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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)phosphoranidite 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)phosphoranidite 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<sub>m</sub> measurements showed that, at low salt with Mg<sup>2+</sup> 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  $\sim$ +5° to +6.5° 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  $\sim$ +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<sup>2+</sup>, 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  $\sim$ +1° C, respectively, over 30 at low salt with Mg<sup>2+</sup> within the pH range 5.5 - 7.6. Convript © 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.<sup>1,2</sup> 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,<sup>3a,b</sup> alkyl groups<sup>4a,b,c</sup> and by derivatising the internucleotide phosphate linkages<sup>5a,b</sup> or by substituting it with various internucleoside aliphatic linkers.<sup>5b</sup>

External tetracationic spermine has been shown to stabilise DNA duplexes and triplexes<sup>6a,b</sup> 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*<sup>7</sup> introduced the spermine to the 5'-phosphate of their oligonucleotide in a post-solid phase synthetic step by



NH(CH<sub>2</sub>)<sub>3</sub>NH(CH<sub>2</sub>)<sub>4</sub>NH(CH<sub>2</sub>)<sub>3</sub>NH<sub>2</sub>







reacting the 5'-iodoacetamidoalkyl linked oligo-DNA with N<sup>1</sup>-(3-mercaptopropyl)spermine (1) in aqueous solution. Prakash *et al*<sup>8</sup> conjugated the spermine to the C4 of the 5-methylcytosine ( $C^{5-Me}$ ) moiety, as in 2, and introduced the spermine-conjugated block directly into oligo-DNA through the phosphoramidite based solid-phase synthesis. Schmid *et al*<sup>9</sup> 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*<sup>10</sup> 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<sup>5-Me</sup> 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<sup>2+</sup>, whereas no triplex was formed with the underivatised oligomer.<sup>7</sup> 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<sup>2+</sup>), whereas the underivatised oligomer gave no triplex. But at pH 6.0, the  $\Delta T_m$  was +3° to +6° C for the mono or di-spermine containing oligonucleotides compared to the underivatised oligomer and the trispermine containing oligomer had a  $\Delta T_m$  of - 5° C.<sup>11</sup> Oligo-DNA conjugates of type **2** was shown to give destabilised duplexes.<sup>8</sup> 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  $\Delta T_m$  varying between +15° to +25° C at pH 6.8 at low salt and no Mg<sup>2+</sup> 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<sup>5-Me</sup> 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<sup>2+</sup> and free spermine. The  $\Delta T_m$  was +12° C compared to the conjugate where the spermine was replaced by an aliphatic diamino linker.<sup>10</sup>

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 oligo-DNA. (ii) 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<sub>26</sub> (31) alone or with a 1 : 1 complex of dA<sub>26</sub> : dT<sub>26</sub> 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<sup>12a-c</sup> (8) was reduced with LiAlH<sub>4</sub> in refluxing diethylether to give 1,4,7-heptanetriol (9) (36%).<sup>13</sup> Triol 9 was then selectively monotritylated at one of the primary hydroxyls with MMTr-Cl in pyridine and CH<sub>2</sub>Cl<sub>2</sub> to give the racemic mixture of compound 10 (78%), which was converted to its bis-mesylate 11 (59%).<sup>14</sup> Bis-mesylate 11 was reacted with NaN<sub>3</sub> in DMF<sup>15</sup> 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<sup>16</sup> 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)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8%).  $N^4$ ,  $N^7$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8\%).  $N^4$ ,  $N^4$ -bis(2-cyanoethyl)-4,7-diamino-1-O-MMTr-heptane (~8\%).



(a) LiAlH<sub>4</sub> / diethylether, reflux 45 min; (b) MMTrCl / pyridine / CH<sub>2</sub>Cl<sub>2</sub>, 18 h, 20°C; (c) MsCl / pyridine / CH<sub>2</sub>Cl<sub>2</sub>, 1 h, 0-20°C; (d) NaN<sub>3</sub> / DMF, 18 h, 20°C; (e) H<sub>2</sub> / Pd / C, MeOH, 1 h, 20°C; (f) CH<sub>2</sub>=CHCN / DMF, 14 h, 20°C; (g) LiAlH<sub>4</sub> / AlCl<sub>3</sub> (1:1) / THF / diethylether, 15 h, 20°C; (h) (Pac)<sub>2</sub>O / pyridine, 1 h, 20°C; (i) NH<sub>3</sub> / MeOH, 3 h, 20°C; (j) (2-CeO)-((ipr)<sub>2</sub>N)-PCl / (ipr)<sub>2</sub>EtN / THF, 45 min, 20°C.

diaminoheptan-1-ol (14b) was then reduced with LiAlH<sub>4</sub> / AlCl<sub>3</sub> in tetrahydrofuran and diethylether<sup>17</sup> 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<sub>3</sub> 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.<sup>18</sup>

(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<sup>20</sup> and others.<sup>20</sup> 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)<sup>21</sup> to the corresponding 2'-phosphoramidte block 20 (90%) in the conventional way.<sup>18</sup> Then the spermine hydroxy block 17 was condensed with 20 in a tetrazole mediated reaction<sup>22</sup> 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)  $(MeO)-((ipr)_2N)-PCI / (ipr)_2EtN / THF, 1 h, 20^{\circ}C; (b) (i) 17 (0.7eq.) / tetrazole / MeCN, 45 min, 20^{\circ}C, (ii) 1<sub>2</sub> / THF / H<sub>2</sub>O / pyridine, 10 min, 20^{\circ}C; (c) TBAF.H<sub>2</sub>O / THF, 30 min, 0^{\circ}C; (d) DMTrCl / pyridine, 3 h, 20^{\circ}C; (e) (2-CeO)-((ipr)_2N)-PCl / (ipr)_2EtN / THF, 45 min, 20^{\circ}C; (f) succinic anhydride / DMAP / CH<sub>2</sub>Cl<sub>2</sub>, 1.5 h, 20^{\circ}C; (g), (i) 4-NO<sub>2</sub>PhOH / DCC / pyridine / THF, 16 h, 20^{\circ}C, (ii) DMF / MeIm / aminopropyl CPG, 48 h, 20^{\circ}C.$ 

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

anhydride and DMAP in CH<sub>2</sub>Cl<sub>2</sub> to give 25 (92%). The succinate block 25 was then converted into its 4nitrophenyl ester with DCC in tetrahydrofuran and pyridine,<sup>23</sup> which was then reacted with 6aminohexanamido-N<sup>3</sup>-propyl-CPG in DMF together with *N*-methylimidazole to give, after capping with acetic anhydride, 20  $\mu$ mol 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<sub>14</sub> (30) ( $R_t = 14.7$  min) and dA<sub>26</sub> (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



Figure 1. The Hplc profiles of purified oligonuclcotides 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 (Rt) 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<sup>24</sup> as well as participate in the hydrogen bonding to adjacent and complementary base pairs.<sup>25</sup> (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.<sup>25</sup> 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.<sup>4a</sup> (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.<sup>26</sup> 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<sub>2</sub> concentrations. It is clear that only in the case of 27+31, there is a small but distinct duplex stabilisation  $(\Delta T_m \sim +2^{\circ} C)$  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 ( $\Delta T_m \sim -30^{\circ} C$ ) 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<sub>2</sub>. Again the same consistent stabilisation  $(\Delta T_m \sim +2^\circ C)$  is observed for 27+31 over 0.05 to 0.8M NaCl range, but for 29+31 a slight destabilisation  $(\Delta T_m \sim -1^\circ C)$  is now observed, compared to 30+31. For the 28+31 complex a large destabilisation is again observed  $(\Delta T_m \sim -20^\circ \text{ to } -30^\circ \text{ C})$  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<sub>2</sub> is more or less equivalent to the phosphate-buffer containing 0.8M NaCl without MgCl<sub>2</sub>.

(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<sub>2</sub> concentrations. Here the stabilising effects of the tethered spermine are more positive and pronounced than in

Table 1. Melting temperatures (° C) (heating  $T_m$  (<sup>h</sup> $T_m$ ) & cooling  $T_m$  (<sup>c</sup> $T_m$ ) ) of duplexes and triplexes at different pH

Hybrids (µM)	nH 5 5				nH 7.6	
	<sup>b</sup> Tm	°T <sub>m</sub>	հT <sub>m</sub>	<sup>c</sup> Tm	hTm	ν.υ <sup>c</sup> Tm
Duplex						
<b>31+27</b> (1:1)	48.0	а	48.5	а	48.0	а
<b>31+28</b> (1:1)	19.0	а	17.5	а	17.5	а
<b>31+29</b> (1:1)	46.5	а	46.5	а	47.0	а
<b>31+30</b> (1:1)	46.0	a	46.0	а	46.0	<i>a</i>
Triplex <sup>C</sup>						
<b>31+32+27</b> (1:1:2)	19.0	20.0	19.0	19.5	19.0	21.0
<b>31+32+28</b> (1:1:2)	b	b	b	b	b	b
<b>31+32+29</b> (1:1:2)	22.5	21.0	23.0	22.0	21.0	21.5
<b>31+32+30</b> (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<sub>2</sub>; Buffer at pH 7.0 & 7.6: 25 mM TrisHCl, 100 mM NaCl, 20 mM MgCl<sub>2</sub>; a : not measured; b : not detected; c : <sup>h</sup>T<sub>m</sub> 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<sub>2</sub>PO<sub>4</sub> / Na<sub>2</sub>HPO<sub>4</sub>, 0.1 mM EDTA 2Na<sup>+</sup>.

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

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  $\Delta^cT_ms$  are higher than the  $\Delta^hT_ms$  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.<sup>11</sup> 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  ${}^{-1}^{\circ}$  C higher than the  ${}^{h}T_{m}$ , whereas the reverse is true for the [31+32]+29 and [31+32]+30 triplexes ( $\Delta T_{m} \sim 0.5^{\circ}$  to  $-3.5^{\circ}$  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<sub>a</sub>s of normal underivatized spermine (10.97, 10.27, 9.04 and 8.03).<sup>27</sup> 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,<sup>11</sup> which shows increase of T<sub>m</sub> 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<sub>2</sub>. 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  $\sim +5^{\circ}$  C at 0.8M and rises to almost  $\pm 10^{\circ}$  C at 1.4M. In the [31+32]+30 triplex the <sup>h</sup>T<sub>m</sub> has a level-off tendency already from 1M NaCl, whereas in [31+32]+27 and [31+32]+29 triplexes, the <sup>h</sup>T<sub>m</sub> 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<sub>2</sub> moleties and the nucleobases and a decrease of water activity<sup>26</sup> around the ammonium cations giving stronger electrostatic interactions with phosphodiester linkages. The (hTm - cTm) 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)+29triplexes shows a positive increase in the 0.8-1.4M salt range, being  $\pm 1.5^{\circ}$  to  $\pm 2.0^{\circ}$  C for 27 and  $\pm 1.0^{\circ}$  to  $\pm 3.0^{\circ}$ C for 29. This is in accordance with earlier findings<sup>11</sup> that spermine tethered third strand reassociation becomes more hindered towards high salt.

Hybrids (µM)	<sup>հ</sup> T <sub>m</sub> & ՙT <sub>m</sub>	0.2M	0.4M	0.8M	1.0M	1.2M	1.4M
<b>31+32+27</b>	<sup>▶</sup> Tm	b	<7.0	20.0	25.0	32.5	38.0
(1:1:2)	°Tm	b	a	18.5	24.5	31.0	36.0
<b>31+32+28</b>	<sup>հ</sup> Tm	b	b	b	b	b	b
(1:1:2)	°Tm	b	b	b	b	b	b
<b>31+32+29</b>	<sup>ь</sup> Tm	b	<7.0	20.0	25.0	32.5	38.0
(1:1:2)	°Tm	b	a	19.0	23.5	29.5	35.0
<b>31+32+30</b>	<sup>հ</sup> Tm	b	<7.0	15.0	21.5	25.5	28.5
(1:1:2)	°Tm	b	a	12.5	19.5	24.0	29.0

**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<sub>2</sub>PO<sub>4</sub> / Na<sub>2</sub>HPO<sub>4</sub>, 0.1 mM EDTA 2Na<sup>+</sup>.

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 pKas 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<sub>26</sub>, and it did not form a triplex with dA<sub>26</sub>+dT<sub>26</sub> 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  $\Delta^{h}T_{m}s$  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<sup>2+</sup> (Table 1). The corresponding  $\Delta^{c}T_{m}s$  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<sup>6a</sup> or conjugated linear spermine.<sup>7-9</sup> Also the hysteresis ( ${}^{h}T_{m} - {}^{c}T_{m}$ ) showed the same trend. Without Mg<sup>2+</sup>, 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  $\Delta^{h}T_{m}s$  increased from +5° to +10° C for 27 and 29 over the blank 30. The corresponding  $\Delta^{c}T_{m}s$  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  $R_1$  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.<sup>7-9,11</sup> They exhibit, however, a poor efficiency in the stabilisation of the duplexes as found in the type 2 spermine tether.<sup>8</sup> These are in contrast with the guanine-N<sup>2</sup> conjugated spermine derivative 3, which gave a considerable duplex stabilisation.<sup>9</sup>

#### **EXPERIMENTAL**

<sup>1</sup>H-NMR spectra were recorded in  $\delta$  scale on a Jeol JNM-GX 270 spectrometer at 270 MHz, using TMS as an internal standard. <sup>31</sup>P-NMR spectra were recorded at 36 MHz in the same solvent using 85 % phosphoric acid as external standard. <sup>13</sup>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<sub>254</sub> 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<sub>2</sub>Cl<sub>2</sub>:EtOAc:Et<sub>3</sub>N 45:45:10 (v/v/v) (E) and isopropanol:NH<sub>3</sub>:H<sub>2</sub>O 70:20:10 (v/v/v) (F). Dry pyridine was obtained by distillation over 4-toluenesulphonyl chloride. Acetonitrile and dichloromethane were distilled from P<sub>2</sub>O<sub>5</sub> under argon. Dimethylformamide was distilled over CaH. All reactions 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 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 LiAlH4 (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. NH4OH 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<sub>f</sub>: 0.15 (KMnO4 sprayed) (C); <sup>1</sup>H-NMR (CD<sub>3</sub>OD): 3.60 (m, 5H) 2 xCH<sub>2</sub>OH & CHOH; 1.70-1.40 (m, 8H) 4 x CH<sub>2</sub>; <sup>13</sup>C-NMR (CD<sub>3</sub>OD): 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<sub>2</sub>Cl<sub>2</sub> (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<sub>2</sub>Cl<sub>2</sub>. 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%. Rf: 0.55 (C); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.45-6.70 (m, 14H) arom.; 3.77 (s, 3H) -OCH<sub>3</sub>; 3.64 (m, 3H) *CH*<sub>2</sub>OH & *CHO*H; 3.12 (m, 2H) -*CH*<sub>2</sub>OMMTr; 2.58 (br, 2H) 2xOH; 1.65-1.40 (m, 8H) 4 x CH<sub>2</sub>; <sup>13</sup>C-NMR (CDCl<sub>3</sub>): 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 (OCH<sub>3</sub>); 3.4.5, 3.4.4, 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<sub>2</sub>Cl (22 ml, 283 mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (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<sub>2</sub>Cl<sub>2</sub> 90% - 2% ethanol in CH<sub>2</sub>Cl<sub>2</sub>, compound 11 eluted at 0-1% EtOH). Yield 19.2 g, 59%. R<sub>f</sub>: 0.60 (A); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.45-6.70 (m, 14H) arom.; 4.75 (quint., 1H) CH; 4.25 (t, 2H) CH<sub>2</sub>OMs; 3.79 (s, 3H) -OCH<sub>3</sub>; 3.12 (m, 2H) -CH<sub>2</sub>OMMTr; 2.99, 2.97 (2 x s, 6H) 2 x CH<sub>3</sub>SO<sub>2</sub>-; 1.95-1.65 (m, 8H) 4 x-CH<sub>2</sub>-; <sup>13</sup>C-NMR (CDCl<sub>3</sub>): 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 (OCH<sub>3</sub>); 38.6, 37.2 (2 x CH<sub>3</sub>SO<sub>2</sub>O-; 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<sub>3</sub> (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<sub>2</sub>Cl<sub>2</sub> 1:1 v/v) to give 12 (14.3 g, 91%). R<sub>f</sub>: 0.90 (A); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.50-6.78 (m, 14H) arom.; 3.79 (s, 3H) -OCH<sub>3</sub>; 3.30 (t, 2H) CH<sub>2</sub>N<sub>3</sub>; 3.25 (m, 1H) CHN<sub>3</sub>; 3.10 (m, 2H) -CH<sub>2</sub>OMMTr; 1.82-1.55 (m, 8H) 4 x CH<sub>2</sub>; <sup>13</sup>C-NMR (CDCl<sub>3</sub>): 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<u>C</u>H<sub>3</sub>); 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<sub>2</sub> 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<sub>f</sub> 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. <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.45-6.80 (m, 14H) arom.; 3.79 (s, 3H) -OCH<sub>3</sub>; 3.07 (m, 2H) -*CH*<sub>2</sub>OMMTr; 2.68 (m, 3H) *CH*<sub>2</sub>NH<sub>2</sub> & *CH*NH<sub>2</sub>; 1.75-1.25 (m, 12H) 4 x CH<sub>2</sub> & 2 x NH<sub>2</sub>; <sup>13</sup>C-NMR (CDCl<sub>3</sub>): 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 (OCH<sub>3</sub>); 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<sub>2</sub>Cl<sub>2</sub>). Product **14a** (R<sub>f</sub>: 0.45, C) eluted at 4% EtOH and product **14b** (R<sub>f</sub>: 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** <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.45-6.80 (m, 14H) arom.; 3.79 (s, 3H) OCH<sub>3</sub>; 3.07 (t, J = 6.35 Hz, 2H) -*CH*<sub>2</sub>OMMTr; 2.92 (t, J = 6.6 Hz, 2H) NH*CH*<sub>2</sub>CH<sub>2</sub>CN; 2.86 (t, J = 6.6 Hz, 2H) NH*CH*<sub>2</sub>CH<sub>2</sub>CN; 2.63 (t, J = 7.0 Hz, 2H) *CH*<sub>2</sub>NH-; 2.51 (t, J = 6.6 Hz, 2H) NH*CH*<sub>2</sub>*CH*<sub>2</sub>CN; 2.86 (t, J = 6.6 Hz, 2H) NH*CH*<sub>2</sub>*CH*<sub>2</sub>CN; 1.72-1.38 (m, 10H) 4 x CH<sub>2</sub> & 2 x NH; <sup>13</sup>C-NMR (CDCl<sub>3</sub>): 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<u>C</u>H<sub>3</sub>); 50.0 (C7); 44.7, 41.9 (2 x NHCH<sub>2</sub><u>C</u>H<sub>2</sub>CN); 31.3, 30.2, 25.8 (C2,C3,C5,C6); 19.0, 18.4 (2 x NHCH<sub>2</sub>CH<sub>2</sub>CN); 2.66 (t, J = 7.0 Hz, 2H) *CH*<sub>2</sub>NH-; 2.53 (m, 1H) *CH*NH; 2.53 (t, J = 6.6 Hz, 2H) NHCH<sub>2</sub>CH<sub>2</sub>CN; 2.27 (br; 3H) 2 x NH& OH; 1.69-1.46 (m, 8H) 4 x CH<sub>2</sub>; <sup>13</sup>C-NMR (CDCl<sub>3</sub>): 118.5 (2 x CN); 62.3 (C1); 56.4 (C4); 48.8 (C7); 44.6, 41.7 (2 x NHCH<sub>2</sub><u>C</u>H<sub>2</sub>CN); 31.0, 30.7, 28.7, 25.6 (C2,C3,C5,C6); 18.6, 18.3 (2 x NH<u>C</u>H<sub>2</sub>CH<sub>2</sub>CN).

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

Anhydrous AlCl<sub>3</sub> (16.7 g, 125 mmol) was dissolved in dry diethylether (150 ml) and the solution was then carefully added to a suspension of LiAlH<sub>4</sub> (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<sub>2</sub>SO<sub>4</sub> 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<sub>2</sub>Cl<sub>2</sub>) to give penta-Pac derivative **16** (1.93 g, 42% from **14b**). R<sub>f</sub>: 0.75 (C); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.45 (m, 1H) NH; 7.32-6.83 (m, 26H) arom. & NH; 4.62 (m, 6H) 3 x PhOCH<sub>2</sub>; 4.45 (m, 4H) 2 x PhOCH<sub>2</sub>; 4.13 (m, 2H)

-*CH*<sub>2</sub>OPac; 3.80 (m, 1H) CH-6; 3.37-3.05 (m, 10H) CH<sub>2</sub>-2,4,9,11,13; 1.86-1.27 (m, 12H) CH<sub>2</sub>-3,7,8,12 & CH<sub>2</sub>CH<sub>2</sub> of propyl; <sup>13</sup>C-NMR (CDCl<sub>3</sub>): 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 PhOCH<sub>2</sub>CO-); 64.4 (CH<sub>2</sub>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 & CH<sub>2</sub>CH<sub>2</sub> 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<sub>3</sub> / 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<sub>2</sub>Cl<sub>2</sub>) to give 16 (1.65 g, 99%). R<sub>f</sub>: 0.50 (C); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.45 (m, 2H) 2 x NH; 7.32-6.83 (m, 20H) arom.; 4.66 (m, 4H) 2 x PhOCH<sub>2</sub>CO; 4.45 (m, 4H) 2 x PhOCH<sub>2</sub>CO; 3.63 (m, 2H) -CH<sub>2</sub>OH; 3.84 (m, 1H) CH-6; 3.39-3.12 (m, 11H) CH<sub>2</sub>-2,4,9,11,13 & CH-6; 1.90-1.33 (m, 12H) CH<sub>2</sub>-3,7,8,12 & CH<sub>2</sub>CH<sub>2</sub> of propyl; <sup>13</sup>C-NMR (CDCl<sub>3</sub>): 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 PhOCH<sub>2</sub>CO); 62.0 (CH<sub>2</sub>OH); 57.1 (C6); 53.3, 46.8, 45.2, 42.5, 38.2 (C-2,4,9,11,13); 36.7, 36.4, 35.6, 31.0, 30.0, 27.2 (C-3,7,8,12 & CH<sub>2</sub>CH<sub>2</sub> 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  $\mu$ l, 4 mmol) was then added, followed by addition of O-(2-cyanoethyl)-N.N-diisopropylphosphoramidic chloride (290  $\mu$ l, 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  $\mu$ l) and continued stirring for 15 min. The crude material obtained after aqueous saturated NaCl / ethyl acetate work up and drying by filtration through Na<sub>2</sub>SO<sub>4</sub> was then silica gel column chromatographed (Hexane:CH<sub>2</sub>Cl<sub>2</sub>:Et<sub>3</sub>N, 50:48:2 to 0:98:2 v/v/v). Yield 990 mg, 86%. R<sub>f</sub>: 0.65 (E); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.44 (m, 2H) 2 x NH; 7.35-6.80 (m, 20H) arom.; 4.67 (m, 4H) 2 x PhOCH<sub>2</sub>CO; 4.46 (m, 4H) 2 x PhOCH<sub>2</sub>CO; 3.91-3.15 (m, 17H) -CH<sub>2</sub>OP, OCH<sub>2</sub>CH<sub>2</sub>CN, CH of isopropyl & CH<sub>2</sub>-2,4,9,11,13 & CH-6; 2.64 (m, 2H) OCH<sub>2</sub>CH<sub>2</sub>CN; 2.05-1.48 (m, 12H) CH<sub>2</sub>-3,7,8,12 & CH<sub>2</sub>CH<sub>2</sub>CD gropyl; 1.17 (d, 12H) CH<sub>3</sub> of isopropyl. <sup>31</sup>P-NMR (CDCl<sub>3</sub>): +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-disiloxylarauridine(**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<sub>2</sub>SO<sub>4</sub> was then silica gel column chromatographed (Hexane:CH<sub>2</sub>Cl<sub>2</sub>:Et<sub>3</sub>N, 80:20:2 to 60:40:2 v/v/v). Yield 2.71 g, 84%. R<sub>f</sub>: 0.75 (E); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.95 (s, 1H) NH; 7.45, 7.32 (2 x d, J<sub>5,6</sub>= 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<sub>5',5''</sub> = 13.3 Hz, J<sub>4',5'</sub> = 2.56 Hz, 1H) H5'; 3.97 (d,d, J<sub>4',5''</sub> = 2.55 Hz, 1H) H5'; 3.80, 3.65 (2 x m, 1H) H4'; 3.59, 3.54 (2 x s, 3H) CH<sub>3</sub>OP; 3.40 (m, 2H) CH of isopropylamino; 1.25-0.85 (m, 40H) CH<sub>3</sub> of isopropyl & CH of isopropyl-Si; <sup>31</sup>P-NMR (CDCl<sub>3</sub>): +151.8, +149.6 ppm.

## 3',5'-O-1,1,3,3-tetraisopropyl-1,3-disiloxyl-arabinouridine-2'-(O-methyl)-(O-(N<sup>1</sup>,N<sup>5</sup>,N<sup>10</sup>,N<sup>14</sup>-tetraphenoxy-acetyl)-spermine-6-(prop-3-yl)) phosphate (**21**).

Phosphoramidite **20** (900 mg, 1.39 mmol) and 6-(3-hydroxypropyl)- $N^1$ , $N^5$ , $N^{10}$ , $N^{14}$ -tetraphenoxyacetylspermine (**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<sub>2</sub>SO<sub>4</sub> was then silica gel column chromatographed (4%-6%EtOH in CH<sub>2</sub>Cl<sub>2</sub>). Yield 1.34 g, 93%. R<sub>f</sub>: 0.60 (C); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 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<sub>2</sub>CO; 4.48 (m, 4H) PhOCH<sub>2</sub>CO; 4.13-3.67 (m, 9H) H4', H5', H5'', CH<sub>3</sub>OP, POCH<sub>2</sub>- & CH-6 of spermine; 3.48-3.10 (m, 10H) CH<sub>2</sub>-2,4,9,11,13; 1.92-1.37 (m, 12H) CH<sub>2</sub>-3,7,8,12 & CH<sub>2</sub>CH<sub>2</sub> of 6-propyl spermine; 1.09-1.03 (m, 28H) CH<sub>3</sub> of isopropyl & CH of isopropyl-Si; <sup>31</sup>P-NMR (CDCl<sub>3</sub>): +9.86(s), +8.52(s), +0.24(s), -1.61(m) ppm. Arabinouridine-2'-(O-methyl)-(O-(N<sup>1</sup>,N<sup>5</sup>,N<sup>10</sup>,N<sup>14</sup>-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<sub>2</sub>O 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<sub>2</sub>Cl<sub>2</sub>). Yield 1.03 g, 97%. R<sub>f</sub>: 0.45 (C); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 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<sub>2</sub>CO; 4.46 (m, 4H) PhOCH<sub>2</sub>CO; 4.02-3.63 (m, 9H) H4', H5', H5'', CH<sub>3</sub>OP, POCH<sub>2</sub>- & CH-6 of spermine; 3.43-3.13 (m, 10H) CH<sub>2</sub>-2,4,9,11,13; 2.02 (br, 2H) OH; 1.91-1.37 (m, 12H) CH<sub>2</sub>-3,7,8,12 & CH<sub>2</sub>CH<sub>2</sub> of 6-propyl spermine; <sup>31</sup>P-NMR (CDCl<sub>3</sub>): -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<sub>2</sub>Cl<sub>2</sub>, product eluted at 4-6%). Yield 1.1 g, 86%. R<sub>f</sub>: 0.55 (C); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 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<sub>2</sub>CO; 4.47 (m, 4H) PhOCH<sub>2</sub>CO; 4.09-3.70 (m, 4H) H4', POCH<sub>2</sub>-& CH-6 of spermine; 3.77 (s, 6H) OCH<sub>3</sub>; 3.62 (m, 3H), CH<sub>3</sub>OP; 3.46 (m, 2H) H5', H5''; 3.45-3.14 (m, 10H) CH<sub>2</sub>-2,4,9,11,13; 1.91-1.36 (m, 12H) CH<sub>2</sub>-3,7,8,12 & CH<sub>2</sub>CH<sub>2</sub> of 6-propyl spermine; <sup>31</sup>P-NMR (CDCl<sub>3</sub>): +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  $\mu$ l). Then dry diisopropylethylamine (174  $\mu$ l, 1 mmol) was added, followed by addition of O-(2-cyanoethyl)-N,N-diisopropylphosphoramidic chloride (78  $\mu$ l, 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  $\mu$ l) and continued stirring for 15 min. The crude material obtained after aqueous saturated NaCl / ethyl acetate work up and drying by filtration through Na<sub>2</sub>SO<sub>4</sub> was then silica gel column chromatographed (EtOH:CH<sub>2</sub>Cl<sub>2</sub>:Et<sub>3</sub>N, 0:98:2 to 1.5:96.5:2 v/v/v). Yield 407 mg, quant.. R<sub>f</sub>: 0.60 (C); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 7.46-6.80 (m, 37H) arom., H& & 3 x NH; 6.19 (m, 1H) H1'; 5.50 (d, J<sub>5,6</sub> = 8.4 Hz, 1H) H5; 4.93 (m, 1H) H2'; 4.74-4.43 (m, 9H) 2 x PhOCH<sub>2</sub>CO & H3'; 4.22 (m, 1H) H4'; 3.77 (s, 6H) OCH<sub>3</sub>; 3.92-3.45 (m, 10H), CH<sub>3</sub>OP, POCH<sub>2</sub>-, CH-6 of spermine, OCH<sub>2</sub>CH<sub>2</sub>CN & CH of isopropyl; 3.44 (m, 2H) ; 3.45-3.14 (m, 12H) H5', H5'', CH<sub>2</sub>-2,4,9,11,13; 2.67, 2.44 (2 x m, 2H) OCH<sub>2</sub>CH<sub>2</sub>CN; (1.87-1.35 (m, 12H) CH<sub>2</sub>-3,7,8,12 & CH<sub>2</sub>CH<sub>2</sub> of 6-propyl spermine; 1.17 (d, 12H) CH<sub>3</sub> of isopropyl; <sup>31</sup>P-NMR (CDCl<sub>3</sub>): +151.5, +150.9, +150.4, -0.18, -0.30, -1.95 ppm.

## 5'-O-(4,4'-dimethoxytrityl)-arabinouridine-2'-[(O-methyl)-(O-(N<sup>1</sup>,N<sup>5</sup>,N<sup>10</sup>,N<sup>14</sup>-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<sub>2</sub>Cl<sub>2</sub> (1.5 ml). Then 4-(N,N-

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<sub>2</sub>SO<sub>4</sub> 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<sub>f</sub>: 0.40 (C); <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 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<sub>2</sub>CO; 4.20 (m, 1H) H4'; 4.00-3.50 (m, 6H) POCH<sub>2</sub>-, CH<sub>3</sub>OP & CH-6 of spermine; 3.77 (s, 6H) OCH<sub>3</sub>; 3.45-3.09 (m, 12H) H5', H5'', CH<sub>2</sub>-2,4,9,11,13; 2.78-2.37 (m, 8H) CH<sub>2</sub>CH<sub>2</sub> of succinyl & NH<sub>4</sub><sup>+</sup>; 1.86-1.36 (m, 12H) CH<sub>2</sub>-3,7,8,12 & CH<sub>2</sub>CH<sub>2</sub> of 6-propyl spermine; <sup>31</sup>P-NMR (CDCl<sub>3</sub>): -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<sup>3</sup>-propyl-CPG (250 mg, 65 µmol NH<sub>2</sub>/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<sub>2</sub>O<sub>5</sub>. 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  $1.0 \,\mu$ mol scale either with an 8-channel Applied Biosystems

394 DNA / RNA synthesiser or with an 8-channel Pharmacia Gene Assembler Special with conventional  $\beta$ 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. NH3 in the automatic mode. Oligomer 31 was then treated with aqueous NH3 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. NH3 and 40% aq. MeNH<sub>2</sub> (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<sub>2</sub>O) 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<sub>2</sub>O). All the oligomers were subsequently sodium exchanged through a column of Dowex-50 Na<sup>+</sup>-form, the collected solutions evaporated and the A<sub>260</sub> 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<sub>m</sub>s 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<sub>2</sub> (pH 5.5); (ii) 10 mM Na<sub>2</sub>HPO<sub>4</sub> / NaH<sub>2</sub>PO<sub>4</sub>, 0.1-1.6M NaCl, 0.1 mM EDTA<sup>2-</sup> 2Na<sup>+</sup> (pH 7.0); (iii) 20 mM Tris HCl, 100 mM NaCl, 20 mM MgCl<sub>2</sub> (pH 7.0); (iv) 20 mM TrisHCl, 100 mM NaCl, 20 mM MgCl<sub>2</sub> (pH 7.6). The approximate extinction coefficients for oligonucleotides **27** - **32** were calculated as  $A_{260}$  units (OD) / µmol using the approximation formula:<sup>28</sup>  $\varepsilon_{DpEpFpGp....KpL} = 2(\varepsilon_{DpE} + \varepsilon_{EpF} + \varepsilon_{FpG....} + \varepsilon_{KpL}) - \varepsilon_E - \varepsilon_F - \varepsilon_G.....-\varepsilon_K$  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  $\mu$ l H<sub>2</sub>O) of dA<sub>26</sub> (**31**) and 2.7 nmol (0.3 OD / 12-15  $\mu$ l H<sub>2</sub>O) of each of the 14-mers **27** - **30** were added to 2670  $\mu$ l of buffer, giving a solution of ~2.7 ml with concentrations of ~1 $\mu$ M 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<sub>m</sub> were measured with a temperature gradient 2 - 80° C (156 min). The melting curves and T<sub>m</sub>s of the other reactions (**31+27**, **31+29** & **31+30**) were measured between 20 - 80° C (120min). In the triplex reactions, dA<sub>26</sub> (**31**) (2.7 nmol, 0.86 OD / 20  $\mu$ l H<sub>2</sub>O), dT<sub>26</sub> (**32**) (2.7 nmol, 0.57 OD / 20  $\mu$ l H<sub>2</sub>O) and each of the 14-mers **27** - **30** (5.4 nmol, 0.6 OD / 25-30  $\mu$ l H<sub>2</sub>O) were added to 2630  $\mu$ l buffer giving 1 $\mu$ M, 1 $\mu$ M and

 $2\mu$ M 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<sub>m</sub>s (<sup>h</sup>T<sub>m</sub>) and reassociation T<sub>m</sub>s (<sup>c</sup>T<sub>m</sub>) 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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