Synthesis, Enzymatic Stability and Base-pairing Properties of Oligothymidylates Containing Thymidine Dimers with Different *N*-Substituted Guanidine Linkages[1]

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Reaction of 5'-amino-5'-deoxythymidine **1** with different *S,S*-dimethyl-*N*-substituted dithiocarbonimidates **2a–j** afforded the *N*-substituted isothioureas **3a–j** which, on further reaction with 3'-amino-3'-deoxythymidine **4** in the presence of $AgNO_3$, led to thymidine dimers **5a–j** with different *N*-substituted guanidine linkages. The dimer with a thiourea linkage (compound **9**) was also prepared. Dimers **5a–h** were incorporated at different positions in oligothymidylates by using phosphoramidite chemistry. Attempts to incorporate compounds **5i,j** and **9** led to complex mixtures. 3'-Protected oligonucleotides showed somewhat higher stability to snake venom phosphodiesterase. Melting experiments revealed that the *N*-methylsulfonyl-substituted guanidine linkage best mimics the natural phosphodiester bridge. The fluorescence properties of oligonucleotides with dimer **5f** were studied in view of its potential use as a non-radioactive label for DNA.

The discovery in the late seventies that an oligodeoxynucleotide (ODN) complementary to part of the messenger RNA (mRNA) of Rous Sarcoma virus could prevent the translation of this mRNA and eventually stop the virus production[2] provided us with a potential rational drug-design strategy to treat diseases caused by the expression of unwanted genetic information. Based on the knowledge of the sequence of the undesirable gene, one can design oligonucleotides which form stable duplexes with the target mRNA and prevent its translation. The principal targets are viral infections, many cancers and some maladies caused by bacteria and parasites. The publications of Zamecnik[2] have stimulated a lot of research groups to follow this approach.[3] Oligodeoxynucleotides might also interfere with the transcription process by forming a triple helix with a complementary double-stranded DNA target (antigene therapy).[3,4]

To be useful as therapeutics such oligodeoxynucleotides should meet stringent requirements.[3] Besides low toxicity and absence of immunogenicity, good bioavailability and stability are important properties. As unmodified DNA is hydrolysed very fast *in vivo* by different nucleases, the oligonucleotide should be stabilized against this degradation. On the other hand, the ODN must form a stable duplex or triplex with the complementary target sequence without annealing to non-complementary sequences. The oligonucleotide should also easily enter the cell to reach its intracellular target but still has to remain soluble in biological fluids. Therefore oligonucleotides were chemically modified with the aim of improving enzymatic stability and penetration into cells without affecting the hybridizing properties and the solubility in water.[3] The nucleobases are necessary for selective Watson–Crick base-pairing with the target sequence. A lot of sugar modifications are reported in the literature.[5] These sugar modifications usually give less stable duplexes. On the other hand, the internucleoside phosphate group impedes cellular uptake and is also the cleavage site of nucleases. Phosphate backbone modifications seem therefore to be the most logical approach. Here, we report the synthesis and properties of a new internucleoside linkage.[1]

In phosphorothioates,[6] alkylphosphonates,[7] phosphotriesters[8] and phosphoramidates[9] a non-bridging oxygen of the phosphodiester linkage is replaced by sulfur, an alkyl group, an alkoxy group or an amine function, respectively. All of these modifications show increased stability towards enzymatic breakdown. From these analogues, phosphorothioates, which have the best hybridizing properties and are most easily synthesized, have been studied most profoundly. Whereas the last three analogues are uncharged and might therefore enter cells more easily, all of them are chiral at phosphorus and thus lead to a mixture of diastereoisomers which is difficult to purify and characterize. Moreover, from the viewpoint of a medicinal chemist, the use of enantiomeric or diastereoisomeric mixtures of a drug in therapy should be rejected for many reasons and this also holds for oligomers. Achiral phosphorus-containing linkages include phosphorodithioates,[10] 3'-*S*-phosphorothiolates,[11] 5'-*S*-thioates[12] and 5'-*N*-amidates[13] all being negatively charged and therefore not very apt for cellular uptake.

Substitution of the whole phosphodiester linkage resulted in nucleoside dimers with, *e.g.*, siloxane,[14] carbamate,[15] oxyacetamido,[16] formacetal,[17] dimethylene sulfide, sulfoxide or sulfone,[18] sulfonate,[19] sulfonamide,[19] sulfamate,[20] thioether[21] and (methyl)hydroxylamine[22] linkages. All these linkages are achiral, stable to nucleases, and neutral at physiological pH. Besides, uncharged analogues may provide more stable duplexes because of the absence of ionic repulsion forces between the phosphodiester linkages of the two duplex strands.

Recently the synthesis and properties of analogues with a completely modified backbone, the peptide nucleic acids (PNA),[23] have been reported. They hybridize strongly to complementary DNA and RNA, are stable towards enzymatic degradation, achiral, and easy to prepare in large amounts. Their main disadvantage, for the moment, seems to be their low cellular uptake.

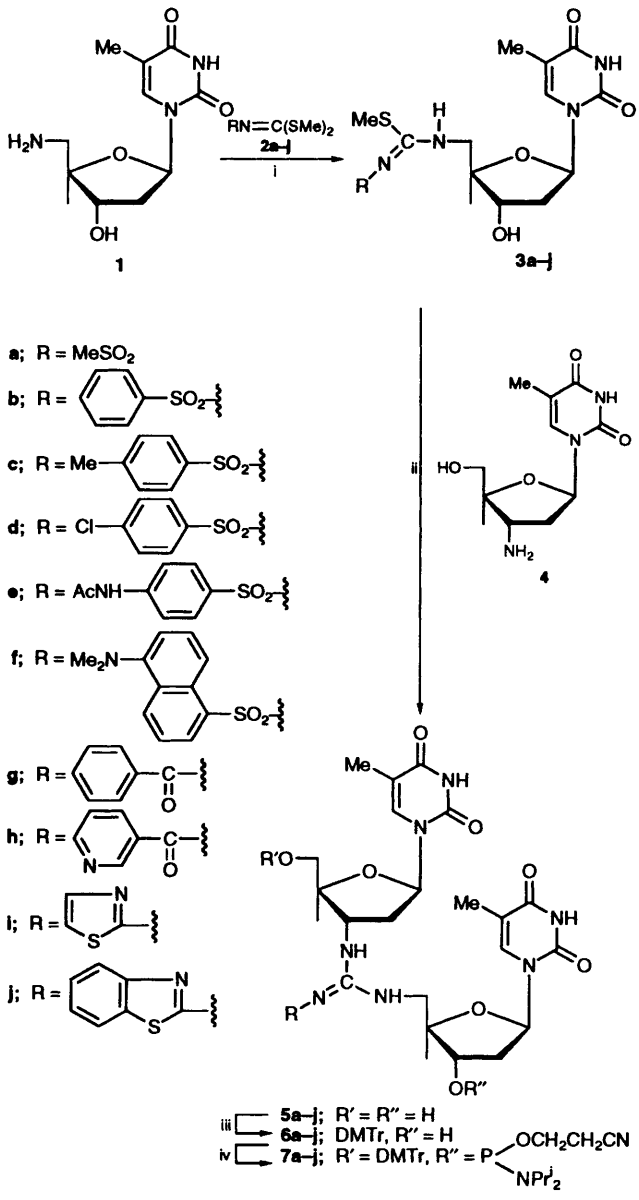
We replaced the 3'-O–PO_2–O-5'-linkage by a 3'-N–CNR–N-5'-functionality and studied the influence on the base-pairing properties of oligonucleotides containing such nucleoside dimers. As non-substituted guanidines are strongly basic (p*K*a

13.6)[24] they will be positively charged *in vivo*, which either may hamper cellular uptake or may lead to aspecific interactions. When incorporation in normal DNA is desirable, the basicity of the guanidine function might lead to side reactions during the preparation of amidite building blocks and problems during classical DNA synthesis. In order to lower the basicity of the guanidine function, we introduced electron-withdrawing substituents (R). Guanidine analogues with such substituents remain uncharged under neutral conditions.[24] An example thereof is the *N*-cyano-substituted guanidine (p*K*a −0.4) present in the anti ulcer drug cimetidine (Tagamet^R).[25] Charton demonstrated a high correlation between the Hammett σ_I-value of a lot of substituents and the p*K*a-value for a series of monosubstituted guanidines.[26] Based on a σ_I-value of 0.59 for the $MeSO_2$ group[27] the p*K*a-value of the sulfonyl-substituted analogues **5a–f** is calculated to be ~1. The sulfonyl-substituted guanidine therefore can be considered as an uncharged, achiral group with a planar π-electron system which is polar and has potential for strong hydrogen bonding. The polarity could be modified by changing the substituent on the sulfonamide function. We also synthesized the *N*-phenylcarbonyl- (**5g**) and *N*-pyridin-3-ylcarbonyl- (**5h**) substituted analogues. The p*K*a-value for the phenylcarbonyl-substituted guanidine is reported to be 7.[24] The *N*-thiazol-2-yl (**5i**) and *N*-benzothiazol-2-yl (**5j**) analogues were prepared to further evaluate the modifications which are allowed during oligonucleotide synthesis. Because of the steric and electronic resemblance with an *N*-cyano-substituted guanidine function,[25] we also included the thiourea-linked dimer **9** in our study. The synthesis and properties of *N*-cyano-substituted guanidine linked dimers have been published elsewhere.[28]

Besides their use as potential therapeutic agents, labelled oligonucleotides are also suitable as probes in genetic analysis tests and for studying biological processes at the molecular level.[29] Owing to the precautions that have to be taken in handling radioactive probes, fluorescently labelled oligonucleotides are more desirable. Attachment of the fluorescent group to the nucleobase[30] might interfere with the base-pairing properties of a probe to the target oligonucleotide. Therefore most 'oligos' have been labelled at the 5′-position *via* different linkers.[29] Recently the introduction of a fluorescent dye at the internucleoside linkage[31] and at the 2′-position of ribose[32] has been reported. Binding of the fluorescent label at the 5′- or 3′-end of an oligonucleotide has the potential disadvantage that it is more easily removed and that the detected fluorescence does not represent the presence of the intact ODN. With the *N*-dansyl-substituted guanidine analogue **5f** we can label oligodeoxynucleotides at several internucleoside linkages. The fluorescence properties as well as the influence on the melting behaviour of those 'oligos' have been studied.

Results

Chemistry.—The different *S*,*S*-dimethyl-*N*-substituted dithiocarbonimidate reagents **2a–j**, used for the introduction of the guanidine linkage were prepared according to literature procedures.[33] Reaction of unprotected 5′-amino-5′-deoxythymidine[34] **1** with one equivalent of an imidate **2a–j** in pyridine at 80 °C afforded the corresponding *N*-substituted-*S*-methyl-*N*′-(5′-deoxythymidin-5′-yl)isothiourea **3a–j** in good yield (70–90%) (Scheme 1). To obtain the guanidine-linked thymidine dimers, the second methylthio group was replaced by 3′-amino-3′-deoxythymidine[35] **4**. Reaction took place only after addition of 1 mol equiv. of $AgNO_3$[28] in either pyridine or dimethylformamide (DMF)–Et_3N as solvent. Under these conditions the *N*-(3′-deoxythymidin-3′-yl)-*N*″-(5′-deoxythymidin-5′-yl)-*N*′-substituted guanidines **5a–j** were obtained in good yield (60–80%) after purification by column chromatography on silica gel. The

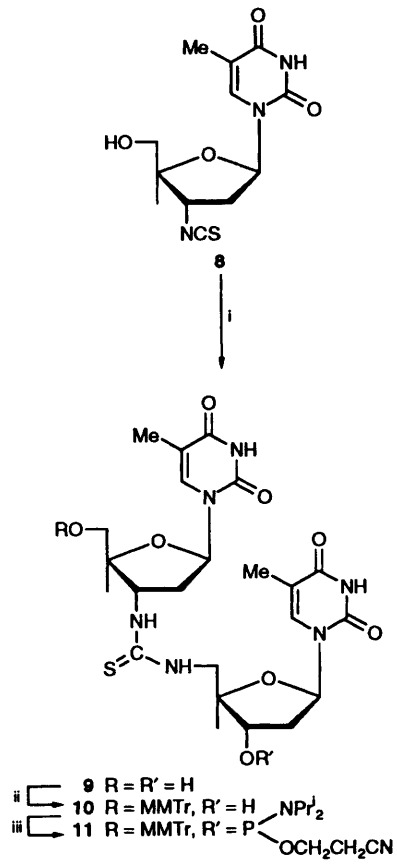


Scheme 1 *Reagents and conditions:* i, pyridine, 80 °C; ii, $AgNO_3$, DMF–Et_3N (in the dark); iii, DMTrCl, pyridine; iv, Pr^i_2NEt, $Pr^i_2NP(Cl)OCH_2CH_2CN$, CH_2Cl_2

dimers can also be prepared starting from 3′-amino-3′-deoxythymidine **4**. As an example, compound **4** was treated with compound **2b** to give *N*′-(3′-deoxythymidin-3′-yl)-*S*-methyl-*N*-(phenylsulfonyl)isothiourea, which on reaction with compound **1** under the same conditions gave the guanidine **5b**. The overall yield, however, was somewhat lower. All dimers were characterized by proton and carbon NMR and mass spectra. However, NMR spectra indicated that the products contained some triethylammonium salts. Analytically pure samples were obtained after purification by reversed-phase (RP) chromatography.

Reaction of 3′-deoxy-3′-isothiocyanatothymidine[36] **8** with compound **1** in DMF afforded *N*-(3′-deoxythymidin-3′-yl)-*N*′-(5′-deoxythymidin-5′-yl)thiourea **9** (Scheme 2), which was also characterized by proton and carbon NMR and mass spectra, and elemental analysis of a RP-HPLC purified sample.

All dimers were protected at their 5′-position by a dimethoxytrityl group (except for **9**, protected with a monomethoxytrityl group) following standard procedures.[37] Some typical signals in the carbon NMR spectrum are given in



Scheme 2 *Reagents:* i, Compd. **1**, DMF; ii, MMTrCl, pyridine; iii, Pr^i_2NEt, $Pr^i_2NP(Cl)OCH_2CH_2CN$, CH_2Cl_2

Table 1 Preparation of the 5′-protected dimers **6a–j,10**: yield (%) and some typical signals in the ^{13}C NMR spectrum ($CDCl_3$) (ppm)

Compound	%	C-5′–O	C-3′–O	C-5	Ph_3C	MeO	Other significant signals
6a	75	63.2	70.9	111.6, 111.3	86.7	55.1	42.3 Me
6b	92	63.4	71.0	111.6, 111.3	86.8	55.2	125.6 Ar, 143.5 Ar
6c	87	63.3	71.1	111.6, 111.3	86.7	55.0	21.2 Me, 125.6 Ar
6d	68	63.5	71.2	111.6, 111.3	86.7	55.1	141.8 Ar, 127.9 Ar
6e	95	63.6	70.8	110.1, 109.8	86.0	55.1	24.2 Me, 141.9 Ar
6f	86			111.6, 111.3	86.7	55.1	45.1 Me, 127.8 Ar
6g	67	63.7	71.0	111.6, 111.2	86.8	55.0	176.9 CO
6h	70	63.4	70.9	111.5, 111.2	86.7	55.0	174.9 CO
6i	50	63.7	70.9	111.5, 111.3	86.8	55.1	175.4 Ar
6j	65	63.7	71.1	111.6, 111.4	86.8	55.0	173.8 Ar
10	*a*	63.9	71.2	109.8, 109.6	86.3	55.0	183.5 CS

[a] 5′-*O*-Monomethoxytritylated dimer.

Table 1. The protected dimers were then phosphitylated by standard procedures[37] to give the amidites **7a–j** and **11** which were characterized by ^{31}P NMR spectroscopy (Table 2).

Using these building blocks, we incorporated the nucleoside dimer analogues in oligothymidylates at different positions by using the standard protocol on a DNA synthesizer. In this way the oligonucleotides listed in Table 4 could be synthesized. Attempts to incorporate dimers **5i,j** and **9** were unsuccessful and resulted in a mixture of products. This might be due to side reactions during the DNA synthesis, or to degradation of the oligonucleotide by alkali on chromatographic purification.

Analysis of the Incorporated Dimers and Enzymatic Stability of the Oligonucleotides.—As it is known that 3′-exonuclease activity is the major cause of degradation of oligonucleotides in serum,[38] it seemed suitable to study the stability of the oligonucleotides in the presence of snake venom phosphodiesterase (SV PDE). During digestion, the increase in absorbance at 260 nm was followed. The results for all oligonucleotides containing dimer **5a** at different positions are given in Table 3. Replacement of one phosphodiester linkage at the 3′-end gives little protection against degradation (1.6 times) whereas substitution with 2 dimers has a more profound influence on the stability (4.1 times). Oligonucleotides with alternating phosphodiester and guanidine linkages show much higher stability (20.4 times). After addition of more SV PDE and alkaline phosphatase, the resulting mixture was analysed by HPLC to identify the degradation products. All oligonucleotides were broken down to the intact dimer **5a** and the nucleosides (thymidine, 2′-deoxycytidine), which were present in the correct ratio of nucleoside dimer to nucleoside. This confirms the stability of the guanidine linkage itself towards enzymatic degradation as well as the suitability of using these dimers during DNA synthesis. This is of particular importance for the fluorescent dimer **5f**.

Hybridizing Properties of the Modified Oligonucleotides.—To study the influence of replacement of the phosphodiester linkage by the uncharged guanidine analogues on the ability to form stable duplexes with an unmodified oligonucleotide, the melting temperatures (T_m) of all oligonucleotides towards the unmodified complementary oligodeoxynucleotides were determined. As, in the antisense approach, the target is an mRNA, we also studied the melting behaviour of some oligonucleotides towards RNA. The results are given in Tables 4 and 5. Substitution of one phosphodiester linkage in the middle of an oligonucleotide has a greater influence than has replacement of two diester bridges, one at the 3′-end and one at the 5′-end, except for the dansyl-substituted dimer **5f**. For substitution in the middle of an oligonucleotide, the (hetero)arylcarbonyl-substituted guanidine linkages give the largest depression in T_m. Replacement of two diester linkages, both at the 3′-end, destabilizes the duplex more than does one substitution in the middle of an oligonucleotide. Oligonucleotides with alternating diester and guanidine linkages do not form duplexes with unmodified DNA unless the substituent R is the small and rather polar $MeSO_2$ group (oligonucleotides with **5a**). For this substitution the depression is only 1.4 °C for each replaced diester bridge. As could be expected, substitution of 2′-deoxycytidine by 2′-deoxy-5-methylcytidine results in more stable duplexes (oligonucleotides with **5h**). An increase in T_m of 1.8 °C is seen per substituted 2′-deoxycytidine. This is analogous to the higher stability of triple helices with 2′-deoxy-5-methylcytidine substituted for 2′-deoxycytidine.[4] When one looks at the influence of the substituent (R) of the guanidine linkage, it is clear that the smallest and most polar substituent ($MeSO_2$) has the least destabilizing effect on the hybridizing properties. The importance of the polarity can also be deduced from the higher T_m for the oligonucleotides with dimer **5e** compared with those of the oligonucleotides with the phenyl-, *p*-methylphenyl-, and *p*-chlorophenyl-sulfonyl-substituted dimers (**5b**, **5c**, **5d**). This is in agreement with the results

Table 2 ^{31}P NMR data ($CDCl_3$) (ppm) and yield (%) of the phosphoramidite building blocks **7a–j,11**

Compound	Yield (%)[a]	^{31}P NMR
7a	51	149.4 and 148.0
7b	75	149.7 and 148.0
7c	53	149.7 and 148.0
7d	70	149.7 and 148.0
7e	51	149.4 and 148.0
7f	70	149.6 and 148.2
7g	52	149.4 and 148.3
7h	70	149.3 and 148.3
7i	55	149.3 and 148.5
7j	39	not determined
11	65	149.3 and 148.9

[a] Yield after precipitation from hexane.

previously found for oligonucleotides with the smaller but less polar cyano substituent which show lower T_ms than do the oligonucleotides with dimer **5a**.[1,28] The stability for the DNA–RNA duplexes of the oligonucleotides with one dimer in the middle is lower than for its DNA–DNA counterpart. The same effect is seen for the unmodified oligothymidylate.

A plot relating absorbance at 260 nm to mole per cent of the completely modified T_{17}-mer containing dimer **5a**, at a given total oligonucleotide concentation of dA_{17} and the modified oligonucleotide (4 μmol dm^{-3}, showed a break at about 65%, indicating a deoxyadenosyl:thymidinyl ratio of 1:2. This is in agreement with mixing curves reported for other non-ionic oligothymidylates.[8,9]

Fluorescence Studies.—The emission and excitation maxima for the *N*-dansyl-substituted isothiourea **3f**, the guanidine-linked dimer **5f** and the oligonucleotides with the dimer at different positions are given in Table 6. The *N*-dansyl-*S*,*S*-dimethyldithiocarbonimidate reagent **2f** is not fluorescent. Fluorescence intensity of the oligonucleotide $T_5(TT)T_6$ increases by ~25% on going from pH 5 to pH 9. To be useful for the detection of nucleic acids in a biological environment, it would be advantageous if the non-hybridized probe could clearly be distinguished from the probe that is hybridized to the target.[32] Therefore the fluorescence intensity of the single-stranded oligonucleotides was compared with the intensity after hybridization with the complementary oligonucleotide. The increase in intensity on hybridization is relatively low, 25–141%, and depends on the position of the fluorescent dimer in the oligonucleotide. Most interestingly, however, the largest increase was seen with the dansyl dimer incorporated in the middle of a sequence (Table 6).

Discussion

Thymidine dimers with *N*-substituted guanidine internucleoside linkages could easily be synthesized in reasonable yield by using different *S*,*S*-dimethyl-*N*-substituted dithiocarbonimidates. The reagents can be prepared in large amounts from common available starting (sulfon)amides and amines. The thiourea-linked dimer likewise is easily available.

Having in view the possible use as new antisense constructs, the different dimers were incorporated into oligodeoxynucleotides. This could be achieved by standard phosphoramidite chemistry on an automated DNA synthesizer. In this way oligonucleotides with alternating phosphodiester and guanidine linkages could be synthesized. Whether the complex mixture obtained after incorporation of dimers **5i,j** and **9** results from side reactions during the synthesis, because of the higher basicity of these analogues, or from degradation during the chromatographic purification at pH 12 was not further investigated.

Table 3 Enzymatic stability of oligodeoxynucleotides with dimer **5a**

'Oligo'[a]	Half-life[b]
T_{17}	1
$(TT)_8T$	20.4
$(TC)_5T_5$	1
$(TC)_5(TT)_2T$	4.1
T_{13}	1
$(TT)T_8(TT)T$	1.6
$T_5(TT)T_6$	1.4

[a] (TT) = **5a**.

[b] Half-life = $\frac{\text{half-life modified 'oligo'}}{\text{half-life unmodified 'oligo'}}$.

Table 4 Melting temperatures (T_m/°C) of the synthesized oligonucleotides towards their respective complementary single-stranded DNA[a]

Oligonucleotide	(TT)	T_m	(TT)	T_m
T_{13}		33.2		
$T_5(TT)_6$	**5a**	31.0	**5e**	29.9
	5b	30.3	**5f**	28.5
	5c	29.3	**5g**	27.1
	5d	28.8	**5h**	27.5
$(TT)T_8(TT)T$	**5a**	31.7	**5e**	30.3
	5b	30.2	**5f**	27.6
	5c	29.8	**5g**	29.3
	5d	29.3	**5h**	30.3
$(TT)T_{11}$	**5f**	31.5		
$T_{10}(TT)T$	**5f**	30.7		
$(TC)_5T_5$		46.3		
$(TC)_5(TT)_2T$	**5a**	44.8	**5g**	40.5
	5c	41.4	**5h**	40.6
	5d	41.1		
$(T^{Me}C)_5(TT)_2T$	**5e**	50.0	**5h**	49.4
T_{17}		43.0		
$(TT)_8T$	**5a**	31.5	**5h**	< 6.5
	5e	< 6.5		

[a] For conditions, see Experimental section.

Table 5 Melting temperature (T_m/°C) of oligonucleotide $T_5(TT)T_6$ towards rA_{12-18}[a]

(TT)	T_m
Unmodified	30.2
5a	25.3
5b	25.3
5c	23.9
5d	24.4
5e	25.6
5f	23.4
5g	22.0
5h	21.8

[a] For conditions, see Experimental section.

The *N*-substituted guanidine linkage has several advantages. As it is achiral, the resulting oligonucleotide is one single product which is easy to purify and characterize.

It is reported in the literature that there are two quite distinct mechanisms of cellular uptake for analogues, namely passive diffusion for the uncharged species and active transport for the charged species.[3,39] The passive mechanism of uptake has the advantage of being unsaturable and without competition by natural DNA. Therefore, we looked for guanidine analogues that would be uncharged at pH 7.4. This was achieved by introduction of the electron-withdrawing groups (R). To confirm the uncharged character of dimer **5a**, a sample of this

Table 6 Fluorescence properties of **3f**,**5f** and the different oligonucleotides containing compound **5f**[a]

Product	Excitation (λ/nm) (RI)[b]		Emission (λ/nm) (RI)[b]	
3f (water)	304 and 364		528	
5f (water)	339		524	
5′ → 3′	Single strand[c]	Duplex[d]	Single strand[c]	Duplex[d]
$(TT)T_{11}$[e]	331 (151)	332 (194)	521 (138)	523 (177)
$T_{10}(TT)T$[e]	331 (141)	331 (260)	520 (127)	523 (242)
$(TT)T_8(TT)T$[e]	332 (255)	331 (380)	520 (255)	523 (378)
$T_5(TT)T_6$[e]	332 (123)	333 (293)	519 (121)	523 (293)

[a] All spectra were taken at 20 °C. [b] RI: relative intensity. [c] Conc. = 2 μmol dm^{-3}. [d] 2 μmol dm^{-3} of each strand. [e] Buffer: 0.1 mol dm^{-3} NaCl, 0.02 mol dm^{-3} phosphate, pH = 7.4, 0.1 mmol dm^{-3} EDTA.

compound in water was titrated with NaOH. As was seen from the titration curve (not shown), there is no transition between pH 4 and 8. A transition at pH 9 results from the deprotonation of the thymine bases and only 2 mol equiv. of NaOH were consumed up to pH 11.5. On the other hand, the analogues should remain soluble in biological fluids. This is of particular importance if one wants to replace all phosphodiester linkages by the guanidine analogues. Even without ionization, however, the hydrogen-bonding capacity of the $MeSO_2$ group is favourable for solubility. The solubility of dimer **5a** in water is ~1 g cm^{-3}. This might be one explanation for the better hybridizing properties of the $MeSO_2$ analogue compared with the cyano analogue.[1,28]

Another reason to select uncharged species is their potentially better hybridizing properties. It is well known that the stability of natural duplexes represents an energetic balance between attractive base–base interactions (*i.e.*, hydrogen bonding between the complementary bases and vertical base stacking), and electrostatic phosphate–phosphate repulsion. Neutralization of the negatively charged phosphate should increase the stability of the complex by decreasing electrostatic repulsion. On the other hand, an apolar substituent may be disadvantageous because of unfavourable hydrophobic forces. This could be another explanation for the better properties of the $MeSO_2$ group compared with the larger aromatic side groups. The concept of using substituted guanidines as internucleoside linkage has the advantage that the polarity of the internucleoside linkage can be modified by changing the substituent (R).

As could be expected, the modified linkage is stable against enzymatic degradation. As is demonstrated before[40] with ODNs containing mixtures of modified and natural linkages, exonucleases that work progressively from one end of the chain can sometimes skip over an isolated phosphonate or triester linkage to cleave the adjacent phosphodiester at a reduced rate. This explains the low stability of oligonucleotides with only one dimer at the 3′-end. Two dimers at the 3′-end lead to a higher stability and oligonucleotides with alternating diester and guanidine linkages show a large increase in stability.

Fluorescently labelled oligonucleotides could be obtained by incorporation of dimer **5f**. The decrease in T_m is ~3 °C if the dimer is situated at the 3′- or 5′-end and ~5 °C if the substitution is in the middle of the sequence. The increase of the fluorescence intensity on hybridization with a complementary oligonucleotide is rather low. The synthesis and incorporation of dimers such as **5f** demonstrate the usefulness of the sulfonylated linkages as anchoring places for reporter groups.

Conclusions.—Although it seems that the methylsulfonyl-substituted guanidine linkage closely mimics the natural phosphodiester linkage, the observed T_m's of the modified ODNs are somewhat lower than the T_m's of the natural ODNs. The incorporation of one thymidine dimer at both ends (4 unnatural thymidines) in a 13-mer resulted in a decrease in T_m of 1.5 °C. The incorporation of one thymidine dimer (2 unnatural thymidines) in the middle of a 13-mer caused a drop in the T_m of 2.2 °C. In the oligonucleotide with alternating diester and guanidine linkages, this drop is 1.4 °C per linkage. This drop could be explained by the different geometry of the internucleoside linkage compared with the normal phosphodiester linkage. Further work to improve the hybridizing properties is in progress.

Experimental

UV spectra were recorded with a Philips PU 8700 UV/Vis spectrophotometer. The ^1H NMR, ^{13}C NMR and ^{31}P NMR spectra were determined with a JEOL FX 90Q spectrometer with tetramethylsilane as internal standard for the ^1H NMR spectra, $(CD_3)_2SO$ (39.6 ppm) or $CDCl_3$ (76.9 ppm) for the ^{13}C NMR spectra, and H_3PO_4 for the ^{31}P NMR spectra. *J* Values are in Hz. Liquid secondary-ion mass spectra (LSIMS) were obtained using a Kratos Concept 1H mass spectrometer. Fluorescence spectra were recorded with a Shimadzu RF-5001PC spectrofluorophotometer. Precoated Macherey-Nagel Alugram^R Sil G/UV_{254} plates were used for TLC and the spots were examined with UV light and sulfuric acid–anisaldehyde spray. Column chromatography was performed on Janssen Chimica silica gel (0.060–200 nm). Anhydrous solvents were obtained as follows: dichloromethane was stored on calcium hydride, refluxed, and distilled; pyridine, triethylamine and *N*,*N*-diisopropylethylamine were refluxed overnight on potassium hydroxide and distilled. MeOH and water for HPLC purification of the dimers and hexane and acetone used in the purification of the amidites were purified by (double) distillation.

Preparation of N-*substituted*-N′-(5′-*deoxythymidin*-5′-*yl*)-S-*methylisothioureas* **3a**–**j**.—A mixture of compound **1** and an *S*,*S*-dimethyl-*N*-substituted dithiocarbonimidate (1.1 mol equiv.) in pyridine (25 cm^3) was refluxed for 18 h, cooled and, after addition of Celite, evaporated and coevaporated with toluene. The residue was applied to a silica gel column which, after elution with CH_2Cl_2–MeOH (95:5), afforded the product as a solid.

Preparation of the Guanidine-linked Dimer **5a**–**j**.—To a mixture of the *N*-substituted-*N*′-(5′-deoxythymidin-5′-yl)-*S*-methylisothiourea and 3′-amino-3′-deoxythymidine (1 mol equiv.) in DMF/Et_3N (20 cm^3/15 cm^3), protected from light, was added $AgNO_3$ (1.2 mol equiv.). After reaction for 18 h at room temperature and addition of Celite, the mixture was evaporated and coevaporated with *m*-xylene. The residue was applied to a column of silica gel and eluted with CH_2Cl_2–MeOH (90:10) to give the guanidine-linked dimer as a solid.

An analytically pure sample was obtained by purification on HPLC. The system consisted of a Gilson Model 303 Pump and Model 802C Manometric Module, a Rogel RP column, a Merck-Hitachi L4000 UV detector and a recorder. A sample (~100 mg) of the product was dissolved in mobile phase

(water–MeOH, 80–90% MeOH depending on the dimer) (1 cm^3), injected and eluated at a flow rate of 5 cm^3 min^{-1}. Detection was by monitoring of the absorbance at 265 nm. The eluent was evaporated, and the residue was dissolved in water containing some MeCN or 1,4-dioxane to enhance solubility, and lyophilized.

N′-(3′-*Deoxythymidin*-3′-*yl*)-N″-(5′-*deoxythymidin*-5′-*yl*)-N′-(*methylsulfonyl*)*guanidine* **5a**. From compound **3a** (624 mg, 1.59 mmol) *compound* **5a** (864 mg, 92%) was obtained, RP-HPLC: 80% MeOH (Found: C, 43.2; H, 5.55; N, 16.05. $C_{22}H_{31}N_7O_{10}S{\cdot}1.5H_2O$ requires C, 43.13; H, 5.59; N, 16.00%); λ_{max}(MeOH)/nm 266 (log ε/dm^3 mol^{-1} cm^{-1} 4.29), ε 260, 80 °C (buffer)* 16600; δ_H[$(CD_3)_2SO$] 11.29 (2 H, s, exch, 2 × N*H*Thym), 7.75 (1 H, s, 6-H), 7.44 (1 H, s, 6-H), 7.2 (2 H, br s, exch, 2 × N*H*Guan), 6.20 (2 H, m, 2 × 1′-H), 5.41 (d, *J* 5.0, exch, 3′-OH), 5.13 (1 H, t, exch, 5′-OH), 4.21 (2 H, m, 2 × 3′-H), 3.84 (2 H, m, 2 × 4′-H), 3.74–3.53 (4 H, m, 2 × 5′-H_2), 2.92 (3 H, s, $MeSO_2$), 2.14 (4 H, m, 2 × 2′-H_2) and 1.79 (6 H, s, 2 × *Me*Thym); δ_C[$(CD_3)_2SO$] 163.7 (C-4), 155.1 (N=C), 150.5 (C-2), 136.2 and 136.0 (2 × C-6), 110.0 and 109.4 (2 × C-5), 85.1 and 83.7 (C-4′ and -1′), 70.8 (C-3′–O), 61.2 (C-5′–O), 51.4 (C-3′–N), 42.8 (C-5′–N), 41.7 ($MeSO_2$), [C-2′ hidden by $(CD_3)_2SO$] and 12.3 and 12.1 (2 × *Me*Thym); *m*/*z* 586 (M + H^+) and 127 (T + H^+).

N′-(3′-*Deoxythymidin*-3′-*yl*)-N″-(5′-*deoxythymidin*-5′-*yl*)-N-(*phenylsulfonyl*)*guanidine* **5b**. Compound **3b** (600 mg, 2.5 mmol) gave *compound* **5b** (925 mg, 58%), RP-HPLC 85% MeOH (Found: C, 48.2; H, 5.4; N, 14.5. $C_{27}H_{33}N_7O_{10}S{\cdot}1.5H_2O$ requires C, 48.06; H, 5.38; N, 14.53%); λ_{max}(MeOH)/nm 266 (log ε 4.28), ε 260, 80 °C (buffer) 16500; δ_H[$(CD_3)_2SO$] 11.26 (2 H, s, exch, 2 × N*H*Thym), 7.73 (3 H, m, 6-H + 2 × ArH), 7.65–7.21 (6 H, m, 2 H exch, 6-H, 3 × ArH and 2 × N*H*Guan), 6.18 (2 H, m, 2 × 1′-H), 5.40 (1 H, d, *J* 4.4, exch, 3′-OH), 5.10 (1 H, t, exch, 5′-OH), 4.37 (1 H, s, 3′-H), 4.14 (1 H, s, 3′-H) 3.76 (2 H, m, 2 × 4′-H), 3.59 (4 H, s, 2 × 5′-H_2), 2.16 (4 H, m, 2 × 2′-H_2) and 1.78 (6 H, s, 2 × *Me*Thym); δ_C[$(CD_3)_2SO$] 163.6 (C-4), 155.0 (N=C), 150.4 (C-2), 144.1 (Ar), 136.1 and 135.9 (2 × C-6), 131.3, 128.6 and 125.4 (Ar), 110.0 and 109.4 (2 × C-5), 85.0 and 83.6 (C-4′ and -1′), 70.8 (C-3′–O), 61.0 (C-5′–O), 51.4 (C-3′–N), 43.0 (C-5′–N) and 12.2 and 12.0 (2 × *Me*Thym); *m*/*z* 648 (M + H^+) and 127 (T + H^+).

N-(3′-*Deoxythymidin*-3′-*yl*)-N″-(5′-*deoxythymidin*-5′-*yl*)-N′-(p-*methylphenylsulfonyl*)*guanidine* **5c**. Compound **3c** (810 mg, 1.7 mmol) afforded *compound* **5c** (1.0 g, 89%), RP-HPLC 85% MeOH (Found: C, 48.6; H, 5.6; N, 14.0. $C_{28}H_{35}N_7O_{10}S{\cdot}1.5H_2O$ requires C, 48.83; H, 5.56; N, 14.24%); λ_{max}(MeOH)/nm 265 (log ε 4.28), ε 260, 80 °C (buffer) 17300; δ_H[$(CD_3)_2SO$] 11.32 and 11.26 (2 H, 2 × s, exch, 2 × N*H*-Thym), 7.74 (1 H, s, 6-H), 7.65 (2 H, d, *J* 8.4, ArH), 7.50–7.20 (5 H, m, 6-H, 2 × ArH and 2 × N*H*Guan), 6.17 (2 H, t, 2 × 1′-H), 5.41 (1 H, d, *J* 4.4, exch, 3′-OH), 5.1 (1 H, br s, exch, 5′-OH), 4.4 (1 H, br s, 3′-H), 4.2 (1 H, br s, 3′-H) 3.77 (2 H, s, 2 × 4′-H), 3.4 (br s, 5′-H, hidden by water signal), 2.34 (3 H, s, *Me*Ar), 2.1 (4 H, br s, 2 × 2′-H_2) and 1.78 (6 H, s, 2 × *Me*Thym); δ_C[$(CD_3)_2SO$] 163.6 (C-4), 154.9 (N=C), 150.3 (C-2), 141.4 (Ar), 136.0 and 135.9 (2 × C-6), 129.0 and 125.5 (Ar), 110.0 and 109.4 (2 × C-5), 85.0 and 83.6 (C-4′ and -1′), 70.8 (C-3′–O), 61.0 (C-5′–O), 51.4 (C-3′–N), 43.0 (C-5′–N), 20.9 (*Me*Ar) and 12.2 and 12.0 (2 × *Me*Thym); *m*/*z* 662 (M + H^+) and 127 (T + H^+).

N′-(p-*Chlorophenylsulfonyl*)-N-(3′-*deoxythymidin*-3′-*yl*)-N″-(5′-*deoxythymidin*-5′-*yl*)*guanidine* **5d**. Compound **3d** (1 g, 2.1 mmol) afforded *compound* **5d** (1.14 g, 82%), RP-HPLC 85% MeOH (Found: C, 45.4; H, 5.1; N, 13.6. $C_{27}H_{32}ClN_7O_{10}S{\cdot}1.5H_2O$ requires C, 45.73; H, 4.97; N, 13.83%); λ_{max}(MeOH)/nm 266 (log ε 4.27), ε 260, 80 °C (buffer) 17500; δ_H[$(CD_3)_2SO$] 11.32 and 11.27 (2 H, 2 × s, exch, 2 × N*H*-*Thym*], *7.73* [*3 H, m, 6-H and 2* ∞ *ArH*], *7.60–7.20* [*5 H, m, 2* ∞ *ArH, 6-H and 2* ∞ *NH*Guan), 6.2 (2 H, br s, 2 × 1′-H), 4.4 (1 H, br s, 3′-H), 4.2 (1 H, br s, 3′-H), 3.8 (br s, 4′- and 5′-H partly hidden by water signal), 2.2 (4 H, br s, 2 × 2′-H_2) and 1.78 (6 H, s, 2 × *Me*Thym); δ_C[$(CD_3)_2SO$] 163.8 (C-4), 155.0 (N=C), 150.5 (C-2), 143.0 (Ar), 136.2 (C-6), 128.9 and 127.5 (Ar), 110.1 and 109.6 (2 × C-5), 84.9 and 83.7 (C-4′ and -1′), 70.9 (C-3′–O), 61.0 (C-5′–O), 51.4 (C-3′–N), 43.2 (C-5′–N) and 12.4 and 12.1 (2 × *Me*Thym); *m*/*z* 682 (M + H^+) and 127 (T + H^+).

N′-(p-*Acetamidophenylsulfonyl*)-N-(3′-*deoxythymidin*-3′-*yl*)-N″-(5′-*deoxythymidin*-5′-*yl*)*guanidine* **5e**. Compound **3e** (990 mg, 1.92 mmol) *compound* **5e** (1.04 g, 74%) RP-HPLC 85% MeOH (Found: C, 47.3; H, 5.4; N, 15.4. $C_{29}H_{36}N_8O_{11}S{\cdot}1.5H_2O$ requires C, 47.60; H, 5.37; N, 15.31%); λ_{max}(MeOH)/nm 263 (log ε 4.68), ε 260, 80 °C (buffer) 44100; δ_H[$(CD_3)_2SO$] 11.29 (2 H, s, exch, 2 × N*H*Thym), 10.23 (1 H, s, exch, N*H*Ar), 7.68 (5 H, m, 6-H and 4 × ArH), 7.56–7.08 (3 H, m, 2 H exch, 6-H and 2 × N*H*Guan), 6.2 (2 H, br s, 2 × 1′-H), 5.42 (1 H, d, *J* 3.5, exch, 3′-OH), 5.1 (1 H, br s, exch, 5′-OH), 4.4 (1 H, br s, 3′-H), 4.2 (1 H, br s, 3′-H), 4.0 (2 H, br s, 4′-H), 3.53 (4 H, m, 5′-H), 2.07 (7 H, m, 2 × 2′-H_2 and MeCO) and 1.78 (6 H, s, 2 × *Me*Thym); δ_C[$(CD_3)_2SO$] 169.2 (CO), 164.0 (C-4), 155.0 (N=C), 150.6 (C-2), 142.0 (Ar), 138.1 (Ar), 136.2 (C-6), 126.7 and 118.6 (Ar), 110.3 and 109.7 (2 × C-5), 85.1 and 83.9 (C-4′ and -1′), 70.9 (C-3′–O), 61.2 (C-5′–O), 51.3 (C-3′–N), 42.4 (C-5′–N), 39.9 (C-2′), 24.2 (*Me*CO) and 12.4 and 12.2 (2 × *Me*Thym); *m*/*z* 705 (M + H^+) and 127 (T + H^+).

N′-*Dansyl*-N-(3′-*deoxythymidin*-3′-*yl*)-N″-(5′-*deoxythymidin*-5′-*yl*)*guanidine* **5f**. Compound **3f** (140 mg, 0.26 mmol) gave *compound* **5f** (190 mg, 61%) as a yellow, fluorescent powder, RP-HPLC 90% MeOH (Found: C, 51.3; H, 5.5; N, 14.25. $C_{33}H_{40}N_8O_{10}S{\cdot}1.5H_2O$ requires C, 51.62; H, 5.64; N, 14.59%); λ_{max}(MeOH)/nm 258 and 332 (log ε 4.49 and 3.67), ε 260, 80 °C (buffer) 25200; δ_H[$(CD_3)_2SO$] 11.28 (2 H, s, exch, 2 × N*H*Thym), 8.38 (2 H, dd, *J* 7.9 and 3.3, ArH), 8.1 (1 H, d, *J* 7.0, ArH), 7.72 (1 H, s, 6-H), 7.6–7.0 (6 H, m, 2 H, exch, 3 × ArH, 6-H and 2 × N*H*Guan), 6.14 (2 H, t, *J* 6.8, 2 × 1′-H), 5.37 (1 H, d, *J* 4.4, exch, 3′-OH), 5.11 (1 H, t, exch, 5′-OH), 4.34 (1 H, m, 3′-H), 4.12 (1 H, m, 3′-H), 3.77 (2 H, m, 4′-H), 3.4 (br s, 5′-H hidden by water signal), 2.80 (6 H, s, Me_2N), 2.09 (4 H, m, 2 × 2′-H_2) and 1.78 and 1.76 (6 H, 2 × s, 2 × *Me*Thym); δ_C[$(CD_3)_2SO$] 163.8 (C-4), 155.0 (N=C), 151.3 (Ar), 150.5 (C-2), 139.3 (Ar), 136.4 and 136.1 (2 × C-6), 129.5, 129.3, 128.7, 127.3, 126.2, 123.3, 120.6 and 115.0 (Ar), 110.3 and 109.6 (2 × C-5), 84.8 and 83.8 (C-4′ and -1′), 70.9 (C-3′–O), 60.9 (C-5′–O), 51.3 (C-3′–N), 45.3 (MeN), 43.2 (C-5′–N) and 12.5 and 12.2 (2 × *Me*Thym); *m*/*z* 741 (M + H^+).

N′-*Benzoyl*-N-(3′-*deoxythymidin*-3′-*yl*)-N″-(5′-*deoxythymidin*-5′-*yl*)*guanidine* **5g**. Reaction of compound **3g** (810 mg, 1.68 mmol) with compound **4** afforded *compound* **5g** (935 mg, 90%), RP-HPLC 90% MeOH (Found: C, 52.7; H, 5.7; N, 15.2. $C_{28}H_{33}N_7O_9{\cdot}1.5H_2O$ requires C, 52.66; H, 5.68; N, 15.35%); λ_{max}(MeOH)/nm 266 (log ε 4.57), ε 260, 80 °C (buffer) 34400; δ_H[$(CD_3)_2SO$] 11.33 (2 H, s, exch, 2 × N*H*Thym), 8.1 (2 H, br s, ArH), 7.9 (1 H, s, 6-H), 7.5 (6 H, br s, 3 × ArH, 6-H and 2 × N*H*Guan), 6.2 (2 H, br s, 2 × 1′-H), 5.5–4.8 (2 H, br s, exch, 3′- and 5′-OH), 4.3 (1 H, br s, 3′-H), 3.9 (3 H, br s, 3′-H and 2 × 4′-H), 3.7 (4 H, br s, 2 × 5′-H_2, partly covered by water signal), 2.25 (4 H, m, 2 × 2′-H_2) and 1.86 and 1.80 (6 H, 2 × s, 2 × *Me*Thym); δ_C[$(CD_3)_2SO$] 175.0 (CO), 163.8 and 163.7 (2 × C-4), 150.5 (C-2), 136.3 (C-6), 136.0, 131.2, 128.6 and 127.9 (Ar), 110.1 and 109.4 (2 × C-5), 84.8 and 83.6 (C-4′ and -1′), 70.8 (C-3′–O), 60.9 (C-5′–O), 50.8 (C-3′–N) and 12.3

* The specific absorption of the dimers in the buffer described in the procedure for the melting experiments was determined at 80 °C to allow for correct determination of the oligomer's concentration.

and 12.0 (2 × *Me*Thym); *m/z* 612 (M + H$^+$) and 127 (T + H$^+$).

N-(3′-*Deoxythymidin*-3′-*yl*)-N″-(5′-*deoxythymidin*-5′-*yl*)-N′-*nicotinoylguanidine* **5h**. Compound **3h** (1.0 g, 2.43 mmol) gave *compound* **5h** (1.4 g, 95%), RP-HPLC 90% MeOH (Found: C, 50.5; H, 5.6; N, 17.4. $C_{27}H_{32}N_8O_9$·1.5H_2O requires C, 50.70; H, 5.52; N, 17.52%); λ_{max}(MeOH)/nm 268 (log ε 4.52), ε 260, 80 °C (buffer) 29200; δ_H[$(CD_3)_2SO$] 11.31 (2 H, s, exch, 2 × N*H*Thym), 9.27 (1 H, d, *J* 1.3, ArH), 8.66 (1 H, dd, *J* 4.8 and 1.8, ArH), 8.38 (1 H, dt, *J* 8.4 and 1.9, ArH), 7.9 (1 H, br s, 6-H), 7.6–7.2 (4 H, m, ArH, 6-H and 2 × N*H*Guan), 6.24 (2 H, m, 2 × 1′-H), 5.5 (1 H, br s, exch, 3′-OH), 5.1 (1 H, br s, exch, 5′-OH), 4.37 (2 H, m, 2 × 3′-H), 3.9 (2 H, br s, 2 × 4′-H) 3.7 (4 H, br s, 2 × 5′-H_2), 2.22 (4 H, m, 2 × 2′-H_2) and 1.84 and 1.79 (6 H, 2 × s, 2 × *Me*Thym); δ_C[$(CD_3)_2SO$] 173.8 (CO), 163.8 and 163.7 (2 × C-4), 159.8 (N=C), 151.4 (Ar), 150.5 (C-2), 150.1 (Ar), 136.4 (Ar), 136.0 (2 × C-6), 133.9 and 123.2 (Ar), 110.1 and 109.5 (2 × C-5), 85.1 and 83.7 (C-4′ and -1′), 70.8 (C-3′–O), 61.2 (C-5′–O), 50.8 (C-3′–N), 12.4 and 12.1 (2 × *Me*Thym); *m/z* 613 (M + H$^+$).

N-(3′-*Deoxythymidin*-3′-*yl*)-N″-(5′-*deoxythymidin*-5′-*yl*)-N′-(*thiazol*-2-*yl*)*guanidine* **5i**. Reaction of compound **3i** (680 mg, 1.71 mmol) afforded *compound* **5i** (920 mg, 90%) as a slightly yellow powder, RP-HPLC 90% MeOH (Found: C, 46.6; H, 5.2; N, 18.0. $C_{24}H_{30}N_8O_8S$·1.5H_2O requires C, 46.67; H, 5.38; N, 18.14%); λ_{max}(MeOH)/nm 274 (log ε 4.40); δ_H[$(CD_3)_2SO$] 11.28 (2 H, s, exch, 2 × N*H*Thym), 9.0 (2 H, br s, N*H*Guan), 7.78 (1 H, s, 6-H), 7.39 (1 H, s, 6-H), 7.17 (1 H, d, *J* 4.0, ArH), 6.82 (1 H, d, *J* 4.4, ArH), 6.21 (2 H, m, 2 × 1′-H), 5.4 (1 H, br s, exch, 3′-OH), 4.44 (1 H, m, 3′-H), 4.23 (1 H, m, 3′-H), 3.88 (2 H, m, 2 × 4′-H), 3.65 (4 H, m, 2 × 5′-H_2), 2.18 (4 H, m, 2 × 2′-H_2), 1.78 and 1.71 (6 H, 2 × s, 2 × *Me*Thym); δ_C[$(CD_3)_2SO$] 163.8 (C-4), 162.3 (Ar), 154.7 (N=C), 150.5 (C-2), 136.2 and 135.9 (2 × C-6), 109.9 and 109.3 (2 × C-5), 84.3 and 83.7 (C-4′ and -1′), 71.0 (C-3′–O), 61.2 (C-5′–O), 51.3 (C-3′–N), 42.9 (C-5′–N) and 12.3 and 12.1 (2 × *Me*Thym); *m/z* 591 (M + H$^+$) and 127 (T + H$^+$).

N′-(*Benzothiazol*-2-*yl*)-N-(3′-*deoxythymidin*-3′-*yl*)-N″-(5′-*deoxythymidin*-5′-*yl*)*guanidine* **5j**. Compound **3j** (1.8 g, 4.1 mmol) gave *compound* **5j** (1.8 g, 70%), RP-HPLC 90% MeOH (Found: C, 50.8; H, 5.4; N, 16.6. $C_{28}H_{32}N_8O_8S$·1.5H_2O requires C, 50.36; H, 5.28; N, 16.78%); λ_{max}(MeOH)/nm 272 and 309 (log ε 4.41 and 4.45); δ_H[$(CD_3)_2SO$] 11.28 (2 H, s, exch, 2 × N*H*Thym), 8.0–7.6 (3 H, m, 2 × ArH and 6-H), 7.57–6.94 (5 H, m, 2 × ArH, 6-H and 2 × N*H*Guan), 6.26 (2 H, m, 2 × 1′-H), 5.5 (1 H, br s, exch, 3′-OH), 4.5 (1 H, br s, 3′-H), 4.3 (1 H, br s, 3′-H), 3.9 (2 H, br s, 2 × 4′-H), 3.82–3.31 (4 H, m, 2 × 5′-H_2, partly covered by water signal), 2.2 (4 H, br s, 2 × 2′-H_2) and 1.80 and 1.59 (2 × 3 H, 2 × s, 2 × *Me*Thym); δ_C[$(CD_3)_2SO$] 175.3 (Ar), 164.0 and 163.8 (2 × C-4), 155.6 (N=C), 150.7 (C-2), 150.1 (Ar), 136.5 and 136.1 (2 × C-6), 125.8, 122.4, 121.2 and 118.3 (Ar), 110.1 and 109.6 (2 × C-5), 85.5 and 84.0 (C-4′ and -1′), 71.4 (C-3′–O), 61.6 (C-5′–O), 51.6 (C-3′–N), 43.2 (C-5′–N) and 12.5 and 12.0 (2 × *Me*Thym); *m/z* 641 (M + H$^+$) and 127 (T + H$^+$).

N-(3′-*Deoxythymidin*-3′-*yl*)-N′-(5′-*deoxythymidin*-5′-*yl*)*thiourea* **9**.—A mixture of 3′-deoxy-3′-isothiocyanatothymidine **8** (440 mg, 1.54 mmol) and of compound **1** (410 mg) in DMF (50 cm^3) was heated for 18 h. After evaporation and coevaporation with *m*-xylene the residue was purified by column chromatography on silica gel [CH_2Cl_2–MeOH (90:10)] to afford *compound* **9** (0.5 g, 62%). An analytically pure sample was obtained by RP-HPLC purification as described for the guanidine dimers (85% MeOH) (Found: C, 46.3; H, 5.6; N, 15.8. $C_{21}H_{28}N_6O_8S$·1H_2O requires C, 46.48; H, 5.57; N, 15.49%); λ_{max}(MeOH)/nm 250 (log ε 4.34); δ_H[$(CD_3)_2SO$] 7.96 (2 H, s, 2 × NHCS), 7.80 (1 H, s, 6-H), 7.49 (1 H, s, 6-H), 6.14 (2 H, t, 2 × 1′-H), 4.7 (1 H, br s, 3′-H), 4.2 (1 H, br s, 3′-H), 3.87 (2 H, m, 2 × 4′-H), 3.7 (4 H, br s, 2 × 5′-H_2), 2.19 (4 H, m, 2 × 2′-H_2) and 1.80 (6 H, s, 2 × *Me*Thym); δ_C[$(CD_3)_2SO$] 182.6 (CS), 164.1 and 162.8 (2 × C-4), 150.7 (C-2), 136.4 (C-6), 110.1 and 109.9 (2 × C-5), 85.6, 84.7, 84.4 and 84.1 (2 × C-1′ and -4′), 71.5 (C-3′–O), 61.8 (C-5′–O), 54.5 (C-3′–N), 46.5 (C-5′–N), 38.6 (C-2′) and 12.6 and 12.4 (2 × *Me*Thym); *m/z* (glycerol) 525 (M + H$^+$).

Dimethoxytritylation of the Dimers **5a–j** *to give Compounds* **6a–j**.—A mixture of the dimer and dimethoxytrityl chloride (1.2 mol equiv.) in dry pyridine (25 cm^3) was kept at room temperature for 18 h. After dilution with EtOAc (25 cm^3) and successive washings with saturated aq. $NaHCO_3$ (25 cm^3) and water (25 cm^3), the organic layer was dried on Na_2SO_4, and evaporated and coevaporated with toluene. Column chromatography of the residue with CH_2Cl_2–MeOH–Et_3N (95:5:1) as the eluting solvent afforded the protected dimer.

Preparation of the Amidite Building Blocks **7a–j** *and* **11**.—A mixture of the 5′-protected dimer **6** or **10** (0.5 mmol), dry *N,N*-diisopropylethylamine (3 mol equiv.) and 1.5 equivalent of 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite in dry CH_2Cl_2 (2.5 cm^3) was stirred at room temperature for 2 h. After addition of EtOH (0.5 cm^3) and further stirring for 25 min, the mixture was washed successively with 5% aq. $NaHCO_3$ (15 cm^3) and saturated aq. NaCl, dried and evaporated. Column chromatography with hexane–acetone–Et_3N (30:70:2) afforded the amidite as a foam, which was dissolved in a minimal volume of dry CH_2Cl_2 and added dropwise to cold (−50 °C) hexane (100 cm^3). The precipitate was isolated, washed with hexane, dried, and used as such for DNA synthesis.

Synthesis of the Oligodeoxynucleotides.—Oligonucleotide synthesis was performed on an ABI 381A DNA synthesizer using the phosphoramidite approach on a 0.5 μmol scale (end dimethoxytrityl off). To ensure efficient coupling yields (estimated by visual control of the released dimethoxytrityl cation), 20–30% supplemental phosphoramidite of the dimers was used. The obtained sequences were deprotected and cleaved from the solid support by treatment with conc. aq. ammonia (55 °C; 18 h). After pre-purification on a NAP-10^R column (Sephadex G25-DNA grade, Pharmacia) eluted with buffer A, purification was effected on a Mono-Q^R HR 10/10 anion-exchange column (Pharmacia) with the following gradient system [A = 10 mmol dm^{-3} NaOH, pH = 12.0, 0.1 mol dm^{-3} NaCl; B = 10 mmol dm^{-3} NaOH, pH = 12.0, 0.9 mol dm^{-3} NaCl; from 40% to 90% B in 40 min depending on the oligonucleotide; flow rate 2 cm^3 min^{-1}]. The low-pressure LC system consisted of a Merck-Hitachi L6200A Intelligent Pump, a Mono-Q^R HR 10/10 column (Pharmacia), a Uvicord SII 2138 UV detector (Pharmacia-LKB) and a recorder. The eluents were desalted on an NAP-10^R column and were lyophilized.

Study of the Enzymatic Stability of Oligonucleotides.—To a solution of 0.4 OD of the oligonucleotide in the following buffer [100 mmol dm^{-3} Tris · HCl pH = 8.6, 100 mmol dm^{-3} NaCl, 14 mmol dm^{-3} $MgCl_2$] (1 cm^3) was added snake venom phosphodiesterase (*Crotalus atrox* venom, Pharmacia) (0.1 or 0.04 U) (solution in the following buffer: 5 mmol dm^{-3} Tris · HCl pH = 7.5, 50% glycerol), at 37 °C. The increase in absorption at 260 nm was followed. The curve could be fitted to an exponential curve from which the half-life could be gathered. The solutions were further used to check the identity of the degradation products.

Analysis of Oligonucleotide Products.—Supplemental snake venom phosphodiesterase (3 U) and calf intestinal alkaline phosphatase (Boehringer Mannheim) (3.4 U) were added to the

previously mentioned solutions which were then kept at 37 °C for a further 18 h. The mixtures were analysed by RP-HPLC.

HPLC Analysis.—The HPLC system consisted of a Merck-Hitachi L-6200A Intelligent Pump, a PRLP-S reversed-phase column, an Uvicord SII 2138 UV detector (Pharmacia-LKB) and an HP 3390A Integrator. Gradient elution with MeOH (25–50% in 25 min) in 0.1 mol dm^{-3} aq. triethylammonium acetate (pH 7.0) at a flow rate of 0.75 cm^3 min^{-1} resulted in elution times of 4.7, 8.5 and 25.3 min for 2′-deoxycytidine, thymidine and the *N*-methylsulfonyl-substituted guanidine dimer, respectively. Detection was effected by monitoring of the absorbance at 254 nm. All oligonucleotides showed the expected signals in the correct ratio.

Melting Temperatures (T_m).—Oligomers were dissolved in the following aqueous buffer: 0.1 mol dm^{-3} NaCl, 0.02 mol dm^{-3} potassium phosphate, pH = 7.5, 0.1 mmol dm^{-3} EDTA. The concentration was determined by measurement of the absorbance at 260 nm at 80 °C and by assuming the following extinction coefficients in the denatured state: T = 8500, C = 7500,[41] ^{5Me}C = 5500 and for the dimers the values reported above.* The concentration in all experiments was 4 μmol dm^{-3} of each strand. Melting curves were determined with a Uvikon 940 Spectrophotometer. Cuvettes were thermostatted with water circulating through the cuvette holder and the temperature of the solution was measured with a thermistor directly immersed in the cuvette. Temperature control and data acquisition were acquired automatically with an IBM/PC AT compatible computer. The samples were heated and cooled at a rate of 0.2 °C min^{-1} and no difference could be observed between heating and cooling melting curves. Melting curves were evaluated according to a simple bimolecular 'All or None' mechanism.[42] Theoretical melting curves according to this mechanism were fitted to the data with VA05A, a non-linear least-squares algorithm[43] taken from the Harwell Subroutine Library. Variation of the T_m of the same mixture was less than 0.5 °C.

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* The specific absorption of the dimers in the buffer described in the procedure for the melting experiments was determined at 80 °C to allow for correct determination of the oligomer's concentration.

References

1 F. Vandendriessche, M. Voortmans, J. Hoogmartens, A. Van Aerschot and P. Herdewijn, *Biorg. Med. Chem. Lett.*, 1993, **3**, 193.
2 P. C. Zamecnik and M. L. Stephenson, *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 280; M. L. Stephenson and P. C. Zamecnik, *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 285.
3 (*a*) *Oligodeoxynucleotides. Antisense Inhibitors of Gene Expression. Topics in Molecular and Structural Biology*, ed. J. S. Cohen, Macmillan, London, 1989, vol. 12; (*b*) G. Zon, *Nucleotide Analogues as Antiviral Agents*, ed. J. C. Martin, ACS Symposium Series 1989, vol. 401, ch. 12, pp. 170–184; (*c*) M. Rothenberg, G. Johnson, C. Laughlin, I. Green, J. Cradock, N. Sarver and J. S. Cohen, *J. Natl. Cancer Inst.*, 1989, **81**, 1539; (*d*) E. Uhlmann and A. Peyman, *Chem. Rev.*, 1990, **90**, 543; (*e*) C. Hélène, *Eur. J. Cancer*, 1991, **27**, 1466.
4 P. B. Dervan, in ref. 3*a*, ch. 9, pp. 197–210.
5 F. Morvan, B. Rayner, J.-L. Imbach, D.-K. Chang and J. W. Lown, *Nucleic Acids Res.*, 1986, **14**, 5019; M. Perbost, M. Lucas, C. Chavis, A. Pompon, H. Baumgartner, B. Rayner, H. Griengl and J.-L. Imbach, *Biochem. Biophys. Res. Commun.*, 1989, **165**, 742; N. I. Sokolova, N. G. Dolinnaya, N. F. Krynetskaya and Z. A. Shabarova, *Nucleosides, Nucleotides*, 1990, **9**, 515; F. Morvan, C. Génu, B. Rayner, G. Gosselin and J.-L. Imbach, *Biochem. Biophys. Res. Commun.*, 1990, **172**, 537; J. Sagi, A. Szemzo, J. Szecsi and L. Ötvös, *Nucleic Acids Res.*, 1990, **18**, 2133; K. C. Schneider and S. A. Benner, *J. Am. Chem. Soc.*, 1990, **112**, 453; H. Rosemeyer, M. Krecmerova and F. Seela, *Helv. Chim. Acta*, 1991, **74**, 2054; H. Urata, K. Shinohara, E. Ogura, Y. Ueda and M. Akagi, *J. Am. Chem. Soc.*, 1991, **113**, 8174; M. J. Damha, P. A. Giannaris, P. Marfey and L. S. Reid, *Tetrahedron Lett.*, 1991, **32**, 2573; B. S. Sproat, A. Iribarren, B. Beijer, U. Pieles and A. I. Lamond, *Nucleosides, Nucleotides*, 1991, **10**, 25; K. Augustijns, A. Van Aerschot, A. Van Schepdael, C. Urbanke and P. Herdewijn, *Nucleic Acids Res.*, 1991, **19**, 2587; J.-M. Henlin, K. Jaekel, P. Moser, H. Rink, E. Spieser and G. Baschang, *Angew. Chem.*, 1992, **104**, 492; L. Bellon, F. Morvan, J.-L. Barascut and J.-L. Imbach, *Biochem. Biophys. Res. Commun.*, 1992, **184**, 797; K. Augustyns, F. Vandendriessche, A. Van Aerschot, R. Busson, C. Urbanke and P. Herdewijn, *Nucleic Acids Res.*, 1992, **20**, 4711; M. Azymah, C. Chavis, M. Lucas, F. Morvan and J.-L. Imbach, *Nucleosides, Nucleotides*, 1992, **11**, 1241.
6 C. A. Stein and J. S. Cohen, in ref. 3*a*, ch. 5, pp. 97–118.
7 P. S. Miller, in ref. 3*a*, ch. 4, pp. 79–96; P. O. P. Ts'o, *Antisense Res. Dev.*, 1991, **1**, 273.
8 P. S. Miller, K. N. Fang, N. S. Kondo and P. O. P. Ts'o, *J. Am. Chem. Soc.*, 1971, **93**, 6657.
9 R. L. Letsinger, S. A. Bach and J. S. Eadie, *Nucleic Acids Res.*, 1986, **14**, 3487.
10 W. K. D. Brill, J. Nielsen and M. H. Caruthers, *J. Am. Chem. Soc.*, 1991, **113**, 3972; K. Bjergarde, B. H. Dahl and O. Dahl, *Nucleosides, Nucleotides*, 1991, **10**, 461.
11 X. Li, D. M. Andrews and R. Cosstick, *Tetrahedron*, 1992, **48**, 2729.
12 M. Mag, S. Lueking and J. Engels, *Nucleic Acids Res.*, 1991, **19**, 1437.
13 W. S. Mungall, G. L. Greene, G. A. Heavner and R. L. Letsinger, *J. Org. Chem.*, 1975, **40**, 1659; M. Mag and J. W. Engels, *Nucleic Acids Res.*, 1989, **17**, 5973.
14 K. K. Ogilvie and J. F. Cormier, *Tetrahedron Lett.*, 1985, **26**, 4159.
15 J. M. Coull, D. V. Carlson and H. L. Weith, *Tetrahedron Lett.*, 1987, **28**, 745; E. P. Stirchak, J. E. Summerton and D. D. Weller, *J. Org. Chem.*, 1987, **52**, 4202.
16 A. Nyilas, C. Glemarec and J. Chattopadhyaya, *Tetrahedron*, 1990, **46**, 2149.
17 G. H. Veeneman, G. A. van der Marel, H. van den Elst and J. H. van Boom, *Recl. Trav. Chim. Pays-Bas*, 1990, **109**, 449; M. Matteucci, *Tetrahedron Lett.*, 1990, **31**, 2385.
18 Z. Huang, K. C. Schneider and S. A. Benner, *J. Org. Chem.*, 1991, **56**, 3869.
19 R. C. Reynolds, P. A. Crooks, J. A. Maddry, M. S. Akhtar, J. A. Montgomery and J. A. Secrist III, *J. Org. Chem.*, 1992, **57**, 2983.
20 E. M. Huie, M. R. Kirshenbaum and G. L. Trainor, *J. Org. Chem.*, 1992, **57**, 4569.
21 S. H. Kawai and G. Just, *Nucleosides, Nucleotides*, 1991, **10**, 1485.
22 J.-J. Vasseur, F. Debart, Y. S. Sanghvi and P. D. Cook, *J. Am. Chem. Soc.*, 1992, **114**, 4006; F. Debart, J.-J. Vasseur, Y. S. Sanghvi and P. D. Cook, *Tetrahedron Lett.*, 1992, **33**, 2645.
23 P. E. Nielsen, M. Egholm, R. H. Berg and O. Buchardt, *Science*, 1991, **254**, 1497; M. Egholm, O. Buchardt, P. E. Nielsen and R. H. Berg, *J. Am. Chem. Soc.*, 1992, **114**, 1895.
24 Y. Yamamoto and S. Kojima in *The Chemistry of Amidines and Imidates*, ed. S. Patai and Z. Rappoport, Wiley, New York, 1991, vol. 2, pp. 485–526.
25 G. J. Durant, J. C. Emmett, C. R. Ganellin, P. D. Miles, M. E. Parsons, H. D. Prain and G. R. White, *J. Med. Chem.*, 1977, **20**, 901.
26 M. Charton, *J. Org. Chem.*, 1965, **30**, 969.
27 R. W. Taft and L. C. Lewis, *J. Am. Chem. Soc.*, 1958, **80**, 2436.
28 C. Pannecouque, P. Wigerinck, A. Van Aerschot and P. Herdewijn, *Tetrahedron Lett.*, 1992, **33**, 7609.
29 J. Goodchild, *Bioconjugate Chem.*, 1990, **1**, 165.
30 A. Roget, H. Bazin and R. Teoule, *Nucleic Acids Res.*, 1989, **17**, 7643.
31 J. A. Fidanza, H. Ozaki and L. W. McLaughlin, *J. Am. Chem. Soc.*,

1992, **114**, 5509; J. A. Fidanza and L. W. McLaughlin, *J. Org. Chem.*, 1992, **57**, 2340.
32 K. Yamana, T. Gokota, H. Ozaki, H. Nakano, O. Sangen and T. Shimidzu, *Nucleosides, Nucleotides*, 1992, **11**, 383.
33 R. Gompper and W. Hägele, *Chem. Ber.*, 1966, **99**, 2885; M. Sato, N. Fukada, M. Kurauchi and T. Takeshima, *Synthesis*, 1981, 554; F. Merchan, J. Garin and E. Meléndez, *Synthesis*, 1982, 590; R. H. Khan and R. C. Rastogi, *J. Chem. Res.* 1991, (*S*), 160.
34 J. P. Horwitz, A. J. Tomson, J. A. Urbanski and J. Chua, *J. Org. Chem.*, 1962, **27**, 3045.
35 J. P. Horwitz, J. Chua and M. Noel, *J. Org. Chem.*, 1964, **29**, 2076.
36 A. Matsuda, M. Satoh, T. Ueda, H. Machida and T. Sasaki, *Nucleosides, Nucleotides*, 1990, **9**, 587.
37 *Oligonucleotide Synthesis: a Practical Approach*, ed. M. J. Gait, IRL Press, Oxford and Washington DC, 1984.
38 J.-P. Shaw, K. Kent, J. Bird, J. Fishback and B. Froehler, *Nucleic Acids Res.*, 1991, **19**, 747.
39 S. L. Loke, C. A. Stein, X. H. Zhang, K. Mori, M. Nakanishi, C. Subasinghe, J. S. Cohen and L. M. Neckers, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 3474.
40 S. Agrawal and J. Goodchild, *Tetrahedron Lett.*, 1987, **28**, 3539.
41 G. Manzini, L. E. Xodo, D. Gasparotto, F. Quadrifoglio, G. A. van der Marel and J. H. van Boom, *J. Mol. Biol.*, 1990, **213**, 833.
42 D. Riesner and R. Römer, in *Physicochemical Properties of Nucleic Acids*, ed. J. Duchesne, Academic Press, London, 1973, vol. 2, pp. 237–318.
43 M. J. D. Powelll, *Comput. J.*, 1965, **7**, 303.

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