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Synthesis of Novel N-Substituted Guanidine Linked Nucleoside Dimers and their Incorporation into Oligonucleotides

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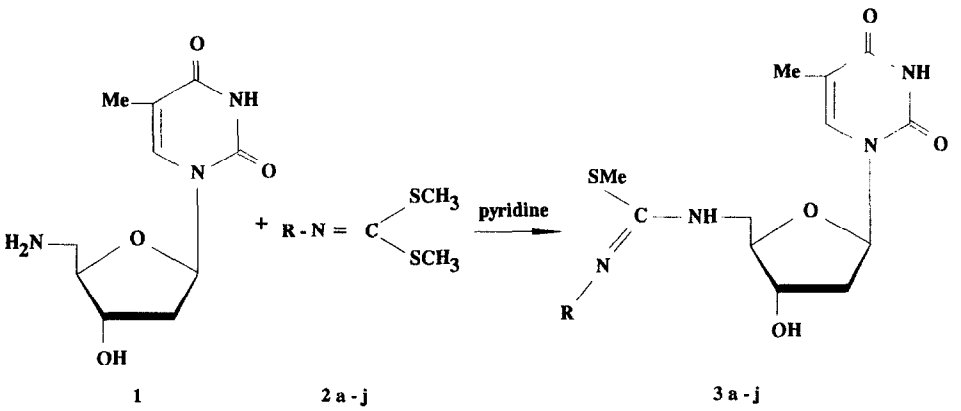
Abstract: *Dimer building blocks (5a-j) with an N-substituted guanidine function were prepared by reaction of 5'-amino-5'-deoxythymidine (1) with S,S-dimethyl-N-substituted iminodithiocarbonimidates (2a-j) followed by reaction of the isothiourea (3a-j) with 3'-amino-3'-deoxythymidine (4). All dimers were protected at the primary hydroxyl group with a dimethoxytrityl group, converted to the phosphoramidite building blocks and incorporated into DNA.*

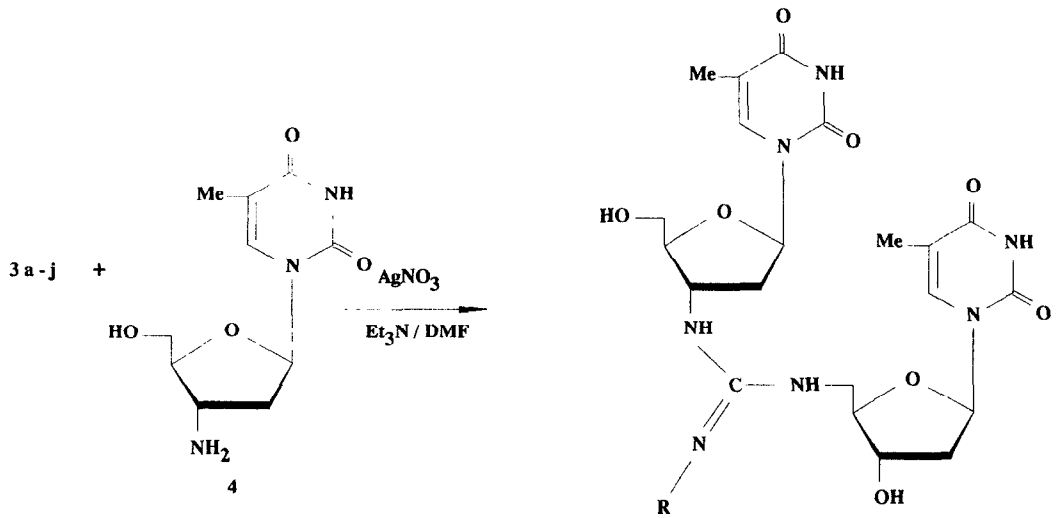
Antisense oligodeoxyribonucleotides can be considered as a new class of potential therapeutic agents that function by preventing the synthesis of specific proteins by either physical blockage of the translation process or inducing RNaseH mediated cleavage of m-RNA[1,2]. Alternatively, oligodeoxyribonucleotides could also be directed against double-stranded DNA (antigene therapy) interfering with the transcription process. Taking their mode of action in consideration, oligodeoxyribonucleotides could be used to treat virtually every disease involving the unwanted expression of genetic information including viral infections, many cancers and some bacterial and parasitic maladies. To be useful such oligo's should be resistant to enzymatical breakdown, soluble in biological fluids, penetrate into cells and form stable duplexes with the complementary oligonucleotide. Besides their potential therapeutic applications, modified oligo's may be useful as diagnostic agents and as probes for studying biological processes at the molecular level[1,2]. Fluorescently labeled oligo's are of particular value in the latter applications.

One way to obtain enzymatically stable oligo's is to modify the natural phosphodiester linkage[1,2]. In recent years there has been a growing interest in oligonucleotides with non-ionic, achiral spacers between the nucleosides[3]. Being uncharged, such analogues could more easily penetrate cell membranes. The absence of ionic repulsion forces, due to the replacement of the phosphate groups by non-ionic linkages, may provide more stable duplexes with the target nucleic acids. Achiral linkages have the advantage of not giving diastereoisomeric mixtures which lead to difficulties in purification and characterization of the compounds.

A substituted guanidine linkage could theoretically fulfill these requirements and therefore we



1 2 a - j 3 a - j



4 5 a - j

a : R = $MeSO_2$

b : R = SO_2

c : R = Me SO_2

d : R = Cl SO_2

e : R = AcNH SO_2

f : R = Me_2N SO_2

g : R = CO

h : R = N CO

i : R =

j : R =

decided to replace the natural phosphodiester linkage by a guanidine linkage. Non substituted guanidines, however, are strongly basic (pKa = 13.6)[4]. This could give rise to several side reactions during the synthesis of amidite building blocks and leads to analogues with a positively charged internucleoside linkage at physiological pH. Guanidine basicity is however markedly reduced by introduction of electron-withdrawing substituents[4]. Charton demonstrated a high correlation between the inductive substituent σ_I and pKa for a series of monosubstituted guanidines[5]. Based on a σ_I value of 0.59 for the $MeSO_2$ group[6], the pKa value of the guanidinium salt was calculated to be about 1. This is in the same range as the reported pKa value of -0.4 for *N*-cyano substituted guanidine[5] .Therefore, we synthesized guanidines with the electron-withdrawing substituents (R). Those lead to a non-charged linkage[7] with a planar π-electron system which is polar and has potential for strong hydrogen bonding. The polarity could be modified by the nature of the substituent R.

Six different *N*-sulfonyl substituted dimers (**5a-f**) were prepared including the fluorescent dansyl substituted analogue (**5f**). Further we synthesized the *N*-phenylcarbonyl (**5g**), the *N*-pyridin-3-ylcarbonyl (**5h**), the *N*-thiazol-2-yl (**5i**) and the *N*-benzothiazol-2-yl (**5j**) substituted analogues.

S,S-Dimethyl-*N*-sulfonyldithiocarbonimidates (**2a-f**), *S,S*-dimethyl-*N*-carbonyldithiocarbonimidates (**2g,h**) and *S,S*-dimethyl-*N*-(benzo)thiazolyldithiocarbonimidates (**2i,j**) were used as reagents to introduce the internucleoside linkage. These were prepared from the corresponding amides and amines following published procedures[8,9,10]. Reaction of unprotected 5'-amino-5'-deoxythymidine[11] (**1**) with the imidates in pyridine at 80°C afforded the *N*-substituted-*S*-methyl-isothiourea (**3a-j**) in high yields (70-90%). The second methylthio group could be replaced by 3'-amino-3'-deoxythymidine[12] (**4**) in the presence of 1 equivalent of $AgNO_3$ and pyridine or Et_3N/DMF[13] as solvent. This reaction led to the dimers (**5a-j**) in good yields (60-80%). All compounds were characterized by ^{1}H-NMR, ^{13}C-NMR, MS and UV. Satisfactory elemental analysis were obtained for all dimers after purification by reversed phase HPLC[14].

The dimers were converted to their properly protected amidites by standard procedures[15]. The accessibility of those phosphitylated dimers enabled us to prepare 13-mers containing dimers at different positions and 17-mers containing 8 consecutive dimers by a conventional protocol using an automated DNA-synthesizer. Side reactions were observed during the synthesis and deprotection of oligonucleotides containing the dimers **5i** and **5j**. The incorporation of the other dimers went smoothly without side reactions.

In conclusion, the results presented here show that dimers having an internucleoside (3'-5')-guanidine linkage between thymidine units can easily be prepared in good yields and incorporated into DNA. Further experiments indicate that some of the modified oligo's form stable duplexes with a complementary non-modified oligo, the methylsulfonyl substituted linkage being the best[16]. For all oligo's, the melting temperature of the DNA-DNA duplex was higher than for the DNA-RNA duplex.

Oligo	Tm (DNA) (°C)	Tm (RNA) (°C)
T_{13}	33.2[b]	30.2[d]
$T_5(TT)T_6$	31.0[b]	25.3[d]
$(TT)T_8(TT)T$	31.7[b]	nd[e]
T_{17}	43.0[c]	nd
$(TT)_8T$	31.5[c]	nd

(TT) = dimer **5a**

[a] Buffer: NaCl 0.1M, NaEDTA 0.1mM, Phosphate 0.02 M pH = 7.5, 4 μ M of each strand.

[b] Tm in the presence of unmodified dA_{13}.

[c] Tm towards dA_{17}.

[d] Melting temperature towards $rA_{12\text{-}18}$.

[e] nd = not determined.

Further studies on the influence on duplex stability and resistance towards nucleases are in progress and will be published later together with a complete description of the experimental procedures.

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14. **3a**: UV (MeOH) : λ_{max} = 265 (logε = 3.98); MS (CIMS : NH_3): 393 ($M+H^+$), 345 (M-SMe), 127 (Thym + H^+); ^{1}H-NMR (DMSO-d_6): 11.28 (s, 1H, NH), 7.97 (br s, 1H, NH-5'), 7.41 (s, 1H, H6), 6.14 (t, J=7.0Hz, 1H, H-1'), 5.36 (d, J=4.4Hz, 1H, 3'OH), 4.26 (m, 1H, H-3'), 3.89 (m, 1H, H-4'), 3.45 (d, 2H, H-5'), 2.94 (s, 3H, *Me*SO_2), 2.51 (s, *Me*S, covered by DMSO-d_6), 2.15 (m, 2H, H-2') and 1.80 (s, 3H, *Me*Th); ^{13}C-NMR (DMSO-d_6): 163.6 (C4), 150.4 (C2), 136.1 (C6), 109.8 (C5), 84.4 and 84.1 (C1' and C4'), 71.0 (C3'), 45.4 (C5'), 41.6 (*Me*SO_2), 38.2 (C2'), 14.1 (*Me*S) and 12.0 (*Me*Th)ppm.
5a: UV (MeOH): λ_{max} = 266 (logε = 4.29); MS (FAB: glycerol) : 586 ($M+H^+$), 127 (Thym + H^+); ^{1}H-NMR (DMSO-d_6): 11.29 (s, 2H, NH), 7.75 (s, 1H, H6), 7.44 (s, 1H, H6), 7.25 (br s, 2H, NH-5' and NH-3'), 6.20 (m, 2H, 2xH-1'), 5.41 (d, J=5.0Hz, 1H, 3'OH), 5.13 (t, 1H, 5'OH), 4.21 (m, 2H, H3'-O and H3'-N), 3.84 (m, 2H,H-4'), 3.74-3.53 (m, 4H, 2xH-5'), 2.92 (s, 3H, *Me*SO_2), 2.14 (m, 4H, 2xH-2'), 1.79 (s, 6H, 2x*Me*Th); ^{13}C-NMR (DMSO-d_6): 163.7 (C4), 155.1 (N=C), 150.5 (C2), 136.3 and 136.1 (2xC6), 110.0 and 109.5 (2xC5), 85.1 and 83.7 (C1' and C4'), 70.8 (C3'-

O), 61.2 (C5'-O), 51.4 (C3'-N), 42.8 (C5'-N), 41.7 (*Me*SO_2), 12.3 and 12.1 (2x*Me*Th) ppm; Anal.: calculated for $C_{22}H_{31}N_7O_{10}S$ 1.5H_2O : C: 43.13, H: 5.59, N: 16.00; found: C: 43.24, H: 5.55, N: 16.05.

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16. Comparison with the *N*-cyanoguanidine analogues[13] (A_{13}/T_5*(TT)*T_6 : 29.8°C; A_{17}/*(TT)*$_8$T : 24°C) indicates that the polarity of the substituent R, rather than its size, might have a greater influence on the ability to hybyridize with complementary DNA.