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Synthesis of Base-Modified 2′-Deoxyribonucleoside Triphosphates and Their Use in Enzymatic Synthesis of Modified DNA for Applications in Bioanalysis and Chemical Biology

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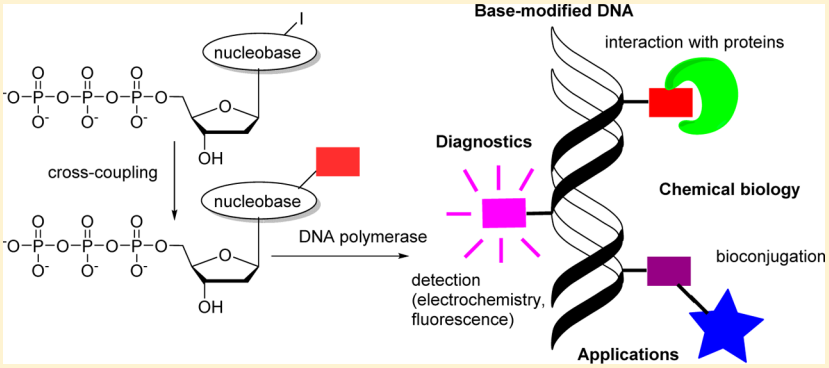

ABSTRACT: The synthesis of 2′-deoxyribonucleoside triphosphates (dNTPs) either by classical triphosphorylation of nucleosides or by aqueous cross-coupling reactions of halogenated dNTPs is discussed. Different enzymatic methods for synthesis of modified oligonucleotides and DNA by polymerase incorporation of modified nucleotides are summarized, and the applications in redox or fluorescent labeling, as well as in bioconjugations and modulation of interactions of DNA with proteins, are outlined.

Nucleosides containing modified base (purine, pyrimidine or their analogues) have been extensively studied since the 1950s and found many applications as clinically used antiviral and antitumor drugs.[1] In addition, base-modified oligonucleotides (ONs) and nucleic acids have become a very popular target, and apart medicinal chemistry, they were applied in various areas of chemical biology[2] and material science.[3] Most of them are synthesized chemically by the classical phosphoramidite method on solid support.[4] The chemical synthesis of ONs is facile, robust, and scalable. However, it has certain disadvantages, i.e., laborious multistep syntheses of some base-modified phosphoramidite intermediates, difficult synthesis of long ONs (>100 nt), or limited compatibility of some functional groups with the phosphoramidite protocol (any groups prone to oxidation or reactive with nucleophiles). An alternative method of the preparation of base-modified nucleic acids is enzymatic synthesis by DNA or RNA polymerases using modified (2′-deoxy)ribonucleoside triphosphates (dNTPs or NTPs) as substrates. Several reviews were published[5] on this topic within the past decade, but this account summarizes the most recent results in the synthesis of modified dNTPs, polymerase synthesis of base-modified DNA, and their applications in bioanalysis and chemical biology. This work focuses only on modified derivatives and analogues of natural A, T, G, or C bases with intact Watson−Crick base-pairing, whereas the important area of artificial base-pairs and extension of the genetic alphabet is not covered here (but has been thoroughly reviewed by others[6]).

SYNTHESIS OF BASE-MODIFIED NUCLEOSIDE TRIPHOSPHATES

General methodology for the preparation of (d)NTPs is by chemical triphosphorylation of nucleosides by the reaction with $POCl_3$ followed by pyrophosphate and triethylamonium bicarbonate (Scheme 1A).[7] The primary OH (at position 5′) is usually sufficiently more reactive than the secondary hydroxyl(s) at position 3′ (and 2′ in ribonucleosides), and hence, no protection is needed in most cases. The resulting (d)NTPs are typically isolated by ionex chromatography and/or preparative HPLC. Some highly nucleophilic functional groups (e.g., aliphatic NH_2, guanidine, etc., but not amino groups at nucleobases) are not compatible with the triphosphorylation protocol and must be protected. Therefore, the synthesis of some highly modified (d)NTPs may be a laborious multistep procedure, and in some special cases, even finding the right orthogonal protection group cleavable without hydrolysis of the triphosphate might be problematic. Alter-

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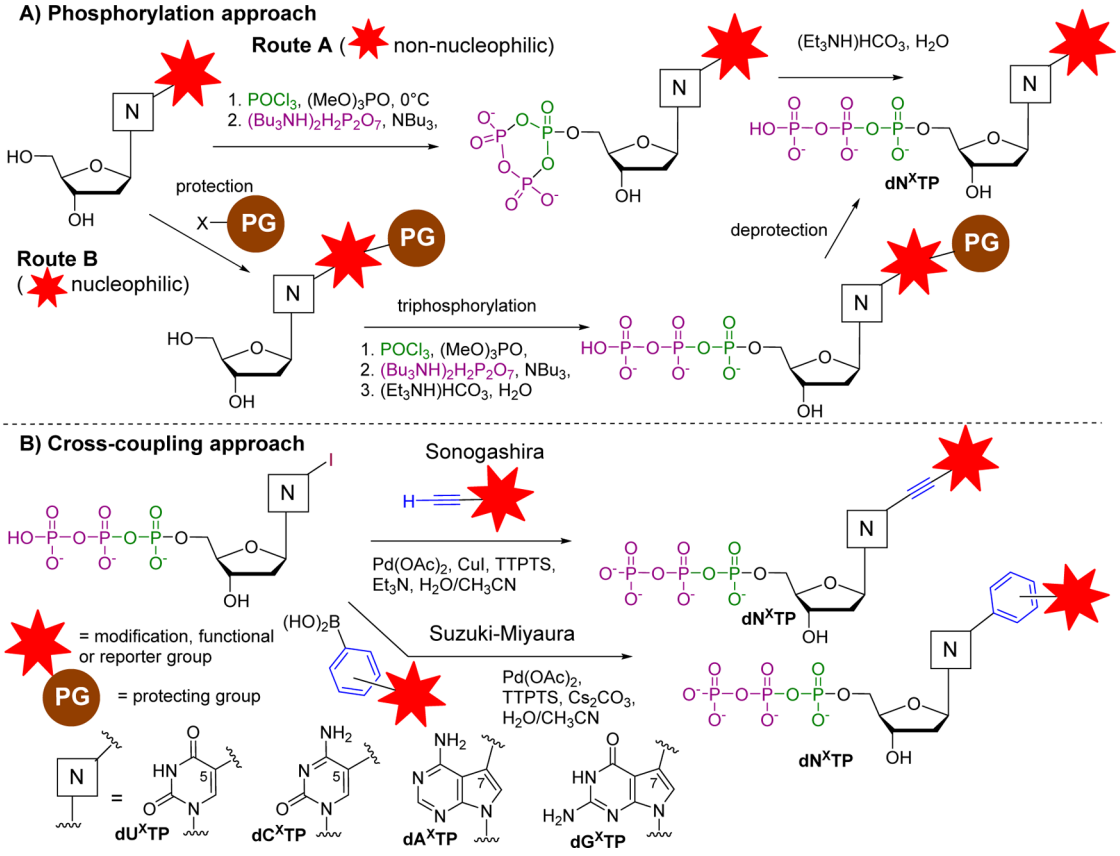
Scheme 1. Triphosphorylation (A) and Cross-Coupling (B) Approaches to the Synthesis of Base-Modified dNTPs



native triphosphorylation methods based on other reagents (e.g., salicyl phosphochloridite) are also available[8,9] but have been scarcely used for syntheses of base-modified dNTPs.

Pd-catalyzed cross-coupling reactions are one of the most useful methods for C−C bond formation and are widely used in modification of nucleobases and nucleosides, i.e., attachment of carbon substituents at carbon atoms of the heterocycle.[10] Classical protocol for these reactions used protected nucleobases or nucleosides in organic solvents.[10] More recently, aqueous-phase cross-coupling reactions have been developed using water-soluble ligands, e.g., triphenylphosphan-3,3′,3″-trisulfonate (TPPTS).[11] These reactions are perfect for direct modification of polar unprotected nucleosides, and Shaughnessy has pioneered their use for arylation of purine nucleosides at position 8.[12] These reactions have been used for direct modification of other nucleosides and nucleotides.[13] The first direct cross-coupling reaction on dNTPs was reported by the Burgess group for the Sonogashira alkynylations of dUTP to afford fluorescent derivatives.[14] Later on, we published the first Suzuki−Miyaura cross-coupling of halogenated dNTPs and started a systematic study of those reactions (Scheme 1B).[15] A major challenge in aqueous-phase cross-coupling reactions of (d)NTPs is their susceptibility to hydrolysis. The cross-coupling reactions require elevated temperatures under which the hydrolysis of (d)NTPs is significant. Fortunately, the base used for the Suzuki (K_2CO_3 or Cs_2CO_3) or Sonogashira (Et_3N) couplings stabilizes the triphosphates due to a higher degree of ionization. Nevertheless, the reactions must be optimized for short reaction times (typically 30−60 min) and also efficient and rapid isolation of the products by reversed-phase HPLC must be used to separate hydrolytic byproducts. The tributylammonium salts of dNTPs are often converted to sodium salts by ion-exchange chromatography. So far, general and efficient protocols for the Suzuki−Miyaura reactions of halogenated (d)NTPs with arylboronic acid was developed for introduction of substituted aryl groups and for the Sonogashira reactions with terminal acetylenes for alkynylations have been developed (Scheme 1B).[5a,c] Recently, we have also reported[16] the first Heck coupling on halogenated dNTPs with butyl acrylate, but this reaction only worked for deazapurine (not for pyrimidine) dNTPs and is yet far from general. These aqueous cross-coupling reactions are highly tolerant to the presence of most organic functional groups, including reactive NH_2, COOH, COOR, CHO, and OH, etc. (but not free SH group which poisons the catalyst), as well as some inorganic functions. Therefore, these reactions are used for a direct single-step introduction of complex highly functionalized substituents without any need for protecting groups. The isolated yields are typically 20−60%, but the procedure is short and straightforward which compensates for moderate yields.

The substituents are usually attached at position 5 of pyrimidines or at position 7 of 7-deazapurines because those substituents point out to the major groove of DNA.[17] Some 8-substituted purine dNTPs were also prepared but were poor substrates for polymerases.[18] For easiest accessibility, most base-modified dNTPs in the literature were 5-substituted uracil derivatives, whereas the 7-deazaguanine dNTPs were only scarcely reported due to difficult multistep synthesis of the nucleoside intermediates.

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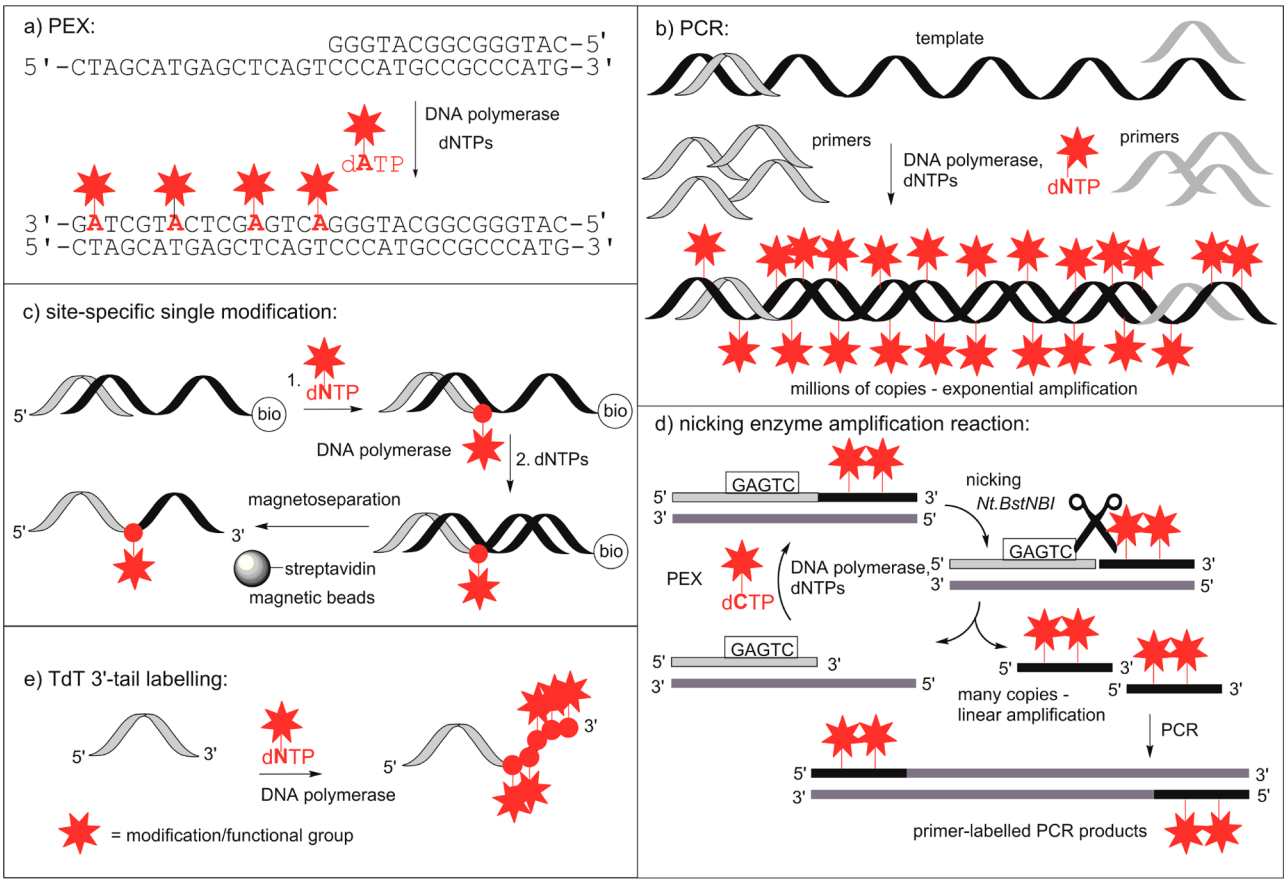
Scheme 2. Enzymatic Methods for Syntheses of Modified ONs or DNA



METHODS FOR SYNTHESIS OF BASE-MODIFIED DNA BY DNA POLYMERASES

Diverse polymerases were tested and used for the enzymatic synthesis of base-modified DNA by incorporation of modified nucleotides.[19] Polymerases lacking the 3′-exonuclease activity (exo-) are typically more promiscuous and tolerant to modifications but also tend to misincorporate, but even many exo+ polymerases are capable of efficient incorporation of modified **dN^{X}TP**s. The most general and efficient seem to be thermostable polymerases (typically used in PCR), such as KOD XL,[20] Vent (exo-), or Pwo operating at temperatures of 50−70 °C. The Holliger and Marx groups have even engineered new polymerases[21] with improved efficiency for incorporation of modified nucleotides.

As mentioned above, 5-substituted pyrimidine or 7-substituted 7-deazapurine dNTPs are mostly good to excellent substrates for DNA polymerases since those substituents point out to the major groove of DNA and, especially when using alkyne tethers, can accommodate well into B−DNA duplex. Melting temperature measurements of the corresponding modified DNA duplexes usually showed moderate destabilization (1−2 °C per base for more bulky groups) but in some cases even certain stabilization (in particular for 7-alkynyl-7-deazapurines).[22] On the other hand, 8-substituted purine dNTPs were repeatedly shown to be rather poor substrates[17,18] for polymerases due to preference of unfavorable *syn*-conformation of the base, although the Perrin group has used[23] some of them for in vitro selections of DNAzymes. The Marx group published a series of seminal papers on crystal structures of DNA polymerases in complex with template, primer, and modified **dN^{X}TP**.[24] Those studies confirmed that there is enough space in those complexes even for relatively bulky substituents in the major-groove edge of the **dN^{X}TP**. What remains unclear is how the polymerase moves forward from the modified active site during further extension of the primer chain. Very recently, we have reported[25] a study of competitive enzymatic incorporations of a series of 5-substituted dCTPs and 7-substituted 7-deazaadenine dNTPs which showed surprising results that 7-aryl-7-deaza-dATPs are better substrates for most DNA polymerases than the natural counterpart (dATP). The detailed kinetic and modeling study revealed that those 7-aryl-modified dATP analogues bind to the active site with higher affinity than dATP due to increased $\pi-\pi$-stacking with the neighboring nucleobase and amino acids. This finding can be applied in metabolic labeling or in in vivo synthesis of modified DNA.

The simplest experiment to test the substrate activity of modified **dN^{X}TP**s to polymerases is primer extension (PEX, Scheme 2a). Typically, a radioactively or fluorescently labeled primer is hybridized with complementary template (having 5′-overhang). DNA polymerase then extends the primer by incorporation of nucleotides complementary to the template sequence. If one (or more) of the natural dNTP(s) is replaced by a modified analogue or derivative, the polymerase incorporates the modified nucleotide to every position opposite to the complementary base of the template. In this way, one can program the template for just one or several modifications, which can be either separated by natural bases or in adjacent positions (which is more challenging). The polymerase not only must be able to recognize the modified **dN^{X}TP** as

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substrate and incorporate it into the growing primer but also continue in further extension by incorporation of another forthcoming nucleotide. The outcome is typically followed by denaturing polyacrylamide gel electrophoresis (PAGE) and compared to positive control (PEX using all four natural dNTPs) and negative control (PEX in the absence of one natural dNTP). The reaction conditions, amount of polymerase, and amount and ratios of nonmodified versus modified dNTPs often have to be optimized to achieve sufficiently negative result (lack of extension) in negative control experiments to ensure that there is no significant misincorporation of noncomplementary natural dNTPs instead of the modified nucleotide. The identity of the PEX product is easily verified by MALDI-TOF spectrometry (but extra care must be given to desalting of the samples).

Most 5-substituted pyrimidine and 7-substituted 7-deazapurine **dN^{X}TP**s were good to excellent substrates for at least some DNA polymerases in PEX experiments. Proper optimization of conditions and linker and choice of polymerase enabled incorporation nucleotides bearing as bulky substituents as grafted polymers[26] or oligonucleotides.[27] However, some bulky **dN^{X}TP**s were difficult for incorporation at adjacent positions to another modification. In some cases, the PEX products (especially when using KOD XL polymerase) contained certain amounts of $n + 1$ extended products resulting from nontemplated incorporation of another nucleotide (usually dA) at the 3′-end. The PEX is suitable for synthesis of middle-sized DNA (15−100 bp) bearing modifications in one strand.

In order to prepare modified single-stranded ONs (ssONs), the PEX can be performed using biotinylated template and coupled with magnetoseparation.[28] The biotinylated PEX product is then bound to streptavidin-coated magnetic beads and fished-out with a magnet, and the rest of the reaction mixture is washed out. After that, the modified ssON can be released by denaturation. The method is efficient for chromatography-free isolation of the modified ssON. However, in some cases (with certain batches of the beads from certain suppliers), we faced problems with insufficient stability of the streptavidin-linked magnetic beads and/or stability of the streptavidin−biotin complex and observed some release of the biotinylated template as well.

The second major enzymatic methodology for synthesis of modified DNA is polymerase chain reaction (PCR)[29] which is commonly used for DNA amplification. Typically, a longer template (100−1000 bp) is used together with forward and reverse primers complementary to the flanking sequences. The reaction is run in cycles (20−40) of repeated denaturation, annealing, and extension in the presence of a thermostable DNA polymerase. If one (or more) natural dNTP(s) is replaced by modified **dN^{X}TP**, the PCR can produce DNA duplex modified in a major groove (Scheme 2b) along the whole sequence in both strands. The PCR using modified **dN^{X}TP** is more challenging for the polymerase since it not only must be able to incorporate the modified nucleotide to any sequence context (including adjacent positions), but also the enzyme must be able to read through the modified template. Not surprisingly, only some modified **dN^{X}TP**s, usually the ones bearing smaller modifications or those bearing bulkier modification attached through a slim and flexible tether (i.e., propargyl), work well in PCR amplification.[17,30] Even when using only one modified **dN^{X}TP** (and three natural ones), ca. 25% of the nucleobases of the resulting PCR product are bearing a modification which results in DNA duplex where the major groove is heavily covered by modifications. Therefore, PCR is suitable for synthesis of large dsDNA containing a high number of modifications in the major groove.

Both PEX and PCR approaches result in DNA modified along all the newly synthesized stretches and do not allow for site-specific single labeling of internal parts of DNA sequences. Therefore, we have developed modified procedures[31] based on single-nucleotide incorporation followed by PEX (Scheme 2c). In an easier case, when the modified nucleotide is followed by a different nucleobase, the template, primer, and polymerase are mixed with ca. 1.1 equiv of modified **dN^{X}TP** for single nucleotide incorporation, and then the mixture of all four natural dNTPs is added to finish the PEX. However, when the modified nucleotide should be followed by nonmodified nucleobase of the same kind, the procedure[31] needs to be more complex using two different templates. First, the primer is annealed with a biotinylated template one-nucleotide longer and the PEX using modified **dN^{X}TP** is performed followed by magnetoseparation of the ssON containing modified nucleotide at the 3′-end. This is then annealed with the longer template, and a standard PEX is performed to synthesize the nonmodified part of the sequence.

None of the above-mentioned methods which require stable hybridization of primer with template are suitable for the synthesis of shorter ONs (<15 nt), which are very useful as primers for PCR. Therefore, we developed a methodology of their enzymatic synthesis based on the nicking enzyme amplification reaction (NEAR).[32] The catalytic cycle consists of two enzymatic reactions (Scheme 2d). A template and primer (containing a recognition sequence for a nicking endonuclease) are annealed, and DNA polymerase catalyzes PEX to synthesize an 8−20 nt stretch according to the template sequence. Then the nicking endonuclease recognizes the sequence of the duplex and cleaves the primer strand next to it. The cleaved newly synthesized modified stretch is too short to stay hybridized under the elevated temperature of the reaction (ca. 50 °C) and falls apart to restore the primer−template complex which undergoes another cycle of PEX, nicking and splitting and the cycles continue until the enzymes are inactive or the dNTPs consumed. The reaction is isothermal (unlike PCR) and releases one copy of the modified ssON in each cycle (linear amplification). The procedure worked best for modified **dC^{X}TP**s, whereas **dA^{X}TP**s or **dU^{X}TP**s performed somewhat worse and **dG^{X}TP**s did not work at all. This method was found suitable for synthesis of short (8−22 nt) ssONs bearing one or several modifications suitable as primers for PEX (to prepare 5′-end labeled ONs) or PCR (applied for fluorescent staining of PCR products).[33] As the only stoichiometric reagents are the dNTPs (enzymes, primer, and template are catalytic), after the reaction, the only DNA present in significant amounts is the modified ON product which can be easily isolated or even used without purification.

Finally, terminal deoxynucleotidyl transferase (TdT) can be used for nontemplated 3′-tail labeling of ONs.[34] By proper optimization of the conditions and concentrations of the enzyme and **dN^{X}TP** (no natural dNTPs are used) one can achieve relatively narrow dispersion of the oligomeric products containing 10−20 modifications at the 3′-end (Scheme 2e). It can be used for simple and robust tail-labeling of ONs not requiring precise quantification of the labels.

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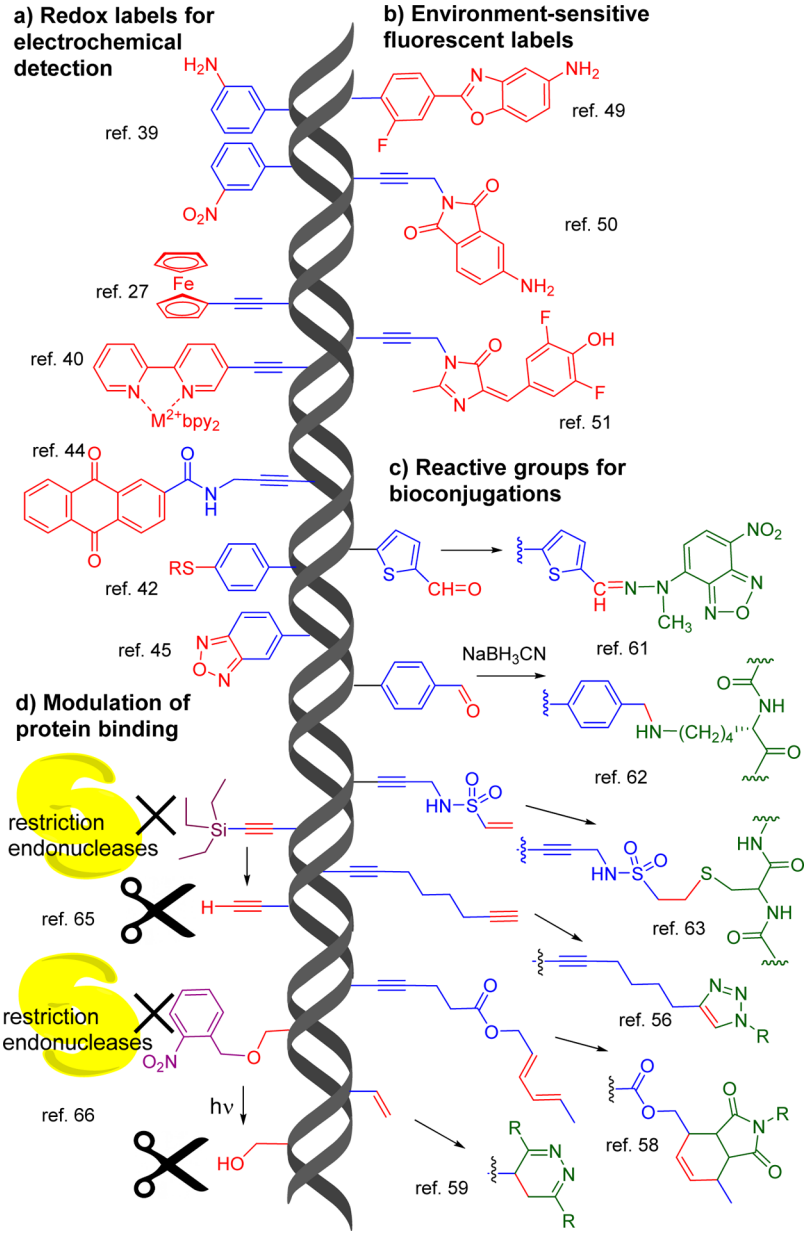
Scheme 3. Examples of DNA Major-Groove Modifications and Their Applications



APPLICATIONS OF THE BASE-MODIFIED ONS OR DNA IN BIOANALYSIS

The enzymatic syntheses of ONs or DNA are suitable for labeling of those nucleic acids by fluorescent,[35] redox,[5a,c,36] or spin[37] markers (Scheme 3). In collaboration with the Fojta group, we have been particularly interested in development of redox labeling and redox coding for electrochemical detection. Although some covalent DNA redox labels were published by others,[38] we have systematically studied diverse oxidizable and reducible functional groups attached to nucleobases in nucleosides and nucleotides, their enzymatic incorporation to DNA, and electrochemistry of the labeled DNA. We reported dNTPs and DNA modified by ferrocene,[28] amino- and nitrobenzene,[39] Ru- or Os(bpy)$_3$ complexes,[40] tetrathiafulvalene,[41] sulfides,[42] hydrazones,[43] anthraquinone,[44] benzofurazane,[45] or methoxyphenol.[46] Each of the labels has a distinct redox potential by which the label can be identified in voltammetry. However, for analysis of longer sequences in one experiment, we need a set of four fully orthogonal and ratiometric labels (for four nucleobases in DNA), readable in the presence of all others. So far we succeeded in combination of benzofurazane with nitrophenyl group and are working on design and study of other orthogonal redox labels.[45]

Fluorescent labeling of nucleic acids is an extensively studied area, and fluorescent dideoxynucleoside triphosphates are used in Sanger sequencing.[47] A number of fluorescent dNTPs are known[14,30b,48] or even commercially available. We are interested in design of environmentally sensitive labels for study of DNA–protein interactions or for time-resolved monitoring of biological processes. Recently, we reported[49] fluorinated biaryl-linked nucleotides which exerted solvatochromic and pH-dependent fluorescence and were suitable for detection of changes in secondary structures of DNA both by fluorescence and by ^{31}P NMR spectroscopy. A solvatochromic aminophthalimide label was also incorporated[50] to DNA and used for detection of interactions with proteins (single-strand-

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binding protein and p53). The change of the polarity around the label resulted in ca. 2.5-fold enhancement of fluorescence intensity. The GFP−fluorophore was selected as an example of a molecular rotor which lights up due to steric hindrance of the rotation. It was attached to dNTP, incorporated to DNA, and apart from application in protein-binding assays, was used for time-resolved study of primer extension.[51] Studies of other solvatochromic fluorophores and molecular rotors continue in our laboratory in order to identify better labels with pronounced response to protein binding.

APPLICATIONS OF THE BASE-MODIFIED ONS OR DNA IN CHEMICAL BIOLOGY

Chemical biology is a rapidly growing field where base-modified nucleic acids find many applications. The Williams[52] and Perrin[23] groups and more recently the Hollenstein laboratory[53] used base-modified **dN^{X}TP**s for selection of DNAzymes, whereas the Kuwahara group developed[54] several modified DNA aptamers. In our laboratory, we use base modifications in the major groove of DNA either for bioconjugations and cross-linking with proteins or for modulation of recognition and binding of proteins.

Modification of DNA by reactive groups has extensively been studied for postsynthetic bioconjugations.[55] Diverse ethynyl- or octadiynyl-modified ONs or DNA were synthesized both chemically and enzymatically, and the Cu-catalyzed click reactions with azido-derivatives were used for attachment of various useful functional groups or molecules.[56] Staudinger ligation of azido-modified ONs was developed to form amides,[57] whereas diene-modified DNA was used for Diels−Alder (DA) cycloadditions with alkenes.[58] Recently, reverse-demand DA cycloadditions of vinyl-modified DNA with tetrazines were reported.[59] Aqueous Suzuki−Miyaura cross-coupling reactions of base-halogenated DNA with aryl- or alkenylboronic acids was developed for attachment of aryl- or alkenyl groups.[60] We developed a synthesis of aldehyde-modified dNTPs and polymerase synthesis of aldehyde-modified DNA which was stained by hydrazone formation[61] or used for preparation of peptide-ON conjugates through reductive amination with lysine.[62] Vinylsulfonamide was identified as a suitable Michael acceptor for specific reactions with cystein, and after enzymatic incorporation to DNA, this reactive group was used for cross-linking with p53 protein.[63]

Our systematic study of the influence of diverse modification in the major groove on the recognition and cleavage of DNA by type-II restriction endonucleases (RE) revealed[64] a surprising tolerance to modifications at T and A, whereas almost no modification was tolerated at C (influence of G-modifications still remains to be investigated). Based on this knowledge, we have developed the first chemical trigger of DNA cleavage by RE. We used 7-(triethylsilyl)ethynyl-7-deaza-dATP for enzymatic synthesis of DNA protected in the major groove by the bulky silyl groups.[65] This silylated DNA was not recognized and cleaved by RE, whereas after desilylation with ammonia, the ethynyl-modified DNA was cleavable again. Later on, we improved the approach by using major-groove-photocaged DNA.[66] Photocleavable 5-[(2-nitrobenzyl)oxymethyl]uracil dNTP was used for polymerase synthesis of the photocaged DNA which was not cleavable by RE. Irradiation by UV (365 nm) deprotected the DNA to release 5-hydroxymethyluracil bases, and such modified DNA was cleaved by most RE. The major-groove protection approach was also utilized[67] in a novel strategy for gene cloning and expression in cases where the gene contains the same sequence, which need to be cleaved by a RE. The protocol is based on protection of central part of the gene by major-groove modifications (i.e., triethylsilylethynyl) while leaving flanking sequences unmodified (such DNA can be easily prepared by a series of 3 PCR experiments). Then, the partly modified DNA can be cleaved by any RE and cloned to a plasmid without any cleavage within the gene. Now, we are working on application of the major-groove protection/deprotection chemistry for regulation of gene expression.

CONCLUSIONS AND OUTLOOK

Enzymatic synthesis of base-modified DNA is a very powerful technology which makes a useful alternative to the classical phosphoramidite chemistry. The major advantages are the tolerance to virtually any functional groups (including strong electrophiles or nucleophiles), possibility to make very long DNA, and potential for selections and amplifications. Major drawbacks are the price (all biochemicals, enzymes, and separation columns are expensive) and limited scale (picomolar to nanomolar). However, the simple and straightforward synthesis of modified **dN^{X}TP**s by aqueous cross-couplings makes them affordable. Further development of methodologies (i.e., solid-phase PEX etc.) and separation techniques, as well as further engineering of even more efficient polymerases, are needed to scale up the enzymatic synthesis. The base-substituted ONs and major-groove-modified DNA are of interest for diverse applications in diagnostics (redox or fluorescent labeling) as well as for applications in bioconjugations and chemical biology.

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Notes

The authors declare no competing financial interest.

Biography

Michal Hocek is a professor of organic chemistry at the Charles University in Prague and the Senior Group Leader at the Institute of Organic Chemistry and Biochemistry ASCR. His research interests include bioorganic and medicinal chemistry of nucleosides, nucleotides, and nucleic acids as well as synthetic methodology of cross-coupling reactions, C−H activations, and bioconjugations.

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