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Synthesis of Thymidine Dimers Containing Piperazine in the Internucleoside Linkage and their Incorporation into Oligodeoxynucleotides

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Abstract: The synthesis of thymidine dimers in which the natural phosphodiester linkage has been replaced by piperazine (3'-(N(CH_2CH_2)$_2$N)-5', **9** and 3'-(N(CH_2CH_2)$_2$N)-CO-4', **10**) are described. These new dimers were incorporated into oligodeoxynucleotides on an automated DNA-synthesizer using the phosphoramidite approach. The thermal stability of DNA/DNA duplexes and the enzymatic stability was studied by UV experiments. 17-Mers with **9** incorporated once or twice in the middle exhibited a pronounced decrease in thermal stability (ΔT_m~ -11 °C per modification) while 17-mers with **10** incorporated once or twice in the middle exhibited only a slight decrease in thermal stability (ΔT_m~ -2 °C per modification) when compared to unmodified 17-mers. Furthermore, end-modified oligodeoxynucleotides containing either **9** or **10** displayed five to six fold increased stability towards snake venom phosphodiesterase.

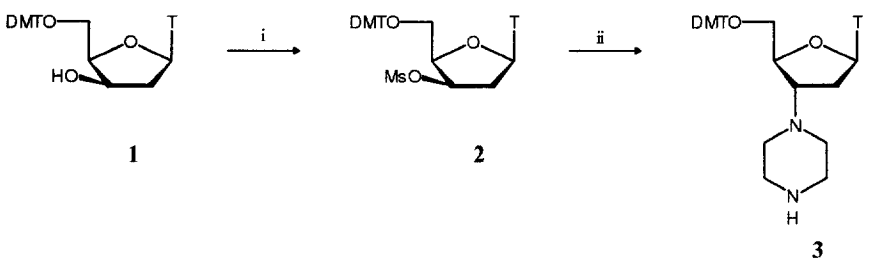
INTRODUCTION

The rational drug design approach offered by antisense oligodeoxynucleotides specifically designed to bind to complementary RNA/DNA, has stimulated into increased activity in this research area in recent years. Naturally occurring oligonucleotides are unsuitable for this purpose as they do not display the necessary stability towards destructive nucleases and ability to penetrate cell membranes. To overcome these problems, oligodeoxynucleotides need to be chemically modified in the carbohydrate moiety, the nucleobase or the internucleoside linkage.[1] Several analogues containing modifications in the phosphodiester linkage have been prepared (e.g. phosphorothioates,[2] phosphoramidates,[3] and methylphosphonates[4]), but while resistance towards nucleases often has been acheived, the chirality at phosphorus leads to heterogenous oligodeoxynucleotides, ultimately lowering the affinity for the target. A second class of linkage modified congeners replace the phosphodiester linkage with achiral, neutral moieties. The nonionic character of these facilitate cell membrane penetration and nuclease stability but in most cases a lowered affinity towards the target sequence compared to the phosphodiester reference has been obtained. This class include sulfonate,[5] sulfone,[6] sulfide,[7] formacetal,[8]

carbamate,[9] amide,[10] and amine[11] linkages. Here we wish to report the synthesis of two novel piperazino-linked thymidine dimers **7** and **8** and their incorporation into oligodeoxynucleotide sequences on which hybridization and enzymatic stability experiments were performed.

RESULTS AND DISCUSSION

We decided to synthesize the two T*T dimers by introduction of piperazine at 3'-*C* of 5'-*O*-(4,4'-dimethoxytrityl)thymidine to give derivative **3** followed by coupling with either the 5'-aldehyde or the 5'-carboxylic acid derivative of thymidine. Introduction of a methanesulfonyl group at 3'-*O* of 5'-*O*-(4,4'-dimethoxytrityl)thymidine[12] was accomplished using methanesulfonylchloride in dry pyridine.[13] Inversion of the configuration around 3'-C was achieved using NaOH in aqueous ethanol according to the procedure described by Fox and Miller[14] giving the *threo*-configurated nucleoside **1** in 96 % yield. The 3'-*O*-methanesulfonyl derivative **2** was obtained in 97 % yield by treating **1** with methanesulfonylchloride in dry pyridine. 3'-Deoxy-5'-*O*-(4,4'-dimethoxytrityl)-3'-piperazinothymidine (**3**) was synthesized in 30 % yield by refluxing **2** with piperazine (Scheme 1). The 5'-aldehyde **4**[15] and 5'-carboxylic acid **5**[16] were prepared from thymidine as previously reported.

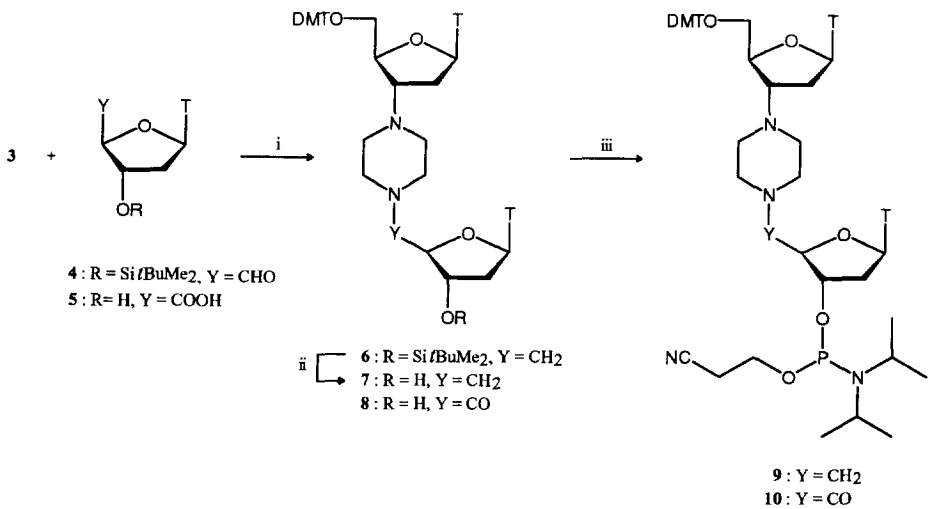


1 **2** **3**

i) MsCl, pyridine; ii) Piperazine, pyridine. DMT = 4,4'-dimethoxytrityl, Ms = methanesulfonyl, T = thymin-1-yl

Scheme 1

The dimer **6** was synthesized in 33 % yield by reductive amination[17,18] of **4** with **3** using sodium cyanoborohydride and titanium tetraisopropoxide in toluene. Desilylation was accomplished by reacting **6** with tetrabutylammoniumfluoride (TBAF) in THF affording **7** in 46 % yield. The amide linkage in the dimer **8** was formed by dicyclohexylcarbodiimide (DCC)/*N*-hydroxysuccinimide (NHS) activation[19] of **5** followed by addition of 3'-piperazino nucleoside **3** to give **8** in 60 % yield. The two dinucleoside phosphoramidites **9** and **10** to be used for preparation of the modified oligodeoxynucleotides were obtained by reacting **7** and **8** with 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite ($NCCH_2CH_2OP(Cl)N{i}Pr_2$) in the presence of *N*,*N*-diisopropylethylamine[20,21] thus affording **9** and **10** in yields of 84 % and 82 %, respectively, after precipitation from petroleum ether (Scheme 2).



i) for **4**: 1) $Ti(OiPr)_4$, toluene, 3A sieves, 2) $NaBH_3CN$, ethanol; for **5**: NHS, DCC, DMF; ii) TBAF, THF; iii) $NCCH_2CH_2OP(Cl)NiPr_2$, $EtNiPr_2$, CH_2Cl_2. DMT = 4,4'-dimethoxytrityl, T = thymin-1-yl

Scheme 2

Structural assignments of nucleosides **1**, **2**, and **3** were done by comparison of ^1H NMR and ^{13}C NMR data with data for similar compounds.[22,23,24] The structures of the thymine dimers **6**, **7** and **8** were confirmed by FAB-MS, ^1H NMR, ^{13}C NMR and ^1H-^1H COSY experiments. Judged from the ^1H NMR experiments, **8** exists as only one rotamer. The structure of **9** and **10** were confirmed by ^1H NMR and ^{31}P NMR experiments.

The phosphoramidites **9** and **10** were incorporated into different oligodeoxynucleotide sequences (Table 1) on an automated solid phase DNA synthesizer using standard phosphoramidite methodology as described in the experimental part. The composition of the oligodeoxynucleotides with the sequence **A** were verified by matrix assisted laser desorption mass spectrometry[25,26] as follows: Oligomer **A** containing the dinucleotide derived from **9** gave a relative molecular mass of 5024.6 Da (calc. 5023.5 Da) and the oligomer **A** containing the dinucleotide derived from **10** gave a relative molecular mass of 5040.1 Da (calc. 5038.4 Da). Because of the homogeneous results from the syntheses of all the modified oligodeoxynucleotides we consider this as a verification of the composition of **A**, **B**, **C** and **D**.

The thermal stability of the duplexes formed by the modified oligodeoxynucleotides and their DNA complements were determined by melting experiments as previously described.[26] It is evident (Table 1), that incorporation of **9** once or twice in the middle of an oligodeoxynucleotide results in a considerable lowering of the melting temperature T_m (approximately 11 °C per modification). Contrary to this, incorporation of the amide **10** once or twice in the middle causes only a slight decrease in T_m (approximately 2 °C per modification). This pronounced effect on the thermal stability upon changing the hybridization of 5'-C from sp^3 to sp^2 might be due to a combination of the increased rigidity of the 5'-C-N bond and the overall shortening of

the linkage. Molecular mechanics investigations have been initiated to shed light onto this aspect. As expected, incorporation of the modified dimers **9** and **10** at the ends do not result in large decreases in T_m values. The hyperchromicities of all the mid-modified oligodeoxynucleotides deviate to some extent from the wild type oligodeoxynucleotides indicating, especially for the amine modified sequences **A** and **B**, a distortion of the secondary structure of the DNA duplexes formed compared to the wild type duplexes. However, in all hybridization experiments sharp melting transitions were detected.

Table 1. Oligodeoxynucleotides Synthesized and Melting Experiments

Sequence (5'→3')	T_m (°C) / h_T	ΔT_m (°C)		h_T	
	w.t.	**9**	**10**	**9**	**10**
A CACCAACT*TCTTCCACA	64/1.34	-10.8	-1.6	1.25	1.28
B CACCAACT*TCT*TCCACA	64/1.34	-11.1	-2.0	1.23	1.26
C TTAACTTCTTCACAT*TC	55/1.34	-2.8	-2.4	1.33	1.34
D T*TAACTTCTTCACAT*TC	55/1.34	-2.0	-1.6	1.31	1.30

T*T = modified thymidine dimer, w.t. = wild type DNA, ΔT_m = decrease in T_m per modification, h_T = thermal hyperchromicity

The fact that 3'-phoshpodiesterases play a predominant role in the *in vivo* degradation of natural oligodeoxynucleotides[27] prompted us to study the enzymatic stability of the modified oligodeoxynucleotides towards the 3'-exonuclease snake venom phosphodiesterase (SV PDE). The results are shown in Table 2. Incorporation of **9** or **10** in the ends induces an increase in the stability towards SV PDE by a factor of 5 to 6, while incorporation in the middle apparently leads to rapid degradation of the 17-mer to give, probably, a stabilized 9-mer (one modification) or 12-mer (two modifications). These observations correspond to previously reported results supported by denaturing gel analyses.[28]

Table 2. Enzymatic Stability of the Modified Oligodeoxynucleotides

Sequence (5'→3')	$t_{1/2}$ (s)		
	w.t.	**9**	**10**
A CACCAACT*TCTTCCACA	60	60	60
B CACCAACT*TCT*TCCACA	60	60	60
C TTAACTTCTTCACAT*TC	60	540	450
D T*TAACTTCTTCACAT*TC	60	540	450

T*T = modified thymidine dimer, w.t. = wild type DNA, $t_{1/2}$ = hyperchromicity half-life

In conclusion, synthesis of phosphoramidite building blocks of the novel piperazino-linked nucleoside dimers **9** and **10** has been accomplished. Oligodeoxynucleotides containing amine **9** exhibit significantly lowered ability to hybridize to a complementary DNA sequence compared to unmodified analogues and are thus probably of no use in antisense oligodeoxynucleotides. On the contrary, incorporation of amide **10** into oligodeoxynucleotides only slightly weakens the duplex stability while considerably increasing the stability towards 3'-exonucleolytic degradation. Hence, this nonionic linkage opens new possibilities for increasing the *in vivo* stability of antisense oligodeoxynucleotides, and it may prove valuable as a lead structure for design of conformationally restricted phosphodiester substitutes capable of hybridizing with high affinity to target nucleic acids.

EXPERIMENTAL

NMR spectra were recorded on a Bruker AC 250 FT NMR spectrometer at 250 MHz for ^{1}H NMR, 62.5 MHz for ^{13}C NMR and 101.3 MHz for ^{31}P NMR. δ-Values are in ppm relative to tetramethylsilane as internal standard (^{1}H NMR and ^{13}C NMR) and relative to 85 % H_3PO_4 as external standard (^{31}P NMR). In the assignments of the spectra for the dimers the following designations are used: ′ ′ ′ (^{1}H NMR) and ′ a ′ (^{13}C NMR) denotes the 5'-nucleoside; ′ " ′ (^{1}H NMR) and ′ b ′ (^{13}C NMR) denotes the 3'-nucleoside. Positive FAB mass spectra were recorded on a Kratos MS 50 RF spectrometer using 8-9 kV xenon atoms.. Merck silica gel 230-400 Mesh was used for column chromatography. Snake venom phosphodiesterase (*Crotalus adamanteus*) was obtained from Pharmacia. Matrix assisted laser desorption mass spectra were obtained on a prototype laser desorption mass spectrometer from Applied Biosystem Sweeden AB, Uppsala, Sweeden.

1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-β-D-*threo*-pentofuranosyl]thymine (1)

5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-methanesulfonylthymidine[13] (9.90 g, 14.30 mmol) was dissolved in ethanol (200 mL) containing 1 M NaOH (57 mL). Water (200 mL) was added and the solution refluxed for 2.5 h. The solution was concentrated under reduced pressure until precipitation commenced (approximate volume 200 mL). It was cooled by addition of ice and allowed to stand at 5 °C overnight. The white precipitate was filtered off, washed with ice-cold water and dried *in vacuo* over P_2O_5 giving 7.52 g (96 %) of **1**. ^{1}H NMR and ^{13}C NMR data were in agreement with previously reported data.[22]

1-[2-Deoxy-5-*O*-(4,4'-dimethoxytrityl)-3-*O*-methanesulfonyl-β-D-*threo*-pentofuranosyl]thymine (2)

Nucleoside **1** (3.5 g, 6.4 mmol) was dissolved in dry pyridine (50 mL) and cooled to 0 °C. Methanesulfonyl-chloride (1.57 mL, 19.2 mmol) was added dropwise and the mixture stirred at 0 °C for 2 h. The temperature was allowed to rise to r.t. and stirring was continued for additional 2 h. The solution was cooled to 0 °C and water (4 mL) was carefully added. Stirring was continued for 30 min and the mixture was poured slowly into a vigorously stirred ice/water mixture (600 mL). The precipitate was filtered off and washed with ice-cold

water and dried *in vacuo* over P_2O_5 yielding 3.87 g (97 %) of **2** which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$) δ: 1.81 (s, 3H, CH_3), 2.46 (dd, 1H, J = 3.0 Hz and 17.2 Hz, H-2'β), 2.74-2.84 (m, 4H, H-2'α, SCH_3), 3.33-3.40 (m, 1H, H-5'a), 3.60-3.67 (m, 1H, H-5'b), 3.79 (s, 6H, 2 x OCH_3), 4.18 (m, 1H, H-4'), 5.26-5.29 (m, 1H, H-3'), 6.27 (dd, 1H, J = 3.3 Hz and 7.9 Hz, H-1'), 6.84 (d, 4H, J = 8.7 Hz, Ar), 7.15-7.32 (m, 8H, Ar), 7.40-7.43 (m, 2H, H-6, Ar), 8.79 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 12.44 (CH_3), 38.44 (C-2'), 39.51 (SCH_3), 55.23 (OCH_3), 60.70 (C-5'), 78.61 (C-3'), 81.02 (C-1'), 83.51 (C-4'), 86.98 (C-Ar_3), 111.24 (C-5), 113.26, 127.81, 127.98, 128.08, 129.11, 129.95, 129.98 (Ar), 135.26 (C-6), 144.08 (Ar), 150.39 (C-2), 158.73 (Ar), 163.48 (C-4).

3'-Deoxy-5'-*O*-(4,4'-dimethoxytrityl)-3'-piperazinothymidine (3)

Nucleoside **2** (1.0 g, 1.61 mmol), piperazine (15 g, 180 mmol) and pyridine (10 mL) was stirred for 55 h at 150 °C. The hot mixture was poured into a saturated aqueous solution of $NaHCO_3$ (400 mL) and extracted with $CHCl_3$ (5 x 150 mL). The combined organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was purified by column chromatography (120 g gel; $CH_2Cl_2/CH_3OH/(CH_3CH_2)_3N$, 98.5:1:0.5 → 84.5:15:0.5, v/v/v) giving **3** as a foam. Yield: 291 mg (30 %). ^{1}H NMR (CD_3OD) δ: 1.31 (s, 3H, CH_3), 2.01-2.09 (m, 1H, H-2'β), 2.37-2.51 (m, 5H, H-2'α, 2 x CH_2), 2.77-2.85 (m, 4H, 2 x CH_2), 3.16 (dd, 1H, J = 7.9 Hz and 10.9 Hz, H-5'a), 3.29-3.39 (m, 2H, H-5'b, H-3'), 3.62 (s, 6H, 2 x OCH_3), 4.02 (m, 1H, H-4'), 5.99 (t, 1H, J = 6.4 Hz, H-1'), 6.70 (d, 4H, J = 8.8 Hz, Ar), 7.07-7.31 (m, 9H, Ar), 7.58 (d, 1H, J = 0.9 Hz, H-6). ^{13}C NMR (CD_3OD) δ: 12.47 (CH_3), 34.30 (C-2'), 46.22, 48.27 (CH_2-piperazino), 56.08 (OCH_3), 65.76, 66.39 (C-3', C-5'), 82.91 (C-1'), 87.06 (C-4'), 88.32 (C-Ar_3), 111.85 (C-5), 114.53, 128.41, 129.21, 129.75, 131.70, 137.21 (Ar), 137.94 (C-6), 146.33 (Ar), 152.70 (C-2), 160.65 (Ar), 166.5 (C-4). FAB MS (CH_3OH/Glycerol): 613 $(M+H)^+$.

3'-(*N*-[3-*O*-(*tert*-Butyldimethylsilyl)-1-(thymine-1-yl)-1,2,5-trideoxy-β-D-*erythro*-pentofuranos-5-yl]piperazino-3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)thymidine (6)

To a mixture of 5'-aldehyde **4**[15] (96 mg, 0.27 mmol) and **3** (150 mg, 0.24 mmol) in toluene (4 mL) containing 3A molecular sieves was added $Ti(OiPr)_4$ (0.11, 0.31 mmol) and the mixture was stirred at r.t. for 2 h. $NaBH_3CN$ (23 mg, 0.37 mmol) dissolved in absolute ethanol (4 mL) was added and stirring at r.t. was continued for 48 h. After addition of a saturated aqueous solution of $NaHCO_3$ (25 mL) the mixture was extracted with $CHCl_3$ (3 x 40 mL). The combined organic phase was washed with water (2 x 50 mL), dried (Na_2SO_4) and evaporated to dryness. The residue was purified by preparative TLC eluting four times in $CH_3OH/CH_2Cl_2/(CH_3CH_2)_3N$ (5:84.5:0.5, v/v/v) giving **6** as a white solid. Yield: 76 mg (33 %). ^{1}H NMR ($CDCl_3$) δ: 0.01 (s, 6H, 2 x CH_3Si), 0.81 (s, 9H, *t*BuSi), 1.36 (s, 3H, CH_3), 1.84 (s, 3H, CH_3), 1.96-2.02 (m, 2H, H-2'β, H-2"β), 2.18-2.24 (m, 2H, H-2'α, H-2"α), 2.47-2.51 (m, 10H, 4 x CH_2, H-5"a, H-5"b), 3.22 (m, 1H, H-3'), 3.39-3.43 (m, 2H, H-5'a, H-5'b), 3.70 (s, 6H, 2 x OCH_3), 3.85-3.88 (m, 1H, H-3"), 4.06-4.08 (m, 2H, H-4', H-4"), 6.06-6.10 (m, 2H, H-1', H-1"), 6.74 (d, 4H, J = 8.8 Hz, Ar), 7.12-7.24 (m, 8H, Ar), 7.31-7.34 (m,

2H, H-6, Ar), 7.60 (s, 1H, H-6). ^{13}C NMR ($CDCl_3$) δ: -4.82, -4.56 (2 x CH_3Si), 11.77, 12.64 (2 x CH_3), 17.88 ($(CH_3)_3\underline{C}Si$), 25.66 ($(\underline{C}H_3)_3CSi$), 33.86 (C-2'a), 40.28 (C-2'b), 49.61, 53.68 (CH_2-piperazino), 55.19 (OCH_3), 60.33 (C-5'b), 64.11, 64.34 (C-3'a, C-5'a), 73.50 (C-3'b), 81.35, 83.86, 85.23, 86.69 (C-1'a, C-1'b, C-4'a, C-4'b), 87.57 (C-Ar_3), 110.83, 110.87 (C-5a, C-5b), 113.21, 127.06, 127.90, 128.14, 130.04 (Ar), 135.41, 135.47 (C-6a, C-6b), 144.33 (Ar), 150.17, 150.40 (C-2a, C-2b), 158.67 (Ar), 163.85, 163.95 (C-4a, C-4b). FAB MS ($CHCl_3$/NBA): 951 $(M+H)^+$.

3'-Deoxy-5'-*O*-(4,4'-dimethoxytrityl)-3'-(*N*-[1-(thymine-1-yl)-1,2,5-trideoxy-β-D-*erythro*-pentofuranos-5-yl]piperazino)thymidine (7)

Dimer **6** (65 mg, 0.07 mmol) was dissolved in anhydrous THF (1.5 mL) and a 1.1 M solution of tetra-*n*-butylammoniumfluoride (TBAF) in THF (0.25 mL, 0.28 mmol) was added. Stirring was continued at r.t. for 2.5 h. The mixture was evaporated to dryness under reduced pressure and the resulting oil was purified by column chromatography (4 g gel; $CH_2Cl_2/CH_3OH/(CH_3CH_2)_3N$, 99.5:0:0.5 → 94.5:5:0.5, v/v/v) affording **7** as a white glass. Yield: 26 mg (46 %). ^{1}H NMR ($CDCl_3$) δ: 1.44 (s, 3H, CH_3), 1.90 (s, 3H, CH_3), 2.05-2.20 (m, 2H, H-2'β, H-2"β), 2.38-2.66 (m, 12H, 4 x CH_2, H-2'α, H-2"α, H-5"a, H-5"b), 3.26-3.29 (m, 1H, H-3'), 3.50-3.54 (m, 2H, H-5'a, H-5'b), 3.77 (s, 6H, 2 x OCH_3), 3.98-4.00 (m, 1H, H-3"), 4.15-4.23 (m, 2H, H-4', H-4"), 6.14-6.21 (m, 2H, H-1', H-1"), 6.82 (d, 4H, J = 8.8 Hz, Ar), 7.21-7.42 (m, 10H, H-6, Ar), 7.65 (s, 1H, H-6). ^{13}C NMR ($CDCl_3$) δ: 11.79, 12.62 (2 x CH_3), 33.56 (C-2'a), 39.63 (C-2'b), 49.84, 53.71 (CH_2-piperazino), 55.22 (OCH_3), 60.12 (C-5'b), 64.08, 64.55 (C-3'a, C-5'a), 73.52 (C-3'b), 81.17, 82.84, 84.11, 84.75 (C-1'a, C-1'b, C-4'a, C-4'b), 86.73 (C-Ar_3), 110.93, 110.99 (C-5'a, C-5'b), 113.24, 127.07, 127.92, 128.16, 130.70 (Ar), 135.45, 135.50 (C-6a, C-6b), 144.34 (Ar), 150.40, 150.54 (C-2a, C-2b), 158.69 (Ar), 163.98, 164.13 (C-4a, C-4b). FAB MS ($CHCl_3$/NBA): 837 $(M+H)^+$.

3'-Deoxy-3'-(*N*-[1,2-dideoxy-5-oxo-1-(thymine-1-yl)-β-D-*erythro*-pentofuranos-5-yl]piperazino-5'-*O*-(4,4'-dimethoxytrityl)thymidine (8)

Nucleoside **3** (100 mg, 0.16 mmol) and 5'-carboxylic acid **5**[16] (42 mg, 0.16 mmol) were dissolved in anhydrous DMF (5 mL) and cooled to -20 °C. *N*-Hydroxysuccinimide (NHS) (18 mg, 0.16 mmol) and dicyclohexylcarbodiimide (DCC) (34 mg, 0.16 mmol) was added and stirring at r.t. was continued for 72 h. The mixture was filtered and the solid washed with EtOAc (3 x 2 mL). The combined filtrate was evaporated to dryness under reduced pressure. The residue was purified by column chromatography (20 g gel; $CH_2Cl_2/CH_3OH/(CH_3CH_2)_3N$, 98.5:1:0.5 → 96.5:3:0.5, v/v/v) giving **8** as a white glass. Yield: 81 mg (60 %). ^{1}H NMR ($CDCl_3$) δ: 1.44 (s, 3H, CH_3), 1.90 (s, 3H, CH_3), 1.96-2.14 (m, 2H, H-2'β, H-2"β), 2.38-2.68 (m, 6H, 2 x CH_2, H-2'α, H-2"α), 3.25-3.28 (m, 1H, H-5'a), 3.42-3.65 (m, 5H, H-5'b, 2 x CH_2), 3.78 (s, 6H, 2 x OCH_3), 4.13 (m, 1H, H-3'), 4.61 (m, 2H, H-3", H-4'), 4.80 (m, 1H, H-4'), 6.11-6.14 (m, 1H, H-1"), 6.55-6.58 (m, 1H, H-1'), 6.82 (d, 4H, J = 8.6 Hz, Ar), 7.18-7.41 (m, 9H, Ar), 7.64 (s, 1H, H-6), 8.08 (s, 1H, H-6), 9.88 (s, 2H, 2 x NH). ^{13}C NMR ($CDCl_3$) δ: 11.86, 12.65 (2 x CH_3), 33.06, 33.13 (C-2'a, C-2'b), 50.20, 53.37 (CH_2-

piperazino), 55.24 (OCH_3), 63.95, 64.69 (C-3'a, C-5'a), 74.03 (C-3'b), 81.35, 82.98, 85.52, 85.89, 86.80 (C-1'a, C-1'b, C-4'a, C-4'b, C-Ar_3), 111.15, 111.28 (C-5a, C-5b), 113.27, 127.12, 127.96, 128.13, 130.05 (Ar), 135.39 (C-6a, C-6b), 144.28 (Ar), 150.71, 151.04 (C-2a, C-2b), 158.70 (Ar), 164.11, 164.23 (C-4a, C-4b), 168.35 (C-5'b). FAB MS ($CHCl_3$/NBA): 851 $(M+H)^+$.

3'-(*N*-[3-*O*-(2-Cyanoethoxy(diisopropylamino)phosphino)-1-(thymine-1-yl)-1,2,5-trideoxy-β-D-*erythro*-pentofuranos-5-yl]piperazino)-3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)thymidine (9)

Dimer **7** (50 mg, 0.06 mmol) was dried by coevaporation with anhydrous CH_3CN (2 mL) and dissolved under nitrogen in anhydrous CH_2Cl_2 (0.17 mL). *N*,*N*-Diisopropylethylamine (0.05 mL) was added followed by dropwise addition of 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite (0.02 mL, 0.10 mmol). After 90 min analytical TLC showed no more starting material and the reaction was quenched with CH_3OH (0.02 mL) followed by addition of EtOAc (1.3 mL) and triethylamine (0.08 mL). The mixture was washed with saturated aqueous solutions of $NaHCO_3$ (3 x 2 mL) and NaCl (3 x 2 mL). The organic phase was dried (Na_2SO_4) and the solvents removed under reduced pressure. The residue was redissolved in anhydrous toluene (0.25 mL) and precipitated from petroleum ether (60 mL) at -20 °C affording **9** as white powder. Yield: 52 mg (84 %).
^{1}H NMR ($CDCl_3$) δ: 1.17-1.43 (m, $CH(C\underline{H}_3)_2$, CH_3), 1.91 (s, CH_3), 2.03-2.21 (m, H-2'β, H-2"β), 2.56-2.78 (m, H-2'α, H-2"α, H-5"a, H-5"b, 4 x CH_2, CH_2CN), 3.26-3.29 (m, H-3'), 3.43-3.63 (m, H-5'a, H-5'b, NCH, OCH_2), 3.79 (s, OCH_3), 4.07-4.24 (m, H-3", H-4', H-4"), 6.16-6.18 (m, H-1', H-1"), 6.81-6.84 (m, Ar), 7.23-7.42 (m, H-6, Ar), 7.68 (s, H-6).
^{31}P NMR ($CDCl_3$) δ: 148.9, 149.6.

3'-(*N*-[3-*O*-(2-Cyanoethoxy(diisopropylamino)phosphino)-1,2-dideoxy-5-oxo-1-(thymine-1-yl)-β-D-*erythro*-pentofuranos-5-yl]piperazino)-3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)thymidine (10)

Dimer **8** (60 mg, 0.07 mmol) was dried by coevaporation with anhydrous CH_3CN (2 mL) and dissolved under nitrogen in anhydrous CH_2Cl_2 (0.75 mL). *N*,*N*-Diisopropylethylamine (0.1 mL) was added followed by dropwise addition of 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite (0.03 mL, 0.14 mmol). After 3 h analytical TLC showed no more starting material and the reaction was quenched with CH_3OH (0.03 mL) followed by addition of EtOAc (1.5 mL) and triethylamine (0.05 mL). The mixture was washed with saturated aqueous solutions of $NaHCO_3$ (3 x 2 mL) and NaCl (3 x 2 mL), dried (Na_2SO_4) and evaporated to dryness under reduced pressure. The residue was redissolved in anhydrous toluene (0.3 mL) and precipitated from petroleum ether (55 mL) at -45 °C affording **10** as a white powder. Yield: 61 mg (82 %).
^{31}P NMR ($CDCl_3$) δ: 149.5 (br s).

Synthesis of T*T modified oligodeoxynucleotides

The modified oligodeoxynucleotides were synthesized on a Pharmacia Gene Assembler® Special synthesizer in 0.2 μmol-scale (7.5 μmol embedded per cycle, Pharmacia primer support™) using commercially available

2-cyanoethylphosphoramidites and **9** and **10**. The syntheses followed the regular protocol of the DNA-synthesizer for 2-cyanoethylphosphoramidites except that the coupling time for the modified amidites **9** and **10** was increased from 2 to 12 minutes. The 5'-*O*-DMT-ON oligodeoxynucleotides were removed from the solid support and deprotected with concentrated aqueous ammonia at room temperature for 72 **h** and then subjected to purification on oligodeoxynucleotide purification cartridges (COP, Cruachem) which includes detritylation.

Melting experiments

Melting experiments were carried out on a Perkin-Elmer UV/VIS spectrometer fitted with a PTP-6 Peltiér temperature programming element. The absorbance at 260 nm was measured from 20 °C to 80 °C in 1 cm cuvettes. The melting temperature was determined as the maximum of the first derivative plots of the melting curve. The oligodeoxynucleotides were dissolved in a medium salt buffer (pH = 7.2, 1 mM EDTA, 10 mM Na_2HPO_4, 140 mM NaCl) to a concentration of 2.5 μM for each strand. Melting hyperchromicity values h_T were calculated as the value of the final absorbance divided by the initial absorbance.

Enzymatic stability of the modified oligodeoxynucleotides

A solution of the oligodeoxynucleotides (0.2-0.3 OD) in 2.0 mL of the following buffer (0.1 M Tris·HCl, pH = 8.6, 0.1 M NaCl, 14 mM $MgCl_2$) was degraded with 1.2 U snake venom phosphodiesterase (34 μL of a solution of the enzyme in the following buffer (5mM Tris·HCl, pH = 7.5, 50 % glycerol (v/v)) at 25 °C. During degradation the increase in absorption at 260 nm was measured and the half-life ($t_{1/2}$) of the full-lenght oligomer estimated.

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REFERENCES

1. (a) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 6123. (b) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543. (c) Tidd, D. M. *Br. J. Cancer* **1991**, *63*, 6.
2. Stein, C. A.; Subasinghe, C.; Shinozuka, K.; Cohen, J. S. *Nucleic Acids Res.* **1988**, *16*, 3209.
3. Uznánski, B.; Wilk, A.; Stec, W. J. *Tetrahedron Lett.* **1987**, *28*, 3401.

4. (a) Löschner, T.; Engels, J. W. *Nucleosides Nucleotides* **1988**, *7*, 729. (b) Miller, P. S.; Dreon, N.; Pulford, S. M.; McParland, K. B. *J. Biol. Chem.* **1980**, *255*, 9659.

5. (a) Reynolds, R. C.; Crooks, P. A.; Maddry, J. A.; Akhtar, M. S.; Montgomery, J. A.; Secrist, J. A., III *J. Org. Chem.* **1992**, *57*, 2983. (b) Musicki, B.; Widlanski, T. S. *Tetrahedron Lett.* **1991**, *32*, 1267.

6. Huang, K. Z.; Schneider, K. C.; Benner, S. A. *J. Org. Chem.* **1991**, *56*, 3869.

7. Kawai, S. H.; Wang, D.; Giannaris, P. A.; Damha, M. J.; Just, G. *Nucleic Acids Res.* **1993**, *21*, 1473.

8. (a) Quaedflieg, P. J. L. M.; Timmers, C. M.; van der Marel, G. A.; Kuyl-Yeheskiely, E.; van Boom, J. H. *Synthesis* **1993**, 627. (b) Jones, R. J.; Lin, K.-Y.; Milligan, J. F.; Wadwani, S.; Matteucci, M. D. *J. Org. Chem.* **1993**, *58*, 2983. (c) Matteucci, M. *Tetrahedron Lett.* **1990**, *31*, 2385.

9. (a) Kutterer, K. M. K.; Just, G. *Biomed. Chem. Lett.* **1994**, *4*, 435. (b) Waldner, A.; De Mesmaeker, A.; Lebreton, J. *Biomed. Chem. Lett.* **1994**, *4*, 405.

10. (a) De Mesmaeker, A.; Lebreton, J.; Waldner, A.; Fritsch, V.; Wolf, R. M.; Freier, S. M. *Synlett* **1993**, 733. (b) Lebreton, J.; De Mesmaeker, A.; Waldner, A.; Fritsch, V.; Wolf, R. M.; Freier, S. M. *Tetrahedron Lett.* **1993**, *34*, 6383. (c) Idziak, I.; Just, G.; Damha, M. J.; Giannaris, P. A. *Tetrahedron Lett.* **1993**, *34*, 5417. (d) Chur, A.; Holst, B.; Dahl, O.; Valentin-Hansen, P.; Pedersen, E. B. *Nucleic Acids Res.* **1993**, *21*, 5179.

11. (a) Saha, A. K.; Schairer, W.; Waychunas, C.; Prasad, C. V. C.; Sardaro, M.; Upson, D. A.; Kruse, L. I. *Tetrahedron Lett.* **1993**, *34*, 6017. (b) De Mesmaeker, A.; Waldner, A.; Sanghvi, Y. S.; Lebreton, J. *Biomed. Chem. Lett.* **1994**, *4*, 395.

12. Schaller, H.; Weimann, G.; Lerch, B.; Khorana, H. G. *J. Am. Chem. Soc.* **1963**, *85*, 3821.

13. Zuckermann, R.; Corey, D.; Schultz, P. *Nucleic Acids Res.* **1987**, *15*, 5305.

14. Fox, J. J.; Miller, N. C. *J. Org. Chem.* **1963**, *28*, 936.

15. Weis, A. L.; Hausheer, F. H.; Chaturvedula, P. V. C.; Delecki, D. J.; Cavanaugh, P. F., Jr.; Moskwa, P. S.; Oakes, F. T. *Compounds and Methods for Inhibiting Gene Expression*, International Patent WO 92/02534 **1992**, CA 117:105700.

16. Moss, G. P.; Reese, C. B.; Schofield, K.; Shapiro, R.; Todd, Lord *J. Chem. Soc.* **1963**, 1149.

17. Matteucci, M.; Jones, R. J.; Munger, J. *Modified Internucleoside Linkages*, International Patent WO92/05186 **1992**, CA 119:139699.

18. Borch, R. F.; Bernstein, M. D.; Durst, H. D. *J. Am. Chem. Soc.* **1971**, *93*, 2897.

19. Wengel, J.; Motawia, M. S.; Pedersen, E. B.; Nielsen, C. M. *J. Heterocycl. Chem.* **1992**, *29*, 5.

20. McBride, L. J.; Caruthers, M. H. *Tetrahedron Lett.* **1983**, *24*, 245.

21. Sinha, N. D.; Biernat, J.; Köster, H. *Tetrahedron Lett.* **1983**, *24*, 5843.

22. Rosemeyer, H.; Seela, F. *Helv. Chim. Acta* **1991**, *74*, 748.

23. Celewicz, L.; Urjasz, W.; Golankiewicz, K. *Nucleosides Nucleotides* **1993**, *12*, 951.

24. Petersen, G. V.; Platz, J.; Wengel, J.; Nielsen, C. M. *Synthesis* **1994**, 823.

25. Pieles, U.; Zürcher, W.; Schär, M.; Moser, H. E. *Nulceic Acids Res.* **1993**, *21*, 3191.

26. Svendsen, M. L.; Wengel, J.; Dahl, O.; Kirpekar, F.; Roepstorff, P. *Tetrahedron* **1993**, *49*, 11341.

27. Tidd, D. M.; Warenius, H. M. *Br. J. Cancer*, **1989**, *60*, 343.

28. Nielsen, P.; Kirpekar, F.; Wengel, J. *Nucleic Acids Res.* **1994**, *22*, 703.

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