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Synthesis of *C*-Branched Spermine Tethered Oligo-DNA and the Thermal Stability of the Duplexes and Triplexes

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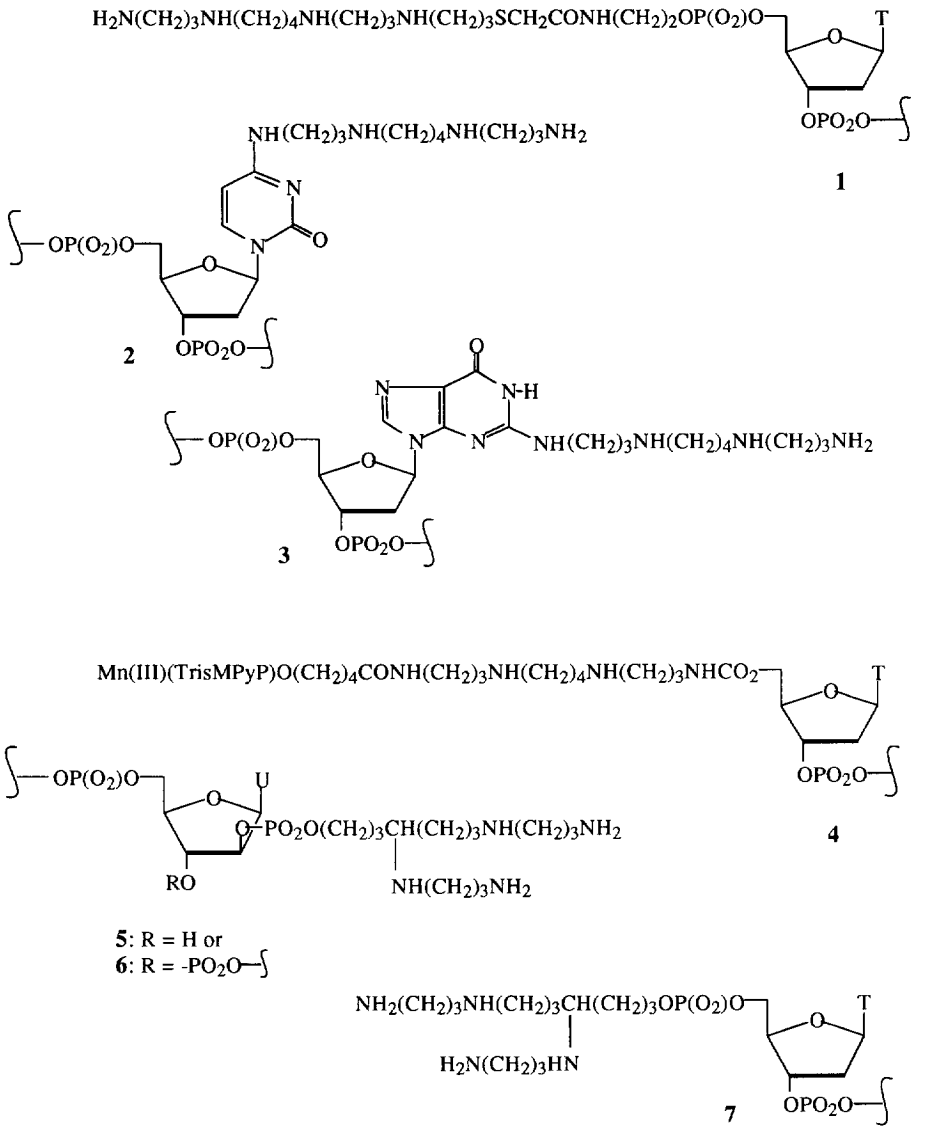
Abstract: The first synthesis of the new *C*-branched spermine derivative **17**, as well as its ability to stabilise DNA duplexes and triplexes, are reported. The *C*-branched spermine block **17** was converted into the corresponding *O*-(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite block **18** for incorporation at the 5'-end of DNA. It was also coupled to the 2' of *ara*-U through a phosphate bridge, leading to the partially protected 3'-hydroxy block **23**, which was either converted to the *O*-(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite **24** or to the 3'-succinate block **25**, leading to the synthesis of three model DNA 14-mers: **27** with tethered spermine at the 5'-end, **28** with tethered spermine at the middle of the DNA strand, and **29** with spermine at the 2'-end, using standard automated solid-phase chemistry and deprotection procedures. The T_m measurements showed that, at low salt with Mg^{2+} within the pH range of 5.5 - 7.6, the 5'-spermine-DNA conjugate **27** gives stabilised DNA triplexes with $\Delta^h T_m$s of ~+3 °C and $\Delta^c T_m$s of +5.5° to +8.5° C and the 2'-spermine-DNA conjugate **29** gives $\Delta^h T_m$s of ~+5° to +6.5 °C and $\Delta^c T_m$s of +7.5° to +9 °C over the underivatised DNA counterpart **30**. In contrast, the spermine block conjugated to the middle of the DNA as in **28** gave no triplex formation. Without Mg^{2+}, these short T-rich oligonucleotides (**27**, **29** & **30**) gave triplex formation only above 0.4M NaCl. At 1.4M, the $\Delta^h T_m$s for **27** and **29** over **30** were +9.5 °C while the $\Delta^c T_m$s were +6° to +7° C. Oligonucleotide conjugates **27** & **29** gave only weak duplex stabilisations with $\Delta^h T_m$s of ~+2° and ~+1° C, respectively, over **30** at low salt with Mg^{2+} within the pH range 5.5 - 7.6. Copyright © 1996 Elsevier Science Ltd

Much attention has been directed towards the design of short oligo-DNA analogues to improve their efficiency as repressors at the transcriptional (antigene) and translational (antisense) level of gene expression.[1,2] This ability is based on the strength and efficiency of specific base pairing, which stabilises their duplex formation with mRNA through Watson-Crick hybridisation and their triplex formation with duplex DNA through Hoogsteen hybridisation. One strategy to improve the efficiency and specificity of the antisense and antigene oligonucleotides is to increase the stability of the DNA/RNA (DNA) duplex and the DNA triplex. This has been achieved mainly by introducing polyaromatic systems,[3a,b] alkyl groups[4a,b,c] and by derivatising the internucleotide phosphate linkages[5a,b] or by substituting it with various internucleoside aliphatic linkers.[5b]

External tetracationic spermine has been shown to stabilise DNA duplexes and triplexes[6a,b] and this has raised the interest in spermine conjugated oligo-DNAs as non-cytotoxic candidates for the antisense and antigene strategies. A few reports on spermine conjugated oligo-DNAs have emerged over recent years where different methods have been employed for conjugation of the spermine to the oligonucleotide. Tung *et al*[7] introduced the spermine to the 5'-phosphate of their oligonucleotide in a post-solid phase synthetic step by



reacting the 5'-iodoacetamidoalkyl linked oligo-DNA with N^1-(3-mercaptopropyl)spermine (**1**) in aqueous solution. Prakash *et al*[8] conjugated the spermine to the C4 of the 5-methylcytosine ($C^{5\text{-Me}}$) moiety, as in **2**, and introduced the spermine-conjugated block directly into oligo-DNA through the phosphoramidite based solid-phase synthesis. Schmid *et al*[9] conjugated the spermine to the C2 of a guanine base, as in **3**, in a post-synthetic deprotection step using a fully protected 2-fluoro-2'-deoxyinosine phosphoramidite block in the solid-phase synthesis. Bigey *et al*[10] used spermine as a linker between the 5'-hydroxyl of their oligomer and a tris(methylpyridiniumyl)porphyrinato-manganese(III) motif (**4**) in a post solid-phase synthetic step for the stabilisation of a triplex and for subsequent cleavage of the duplex component.

A 21-mer 5'-spermine-DNA conjugate of type **1**, consisting of T and $C^{5\text{-Me}}$ blocks gave a triplex with

T_m of 42° C with a 36-mer DNA duplex at low salt at pH 7.0 and no Mg^{2+}, whereas no triplex was formed with the underivatised oligomer.[7] Mono-, di- and tri-spermine conjugated 18-mer DNAs of the type **2**, consisting of T and C blocks, gave a triplex with T_m within the range of 25° - 40° C with a 24-mer duplex at pH 7.3 (at low salt and no Mg^{2+}), whereas the underivatised oligomer gave no triplex. But at pH 6.0, the ΔT_m was +3° to +6° C for the mono or di-spermine containing oligonucleotides compared to the underivatised oligomer and the tri-spermine containing oligomer had a ΔT_m of - 5° C.[11] Oligo-DNA conjugates of type **2** was shown to give destabilised duplexes.[8] An 11-mer DNA of type **3**, containing T, A and G nucleotides with two conjugated spermines around the middle of the oligomer, gave a duplex with ΔT_m varying between +15° to +25° C at pH 6.8 at low salt and no Mg^{2+} compared to the underivatised oligomer. The oligo-DNA of type **3** has to the best of our knowledge not been used for triplex formation. A 16-mer DNA of type **4**, containing T, $C^{5\text{-Me}}$ and G blocks gave a triplex of T_m of 42° C with a 29-mer DNA duplex at low salt at pH 7.0 with Mg^{2+} and free spermine. The ΔT_m was +12° C compared to the conjugate where the spermine was replaced by an aliphatic diamino linker.[10]

In this work, we present a hitherto unexplored use of *C*-branched spermine conjugate at the 3'-end or in the middle or at the 5'-end of an oligo DNA, as in **5** - **7**, for the stabilisation of the duplexes and triplexes. This new type of hydroxyalkyl linked *C*-branched spermine tether is unique in that all the amino functions are underivatised and their pK_as unaltered compared to natural spermine. The novel nature of *C*-branching of the spermine residue also allowed its coupling to the pentofuranose ring, as in **5** and **6**, that enabled us to stereochemically orientate it away from both the minor and major groove of DNA duplex, and thereby placing it in the steric proximity of the phosphate backbone. In the present approach, the spermine is introduced directly during solid-phase synthesis in three ways: (i) The synthesis of the fully protected spermine phosphoramidite block **18**, and subsequently its incorporation at the 5'-end of the oligo-DNA. (ii) The synthesis of fully protected *ara*-U phosphoramidite **24** and its incorporation into the oligo-DNA chain, and (iii) the synthesis of fully protected *ara*-U 3'-succinylaminoalkyl CPG **26** for incorporation of the spermine residue to the 2'-end of the oligo-DNA chain. All through this work, a racemic mixture of *C*-(3-hydroxypropyl)spermine has been used for the oligo-DNA derivatisation.

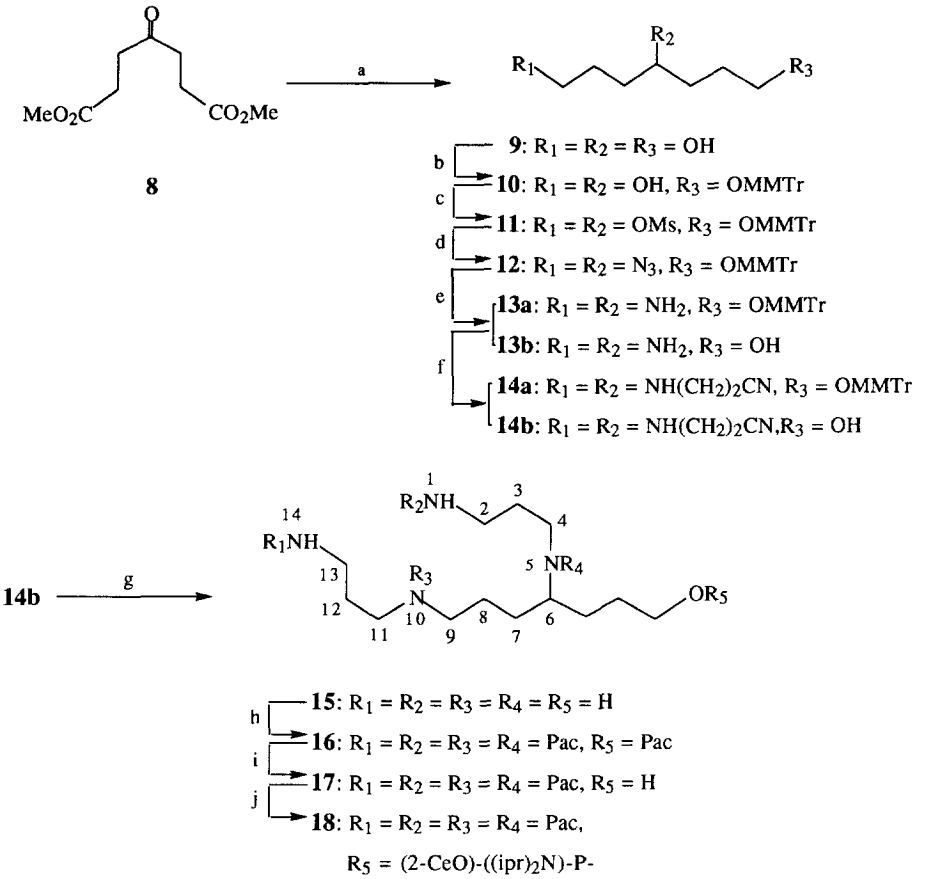
By using the above building blocks, we have synthesised simple T-rich *C*-branched spermine conjugated 14-mers **27**, **28**, **29**, as well as the blank T_{14} (**30**) and hybridised with either the dA_{26} (**31**) alone or with a 1 : 1 complex of dA_{26} : dT_{26} at different pH and salt concentrations, and subsequently studied the melting behavior (T_m) of the resulting double- and triple helical complexes.

RESULTS AND DISCUSSION

(A) Synthesis of 6-(3-hydroxypropyl)spermine block (17) and its phosphoramidite 18.

The starting dimethyl 3-oxopimelate[12a-c] (**8**) was reduced with $LiAlH_4$ in refluxing diethylether to give 1,4,7-heptanetriol (**9**) (36%).[13] Triol **9** was then selectively monotritylated at one of the primary hydroxyls with MMTr-Cl in pyridine and CH_2Cl_2 to give the racemic mixture of compound **10** (78%), which was converted to its bis-mesylate **11** (59%).[14] Bis-mesylate **11** was reacted with NaN_3 in DMF[15] to give the diazido analog **12** (90%), which was subsequently reduced by hydrogenation on 10% Pd/C in methanol to give the mixture of the corresponding diamino compounds **13a** and **13b** (4:6 ratio by Tlc (system F, ~85% crude). The crude racemic mixture of 4,7-diaminoheptan-1-ol (**13b**) and its 1-*O*-MMTr analog **13a** was reacted with acrylonitrile in

DMF[16] to give four products: N^4, N^7-bis(2-cyanoethyl)diaminoheptan-1-ol (**14b**) (35%), 1-*O*-MMTr-N^4, N^7-bis(2-cyanoethyl)diaminoheptane (**14a**) (13%), N^4,N^7,N^7-tris(2-cyanoethyl)-4,7-diamino-1-*O*-MMTr-heptane (~10%) and N^7,N^7-bis(2-cyanoethyl)-4,7-diamino-1-*O*-MMTr-heptane (~8%). N^4, N^7-bis(2-cyanoethyl)-

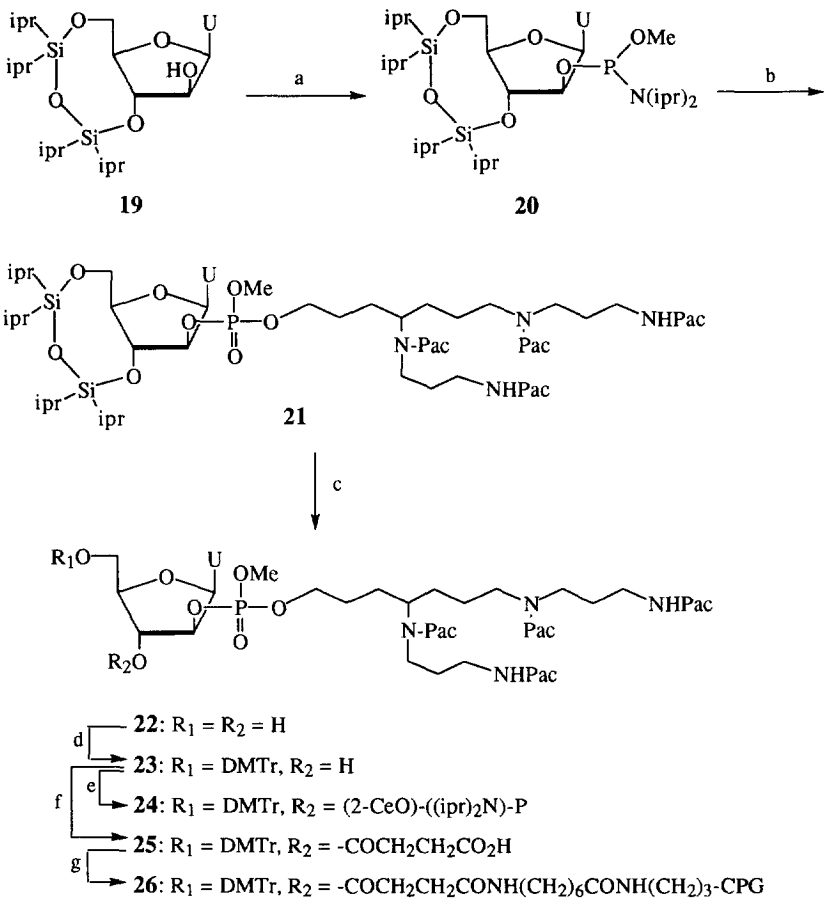


(a) $LiAlH_4$ / diethylether, reflux 45 min; (b) MMTrCl / pyridine / CH_2Cl_2, 18 h, 20°C; (c) MsCl / pyridine / CH_2Cl_2, 1 h, 0-20°C; (d) NaN_3 / DMF, 18 h, 20°C; (e) H_2 / Pd / C, MeOH, 1 h, 20°C; (f) CH_2=CHCN / DMF, 14 h, 20°C; (g) $LiAlH_4$ / $AlCl_3$ (1:1) / THF / diethylether, 15 h, 20°C; (h) $(Pac)_2O$ / pyridine, 1 h, 20°C; (i) NH_3 / MeOH, 3 h, 20°C; (j) (2-CeO)-((ipr)$_2$N)-PCl / $(ipr)_2EtN$ / THF, 45 min, 20°C.

diaminoheptan-1-ol (**14b**) was then reduced with $LiAlH_4$ / $AlCl_3$ in tetrahydrofuran and diethylether[17] mixture to generate the racemic 6-(3-hydroxypropyl)spermine (**15**) (95% as crude oil), which was then reacted directly with phenoxyacetic (Pac) anhydride in pyridine to generate the penta-Pac intermediate **16** (44%). Compound **16** was then selectively hydrolysed with dry NH_3 in dry MeOH to give the N^1,N^5,N^{10},N^{14}-tetra-Pac block **17** in a quantitative yield. An aliquot of **17** was converted into the *O*-(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite block **18** (80%) in a standard way.[18]

*(B) Synthesis of 5'-O-DMTr-2'-(tetra-Pac-spermine)phosphate (**23**), its 3'-phosphoramidite block **24** and its 3'-succinamido anchored CPG **26**.*

The hydroxy block **17** was coupled to the 2'-hydroxyl of *ara*-uridine (*ara*-U)[19a,b] via a phosphate linkage, since selective preparation of asymmetric 2',3'-bis-phosphate of a ribonucleoside is much more complex as it has been demonstrated by us[20] and others.[20] The synthesis of **23** started with the conversion of 3', 5'-*O*-(1,1,3,3-tetraisopropyl-1,3-disilyl,1,3-yl) (TipDSi) *ara*-U (**19**)[21] to the corresponding 2'-phosphoramidte block **20** (90%) in the conventional way.[18] Then the spermine hydroxy block **17** was condensed with **20** in a tetrazole mediated reaction[22] in acetonitrile, followed by iodine oxidation of the intermediary phosphitetriester to give the phosphotriester block **21** (93%), which was then treated with *n*-tetrabutylammonium fluoride monohydrate in dry tetrahydrofuran at 0° C for 30 min to give the 3', 5'-dihydroxy-2'-phosphotriester block **22** (>90%), which, as expected, was found to be completely stable at room temperature. Compound **22** was then converted



(a) (MeO)-((ipr)$_2$N)-PCl / (ipr)$_2$EtN / THF, 1 h, 20°C ; (b) (i) **17** (0.7eq.) / tetrazole / MeCN, 45 min, 20°C, (ii) I_2 / THF / H_2O / pyridine, 10 min, 20°C ; (c) TBAF.H_2O / THF, 30 min, 0°C; (d) DMTrCl / pyridine, 3 h, 20°C; (e) (2-CeO)-((ipr)$_2$N)-PCl / (ipr)$_2$EtN / THF, 45 min, 20°C; (f) succinic anhydride / DMAP / CH_2Cl_2, 1.5 h, 20°C; (g), (i) 4-NO_2PhOH / DCC / pyridine / THF, 16 h, 20°C, (ii) DMF / MeIm / aminopropyl CPG, 48 h, 20°C.

to 5'-*O*-DMTr-block **23** (86%). An aliquot of this material was converted in the usual way[18] into the phosphoramidite block **24** in a quantitative yield. A second aliquot of **23** was 3'-succinylated with succinic

anhydride and DMAP in CH_2Cl_2 to give **25** (92%). The succinate block **25** was then converted into its 4-nitrophenyl ester with DCC in tetrahydrofuran and pyridine,[23] which was then reacted with 6-aminohexanamido-N^3-propyl-CPG in DMF together with *N*-methylimidazole to give, after capping with acetic anhydride, 20 μmol of bound **25** per gram CPG (**26**).

(C) Preparation of Oligonucleotides 27 - 32.

Synthesis, deprotection and purification of oligonucleotides **27** - **32** are described in the experimental section. Examination of the hplc profiles of the purified sodium exchanged oligomers reveals (Fig. 1) that having the spermine residue **15** attached either to the 5'- or 3'-end of a short oligonucleotide give strongly retarded retention times (R_t) compared to the corresponding underivatized oligomer. Thus, the spermine tethered oligomers **27** and **29** have R_t = 26.1 min and 27.5 min, respectively, which are longer than the underivatized dT_{14} (**30**) (R_t = 14.7 min) and dA_{26} (**31**) (R_t = 22.5 min). In contrast, the 14-mer oligomer **28**, in which the spermine residue **15** is attached to the middle part of the oligomer, shows a retention time of only

27: 5'-**sp**-d(TTTTTTTTTTTTTT)-3'

28: 5'-d(TTTTTT)aU(2'-**sp**)d(TTTTTTT)-3'

29: 5'-d(TTTTTTTTTTTTT)aU(2'-**sp**)-3'

30: 5'-d(TTTTTTTTTTTTT)-3'

31: 5'-d(AAAAAAAAAAAAAAAAAAAAAAAAA)-3'

32: 5'-d(TTTTTTTTTTTTTTTTTTTTTTTTT)-3'

sp = spermine

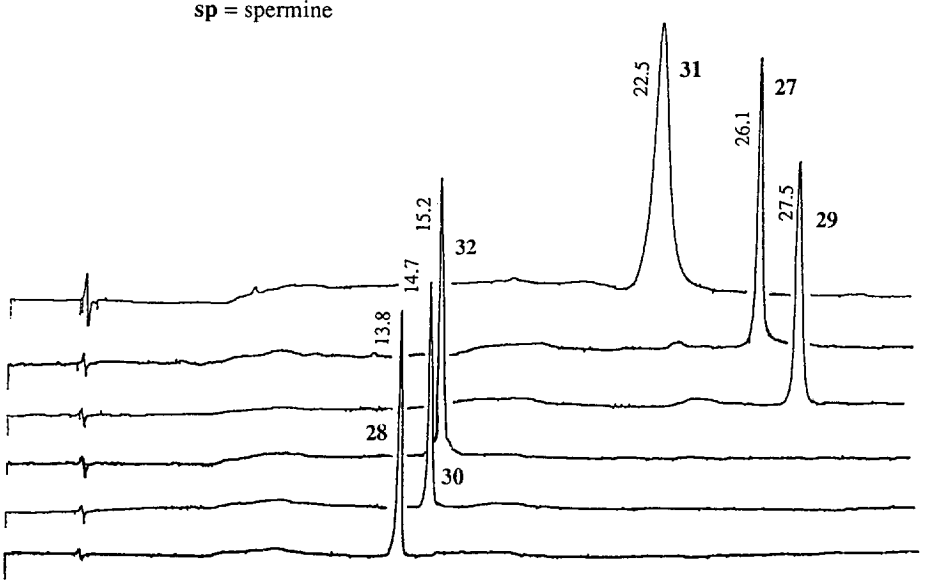


Figure 1. The Hplc profiles of purified oligonucleotides 27-32 using the gradient 0-100% B in 30 min at 1 ml / min. Buffer A 0.1M TEAA, 5% MeCN; Buffer B 0.1M TEAA, 50% MeCN. The analytical column Nucleosil NC100-5C18. Retention times (R_t) given in minutes.

13.8 min, which is similar to underivatised dT_{14} (**30**) or dT_{26} (**32**, R_t = 15.2 min). This means that when the spermine moiety is located at either of the ends of oligo-DNA (*i.e.* **27** and **29**), it has a retarding effect on the

hydrophobic column, whereas when it is located in the middle (*i.e.* **28**) it has a very little effect on its R_t compared to the natural counterpart **30**. This presumably results from a varied degree of the intramolecular electrostatic interactions between spermine cation and the phosphodiesters in **27**, **28** and **29**: (i) When the spermine moiety is located at either end of oligo-DNA, as in **27** and **29**, it can effectively ion-pair with the phosphodiester(s), and thereby neutralizing the intramolecular phosphate charge with a net increase of the hydrophobic character of the oligo-DNA, causing its larger retention on the hydrophobic column. (ii) When however spermine is located in the middle of the oligo-DNA, as in **28**, this intramolecular ion-pairing with the phosphate is minimal, and thus the hydrophobic character of **28** changes almost negligibly, causing its R_t to be very comparable to the non-tethered counterpart **30**.

(D) Melting experiments.

Three major effects most probably influence the stability of the duplexes and triplexes in a oligonucleotide system involving spermine-DNA conjugates: (i) *Electrostatic effect*, where the tetracationic part of the spermine residue can have electrostatic binding to the anionic phosphodiester linkages[24] as well as participate in the hydrogen bonding to adjacent and complementary base pairs.[25] (ii) *Hydrophobic effect*, where the hydrophobic methylenes (altogether 13 in our spermine residue) have van der Waals interactions with the nucleobases in the hydrophobic interior of a duplex or triplex.[25] This effect also involves an entropic factor where the hydrophobic residue releases structured water from the interior of a duplex or triplex to the bulk and this would be a source of positive entropy change.[4a] (iii) *Steric effect*. These effects are influenced by the structural factors such as sugar puckerings, base orientations and structural orientations along the phosphodiester linkages as well as hydration states in and around the oligonucleotide complexes.[26] One might expect that these three effects act in synergistic manner when the tethered spermine residue consists of both polar and nonpolar groups and their populations may change as the pH, salt concentration, solvent or temperature is altered. What we however observe in each melting curve is the net effect of all these three effects .

(a) Duplex formation with 27-30. Table 1 shows the melting temperatures of duplexes consisting of **27-30** and **31** at three different pH at the same NaCl and $MgCl_2$ concentrations. It is clear that only in the case of **27+31**, there is a small but distinct duplex stabilisation (ΔT_m ~+2° C) over the pH range (5.5-7.6) compared to **30+31**, whereas **29+31** shows no increased stabilisation. The oligomer **28** forms, however, a weakly associated duplex with **31** (ΔT_m ~-30° C) compared to **30+31**. This is probably because of a distorted oligomer conformation of **28**, giving steric distortions around the tethered spermine residue at the center of the duplex. It is found that the changes in pH has no effect on the stabilities of these duplexes except for **28+31** where the stability appears to decrease towards a higher pH.

Table 2 shows the melting temperatures of these duplexes at various NaCl concentrations at pH 7.0 without $MgCl_2$. Again the same consistent stabilisation (ΔT_m ~+2° C) is observed for **27+31** over 0.05 to 0.8M NaCl range, but for **29+31** a slight destabilisation (ΔT_m ~-1° C) is now observed, compared to **30+31**. For the **28+31** complex a large destabilisation is again observed (ΔT_m ~-20° to -30° C) over the 0.05 to 0.8M salt concentration. The comparison of T_m of the duplexes and the triplexes *(vide infra)* under the above conditions show that the Tris-buffer containing 0.1M NaCl, 20mM $MgCl_2$ is more or less equivalent to the phosphate-buffer containing 0.8M NaCl without $MgCl_2$.

(b) Triplex formation of ***27-30*** *with duplex [31+32].* Table 1 shows also the melting temperatures of triplexes consisting of **27-30** and the duplex **[31+32]** at three different pHs at the same NaCl and $MgCl_2$ concentrations. Here the stabilising effects of the tethered spermine are more positive and pronounced than in

Table 1. Melting temperatures (° C) (heating T_m (^hT_m) & cooling T_m (^cT_m)) of duplexes and triplexes at different pH

Hybrids (μM)	pH 5.5		pH 7.0		pH 7.6	
	^hT_m	^cT_m	^hT_m	^cT_m	^hT_m	^cT_m
Duplex						
31+27 (1:1)	48.0	*a*	48.5	*a*	48.0	*a*
31+28 (1:1)	19.0	*a*	17.5	*a*	17.5	*a*
31+29 (1:1)	46.5	*a*	46.5	*a*	47.0	*a*
31+30 (1:1)	46.0	*a*	46.0	*a*	46.0	*a*
***Triplex**[c]*						
31+32+27 (1:1:2)	19.0	20.0	19.0	19.5	19.0	21.0
31+32+28 (1:1:2)	*b*	*b*	*b*	*b*	*b*	*b*
31+32+29 (1:1:2)	22.5	21.0	23.0	22.0	21.0	21.5
31+32+30 (1:1:2)	16.0	13.5	16.5	14.0	16.0	12.5

Buffer at pH 5.5 : 50 mM AcONa/AcOH, 100 mM NaCl, 20 mM $MgCl_2$; Buffer at pH 7.0 & 7.6: 25 mM TrisHCl, 100 mM NaCl, 20 mM $MgCl_2$; *a* : not measured; *b* : not detected; *c* : ^hT_m of duplex **31+32** was ~58° C for all pH.

Table 2. Melting temperatures (° C) (heating T_m (^hT_m)) of duplexes at various NaCl concentrations at pH 7.0 in 10 mM NaH_2PO_4 / Na_2HPO_4, 0.1 mM EDTA $2Na^+$.

Hybrids (μM)	0.05M	0.1M	0.2M	0.4M	0.8M	1.2M
31+32 (1:1)	44.0	48.0	53.0	58.0	62.0	64.5
31+27 (1:1)	31.0	36.0	40.5	46.0	50.0	*a*
31+28 (1:1)	*a*	8.0	13.5	18.0	21.5	*a*
31+29 (1:1)	29.0	33.5	38.5	43.5	48.0	*a*
31+30 (1:1)	29.5	34.0	39.0	44.0	48.0	*a*

a : not measured.

the cases of the duplexes discussed above. We measured both the heating T_m (^hT_m) and cooling T_m (^cT_m) in these triplexes, showing the effect of the tethered spermine in **27-29** on the dissociation and reassociation of Hoogsteen base pair: The **[31+32]+27** and **[31+32]+28** complexes give triplex ^hT_ms which are 2.5-6.5° C higher than that for **[31+32]+30**, while the corresponding ^cT_ms are 6.5-9.0° C higher. This observation that the Δ^cT_ms are higher than the Δ^hT_ms at all three pHs with **27** and **29** compared to underivatized **30** shows that the tethered spermine residue has a stronger influence on the reassociation than during the dissociation of the third strand spermine conjugate at low salt, which is consistent with the earlier observations.[11] The hysteresis

analysis ($^{h}T_m$ - $^{c}T_m$ for a given complex) also shows that the $^{c}T_m$ in the **[31+32]+27** triplex is consistently ~1° C higher than the $^{h}T_m$, whereas the reverse is true for the **[31+32]+29** and **[31+32]+30** triplexes (ΔT_m ~-0.5° to -3.5° C), indicating that the tethered spermine in **27** orientates itself somewhat in a more sterically favored position during the reassociation of **27** to **[31+32]**.

It can be seen that the $^{h}T_m$s and the $^{c}T_m$s are largely independent of the pH range of 5.5 to 7.6. Since the stability of the TAT triad is expected to be independent of pH within this range, the observed pH independency of the third strand dissociation indicates that the spermine residue stays fully protonated in this pH range, which complies with the known pK_as of normal underivatized spermine (10.97, 10.27, 9.04 and 8.03).[27] This should mean that the electrostatic effect of our tethered *C*-branched spermine remains unaltered within this pH range, which is in serious contrast with the melting behaviour of type **2** spermine tether,[11] which shows increase of T_m as the pH is raised from 5.5 to 7.5.

Table 3 shows how the tethered spermine in **27** and **29** influences the T_ms of their triplexes with **[31+32]** at pH 7.0 at various NaCl concentrations without $MgCl_2$. No triplex formation was observed below 0.4M for **27**, **29** and **30**. Oligomer **28** did not form any detectable triplex with **[31+32]** at any salt concentration. $\Delta^{h}T_m$ between **[31+32]+27** / **[31+32]+29** and **[31+32]+30** is ~+5° C at 0.8M and rises to almost +10° C at 1.4M. In the **[31+32]+30** triplex the $^{h}T_m$ has a level-off tendency already from 1M NaCl, whereas in **[31+32]+27** and **[31+32]+29** triplexes, the $^{h}T_m$ rises more steeply and only with a slight levelling-off above 1.2M NaCl. This increase of spermine induced stabilisation of the triple helices at a higher salt concentration is possibly indicative of both a "salting out effect" giving stronger interactions between the hydrophobic spermine CH_2 moieties and the nucleobases and a decrease of water activity[26] around the ammonium cations giving stronger electrostatic interactions with phosphodiester linkages. The ($^{h}T_m$ - $^{c}T_m$) also show different tendencies for **[31+32]+27** / **[31+32]+29** versus **[31+32]+30**. In the **[31+32]+30** triplex, the ($^{h}T_m$ - $^{c}T_m$) decreases steadily from +2.5° to +0.5° C and then switches to -0.5° C at 1.4M, showing that the rate of reassociation of the third strand **30** is increased towards high salt. On the other hand, the ($^{h}T_m$ - $^{c}T_m$) for **[31+32]+27** and **[31+32]+29** triplexes shows a positive increase in the 0.8-1.4M salt range, being +1.5° to +2.0° C for **27** and +1.0° to +3.0° C for **29**. This is in accordance with earlier findings[11] that spermine tethered third strand reassociation becomes more hindered towards high salt.

Table 3. Melting temperatures (° C) (heating T_m ($^{h}T_m$) & cooling T_m ($^{c}T_m$)) of triplexes at various NaCl concentrations at pH 7.0 in 10 mM NaH_2PO_4 / Na_2HPO_4, 0.1 mM EDTA $2Na^+$.

Hybrids (μM)	$^{h}T_m$ & $^{c}T_m$	0.2M	0.4M	0.8M	1.0M	1.2M	1.4M
31+32+27	$^{h}T_m$	*b*	<7.0	20.0	25.0	32.5	38.0
(1:1:2)	$^{c}T_m$	*b*	*a*	18.5	24.5	31.0	36.0
31+32+28	$^{h}T_m$	*b*	*b*	*b*	*b*	*b*	*b*
(1:1:2)	$^{c}T_m$	*b*	*b*	*b*	*b*	*b*	*b*
31+32+29	$^{h}T_m$	*b*	<7.0	20.0	25.0	32.5	38.0
(1:1:2)	$^{c}T_m$	*b*	*a*	19.0	23.5	29.5	35.0
31+32+30	$^{h}T_m$	*b*	<7.0	15.0	21.5	25.5	28.5
(1:1:2)	$^{c}T_m$	*b*	*a*	12.5	19.5	24.0	29.0

a : not measured; *b* : not detected.

CONCLUSION

The new *C*-branched 6-(3-hydroxypropyl)-N^1,N^5,N^{10},N^{14}-tetraphenoxyacetylspermine **17** provides a convenient way of attaching the spermine residue to oligonucleotides through a phosphate linkage using standard solid-phase techniques and can be relatively easily prepared in a large quantity. The present *C*-branched spermine conjugates are the first examples of the spermine tether, wherein all four nitrogens have the same substitution level as in the natural spermine, and hence the pK_as of all four constituent nitrogens are expected to be unchanged, which is proved by unchanged triplex stabity from pH 5.5 to 7.6. Although the chirality introduced by the 6-(3-hydroxy propyl) linker at C6 makes the present *C*-branched spermine tether **17** a racemic mixture, it does not seem to have much effect on the ability of **17** to be involved in the intra and intermolecular electrostatic interaction with the phosphate.

Our melting studies have shown that the thermal stability of duplexes and triplexes decreased when the *ara*-U 2'-phosphoropropylspermine unit was incorporated into the middle of the oligonucleotide probe. The 14-mer **28** generated a destabilised duplex with dA_{26}, and it did not form a triplex with dA_{26}+dT_{26} duplex under any of our conditions. The 6-(3-propyl)spermine conjugation at the 2'- or 5'-end of oligo-DNA (*i.e.* **27** and **29**) had only a minor duplex stabilising effect compared to the underivatised **30** (Tables 1 & 2). In contrast, the spermine conjugation of **27** and **29** resulted in the triplex stabilisations with Δ^hT_ms of +2.5° to +6.5° C compared to **30**, when complexed with the dA_{26}+dT_{26} duplex, over the pH range 5.5 - 7.6 at low salt and with Mg^{2+} (Table 1). The corresponding Δ^cT_ms were consistently larger (+6.5° to +9.5° C) under these conditions and shows that the 2'- and 5'-conjugated spermine residue favourably influences the reassociation of the third strand in a very similar way as the free[6a] or conjugated linear spermine.[7-9] Also the hysteresis (^hT_m - ^cT_m) showed the same trend. Without Mg^{2+}, none of our 14-mers gave any triplex formation below 0.4M NaCl (Table 3). Over the NaCl concentration range 0.8-1.4M, the Δ^hT_ms increased from +5° to +10° C for **27** and **29** over the blank **30**. The corresponding Δ^cT_ms showed a smaller increase of +5° to +7° C, which demonstrates that the high concentrations of NaCl has a negative effect on the reassociation of these spermine tethered third strands. Also the hysteresis shows the same trend and these observations seem to be similar to those found with conjugate **2**.[11]

It has not escaped our attention that there is a direct parallel in the higher retention of 2'- or 5'-tethered oligo-DNA, **27** and **29**, on the reverse phase hplc column (presumably because of higher intramolecular electrostatic interaction between spermine-ammonium and the phosphate anion) and their enhanced abilities to stabilize the duplex and triplex than the centrally-tethered oligo-DNA **28** which has much lower R_t on the corresponding hplc column under an identical elution medium. This means that the 2'- or 5'-tethered oligo-DNA, **27** and **29**, unlike the centrally-tethered oligo DNA **28**, can also successfully induce intermolecular electrostatic interaction between spermine-ammonium and the phosphate moieties of the Watson-Crick or Hoogsteen base-paired opposite strand.

In conclusion, our present study shows that short *C*-branched spermine-tethered oligo-DNA, exemplified in our work by **27** and **29**, indeed promote uniform triple helix stabilisation over the whole pH range of 5.5 to 7.6 unlike other spermine oligonucleotide conjugates reported earlier.[7-9,11] They exhibit, however, a poor efficiency in the stabilisation of the duplexes as found in the type **2** spermine tether.[8] These are in contrast with the guanine-N^2 conjugated spermine derivative **3**, which gave a considerable duplex stabilisation.[9]

EXPERIMENTAL

^{1}H-NMR spectra were recorded in δ scale on a Jeol JNM-GX 270 spectrometer at 270 MHz, using TMS as an internal standard. ^{31}P-NMR spectra were recorded at 36 MHz in the same solvent using 85 % phosphoric acid as external standard. ^{13}C-NMR spectra were recorded at 22.5 MHz and 69 MHz in the same solvent using the solvent resonance as the internal standard. TLC was carried out using pre-coated silica gel F_{254} plates in the following dichloromethane-methanol mixtures: (A) 98: 2 (v/v), (B) 95:5 (v/v), (C) 90:10 (v/v), (D) 80:20 (v/v). Other eluents were CH_2Cl_2:EtOAc:Et_3N 45:45:10 (v/v/v) (E) and isopropanol:NH_3:H_2O 70:20:10 (v/v/v) (F). Dry pyridine was obtained by distillation over 4-toluenesulphonyl chloride. Acetonitrile and dichloromethane were distilled from P_2O_5 under argon. Dimethylformamide was distilled over CaH. All reactions were carried out at room temperature (20-23° C) unless otherwise stated. The column chromatographic separations of all the protected intermediates were carried out using Merck G 60 silica gel. A Gilson equipment with Pump Model 303, Manometric Module Model 802C and Dynamic Mixer 811B connected to a Dynamax computer program for gradient control was used for semi-preparative RP-HPLC separations on Spherisorb 5ODS2. Melting measurments were carried out with a PC-computer interfaced Hitachi Model U-3300 Spectrophotometer equipped with a thermoelectrically controlled cell holder connected to a Hitachi Temperature Controller SPR-10.

*1,4,7-heptanetriol (**9**).*

Diethyl 3-oxopimelate **8** (30 g, 148 mmol) was dissolved in dry diethylether (500 ml). Powdered $LiAlH_4$ (16.9 g, 446 mmol) was then added portionwise to the solution of **8** under reflux for 15 min. The reaction mixture was refluxed for a further period of 30 min. The suspension was filtered and washed with ether and the solid was then carefully added portionwise to water (~600 ml). The aqueous filtrate was acidified with conc. HCl to ~pH 2.5. The insoluble material was filtered off through Celite. The aqueous filtrate was then basified with conc. NH_4OH to ~pH 8.5 and the precipitate was filtered off through Celite. The aqueous filtrate was then evaporated to dryness and coevaporated with ethanol (2 x 200 ml). The brown oily residue obtained after evaporation was transfered to a conical distillation flask equipped with a short Vigreux column and distilled under vacum. The fraction distilled at ~190-205 ° C at ~0.5 mmHg was collected (8.05 g, 36%). R_f: 0.15 ($KMnO_4$ sprayed) (C); ^{1}H-NMR (CD_3OD): 3.60 (m, 5H) 2 x*C*H_2OH & *C*HOH; 1.70-1.40 (m, 8H) 4 x CH_2; ^{13}C-NMR (CD_3OD): 72.3 (C4); 63.3 (C1, C7); 35.0, 30.1 (C2, C3, C5, C6).

*4,7-dihydroxy-1-O-(4-methoxytrityl)heptane (**10**).*

Pyridine coevaporated triol **9** (14.8 g, 100 mmol) was dissolved in dry pyridine (900 ml). MMTr-Cl (40 g, 130 mmol) was dissolved in dry CH_2Cl_2 (150 ml) in a dropping funnel and was added dropwise over 4 h to the solution of **9**, under vigorous stirring and then left stirring overnight. The reaction was quenched with MeOH, the pyridine concentrated on rotavapor and the resulting concentrate extracted with aqueous ammonium bicarbonate and dichloromethane. The oily residue obtained after evaporation of the organic phase and coevaporation with toluene was then chromatographed through silica gel using 0-4% ethanol in CH_2Cl_2. The bis-MMTr product eluted at 1% EtOH and the mono-MMTr compound **10** eluted at 4% EtOH (18.2 g, 45%). The bis-MMTr product (28 g, 40.5 mmol) was treated with 200 ml 80% AcOH for 3 h and then evaporated to dryness and coevaporated with water. The residue was partitioned between water and diethylether. The aqueous phase was evaporated to dryness and the residue coevaporated with water and then pyridine, and the above tritylation reaction was repeated once more to give **10** (5.6 g, ~33%). Combined yield 78%. R_f: 0.55 (C); ^{1}H-NMR ($CDCl_3$): 7.45-6.70 (m, 14H) arom.; 3.77 (s, 3H) -OCH_3; 3.64 (m, 3H) *C*H_2OH & *C*HOH; 3.12 (m, 2H) -*C*H_2OMMTr; 2.58 (br, 2H) 2xOH; 1.65-1.40 (m, 8H) 4 x CH_2; ^{13}C-NMR ($CDCl_3$): 158.3, 144.0, 135.7, 130.1, 128.2, 127.6, 126.6, 112.8 (arom.); 86.2 (MMTr-tert-C); 71.4 (C4); 63.5(C7); 62.7 (C1); 55.0 (O<u>C</u>H_3); 34.5, 34.4, 29.1, 26.3 (C2, C3, C5, C6).

*4,7-di-O-methanesulfonyl-1-O-(4-methoxytrityl)heptane (**11**).*

Pyridine coevaporated 4,7-dihydroxy-1-*O*-MMTr-heptane **10** (23.8 g, 56.7 mmol) was dissolved in pyridine (450 ml). $MeSO_2Cl$ (22 ml, 283 mmol) was dissolved in dry CH_2Cl_2 (60 ml) in a dropping funnel and was added dropwise over 10 min to the solution of **10** under vigorous stirring at 0 °C, and then left stirring for 1 h during which time the temperature had risen to 20 °C. The reaction was quenched with MeOH, the pyridine concentrated on a rotavapor and the resulting mixture extracted with aqueous ammonium bicarbonate and dichloromethane. The oily residue after evaporation and coevaporation with toluene was then chromatographed through silica gel (Petroleum ether 10% / CH_2Cl_2 90% - 2% ethanol in CH_2Cl_2, compound **11** eluted at 0-1% EtOH). Yield 19.2 g, 59%. R_f: 0.60 (A); ^{1}H-NMR ($CDCl_3$): 7.45-6.70 (m, 14H) arom.; 4.75 (quint., 1H) CH; 4.25 (t, 2H) *C*H_2OMs; 3.79 (s, 3H) -OCH_3; 3.12 (m, 2H) -*C*H_2OMMTr; 2.99, 2.97 (2 x s, 6H) 2 x CH_3SO_2-; 1.95-1.65 (m, 8H) 4 x-CH_2-; ^{13}C-NMR ($CDCl_3$): 158.4, 144.5, 135.7, 130.1, 128.2, 127.6, 126.7, 112.9

(arom.); 86.1 (MMTr-tert-C); 82.2 (C4); 69.1 (C7); 62.5 (C1); 55.1 ($O\underline{C}H_3$); 38.6, 37.2 (2 x $\underline{C}H_3SO_2O$-; 31.3, 30.3, 25.4, 24.6 (C2, C3, C5, C6).

*4,7-diazido-1-O-(4-methoxytrityl)heptane (**12**).*

Compound **11** (19.2 g, 33.3 mmol) was dissolved in distilled DMF (260 ml), then NaN_3 (10.8 g, 166.5 mmol) was added and the resulting suspension was stirred for 18 h. The reaction mixture was filtered through Celite and the filtrate evaporated to dryness. The residue was then partitioned between water and dichloromethane. The organic phase was evaporated and coevaporated with toluene and the residue was subjected to silica gel chromatography (Petroleum ether / CH_2Cl_2 1:1 v/v) to give **12** (14.3 g, 91%). R_f: 0.90 (A); ^{1}H-NMR ($CDCl_3$): 7.50-6.78 (m, 14H) arom.; 3.79 (s, 3H) -OCH_3; 3.30 (t, 2H) CH_2N_3; 3.25 (m, 1H) CHN_3; 3.10 (m, 2H) -CH_2OMMTr; 1.82-1.55 (m, 8H) 4 x CH_2 ; ^{13}C-NMR ($CDCl_3$): 158.3, 144.0, 135.7, 130.1, 128.2, 127.6, 126.6, 112.8 (arom.); 86.2 (MMTr-tert-C); 62.5 (C1); 61.7 (C4); 54.8 ($O\underline{C}H_3$); 50.8 (C7); 31.2, 31.0, 26.3, 25.3 (C2, C3, C5, C6).

*4,7-diamino-1-O-(4-methoxytrityl)heptane (**13a**) & 4,7-diaminoheptane-1-ol (**13b**).*

Compound **12** (14.3 g, 30 mmol) was dissolved in distilled MeOH (500 ml), then 10% Pd on charcoal (3.2 g) was added. H_2 gas was then bubbled at moderate speed through the suspension for 1 h. The reaction mixture was filtered through Celite and the filtrate evaporated to dryness and the residue was then coevaporated with toluene. This crude mixture (12.5 g) contained an approximately 4:6 ratio of products **13a** and **13b** judged from TLC (R_f **13a**: 0.55 & **13b**: 0.00, F), and was used directly for the next step. NMR-data for **13a** was obtained from a small scale reaction (<10 mmol), where **13a** was the sole product. ^{1}H-NMR ($CDCl_3$): 7.45-6.80 (m, 14H) arom.; 3.79 (s, 3H) -OCH_3; 3.07 (m, 2H) -CH_2OMMTr; 2.68 (m, 3H) CH_2NH_2 & $CHNH_2$; 1.75-1.25 (m, 12H) 4 x CH_2 & 2 x NH_2; ^{13}C-NMR ($CDCl_3$): 158.3, 144.0, 135.7, 130.1, 128.2, 127.6, 126.6, 112.8 (arom.); 86.2 (MMTr-tert-C); 63.3 (C1); 54.9 ($O\underline{C}H_3$); 50.8 (C4); 42.1 (C7); 35.0, 34.6, 30.1, 26.5 (C2, C3, C5, C6).

*N^4,N^7-bis(2-cyanoethyl)-4,7-diamino-1-O-(4-methoxytrityl)heptane (**14a**) & N^4,N^7-bis(2-cyanoethyl)-4,7-diaminoheptan-1-ol (**14b**).*

The mixture of **13a** and **13b** (12.5 g, ~29.8 mmol) was dissolved in distilled DMF (5.5 ml) and then acrylonitrile (4.3 ml, 65.5 mmol) was added portionwise during 15 min and the solution was stirred 14 h. The volatile matter was then evaporated and the residue coevaporated with toluene, and applied to silica gel chromatography (0-50% EtOH in CH_2Cl_2). Product **14a** (R_f: 0.45, C) eluted at 4% EtOH and product **14b** (R_f: 0.20, D) at 30-50% EtOH. Yield **14a** (2.0 g, 13%), **14b** (2.67 g, 35%). Total N^4,N^7-bisalkylation yield 48%. **14a** ^{1}H-NMR ($CDCl_3$): 7.45-6.80 (m, 14H) arom.; 3.79 (s, 3H) OCH_3; 3.07 (t, J = 6.35 Hz, 2H) -CH_2OMMTr; 2.92 (t, J = 6.6 Hz, 2H) NHCH_2CH_2CN; 2.86 (t, J = 6.6 Hz, 2H) NHCH_2CH_2CN; 2.63 (t, J = 7.0 Hz, 2H) CH_2NH-; 2.51 (t, J = 6.6 Hz, 2H) NHCH$_2CH_2$CN; 2.51 (m, 1H) $CHNH_2$; 2.43 (t, J = 6.6 Hz, 2H) NHCH$_2CH_2$CN; 1.72-1.38 (m, 10H) 4 x CH_2 & 2 x NH; ^{13}C-NMR ($CDCl_3$): 158.2, 144.6, 135.8, 130.0, 128.2, 127.5, 126.5, 112.7 (arom.); 118.7 (CN); 118.5 (CN); 85.8 (MMTr-tert-C); 63.1 (C1), 56.2 (C4); 54.9 ($O\underline{C}H_3$); 50.0 (C7); 44.7, 41.9 (2 x NHCH$_2\underline{C}H_2$CN); 31.3, 30.2, 25.8 (C2,C3,C5,C6); 19.0, 18.4 (2 x NHCH$_2CH_2$CN). **13b** ^{1}H-NMR ($CDCl_3$): 3.60 (t, J = 5.5 Hz, 2H) -CH_2OH; 2.93 (m, 4H) 2 x NHCH_2CH_2CN; 2.66 (t, J = 7.0 Hz, 2H) CH_2NH-; 2.63 (m, 1H) CHNH; 2.53 (t, J = 6.6 Hz, 4H) 2 x NHCH$_2CH_2$CN; 2.27 (br, 3H) 2 x NH & OH; 1.69-1.46 (m, 8H) 4 x CH_2; ^{13}C-NMR ($CDCl_3$): 118.5 (2 x CN); 62.3 (C1); 56.4 (C4); 48.8 (C7); 44.6, 41.7 (2 x NHCH$_2\underline{C}H_2$CN); 31.0, 30.7, 28.7, 25.6 (C2,C3,C5,C6); 18.6, 18.3 (2 x NH$\underline{C}H_2CH_2$CN).

*6-(3-O-(phenoxyacetyl)propyl)-(N^1,N^5,N^{10},N^{14}-tetraphenoxyacetyl)-spermine (**16**).*

Anhydrous $AlCl_3$ (16.7 g, 125 mmol) was dissolved in dry diethylether (150 ml) and the solution was then carefully added to a suspension of $LiAlH_4$ (4.73 g, 125 mmol) in dry diethylether (250 ml) and stirred for 15 min under argon. The resulting mixture was then added dropwise into an anhydrous tetrahydrofuran solution (150 ml) of compound **14b** (1.26 g, 5 mmol). After stirring for 15 h, the reaction mixture was evaporated to dryness and then carefully treated with 20 ml of ice-cooled aqueous 30% KOH solution. The insoluble materials were removed by filtration through Celite, followed by filtration and washed with cold water (~20 ml). The clear alkaline filtrate was concentrated to about 20 ml and then extracted with 20% ethanol in dichloromethane (3x50 ml). The organic phase was filtered through Na_2SO_4 and evaporated. The residue (1.24 g) containing crude 6-(3-hydroxypropyl)spermine **15** was coevaporated with dry pyridine and dissolved in 40 ml dry pyridine. Then solid phenoxyacetic anhydride (13.65 g, 47.7 mmol, ~10 eq.) was added in portions and the solution stirred for 1 h. The reaction was quenched with MeOH, poured into aqueous ammonium bicarbonate and extracted with dichloromethane. The oily residue obtained after evaporation of the organic phase and coevaporation with toluene was then chromatographed through silica gel (0-2% ethanol in CH_2Cl_2) to give penta-Pac derivative **16** (1.93 g, 42% from **14b**). R_f: 0.75 (C); ^{1}H-NMR ($CDCl_3$): 7.45 (m, 1H) NH; 7.32-6.83 (m, 26H) arom. & NH; 4.62 (m, 6H) 3 x PhOCH_2; 4.45 (m, 4H) 2 x PhOCH_2; 4.13 (m, 2H)

-CH_2OPac; 3.80 (m, 1H) CH-6; 3.37-3.05 (m, 10H) CH_2-2,4,9,11,13; 1.86-1.27 (m, 12H) CH_2-3,7,8,12 & CH_2CH_2 of propyl; ^{13}C-NMR ($CDCl_3$): 168.9, 168.4, 168.3 (3 x C=O), 157.7, 157.3 (2 x C=O), 129.5, 121.7, 114.4 (arom.); 67.9, 67.1 (4 x PhO$\underline{C}H_2$CO-); 64.4 ($\underline{C}H_2$OPac); 57.0 (C6); 46.6, 45.2, 42.3, 38.1, 36.5 (C-2, 4, 9, 11, 13); 31.0, 29.9, 28.9, 27.1, 26.0, 25.6 (C-3, 7, 8, 12 & $\underline{C}H_2\underline{C}H_2$ of 6-propyl).

*6-(3-hydroxypropyl)-(N^1,N^5,N^{10},N^{14}-tetraphenoxyacetyl)-spermine (**17**).*

Compound **16** (1.93 g, 2.1 mmol) was dissolved in dry NH_3 / MeOH solution (150 ml) and stirred for 3 h, after which it was evaporated and coevaporated with toluene and the residue column chromatographed (0-6% EtOH in CH_2Cl_2) to give **16** (1.65 g, 99%). R_f: 0.50 (C); ^{1}H-NMR ($CDCl_3$): 7.45 (m, 2H) 2 x NH; 7.32-6.83 (m, 20H) arom.; 4.66 (m, 4H) 2 x PhOCH_2CO; 4.45 (m, 4H) 2 x PhOCH_2CO; 3.63 (m, 2H) -CH_2OH; 3.84 (m, 1H) CH-6; 3.39-3.12 (m, 11H) CH_2-2,4,9,11,13 & CH-6; 1.90-1.33 (m, 12H) CH_2-3,7,8,12 & CH_2CH_2 of propyl; ^{13}C-NMR ($CDCl_3$): 168.5, 168.3 (2 x C=O); 157.7, 157.2 (2 x C=O); 129.6, 121.7, 114.6, 114.4 (arom.); 68.0, 67.1 (4 x PhO$\underline{C}H_2$CO); 62.0 ($\underline{C}H_2$OH); 57.1 (C6); 53.3, 46.8, 45.2, 42.5, 38.2 ($\underline{C}$-2,4,9,11,13); 36.7, 36.4, 35.6, 31.0, 30.0, 27.2 (C-3,7,8,12 & $\underline{C}H_2\underline{C}H_2$ of propyl.

*(N^1,N^5,N^{10},N^{14}-tetraphenoxyacetyl)-spermine-6-(propyl-3-(O-2-cyanoethyl)-(N,N-diisopropyl)phosphoramidite) (**18**).*

The tetraphenoxyacetyl spermine block **17** (792 mg, 1 mmol) was dissolved in dry tetrahydrofuran (3 ml). Then dry diisopropylethylamine (700 µl, 4 mmol) was then added, followed by addition of *O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidic chloride (290 µl, 1.3 mmol) under vigorous stirring and the stirring was continued for 45 min under argon. The reaction was then quenched by addition of dry MeOH (400 µl) and continued stirring for 15 min. The crude material obtained after aqueous saturated NaCl / ethyl acetate work up and drying by filtration through Na_2SO_4 was then silica gel column chromatographed (Hexane:CH_2Cl_2:Et_3N, 50:48:2 to 0:98:2 v/v/v). Yield 990 mg, 86%. R_f: 0.65 (E); ^{1}H-NMR ($CDCl_3$): 7.44 (m, 2H) 2 x NH; 7.35-6.80 (m, 20H) arom.; 4.67 (m, 4H) 2 x PhOCH_2CO; 4.46 (m, 4H) 2 x PhOCH_2CO; 3.91-3.15 (m, 17H) -CH_2OP, OCH_2CH_2CN, CH of isopropyl & CH_2-2,4,9,11,13 & CH-6; 2.64 (m, 2H) OCH_2CH_2CN; 2.05-1.48 (m, 12H) CH_2-3,7,8,12 & CH_2CH_2 of propyl; 1.17 (d, 12H) CH_3 of isopropyl. ^{31}P-NMR ($CDCl_3$): +147.3 ppm.

*3',5'-O-1,1,3,3-tetraisopropyl-1,3-disiloxylarabinouridine-2'-(O-Methyl)-(N,N-diisopropyl)phosphoramidite (**20**).*

3',5'-*O*-1,1,3,3-tetraisopropyl-1,3-disiloxyl*ara*uridine(**19**) (2.43 g, 5 mmol) was dissolved in dry tetrahydrofuran (10 ml). Then dry diisopropylethylamine (3.5 ml, 20 mmol) was added, followed by addition of *O*-methyl-*N,N*-diisopropylphosphoramidic chloride (1.1 ml, 5.5 mmol) under vigorous stirring and the stirring was continued for 1 h under argon. Then the reaction was quenched by addition of dry MeOH (2 ml) and kept stirring for 15 min. The crude material obtained after aqueous saturated NaCl / ethyl acetate work up and drying by filtration through Na_2SO_4 was then silica gel column chromatographed (Hexane:CH_2Cl_2:Et_3N, 80:20:2 to 60:40:2 v/v/v). Yield 2.71 g, 84%. R_f: 0.75 (E); ^{1}H-NMR ($CDCl_3$): 7.95 (s, 1H) NH; 7.45, 7.32 (2 x d, $J_{5,6}$= 7.9 Hz, 1H) H6; 6.19 (m, 1H) H1'; 5.60, 5.58 (2 x d, 1H) H5; 4.55-4.35 (m, 2H) H2', H3'; 4.08 (d,d, $J_{5',5''}$ = 13.3 Hz, $J_{4',5'}$ = 2.56 Hz, 1H) H5'; 3.97 (d,d, $J_{4',5''}$ = 2.55 Hz, 1H) H5''; 3.80, 3.65 (2 x m, 1H) H4'; 3.59, 3.54 (2 x s, 3H) CH_3OP; 3.40 (m, 2H) CH of isopropylamino; 1.25-0.85 (m, 40H) CH_3 of isopropyl & CH of isopropyl-Si; ^{31}P-NMR ($CDCl_3$): +151.8, +149.6 ppm.

*3',5'-O-1,1,3,3-tetraisopropyl-1,3-disiloxyl-arabinouridine-2'-(O-methyl)-(O-(N^1,N^5,N^{10},N^{14}-tetraphenoxyacetyl)-spermine-6-(prop-3-yl)) phosphate (**21**).*

Phosphoramidite **20** (900 mg, 1.39 mmol) and 6-(3-hydroxypropyl)-N^1,N^5,N^{10},N^{14}-tetraphenoxyacetyl-spermine (**17**) (847 mg, 1.07 mmol) were coevaporated together with dry acetonitrile and then dissolved in dry acetonitrile (7.7 ml). Then tetrazole (487 mg, 6.95 mmol) was added and the resulting reaction solution was stirred under argon for 45 min. Then the reaction was quenched by addition of 1.5 ml (1.5 mmol) of 0.1M iodine in THF / pyridine / water (7:2:1 v/v/v) and stirred for 10 min. The crude residue obtained after aqueous ammonium bicarbonate / sodium thiosulfate / dichloromethane work up and drying by filtration through Na_2SO_4 was then silica gel column chromatographed (4%-6%EtOH in CH_2Cl_2). Yield 1.34 g, 93%. R_f: 0.60 (C); ^{1}H-NMR ($CDCl_3$): 8.05 (s, 1H) NH; 7.41 (m, 3H) 2 x NH & H6; 7.35-6.87 (m, 20H) arom.; 6.19 (m, 1H) H1'; 5.65 (m, 1H) H5; 5.10 (m, 1H) H3'; 4.83 (m, 1H) H2'; 4.78 (m, 4H) PhOCH_2CO; 4.48 (m, 4H) PhOCH_2CO; 4.13-3.67 (m, 9H) H4', H5', H5'', CH_3OP, $POCH_2$- & CH-6 of spermine; 3.48-3.10 (m, 10H) CH_2-2,4,9,11,13; 1.92-1.37 (m, 12H) CH_2-3,7,8,12 & CH_2CH_2 of 6-propyl spermine; 1.09-1.03 (m, 28H) CH_3 of isopropyl & CH of isopropyl-Si; ^{31}P-NMR ($CDCl_3$): +9.86(s), +8.52(s), +0.24(s), -1.61(m) ppm.

*Arabinouridine-2'-(O-methyl)-(O-(N^1,N^5,N^{10},N^{14}-tetraphenoxyacetyl)-spermine-6-(prop-3-yl))phosphate (**22**).*

Tetrahydrofuran coevaporated **21** (1.3 g, 0.96 mmol) was dissolved in dry tetrahydrofuran (6 ml). The solution was cooled to 0 °C and then 1.54 ml (1.54 mmol) 1M TBAF x H_2O was added and the resulting solution was stirred for 30 min at 0° C. The solution was evaporated and coevaporated with toluene and the residue triturated with hexane and silica gel column chromatographed (4%-8%EtOH in CH_2Cl_2). Yield 1.03 g, 97%. R_f: 0.45 (C); ^{1}H-NMR ($CDCl_3$): 7.70 (m, 1H) H6; 7.46 (m, 2H) NH; 7.35-6.84 (m, 21H) arom. & NH; 6.22 (m, 1H) H1'; 5.65 (m, 1H) H5; 5.05 (m, 1H) H3'; 4.85 (m, 1H) H2'; 4.66 (m, 4H) PhOCH_2CO; 4.46 (m, 4H) PhOCH_2CO; 4.02-3.63 (m, 9H) H4', H5', H5", CH_3OP, $POCH_2$- & CH-6 of spermine; 3.43-3.13 (m, 10H) CH_2-2,4,9,11,13; 2.02 (br, 2H) OH; 1.91-1.37 (m, 12H) CH_2-3,7,8,12 & CH_2CH_2 of 6-propyl spermine; ^{31}P-NMR ($CDCl_3$): -0.15 to -0.68 (m) ppm.

*5'-O-(4,4'-dimethoxytrityl)-arabinouridine-2'-(O-methyl)-(O-(N^1,N^5,N^{10},N^{14}-tetraphenoxyacetyl)-spermine-6-(prop-3-yl))phosphate (**23**).*

Pyridine coevaporated dihydroxy block **22** (1.0 g, 0.9 mmol) was dissolved in dry pyridine (9 ml) and then DMTr-Cl (532 mg, 1.62 mmol) was added in four portions every 30 min. Within 2.5 h almost all starting compound **22** had reacted and the reaction was quenched by addition of MeOH. After 15 min stirring, the reaction mixture was poured into aqueous ammonium bicarbonate and extracted with dichloromethane. The residue obtained from evaporation of the organic phase was then subjected to silica gel column chromatographed (0-6%EtOH in CH_2Cl_2, product eluted at 4-6%). Yield 1.1 g, 86%. R_f: 0.55 (C); ^{1}H-NMR ($CDCl_3$): 7.72 (m, 1H) H6; 7.45-6.79 (m, 36H) arom. & 3 x NH; 6.25 (m, 1H) H1'; 5.42 (m, 1H) H5; 4.86-4.72 (m, 2H) H2', H3'; 4.65 (m, 4H) PhOCH_2CO; 4.47 (m, 4H) PhOCH_2CO; 4.09-3.70 (m, 4H) H4', $POCH_2$- & CH-6 of spermine; 3.77 (s, 6H) OCH_3; 3.62 (m, 3H), CH_3OP; 3.46 (m, 2H) H5', H5"; 3.45-3.14 (m, 10H) CH_2-2,4,9,11,13; 1.91-1.36 (m, 12H) CH_2-3,7,8,12 & CH_2CH_2 of 6-propyl spermine; ^{31}P-NMR ($CDCl_3$): +0.37 to -0.32 (m) ppm.

*5'-O-(4,4'-dimethoxytrityl)-arabinouridine-2'-(O-methyl)-(O-(N^1,N^5,N^{10},N^{14}-tetraphenoxyacetyl)-spermine-6-(prop-3-yl))phosphate-3'-(O-(2-cyanoethyl)-(N,N-diisopropyl)phosphoramidite (**24**).*

3'-hydroxy block **23** (353 mg, 0.25 mmol) was dissolved in dry tetrahydrofuran (800 µl). Then dry diisopropylethylamine (174 µl, 1 mmol) was added, followed by addition of *O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidic chloride (78 µl, 0.35 mmol) under vigorous stirring and the stirring was continued for a further period of 45 min under argon. The reaction was quenched by addition of dry MeOH (100 µl) and continued stirring for 15 min. The crude material obtained after aqueous saturated NaCl / ethyl acetate work up and drying by filtration through Na_2SO_4 was then silica gel column chromatographed (EtOH:CH_2Cl_2:Et_3N, 0:98:2 to 1.5:96.5:2 v/v/v). Yield 407 mg, quant.. R_f: 0.60 (C); ^{1}H-NMR ($CDCl_3$): 7.46-6.80 (m, 37H) arom., H& & 3 x NH; 6.19 (m, 1H) H1'; 5.50 (d, $J_{5,6}$ = 8.4 Hz, 1H) H5; 4.93 (m, 1H) H2'; 4.74-4.43 (m, 9H) 2 x PhOCH_2CO & H3'; 4.22 (m, 1H) H4'; 3.77 (s, 6H) OCH_3; 3.92-3.45 (m, 10H), CH_3OP, $POCH_2$-, CH-6 of spermine, OCH_2CH_2CN & CH of isopropyl; 3.44 (m, 2H) ; 3.45-3.14 (m, 12H) H5', H5", CH_2-2,4,9,11,13; 2.67, 2.44 (2 x m, 2H) OCH_2CH_2CN; (1.87-1.35 (m, 12H) CH_2-3,7,8,12 & CH_2CH_2 of 6-propyl spermine; 1.17 (d, 12H) CH_3 of isopropyl; ^{31}P-NMR ($CDCl_3$): +151.5, +150.9, +150.4, -0.18, -0.30, -1.95 ppm.

*5'-O-(4,4'-dimethoxytrityl)-arabinouridine-2'-[(O-methyl)-(O-(N^1,N^5,N^{10},N^{14}-tetraphenoxyacetyl)-spermine-6-(prop-3-yl))phosphate]-3'-ammonium succinate (**25**).*

3'-hydroxy block **23** (395 mg, 0.28 mmol) was dissolved in dry CH_2Cl_2 (1.5 ml). Then 4-(*N*,*N*-dimethylamino)pyridine (68 ml, 0.56 mmol) was added, followed by addition of succinic anhydride (56 ml, 0.56 mmol) and the solution was stirred for 90 min. Then the reaction mixture was extracted first with 0.1M citric acid and then with aqueous ammonium bicarbonate. The organic phase was dried by filtration through Na_2SO_4 and then evaporated to dryness. The residue consisting of **25** was used directly for coupling to CPG without further purification. Yield 393 mg, 92%). R_f: 0.40 (C); ^{1}H-NMR ($CDCl_3$): 7.46-6.76 (m, 37H) arom. H6 & 3 x NH; 6.07 (m, 1H) H1'; 5.45 (m, 1H) H5; 5.26 (m, 1H) H3'; 4.88 (m, 1H) H2'; 4.63 (m, 4H) PhOCH_2CO; 4.45 (m, 4H) PhOCH_2CO; 4.20 (m, 1H) H4'; 4.00-3.50 (m, 6H) $POCH_2$-, CH_3OP & CH-6 of spermine; 3.77 (s, 6H) OCH_3; 3.45-3.09 (m, 12H) H5', H5", CH_2-2,4,9,11,13; 2.78-2.37 (m, 8H) CH_2CH_2 of succinyl & NH_4^+; 1.86-1.36 (m, 12H) CH_2-3,7,8,12 & CH_2CH_2 of 6-propyl spermine; ^{31}P-NMR ($CDCl_3$): -0.37 to -1.93 (m) ppm.

*5'-O-(4,4'-dimethoxytrityl)-arabinouridine-2'-[(O-methyl)-(O-(N^1,N^5,N^{10},N^{14}-tetraphenoxyacetyl)-spermine-6-(prop-3-yl))phosphate]-3'-succinamido-N^6-hexanamido-N^3-propyl-CPG (**26**).*

3'-succinate block **25** (393 mg, 0.256 mmol) and 4-nitrophenol (43 mg, 0.307 mmol) were coevaporated

with pyridine and then dissolved in dry tetrahydrofuran (2 ml) and dry pyridine (200µl). Then DCC (74 mg, 0.358 mmol) was added and the solution was stirred for 16 h. The suspension was rapidly vacuum filtered, the filtrate evaporated and dissolved in dry DMF (2 ml) followed by addition of N-methylimidazole (223 µl, 1.28 mmol) and 6-aminohexanamido-N^3-propyl-CPG (250 mg, 65 µmol NH_2 / g). The suspension was shaken for 48 h, then filtered and thoroughly washed with DMF, pyridine, methanol, dichloromethane and tertrahydrofuran. The support was then suspended in dry tetrahydrofuran (2.5 ml) and then MeIm (150 µl), lutidine (250 µl) and acetic anhydride (250 µl) were added and the suspension shaken for 1 h, after which the suspension was filtered and thoroughly washed with pyridine, methanol, dichloromethane and diethylether and then vacuum dried over P_2O_5. DMTr release with acid and measurment at 498 nm showed a loading of 20 µmol / g CPG.

Synthesis, deprotection and purification of oligonucleotides **27 - 32** .

All oligonucleotides were synthesised on 1.0 µmol scale either with an 8-channel Applied Biosystems 394 DNA / RNA synthesiser or with an 8-channel Pharmacia Gene Assembler Special with conventional β-cyanoethyl phosphoramidite chemistry. For synthesis of oligomers **27**, **28** and **30** - **32** standard CPG support was used, while support **26** was used for synthesis of oligomer **29**. The standard amidites (of dA & dT) and amidite blocks **18** and **24** were dissolved in dry acetonitrile with final concentration of 0.1M (for Gene Assembler) or 0.05M (for ABI 394). Standard coupling times were used (1.5 min for Gene Assembler, 30 s for ABI) for all amidites. The coupling efficiency, judged from DMTr release, was shown to be the same for all amidites (>98%). All oligomers except for oligomer **27** and **31**, were synthesised "5'-*O*-trityl on". After synthesis, oligomers **30** - **32** were treated with conc. aq. NH_3 in the automatic mode. Oligomer **31** was then treated with aqueous NH_3 at 60° C for 16h. In each case of oligomers **27** - **29**, the solid support was transfered directly out from the cassette to a 100 ml RB flask containing 40 ml of a mixture of conc. aq. NH_3 and 40% aq. $MeNH_2$ (1:1 v/v) and stirred for 24 h. The crude non-(**27**, **31**) and 5'-*O*-tritylated (**28** - **30** & **32**) oligomers were purified on reverse-phase HPLC (semi-preparative column Spherisorb 5ODS2) using the following gradient solvents: **A** (0.1M triethylammonium acetate, 5% MeCN, pH 7.0; **B** (0.1M triethylammonium acetate, 50% MeCN, pH 7.0). For oligomers **27** (5'-sp), **29** (5'-*O*-DMTr), **31** and **32** (5'-*O*-DMTr), a linear gradient of 0 - 100% buffer **B** over 50 min at a flow of 1 ml / min was used. For oligomers **28** (5'-DMTr) and **30**, a linear gradient of 0 - 35% buffer **B** over 40 min was used. After collection, the solutions of the 5'-*O*-tritylated oligomers were evaporated and lyophilized (4 x 1ml H_2O) and then dissolved in 2-3 ml 80% AcOH and stirred for 15 min and then evaporated and neutralized with water and triethylamine, followed by evaporation. The residue was dissolved in water and extracted with diethylether. The water phase was then evaporated and the residue lyophilized (6 x 1 ml H_2O). The non-tritylated oligomers **27** and **31** were evaporated and directly lyophilized (7 x 1ml H_2O). All the oligomers were subsequently sodium exchanged through a column of Dowex-50 Na^+-form, the collected solutions evaporated and the A_{260} optical units were determined.

Melting measurments.

UV melting profiles were obtained by scanning A_{260} absorbance versus time at a heating / cooling rate of 0.5° C / min. The T_ms were calculated from the culmination point of the first derivative of the melting curves with an accuracy of +/-0.5° C. Duplex and triplex melting experiments were carried out in the following buffers: (i) 50 mM AcONa / AcOH, 100 mM NaCl, 20 mM $MgCl_2$ (pH 5.5); (ii) 10 mM Na_2HPO_4 / NaH_2PO_4, 0.1-1.6M NaCl, 0.1 mM $EDTA^{2-}$ $2Na^+$ (pH 7.0); (iii) 20 mM Tris HCl, 100 mM NaCl, 20 mM $MgCl_2$ (pH 7.0); (iv) 20 mM TrisHCl, 100 mM NaCl, 20 mM $MgCl_2$ (pH 7.6). The approximate extinction coefficients for oligonucleotides **27** - **32** were calculated as A_{260} units (OD) / µmol using the approximation formula:[28] $\varepsilon_{DpEpFpGp....KpL} = 2(\varepsilon_{DpE} + \varepsilon_{EpF} + \varepsilon_{FpG}.....+ \varepsilon_{KpL}) - \varepsilon_E - \varepsilon_F - \varepsilon_G.....-\varepsilon_K$ for an oligonucleotide sequence DpEpFpGp....KpL, giving the OD value / µmol with an estimated accuracy of 10%.

In the duplex reactions, 2.7 nmol (0.86 OD / 20 µl H_2O) of dA_{26} (**31**) and 2.7 nmol (0.3 OD / 12-15 µl H_2O) of each of the 14-mers **27** - **30** were added to 2670 µl of buffer, giving a solution of ~2.7 ml with concentrations of ~1µM of each oligomer. The solutions were heated to 70° C for 3 min and then allowed to stand for 30 min at RT. The reaction containing **31+28** was cooled at 0° C over night and the melting curve and dissociation T_m were measured with a temperature gradient 2 - 80° C (156 min). The melting curves and T_ms of the other reactions (**31+27**, **31+29** & **31+30**) were measured between 20 - 80° C (120min). In the triplex reactions, dA_{26} (**31**) (2.7 nmol, 0.86 OD / 20 µl H_2O), dT_{26} (**32**) (2.7 nmol, 0.57 OD / 20 µl H_2O) and each of the 14-mers **27** - **30** (5.4 nmol, 0.6 OD / 25-30 µl H_2O) were added to 2630 µl buffer giving 1µM, 1µM and

2μM concentrations respectively in a solution of ~2.7 ml. The solutions were heated to 70° C for 3 min, and then allowed to stand for 30 min at RT and then allowed to stand at 0° C overnight. The melting curves and the dissociation T_ms (^hT_m) and reassociation T_ms (^cT_m) of the third strand with the duplex were measured using a temperature gradient of type X° C - Y° C (Z min) - Y° C (5 min) - X° C (Z min). Gradient 1: X = 2, Y = 35, Z = 66; Gradient 2: X = 10, Y = 45, Z = 70. The melting curve of **31+32** duplex dissociation was measured with the 20 - 80° C (120min) gradient. At temperatures below ~15° C, nitrogen gas was continously passed through the sample compartment to prevent moisture condensation.

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