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Synthesis, Enzymatic Stability And Physicochemical Properties Of Oligonucleotides Containing A N-Cyanoguanidine Linkage.

C. Pannecouque, F. Vandendriessche, J. Rozenski, G. Janssen, R. Busson, A. Van Aerschot, P. Claes and P. Herdewijn*

Laboratory of Medicinal Chemistry (F.F.W.), Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium.

Abstract : Nucleoside dimers with a N-cyanoguanidine linkage were synthesized and used as building blocks for oligonucleotide synthesis. Oligonucleotides composed of alternating phosphodiester and cyanoguanidine functions are still able to hybridize with a complementary natural oligodeoxynucleotide.

INTRODUCTION

The development of modified oligonucleotides is of great current interest because of their potential use as antiviral and antitumoral agents (antisense and antigene approach).[1] Natural oligonucleotides suffer from two serious limitations, namely the instability of the constructs against enzymatic degradation and the poor cellular uptake due to their polyanionic character.[2]

In an effort to overcome these problems we decided to evaluate dimeric building blocks with an uncharged [3], enzymatically stable and achiral *N*-cyanoguanidine linkage as potential analogues. Here we describe the synthesis of dimers consisting of two nucleosides linked by a *N*-cyanoguanidine functionality. These dimers are incorporated in oligonucleotides and the biochemical and physical properties of these oligonucleotides are studied.

In order to obtain sufficient amounts of these building blocks, the separate steps for their synthesis have to be high yielded and the purification steps have to be simple. A satisfactory method was developed for the synthesis of N^1-(3'-deoxythymidin-3'-yl)-N^2-cyano-N^3-(5'-deoxythymidin-5'-yl)guanidine. This method was successfully used for the synthesis of other dimers containing adenine, cytosine and guanine bases providing the base moiety was protected with the acid labile dimethoxytrityl group which could easily be removed by dilute acid without hydrolysis of the cyano function.

MOLECULAR MODELLING

In order to investigate whether or not a cyanoguanidine is a good isostere for the phosphodiester linkage in natural B-DNA a molecular modelling study was undertaken.

Contour plots of the energy as a function of torsion angles α' and ζ (figure 1) were generated for the cyanoguanidine linked deoxyribose dimers using molecular mechanics (MM+ parametrization in the Hyperchem^{TM} computer package), after calculating the charges on the atoms with semi-empirical quantumchemical method AM1. Two structures were compared: the first with the cyano group towards the 5'-direction, the second one with the cyano group towards the 3'-direction. The maps (figure 2) show the flexibility of the linkage. For both possible directions, nearly all conformations have an energy within 6 kcal/mol.

The energy required to bring the dimer into a B-DNA like conformation (figure 3), *i.e.* the energy difference between the fit and the fully minimized structure, was also calculated using the same method as described above. The energy is found to be 4.3 kcal/mol for the stucture with the cyano group towards 5', and 4.4 kcal/mol with the cyano group towards 3'. Both structures are thus comparable as far as their energies and conformations are considered (table1).

When the cyanoguanidine group is incorporated as a linker in DNA, the cyano group points away from the bases and does not sterically inhibit a possible duplex formation.

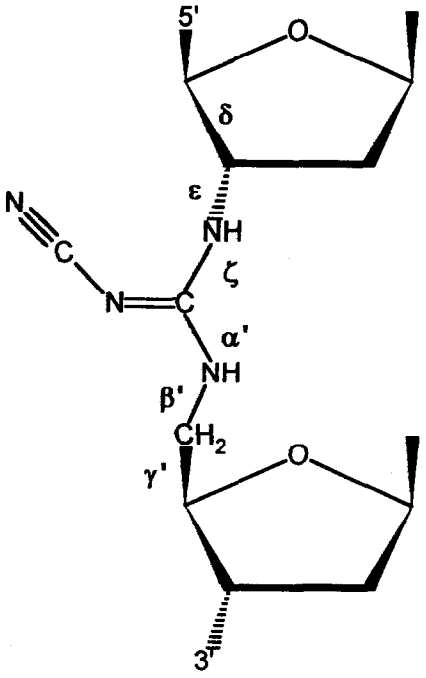


Figure 1: Definition of torsion angles in the cyanoguanidine linked dimer.

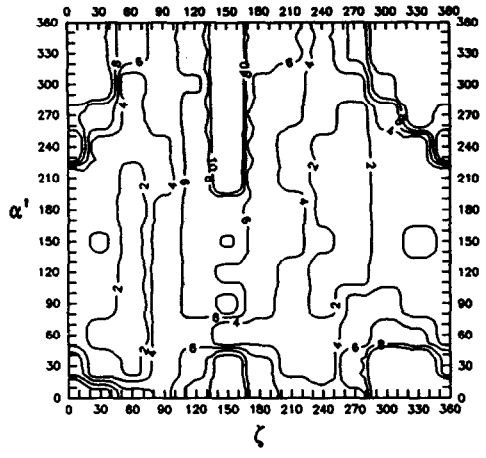


fig. 2a

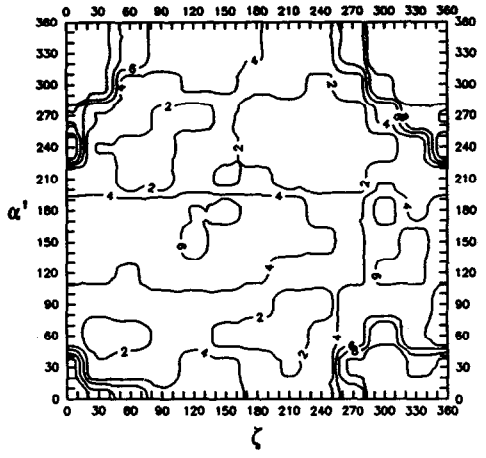


fig. 2b

Figure 2: Contour plots of the energy calculated using MM+ for cyanoguanidine linked dimers, with the cyano function towards 5' end (fig 2a) and towards 3' end (fig 2b) of the oligonucleotide.
Energies (kcal/mol) are expressed relative to the minimum energy of each structure.

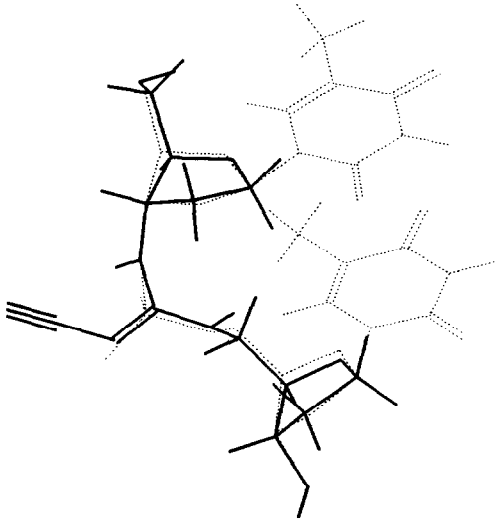


Figure 3: Overlay of a modelled cyanoguanidine linked dimer (solid structure) with a TT dimer from B- DNA (dashed). Atoms C-1', C-1' and P are superimposed on the corresponding atoms of the model.

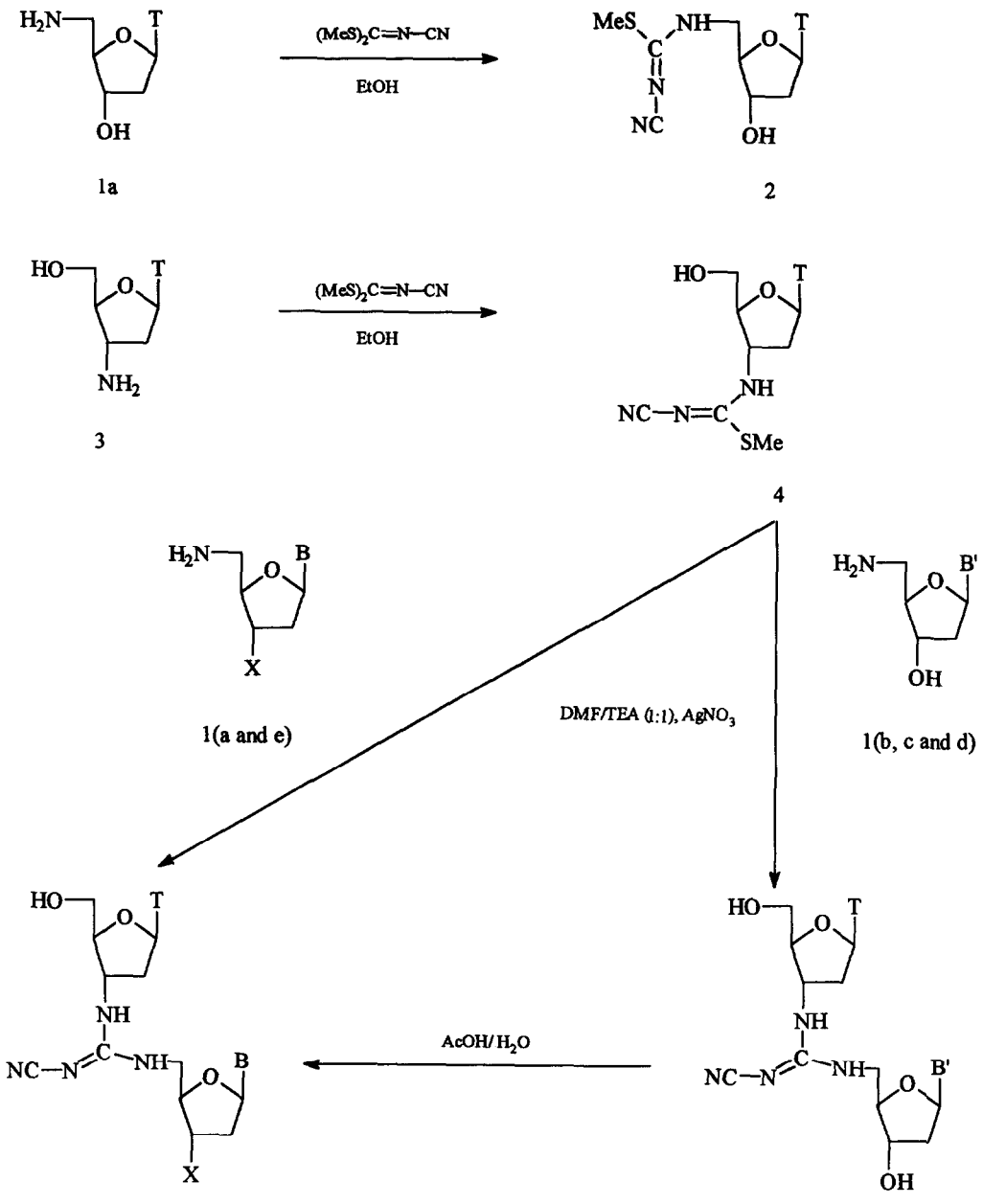
Torsion angles (°)	CN(→5')	CN(→3')
ε	145.5	144.5
ζ	-86.3	-85.5
α'	-54.1	-54.6
β'	-165.8	-165.1
γ'	47.1	47.6
Energies (kcal/mol)		
fit	8.130	7.446
minimum	3.840	3.021

Table 1: Torsion angles and energies of the fitted cyanoguanidine linked deoxyribose dimers.

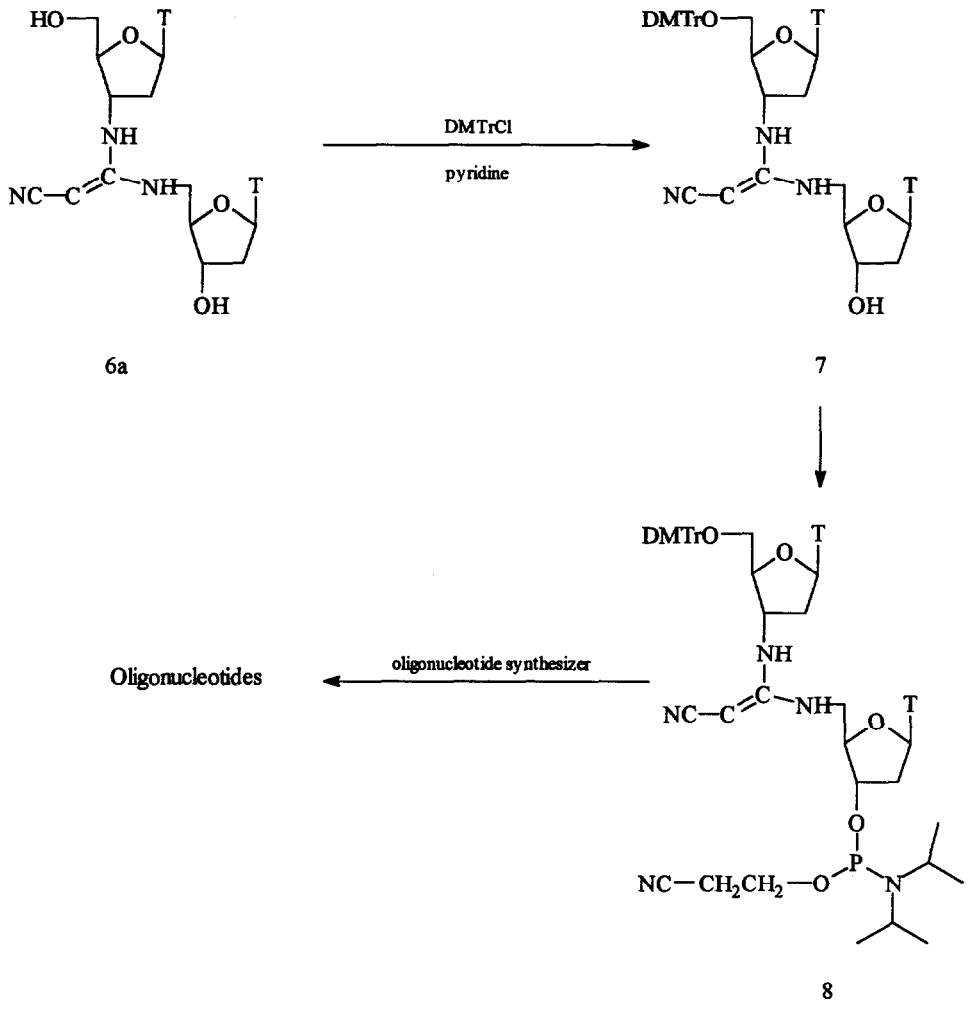
CHEMISTRY

S,S-Dimethyl-*N*-cyanodithioimidocarbonate easily reacts with aliphatic primary amines with formation of *N*-cyanoisothiourea in good yields.[4] Reaction of this reagent with 5'-amino-5'-deoxythymidine **1a** or with 3'-amino-3'-deoxythymidine **3** in ethanol at room temperature afforded the respective methylisothiourea derivatives **2** or **4** in high yields [5]. The second thiomethyl group can be replaced by an aliphatic amine resulting in the formation of a cyanoguanidino derivative. This reaction is generally carried out with a high excess of the primary amine at elevated temperature. However, the reaction of 5'-amino-5'-deoxythymidine **1a** and 3'-amino-3'-deoxythymidine **3** with the above mentioned isothiourea **4** and **2** respectively, did not take place under the conditions normally described for the formation of the cyanoguanidine derivatives. After several attempts the best reaction conditions seem to be a mixture of DMF and TEA (1:1) as solvent, to which silver nitrate was added. This method yielded 80% of N^1-(3'-deoxythymidin-3'-yl)-N^2-cyano-N^3-(5'-deoxythymidin-5'-yl) guanidine. The function of silver nitrate can be explained by the intermediate formation of a reactive carbodiimide [6].

For the synthesis of dimers containing 5'-amino-2',5'-dideoxyadenosine, 5'-amino-2',5'-dideoxycytidine or 5'-amino-2',5'-dideoxyguanosine, protection of the base moiety is necessary. The 5'-amino-2',5'-dideoxynucleosides were obtained as follows. Transient protection of the sugar hydroxyl groups using trimethylsilyl chloride in pyridine allowed protection of the base moiety of the 2'-deoxynucleosides (2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine) with dimethoxytrityl chloride.[7] A tosyl group was selectively introduced at the 5'-position, followed by substitution of the tosyl group with sodium azide in DMF and reduction of the azido function to yield the (base protected) 5'-amino-2',5'-dideoxynucleosides. Reaction of these 5'-amino-2',5'-dideoxynucleosides with 1-cyano-3-(3'-deoxythymidin-3'-yl)-2-methylisothiourea **4** in a mixture of DMF and TEA (1:1) in the presence of silver nitrate as activator gave the protected dimers **5(b-d)**.



series a: B= thymin-1-yl, X= OH
series b: B= cytosin-1-yl; B'= N^4-(dimethoxytrityl) cytosin-1-yl.
series c: B= guanin-9-yl; B'= N^2-(dimethoxytrityl) guanin-9-yl.
series d: B= adenin-9-yl; B'= N^6-(dimethoxytrityl) adenin-9-yl.
series e: B= thymin-1-yl, X= F



Deprotection was accomplished with 50% aqueous acetic acid for 3 minutes on a steam bath. This way no hydrolysis of the cyano function was observed. When the monomethoxytrityl group was used to protect the base moiety of the nucleosides (adenine, cytosine, guanine) we did not succeed in a selective deprotection, as the cyano group was concomitantly hydrolysed to a carboxamide, yielding the N^2-carboxamido substituted guanidine linked dimers.

As nucleoside analogues lacking the 3'-hydroxyl group can function as chain terminators and, hence, are potential anti-HIV agents, we were interested in the synthesis of dimers containing one of these very active nucleoside analogues : 3'-fluoro-3'-deoxythymidine. Therefore, 3'-fluoro-5'-amino-3',5'-dideoxythymidine **1e** [8] was reacted with 1-cyano-3-(3'-deoxythymidin-3'-yl)-2-methylisothiourea giving **6e** in 78 % yield.
This dimer however did not show anti-HIV activity, most probably due to lack of phosphorylation.

When used as an antisense oligonucleotide, the molecule should be at the same time water soluble and able to penetrate cells. Oligonucleotides containing alternating phosphodiester and neutral linkages could fulfill these requirements. Moreover a combination of internucleotide linkages could make the construct more stable against enzymatic degradation. In order to be able to use the dimer building blocks for oligonucleotide synthesis, the thymidine dimer was protected in the 5'-position with a dimethoxytrityl group and phosphitylated in the 3'-position with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite without any side reaction to give the building block **8**.

Using the standard protocol on a DNA synthesizer this building block was incorporated in oligothymidylates at different positions.

ENZYMATIC DEGRADATION EXPERIMENTS

An hexamer with sequence TT(TT)TT, where (TT) represents the dimer with the guanidine linkage, was enzymatically broken down with snake venom phosphodiesterase (SV PDE) and then treated with calf intestinal alkaline phosphatase. The degradation products were analysed by HPLC on a reversed phase column, showing only the presence of thymidine and the intact dimer. The experiments clearly prove the *N*-cyanoguanidine linkage to be stable under the usual oligonucleotide synthesis conditions, and to withstand enzymatic degradation.

HYBRIDIZING PROPERTIES OF MODIFIED OLIGONUCLEOTIDES

To study the influence of the *N*-cyano substituted guanidine linkage on the hybridizing properties, DNA sequences were synthesized with the modified dimer at different positions. Table 2 gives the results of melting point experiments. As could be expected, substitution in the middle of a sequence gives more destabilisation than substitution with two modified dimers one at the 3'- and one at the 5'-end. A 17-mer containing alternating diester and cyanoguanidine linkages still forms a duplexe with unmodified DNA, but the melting temperature decreases from 43.0°C for a normal $d(T)_{17}.d(A)_{17}$ duplex to 24°C for the duplex containing the modified oligonucleotide $(TT)_8T$.

oligo 5' → 3'	Tm (°C)
T_{13}	33.0
$T_5(TT)T_6$	29.8
$(TT)T_8(TT)T$	31.2
T_{17}	43.0
$(TT)_8T$	24.0

Table 2 : Melting temperatures towards their respective complementary DNA.

This amounts to 2.4°C per subtituted linkage. This drop in stability might be explained by the different geometry of the guanidine linkage compared to the normal phosphodiester linkage. As antisense oligonucleotides are primarily targeted against mRNA, the melting temperature of 5'-T_5(TT)T_6-3' was also determined towards rA_{13-18} (22.8°C) which was lower than for its DNA:DNA counterpart (29.8°C).

SYNTHESIS OF OLIGONUCLEOTIDES FOR BIOLOGICAL EVALUATION.

In search for their biological evaluation, mixed oligonucleotides were synthesized targeted at either the PBS or next to PBS site of HIV-1 [9] or at the c-*fos* protein synthesis, the cellular homolog of v-*fos*, which is the transforming gene product of a murine osteosarcoma virus [10,11].
As an example, the HPLC profile of the 20-mer C-Fos start 277-297 sense is shown in fig 4.

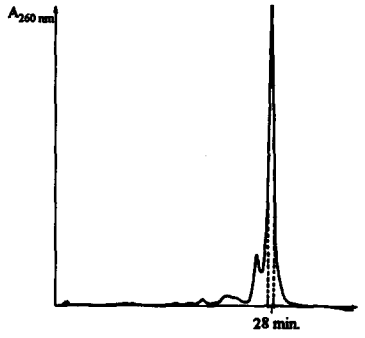


Figure 4: HPLC profile of the crude oligonucleotide (anion exchange chromatography Mono Q HR 10/10, Pharmacia). Eluens buffer A=10mM NaOH + 0.1M NaCl; buffer B 10mM NaOH + 0.9M NaCl. pH=12.0
Table 3 describes the synthesized sequences, their length (n) and their target sequence.

sequence 5' → 3'	n	target
CCTG(TT)CGGGCGCCA	16	PBS antisense
G(TT)CCTCACCTG(TT)CCACC(TT)GC	23	C-Fos splice acceptor 1421-1443 antisense
CTGCTAGAGA(TT)(TT)CCACAC	20	next to PBS antisense
CTAACCGCCACGATGATG(TT)C	20	C-Fos start 277-297 sense
GAACATCATCGTGGCGG(TT)AG	21	C-Fos start 277-297 antisense

Table 3

Unfortunately, no inhibition of cell proliferation was noticed neither with the modified nor with natural oligonucleotides [12]. Neither could the results of Goodchild *et al.* [9] be duplicated: no inhibition of HIV replication could be detected with any of the synthesized modified oligonucleotides nor with their control sequences.

The biological effect of oligonucleotides containing some cyanoguanidine linkages instead of the natural phosphate linkage remains to be determined.

EXPERIMENTAL SECTION

Melting points were determined in capillary tubes with a Büchi-Tottoli apparatus and are uncorrected. Ultra-violet spectra were recorded with a Philips PU 8740 UV/Vis spectrophotometer. The ^{13}C NMR spectra were recorded with a Jeol FX 90 Q spectrometer.
Molecular modelling was performed on Hyperchem ^{TM}, Release 3 for Windows ^{TM}, Autodesk Inc..
Exact mass measurements by liquid secondary ion mass spectrometry were obtained using a Kratos Concept 1H mass spectrometer.

Precoated Macherey - Nagel Alugram® 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 mm).

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 H_2O for HPLC purification, n-hexane and acetone for purification of the amidites were doubly distilled.

The products **1a** [13,15], **3** [14,15] and **1e** [8] were synthesized according to literature procedures.

1-Cyano-3-(5'-deoxythymidin-5'-yl)-2-methylisothiourea (2)

A solution of 2.00 g (8.3 mmol) of **1a** in 100 mL of ethanol was added dropwise to a solution of 4.00 g (27.4 mmol) of *S,S*-dimethyl-*N*-cyanodithioimidocarbonate in 70 mL of ethanol over a period of 1 hour. The reaction mixture was stirred for 24 h and the white precipitate was collected by filtration giving 2.7 g (7.96 mmol, 95% yield) of 1-cyano-3-(5'-deoxythymidin-5'-yl)-2-methylisothiourea. An analytical sample was obtained by crystallisation in methanol.
mp : 224°C
U.V. (MeOH) λ_{max} = 251 nm (ε = 17,700); 220 nm (ε = 18,800)
^{13}C NMR (DMSO-d_6) : δ 12.1 (CH_3); 14.2 (SCH_3); 38.3 (C-2'); 45.4 (C-5'); 71.2 (C-3'); 83.6 and 84.4 (C-1' and C-4'): 109.8 (C-5); 115.6 (C ≡ N); 136.3 (C-6); 150.5 (C-2); 163.7 (C-4); 170.6 (C = N) ppm.
element. anal : ($C_{13}H_{17}N_5SO_4$)
Calculated : C : 46.01; H : 5.05; N : 20.64
Found : C : 45.93; H : 5.06; N : 20.66

1-Cyano-3-(3'-deoxythymidin-3'-yl)-2-methylisothiourea (4)

A solution of 1.20 g (5 mmol) of **3** in 30 mL ethanol was added dropwise to a solution of 2.20 g (15 mmol) of *S,S*-dimethyl-*N*-cyanodithioimidocarbonate in 10 mL of ethanol. The reaction mixture was stirred for 24 h at room temperature. The white precipitate was collected by filtration affording 1.35 g (4 mmol, 80% yield) of the title compound.
mp : 220-221°C
U.V. (MeOH) λ_{max} = 252 nm (ε = 17,990); 231 nm (ε = 15,681)

^{13}C NMR (DMSO-d_6) : δ 12.3 (CH_3); 14.4 (SCH_3); 37.8 (C-2'); 53.6 (C-3'); 61.4 (C-5'); 83.7 and 84.1 (C-1' and C-4'); 109.6 (C-5); 115.6 (C ≡ N); 136.3 (C-6); 150.5 (C-2); 163.7 (C-4); 170.4 (C = N) ppm.
element. anal. : ($C_{13}H_{17}N_5SO_4$)
Calculated : C : 46.01; H : 5.05; N : 20.64
Found : C : 45.80; H : 5.08; N : 20.58.

5'-Amino-4-N-dimethoxytrityl-2',5'-dideoxycytidine (**1b**)

The hydrochloride salt of 2'-deoxycytidine (3.5 g, 13.25 mmol) was dried by coevaporation with dry pyridine and taken up in pyridine. After addition of trimethylsilyl chloride (TMSCl, 8.4 mL) and stirring for 15 minutes, followed by addition of dimethoxytrityl chloride (9.00 g, 26.50 mmol), the reaction mixture was stirred for 18 hours at room temperature. After addition of dichloromethane and extraction with a saturated sodium bicarbonate solution, the organic layer was evaporated and taken up in dioxane (100 mL). Aqueous ammonia (20 mL) was added and the reaction mixture was stirred for 2 days. After evaporation the solid residue was purified on silica gel, giving 6.3 g of the desired compound (11.93 mmol, 90%).
The dimethoxytritylated product was further treated as for synthesis of **1a**.
Overall yield on dimethoxytritylation, tosylation, introduction of an azide and reduction to the amino derivative was 55 %.
U.V. (MeOH) λ_{max} = 278.4 nm (ε = 13,500)
^{13}C NMR ($CDCl_3$) : δ 39.3 (C-2'); 41.9 (C-5'); 54.7 (DMTr); 69.7 (DMTr); 70.7 (C-3'); 84.8 and 87.6 (C-1' and C-4'); 94.7 (C-5); 113.1, 127.0, 127.9, 129.3, 135.6 (DMTr); 141.5 (C-6); 143.8 (DMTr); 155.0 (C-2); 158.1 (DMTr) ; 164.9 (C-4) ppm.
Exact mass (thioglycerol doped with NaCl) calcd for $C_{30}H_{32}N_4O_5Na$ $[M + Na]^+$ 551.2270, found 551.2251.

5'-Amino-2-N-dimethoxytrityl-2',5'-dideoxyguanosine (**1c**)

Compound **1c** was obtained according to the procedure described for **1b**. Overall yield 40 %.
U.V. (MeOH) λ_{max} = 278 nm (ε = 25,600)
^{13}C NMR ($CDCl_3$) : δ 38.5 (C-2'); 43.0 (C-5'); 54.8 (DMTr); 69.9 (DMTr); 71.2 (C-3'); 83.6 and 86.1 (C-1' and C-4'); 112.8 (DMTr); 117.2 (C-5); 126.4, 127.4, 128.2, 129.6 (DMTr); 136.2 (C-8); 136.5 and 144.4 (DMTr); 149.5 (C-4); 150.7 (C-2), 157.7 (C-6); 157.9 (DMTr) ppm.
Exact mass (thioglycerol doped with NaCl) calcd for $C_{31}H_{32}N_6O_5Na$ $[M + Na]^+$ 591.2332, found 591.2316.

5'-Amino-6-N-dimethoxytrityl-2',5'-dideoxyadenosine (**1d**)

Compound **1d** was obtained according to the procedure described for **1b**. Overall yield 48 %.
U.V. (MeOH) λ_{max} = 275 nm (ε = 23,900)
^{13}C NMR ($CDCl_3$); δ 39.7 (C-2'); 43.2 (C-5'); 54.9 (DMTr); 70.4 (DMTr); 71.5 (C-3'); 84.4 and 87.0 (C-1' and C-4'); 112.9 (DMTr); 121.3 (C-5); 126.6, 127.5, 128.5, 129.8, 137.1 (DMTr); 138.5 (C-8); 145.1 (DMTr); 148.0 (C-4); 151.5 (C-2); 153.9 (C-6); 158.0 (DMTr) ppm.
Exact mass (thioglycerol doped with NaCl) calcd for $C_{31}H_{32}N_6O_4Na$ $[M + Na]^+$ 575.2383, found 575.2396.

^{13}C NMR (DMSO-d_6); δ 12.3 (CH_3); 37.3 (C-2'); 38.5 (C-2'); 43.5 (N-C-5'); 51.2 (N-C-3'); 60.7 (C-5'); 71.2 (C-3'); 83.6, 84.5, 85.8 (2 x C-1' and 2 x C-4'); 94.4 (C-5 cytosine); 109.5 (C-5 thymine); 117.5 (C ≡ N); 136.3 (C-6 thymine); 141.5 (C-6 cytosine); 150.5 (C-2 thymine); 155.2 (C-2 cytosine); 159.2 (C = N); 163.8 (C-4 thymine); 165.6 (C-4 cytosine) ppm.

Exact mass (thioglycerol) calcd for $C_{21}H_{28}N_9O_7$ $[M + H]^+$ 518.2111, found 518.2101.

element anal. ($C_{21}H_{27}N_9O_7.2H_2O.MeOH$)

Calculated C : 45.13; H : 6.02; N : 21.53

Found C : 45.26; H : 5.61; N : 21.75

N^1-(2',5'-dideoxy-2-N-dimethoxytritylguanosin-5'-yl)-N^2-cyano-N^3-(3'-deoxythymidin-3'-yl)guanidine (**5c**)

To a solution of 390 mg (1.14 mmol) of **4** and 650 mg (1.14 mmol) of **1c** in 40 mL of a mixture of Et_3N-DMF (1:1), 195 mg (1.14 mmol) of silver nitrate was added.

Reaction for 24 h and work-up as described for **5b** yielded 690 mg of the title compound (0.79 mmol, 80%).

U.V. (MeOH) λ_{max} = 264 nm (ε = 21,900)

^{13}C NMR ($CDCl_3$ and CD_3OD): δ 11.7 (CH_3); 37.6 (2 x C-2'); 43.7 (N-C-5'); 51.2 (N-C-3'); 54.7 (DMTr); 60.4 (C-5'); 69.8 (DMTr); 71.0 (C-3'); 83.8, 84.1, 84.6, 85.1 (2 x C-1' and 2 x C-4'); 110.5 (C-5 thymine); 112.7 (DMTr); 117.2 (C-5 guanosine); 117.7 (C ≡ N); 126.5, 127.4, 128.1, 129.5 (DMTr); 136.2 (C-8); 136.5 (C-6 thymidine + DMTr); 144.3 (DMTr); 149.5 (C-4 guanosine); 150.6 (C-2 thymine); 150.9 (C-2 guanosine); 157.9 (DMTr and C-6 guanosine); 159.4 (C = N); 164.5 (C-4 thymine) ppm.

Exact mass (thioglycerol doped with NaCl) calcd for $C_{43}H_{45}N_{11}O_9Na$ $[M + Na]^+$ 882.3299, found 882.3287.

N^1-(2',5'-dideoxyguanosin-5'-yl)-N^2-cyano-N^3-(3'-deoxythymidin-3-yl)-guanidine (**6c**)

Product **5c** was taken up in 10 mL of a 50% solution of acetic acid in water. The solution was heated for 3 minutes in a steam bath.

The solution was evaporated to dryness under reduced pressure. The residue was taken up in 30 mL water and extracted 3 times with diethyl ether.

The aqueous layer containing the deprotected dimer was evaporated to dryness and the residue was crystallized from a mixture of methanol and diethylether as acetate.

U.V. (MeOH) λ_{max} = 258 nm (ε = 19,200)

^{13}C NMR (DMSO-d_6): δ 12.4 (CH_3); 23.9 ($\underline{C}H_3COOH$); 43.8 (N-C-5'); 51.2 (N-C-3'); 60.7 (C-5'); 71.5 (C-3'); 82.8, 83.5, 84.4, 84.8 (2 x C-1' and 2 x C-4'); 109.4 (C-5 thymine); 116.9 (C-5 guanosine); 117.6 (C ≡ N); 135.3 (C-8 guanosine); 136.3 (C-6 thymidine); 150.5 (C-2 thymidine); 151.1 (C-4 guanosine); 154.1 (C-2 guanosine); 157.2 (C-6 guanosine); 158.8 (C = N); 163.8 (C-4 thymin); 182.5 (CH_3-$\underline{C}OOH$) ppm.

Exact mass (thioglycerol) calcd for $C_{22}H_{28}N_{11}O_7$ $[M + H]^+$ 558.2173, found 558.2180.

element anal. ($C_{22}H_{27}N_{11}O_7.CH_3COOH.3H_2O$)

Calculated : C : 42.92; H : 5.55; N : 22.94

Found : C : 42.60; H : 5.31; N : 23.36

N^1-(3'-deoxythymidin-3'-yl)-N^2-cyano-N^3-(5'-deoxythymidin-5-yl)guanidine (**6a**)

To a solution of 1.7 g (5 mmol) of **2** and 1.3 g (5.4 mmol) **3** in 100 mL of a mixture of Et_3N-DMF (1:1), 850 mg (5 mmol) of silver nitrate was added.

The reaction mixture was stirred at room temperature protected from light for 16 h and TLC analysis (CH_2Cl_2 - MeOH 9:1) revealed that all starting material had disappeared. The mixture was evaporated, coevaporated with m-xylene, adsorbed on celite and purified by column chromatography (CH_2Cl_2 - MeOH 85:15) giving 2.0 g (3.76 mmol, 75% yield) of the title compound as a solid.

U.V. (MeOH) λ_{max} = 267 (ε = 19,000)

^{13}C NMR (DMSO-d_6) : δ 12.0 (CH_3); 12.1 (CH_3); 37.2 (C-2'); 38.3 (C-2'); 43.4 (N-C-5'); 51.2 (N-C-3'); 60.7 (C-5'); 71.0 (C-3'); 83.4, 83.8, 84.2, 84.6 (2 x C-1' and 2 x C-4'); 109.3 (C-5); 109.8 (C-5); 117.4 (C $\equiv$ N); 135.9 (C-6), 136.1 (C-6), 150.4 (2 x C-2), 159.2 (C=N); 163.7 (2 x C-4) ppm.

Exact mass (glycerol) calcd for $C_{22}H_{29}N_8O_8$ $[M + H]^+$ 533.2108, found 533.2102.

element. anal. ($C_{22}H_{28}N_8O_8.H_2O$)

Calculated : C : 48.00; H : 5.49; N : 20.35

Found : C : 48.07; H : 5.52; N : 19.86.

N^1-(2',5'-dideoxy-4-N-dimethoxytritylcytidin-5'-yl)-N^2-cyano-N^3-(3'-deoxythymidin-3'-yl)guanidine (**5b**)

To a solution of 170 mg (0.5 mmol) of **4** and 265 mg of **1b** in 40 mL of a mixture of Et_3N-DMF (1:1), 85 mg (0.5 mmol) of silver nitrate was added.

The reaction mixture was stirred at room temperature protected from light for 24 h.

The mixture was evaporated, the residue was adsorbed on celite and purified by column chromatography (CH_2Cl_2 : MeOH : TEA 94:5:1) giving 212 mg (0.26 mmol, 52% yield) of the title compound as a solid.

U.V. (MeOH) λ_{max} = 272 nm (ε = 18,800)

^{13}C NMR ($CDCl_3$ and CD_3OD) δ 11.8 (CH_3); 37.4 (C-2'); 38.2 (C-2'); 43.5 (N-C-5'); 51.4 (N-C-3'); 54.8 (DMTr); 60.5 (C-5'); 70.0 (DMTr); 71.0 (C-3'); 84.3, 84.6, 85.4 (2 x C-1' and 2 x C-4'); 95.3 (C-5 cytosine); 110.4 (C-5 thymine); 113.3 (DMTr); 117.8 (C $\equiv$ N); 127.2, 128.0, 129.5, 135.6 (DMTr); 136.3 (C-6 thymine); 142.6 (C-6 cytosine); 143.7 (DMTr); 150.3 (C-2 thymine); 155.5 (C-2 cytosine); 158.4 (DMTr); 159.5 (C = N); 164.3 (C-4 thymine); 165.5 (C-4 cytosine) ppm.

Exact mass (thioglycerol doped with NaCl) calcd for $C_{42}H_{45}N_9O_9Na$ $[M + Na]^+$ 842.3238, found 842.3222.

N^1-(2',5'-dideoxycytidin-5'-yl)-N^2-cyano-N^3-(3'-deoxythymidin-3'-yl)guanidine (**6b**)

The protected dimer **5b** (215 mg, 0.27 mmol) was taken up in 10 mL of 50% aqueous solution of acetic acid. The solution was heated for 3 minutes in a steam bath upon which the colour turned to orange. The solution was evaporated to dryness under reduced pressure. The residue was taken up in 30 mL of water and extracted 3 times with 30 mL diethyl ether. The water layer, containing the unprotected dimer, was evaporated, the residue was adsorbed on celite and purified on silica gel giving 70 mg (0.14 mmol, 50% yield) of **6b**.

U.V. (MeOH) λ_{max} = 268 nm (ε = 16,400)

N^1-(2',5'-dideoxy-6-N-dimethoxytrityladenin-5'-yl)-N^2-cyano-N^3-(3'-deoxythymidin-3'-yl)guanidine (**5d**)

To a solution of 552 mg (1 mmol) of **1d** and 340 mg (1 mmol) of **4** in a mixture of 20 mL of DMF and 20 mL of TEA, 170 mg (1 mmol) of silver nitrate was added. The reaction was protected from light and stirred at room temperature for 24 h. Work-up as for **5b** yielded 660 mg (0.781 mmol, 78 %) of **5d**.

Exact mass (triethanolamine doped with NaCl) calcd for $C_{43}H_{45}N_{11}O_8Na$ $[M + Na]^+$ 866.3350, found 866.3404.

N^1-(2',5'-dideoxyadenin-5'-yl)-N^2-cyano-N^3-(3'-deoxythymidin-3'-yl)guanidine (**6d**)

The protected dimer **5d** (540 mg, 0.64 mmol) was taken up in 10 mL of a 50% solution of acetic acid in water. The solution was evaporated to dryness under reduced pressure. The residue was taken up in 30 mL water and extracted 3 times with diethylether. The aqueous layer, containing the deprotected dimer was evaporated giving 310 mg of the title compound (0.57 mmol, 90% yield). An analytical sample was obtained by reversed phase chromatography, followed by lyophylization.

UV (MeOH) λ_{max} = 262 nm (ε = 19,000)

^{13}C NMR (DMSO-d_6): δ 12.2 (CH_3); 36.8 (C-2'); 37.4 (C-2'); 43.7 (N-C-5'); 51.3 (N-C-3'); 60.7 (C-5'); 71.5 (C-3'), 83.4, 84.4 and 84.9 (2 x C-1' and 2 x C-4'); 109.3 (C-5 thymine); 117.4 (C-5 adenine); 119.3 (C $\equiv$ N); 136.1 (C-6 thymine); 139.6 (C-8 adenine); 149.1 (C-4 adenine); 150.4 (C-2 thymine); 152.6 (C-2 adenine); 156.0 (C-6 adenine); 159.1 (C=N); 163.7 (C-4 thymine) ppm.

Exact mass (glycerol) calcd for $C_{22}H_{28}N_{11}O_6$ $[M + H]^+$ 542.2224, found 542.2206.

element. anal. ($C_{22}H_{27}N_{11}O_6.2H_2O.MeOH$)

Calculated : C : 45.32; H : 5.79; N : 25.27

Found : C : 44.88; H : 5.52; N : 25.04

N^1-(3'-deoxythymidin-3'-yl)-N^2-cyano-N^3-(3'-fluoro-3',5'-dideoxythymidin-5'-yl)guanidine (**6e**)

To a solution of 148 mg (0.53 mmol) of **1e** and 180 mg (0.53 mmol) of **4** in a mixture of 10 mL DMF and 10 mL TEA, 100 mg (0.60 mmol) of silver nitrate was added. The reaction was protected from light and stirred at room temperature for 24 hours. The reaction mixture was evaporated, adsorbed on celite and chromatographically purified on silica gel, yielding 220 mg (0.41 mmol, 78%). The product was precipitated from a mixture of ethanol-diethyl ether.

UV (MeOH) λ_{max} = 265 nm (ε = 18,800)

^{13}C NMR (DMSO-d_6) : δ 12.1 and 12.3 (2 x CH_3); 35.6 (d, $^2J_{2',F}$ = 21.9 Hz, C-2'); 37.3 (C-2'); 42.4 (N-C-5'); 51.3 (N-C-3'); 60.7 (C-5'), 82.2 (d, $^2J_{4',F}$ = 24.4 Hz, C-4'); 83.4 (C-4'); 84.2 (2 x C-1'); 94.1 (d, $^1J_{3',F}$ = 175.7 Hz, C-3'); 109.4 and 110.0 (2 x C-5); 136.1 (2 x C-6); 150.5 (2 x C-2); 159.2 (C=N); 163.6 (2 x C-4) ppm.

Exact mass (glycerol) calcd for $C_{22}H_{28}N_8O_7F$ $[M + H]^+$ 535.2065, found 535.2048.

element. anal. ($C_{22}H_{27}N_8O_7.2^1/_2\ H_2O$)

Calculated : C : 45.58; H : 5.57; N : 19.34

Found : C : 45.55; H : 5.37; N : 19.19

Dimethoxytritylation of the dimer (7)

Product **6a**, 530 mg (1 mmol) was dissolved in pyridine and 410 mg (1.2 mmol) of dimethoxytritylchloride was added. The reaction was stirred for 3 days at room temperature (TLC evaluation on silica gel; mobile phase CH_2Cl_2-MeOH 9:1). The reaction mixture was concentrated, taken up in ethyl acetate and washed with a saturated bicarbonate solution. The organic layer was dried on anhydrous sodium sulphate and evaporated to dryness. This gave 760 mg of the 5'-O-dimethoxytritylated dimer 7 (90% yield). U.V. (MeOH) λ_{max} = 265 nm (ε = 17,600)

Preparation of the amidite building block (8)

A mixture of 510 mg (0.613 mmol) of the 5'-O-protected dimer 7, 3 equivalents of dry N,N-diisopropylethylamine and 1.5 equivalents of 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite in 2.5 mL of dry CH_2Cl_2 was stirred at room temperature for 2 hours. After addition of 0.5 mL of ethanol and further stirring for 25 min., the mixture was washed with 5% $NaHCO_3$ solution (15 mL) and saturated NaCl solution (twice 15 mL), dried and evaporated. Column chromatography with n-hexane-acetone-Et_3N as eluent afforded the amidite. The obtained product was dissolved in 1 ml of dry CH_2Cl_2 and added dropwise to 100 mL of cold (-40°C) n-hexane. The precipitate was isolated, washed with n-hexane, dried and used as such for DNA synthesis.

Exact mass (*m*-nitrobenzyl alcohol doped with CH_3COONa) calcd for $C_{52}H_{63}N_{10}O_{11}PNa$ $[M + Na]^+$ 1057.4318, found 1057.4332.

Synthesis of the oligodeoxynucleotides

Oligonucleotide synthesis was performed on an ABI 381 A DNA synthesizer (Applied Biosystems) using the phosphoramidite approach.

The obtained sequences were deprotected and cleaved from the solid support by treatment with concentrated ammonia at room temperature for 4 h.

After a first purification on a NAP-10^R column (Sephadex G25-DNA grade, Pharmacia), eluted with buffer A, a final purification was done on a Mono-Q^R HR 10/10 anion exchange column (Pharmacia) with the following gradient system (A = 10 mM NaOH, pH = 12.0, 0.1 M NaCl; B = 10 mM NaOH, pH = 12.0, 0.9 M NaCl; gradient used depended on the oligo; flow rate 2 mL/min).

The low pressure liquid chromatography system consisted of a Merck-Hitachi L6200 A Intelligent pomp, a Mono-Q^R HR 10/10 column, an Uvicord SII 2138 UV detector (Pharmacia - LKB) and a recorder. The eluent was desalted on a NAP-10^R column and lyophilized.

Enzymatic degradation

To a solution of 0.5 OD of the oligonucleotide TT(TT)TT in 150 µl of the following buffer : 70 mM Tris HCl pH = 8.6, 70 mM NaCl, 9 mM $MgCl_2$, 3U snake venom PDE were added.

After reaction at 37°C for 15 h, 3U of calf intestinal alkaline phosphatase were added and the mixture was kept at 37°C for a further 4 h.

HPLC-analysis of the obtained nucleoside mixture was done on a PLRP-S reversed phase column (250 x 4.6 mm). The mobile phase consisted of solution A : 5% methanol in 0.1 M TEA.HOAc pH 7.0 and

solution B 75% MeOH in 0.1 M TEA.HOAc pH 7.0, with a gradient from 30 to 60 % B within 30 minutes at 0.75 mL/min. Detection was done with a UV detector at 254 nm.

After injection of the sample, only 2 peaks could be detected, one with a retention time of 24 min and one with a retention time of 6.5 min.

In a second experiment an aliquot of the dimer and of thymidine were co-injected with the degradation mixture. Also here only 2 peaks could be detected with the same retention time as in the previous chromatogram.

Melting temperatures

Oligomers were dissolved in the following buffer : 0.1 M NaCl, 0.02 M potassium phosphate pH = 7.5, 0.1 mM EDTA. The concentration was determined by measuring the absorbance at 260 nm at 80°C and assuming the following coefficients in the denaturated state : T = 8,500, dA = 15,000 and (TT) = 14,600. The concentration in all experiments was 4 μM of each strand.

Melting curves were determined with a Uvikon 940 Spectrophotometer. Cuvettes were thermostated 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 done automatically with an IBM/PC AT compatible computer. The samples were heated and cooled at a rate of 0.2 °C/min. Melting curves were evaluated according to a simple bimolecular All or None mechanism. Theoretical melting curves according to this mechanism were fitted to the data with VAO5A, a non linear least squares algorithm taken from the Harwell Subroutine Library. Variability of the Tm of the same mixture was less than 0.5°C.

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