and proteins activated with bifunctional crosslinking reagents such as sulfosuccinyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) [100, 101] (c) native ligation between cysteine-oligonucleotide s and protein thioesters [102] (d) copper-free click reaction [103], (e) use of a homofunctional crosslinker between the protein and an amino-oligonucleotide [104] and (f) reductive amination of IO<sub>4</sub>-oxidized RNA dialdehydes [105] or aldehyde containing oligonucleotides [106]. Recently, DNA-templated

protein conjugation (DTPC) has been reported for the site-selective conjugation of DNA to proteins [107]. These strategies for designing site-specific coupling of DNA oligomers to proteins are needed in order to allow for precise control over stoichiometry and position for the assembly of protein-DNA complexes with precise molecular recognition properties [108].

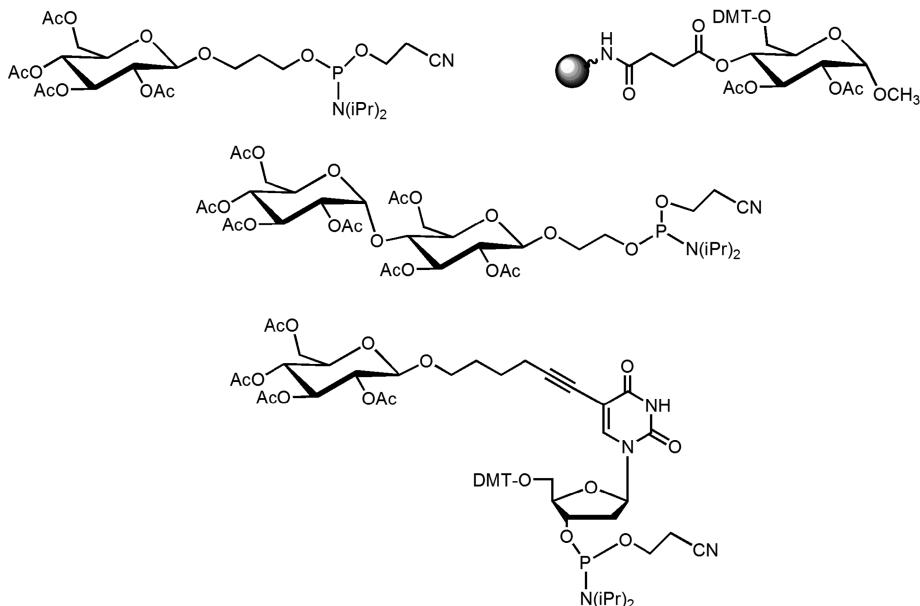
The widespread use of monoclonal antibodies in cancer therapy has triggered the interest for the functionalization of antibodies with oligonucleotides [109]. Antibodies-oligonucleotide conjugates (AOCs) together with antibody-drug conjugates (ADCs) are between the most interesting compounds for the use of personalized medicine for cancer treatment [110, 111]. Typically, antibodies are functionalized with therapeutic oligonucleotides such as antisense siRNA designed to inhibit some over-expressed protein [111], but also DNA duplexes are added to monoclonal antibodies as doxorubicin-binding compounds [112]. In this way, specific antibodies are functionalized with doxorubicin by non-covalent intercalation allowing the delivery of the drug to specific cell types [112, 113].

In addition to antibodies, multifunctional proteins have emerged as powerful engineered chimeric proteins that incorporate several selected domains. Among them, protein-based nanocarriers based on the concept of virus-mimetic vehicles have been studied for drug delivery [114]. Protein nanoparticles carrying a peptide with affinity to the chemokine receptor CXCR4 have been functionalized with floxuridine oligomers (Section 7.1.8) to treat colorectal metastatic tumors [115, 116]. This targeted drug delivery approach yielded a potent *in vivo* antimetastatic effect, through selective depletion of metastatic CXCR4<sup>+</sup> cancer cells [115].

### 8.1.4 Carbohydrate-oligonucleotide conjugates (COCs)

The addition of carbohydrates to oligonucleotides has been subject of intense research due to the important role of carbohydrate-protein interactions that can be used for the enhancement of the cellular uptake of therapeutic oligonucleotides [117, 118]. The preparation of COCs can be achieved by several strategies that have been recently reviewed [117–119]. The most popular route is based on the preparation of protected carbohydrate phosphoramidites yielding 5'-carbohydrate DNA [120–122] and siRNA [123]. Examples of some of the reported carbohydrate phosphoramidites are shown in Figure 8.6. Alcohol functions are usually protected with acetyl or toluoyl groups that are removed in the standard ammonia treatment used in oligonucleotide synthesis. There have also been some reports for the incorporation of carbohydrates at the 3'-end [124–127] or at the nucleobase [128–130] (Figure 8.6).

In addition to solid-phase phosphoramidite chemistry, there are several methods for the postsynthetic conjugation of carbohydrates and oligonucleotides. As described



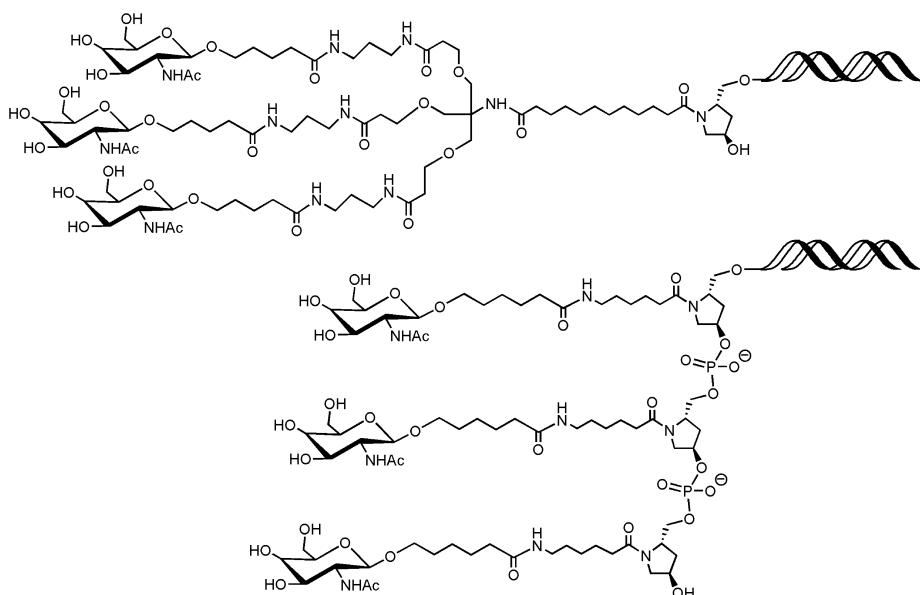
**Figure 8.6:** Chemical structures of reagents used for the synthesis of oligonucleotide-carbohydrate conjugates.

for peptide-oligonucleotide conjugates (see also Chapter 4), postsynthetic conjugations required the introduction of reactive groups in both oligonucleotide and carbohydrate molecules in order to achieve a conjugation reaction either on the solid-support or in aqueous solutions (Figure 8.4). The copper-catalyzed 2 + 2 cycloaddition between azides and alkynes has been widely used for the synthesis of COCs. Usually, glycosyl-azides are reacted with alkyne-oligonucleotides [131–135]. The copper free 2 + 2 cycloaddition between azides and alkynes using cyclooctyne phosphoramidite has also been described for the coupling of hyaluronic acid and peptides to oligonucleotides [136]. Oxime formation (Figure 8.4) was also used for linking hydroxylaminoglycosides and aldehyde-oligonucleotides [137–139]. An interesting method designed to functionalize salmon DNA is the use of the diazo coupling [140]. In this method, lactose and cellobiose were functionalized with 4-nitrobenzoyl groups that were hydrogenated to produce 4-aminobenzoyl compounds that were treated with sodium nitrite to produce the diazonium salt that reacted with the C-8 of purines of DNA. Another unusual method to functionalize oligonucleotides with carbohydrates is based on the use of 3,4-ethoxy-3-cyclobutene-1,2-dione as a crosslinker connecting amino-sugars with amino-oligonucleotides [141].

A large number of applications can be found for COCs including (1) the study of lectin binding using DNA-immobilized glycoarrays [135] or glycoclusters [139], (2) the structural elucidation of molecular interactions [121, 142–144] as well as the discovery

of novel carbohydrate: DNA base pairs [145] and 3) improving cellular uptake and *in vivo* biodistribution [146–154].

The leading oligonucleotide conjugates for clinical use and clinical development (Givlaari, Inclisiran, Chapter 7) are carbohydrate-siRNA conjugates composed of chemically stabilized siRNA carrying a trivalent liver-targeting ligand [155, 156]. In these conjugates, the 3' terminus of the siRNA sense strand is linked to three molecules of *N*-acetylgalactosamine (GalNAc), an asialoglycoprotein receptor ligand, by means of a tribranched spacer [150] or by successive additions of a GalNAc monomer [153, 154] (Figure 8.7).



**Figure 8.7:** Chemical structures of siRNA conjugates carrying *N*-acetylgalactosamine (GalNAc) used for hepatocyte delivery through the asialoglycoprotein receptors.

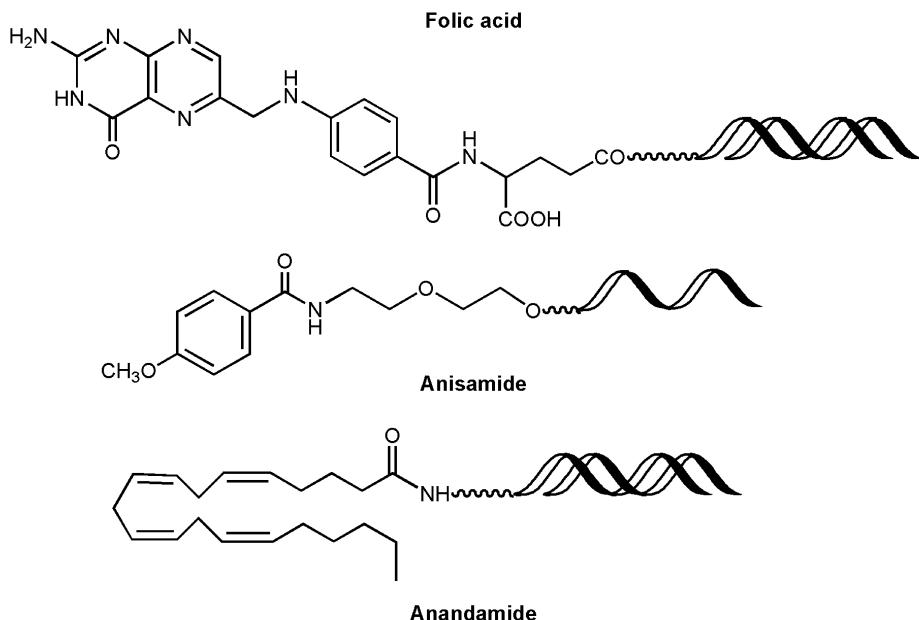
The asialoglycoprotein receptor is mainly found in hepatocytes exhibiting a high affinity for galactose glycoproteins and trivalent GalNAc oligonucleotides with a rapid internalization rate via the clathrin-mediated pathway. The modification of antisense oligonucleotides [147–149] and especially siRNA molecules [150–154] with trivalent GalNAc compounds has made a strong impact on the therapeutic use of siRNA due to their active transport properties to the liver by effective delivery via subcutaneous administration. The GalNAc-siRNA conjugates are hepatotropic and long-acting having the potential to treat a wide range of diseases involving liver-expressed genes [150].

Besides GalNAc, mannose 6-phosphate [157], sialyl [158] and hyaluronic acid [136, 159, 160] have been linked to oligonucleotides for targeted delivery to cells using the

mannose 6-phosphate/insulin-like growth factor-II receptor [157], CD22 [158] or CD44 [159] receptors.

### 8.1.5 Oligonucleotide functionalized with substrates for receptor-mediated recognition

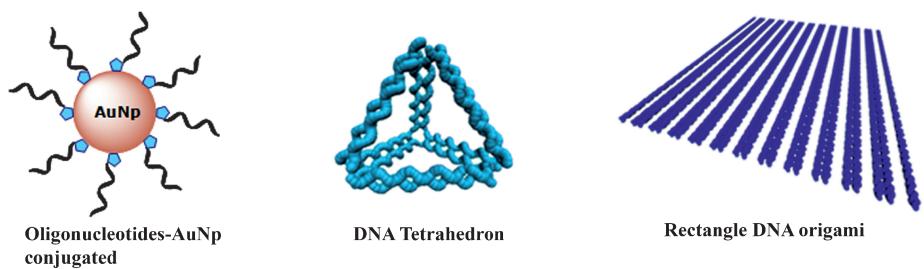
Although efficient transfecting agents are available for cell culture experiments, delivery to the right tissue is still one of the most important limitations in oligonucleotide therapeutics. The most critical challenge is to achieve *in vivo* tissue distribution following systemic administration. In this way, targeting the right tissue, and especially in tissues other than the liver, becomes crucial [161]. In addition to the successful examples of peptides, carbohydrates and aptamers, there are a few small molecules that have been proposed for receptor mediated cell targeting. Oligonucleotides functionalized with folic acid [162–166] (Figure 8.8) pursued the internalization of therapeutic oligonucleotides in tumor cells that overexpress the folate receptors [162–166]. Other small ligands such as anisamide [167] and anandamide [168] (Figure 8.8) aimed to bind the sigma and the cannabinoid receptors, respectively. All of these conjugates showed significant cellular uptake enhancement in the absence of transfecting reagents.



**Figure 8.8:** Chemical structures of antisense and siRNA conjugates carrying substrates for receptor-mediated recognition.

## 8.2 DNA nanotechnology

The large interest in the field of DNA nanotechnology arose after pioneering studies at the end of the last century. Synthetic oligonucleotides were successfully attached to gold nanoparticles (Au NPs) to direct the assembly of discrete gold nanoparticles assemblies [169, 170] but also DNA-functionalized Au NPs were able to form tridimensional networks useful for detection of DNA sequences [171, 172]. Previously, the group of Ned Seeman demonstrated that synthetic oligonucleotides can be used to assemble precise large nanostructures with an exquisite control of size and shape [173–175]. Moreover, it was demonstrated that DNA can be used as a template for the fabrication of tiny silver [176] or gold [177] conducting nanowires. These pioneering works have generated several research directions including structural DNA nanotechnology [178, 179], DNA-directed functionalization of surfaces for potential nanoelectronic purposes [180, 181], the development of highly efficient biosensors [182, 183] and the synthesis of defined molecular devices for drug delivery [184, 185]. In this chapter, we will focus on the use of gold nanoparticles and DNA nanostructures for drug delivery (Figure 8.9).



**Figure 8.9:** Schematic representation of DNA-gold nanoparticles, DNA tetrahedron and DNA origami.

### 8.2.1 Gold nanoparticles functionalized with oligonucleotides

Inorganic NPs such as colloidal gold nanoparticles (AuNPs) have become attractive materials due to an increasing interest in their potential applications in biotechnology, molecular diagnostics and biomedicine [186–188]. Because of their unique properties such as size and shape-dependent optical and electronic features, good biocompatibility and the ability to bind ligands containing thiols, phosphines and amines, they have emerged as useful tools for the design of biosensors [189], the development of methods for cancer detection [190] and potential vehicles for drug delivery [187]. AuNPs are able to protect their cargo from degradation in serum and they can be used for passive or active targeting delivery. Passive delivery by the enhanced permeability

and retention effect (EPR) has been reported for the delivery of AuNPs to tumor cells due to the differences in the vascularization of normal or tumor cells [187].

The conjugation of AuNPs with oligonucleotides can be carried out through the reaction of thiolated-oligonucleotides with citrate-stabilized AuNPs due to the strong affinity of thiolated species for gold surfaces [171, 191] (Figure 8.9). The modification of nucleic acids with thiolated derivatives was explained in Chapter 4. The oligonucleotide loading can be determined by hybridization with complementary fluorescent oligonucleotides [192]. It has been described that the size of the nanoparticle aggregates are important to control nanoparticle assembly [193]. Also, oligonucleotide functionalization at acid pH can be accelerated due to the protonation of the phosphate bonds that help the approximation of the oligonucleotide to the citrate-stabilized AuNPs [194]. The use of oligonucleotides carrying lipoic or thioctic acid derivatives can increase the loading of AuNPs [195–197] and allows the introduction of cleavable peptide sequences between the AuNPs and oligonucleotide [197].

Gold nanoparticles functionalized with antisense [198] or siRNA [199, 200] oligonucleotides were demonstrated to be able to enter the cells without transfecting agents and exhibit gene knockdown. When the gold nanoparticles are densely packed with oligonucleotides the resulting NPs were designed as spherical nucleic acids (SNAs) [201]. SNAs can enter more than 50 different cell types without the aid of transfection agents and can produce antisense and RNAi activity [202, 203]. Mechanism studies have been followed to elucidate endocytosis pathways of SNAs [204, 205]. SNAs can bind strongly to class A scavenger receptors and undergo rapid cellular uptake via a lipid-raft-dependent, caveolae-mediated pathway. Evidence also showed that the SNAs enter early endosomes. The conjugation of a HER2 antibody to SNAs produced cell-selective delivery and enhanced RNAi activity in HER2 positive cells [206]. The cellular uptake behavior of SNAs carrying several backbone modifications has been reported demonstrating that the uptake efficiencies are different for each backbone opening the possibility of tuning the cellular uptake behavior of SNAs [207].

### 8.2.2 DNA nanostructures and DNA origami

The remarkable specificity of the molecular recognition between complementary nucleotides in the DNA base pairing has made DNA an attractive molecule for scientists and engineers interested in micro and nanofabrication. The canonical double helix B-form based on Watson–Crick base pairing has well defined parameters that are repeated along the structure having a helical turn of approximately 3.4 nm, and a helical diameter of 2.0 nm in solution [208]. The rigidity and precise structural control, as well as the creation of algorithms for *de novo* design of new self-assembled structures [209] make it a valuable building material to develop different kinds of nanotechnological platforms. Compared to other self-assembling molecules, DNA nanostructures

offer programmable interactions and surface features for the precise positioning of nanoparticles and other biomolecules [210, 211].

The field of DNA nanostructures was pioneered by Ned Seeman, who set the foundations for the use of DNA as a scaffold for nanoscale building material [212]. Seeman's original goal was the creation of regular 3D lattices of DNA which could be used as scaffolding for the rapid, orderly binding of biological macromolecules to speed the formation of suitable crystals for 3D protein structure elucidation in X-ray diffraction studies [212]. This concept gave rise to the tile-based assembly method, used to synthesize two-dimensional periodic lattices [175] and other three-dimensional architectures [174, 176] (Figure 8.9).

Another important breakthrough in the structural DNA nanotechnology field has been the development of DNA origami by Paul Rothemund [213], where a long scaffold strand is folded with the help of hundreds of short "staples" to create the desired two-dimensional shape (Figure 8.9). Since then, various DNA motifs have been designed in 2D and 3D, and extensive studies are currently ongoing to apply these nanostructures to a large amount of structural, computational and molecular motor purposes [181].

### 8.2.3 DNA nanostructures in drug delivery

Self-assembled 2D-and 3D-DNA nanostructures are increasingly employed as agents for delivering functional oligonucleotides and drugs into cells [214–221]. In comparison to other methods, DNA nanotechnology is a highly efficient and controllable strategy for creating structures of defined shape and size through rational design and construction [217]. These DNA nanostructures with different sizes, shapes and geometries have characteristic uniform size, precise addressability, excellent water solubility and biocompatibility. Hence, they offer new opportunities for the construction of nanostructures for biomedical applications [218–221].

The first study of targeted DNA nanostructures was reported in 2008 and concerned DNA nanotubes assembled from a single DNA strand tile modified with folate or the fluorophore Cy3. Cells overexpressing the folate receptor showed increased internalization of folate-modified DNA nanotubes [222]. In another study, a defined DNA icosahedron, encapsulating fluorescent polymers, showed colocalization upon uptake through anionic ligand-binding receptors in *Drosophila hemocytes* and *C. elegans* [223]. These experiments demonstrated the intracellular delivery of a molecular cargo using a synthetic DNA nanostructure. Later, Goodman et al. [224] investigated the uptake and structural integrity of a DNA tetrahedron in HEK cells. More recently, it was shown that the DNA tetrahedron can act as a carrier for doxorubicin in the treatment of drug-resistant cancer cells [225] and for the targeted delivery of folate-conjugated siRNA [226]. Recently, DNA tetrahedron conjugated with various numbers

of the mitochondria-targeting peptide sequence, D-(KLAKLAK)<sub>2</sub>, was able to deliver doxorubicin to cancer cells activating the mitochondria-mediated, programmed apoptosis to enhance anticancer efficiency *in vivo* [227].

An interesting DNA-minimal cage capable of encapsulating siRNA molecules for *in vitro* assays has been described [228]. The siRNA cargo is located in the inside of a DNA parallelogram protecting the cargo against nuclease degradation. Release of the cargo is done as response of the complex cellular environment [228]. DNA cages carrying dendritic alkyl chains have been developed to exert a strong binding affinity to human serum albumin resulting in DNA systems with enhanced serum stability [229]. In another approach, spherical nucleic acids constituted by spontaneous self-assembly of DNA-polymer conjugates were used as a general platform to deliver anticancer drugs *in vivo* [230].

Origami structures have also been used to deliver therapeutic cargo to cells. For example, doxorubicin has been delivered and released in breast cancer cells [231, 232]. DNA nanostructures have been also used for delivery of immunostimulatory CpG oligonucleotides [233] and 5-fluoro-2'-deoxyuridine oligomers for colorectal cancer therapy [234]. A DNA origami has been described for the specific delivery of thrombin to the tumor site [235]. The DNA origami acted as a nanorobot with a molecular triggered release. The toxic cargo was kept inside the nanostructure. The external part of the nanorobot was functionalized with a nucleolin-binding aptamer. As nucleolin is overexpressed in cancer cells, the nanorobot was able to deliver thrombin near the tumor site provoking the coagulation of the blood vessels of the tumor [235]. Another outstanding result was the development of a DNA origami for the organization of antigens to induce an efficient B-cell activation [236]. These promising results may facilitate the fabrication of novel and more efficient vaccines.

In addition, DNA nanostructures functionalized with lipids have been shown to interact with lipid bilayer membranes [237]. Under certain conditions, DNA nanostructures remain mobile on membranes and dynamically associate into higher-order structures. Appropriate design along with chemical modification allows the insertion of DNA structures into lipid bilayer membranes, resulting in artificial ion channel mimics made from DNA [237]. For these reasons, membrane-embedded DNA nanostructures are expected to find applications in diverse areas ranging from basic biological research to synthetic biology [238].

## Bibliography

- [1] Manoharan M. Oligonucleotide conjugates as potential antisense drugs with improved uptake, biodistribution, targeted delivery, and mechanism of action. *Antisense Nucleic Acid Drug Dev.* 2002;12:103–28.
- [2] Lebedeva I, Benimetskaya L, Stein CA, Vilenchik M. Cellular delivery of antisense oligonucleotides. *Eur J Pharmacol Biopharmacol.* 2000;50:101–19.
- [3] Beaucage SL, Iyer RP. The functionalization of oligonucleotides via phosphoramidite derivatives. *Tetrahedron.* 1993;49:1925–63.
- [4] Agrawal S, editor. *Protocols for oligonucleotide conjugates.* New Jersey: Humana Press; 1994.
- [5] Lönnberg H. Solid-phase synthesis of oligonucleotide conjugates useful for delivery and targeting of potential nucleic acid therapeutics. *Bioconjug Chem.* 2009;20:1065–94.
- [6] Grijalvo S, Alagia A, Jorge AF, Eritja R. Covalent strategies for targeting messenger and non-coding RNAs. An updated review on siRNA, miRNA and antimiR conjugates. *Genes.* 2018;9:74.
- [7] Ming X, Laing B. Bioconjugates for targeted delivery of therapeutic oligonucleotides. *Adv Drug Deliv Rev.* 2015;87:81–9.
- [8] Winkler J. Oligonucleotide conjugates for therapeutic applications. *Ther Deliv.* 2013;4:791–809.
- [9] Juliano RL, Ming X, Nakagawa O. The chemistry and biology of oligonucleotide conjugates. *Acc Chem Res.* 2012;45:1067–76.
- [10] Goodchild J. Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. *Bioconjug Chem.* 1990;1:165–87.
- [11] Xia X, Pollock N, Zhou J, Rossi J. Tissue-specific delivery of oligonucleotides. In: Gissberg O, Zain R, Lundin KE, editors. *Oligonucleotide-based therapies: methods and protocols. Methods in Molecular Biology.* vol. 2036. Humana Press; 2019. p. 17–50.
- [12] Patwa A, Gissot A, Bestel I, Barthélémy P. Hybrid lipid oligonucleotide conjugates: synthesis, self-assemblies and biomedical applications. *Chem Soc Rev.* 2011;40:5844–54.
- [13] Pokholenko O, Gissot A, Vialet B, Bathany K, Thiery A, Barthélémy P. Lipid oligonucleotide conjugates as responsive nanomaterials for drug delivery. *J Mater Chem B.* 2013;1:5329–34.
- [14] Zhao B, Tian Q, Bagheri Y, You M. Lipid-oligonucleotide conjugates for simple and efficient cell membrane engineering and bioanalysis. *Curr Opin Biomed Eng.* 2020;13:76–83.
- [15] Soutscheck J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Röhrl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliansky V, Limmer S, Manoharan M, Vornlocher HP. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature.* 2004;432:173–8.
- [16] Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, Rajeev KG, Nakayama T, Charrise K, Ndungo EM, Zimmermann T, Koteliansky V, Manoharan M, Stoffel M. *Nat Biotechnol.* 2007;25:1149–57.
- [17] Ueno Y, Kawada K, Naito T, Shibata A, Yoshikawa K, Kim H-S, Wataya Y, Kitade Y. Synthesis and silencing properties of siRNAs possessing lipophilic groups at their 3'-termini. *Bioorg Med Chem.* 2008;16:7698–704.
- [18] Manoharan M, Johnson LK, Bennett CF, Vickers TA, Ecker DJ, Cowser LM, Freier SM, Dan Cook P. Cholic acid-oligonucleotide conjugates for antisense applications. *Bioorg Chem Lett.* 1994;4:1053–60.
- [19] Lorenz C, Hadwiger P, John M, Vornlocher HP, Unverzagt C. Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells. *Bioorg Med Chem Lett.* 2004;14:4975–7.

- [20] Tort N, Salvador JP, Aviñó A, Eritja R, Comelles J, Martínez E, Samitier J, Marco MP. Synthesis of steroid-oligonucleotide conjugates for a site-encoded SPR immunosensor. *Bioconjug Chem.* 2012;23:2183–91.
- [21] Nishina T, Numata J, Nishina K, Yoshida-Tanaka K, Nitta K, Piao W, Iwata R, Ito S, Kuwahara H, Wada T, Mizusawa H, Yokota T. Chimeric antisense oligonucleotide conjugated to  $\alpha$ -tocopherol. *Mol Ther Nucleic Acids.* 2015;4:e220.
- [22] Acedo M, Tarrason G, Piulats J, Mann M, Wilm M, Eritja R. Preparation of oligonucleotide-dexamethasone conjugates. *Bioorg Med Chem Lett.* 1995;5:1577–80.
- [23] Aviñó A, Ocampo SM, Perales JC, Eritja R. Synthesis and in vitro inhibition properties of siRNA conjugates carrying acridine and quindoline moieties. *Chem Biodivers.* 2012;9:557–66.
- [24] Østergaard ME, Jackson M, Low A, Chappell AE, Lee RG, Peralta RQ, Yu J, Kinberger GA, Dan A, Carty R, Tanowitz M, Anderson P, Kim TW, Fradkin L, Mullick AE, Murray S, Rigo F, Prakash TP, Bennett CF, Swayze EE, Gaus HJ, Seth PP. Conjugation of hydrophobic moieties enhances potency of antisense oligonucleotides in the muscle of rodents and non-human primates. *Nucleic Acids Res.* 2019;60:45–58.
- [25] Prakash TP, Mullick AE, Lee RG, Yu J, Yeh ST, Low A, Chappell AE, Østergaard ME, Murray S, Gaus HJ, Swayze EE, Seth PP. Fatty acid conjugation enhances potency of antisense oligonucleotides in muscle. *Nucleic Acids Res.* 2019;47:6029–44.
- [26] Godeau G, Staedel C, Barthélémy P. Lipid-conjugated oligonucleotides via “click chemistry” efficiently inhibit hepatitis C virus translation. *J Med Chem.* 2008;51:4374–6.
- [27] Grijalvo S, Ocampo SM, Perales JC, Eritja R. Synthesis of lipid-oligonucleotide conjugates for RNA interference studies. *Chem Biodivers.* 2011;8:287–99.
- [28] Grijalvo S, Ocampo SM, Perales JC, Eritja R. Synthesis of oligonucleotides carrying amino lipid groups at the 3'-end for RNA interference studies”. *J Org Chem.* 2010;75:6806–13.
- [29] Ugarte-Uribe B, Grijalvo S, Bustos JV, Martín C, Eritja R, Goñi FM, Alkorta I. Double-tailed lipid modification as a promising candidate for oligonucleotide delivery in mammalian cells. *Biochim Biophys Acta.* 2013;1830:4872–84.
- [30] Ugarte-Uribe B, Grijalvo S, Nuñez Pertíñez S, Bustos JV, Martín C, Alagia A, Goñi FM, Eritja R, Alkorta I. Lipid modified oligonucleotides conjugates: insights into gene silencing, interaction with model membranes and cellular uptake mechanisms. *Bioorg Med Chem.* 2017;25:175–86.
- [31] Ries O, Löffler PMG, Vogel S. Convenient synthesis and application of versatile nucleic acid lipid membrane anchors in the assembly and fusion of liposomes. *Org Biomol Chem.* 2015;13:9673–80.
- [32] Nikan M, Osborn MF, Coles AH, Godinho BM, Hall LM, Haraszti RA, Hassler MR, Echeverria D, Aronin N, Khvorova A. Docosahexaenoic acid conjugation enhances distribution and safety of siRNA upon local administration in mouse brain. *Mol Ther Nucleic Acids.* 2016;5:e344.
- [33] Alterman JF, Hall LM, Coles AH, Hassler MR, Didiot MC, Chase K, Abraham J, Sottosanti E, Johnson E, Sapp E, Sapp E, Osborn MF, Difiglia M, Aronin N, Khvorova A. Hydrophobically modified siRNAs silence huntingtin mRNA in primary neurons and mouse brain. *Mol Ther Nucleic Acids.* 2015;4:e266.
- [34] Biscans A, Coles A, Haraszti RA, Echeverria D, Hassler MR, Osborn M, Khvorova A. Diverse lipid conjugates for functional extra-hepatic siRNA delivery in vivo. *Nucleic Acids Res.* 2019;47:1070–81.
- [35] Biscans A, Caiazzi J, Davis S, McHugh N, Sousa J, Khvorova A. The chemical structure and phosphorothioate content of hydrophobically modified siRNAs impact extrahepatic distribution and efficacy. *Nucleic Acids Res.* 2020;48:7665–80.
- [36] Osborn MF, Khvorova A. Improving siRNA delivery in vivo through lipid conjugation. *Nucleic Acid Ther.* 2018;28:128–36.

- [37] Biscans A, Coles A, Echeverria D, Khvorova A. The valence of fatty acid conjugates impacts siRNA pharmacokinetics, distribution, and efficacy in vivo. *J Control Release*. 2019;302:116–25.
- [38] Osborn MF, Coles A, Biscans A, Haraszti RA, Roux L, Davis S, Ly S, Echeverria D, Hassler MR, Godinho BMDC, Nikan M, Khvorova A. Hydrophobicity drives the systemic distribution of lipid-conjugated siRNAs via lipid transport pathways. *Nucleic Acids Res*. 2019;47:1070–81.
- [39] Ly S, Echeverria D, Sousa J, Khvorova A. Single-stranded phosphorothioated regions enhance cellular uptake of cholesterol-conjugated siRNA but not silencing efficacy. *Mol Ther Nucleic Acids*. 2020;21:991–1005.
- [40] Koutsoudakis G, Paris de León A, Herrera C, Dorner M, Pérez-Vilaró G, Lyonnais S, Grijalvo S, Eritja R, Meyerhans A, Mirambeau G, Díez J. Oligonucleotide-lipid conjugates forming G-quadruplex structures are potent and pangenotypic hepatitis C virus entry inhibitors in vitro and ex vivo. *Antimicrob Agents Chemother*. 2017;61:e02354–16.
- [41] Musumeci D, Montesarchio D. Synthesis of a cholesterol-HEG phosphoramidite derivative and its application to lipid-conjugates of the anti-HIV 5' TGGGAG3' Hotoda's sequence. *Molecules*. 2012;17:12378–92.
- [42] Wolfe JL, Goodchild J. Modulation of tetraplex formation by chemical modifications of a G4-containing phosphorothioate oligonucleotide. *J Am Chem Soc*. 1996;118:6301–2.
- [43] Tung CH, Stein S. Preparation and applications of peptide-oligonucleotide conjugates. *Bioconjug Chem*. 2000;11:605–18.
- [44] Grandas A, Marchán V, Debéthune L, Pedroso E. Stepwise solid-phase synthesis of nucleopeptides. *Curr Protocols Nucleic Acid Chem*. 2004;4,22.1–54.
- [45] Eritja R. Synthesis of oligonucleotide-peptide conjugates and nucleopeptides. Solid-phase synthesis. A practical guide. New York: Marcel Dekker; 2000. p. 529–48.
- [46] Aviñó A, Grijalvo S, Pérez-Rentero S, Garibotti A, Terrazas M, Eritja R. Synthesis of oligonucleotide-peptide conjugates for biomedical and technological applications. In: Mark S, editor. *Bioconjugation protocols, part 2*. 2nd ed. Methods in Molecular Biology vol. 751. New York: Springer; 2011. p. 223–38.
- [47] Lu K, Duan QP, Ma L, Zhao D-X. Chemical strategies for the synthesis of peptide-oligonucleotide conjugates. *Bioconjug Chem*. 2010;21:187–202.
- [48] Gait MJ. Peptide-mediated cellular delivery of antisense oligonucleotides and their analogues. *Cell Mol Life Sci*. 2003;60:844–53.
- [49] Venkatesan N, Kim BH. Peptide conjugates of oligonucleotides: synthesis and applications. *Chem Rev*. 2006;106:3712–61.
- [50] Eritja R, Pons A, Escarceller M, Giralt E, Albericio F. Synthesis of defined peptide-oligonucleotide hybrids containing a nuclear transport signal sequence. *Tetrahedron*. 1991;47:4113–20.
- [51] De la Torre BG, Aviñó A, Tarrason G, Piulats J, Albericio F, Eritja R. Stepwise solid-phase synthesis of oligonucleotide-peptide hybrids. *Tetrahedron Lett*. 1994;35:2733–6.
- [52] de la Torre BG, Albericio F, Saison-Behmoaras E, Bachi A, Eritja R. Synthesis and binding properties of oligonucleotides carrying nuclear localization sequences. *Bioconjug Chem*. 1999;10:1005–12.
- [53] Arar K, Monsigny M, Royer M. Synthesis of oligonucleotide-peptide conjugates containing a KDEL signal sequence. *Tetrahedron Lett*. 1993;34:8087–90.
- [54] Arar K, Aubertin AM, Roche AC, Monsigny M, Mayer R. Synthesis and antiviral activity of peptide-oligonucleotide conjugates prepared by using  $N^{\alpha}$ -(bromoacetyl)peptides. *Bioconjug Chem*. 1995;6:573–7.
- [55] Forget D, Boturyn D, Defrancq E, Lhomme J, Dumy P. Highly efficient synthesis of peptide-oligonucleotide conjugates: chemoselective oxime and thiazolidine formation. *Chem Eur J*. 2001;7:3976–84.

- [56] Singh Y, Defrancq E, Dumy P. New method to prepare peptide-oligonucleotide conjugates through glyoxylic oxime formation. *J Org Chem.* 2004;69:8544–6.
- [57] Stetsenko DA, Gait MJ. Efficient conjugation of peptides to oligonucleotides by “Native Ligation”. *J Org Chem.* 2000;65:4900–8.
- [58] Marchán V, Ortega S, Pulido D, Pedroso E, Grandas A. Diels–Alder cycloadditions in water for the straightforward preparation of peptide–oligonucleotide conjugates. *Nucleic Acids Res.* 2006;34:e24.
- [59] Steven V, Graham D. Oligonucleotide conjugation to a cell-penetrating (TAT) peptide by Diels–Alder cycloaddition. *Org Biomol Chem.* 2008;6:3781–7.
- [60] Gandioso A, Massaguer A, Villegas N, Salvans C, Sánchez D, Brun-Heath I, Marchán V, Orozco M, Terrazas M. Efficient siRNA-peptide conjugation for specific targeted delivery into tumor cells. *Chem Commun.* 2017;53:2870–3.
- [61] Peyrottes S, Mestre B, Burlina F, Gait MJ. The synthesis of peptide-oligonucleotide conjugates by a fragment coupling approach. *Tetrahedron.* 1998;54:12513–22.
- [62] Zaramella S, Yeheskiely E, Stromberg R. A method for solid-phase synthesis of oligonucleotide 5'-peptide conjugates using acid-labile alpha-amino protections. *J Am Chem Soc.* 2004;126:14029–35.
- [63] Ocampo SM, Albericio F, Fernández I, Vilaseca M, Eritja R. A straightforward synthesis of 5'-peptide oligonucleotide conjugates using N<sup>α</sup>-Fmoc-protected amino acids. *Org Lett.* 2005;7:4349–52.
- [64] Aviñó A, Jorge AF, Huertas CS, Cova TFGG, Pais A, Lechuga LM, Eritja R, Fàbrega C. Aptamer-peptide conjugates as a new strategy to modulate human  $\alpha$ -thrombin binding affinity. *Biochim Biophys Acta.* 2019;1863:1610–30.
- [65] Antopolksky M, Azhayev A. Stepwise solid-phase synthesis of peptide-oligonucleotide conjugates on new solid supports. *Helv Chim Acta.* 1999;82:2130–40.
- [66] Astriab-Fisher A, Sergueev D, Fisher M, Ramsay Shaw B, Juliano RL. Conjugates of antisense oligonucleotides with the Tat and antennapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences, and biologic actions. *Pharm Res.* 2002;19:744–54.
- [67] Muratovska A, Eccles MR. Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. *FEBS Lett.* 2004;558:63–8.
- [68] Turner JJ, Arzumanov AA, Gait MJ. Synthesis, cellular uptake and HIV-1 Tat-dependent trans-activation inhibition activity of oligonucleotide analogues disulphide-conjugated to cell-penetrating peptides. *Nucleic Acids Res.* 2005;33:27–42.
- [69] Moschos SA, Jones SW, Perry MM, Williams AE, Erjefalt JS, Turner JJ, Barnes PJ, Sproat BS, Gait MJ, Lindsay MA. Lung delivery studies using siRNA conjugated to TAT(48-60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. *Bioconjug Chem.* 2007;18:1450–9.
- [70] Ishihara T, Goto M, Kodera K, Kanazawa H, Murakami Y, Mizushima Y, Higaki M. Intracellular delivery of siRNA by cell-penetrating peptides modified with cationic oligopeptides. *Drug Deliv.* 2009;16:153–9.
- [71] Antopolksky M, Azhayeva E, Tengvall U, Auriola S, Jääskeläinen I, Rönkkö S, Honkakoski P, Urtti A, Lönnberg H, Azhayev A. Peptide-oligonucleotide phosphorothioate conjugates with membrane translocation and nuclear localization properties. *Bioconjug Chem.* 1999;10:598–606.
- [72] Reed MW, Fraga D, Schwartz DE, Scoller J, Hinrichsen RD. Synthesis and evaluation of nuclear targeting peptide-antisense oligodeoxynucleotide conjugates. *Bioconjug Chem.* 1995;6:101–8.
- [73] Zanta MA, Belgique-Balladier P, Behr JP. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to cell nucleus. *Proc Natl Acad Sci USA.* 1999;96:91–6.

- [74] Aviñó A, Ocampo SM, Caminal C, Perales JC, Eritja R. Stepwise synthesis of RNA conjugates carrying peptide sequences for RNA interference studies. *Mol Divers.* 2009;13:287–93.
- [75] Grijalvo S, Terrazas M, Aviñó A, Eritja R. Stepwise synthesis of oligonucleotide-peptide conjugates containing guanidinium or lipophilic groups in their 3'-termini. *Bioorg Med Chem Lett.* 2010;20:2144–7.
- [76] Grijalvo S, Eritja R. Synthesis and in vitro inhibition properties of oligonucleotide conjugates carrying amphiphatic proline-rich peptide derivatives of the sweet arrow peptide (SAP). *Mol Divers.* 2012;16:307–17.
- [77] Ye J, Liu E, Gong J, Wang J, Huang Y, He H, Yang VC. High-yield synthesis of monomeric LMWP(CPP)-siRNA covalent conjugate for effective cytosolic delivery of siRNA. *Theranostics.* 2017;7:2495–508.
- [78] Ivanova GD, Arzumanov A, Abes R, Yin H, Wood MJ, Lebleu B, Gait MJ. Improved cell-penetrating peptide-PNA conjugates for splicing redirection in HeLa cells and exon skipping in mdx mouse muscle. *Nucleic Acids Res.* 2008;36:6418–28.
- [79] Yin H, Saleh AF, Betts C, Camelliti P, Seow Y, Ashraf S, Arzumanov A, Hammond S, Merritt T, Gait MJ, Wood MJ. Pip5 transduction peptides direct high efficiency oligonucleotide-mediated dystrophin exon skipping in heart and phenotypic correction in mdx mice. *Mol Ther.* 2011;19:1295–303.
- [80] Saleh AF, Arzumanov AA, Gait MJ. Overview of alternative oligonucleotide chemistries for exon skipping. *Methods Mol Biol.* 2012;867:365–78.
- [81] Alam MR, Dixit V, Kang H, Li ZB, Chen X, Trejo JA, Fisher M, Juliano RL. Intracellular delivery of an anionic antisense oligonucleotide via receptor-mediated endocytosis. *Nucleic Acids Res.* 2008;36:2764–76.
- [82] Michael J, Schönenhart L, Israel I, Beutner R, Scharnweber D, Worch H, Hempel U, Schwenzer B. Oligonucleotide-RGD peptide conjugates for surface modification of titanium implants and improvement of osteoblast adhesion. *Bioconjug Chem.* 2009;20:710–8.
- [83] Nakagawa O, Ming X, Carver K, Juliano R. Conjugation with receptor-targeted histidine-rich peptides enhances the pharmacological effectiveness of antisense oligonucleotides. *Bioconjug Chem.* 2014;25:165–70.
- [84] Mier W, Eritja R, Mohammed A, Haberkorn U, Eisenhut M. Preparation and evaluation of tumor-targeting peptide-oligonucleotide conjugates. *Bioconjug Chem.* 2000;11:855–60.
- [85] Mier W, Eritja R, Mohammed A, Haberkorn U, Eisenhut M. Peptide-PNA conjugates: targeted transport of antisense therapeutics into tumors. *Angew Chem, Int Ed Engl.* 2003;42:1968–71.
- [86] Gottschling D, Seliger H, Tarrasón G, Piulats J, Eritja R. Synthesis of oligodeoxynucleotides containing N<sup>4</sup>-mercaptoproethylcytosine and their use in the preparation of oligonucleotide-peptide conjugates carrying c-myc tag sequence. *Bioconjug Chem.* 1998;9:831–7.
- [87] Frieden M, Aviñó A, Tarrasón G, Escorihuela M, Piulats J, Eritja R. Synthesis of oligonucleotide-peptide conjugates carrying the c-myc peptide epitope as recognition system. *Chem Biodivers.* 2004;1:930–8.
- [88] Gottschling D, Seliger H, Tarrasón G, Piulats J, Wiersma M, Eritja R. Synthesis of peptide nucleic acid-peptide chimeras carrying c-myc TAG-sequence. *Lett Pept Sci.* 2000;7:35–9.
- [89] Merkoçi A, Aldavert M, Tarrasón G, Eritja R, Alegret S. Toward an ICPMS-linked DNA assay based on gold nanoparticles immunoconnected through peptide sequences. *Anal Chem.* 2005;77:6500–3.
- [90] Aviñó A, Gómara MJ, Malakoutikhah M, Haro I, Eritja R. Oligonucleotide-peptide conjugates: solid-phase synthesis under acidic conditions and use in ELISA assays. *Molecules.* 2012;17:13825–43.

- [91] Portela C, Albericio F, Eritja R, Castedo L, Mascareñas JL. Ds-Oligonucleotide-peptide conjugates featuring peptides from the leucine zipper region of fos as switchable receptors for the oncoprotein *Jun*. *ChemBioChem*. 2007;8:1110–4.
- [92] Niemeyer CM. Semisynthetic DNA-protein conjugates for biosensing and nanofabrication. *Angew Chem, Int Ed Engl*. 2010;49:1200–16.
- [93] Niemeyer CM. Functional devices from DNA and proteins. *Nano Today*. 2007;2:42–52.
- [94] Niemeyer CM. The developments of semisynthetic DNA-protein conjugates. *Trends Biotechnol*. 2002;20:395–401.
- [95] McMillan JR, Brodin JD, Millan JA, Lee B, Olvera de la Cruz M, Mirkin CA. Modulating nanoparticle superlattice structure using proteins with tunable bond distributions. *J Am Chem Soc*. 2017;139:1754–7.
- [96] Akter F, Mie M, Kobatake E. DNA-based immunoassays for sensitive detection of protein. *Sens Actuators B, Chem*. 2014;202:1248–56.
- [97] Rajur SB, Roth CM, Morgan JR, Yarmush ML. Covalent protein- oligonucleotide conjugates for efficient delivery of antisense molecules. *Bioconjug Chem*. 1997;8:935–40.
- [98] Kang H, Alam MR, Dixit V, Fisher M, Juliano RL. Cellular delivery and biological activity of antisense oligonucleotides conjugated to a targeted protein carrier. *Bioconjug Chem*. 2008;19:2182–8.
- [99] Corey DR, Pei D, Schultz PG. Generation of a catalytic sequence-specific hybrid DNase. *Biochemistry*. 1989;28:8277–86.
- [100] Reyes RA, Cockerell GL. Preparation of pure oligonucleotide-alkaline phosphatase conjugates. *Nucleic Acids Res*. 1993;21:5532–3.
- [101] Cordero MJ, Murillo MI, Arroyo D, Eritja R, San Segundo B. Use of oligonucleotide-alkaline phosphatase conjugates as non-radioactive probes for rapid analysis of a proteinase inhibitor gene from Zea mays. *Plant Mol Biol Rep*. 1994;22:265–73.
- [102] Lovrinovic M, Niemeyer CM. Microtiter plate-based screening for the optimization of DNA–protein conjugate synthesis by means of expressed protein ligation. *ChemBioChem*. 2007;8:61–7.
- [103] Khatwani SL, Kang JS, Mullen DG, Hast MA, Beese LS, Distefano MD, Taton TA. Covalent protein-oligonucleotide conjugates by copper-free click reaction. *Bioorg Med Chem*. 2012;20:4532–9.
- [104] Glynou K, Ioannou PC, Christopoulos TK. Affinity capture-facilitated preparation of aequorin-oligonucleotide conjugates for rapid hybridization assays. *Bioconjug Chem*. 2003;14:1024–9.
- [105] Leonetti JP, Rayner B, Lemaitre M, Gagnor C, Milhaud PG, Imbach JL, Lebleu B. Antiviral activity of conjugates between poly(L-Lysine) and synthetic oligodeoxyribonucleotides. *Gene*. 1988;72:323–32.
- [106] Haralambidis J, Lagunita L, Tregear GW. The preparation of enzyme-labelled oligonucleotides by reductive amination. *Bioorg Med Chem Lett*. 1994;4:1005–10.
- [107] Trads JB, Tørring T, Gothelf KV. Site-selective conjugation of native proteins with DNA. *Acc Chem Res*. 2017;50:1367–74.
- [108] Zhou K, Dong J, Zhou Y, Dong J, Wang M, Wang Q. Toward precise manipulation of DNA–protein hybrid nanoarchitectures. *Small*. 2019;1804044.
- [109] Sievers EL, Senter PD. Antibody-drug conjugates in cancer therapy. *Annu Rev Med*. 2013;64:15–29.
- [110] Dovgan I, Koniev O, Kolodych S, Wagner A. Antibody–oligonucleotide conjugates as therapeutic, imaging, and detection agents. *Bioconjug Chem*. 2019;30:2483–501.
- [111] Tsuchikama K, An Z. Antibody-drug conjugates: recent advances in conjugation and linker chemistries. *Protein Cell*. 2018;9:33–46.

- [112] Ma Y, Kowolik CM, Swiderski PM, Kortylewski M, Yu H, Horne DA, Jove R, Caballero OL, Simpson AJ, Lee FT, Pillay V, Humanized SAM. Lewis-Y specific antibody based delivery of STAT3 siRNA. *ACS Chem Biol.* 2011;6:962–70.
- [113] Liu L, Song P, Märcher A, Kjems J, Yang H, Gothelf KV. Selective delivery of doxorubicin to EGFR<sup>+</sup> cancer cells by Cetuximab-DNA conjugates. *ChemBioChem.* 2019;20:1014–18.
- [114] Unzueta U, Céspedes MV, Vázquez E, Ferrer-Miralles N, Mangues R, Villaverde A. Towards protein-based viral mimetics for cancer therapies. *Trends Biotechnol.* 2015;33:253–8.
- [115] Céspedes MV, Unzueta U, Aviñó A, Gallardo A, Álamo P, Sala R, Sánchez-Sardi A, Casanova I, Mangues MA, Lopez-Pousa A, Eritja R, Villaverde A, Vázquez E, Mangues R. Selective depletion of metastatic stem cells as therapy for human colorectal cancer. *EMBO Mol Med.* 2018;10:e8772.
- [116] Aviñó A, Unzueta U, Céspedes MV, Casanova I, Vázquez E, Villaverde A, Mangues R, Eritja R. Efficient bioactive oligonucleotide-protein conjugation for cell-targeted cancer therapy. *ChemistryOpen.* 2019;8:382–7.
- [117] Zatsepina TS, Oretskaya TS. Synthesis and applications of oligonucleotide-carbohydrate conjugates. *Chem Biodivers.* 2004;1:1401–17.
- [118] Morales JC. Synthesis of carbohydrate-oligonucleotide conjugates and their applications. In: Fernández-Lucas J, Camarasa Rius MJ, editors. Enzymatic and chemical synthesis of nucleic acid derivatives. Weinheim: Wiley-VCH Verlag; 2019. p. 259–89.
- [119] Singh Y, Murat P, Defrancq E. Recent developments in oligonucleotide conjugation. *Chem Soc Rev.* 2010;39:2054–70.
- [120] Akhtar S, Routledge A, Patel R, Gardiner JM. Synthesis of mono- and dimannoside phosphoramidite derivatives for solid-phase conjugation to oligonucleotides. *Tetrahedron Lett.* 1995;36:7333–6.
- [121] Morales JC, Reina JJ, Díaz I, Aviñó A, Nieto PM, Eritja R. Experimental measurement of carbohydrate-aromatic stacking in water by using a dangling-ended DNA model system. *Chem Eur J.* 2008;14:7828–35.
- [122] Ugarte-Uribe B, Pérez-Rentero S, Lucas R, Aviñó A, Reina JJ, Alkorta I, Eritja R, Morales JC. Synthesis, cell-surface binding and cellular uptake of fluorescently labelled glucose-DNA conjugates with different carbohydrate presentation. *Bioconjug Chem.* 2010;21:1280–7.
- [123] Aviñó A, Ocampo SM, Lucas R, Reina JJ, Morales JC, Perales JC, Eritja R. Synthesis and in vitro inhibition properties of siRNA conjugates carrying glucose and galactose with different presentation. *Mol Divers.* 2011;15:751–7.
- [124] D'Onofrio J, de Champdoré M, De Napoli L, Montesarchio D, Di Fabio G. Glycomimetics as decorating motifs for oligonucleotides: solid-phase synthesis, stability, and hybridization properties of carbopeptoid-oligonucleotide conjugates. *Bioconjug Chem.* 2005;16:1299–309.
- [125] D'Onofrio J, Petraccone L, Martino L, Di Fabio G, Iadonisi A, Balzarini J, Giancola C, Montesarchio D. Synthesis, biophysical characterization, and anti-HIV activity of glyco-conjugated G-quadruplex-forming oligonucleotides. *Bioconjug Chem.* 2008;19:607–16.
- [126] Ikeda Y, Kubota D, Nagasaki Y. Simple solid-phase synthesis and biological properties of carbohydrate-oligonucleotide conjugates modified at the 3'-terminus. *Bioconjug Chem.* 2010;21:1685–90.
- [127] Adinolfi M, De Napoli L, Di Fabio G, Iadonisi A, Montesarchio D. Modulating the activity of oligonucleotides by carbohydrate conjugation: solid phase synthesis of sucrose-oligonucleotide hybrids. *Org Biomol Chem.* 2004;2:1879–86.
- [128] Matsura K, Hibino M, Et KM et al. Phosphoramidite solid-phase synthesis of site-specifically glycosylated oligodeoxynucleotides. *Tetrahedron Lett.* 2000;41:7529–33.

- [129] Matsuura K, Hibino M, Yamada Y, Kobayashi K. Construction of glyco-clusters by self-organization of site-specifically glycosylated oligodeoxynucleotides and their cooperative amplification of lectin-recognition. *J Am Chem Soc.* 2001;123:357–8.
- [130] Charles I, Xi H, Arya DP. Sequence-specific targeting of RNA with an oligonucleotide-neomycin conjugate. *Bioconjug Chem.* 2007;18:160–9.
- [131] Pourceau G, Meyer A, Vasseur JJ, Morvan F. Synthesis of mannose and galactose oligonucleotide conjugates by bi-click chemistry. *J Org Chem.* 2009;74:1218–22.
- [132] Pourceau G, Meyer A, Chevrolot Y, Souteyrand E, Vasseur JJ, Morvan F. Oligonucleotide carbohydrate-centered galactosyl cluster conjugates synthesized by click and phosphoramidite chemistries. *Bioconjug Chem.* 2010;21:1520–9.
- [133] Yamada T, Peng CG, Matsuda S, Addepalli H, Jayaprakash KN, Alam R, Mills K, Maier MA, Charisse K, Sekine M, Manoharan M, Rajeev KG. Versatile site-specific conjugation of small molecules to siRNA using click chemistry. *J Org Chem.* 2011;76:1198–211.
- [134] Bouillon C, Meyer A, Vidal S, Jochum A, Chevrolot Y, Cloarec JP, Praly JP, Vasseur JJ, Morvan F. Microwave assisted “Click” chemistry for the synthesis of multiple labeled-carbohydrate oligonucleotides on solid support. *J Org Chem.* 2006;71:4700–2.
- [135] Chevrolot Y, Bouillon C, Vidal S, Morvan F, Meyer A, Cloarec JP, Jochum A, Praly JP, Vasseur JJ, Souteyrand E. DNA-based carbohydrate biochips: a platform for surface glyco-engineering. *Angew Chem.* 2007;119:2450–4.
- [136] van Delft P, Meeuwenhoord NJ, Hoogendoorn S, Dinkelaar J, Overkleef HS, van der Marel GA, Filippov DV. Synthesis of oligoribonucleic acid conjugates using a cyclooctyne phosphoramidite. *Org Lett.* 2010;12:5486–9.
- [137] Forget D, Renaudet O, Botury D, Defrancq E, Dumy P. 3-Oligonucleotides conjugation via chemoselective oxime bond formation. *Tetrahedron Lett.* 2001;42:9171–4.
- [138] Katajisto J, Virta P, Lönnberg H. Solid-phase synthesis of multiantennary oligonucleotide glycoconjugates utilizing on-support oximation. *Bioconjug Chem.* 2004;15:890–6.
- [139] Singh Y, Renaudet O, Defrancq E, Dumy P. Preparation of a multitopic glycopeptide-oligonucleotide conjugate. *Org Lett.* 2005;7:1359–62.
- [140] Matsuura K, Akasaka T, Hibino M, Kobayashi K. Facile synthesis of stable and lectin-recognizable DNA-carbohydrate conjugates via diazo coupling. *Bioconjug Chem.* 2000;11:202–11.
- [141] Yan H, López Aguilar A, Zhao Y. Preparation of carbohydrate–oligonucleotide conjugates using the squareate spacer. *Bioorg Med Chem Lett.* 2007;17:6535–8.
- [142] Lucas R, Gómez-Pinto I, Aviñó A, Reina JJ, Eritja R, González C, Morales JC. Highly polar carbohydrates stack onto DNA duplexes via CH/π interactions. *J Am Chem Soc.* 2011;133:1909–16.
- [143] Lucas R, Vengut-Climent E, Gómez-Pinto I, Aviñó A, Eritja R, González C, Morales JC. Apolar carbohydrates as DNA capping agents. *Chem Commun.* 2012;48:2991–3.
- [144] Gómez-Pinto I, Vengut-Climent E, Lucas R, Aviñó A, Eritja R, González C, Morales JC. Carbohydrate DNA interaction at G-quadruplexes: folding and stability changes by attaching sugars at the 5'-end. *Chem Eur J.* 2013;19:1920–7.
- [145] Lucas R, Peñalver P, Gómez-Pinto I, Vengut-Climent E, Mtashobbya L, Cousin J, Maldonado O, Pérez V, Reynes V, Aviñó A, Eritja R, González C, Linclau B, Morales JC. Effects of sugar functional groups, hydrophobicity and fluorination on carbohydrate-DNA stacking interactions in water. *J Org Chem.* 2014;79:2419–29.
- [146] Vengut-Climent E, Terrazas M, Lucas R, Arévalo-Ruiz M, Eritja R, Synthesis MJC. RNAi activity and nuclease-resistant properties of apolar carbohydrates siRNA conjugates. *Bioorg Med Chem Lett.* 2013;23:4048–51.

- [147] Maier MA, Yannopoulos CG, Mohamed N, Roland A, Fritz H, Mohan V, Just G, Manoharan M. Synthesis of antisense oligonucleotides conjugated to a multivalent carbohydrate cluster for cellular targeting. *Bioconjug Chem*. 2003;14:18–29.
- [148] Prakash TP, Yu J, Migawa MT, Kinberger GA, Wan WB, Østergaard ME, Carty RL, Vasquez G, Low A, Chappell A, Schmidt K, Aghajan M, Crosby J, Murray HM, Booten SL, Hsiao J, Soriano A, Machemer T, Cauntay P, Burel SA, Murray SF, Gaus H, Graham MJ, Swayze EE, Seth PP. Comprehensive structure – activity relationship of triantennary *N*-acetylgalactosamine conjugated antisense oligonucleotides for targeted delivery to hepatocytes. *J Med Chem*. 2016;59:2718–33.
- [149] Schmidt K, Prakash TP, Donner AJ, Kinberger GA, Gaus HJ, Low A, Østergaard ME, Bell M, Swayze EE, Seth PP. Characterizing the effect of GalNAc and phosphorothioate backbone on binding of antisense oligonucleotides to the asialoglycoprotein receptor. *Nucleic Acids Res*. 2017;45:2294–306.
- [150] Nair JK, Willoughby JLS, Chan A, Charisse K, Alam R, Wang Q, Hoekstra M, Kandasamy P, Kel'in AV, Milstein S, Taneja N, O'Shea J, Shaikh S, Zhang L, van der Sluis RJ, Jung ME, Akinc A, Hutabarat R, Kuchimanchi S, Fitzgerald K, Zimmermann T, van Berkel TJC, Maier MA, Rajeev KG, Multivalent MM. *N*-Acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. *J Am Chem Soc*. 2014;136:16958–61.
- [151] Janas MM, Schlegel MK, Harbison CE, Yilmaz VO, Jiang Y, Parmar R, Zlatev I, Castoreno A, Xu H, Shulga-Morskaya S, Rajeev KG, Manoharan M, Keirstead ND, Maier MA, Jadhav V. Selection of GalNAc-conjugated siRNAs with limited off-target-driven rat hepatotoxicity. *Nat Commun*. 2018;9:723.
- [152] Hassler MA, Turanov AA, Alterman JF, Haraszti RA, Coles AH, Osborn MF, Echeverria D, Nikan M, Salomon WE, Roux L, Godinho BMDC, Davis SM, Morrissey DV, Zamore PD, Karumanchi SA, Moore MJ, Aronin N, Khvorova A. Comparison of partially and fully chemically-modified siRNA in conjugate-mediated delivery *in vivo*. *Nucleic Acids Res*. 2018;46:2185–96.
- [153] Rajeev KG, Nair JK, Jayaraman M, Charisse K, Taneja N, O'Shea J, Willoughby JLS, Yucus K, Nguyen T, Shulga-Morskaya S, Milstein S, Liebow A, Querbes W, Borodovsky A, Fitzgerald K, Maier MA, Manoharan M. Hepatocyte-specific delivery of siRNAs conjugated to novel non-nucleosidic trivalent *N*-acetylgalactosamine elicits robust gene silencing *in vivo*. *ChemBioChem*. 2015;16:903–8.
- [154] Matsuda S, Keiser K, Nair JK, Charisse K, Manoharan RM, Kretschmer P, Peng CG, Kel'in AV, Kandasamy P, Willoughby JLS, Liebow A, Querbes W, Yucus K, Nguyen T, Milstein S, Maier MA, Rajeev KG, Manoharan M. siRNA conjugates carrying sequentially assembled trivalent *N*-acetylgalactosamine linked through nucleosides elicit robust gene silencing *in vivo* in hepatocytes. *ACS Chem Biol*. 2015;10:1181–7.
- [155] Balwani M, Sardh E, Ventura P, Aguilera Peiró P, Rees DC, Stölzel U, Bissell DM, Bonkovsky HL, Windyga J, Anderson KE, Parker C, Silver SM, Keel SB, Wang JD, Stein PE, Harper P, Vassiliou D, Wang B, Phillips J, Ivanova A, Langendonk JG, Kauppinen R, Minder E, Horie Y, Penz C, Chen J, Liu S, Ko JJ, Sweetser MT, Garg P, Vaishnav A, Kim JB, Simon AR, Gouya L. Phase 3 trial of RNAi therapeutic Givosiran for acute intermittent porphyria. *N Engl J Med*. 2020;382:2289–301.
- [156] Ray KK, Wright RS, Kallend D, Koenig W, Leiter LA, Raal FJ, Bisch JA, Richardson T, Jaros M, Wijngaard PLJ, Kastelein JJP. Two phase 3 trials of inclisiran in patients with elevated LDL cholesterol. *N Engl J Med*. 2020;382:1507–19.
- [157] Zhu L, Mahato RI. Targeted delivery of siRNA to hepatocytes and hepatic stellate cells by bioconjugation. *Bioconjug Chem*. 2010;21:2119–27.

- [158] St-Pierre G, Pal S, Østergaard ME, Zhou T, Yu J, Tanowitz M, Seth PP, Hanessian S. Synthesis and biological evaluation of sialyl-oligonucleotide conjugates targeting leukocyte B trans-membranal receptor CD22 as delivery agents for nucleic acid drugs. *Bioorg Med Chem.* 2016;24:2397–409.
- [159] Karskela M, Virta P, Malinen M, Urtti A, Lönnberg H. Synthesis and cellular uptake of fluorescently labeled multivalent hyaluronan disaccharide conjugates of oligonucleotide phosphorothioates. *Bioconjug Chem.* 2008;19:2549–58.
- [160] Jadhac S, Käkelä M, Mäkilä J, Kiugel M, Liljenbäck H, Virta J, Poijärvi-Virta P, Laitala-Leinonen T, Kyöö V, Jalkanen S, Saraste A, Roivainen A, Lönnberg H, Virta P. Synthesis and in vivo PET imaging of hyaluronan conjugates of oligonucleotides. *Bioconjug Chem.* 2016;27:391–403.
- [161] Marlin F, Simon P, Saïson-Behmoaras T, Giovannangeli C. Delivery of oligonucleotides and analogues: the oligonucleotide conjugate-based approach. *ChemBioChem.* 2010;11:1493–500.
- [162] Dohmen C, Frohlich T, Lachelt U, Rohl I, Vornlocher HP, Hadwiger P, Wagner E. Defined folate-PEG-siRNA conjugates for receptor-specific gene silencing. *Mol Ther Nucleic Acids.* 2012;1:e7.
- [163] Kazanova EV, Zubin EM, Kachalova AV, Volkov EM, Oretskaya TS, Stetsenko DA, Gottikh MB. A convenient solid-phase method for the synthesis of novel oligonucleotide-folate conjugates. *Nucleosides Nucleotides Nucleic Acids.* 2007;26:1273–6.
- [164] Harrison JG, Balasubramanian S. A convenient synthetic route to oligonucleotide conjugates. *Bioorg Med Chem.* 1997;7:1041–6.
- [165] Matulic-Adamic J, Serebryany V, Haeberli P, Mokler VR, Beigelman L. Synthesis of *N*-acetyl-D-galactosamine and folic acid conjugated ribozymes. *Bioconjug Chem.* 2002;13:1071–8.
- [166] Habus I, Xie J, Iyer RP, Zhou WQ, Shen LX, Agrawal S. A mild and efficient solid-support synthesis of novel oligonucleotide conjugates. *Bioconjug Chem.* 1998;9:283–91.
- [167] Nakagawa O, Ming X, Huang L, Juliano RL. Targeted intracellular delivery of antisense oligonucleotides via conjugation with small-molecule ligands. *J Am Chem Soc.* 2010;132:8848–9.
- [168] Willibald J, Harder J, Sparrer K, Conzelmann KK, Carell T. Click-modified anandamide siRNA enables delivery and gene silencing in neuronal and immune cells. *J Am Chem Soc.* 2012;134:12330–3.
- [169] Alivisatos AP, Johnsson KP, Peng X, Wilson TE, Loweth CJ, Bruchez MP Jr., Schultz PG. Organization of “nanocrystal molecules” using DNA. *Nature.* 1996;382:609–11.
- [170] Loweth CJ, Caldwell WB, Peng X, Alivisatos AP, Schultz PG. DNA-based assembly of gold nanocrystals. *Angew Chem, Int Ed Engl.* 1999;38:1808–12.
- [171] Mirkin CA, Letsinger RL, Mucic RC, Storhoff JJ. A DNA-based method for rationally assembling nanoparticles into macroscopic materials. *Nature.* 1996;382:607–9.
- [172] Storhoff JJ, Elghanian R, Mucic RC, Mirkin CA, Letsinger RL. One-pot colorimetric differentiation of polynucleotides with single base imperfections using gold nanoparticle probes. *J Am Chem Soc.* 1998;120:1959–64.
- [173] Chen J, Seeman NC. Synthesis from DNA of a molecule with the connectivity of a cube. *Nature.* 1991;350:631–3.
- [174] Winfree E, Liu F, Wenzler LA, Seeman NC. Design and self-assembly of two-dimensional DNA crystals. *Nature.* 1998;394:539–44.
- [175] Zhang Y, Seeman NC. Construction of a DNA-truncated octahedron. *J Am Chem Soc.* 1994;116:1661–9.
- [176] Braun E, Eichen Y, Sivan U, Ben-Yoseph G. DNA-templated assembly and electrode attachment of a conducting silver wire. *Nature.* 1998;391:775–8.

- [177] Keren K, Krueger M, Gilad R, Ben-Yoseph G, Sivan U, Braun E. Sequence-specific molecular lithography on single DNA molecules. *Science*. 2002;297:72–5.
- [178] Aldaye FA, Palmer AL, Sleiman HF. Assembling materials with DNA as the guide. *Science*. 2008;321:1795–9.
- [179] Seeman NC. Nucleic acid nanostructures and topology. *Angew Chem Int Ed*. 1998;3220–8.
- [180] Kershner RJ, Bozano LD, Micheel CM, Hung AM, Fornof AR, Cha JN, Rettner CT, Bersani M, Frommer J, Rothmund PW, Wallraff GM. Placement and orientation of individual DNA shapes on lithographically patterned surfaces. *Nat Nanotechnol*. 2009;4:557–61.
- [181] Gállego I, Manning B, Prades JD, Mir M, Samitier J, Eritja R. DNA origami-driven lithography for patterning on gold surfaces with sub-10 nanometer resolution. *Adv Mater*. 2017;29:1603233.
- [182] Bell NM, Mickefield J. Chemical modification of oligonucleotides for therapeutic, bioanalytical and other applications. *ChemBioChem*. 2009;10:2691–703.
- [183] Yang YR, Liu Y, Yan H. DNA nanostructures as programmable biomolecular scaffolds. *Bioconjug Chem*. 2015;26:1381–95.
- [184] Tintoré M, Eritja R, Fàbrega C. DNA nanoarchitectures: steps towards biological applications. *ChemBioChem*. 2014;15:1374–90.
- [185] Hu Q, Wang S, Wang L, Gu H, Fan C. DNA nanostructure-based systems for intelligent delivery of therapeutic oligonucleotides. *Adv Healthcare Mater*. 2018;1701153.
- [186] Giljohann DA, Seferos DS, Daniel WL, Massich MD, Patel PC, Mirkin CA. Gold nanoparticles for biology and medicine. *Angew Chem, Int Ed Engl*. 2012;49:3280–94.
- [187] Shen J, Zhang W, Qi R, Mao ZW, Shen H. Engineering functional inorganic-organic hybrid systems: advances in siRNA therapeutics. *Chem Soc Rev*. 2018;47:1969–95.
- [188] Mahmoodi Chalbatani G, Dana H, Gharagouzloo E, Grijalvo S, Eritja R, Logsdon CD, Memari F, Miri SR, Rad MR, Marmari V. The small interfering RNAs (siRNAs) in cancer therapy: nano-based approach. *Int J Nanomed*. 2019;14:3111–28.
- [189] Saha K, Agasti SS, Kim C, Li X, Rotello VM. Gold nanoparticles in chemical and biological sensing. *Chem Rev*. 2012;112:2739–79.
- [190] Llevot A, Astruc D. Applications of vectorized gold nanoparticles to the diagnosis and therapy of cancer. *Chem Soc Rev*. 2012;41:242–57.
- [191] Taton TA. Preparation of gold-nanoparticle-DNA conjugates. *Curr Protoc Nucleic Acid Chem*. 2002;12.2.1–12.
- [192] Demers LM, Mirkin CA, Mucic RC, Reynolds RA, Letsinger RL, Elghanian R, Viswanadham G. A fluorescence-based method for determining the surface coverage and hybridization efficiency of thiol-capped oligonucleotides bound to gold thin films and nanoparticles. *Anal Chem*. 2000;72:5535–41.
- [193] Storhoff LL, Lazarides AA, Mucic RC, Mirkin CA, Letsinger RL, Schatz GC. What controls the optical properties of DNA-linked gold nanoparticle assemblies? *J Am Chem Soc*. 2000;122:4640–50.
- [194] Dam DHM, Lee H, Lee RC, Kim KH, Kelleher NL, Odom TW. Tunable loading of oligonucleotides with secondary structure on gold nanoparticles through a pH-driven method. *Bioconjug Chem*. 2015;26:279–85.
- [195] Dougan JA, Karlsson C, Smith WE, Graham D. Enhanced oligonucleotide-nanoparticle conjugate stability using thiocytic acid modified oligonucleotides. *Nucleic Acids Res*. 2007;35:3668–75.
- [196] Dougan JA, Reid AK, Graham D. Thiocytic acid modification of oligonucleotides using an H-phosphonate. *Tetrahedron Lett*. 2010;51:5787–90.

- [197] Pérez-Rentero S, Grijalvo S, Peñuelas G, Fàbrega C, Eritja R. Thioctic acid derivatives as building blocks to incorporate DNA oligonucleotides onto gold nanoparticles. *Molecules*. 2014;19:10495–523.
- [198] Rosi NL, Giljohann DA, Thaxton CS, Lytton-Jean AKR, Han MS, Mirkin CA. Oligonucleotide-modified gold nanoparticles for intracellular gene regulation. *Science*. 2006;312:1027–30.
- [199] Giljohann DA, Seferos DS, Prigodich AE, Patel PC, Mirkin CA. Gene regulation with polyvalent siRNA – nanoparticle conjugates. *J Am Chem Soc*. 2009;131:2072–3.
- [200] Singh N, Agrawal A, Leung AKL, Sharp PA, Bhatia SN. Effect of nanoparticle conjugation on gene silencing by RNA interference. *J Am Chem Soc*. 2010;132:8241–3.
- [201] Cutler JI, Auyeung E, Mirkin CA. Spherical nucleic acids. *J Am Chem Soc*. 2012;134:1376–91.
- [202] Zheng D, Giljohann DA, Chen DL, Massich MD, Wang XQ, Iordanov H, Mirkin CA, Paller AS. Topical delivery of siRNA-based spherical nucleic acid nanoparticle conjugates for gene regulation. *Proc Natl Acad Sci USA*. 2012;109:11975–80.
- [203] Vigderman L, Zubarev ER. Therapeutic platforms based on gold nanoparticles and their covalent conjugates with drug molecules. *Adv Drug Deliv Rev*. 2013;65:663–76.
- [204] Choi CH, Hao L, Narayan SP, Auyeung E, Mirkin CA. Mechanism for the endocytosis of spherical nucleic acid nanoparticle conjugates. *Proc Natl Acad Sci USA*. 2013;110:7625–30.
- [205] Patel PC, Giljohann DA, Daniel WL, Zheng D, Prigodich AE, Mirkin CA. Scavenger receptors mediate cellular uptake of polyvalent oligonucleotide-functionalized gold nanoparticles. *Bioconjug Chem*. 2010;21:2250–6.
- [206] Zhang K, Hao L, Hurst SJ, Mirkin CA. Antibody-linked spherical nucleic acids for cellular targeting. *J Am Chem Soc*. 2012;134:16488–91.
- [207] Song WC, Kim KR, Park M, Lee KE, Ahn DR. Backbone-modified oligonucleotides for tuning the cellular uptake behavior of spherical nucleic acids. *Biomater Sci*. 2017;5:412–6.
- [208] Watson JD, Crick FH. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature*. 1953;171:737–8.
- [209] Rothemund PW, Papadakis N, Winfree E. Algorithmic self-assembly of DNA Sierpinski triangles. *PLoS Biol*. 2004;2:e424.
- [210] Fu J, Liu M, Liu Y, Yan H. Spatially-interactive biomolecular networks organized by nucleic acid nanostructures. *Acc Chem Res*. 2012;45:1215–26.
- [211] Seeman NC, Sleiman HF. DNA nanotechnology. *Nat Rev*. 2017;3:17068.
- [212] Seeman NC. Nucleic acid junctions and lattices. *J Theor Biol*. 1982;99:237–47.
- [213] Rothemund PW. Folding DNA to create nanoscale shapes and patterns. *Nature*. 2006;440:297–302.
- [214] de Vries JW, Zhang F, Herrmann A. Drug delivery systems based on nucleic acid nanostructures. *J Control Release*. 2013;172:467–83.
- [215] Pinheiro AV, Han D, Shih WM, Yan H. Challenges and opportunities for structural DNA nanotechnology. *Nat Nanotechnol*. 2011;6:763–72.
- [216] Lo PK, Metera KL, Sleiman HF. Self-assembly of three-dimensional DNA nanostructures and potential biological applications. *Curr Opin Chem Biol*. 2010;14:597–607.
- [217] Hu Q, Li H, Wang L, Gu H, Fan C. DNA nanotechnology-enabled drug delivery systems. *Chem Rev*. 2019;119:6459–506.
- [218] Li J, Fan C, Pei H, Shi J, Huang Q. Smart drug delivery with self-assembled DNA nanostructures. *Adv Mater*. 2013;25:4386–96.
- [219] Tibbitt MW, Dahlman JE, Langer R. Emerging frontiers in drug delivery. *J Am Chem Soc*. 2016;138:704–11.
- [220] Bujold KE, Lacroix A, Sleiman HF. DNA nanostructures at the interface with biology. *Chem*. 2018;4:495–521.

- [221] Angell C, Xie S, Zhang L, Chen Y. DNA nanotechnology for precise control over drug delivery and gene therapy. *Small*. 2016;12:1117–32.
- [222] Ko S, Liu H, Chen Y, Mao C. DNA nanotubes as combinatorial vehicles for cellular delivery. *Biomacromolecules*. 2008;9:3039–43.
- [223] Bathia D, Surana S, Chakraborty S, Sandhya P, Koushika SP, Krishnan Y. A synthetic icosahedral DNA-based host–cargo complex for functional *in vivo* imaging. *Nat Commun*. 2011;2:339.
- [224] Goodman RP, Schaap IAT, Tardin CF, Erben CM, Berry RM, Schmidt CF, Turberfield AJ. Rapid chiral assembly of rigid DNA building blocks for molecular nanofabrication. *Science*. 2005;310:1661–5.
- [225] Kim KR, Kim DR, Lee T, Yhee JY, Kim BS, Kwon IC, Ahn DR. Drug delivery by a self-assembled DNA tetrahedron for overcoming drug resistance in breast cancer cells. *Chem Commun*. 2013;49:2010–2.
- [226] Lee H, Lytton-Jean AKR, Chen Y, Love KT, Park AI, Karagiannis ED, Sehgal A, Querbes W, Zurenko CS, Jayaraman M, Peng CG, Charisse K, Borodovsky A, Manoharan M, Donahoe JS, Truelove J, Nahrendorf M, Langer R, Anderson DG. Molecularly self-assembled nucleic acid nanoparticles for targeted *in vivo* siRNA delivery. *Nat Nanotechnol*. 2012;7:389–93.
- [227] Yan J, Chen J, Zhang N, Yang Y, Zhu W, Li L, He B. Mitochondria-targeted tetrahedral DNA nanostrucutures for doxorubicin delivery and enhancement of apoptosis. *J Mat Chem B*. 2020;8:492–503.
- [228] Bujold KE, Hsu JCC, Sleiman HF. Optimized DNA “nanosuitcases” for encapsulation and conditional release of siRNA. *J Am Chem Soc*. 2016;138:14030–8.
- [229] Lacroix A, Edwardson TGW, Hancock MA, Dore MD, Sleiman HF. Development of DNA nanostructures for high-affinity binding to human serum albumin. *J Am Chem Soc*. 2017;139:7355–62.
- [230] Bousmail D, Amrein L, Fakhoury JJ, Fakih HH, Hsu JCC, Panasci L, Sleiman HF. Precision spherical nucleic acids for delivery of anticancer drugs. *Chem Sci*. 2017;8:6218–29.
- [231] Jiang Q, Song C, Nangrave J, Liu X, Lin L, Qiu D, Wang ZG, Zou G, Liang X, Yan H, Ding B. DNA origami as a carrier for circumvention of drug resistance. *J Am Chem Soc*. 2012;134:13396–403.
- [232] Zhao YX, Shaw A, Zeng X, Benson A, Nystrom AM, Höglberg B. DNA origami delivery system for cancer therapy with tunable release properties. *ACS Nano*. 2012;6:8684–91.
- [233] Li J, Pei H, Zhu B, Liang L, Wei M, He Y, Chen N, Li D, Huang Q, Fan C. Self-assembled multivalent DNA nanostructures for noninvasive intracellular delivery of immunostimulatory CpG oligonucleotides. *ACS Nano*. 2011;5:8783–9.
- [234] Jorge AF, Aviñó A, Pais AAC, Eritja R, Fàbrega C. DNA-based nanoscaffolds as vehicles for 5-fluoro-2'-deoxyuridine oligomers in colorectal cancer therapy. *Nanoscale*. 2018;10:7238–49.
- [235] Li S, Jiang Q, Liu S, Zhang Y, Tian Y, Song C, Wang J, Zou Y, Anderson GJ, Han JY, Chang Y, Liu Y, Zhang C, Chen L, Zhou G, Nie G, Yan H, Ding B, Zhao Y. A DNA nanorobot functions as a cancer therapeutic in response to a molecular trigger *in vivo*. *Nat Biotechnol*. 2018;36:258–64.
- [236] Veneziano R, Moyer TJ, Stone MB, Wamhoff EC, Read BJ, Mukherjee S, Shepherd TS, Das J, Schief WR, Irvine DJ, Bathe M. Role of nanoscale antigen organization on B-cell activation probed using DNA origami. *Nat Nanotechnol*. 2020;15:716–23.
- [237] Langecker M, Arnout V, Martín TG, List J, Renner S, Mayer M, Dietz H, Simmel FC. Synthetic lipid membrane channels formed by designed DNA nanostructures. *Science*. 2012;338:932–6.
- [238] Langecker M, Arnout V, List J, Simmel FC. DNA nanostructures interacting with lipid bilayer membranes. *Acc Chem Res*. 2014;47:1807–15.

# Index

- 1,2,4-dithiazoline-3,5-dione 24  
1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indol-7-carboxylate tripeptide 108  
1,3-dipolar cycloaddition 155  
1,3-dithian-2-yl-methoxy 19  
1,3-dithian-2-yl-methoxycarbonyl 15  
1,3-propandiol linker 244  
1,3-propanediol 201  
1,4-anhydro-2-deoxyribitol 64  
1,4-anhydro-D-ribitol 19  
1,8-diaza[5.4.0]bicyclo[4.1.0]undec-7-ene 50  
1,8-diazabicyclo[5.4.0]undec-7-ene 19, 89, 284, 291  
1,*N*<sup>2</sup>-(1,3-propano)-2'-deoxyguanosine 67  
1,*N*<sup>2</sup>-etheno-2'-deoxyguanosine 68  
1,*N*<sup>2</sup>-propano-2'-deoxyguanosine 68  
1,*N*<sup>6</sup>-etheno-2'-deoxyadenosine 67  
1-(2-chloro-4-tolyl)-4-methoxypiperidin-4-yl 11  
1-(2-cyanoethoxy)ethyl 11  
1-(2-fluorophenyl)-4-methoxypiperidin-4-yl 11  
1-(3,4-dihydroxypropyl)-dG 68  
1-deaza-2'-deoxyadenosine 118  
1-hydroxy-benzotriazole 23  
1-hydroxyisoquinoline 112  
1-pyrenylethynyl 208  
2,3,4,5-tetrafuorobenzene 112  
2',3'-seco-RNA 299  
2,4-dibromotoluene 111  
2,4-dichlorobenzyl 286  
2,4-dichlorotoluene 111  
2,4-difluorotoluene 110, 111  
2,4-diiodotoluene 111  
2,4-dinitrophenyl 97, 104  
2,6-diaminopurine 47, 103, 118  
2,7-dioxopyrido[2,3-d]pyrimidine 107  
2-(4-methoxybenzamido)ethyl 161  
2-(4-tolylsulfonyl)ethoxymethyl 11  
2-(acetoxymethyl)benzoyl 67  
2-acetylaminofluorene 71  
2-amino-1,3-butane diol 300  
2-amino-1,3-propanediol 300  
2-amino-3-cyano-4-halopyridine-C-nucleoside 121  
2-amino-4-halopyridine-C-nucleoside 121  
2-amino-6-methoxyaminopurine 102  
2'-amino-RNA 295  
2-aminoadenine 103  
2-aminopurine 88, 92, 93, 168, 195  
2-aminopyridine 194  
2-aminoquinazoline 200  
2-aza-2'-deoxyinosine 101  
2-azido-2'-deoxyinosine 119  
2-(azidomethyl)benzoyl 8, 28  
2'-C-methylribonucleoside 296  
2'-C-piperazino-UNA 240  
2-chlorophenyl 4, 16, 25, 26  
2-cyano-1,1-dimethylethyl 17  
2-cyano-1,1-dimethylethyl ester 291  
2-cyanoethoxymethyl 11  
2-cyanoethyl 16, 25, 26, 29, 47, 61, 95–98, 291  
2-dehydro-thymidine 119  
2-deoxy-1,4-anhydroribitol 201  
2'-deoxy-2'-fluoro arabinocytosine 256  
2'-deoxy-2'-fluoro-5-methyl-arabinocytidine 257  
2'-deoxy-2'-fluoro-D arabino 238  
2'-deoxy-2'-fluoro-riboguanosine 239  
2'-deoxy-2'-fluorouridine 242  
2'-deoxy-4-desmethylwosine 70  
2'-deoxy-5-iodouridine 98  
2'-deoxy-6-thioinosine 97  
2'-deoxyinosine 100  
2-deoxyribonolactone 64, 65  
2'-deoxyribosyl formamidine 63  
2'-deoxyribosyl urea 63  
2'-deoxytubercidin 118  
2'-deoxyxanthosine 101, 114  
2'-deoxyzebularine 121  
2-(dibromomethyl)benzoyl 8  
2'-F-RNA 275  
2'-fluoro-2'-deoxycytidine 256  
2'-fluoro-RNA 295, 296  
2'-fluoroarabino 295  
2'-fluoroarabino nucleoside 296  
2-fluorohypoxanthine 164  
2-fluoropurine-2'-deoxynucleoside 94  
2-hydroxyethylphosphate 245  
2-(isopropylthiomethoxymethyl)benzoyl 8  
2'-methylarabino 295  
2-(*N*-isopropyl-4-methoxybenzamido)-ethyl 161  
2-nitrobenzyl 11, 63, 147, 149, 151  
(2-nitrobenzyl)oxymethyl 11  
2-nitrophenoylethoxycarbonyl 9  
2-nitrophenylpropoxycarbonyl 9

- 2-nitrophenylsulfenyl 15  
 2'-*O*-(2-aminoethyl)-RNA 206  
 2'-*O*-alkyl-RNA 294  
 2'-*O*-benzyl 295  
 2'-*O*-methyl-4-pyridine 295  
 2'-*O*-methyl-RNA 205, 238, 241, 253, 275, 294  
 2'-*O*-methylribonucleosides 194  
 2-pyridinone-2'-deoxyriboside 119  
 2-pyrimidinone 168  
 2-pyrimidinone-2'-deoxyriboside 119  
 2-(trimethylsilyl)ethoxymethyl 11  
 (2'S)-2'-deoxy-2'-methyl-cytidine 257  
 3,4-dibenzoyloxybenzyl 245  
 3',5'-dimethoxybenzoinoxycarbonyl 9  
 3,*N*<sup>4</sup>-etheno-2'-deoxycytidine 67  
 3-amino-propan-1,2-diol 19  
 3-deaza-2'-deoxyadenosine 118  
 3-deaza-2'-deoxyguanosine 118  
 3-deaza-thymidine 119  
 3-deazaadenine 105  
 3-((dimethylaminomethylidene)amino-3*H*-1,2,4-dithiazole-3-thione 24  
 3-ethoxy-1,2,4-dithiazoline-5-one 24  
 3'-fluoro hexitol nucleic acid 296  
 3'-fluoro hexitol nucleoside 295  
 3-fluoro-4-[2-(4-nitrophenylethoxy)-carbonyl]oxybenzoyloxyethyl 11  
 3-hydroxypropyl-thiophosphoryl 21  
 3-(*N,N*-dimethylamino)propyl 294  
 3-nitro-1,2,4-triazol-1-yl-tris(pyrrolidin-1-yl)-phosphonium hexafluorophosphate 293  
 3-nitro-1,2,4-triazole 23  
 3-nitopyrrole 102, 200  
 3*H*-1,2-benzodithiol-3-one-1,1-dioxide 24, 291  
 3' untranslated region 278  
 4,5,6,7-tetrafluoroindole 112  
 4,5-*bis*(ethoxycarbonyl)-1,3-dioxolan-2-yl 12  
 4,5-dicyanoimidazole 23  
 4-(1,2,4-triazol) thymidine 50  
 4-(1,2,4-triazolyl)-2'-deoxyuridine 162  
 4-[2-(4-nitrophenylethoxy carbonyl)-oxybenzoyloxyethyl 11  
 4-(3-benzamidophenyl)imidazole 197  
 4-aminobenzimidazole 105  
 (4-aminobutyl) propane-1,3-diol 200  
 4-azidobenzyl 293  
 4-guanidino-5-methylcytosine 195  
 4-isopropylphenoxyacetyl 14  
 4-methoxytetrahydropyran-4-yl 11  
 4-methylbenzimidazole 110  
 4-methylindole 110  
 4-(*N*-dichloroacetyl-*N*-methylamino)-benzyloxymethyl 12  
 4-(*N*-methylamino)benzyloxymethyl 12  
 4-nitrobenzimidazole 102  
 4-nitrobenzoyl 339  
 4-nitroindazole 102  
 4-nitrophényl ethyl 89  
 4-nitrophénylethyoxy carbonyl 15  
 4-nitropyridine 102  
 4-phenoxyphenyl 107  
 4-*tert*-butylphenoxyacetyl 14  
 4-thio-2'-deoxyuridine 99  
 4-thiouracil UNA 244  
 4(1,2,4-triazolyl)thymidine 162  
 5,6-dihydro-5-azacytosine 121  
 5,6-dihydrothymine 63  
 5,6-dimethyl-2'-deoxyuridine 105  
 5-(2,4-diamino)pyrimidine 114  
 5-(2-nitrophenyl)-1*H*-tetrazole 23  
 5-(4-nitrophenyl)-1*H*-tetrazole 23  
 5'-5' inversion polarity 237  
 5'-acylamino caps 109  
 5-aminoimidazole-4-carboxamide 101  
 5-(aminopropargyl)-uracil 191  
 5-(aminopropargyl)uridine 206  
 5-aminouracil 90, 190  
 5'-aryloxy carbonates 9  
 5-aza-2'-deoxycytidine 120, 121  
 5-azacytidine 120  
 5-azacytosine 252  
 5-benzylthio-1*H*-tetrazole 23  
 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole 23  
 5-bromo-2'-deoxyuridine 88, 90  
 5-bromo-dC 252  
 5-bromo-dU 244  
 5-bromo-U 200  
 5-bromocytosine 190  
 5-bromouracil 190  
 5'-*C*-malonyl-modified nucleotide 305  
 5'-*C*-methyl analog 305  
 5'-carbohydrate DNA 338  
 5-carboxy-2'-deoxycytidine 61  
 5-carboxy-dC 61  
 5-chloro-2'-deoxycytosine 91  
 5-chloro-2'-deoxyuridine 90  
 5-chloro-4-*O*-ethyl-2'-deoxyuridine 91

- 5-chloro-2'-deoxycytidine 163  
 5'-(E)-vinylphosphate 305  
 5-ethylthio-1*H*-tetrazole 23  
 5-fluoro-2'-deoxycytidine 90, 120, 121, 162  
 5-fluoro-2'-deoxyuridine 88, 283  
 5-fluoro-4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine 91  
 5-fluoro-4-*O*-ethyl-2'-deoxyuridine 91  
 5-fluoro-4-triazol-2'-deoxyuridine 91  
 5-fluorouracil 200  
 5-formyl-2'-deoxycytidine 61  
 5-formyl-2'-deoxyuridine 61  
 5-heteroaryl-2'-deoxyuridines 107  
 5-hydroxy-5-methylhydantoin 63  
 5-hydroxycytosine 61  
 5-hydroxymethyl-2'-deoxycytidine 61  
 5-hydroxymethyl-2'-deoxyuridine 61  
 5-hydroxymethyl-dC 251  
 5-hydroxymethyl-dU 244  
 5-hydroxyuracil 61  
 5-iodo-2'-deoxycytidine 91  
 5-iodo-2'-deoxyuridine 90, 165  
 5-iodocytosine 190  
 5-iodouracil 190  
 5'-isothiocyanatenucleosides 304  
 5'-methyl phosphonate 305  
 5-methyl-2'-deoxycytidine 119  
 5-methyl-2-pyrimidinone 168  
 5-methyl-4-pyrimidinone-2'-deoxyriboside 119  
 5-methyl-cytosine 50  
 5-methyl-dC 251  
 5-methyl-*isocytidine* 114  
 5-methylcytosine 190  
 5-methylpyrimidone 114  
 5-methylthio-1*H*-tetrazole 23  
 5'-modified guide strand 305  
 5-nitroindazole 102  
 5-nitroindole 102  
 5'-*O*-[(*cis*-2,6-dimethylpiperidino)(2-cyanoethyl)phosphoramidite 289  
 5'-phosphate 305  
 5'-phosphate mimics 305  
 5'-phosphorothioate 305  
 5-propyne pyrimidine 105  
 5-propyne-dU 106  
 5-propynyl-C 190, 200  
 5-propynyl-dC 253  
 5-propynyl-U 190  
 5-substituted 2-aminoquinolines 197  
 5-substituted pyrimidines 190  
 5-thiocyanate 2'-deoxyuridine 99  
 5-tripropargylamine-dU 158  
 (6-4) photoproduct 53, 55  
 6-aminocytosine 195  
 6-aza-2'-deoxycytidine 105  
 6-azathymidine 105  
 6-chloroguanosine 94  
 6-chloropurine 69  
 6-fluoropurine 69  
 6-mercaptopurine 97  
 6-methyl-2'-deoxycytidine 105  
 6-methylthioguanine 97  
 6-oxocytosines 193  
 6-thio-7-deazaguanine 196  
 6-thioguanine 88, 95, 114, 236  
 6-thioguanosine 94  
 6*H*, 8*H*-3,4-dihydropyrimidino[5,4-c]  
 [1,2]-oxazin-7-one 102  
 7-chloro-7-deaza guanine 196  
 7-deaza-2'-deoxyadenosine 118  
 7-deaza-2'-deoxyguanosine 118  
 7-deaza-2'-deoxyinosine 101, 118  
 7-deaza-2'-deoxynucleobarine 118  
 7-deaza-8-aza-2'-deoxyadenosine 118  
 7-deaza-8-aza-2'-deoxyguanosine 118  
 7-deazapurines 157  
 7-deazaxanthines 196  
 7-nitroindole 64  
 7-propynyl isocarbostyril 112  
 7-substituted 7-deazaadenine 105  
 8-amino-2'-deoxyinosine 101  
 8-amino-guanine 237  
 8-aminopurine 203  
 8-aza-7-deazapurine 102  
 8-azido-2'-deoxyadenine 119  
 8-azido-2'-deoxyadenosine 92  
 8-bromo-2'-deoxyadenosine 92  
 8-bromo-2'-deoxyguanosine 92  
 8-bromo-dG 236  
 8-bromo-guanine 237  
 8-methoxy-2'-deoxyguanosine 118  
 8-methoxy-guanine 237  
 8-methyl-dG 236  
 8-oxo-2'-deoxyguanosine 118  
 8-oxo-dA 57, 254  
 8-oxo-dG 57, 58, 236  
 8-oxo-guanine 237  
 8-oxoadenine 194, 254

- 8-oxodA 57  
 8-oxopurines 56  
 8-thioguanine 97  
 9-deazaguanine 196  
 9-fluorenylmethoxycarbonyl 8, 335  
 9-fluorenylmethyl 335  
 9-methyl-1*H*-imidazo[4,5-*d*]pyridine 110  
 10-camphorsulfonyloxaziridine 24, 291  
 93del aptamer 245  
 $\alpha$ -anomers 206  
 $\alpha$ -methyl-*o*-nitropiperonyloxycarbonyl 9  
 $\alpha$ -nucleoside 295  
 $\beta$ -thiobenzoylethyl 286
- abasic site 64, 100, 111, 121, 171, 254  
 aberrant splicing 298  
 ACE 12  
 acetyl 3, 10, 14, 50, 61, 63, 71, 95, 338  
 acetyloxymethyl 12  
 acridine 107, 108, 195, 247, 332  
 acrolein 66  
 acute lymphocytic leukemia 283  
 acute myeloid leukemia 283  
 acyclic threoninol nucleic acid 257, 300  
 acyclic thymine 242  
 adamantoyl chloride 6  
 AFM 211  
 AIDS patients 275  
 aldehyde-oligonucleotides 339  
 aldoximate 4  
 alkyl phosphorothioate 244  
 alkylating agents 208  
 alkyne-azide cycloaddition 255  
 Alkynyl-dU 157  
 allopurinol 101  
 allyl 69  
 allyloxycarbonyl 15, 52  
 Alnylam Pharmaceuticals 279  
 alternate-strand 201  
 altritol nucleoside 295  
 amino-oligonucleotide 144, 148, 153, 332, 337, 339  
 aminobenzimidazole 200  
 aminolevulinic acid synthase 1 279  
 aminoxy-oligonucleotides 154  
 aminophenyl-thiazole 199  
 aminotetraethyleneglycol 190  
 amphipathic peptides 336  
 amphiphilic dendrons 259
- anandamide 341  
 Angelman syndrome 280  
 anisamide 341  
 antagonists 279  
 anthracene 171  
 anthraquinone 192, 201, 202, 257  
 anti-HIV-1 activity 245, 246  
 anti-HIV-1 inhibition 244  
 anti-lncRNA 280  
 anti-miRNA 279  
 anti-proliferative oligonucleotides 283  
 antibodies-oligonucleotide conjugates 338  
 antibody-drug conjugates 338  
 anticoagulant 243, 244  
 anticoagulant activity 242  
 antimetabolite 283  
 antimirRNA 279, 296  
 antiparallel quadruplex 236, 247  
 antiparallel triplex 189  
 antiproliferative 243, 244  
 antisense 296  
 antisense oligonucleotide 275  
 antiviral 334  
 apolipoprotein 277  
 aptamers 114, 115, 232, 244, 280, 295, 296, 299, 341  
 arabinonucleosides 206, 296  
 arabinonucleotides 255  
 Arenesulfonylazoles 4  
 Argonaute 279  
 Argonaute2 278  
 aromatic sulfonyl chloride 3  
 artificial DNA 114, 159  
 artificial miRNA 278  
 AS1411 248  
 asialoglycoprotein receptor 340  
 aTNA 257, 300  
 atomic force microscopy 211, 236  
 autoimmune disease 279  
 azide-alkyne cycloaddition 304, 332  
 azido-oligonucleotides 159  
 azidomethylbenzoyl 26  
 azidoproflavin 208  
 AZMB 8  
 azobenzene 208
- B-cell activation 345  
 bacterial RNA 212  
 Beaucage 5, 6, 291

- Beaucage's reagent 24  
 Benner 114  
 benzaldehyde 153  
 benzaminoimidazole 197  
 benzhydroxy-*bis*(trimethylsilyloxy)silyl 12, 292  
 benzhydryloxycarbonyl 302  
 benzimidazolium triflate 23, 56  
 benzoimidazole 100  
 benzopyridoindole 208  
 benzopyridoquinoxaline 208  
 benzoyl 10, 13, 15, 62, 90, 93, 95, 97, 103, 153  
 benzyl 15, 96  
 benzyloxycarbonyl 15, 101, 302  
 bicyclo thymidine 206  
 bicyclo-DNA 297, 298  
 bicyclo[2.2.1]diol 19  
 bicyclo[3.1.0]hexane pseudonucleosides 239  
 bifunctional crosslinking reagent 337  
 biodistribution 332, 340  
 bioimaging 259  
 biomedical applications 344  
 biosensors 342  
*bis*-LNA 282  
*bis*-PNA 211, 302  
*bis*(2-acetoxyethoxy)methyl 12  
 bisphosphoramidite 24  
*bis*(trimethylsiloxy)-cyclododecylloxysilyl 9, 66  
*bis*(trimethylsiloxy)-cyclooctyloxysilyl 9  
*bis*(trimethylsilyl)peroxide 24  
 black hole 165  
 blood coagulation 345  
 blueberry quencher 165  
 BNA 206  
 bombesin 336  
 Bonnet 255  
 borane phosphonates 291  
 boranophosphates 291  
 boranophosphoramidite 293  
 boranophosphorothioate 293  
 boron neutron capture therapy 291  
 Bpoc 145, 335  
 branched oligonucleotide 255  
 bromoacetyl-thiol 334  
 butyryloxymethyl 12  
  
*c-myc* proto-oncogene 257  
*c-myc* tag 336  
 C-rich oligonucleotides 248  
 C<sup>8</sup>-alkyl guanine 237  
  
 cancer 279  
 cannabinoid receptors 341  
 carbamate linkage 304  
 carbazole 107  
 carbohydrate DNA base pairs 340  
 carbohydrate-DNA conjugates 247  
 carbohydrate-oligonucleotide conjugate 332, 338  
 carbohydrate-siRNA 340  
 carbohydrates 245  
 carboxamido linkage 304  
 carboxy-dT 151  
 carboxyl-oligonucleotides 149, 153, 160  
 Carell 157  
 Caruthers 6  
 cationic liposomes 336  
 caveolae-mediated pathway 343  
 CD 249, 255  
 CD spectra 256  
 CDPI3 108  
 cell penetration peptide 336  
 cellobiose 339  
 cellular phosphatases 305  
 cellular uptake 332, 343  
 centromeric 250  
 cetyltrimethylammonium bromide 160  
 chemical ligation 155  
 chemical phosphorylation 305  
 chemiluminescence 336  
 chemotherapeutic 252  
 chimeric proteins 338  
 chloroacetaldehyde 67  
 chlorotrityl 151  
 cholesterol 246, 332  
 cholic acid 109, 332  
 circular dichroism 233, 248  
 circular oligonucleotides 255  
*cis-trans* photoisomerization 237  
 citrullinated peptide 337  
 clamp strategy 211  
 clathrin-mediated pathway 340  
 cleavable peptide 343  
 click chemistry 155, 158, 304  
 click reaction 332  
 colorectal cancer 345  
 constrained 2'-O-ethyl 298  
 constrained ethyl 297  
 controlled pore glass 18, 21, 27  
 convertible nucleoside 161

- copper-free click reaction 337  
 CPG 18  
 CpG oligonucleotides 281  
 cross-linking 97  
 cross-links 46, 52  
 crosslinkers 207  
 crowding agents 251  
 Ctmp 11  
 CuAAC 156  
 cumene hydroperoxide 24  
 CXCR4 338  
 cyclic anti-HER2 336  
 cyclic DNA 97  
 cyclic oligonucleotides 210, 212  
 cyclic RGD peptide 336  
 cycloaddition 334, 339  
 cycloaddition reaction 212  
 cyclobutane pyrimidine dimers 53, 54  
 cyclohexene nucleoside 295  
 cyclonucleosides 56, 60  
 cyclooctyne 159, 339  
 cyclopenta uracil 242  
 cyclopropanpyrroloindole 208  
 cysteine-oligonucleotide 337  
 cytarabine 283  
 cytomegalovirus 275  
  
 D-altritol nucleosides 296  
 dabcyl 165  
 dabsyl 165  
 DBU 19, 50–52, 68, 71, 89, 291  
 DCC 3  
 DDTT 24  
 deazaguanine 48  
 decitabine 120  
 Dewar 53, 54, 56  
 dexamethasone 332  
 di-*n*-butylformamidine 103  
 diamidites 286  
 diazaalkanes 51  
 diaziridinylquinone 208  
 diazo coupling 339  
 diazoalkanes 50, 51  
 dibenzocyclooctyne 159  
 Dicer 278  
 dichloroacetic 21  
 dichloroacetic acid 47, 66  
 dicyclohexylcarbodiimide 3  
 Diels–Alder 334  
  
 dimethoxytrityl 8  
 dimethylaminoformamidino 92  
 dimethylformamidino 71, 94  
 diphenylcarbamoyl 293  
 dipyridiyl-2,2'-disulfide 160  
 dipyridophenazine 208  
 dithioate DNA 286  
 dithiophosphate triester 286  
 dithiothreitol 98  
 dmf 15, 94  
 DMT 8, 52, 54, 90  
 DNA alkyltransferase 48  
 DNA biosensors 144  
 DNA cages 345  
 DNA cyclization 159  
 DNA Holliday junction 299  
 DNA labeling 143  
 DNA methylases 2, 118  
 DNA methylation 212  
 DNA methyltransferase 91, 94, 120, 252  
 DNA microarray 2, 9, 144  
 DNA mutagenesis 45, 88  
 DNA nanobiotechnology 3  
 DNA nanocircles 255  
 DNA nanomachine 259  
 DNA nanosprings 259  
 DNA nanostructures 342–345  
 DNA nanotechnology 342, 344  
 DNA nanotubes 344  
 DNA origami 236, 343, 345  
 DNA parallelogram 345  
 DNA polymerase 2, 88, 90, 94, 102, 108,  
     110–115  
 DNA repair 2, 45, 48, 68  
 DNA replication 93  
 DNA sequencing 164  
 DNA synthesizers 5  
 DNA tetrahedron 344  
 DNA-binding molecules 188  
 DNA-binding proteins 20  
 DNA-decoy oligonucleotide 282  
 DNA-dendron conjugates 259  
 DNA-gold nanoparticles 253  
 DNA-methylases 117  
 DNA-minimal cage 345  
 DNA-PNA hybrid 302  
 DNA-polymer conjugates 345  
 DNA-templated protein conjugation 338  
 DNAzymes 282

- DNMT 120  
 docosahexaenoic acid 334  
 docosanoic acid 334  
 DOD 9, 12, 66  
 double-tail lipid 333  
 doxorubicin 338, 344  
 Drosha 278  
 drug delivery 342, 344  
 DtsNH 24  
 Duchenne muscular dystrophy 298, 301  
 Dvorakova 254
- EDITH 24  
 ELISA 337  
 ellipticine 208  
 ENA 206  
 endocytosis pathways 343  
 enediyne antibiotics 64  
 enhanced permeability 342  
 enzymes 143  
 epigenetic modifications 251  
 EPR 343  
 Eritja 93  
 Eteplirsen 301  
 etheno derivatives 67  
 ethyldisulfide 27  
 ethylenediamine 287  
 exon-skipping 276, 277, 298  
 exonucleases 238  
 extended nucleobases 113  
 extrahepatic delivery 334
- F-ANA 238, 242  
 F-ANA-C 256  
 familial hypercholesterolemia 279  
 Fapy 56, 59  
 fatty acid 332, 334  
 Fenna 256  
 Fire 278  
 floxuridine 88  
 floxuridine oligomers 338  
 fluorene 257  
 fluorenlymethoxycarbonyl 15, 287, 302  
 fluorenlymethyl 19, 151  
 fluorescein 259  
 fluorescein-labeled 255  
 fluorescence energy transfer 160  
 fluorescence *in situ* hybridization 290  
 fluorescence quenching 171
- fluorescent chromophores 170  
 fluorescent compounds 143  
 fluorescent DNA nanodevices 252  
 fluorescent nucleobases 169  
 fluorescent phosphoramidites 165  
 fluorophore Cy3 344  
 Fmoc 8, 15, 19, 26, 145, 147  
 Fnebe 11  
 folate receptor 344  
 folate-conjugated siRNA 344  
 folic acid 341  
 footprinting 194, 195  
 formacetal 55, 241  
 formamidinopyrimidine 56, 59  
 formylindole 153  
 Fpmp 11  
 FRET analysis 189  
 fullerene 160, 259
- G-clamp effect 252  
 G-quadruplex 92, 231, 299  
 G-tetrads 232  
 galactose glycoproteins 340  
 GalNAc 279  
 gapmer 277, 279  
 gel electrophoresis 255  
 gemcitabine 283  
 gene knockdown 343  
 Givlaari 294, 296, 340  
 Givosiran 279  
 glioblastoma multiform 283  
 glucose 245  
 glycerol nucleic acids 299  
 glycoarrays 339  
 glycoclusters 339  
 glycol nucleic acid 242  
 glycoprotein gp120 245  
 glycosyl-azides 339  
 glycosylase 59, 64, 66, 90, 91  
 GNA 242, 299  
 gold nanoparticles 342  
 Golodirsen 301  
 gp120 protein 246  
 graphene quantum dots 259  
 Greenberg 151  
 GRO-binding protein 243  
 Guanidine linkage 304  
 guanidine peptides 336

- guanidino 207  
 guanylurea 252  
  
*H*-phosphonate 2, 6, 13, 17, 57, 293  
*H*-phosphonate diester 289  
*H*-phosphonodithioates 286  
 hachimoji 114  
 hAGT 48, 49  
 Halogenated nucleobases 88  
 HEK cells 344  
 helicases 94, 117  
 Hepatitis virus C 279  
 hepatocytes 340  
 HER2 positive cells 343  
 hereditary transthyretin-mediated amyloidosis 279  
 hexitol nucleoside 295  
 hexose nucleic acids 296  
 Hirao 114  
 histidine-rich peptide 336  
 HIV Tat protein 192  
 HIV-1 envelope 245  
 HIV-1 integrase 245  
 HIV-1 reverse transcriptase 244  
 HIV-1 RNase H 244  
 Hoechst 33258 208  
 Holliger 116  
 homofunctional crosslinker 337  
 homopurine-homopyrimidine 187  
 Hoogsteen 170, 189, 197, 204, 208, 212, 252  
 Hotoda 245  
 human cytomegalovirus 247  
 Human Genome Project 164  
 human telomere sequence 247  
 human telomeric 257, 259  
 human telomeric sequence 254  
 human transthyretin amyloidosis 275  
 hyaluronic acid 339  
 HybCPG 18  
 hydrazide-oligonucleotides 152  
 hydroxylamino-glycosides 339  
 hydroxylated-5,6-dihydrothymidines 63  
 hydroxymethyl-dU 242, 254  
 hyperlipidemia 277  
 hypochromicity 189  
 hypoxanthine 101, 194  
  
 i-clamp 253  
 i-motif 231, 248  
  
 ICPMS 336  
 imidazolium triflate 23  
 immunostimulatory CpG oligonucleotide 345  
 Inclisiran 279, 296, 340  
 inosine 100  
 Inotersen 279  
 integrin receptor 336  
 intelligent nanopores 259  
 intercalating agents 207  
 intercalating compounds 246  
 interruptions 196, 200  
 intracellular sensing 259  
 intramolecular quadruplex 233  
 Ionix Pharmaceuticals 279  
 ionized species 88  
 isobutyryl 13, 14, 47, 92, 93, 95, 97, 103  
 isoguanine 58, 114, 237  
 isopropoxyacetyl 14  
 isoxanthopterin 234  
  
 Jones 47  
  
 Kanehara 254  
 Khorana 2, 3, 13  
 Kool 109, 110  
 Kynamro 294  
  
 L-nucleotides 241, 281  
 L-thymidine 242  
 lactose 339  
 LDL receptor 279  
 lectin 339  
 Letsinger 5  
 Leumann 298  
 Lev 8  
 levulinyl 8, 10, 25, 29, 52, 55  
 levulinylloxymethyl 12  
 Li 255  
 lipid bilayer 345  
 lipid nanoparticles 280  
 lipid-oligonucleotide conjugates 247, 332  
 lipoic acid 343  
 Lipoquads 247  
 Liu 259  
 LNA 206, 238, 241, 242, 246, 257, 297, 304  
 LNA aptamers 298  
 LNAszymes 298  
 lncRNA ASBEL 280

- locked nucleic acid 238, 275, 293, 297  
 long non-coding RNAs 280
- m*-chloroperbenzoic acid 24  
 Macugen 281, 294, 296  
 macular degeneration 281  
 MALAT1 280  
 maleimide-oligonucleotide 148  
 maleimide-thiol 334  
 malondialdehyde 66  
 mannose 245  
 mannose 6-phosphate 340  
 mechanochemical synthesis 29  
 Mello 278  
 membrane translocation 336  
 membrane-embedded DNA 345  
 MeNPOC 9  
 Merrifield 5  
 methanocarbanucleosides 239  
 methoxyacetyl 14  
 methoxyethyl 275, 294  
 methylases 211  
 methyl diphenylphosphine 293  
 methylenemethylimino 304  
 methyleneoxy(methylimino) 304  
 methylisothiouronium 207  
 methyloxyethyl 277  
 methylphosphonate 287  
 methylphosphonates 17, 94, 253, 291  
 methylphosphonic *bis*(imidazolide) 287  
 methylphosphoramidites 47  
 methylphosphotriester 287  
 methylsulfinyl 147  
 methyltransferases 163  
 microRNA 278  
 Mipomersen 284  
 Miravirsen 279, 298  
 miRNA 212  
 miRNA mimics 279  
 miRNA-based therapeutics 279  
 mitochondria 344  
 Mitsonobu 49  
 MMT 8, 145  
 MNTP 293  
 Modi 258  
 modulators of gene expression 250  
 MOE 294  
 Molecular Beacons 165  
 molecular devices 342
- molecular dynamics 190  
 molecular logic gates 259  
 molecular motors 250  
 monoclonal antibodies 338  
 monomethoxytrityl 8, 302  
 morpholino oligomers 336  
 morpholino oligonucleotides 207  
 multiple intercalated structures 255  
 multivariate curve resolution 190  
 mustard gas 45
- N,N'*-bis(2-oxo-3-oxazolidinyl)phosphonic chloride 293  
*N,N*-dibutylformamidine 15  
*N,N*-dimethylaminoethyl phosphoramidates 207  
*N,N*-dimethylaminopyridine 13  
*N,N*-dimethylformamidine 15  
*N,N*-diphenylcarbamoyl 101  
*N*-acetylgalactosamine 275, 279, 296, 332, 340  
*N*-methylimidazole 4, 13, 22  
*N*-methylpyrrolidine 15, 103  
*N*-nitrosamines 45  
*N*-nitrosoguanidines 45  
*N*-nitrosoureas 46  
*N*-nitrotriazole 4  
*N*-pent-4-enoyl 287  
*N*-triphenylacetylcystamine 161  
*N*<sup>2</sup>-3-aminopropylguanine 104  
*N*<sup>2</sup>-imidazolylpropyl-2-aminoadenine 104  
*N*<sup>2</sup>-imidazolylpropylguanine 104  
*N*<sup>2</sup>-methyl-2'-deoxyguanosine 119  
*N*<sup>2</sup>-spermineguanine 104  
*N*<sup>3</sup>-alkyl adenine 46  
*N*<sup>3</sup>-alkyl-T 16  
*N*<sup>3</sup>-alkylthymidine 51  
*N*<sup>4</sup>-(6-aminopyridynyl)-C 197  
*N*<sup>4</sup>-(acetamidopropyl)-dC 197  
*N*<sup>4</sup>-amino-2'-deoxycytidine 94  
*N*<sup>4</sup>-hydroxy-2'-deoxycytidine 94  
*N*<sup>4</sup>-methoxy cytosine 102  
*N*<sup>4</sup>-methyl-2'-deoxycytidine 119  
*N*<sup>6</sup>,*N*<sup>6</sup>-dibenzoyl 15  
*N*<sup>6</sup>-methoxy-2,6-diaminopurine 200  
*N*<sup>6</sup>-methoxy-2'-deoxyadenine 94  
*N*<sup>6</sup>-methoxyadenine 102  
*N*<sup>6</sup>-methyl-2'-deoxyadenosine 119  
*N*<sup>6</sup>-methyl-7-deaza-2'-deoxyadenosine 119  
*N*<sup>7</sup>-alkylguanine 46, 49

- nanoelectrospray ionization spectroscopy 258  
 nanofiltration 29  
 nanorobot 345  
 nanostructures 299  
 nanoswitches 250  
 nanotechnology 250  
 nanowires 342  
 Naphthalene-diimide 208  
 naphthimidazole 197  
 naphtopyridopyrimidine 170  
 native ligation 334, 337  
 Nebe 11  
 neurodegenerative disease 279  
 nitrophenylethyl 99  
 nitrosamines 48  
 nitrosoguanidines 49  
 nitrosoureas 49  
 nitroveratryl 65  
 NittoPhase 18  
 NMR 247, 249, 255, 256  
 NOE cross-peaks 233  
 NOESY experiments 233  
 non-coding RNA 2  
*North bicyclo[3.1.0]hexane pseudonucleoside*  
 297  
*North-methanocarbathymidine* 242  
 NOXXON Pharma 281  
 NPE 104  
 Npec 15, 19  
 NPEOC 50, 147  
 Npes 11  
 NPOC 9  
 NPPOC 9  
 nuclear localization 336  
 nuclear magnetic resonance 233, 249  
 nuclease degradation 345  
 nuclease resistance 332  
 nucleolin 345  
 nucleolin binding 248  
 nucleoside 3'-phosphinoamidites 290  
 nucleoside  $\alpha$ -boranotriphosphates 291  
 nucleoside *H*-boranephosphonate 293  
  
 $\alpha$ -nitrobenzyl 10, 19  
 $\alpha$ -nitrophenylethyl 19  
 $O^2,5'$ -cyclothymidine 51  
 $O^2$ -alkylthymidine 50  
 $O^4$ -(2,4,6-trimethylphenyl) thymidine 162  
 $O^4$ -(4-nitrophenyl)-dU 162  
  
 $O^4$ -alkylthymidine 46, 50  
 $O^4$ -alkylthymidines 14  
 $O^4$ -phenyl 13  
 $O^6$ -alkyl-dG 195  
 $O^6$ -alkylated guanines 171  
 $O^6$ -alkylguanine 46–50  
 $O^6$ -alkylguanine-DNA 235  
 $O^6$ -diphenylcarbamoyl 13  
 $O^6$ -methyl-7-deaza-2'-deoxyguanosine 118  
 $O^6$ -methyl-dG 235  
 $O^6$ -phenyl-2'-deoxyinosine 99, 163  
octreotide 336  
Ogilvie 11  
oligonucleoside methylphosphonates 286  
oligonucleoside  $O$ -alkylphosphates 286  
oligonucleotide conjugates 331  
oligonucleotide functionalized proteins 332  
oligonucleotide phosphorothioate 336  
oligonucleotide-directed mutagenesis 2, 26  
oligonucleotide-peptide 148  
oligonucleotide-protein 148  
Oligonucleotides carrying *cis*-aminoalcohol 153  
OligoPrep 18  
oligopurine-oligopyrimidine tracks 201  
Onpatro 294  
origami DNA 211  
osteoblast adhesion 336  
oxalyl 19, 27  
oxathiaphospholane 284  
oxime 334  
oxime condensation 255  
  
 $p$ -azidophenacyl 208  
 $p$ -benzoquinone 68  
 $p$ -nitrobenzyloxymethyl 11  
 $p$ -nitrophenylethyl 50, 69  
 $p$ -nitrophenylethyoxy carbonyl 50  
 $p$ -nitrophenylethylsulfonyl 11  
Pac 14, 24–26, 71, 92, 95, 284  
PACE 290  
parallel clamps 212  
parallel G-quadruplex 245, 247  
parallel triplex 189  
Patisiran 279, 296  
PCR 2, 19, 99, 102, 114, 143, 148, 165, 331  
Pegaptanib 281  
penetratin 336  
pentaerythritol 201  
peptide nucleic acids 162, 207, 240, 301, 336

- peptide-oligonucleotide conjugate 332, 334  
 peptide-TBA conjugates 248  
 peptides 248  
 peracylation method 13  
 personalized medicine 338  
 perylene 171, 208  
 Pfleiderer 9  
 pH-sensitive devices 259  
 pharmacokinetics 331  
 phenoxazine 107, 252  
 phenoxyacetyl 14, 49, 52, 62, 63, 71, 92, 94, 95, 103  
 phenylacetyl 47  
 phenylglycidol 70  
 phenylglycinol 70  
 phenylimidazole 197  
 phosphonoacetate 290  
 phosphite-triester 2, 4, 5, 22, 27  
 phosphodiester 2, 3, 13  
 phosphoramidate 17, 206, 253, 288  
 phosphoramidite chemistry 2, 21, 274  
 phosphoramidite derivatives 143  
 phosphoramidite method 2, 16, 29, 47  
 phosphorodiamidate morpholino 275  
 phosphorodiamidate morpholino oligomers 300  
 phosphorodithioate 284, 286  
 phosphorothioate 24, 29, 149, 160, 206, 241, 253, 275, 279, 280, 284, 334  
 phosphorothiolate 254  
 phosphotriester 2, 4, 5, 13, 16, 18, 19, 23, 26–28, 47, 88, 95  
 phosphotriester linkage 287  
 phosphotriester method 274  
 photocaged 237  
 photocleavable 147  
 photocrosslinking 90, 92  
 photodimers 53–55  
 photodynamic therapy 160  
 photolabile groups 9  
 photolyase 55  
 photoregulation 208, 237  
 photosensitive thymidine 244  
 phthalimide 197  
 phthaloyl 15, 19, 55, 147  
 pivaloyl 97  
 pivaloyl chloride 6  
 pivaloyloxymethyl 12  
 Piwi-interacting RNA 278  
 pixyl 8  
 PMO 300, 336  
 PNA 162, 207, 211, 240, 257, 301, 336  
 PNA beacons 211  
 PNA openers 211  
 PNAs clamps 211  
*Pneumocystis* 212  
 polyacrylmorpholide 21  
 polyamines 190  
 polycyclic aromatic hydrocarbons 68  
 polydimethylacrylamide-kieselguhr 18  
 polyethyleneglycol 18, 21, 259, 281  
 polymerase chain reaction 2, 99, 331  
 polypurine hairpins 282  
 polypurine-polypyrimidine tracks 196  
 polypyrimidine 253  
 polystyrene 18  
 polystyrene supports 287  
 polystyrene-1%-divinylbenzene 18  
 polyvinylacetate 18  
 porphyrin 64, 111, 257  
 postsynthetic conjugation 144, 332, 334, 338  
 PPRH 212  
 primer extension experiments 236  
 programmed apoptosis 345  
 promoter region 250, 257  
 Propeller loops 233  
 propionyloxymethyl 12  
 proprotein convertase subtilisin kexin type 9 275  
 proprotein convertase subtilisin/kexin type 9 279  
 propynoylamino-*p*-toluic acid 157  
 prostate cancer 283  
 protamine 336  
 protein arrays 337  
 protein binding 332  
 protein thioester 337  
 pseudouracil 252  
 pseudouridine 193  
 pseudouridine 193, 203  
 psolaren 66, 71  
 psoralen 208  
 Pteridines 168  
 purine 2'-deoxyriboside 195  
 Purine convertible nucleosides 163  
 pyrazole 194  
 pyrene 171, 257  
 pyrenylethynyl groups 169

- pyrenylmethyl 107, 109  
 pyrenylmethylglycerol 246  
 pyridinium hydrochloride 23  
 pyrido[2,3-*d*]pyrimidine 192  
 Pyrimidine convertible nucleosides 162  
 pyrrole/imidazole polyamines 208  
 Q-linker 19, 27  
 quadruplex 170  
 quantum yields 170, 171  
 quinacridine 208  
 quinazolininedione 192  
 quindoline 247  
 (*R*)-1-(2-nitrophenyl)ethoxymethyl 11  
 rare tautomers 88  
 reductive amination 337  
 restriction enzymes 211  
 retinoic acid-inducible gene 281  
 reverse Hoogsteen 189  
 reverse transcriptase 110  
 RGD tripeptide motif 336  
 rheumatoid arthritis 337  
 Ribonuclease H 276, 282  
 ribozymes 282, 296  
 RISC 300  
 RNA dialdehydes 337  
 RNA interference 2, 278  
 RNA methyltransferases 160  
 RNA splicing 2  
 RNA-decoy 282  
 RNA-Induced Silencing Complex 278  
 RNAi-based therapeutics 278  
 RNase H 276  
 RNase H 284, 291  
 Romesberg 114  
 Rothemund 344  
 S-cyanoethyl methylthiosulfonate 284  
 S-methylthiourea 304  
 sarcosyl-succinyl 19  
 Sasaki 200  
 scorpion probes 165  
 Seela 157  
 Seeman 342, 344  
 SELEX 114, 116, 281  
 self-assembling molecules 343  
 sequencing primers 145  
 serine protease 279  
 serinol 300  
 Serinol Nucleic Acids 300  
 serum protein 274, 284, 332  
 short interfering RNA 278  
 single-walled carbon nanotubes 259  
 siRNA 278, 299  
 small hairpin RNA 278  
 smart surfaces 259  
 SNA 300  
 sobutyryloxymethyl 11  
 solid-phase 2, 5, 8, 9, 13, 18, 21, 24, 25, 27  
 somatostatin 336  
 SOSICS 284  
 SPAAC 159  
 spermine 109, 190  
 spherical nucleic acids 343, 345  
 Spiegelmers 281, 297  
 spine of hydration 204  
 Spinraza 294  
 splice switching 336  
 spore photoproduct 54, 56  
 SPR 248  
 Stephenson 274  
 stereocontrolled synthesis 284  
 steroids 332  
 strand displacement 203, 208, 211  
 succinyl 15, 18, 19, 23  
 sucrose 245  
 sulfo-SMCC 337  
 supramolecular device 337  
 supramolecular structures 250  
 Surface Plasmon Resonance 248  
 SWNT 259  
 synthetic biology 345  
 synthetic miRNA 279  
 synthetic nucleases 332  
*t*-butoxycarbonyl 335  
*t*-butyldimethylsilyl 11, 55  
*t*-butylphenoxyacetyl 50  
*t*-butyl(phenoxy)acetyl 55  
 T30177 246  
 T4 DNA ligase 255  
 T7 RNA polymerase 296  
 tail-clamps 212  
 Taqman 165  
 Tat-peptide 336  
 TBA 236, 237, 241, 243, 244  
 TBA aptamer 248

- TBDMS 11, 25–27, 29  
 Teflon 21  
 Tegsedi 294  
 telomeric 250  
 telomeric G-quadruplex 235, 237  
 telomeric i-motif 251  
 telomeric sequence 238, 240  
 terminal deoxynucleotidyl transferase 7  
*tert*-butoxycarbonyl 302  
*tert*-butyl peroxide 24  
*tert*-butyldimethylsilyl 61, 62  
*tert*-butyldiphenylsilyl 245  
*tert*-butyldithiomethyl 12  
*tert*-butylperoxide 287  
*tert*-butylphenoxyacetyl 67  
*tert*-butylphenoxyacetyl groups 288  
*tert*-butylthio 98, 99  
 TETD 24  
 tetra-end-linked oligonucleotides 246  
 tetrabutylammonium fluoride 11, 97  
 tetrabutylammonium periodate 24  
 tetra(ethyleneglycol) 255  
 tetraethylthiuram disulfide 24  
 tetrafluorophenoxazine 102  
 tetrahydrofuranediol 19  
 tetrahydropyran-2-yl 11  
 tetrameric i-motif 255  
 tetramethylguanidinium aldoxymate 16  
 tetramethylrhodamine 259  
 tetrathiafulvalene 109  
 tetrazine ligation 159  
 tetrazole 6, 11, 18, 22–24  
 TFA 145  
 TFO 282  
 therapeutic oligonucleotide 144, 274, 332, 338  
 thiazol 197  
 thiazolidine 334  
 thieno[3,4-*d*]pyrimidine 170  
 thioctic acid 343  
 thiol-ene chemistry 159  
 thiol-maleimide 337  
 thiol-oligonucleotides 149  
 thiolated nucleobases 88  
 thiolated-oligonucleotide 337  
 thionocarbamate 1,1-dioxo-1λ<sup>6</sup>-  
     thiomorpholine-4-carbothioate 12  
 thiophene 169  
 thiophosphonoacetates 291  
 thiophosphoramidate 288, 289  
 thiophosphoramidites 286  
 Thp 11  
 three-dimensional architectures 344  
 threoninol 300  
 thrombin 345  
 thrombin binding aptamer 236, 237, 242  
 thymine glycol 62, 64  
 TIPS 11  
 TMSE 97, 99  
 tocopherol 332  
 Toll-like receptor 281  
 toluoyl 338  
 Tom 11  
 topoisomerases 117  
 transactivation response RNA-binding proteins 278  
 transcription factors 117, 211, 281, 337  
 transient protection 13  
 transportan 336  
 TRBP 278  
 triazolyl-dinucleoside 304  
 trichloroacetic 21  
*tricyclo*-DNA 297, 298  
 triethylamine hydrofluoride 11  
 triethylammonium hydrofluoride 27  
 trifluoroacetyl 97, 104, 335  
 triisopropylsilyl 11, 29  
 (triisopropylsilyl)oxymethyl 11  
 trimethoxytrityl 292  
 triphenylphosphine 160  
 triple helices 187  
 triplex forming oligonucleotides 282  
 triplex stabilization 101  
 triplex-forming oligonucleotide 188  
 tris(benzyloxy)trityl 8  
 trityl 3, 7, 8, 147, 151  
 Tsvetkov 252  
 UNA 240, 242, 257, 299  
 universal base 100, 200  
 unlocked nucleic acid 240, 257, 299  
 Unylinker 19  
 urea linkage 304  
 UV melting curves 189  
 UV melting experiments 256  
 UV-VIS denaturation 190  
 vaccines 345  
 Van Craynest 200

- vascularization 343  
VEGF 281  
Verdine 91, 162  
Viltepso 301  
Vitravene 275  
  
Watson–Crick 168, 169, 171, 188, 198, 209, 212,  
  233, 274, 282, 299, 343  
Waylivra 294  
wedged 212  
wobble base-pair 88  
Wright 252  
  
X-ray diffraction 90, 344  
xanthine 101  
xanthosine 236  
xenonucleic acids 114  
XNA 114  
Xu 164  
  
Z-DNA 92  
Zamecnick 274  
zebularine 120  
Zintevir 245  
Zip nucleic acids 109