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Synthesis and physicochemical properties of decanucleotides containing (3′ → 5′)-O-CH_2-O-linkages at predetermined positions

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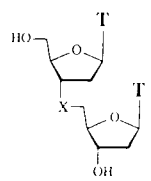
Abstract. Synthesis of the modified decadeoxynucleotides **16–20** having the sequence d($G^1pC^2pG^3pT^4x_1T^5x_2T^6x_3T^7pG^8pC^9pG^{10}$) and containing one (x2 = CH_2, x1 = x3 = p, *i.e.*, **16**), two (x1 = x3 = CH_2, x2 = p, *i.e.*, **17**, x2 = x3 = CH_2, x1 = p, *i.e.*, **18** and x1 = x2 = CH_2, x3 = p, *i.e.*, **19**) or three (x1 = x2 = x3 = CH_2, *i.e.*, **20**) -O-CH_2-O- linkages could be accomplished using the respective mono-, di- or tri(-O-CH_2-O) 3′-O-phosphoramidites **6**, **10** and **14** as the incoming synthons. Hybridization of **16–20** with their native complementary strand afforded stable duplexes, which were studied by 1D- and 2D-^{1}H-NMR and UV-hyperchromicity techniques.

Introduction *

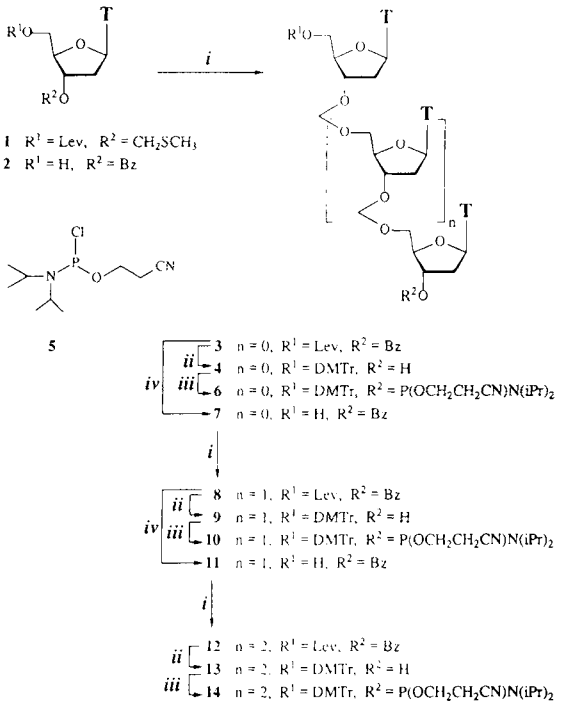
Inhibition of gene expression by antisense oligonucleotides has gained increasing attention as a promising drug design concept[1–5]. The inhibitory effect may be ascribed to the specific binding of antisense molecules to messenger RNAs (sense molecules) as DNA · RNA complexes, thus blocking the translation process. The latter phenomenon suggests a potential application of DNA fragments in the treatment of viral infections, many cancers and most bacterial and parasitic maladies. In order to improve cell membrane permeability and nuclease resistance much effort has been devoted to the development of antisense DNA bearing charged (*i.e.*, **1a.b**) or non-charged (*i.e.*, **1c–e**) modified phosphate linkages[1]. However, all the alterations, except the -O-P(S)(S$^-$)-O- one (*i.e.*, **1b**), introduce additional stereoisomerism, the effect of which on the thermal stability of the proposed DNA · RNA duplexes is still a subject of debate. Several other approaches[1] comprise the replacement of the (3′ → 5′) internucleosidic phosphodiester by a neutral and achiral "dephospho" linkage (*i.e.*, **1f–i**). The -O-CO-O- (*i.e.*, **1f**), -O-CH_2-CO-O- (*i.e.*, **1g**) and -O-CH_2-CO-NH- (*i.e.*, **1h**) linkages possess the serious drawback of intrinsic base-lability. On the other hand, the -O-Si(iPr)$_2$-O- (*i.e.*, **1i**) modification embodies a bulky and highly lipophilic center which poses an obstacle to duplex formation.



1a	X = O-P(O)(S$^-$)-O	**1i**	X = O-Si(iPr)$_2$-O
1b	X = O-P(S)(S$^-$)-O	**1k**	X = CH_2-S-CH_2
1c	X = O-P(O)Me-O	**1l**	X = CH_2-SO-CH_2
1d	X = O-P(O)(O-Alkyl)-O	**1m**	X = CH_2-SO_2-CH_2
1e	X = O-P(O)N(Alkyl)$_2$-O	**1n**	X = O-SO_2-CH_2
1f	X = O-CO-O	**1o**	X = CH_2-N(CH_3)-O
1g	X = O-CH_2-CO-O	**1p**	X = O-CH_2-O
1h	X = O-CH_2-CO-NH		

Recently, other promising types of achiral "dephospho" analogs (*i.e.*, **1k–p**) have been described. Thus, dimers containing a -CH_2-S-CH_2-[6] (*i.e.*, **1k**), -CH_2-SO-CH_2-[6] (*i.e.*, **1l**), -CH_2-SO_2-CH_2-[6] (*i.e.*, **1m**), -O-SO_2-CH_2-[7] (*i.e.*, **1n**) or -CH_2NMe-O- (*i.e.*, **1o**) were prepared; only in the case of **1o** was it established that the modified oligomers hybridized effectively to their complementary RNAs[8]. In addition, we presented[9,12] the synthesis of several (3′ → 5′)-O-CH_2-O- (*i.e.*, **1p**) linked dinucleosides containing the nucleobases T, C and G. Preliminary experiments indicated that the presence of one -O-CH_2-O- linkage in a decanucleotide (*i.e.*, **16**) did not exert a dramatic effect on the hybridization to the native complementary sequence. Later on, *Matteucci* showed[13] that a tetradecamer DNA strand containing four -O-CH_2-O- linkages effectively hybridized to its complementary native RNA oligomer. Furthermore, the modified 14-mer proved to be suitable for sequence-specific triple helix formation[14]. These promising observations stimulated us to investigate in more detail the effect of one or more -O-CH_2-O- linkages in an oligonucleotide on the structure and thermal stability of the hybrid with its native complementary strand. We here report the synthesis of five -O-CH_2-O-containing decanucleotides **16–20**, which hybridized effectively with their native complementary strand d(CpGpCpApApApCpGpC), to afford the corresponding duplexes **D1**, **D2**, **D2a**, **D2b** and **D3**. The stability and conforma-

* **Abbreviations**: DIPEA = diisopropylethylamine. DMTr = 4,4′-dimethoxytrityl. Lev = levulinoyl = 1,4-dioxopentyl. NIS = N-iodosuccinimide. TfOH = trifluoromethanesulfonic acid. TLC = thin-layer chromatography. TMA = tetramethylammonium bromide. TMS = tetramethyl silane. TOCSY = total correlation spectroscopy.



Reagents: (i) NIS/*cat.* TfOH; (ii) aq. NH_3; DMTr-Cl/pyridine; (iii) **5**/DIPEA/1,2-dichloroethane; (iv) NH_2NH_2/CH_3COOH/pyridine.

Scheme 1.

tional properties of the resulting hybrids and the non-altered duplex **D0** were determined by NMR and UV spectroscopy.

Decamer [a]		Duplex
d(GpCpGpTpTpTpTpGpCpG)	**15**	**D0**
d(GpCpGpTpT^TpTpGpCpG)	**16**	**D1**
d(GpCpGpT^TpT^TpGpCpG)	**17**	**D2**
d(GpCpGpTpT^T^TpGpCpG)	**18**	**D2a**
d(GpCpGpT^T^TpTpGpCpG)	**19**	**D2b**
d(GpCpGpT^T^T^TpGpCpG)	**20**	**D3**

[a] p and ^ represent a -O-P(O)(O^-)-O- and -O-CH_2-O- linkage, respectively.

Results and discussion

The preparation of the mono-, di- and tri(-O-CH_2-O) phosphoramidites **6**, **10** and **14** required for the assembly of decamers **16–20**, is illustrated in Scheme 1. Thus, the donor 5′-*O*-levulinoyl-3′-*O*-(methylthiomethyl)thymidine[9] (**1**) was condensed with the acceptor 3′-*O*-benzoylthymidine[15] (**2**) using NIS and catalytic TfOH[9–11] as the promoter, to give the (3′ → 5′) -O-CH_2-O-linked dimer **3**. Ammonolysis of the levulinoyl and benzoyl protective groups from **3**, followed by tritylation (DMTr-Cl/pyridine) of the free 5′-hydroxyl function afforded homogenous **4**. Phosphitylation of **4** with (2-cyanoethoxy)(diisopropylamino)chlorophosphine[16] (**5**) in the presence of DIPEA furnished the desired dimer phosphoramidite **6**.

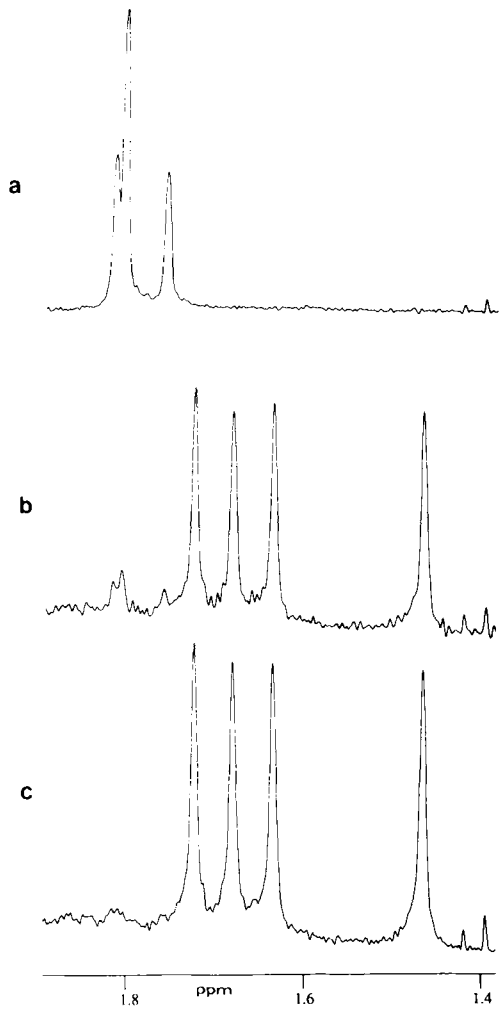


*Figure 1. Thymidine 5-methyl signals in the 300-MHz ^{1}H-NMR spectrum measured at 20°C, of: (a) **16**; (b) **16**, ~0.9 equiv. of d(CpGpCpApApApApCpGpC) added; (c) **16**, 1.0 equiv. of d(CpGpCpApApApApCpGpC) added.*

Elongation of dimer **3** to give trimer **8** was readily accomplished as follows. Selective removal (NH_2NH_2/pyridine/acetic acid)[17] of the levulinoyl group from **3** resulted in the isolation of **7**. Coupling of the latter with donor **1** in the presence of NIS/*cat.*-TfOH furnished **8**. Complete deblocking of **8** by ammonolysis, followed by tritylation of the 5′-terminus gave homogeneous **9**. Subsequent phosphitylation of **9** with **5** yielded the desired trimer-phosphoramidite derivative **10**. The conversion of trimer **8** into tetrameric phosphoramidite **14** was executed by the same consecutive four steps (*i.e.*, iv, i, ii, and iii) as described above for the preparation of the trimeric phosphoramidite derivative **10**.

The accessibility of phosphoramidites **6**, **10** and **14** enabled us to assemble the modified decamers **16–20** on 10-μmol scale by a conventional phosphoramidite protocol[18] using an automated DNA synthesizer (Gene Assembler, Pharmacia). In this respect, it is of interest to note

*Table I. Conditions used in the elongation steps of the solid-phase synthesis of **16 – 20**.*

Phosphoramidite	Equiv. [a]	1*H*-Tetrazole (equiv.) [a]	Coupling time [b]	Efficiency
6	4.0 [c]	30.0 [c]	3.0 [c]	> 99%
10	5.0	37.5	10.0	> 99%
14	5.0	37.5	10.0	> 99%

[a] Relative to immobilized oligonucleotide. [b] In min. [c] As used in the standard protocol for monomeric phosphoramidites[18].

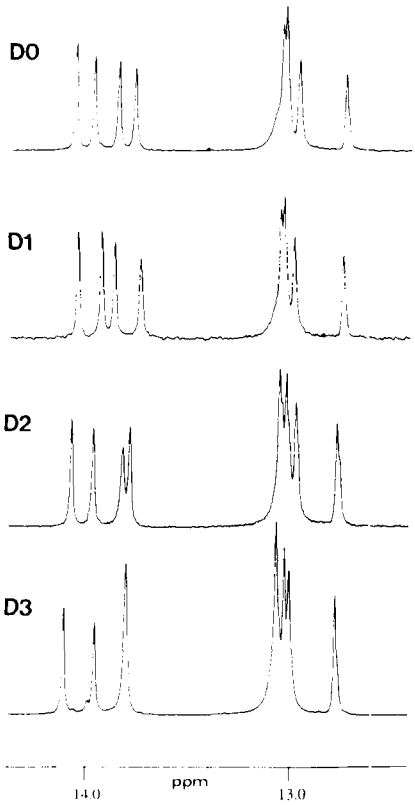


Figure 2. Imino-proton region of the 600-MHz ^{1}H-NMR spectrum measured at 20°C of **D0**, **D1**, **D2** *and* **D3**, *recorded in D_2O/H_2O (9:1, v/v).*

that the elongation of immobilized strands at the 5′-end with **6**, **10** and **14** could be performed with a coupling efficiency of more than 99%, as gauged spectrophotometrically from the released DMTr cation (see Table 1).

Duplexes **D0**, **D1**, **D2** and **D3** were formed by titration[19] of an equimolar amount of d(CpGpCpApApApApCpGpC) to the single stranded decamers **15**, **16**, **17** and **20**. The course of the hybridization process was monitored by integrating the intensities of the 5-methyl group of the thymidine residues in the 300-MHz ^{1}H-NMR spectrum (see Figure 1 exemplifying the formation of duplex **D1**). In the single-stranded case the 5-methyl signals resonate in the spectral region from 1.9 to 1.7 ppm. Upon titration of **15**, **16**, **17** and **20** with the native complementary strand a decrease in intensity of the 5-methyl resonances was observed. Simultaneously, a new set of four signals appeared between 1.8 and 1.4 ppm, indicating the formation of the duplex.

In order to study the process of base pairing within the duplexes **D0**, **D1**, **D2** and **D3** in more detail, attention was focussed on the imino-proton regions of the 600-MHz ^{1}H-NMR spectra (see Figure 2). In each case four narrow signals at approximately 14 ppm, typical for thymidine-imino protons involved in a canonical Watson–Crick A–T base pair, were observed. In addition, four narrow peaks, indicative of guanine-imino protons participating in a standard Watson–Crick G–C base pair, were observed around 13 ppm. In the same region, two broadened signals, hidden underneath the sharp guanine-imino proton signals, could be discerned. On the basis of NOESY spectra, recorded in H_2O (*vide infra*), the latter signals were attributed to the terminating guanine residues G1 and G10. This assignment is supported by the well-known phenomenon[20] that imino-proton resonances of terminating base pairs are broadened due to their enhanced accessibility for exchange with protic solvent molecules. The results of the imino-proton measurements clearly show that all 10 base pairs within **D0**, **D1**, **D2** and **D3** are intact.

In order to study the influence of a (3′ → 5′)-O-CH_2-O-linkage on the duplex conformation, we examined **D0** and **D1** in more detail by 2D-^{1}H-NMR spectroscopy at 500 MHz[21].

The NOESY and clean TOCSY spectra of **D0** and **D1** show a remarkable similarity in their cross-peak patterns which, in turn, are in good accord with a proposed B-DNA geometry. The presence of the intranucleotidic H6/H8-(*n*)–H1′/H2′/H2″(*n*) as well as the internucleotidic H6/H8(*n*)–H1′/H2′/H2″(*n*–1) NOESY cross peaks enabled us to assign the base protons and the sugar H1′, H2′ and H2″ protons of all 20 residues in **D0** and **D1** using conventional procedures[20,22–25].

It can be seen in Table II and Figure 3, that the chemical-shift differences between **D0** and **D1** with respect to the non-exchangeable base protons are small and predominantly confined to the thymidine residues neighbouring the (3′ → 5′)-O-CH_2-O-linkage (*i.e.*, T5 and T6). As chemical shifts of these protons are known[26] to be

Table II. Chemical shifts (ppm) at 20°C of the non-exchangeable base protons of **D0** *(δ_{D0}) and* **D1** *(δ_{D1}) and the corresponding chemical-shift differences ($\delta_{D1}-\delta_{D0}$).*

Residue	δ_{D0}		δ_{D1}		$\delta_{D1}-\delta_{D0}$	
	H8/H6	H2/H5/Me	H8/H6	H2/H5/Me	H8/H6	H2/H5/Me
G1	7.958		7.962		0.004	
C2	7.416	5.362	7.416	5.376	0.001	0.014
G3	7.976		7.970		−0.006	
T4	7.285	1.440	7.276	1.447	−0.009	0.007
T5	7.508	1.606	7.544	1.612	0.036	0.006
T6	7.515	1.653	7.461	1.633	−0.054	−0.020
T7	7.329	1.698	7.306	1.691	−0.023	−0.007
G8	7.940		7.937		−0.003	
C9	7.371	5.430	7.366	5.433	−0.005	0.003
G10	7.956		7.953		−0.003	
C11	7.648	5.915	7.648	5.92	0.000	0.005
G12	7.957		7.953		−0.004	
C13	7.331	5.447	7.331	5.444	0.000	−0.003
A14	8.202	7.195	8.190	7.219	−0.012	0.024
A15	8.114	7.007	8.110	7.009	−0.004	0.002
A16	8.032	7.044	8.006	7.040	−0.026	−0.004
A17	8.001	7.596	7.986	7.602	−0.015	0.006
C18	7.071	5.038	7.074	5.045	0.003	0.007
G19	7.848		7.858		0.010	
C20	7.420	5.388	7.455	5.466	0.035	0.078

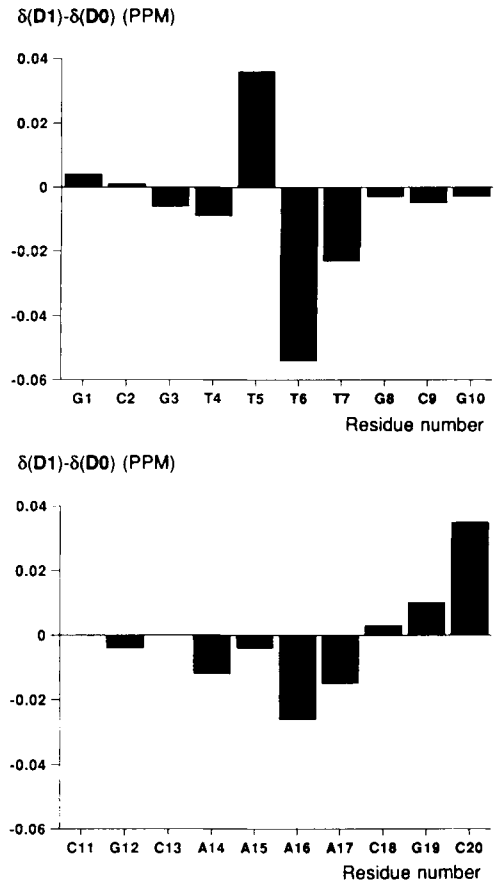


Figure 3. Graphical representation of the chemical-shift differences of the H6/H8 protons between **D0** *and* **D1** *at 20°C (δ_{D1} δ_{D0}), listed in Table II.*

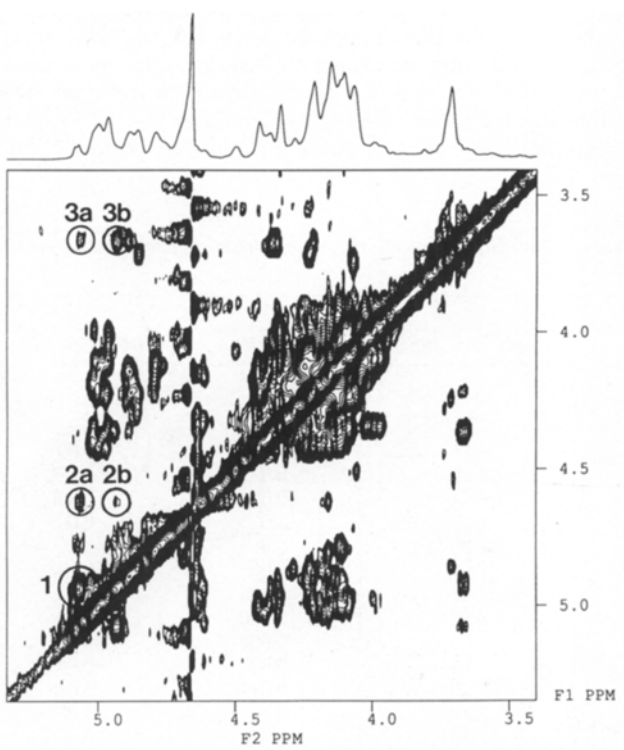


Figure 4. Expansion of the NOESY spectrum (500 MHz, 20°C, mixing time 200 mn) of **D1**, *showing the mutual cross peak of the (3′ → 5′) -O-CH$_2$-O- protons HMA and HMB (1), and their cross peaks with H3′(T5) (2a and 2b) and H5″(T6) (3a and 3b).*

Table III T_m values (± 0.2°C) of the studied duplexes, as determined by UV melting experiments (C_T 10^{-5} M, [Na$^+$] = 200 mM, pH = 7.0).

Duplex	T_m (°C)
D0	55.1
D1	52.7
D2	52.3
D2a	51.1
D2b	50.4
D3	48.6

sensitive to small geometrical changes within the DNA duplex, it is obvious that the conformations of **D0** and **D1** do not differ significantly.

We also observed minor differences (< 0.05 ppm) between **D0** and **D1** with respect to the chemical shifts of the sugar protons (data not shown), except for H3′(T5) (> 0.2 ppm) and H5″(T6) (> 0.3 ppm). The latter two differences are more pronounced as a result of the fact that the change in shielding caused by the substitution of the native phosphodiester by a -O-CH$_2$-O- union mainly effects H3′(T5) and H5″(T6). The resonances fo the methylene protons HMA and HMB (δ 5.068 and 4.928 ppm, respectively) were clearly recognized in the NOESY (see Figure 4) and clean TOCSY spectrum by the presence of the intense mutual cross-peak near the diagonal. In addition, both -O-CH$_2$-O protons displayed NOE contacts with H3′(T5) and H5″(T6). Similar NOE contacts were previously reported for other backbone-modified oligonucleotides (*e.g.*, ethylphosphotriesters[27] and methylphosphonates[28]).

The melting temperatures (T_m) of the duplexes **D0**, **D1**, **D2**, **D2a**, **D2b** and **D3** were determined by UV hyperchromicity. Introduction of one (3′ → 5′)-O-CH$_2$-O- linkage (**D1**) results in a T_m decrease of 2.4°C compared to the native duplex **D0** (see Table III). This destabilizing effect seems relatively small in comparison with some other phosphate modifications[27–30]. Remarkably, duplex **D2** bearing two -O-CH$_2$-O linkages separated by a native phosphodiester displays an almost equal duplex stability (0.4°C decrease relative to **D1**). In contrast, the destabilizing effect of two neighbouring -O-CH$_2$-O- bridges, as reflected in the T_m values of hybrids **D2a** and **D2b**, is much more pronounced (decrease of 1.6 and 2.3°C, respectively, compared to **D1**). Furthermore, the introduction of three neighbouring -O-CH$_2$-O- linkages (**D3**) results in an additional decline of the duplex stability (3.7°C, compared to **D2**).

The close resemblance of the NOESY cross-peak patterns of **D0** and **D1**, together with the observed small differences of the chemical shifts of the non-exchangeable base protons between **D0** and **D1**, reveals that the introduction of one (3′ → 5′)-O-CH$_2$-O- linkage does not have a significant effect on the stability and conformation of the duplex. Apparently, a (3′ → 5′)-O-CH$_2$-O- linkage can be easily accommodated despite its shorter 3′-oxygen to 5′-oxygen distance in a DNA duplex relative to a native internucleotidic phosphodiester. It is also evident that two -O-CH$_2$-O- linkages separated by a phosphodiester (as in **D2**) are more readily adjusted within the duplex than two neighbouring -O-CH$_2$-O- linkages (as in **D2a** and **D2b**). In this respect, it is of interest to note that *Matteucci* studied [13] the hybridization properties of the 14-mer d(TpCp-TpC^{Me}-TpC^{Me}-TpC^{Me}-TpC^{Me}-pTpTpTpT) having four alternating -O-CH$_2$-O linkages (C^{Me} is 5-methyl-deoxycytidine). It was found that the latter fragment showed significantly less affinity (T_m 39.0°C) for a native complementary DNA strand as compared to the diester control (T_m 60.0°C). However, in its binding with native complementary RNA, the -O-CH$_2$-O-compound was comparable (T_m 59.0°C) to the natural counterpart (T_m 60.0°C).

The physicochemical studies presented in this paper * indicate that oligonucleotides in which one or more native (3′ → 5′) internucleotidic phosphodiester bonds have been replaced by a -O-CH_2-O- linkage are promising antisense inhibitors of gene expression. Furthermore, a -O-CH_2-O linkage serves as a powerful tool in fundamental studies of the DNA backbone, and particularly of the conformations around the C5′–O5′ (β) and C3′–O3′ (ϵ) bonds.

Experimental

General methods and materials in synthesis

NMR spectra of compounds **1–14** were recorded with a Jeol JNM-FX200 (^{1}H, ^{13}C and ^{31}P at 200.0, 50.1 and 80.7 MHz, respectively). ^{1}H and ^{13}C chemical-shift values are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard and ^{31}P values in ppm (δ) relative to 85% H_3PO_4 as external standard. In the ^{1}H-NMR data, the 5′-terminal, central and 3′-terminal thymidine residues are designated as T-CH_2, CH_2-T-CH_2 and CH_2-T, respectively.
Pyridine and dioxane were dried by refluxing with CaH_2 (5 g/l), distilled and stored over molecular sieves (4Å). Dichloromethane and 1,2-dichloromethane were distilled from P_2O_5 and stored over molecular sieves (4Å). Diethyl ether was distilled from $LiAlH_4$ and stored over molecular sieves (4Å). Tetrahydrofuran (THF) was freshly distilled from $LiAlH_4$. Acetonitrile (Rathburn, HPLC grade) was stored over molecular sieves (4Å). N-Iodosuccinimide (NIS, Aldrich) and trifluoromethanesulfonic acid (TfOH, Fluka), benzoyl chloride (BAKER, HPLC-grade) and 4,4′-dimethoxytrityl (DMTr) chloride (Janssen) were used as received. 5′-*O*-Levulinoyl-3′-*O*-(methylthiomethyl)thymidine[9] (**1**) and 3′-*O*-benzoylthymidine[15] (**2**) were prepared as previously described.
Reactions were run at ambient temperature unless noted otherwise. Prior to reactions in pyridine or acetonitrile, nucleosides were dried by co-evaporation with two portions of the dry solvent. Prior to NIS/*cat.*-TfOH promoted condensations, nucleosides were dried by co-evaporation with two portions of dry 1,2-dichloroethane.
Column chromatography was performed on columns of silica gel 60 (Merck 70-230 mesh). Gel filtration was performed on Sephadex LH-20 (Pharmacia). TLC was conducted on DC Fertigfolien (Schleicher & Schüll F1500 LS-254). Compounds were detected by charring with 20% sulfuric acid in methanol.

Preparation of dimer 3

A freshly prepared solution of NIS (0.62 g, 2.75 mmol) and TfOH (36.4 μl, 412 μmol) in 1,2-dichloroethane/THF (26 ml, 1:1, v/v) was added to a cooled (0°C) and stirred mixture containing donor **1** (1.10 g, 2.75 mmol) acceptor **2** (0.79 g, 2.29 mmol) and powdered molecular sieves (4Å) in 1,2-dichloroethane/THF (14 ml, 1:1, v/v). After 3 min, the reaction mixture was filtered, diluted with dichloromethane (40 ml), washed with aq. $Na_2S_2O_3$ (1 M, 20 ml) and aq. $NaHCO_3$ (0.9 M, 20 ml), dried ($MgSO_4$) and concentrated. The remaining residue was purified by column chromatography on silica gel, using a gradient of methanol (0 → 7 vol%) in dichloromethane as eluent, yielding dimer **3** as a white foam (1.46 g, 92%). ^{1}H NMR ($CDCl_3$): δ 9.70 (bs, 2NH), 8.05 (m, benzoyl), 7.66–7.10 (m, 2 H6/benzoyl), 6.45 (dd, H1′, J_{12a} 8.2 Hz, J_{12b} 5.9 Hz), 6.26 (t, H1′, J_{12a}, J_{12b} 6.7 Hz), 5.54 (m, H3′ CH_2-T **), 4.86 (AB, OCH_2O), 4.60–4.18 (m, H3′, H4′, H5′, H5″ T-CH_2, H4′ CH_2-T), 3.94 (m, H5′, H5″ CH_2-T), 2.86–2.08 (m, 2 H2′, 2 H2″, 2 CH_2 Lev), 2.15 (s, CH_3 Lev), 1.93 (2 s, 2 CH_3 Thymine). ^{13}C NMR($CDCl_3$): δ 206.3 (C=O Lev), 172.1 (C=O Lev), 165.7 (C=O benzoyl), 163.9 (2 C4), 150.5, 150.3 (2 C2), 135.0 (2 C6), 133.2–124.9 (benzoyl), 110.9, 110.8 (2 C5), 94.6 (OCH_2O), 84.6, 84.4, 83.2, 82.4 (2 Cl′, 2 C4′), 76.8 (C3′ T-CH_2), 74.9 (C3′ CH_2-T), 68.0 (C5′ CH_2-T), 63.5 (C5′ T-CH_2), 37.5, 37.1 (2 C2′, CH_2 Lev), 29.5 (CH_3 Lev), 27.4 ($CH_2\alpha$ Lev), 12.4, 12.3 (2 CH_3 Thymine).

Deblocking of 3 and dimethoxytritylation to give dimer 4

Compound **3** (0.58 g, 0.83 mmol) was dissolved in dioxane (20 ml), whereupon aq. NH_4OH (25%, 40 ml) was added. After stirring for 2 h at 50°C, the mixture was concentrated and the oily residue was washed with dichloromethane (2×50 ml). A solution of the resulting white solid and 4,4′-dimethoxytrityl chloride (340 mg, 1.00 mmol) in pyridine (10 ml) was stirred for 1 h. Water (2.0 ml) was added and the reaction mixture was concentrated, diluted with dichloromethane (25 ml), washed successively with water (10 ml) and aq. $NaHCO_3$ (0.9 M, 2×10 ml), dried ($MgSO_4$) and concentrated. Purification by silica gel chromatography (using a gradient of methanol (0 → 8 vol%) in dichloromethane as eluent) yielded pure **4** (0.50 g, 76%). ^{1}H NMR ($CDCl_3$): δ 9.50 (bs, NH), 9.18 (bs, NH), 7.69–7.12 (m, DMTr, 2 H6), 6.84 (m, DMTr), 6.30 (m, 2 H1′), 4.80 (AB, OCH_2O), 4.43–3.27 (m, 2 H3′, 2 H4′, 2 H5′, 2 H5″), 3.79 (s, 2 CH_3O DMTr), 2.75–2.09 (m, 2 H2′, 2 H2″), 1.89 (s, CH_3 Thymine), 1.49 (s, CH_3 Thymine). ^{13}C NMR ($CDCl_3$/CD_3OD 1:1 v/v): δ 165.2 (2 C4), 159.2 (DMTr), 151.4 and 151.3 (2 C2), 144.8 (DMTr), 136.6, 136.3 (2 C6), 135.8 (DMTr), 130.5–113.7 (DMTr), 111.7, 111.1 (2 C5), 95.6 (OCH_2O), 87.5 (C_{quat} DMTr), 85.8, 85.4, 85.3, 84.9 (2 Cl′, 2 C4′), 79.0 (C3′ T-CH_2), 71.4 (C3′ CH_2-T), 68.7 (C5′ CH_2-T), 64.0 (C5′ T-CH_2), 55.5 (CH_3O DMTr), 40.6, 38.0 (2 C2′), 12.8, 11.9 (2 CH_3 Thymine).

Preparation of dimer phosphoramidite 6

To a stirred solution of **4** (0.50 g, 0.63 mmol) and DIPEA (0.163 g, 1.26 mmol) in 1,2-dichloroethane (10 ml) was added (2-cyanoethoxy)(diisopropylamino)chlorophosphine **5** (0.21 g, 0.89 mmol). After 1 h, the reaction mixture was washed with aq. NaCl (1.5 M, 2×15 ml) and aq. $NaHCO_3$ (0.9 M, 15 ml), dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel (eluent 97:3 (v/v) ethyl acetate/triethyl amine), yielding **6** (0.60 g, 95%). ^{31}P NMR ($CDCl_3$): δ 149.2 (peaks overlap).

Removal of the Lev group of 3 to yield dimer 7

A freshly prepared mixture of hydrazine hydrate (1.0 ml), pyridine (12.0 ml) and acetic acid (8.0 ml) was added to a solution of **3** (1.37 g, 1.96 mmol) in pyridine (10.0 ml). After 5 min, the reaction mixture was diluted with dichloromethane (40 ml), washed with water (2×20 ml) and aq. $NaHCO_3$ (0.9 M, 20 ml), dried ($MgSO_4$), concentrated and co-evaporated with toluene (three times), ethanol (twice) and dichloromethane (twice). Purification by silica-gel-column chromatography (using a gradient of methanol (0 → 8 vol%) in dichloromethane as eluent) yielded pure **7** (0.95 g, 81%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 8.04 (m, benzoyl), 7.65–7.42 (m, 2 H6/benzoyl), 6.45 (dd, H1′, J_{12a} 8.2 Hz, J_{12b} 5.9 Hz), 6.17 (t, H1′, J_{12a}, J_{12b} 6.7 Hz), 5.59 (m, H3′ CH_2-T), 4.85 (AB, OCH_2O), 4.60–3.81 (m, H3′, H4′, H5′, H5″ T-CH_2, H4′, H5′, H5″ CH_2-T), 2.70–2.32 (m, 2 H2′, 2 H2″), 1.92, 1.89 (2 s, 2 CH_3 Thymine). ^{13}C NMR ($CDCl_3$): δ 165.8 (C=O benzoyl), 164.2, 164.0 (2 C4), 150.4 (1 C2), 136.2, 135.2 (2 C6), 133.2–128.2 (benzoyl), 110.8, 110.4 (2 C5), 94.1 (OCH_2O), 85.3, 85.0, 84.4, 83.2 (2 Cl′, 2 C4′), 76.4 (C3′ T-CH_2), 75.0 (C3′ CH_2-T), 67.7 (C5′ CH_2-T), 61.5 (C5′ T-CH_2), 37.2, 37.0 (2 C2′), 12.3, 12.0 (2 CH_3 Thymine).

Preparation of trimer 8

To a cooled (0°C) and stirred mixture of acceptor **7** (0.95 g, 1.58 mmol), donor **1** (0.74 g, 1.85 mmol), and powdered molecular sieves (4Å) in 1,2-dichloroethane/THF (10 ml, 1:1 v/v) was added a solution of NIS (0.45 g, 2.00 mmol) and TfOH (28 μl, 318 μmol) in 1,2-dichloroethane/THF (24 ml, 1:1 v/v). After 5 min, the reaction mixture was processed as described for the synthesis of **3**. The residue was purified by silica-gel chromatography to afford trimer **8** (1.31 g, 80%). ^{1}H NMR ($CDCl_3$): δ 9.76, 9.75, 9.70 (3 s, 3 NH), 8.04 (d, benzoyl), 7.65–7.25 (m, benzoyl/3 H6), 6.43 (t, H1′, J_{12a}, J_{12b} 6.9 Hz), 6.21 (m, 2 H1′), 5.53 (m, H3′), 4.90–4.76 (2 AB, 2 OCH_2O), 4.45–3.70 (m, 2H3′, 3 H4′, 3 H5′, 3 H5″), 2.83–2.03 (m, 3 H2′/3 H2″/2 CH_2 Lev), 2.16 (s, CH_3 Lev), 1.92 (s, CH_3 Thymine), 1.89 (s, 2 CH_3 Thymine). ^{13}C NMR ($CDCl_3$): δ 206.4 (C=O Lev), 172.2 (C=O Lev), 165.8 (C=O benzoyl), 163.9 (bs, 3 C4), 150.6, 150.4 (double int.) (3 C2), 135.1 (bs, 3 C6), 133.4, 129.5, 128.9, 128.3 (benzoyl), 111.0, 110.9, 110.8 (3 C5), 94.6, 94.5 (2 OCH_2O), 85.2, 85.0, 84.7, 83.2 (double int.), 82.3 (3 Cl′, 3 C4′), 77.5, 77.2 (2 C3′, T-CH_2 and CH_2-T-CH_2), 74.9 (C3′ CH_2-T), 68.1 (double int., 2 C5′, CH_2-T-CH_2

* During the preparation of this manuscript, *Gao et al.* independently reported[31] on the physicochemical properties of the duplex between the dodecamer d($C^1pG^2pC^3pG^4pT^5pT^6CH_2T^7pT^8pG^9pC^{10}pG^{11}pC^{12}$) with its non-altered complementary strand. They observed that the structural perturbations imposed by the -O-CH_2-O-linkage were minimal and localized primarily in the region of the two residues immediately adjacent to the -O-CH_2-O- moiety (*i.e.*, T^6 and T^7), thus confirming the outcome of our experiments on duplex **D1**.
** T-CH_2 = 5′-terminal thymidine residue. CH_2-T-CH_2 = central thymidine residue. CH_2-T = 3′-terminal thymidine residue.

and CH_2-T), 63.6 (C5′ T-CH_2), 37.6 (bs, 3 $C_2/CH_2\beta$ Lev), 29.5 (CH_3 Lev), 27.6 ($CH_2\alpha$ Lev), 12.5, 12.4, 12.3 (3 CH_3, Thymine).

Deblocking of 8 and dimethoxytritylation to afford trimer 9

Aq. NH_3 (25%, 55 ml) was added to a solution of compound **8** (1.31 g, 1.42 mmol) in dioxane (20 ml). After stirring for 5 h at 50°C, the mixture was concentrated and the residue was co-evaporated twice with dioxane, washed with dichloromethane (2×30 ml) and dried by co-evaporation (three times) with pyridine. To a solution of the resulting residue in pyridine (6.0 ml) 4,4′-dimethoxytrityl chloride (0.53 g, 1.57 mmol) was added and the mixture was stirred for 40 min. Then, water (3 ml) was added and the reaction mixture was concentrated, diluted with dichloromethane (20 ml) and washed successively with water (20 ml) and aq. $NaHCO_3$ (0.9 M, 35 ml). The aqueous phase was extracted with dichloromethane (2×20 ml), and the combined organic layers were dried ($MgSO_4$) and concentrated. Purification by silica-gel chromatography (using a gradient of methanol (0 → 10 vol%) in dichloromethane as eluent) yielded pure **9** as a white solid (1.03 g, 69%). ^{1}H NMR($CDCl_3$): δ 7.60–7.21 (m, DMTr/2 H6), 6.83 (m, DMTr), 6.38–6.08 (m, 3 H1′), 4.77 (2 H, AB, OCH_2O), 4.51–3.29 (m, 3 H3′, 3 H4′, 3 H5′, 3 H5″), 3.78 (s, 2 CH_3O DMTr), 2.62–2.11 (m, 3 H2′/3 H2″), 1.87 (2 s, 2 CH_3 Thymine), 1.47 (s, CH_3 Thymine). ^{13}C NMR ($CDCl_3$): δ 164.2 (double int.), 164.1 (3 C4), 158.3 (DMTr), 150.5, 150.4 (double int.) (3 C2), 143.9 (DMTr), 135.8, 135.4, 135.3 (3 C6), 134.9 (DMTr), 129.6–112.9 (DMTr), 111.0, 110.6, 110.4 (3 C5), 94.8, 94.6 (2 OCH_2O), 86.6 (C_{quat} DMTr), 85.0, 84.8, 84.7, 84.4, 84.0, 83.1 (3 Cl′, 3 C4′), 78.5, 77.4, 70.7 (3 C3′), 67.8 (double int.), 63.2 (3 C5′), 54.8 (CH_3O DMTr), 39.7, 38.4, 38.0 (3 C2′), 17.6, 12.1, 11.4 (3 CH_3 Thymine).

Preparation of trimer phosphoramidite 10

To a stirred solution of **9** (400 mg, 0.38 mmol) and DIPEA (149 mg, 1.14 mmol) in 1,2-dichloroethane (6.0 ml) was added (2-cyanoethoxy)(diisopropylamino)chlorophosphine **5** (205 mg, 0.87 mmol). After 1 h, the reaction mixture was diluted with dichloromethane (5.0 ml) and washed with aq. NaCl (1.5 M, 10ml) and aq. $NaHCO_3$ (0.9 M, 10 ml). The aqueous phase was extracted with dichloromethane and the combined organic layers were dried ($MgSO_4$) and concentrated. The residue was purified by silica gel chromatography (eluent ethyl-acetate/triethyl-amine, 95:5, v/v, gradient isopropylalcohol 2 → 10 vol%) yielding **10** (400 mg, 84%). ^{31}P NMR ($CDCl_3$): δ 149.2 and 149.0.

Removal of the Lev group of 8 and preparation of tetramer 12

A freshly prepared mixture of hydrazine hydrate (0.3 ml), pyridine (3.6 ml) and acetic acid (2.4 ml) was added to a solution of trimer **8** (0.56 g, 0.59 mmol) in pyridine (3.0 ml). After 3 min of stirring, the reaction mixture was processed as described for the synthesis of **7** giving 0.46 g (0.54 mmol, 92%) of trimer **11**. To a cooled (0°C) and stirred mixture of this product, donor **1** (0.265 g, 0.66 mmol) and molecular sieves (4Å) in 1,2-dichloromethane/THF (8 ml, 1:1 v/v) was added a solution of NIS (149 mg, 0.66 mmol) and TfOH (17.5 μl, 220 μmol) in 1,2-dichloroethane/THF (8 ml, 1:1, v/v). After 5 min of stirring, the reaction mixture was processed as described for the synthesis of **3** and purified on a column of Sephadex LH-20 by elution with dichloromethane/methanol (1:1, v/v). The appropriate fractions were concentrated to yield tetramer **12** (0.52 g, 80%). ^{1}H NMR ($CDCl_3/CD_3OD$ 1:1 v/v): δ 8.02 (d, benzoyl), 7.66–7.10 (m, benzoyl/4 H6), 6.42 (t, H1′), 6.20 (m, 3H1′), 5.55 (m, H3′), 4.93–4.72 (m, 3 OCH_2O), 4.49–3.69 (m, 3 H3′, 4 H4′, 4 H5′, 4 H5″), 2.88–2.05 (m, 4 H2′, 4 H2″, 2 CH_2 Lev), 2.20 (s, CH_3 Lev), 1.93 (bs, 4 CH_3 Thymine). ^{13}C NMR ($CDCl_3/CD_3OD$ 1:1 v/v): δ 206.9 (C=O Lev), 172.3 (C=O Lev), 165.8 (C=O benzoyl), 164.2 (bs, 4 C4), 150.4, 150.3 (4 C2), 135.6, 135.5, 135.3, 135.2 (4 C6), 133.3, 129.2, 128.7, 128.2 (arom. Benzoyl), 110.8, 110.7, 110.6, 110.5 (4 C5), 94.9 (double int.), 94.4 (3 OCH_2O), 85.0, 84.9 (double int.), 84.6, 83.1, 83.0 (double int.), 82.2 (4 Cl′/4 C4′), 77.8, 77.4, 76.9, 74.7 (4 C3′), 67.8 (triple int.), 63.4 (4 C5′), 37.5, 37.3, 37.0 (4 C2′/$CH_2\beta$ Lev), 29.0 (CH_3 Lev), 27.4 ($CH_2\alpha$ Lev), 11.8 (bs, 4 CH_3 Thymine).

Deblocking of 12 and dimethoxytritylation to give tetramer 13

Aq. NH_3 (25%, 100 ml) was added to a solution of **12** (0.52 g, 0.43 mmol) in methanol/dioxane (50 ml, 1:1 v/v). After 1 h of stirring, the solution was concentrated and the oily residue was washed with dichloromethane (50 ml) yielding the completely deprotected tetramer as a white solid (360 mg, 84%). 4,4′-Dimethoxytrityl chloride (102 mg, 0.30 mmol) was added to a solution of part of this product (251 mg, 0.25 mmol) in pyridine (5.0 ml). After 1 h of stirring, water (1.0 ml) was added and the reaction mixture was concentrated, diluted with dichloromethane (10 ml), washed successively with water (2×5 ml) and aq. $NaHCO_3$ (0.9 M, 5 ml), dried ($MgSO_4$), concentrated and co-evaporated with toluene (2 × 10 ml) and dichloromethane (10 ml). The resulting solid was purified by column chromatography on silica gel, using a gradient of methanol (0 → 5%) in dichloromethane as eluent, yielding tetramer **13** as a white solid (229 mg, 70%). ^{1}H NMR (CD_3OD): δ 8.55 (bs, 4 NH), 7.80–7.21 (m, 4 C6/DMTr), 6.85 (d, DMTr), 6.38–6.18 (m, 4 H1′), 4.90–4.70 (m, 3 OCH_2O), 4.70–3.30 (m, 4 H3′, 4 H4′, 4 H5′, 4 H5″), 3.78 (s, 2 CH_3O DMTr), 2.60–2.09 (m, 4 H2′, 4 H2″), 1.90 (s, 2 CH_3 Thymine), 1.88 (s, CH_3 Thymine), 1.85 (s, CH_3 Thymine). ^{13}C NMR (CD_3OD): δ 165.5, 165.3 (4 C4), 151.5, 151.4 (4 C2), 137.0 (double int.), 136.8, 136.7 (4 C6), 159.4–113.8 (DMTr), 111.7, 111.4 (double int.), 111.3 (4 C5), 95.9, 95.7, 95.5 (3 OCH_2O), 86.0, 85.9 (double int.), 85.6, 85.4, 85.1, 84.2 (double int.) (4 C1′/4 C4′), 79.5, 78.8, 78.3, 71.4 (4 C3′), 68.8 (bs, 3 C5′), 64.2 (C5′), 55.6 (2 CH_3O DMTr), 40.4, 39.0, 38.5, 38.4 (4 C2′), 12.8 (triple int.), 12.0 (4 CH_3 Thymine).

Preparation of tetramer phosphoramidite 14

(2-Cyanoethoxy)(diisopropylamino)chlorophosphine **5** (60 mg, 0.25 mmol) was added to a stirred solution of **13** (229 mg, 0.18 mmol) and DIPEA (47 mg, 0.36 mmol) in 1,2-dichloroethane (5.0 ml). After 1 h, the solution was washed with aq. NaCl (10 ml) and aq. $NaHCO_3$ (10 ml), dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography on silica gel, using ethyl acetate/triethyl amine (95:5, v/v) as eluent, yielding **14** (190 mg, 72%). ^{31}P NMR ($CDCl_3$): δ 149.2 and 149.1.

Solid-phase synthesis of the decamers

The polymer-supported synthesis was performed on a fully automated synthesizer (Gene Assembler, Pharmacia) using controlled-pore glass (CPG-AP, 200 mg), covalently linked to the appropriate nucleoside. Oligonucleotides **15–20** and native d(CpGpCpApApA-pApCpGpC) were assembled on a large scale (10 μmol). Cleavage from the resin and complete deprotection was effected by treatment with aq. NH_3 (25%) for 24 h at 50°C. The support was removed by filtration and the filtrate was evaporated under reduced pressure. The crude unprotected decanucleotides were analysed by FPLC and purified on Sephadex G-50 (150 cm×2 cm^2), suspended in and eluted with TEAB buffer (0.05 M). The appropriate fractions were pooled, concentrated to a small volume and co-evaporated with aq. NH_3 (25%) to remove excess TEAB. The decamers were brought into the Na^+ form by elution through a column of Dowex 50W X4 cation-exchange resin (100–200 mesh, Na^+ form). The resulting UV-positive fractions were pooled, concentrated to a small volume and lyophilized.

NMR experiments on duplexes D0, D1, D2 and D3

Samples (concentration ~ 5 mM) of **D0**, **D1**, **D2**, and **D3** for measurements of NMR signals of non-exchangeable protons were prepared by lyophilizing the duplex systems twice from D_2O (99.75%) and finally dissolving in 500 μl D_2O (99.95%). Prior to the final lyophilization, a trace of EDTA (0.1 mM) was added in order to neutralize the effect of paramagnetic impurities. Tetramethylammonium bromide (TMA) was added as an internal chemical-shift reference and the pH values were adjusted with DCl to 7.0 (meter reading). The TMA scale was converted into the sodium 3-(trimethylsilyl)propanesulphonate (DSS) scale by addition of 3.18 ppm to the measured chemical-shift values.

In order to observe the imino-proton resonances in **D0**, **D1**, **D2** and **D3**, a second set of samples was prepared by dissolving the duplexes in 500 μl of H_2O/D_2O (9:1, v/v), containing traces of EDTA and TMA. The pH was adjusted to 6.0 (meter reading). These slightly acidic conditions were chosen in order to suppress proton exchange with the solvent.

300-MHz ^{1}H-NMR experiments were performed on a Bruker WM 300 spectrometer equipped with an ASPECT 2000 computer, 500- and 600-MHz ^{1}H-NMR experiments were conducted on Bruker AM 500 and AM 600 NMR instruments, both interfaced with an ASPECT 3000 computer.

In case of measurements in D_2O the residual HDO signal was suppressed by a weak irradiation at the HDO frequency during the relaxation period. Free-induction decays of 1D-NMR experiments were acquired with 8 K data points and a sweep width of 4808 Hz, apodized with a gaussian window, zero-filled to 32 K points and Fourier transformed. During the imino-proton measurements, sup-

pression of the H_2O signal was achieved by the use of a semi-selective acquisition pulse. The carrier was placed exactly 4032 Hz downfield from the H_2O peak, in the empty region between the imino- and amino-proton resonances. As the spectral width was four times this frequency difference (16128 Hz), the residual H_2O signal could be further reduced by means of data shift accumulation. Approximately 700 free induction decays were acquired with 4 K data points. NOESY[32,34] and clean TOCSY[35] experiments on **D0** and **D1** in D_2O were performed essentially as described elsewhere. Between 450 and 512 free induction decays were collected with 2 K data points and a sweep width of 4808 Hz. Prior to phase-sensitive Fourier transform, the t_1 and t_2 domains were apodized with a $\pi/3$-shifted quadratic sine filter and zero-filled to 4 K.

UV melting experiments

Samples for optical melting experiments on the duplexes **D0**, **D1**, **D2** and **D3** were prepared by dilution of the NMR samples ($\sim$ 5 mM) to 12–14 μM with a H_2O stock buffer (pH 7.0), containing 200mM NaCl, 5mM Na phosphate, 1mM Na cacodylate and 0.1mM EDTA. Samples of the duplexes **D2a** and **D2b** (12–14 μM) were prepared by mixing a solution of **18** and **19**, respectively, in the above described buffer with one molar equivalent (determined by UV absorption) of d(CpGpCpApApApApCpGpC), dissolved in the same stock buffer. UV melting experiments were carried out on a Cary 118C spectrophotometer at 260 nm using a constant temperature cell (Hellma). The temperature was controlled by circulating water from a cryostat (Mettler, WKS), first through the cell holder and then through the cell. For all samples three curves with increasing temperature (ramp $\sim$ 1°C/min) and two curves with decreasing temperature (ramp $\sim$ 0.5°C/min) were recorded. Each melting curve was analyzed separately by means of a non-linear least-squares curve-fitting procedure. We used a three-state model DUPSTAK, in which the parameters of the single-stranded stack state (x) were constrained to average values of $T_{m,x} = 39$°C and $\Delta S^0_x = -89$ J/mol·K[36]. Precise strand concentrations were determined from the high-temperature absorption. In order to make a justified comparison between the thermal stabilities of the examined duplexes, all determined T_m values were adjusted to a concentration of 10 μM.

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