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Synthesis of 1'-Phenazine-Tethered Psicofuranosyl Oligonucleotides: The Thermal Stability and Fluorescence Properties of Their Duplexes and Triplexes

D. Ossipov and J. Chattopadhyaya*

Department of Bioorganic Chemistry, Box 581, Biomedical Center, University of Uppsala, Sweden

E-mail: jyoti@bioorgchem.uu.se

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Abstract: The synthesis of modified oligonucleotides (ODNs), tethered with phenazine (pzn) at C1' of 1-(3'-deoxypsicofuranosyl)uracil, the thermal stability, and fluorescence properties of their duplexes and triplexes are described. The key intermediates, the psicofuranosyluracil derivatives **7** and **8** with phenazine 1'-tethered through a linker composed of a phosphate and two methylene groups, were synthesized from 1-(3'-deoxypsicofuranosyl)uracil **5** [**5** → **6** (26%) → **7** (62%) or **8** (70%)]. Compound **7** was converted into the corresponding 6'-*O*-DMTr protected 4'-phosphoramidite block **13** in three steps [**7** → **9** (83%) → **11** (83%) → **13** (83%)], whereas compound **8** was used in the preparation of the modified solid support **14** in four steps [**8** → **10** (28%) → **12** (70%) → **14**]. Modified 9-mer ODNs **28** - **31** and 18-mer ODNs **22** - **25** were then assembled in a usual manner using automated solid-phase DNA synthesis protocol. The phenazine-tethered 9-mers (**28** - **31**) were tested for their ability to form stable duplexes with target DNA-strands (**19** - **20**). The phenazine-tethered 18-mers (**22** - **25**) were tested for their ability to form stable triplexes with 24-mer and 29-mer duplex targets (**15•16** and **17•18**). Triplexes consisting of modified ODNs with pzn at the 5'-terminal (as in **24**) and at 3',5'-terminals (as in **25**) were more stable than the unmodified parent triplex. No triplex was found to have formed with modified ODNs with pzn attached at 3'- or at the middle of the strand at neutral pH (7.3), but triplex formation was observed at acidic pH (6.0) although they were less stable than the unmodified parent triplex. The same trend was observed for duplexes. The fluorescence intensity of pzn in the modified triplexes was enhanced and blue-shifted by ~13 nm relative to the single strand. In contrast, the changes in fluorescence intensities of pzn in the modified duplexes were relatively less compared to the triplexes. The fluorescence intensity increased proportionally as the thermal stabilities of the triplexes increased. A comparison of the fluorescent intensity changes (ΔF) shows that the fluorophore in duplexes ($\Delta F \approx -1.2$ to $+1.5$) experiences relatively minor change in the microenvironment compared to that of the triplexes ($\Delta F \approx 1.5$ to 4.5). Nevertheless, in both cases the phenazine residue most probably interacts with the neighbouring nucleobases as a weak exterior binder.

Oligonucleotides and their chemically modified analogues are potentially useful for arresting translation by specifically recognising and binding to messenger RNA (antisense approach).[1,2] They may also impede transcription by binding to the major groove of double-stranded DNA (antigene approach).[3,4] In the latter approach, antisense oligodeoxynucleotides forming triplexes (TFOs) may be used as therapeutic agents to artificially control the expression of regulatory genes that have stimulated considerable interest in DNA

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triplexes.[5] Triplex instability under physiological conditions represents a major limiting difficulty to the therapeutic use of TFOs, since the C^+-G•C triplet requires low pH and the T-A•T triplet is only stable under conditions of high ionic strength. One strategy to improve triplex stability is to covalently attach DNA ligands to the third strand.[6,7] To this end, both intercalators and groove binding ligands have been evaluated[8] to assess possible triplex-specific binding properties. The accumulated data reveals that mode and strength of interaction of conjugated ligands with the target duplex depends on many factors such as (i) the type of the intercalator and its sequence specificity, (ii) the type and the length of the linker, (iii) the position of attachment of the linker to the ligand and to the oligonucleotide chain. Usually the base residues, internucleotidic phosphodiester bonds or 3'(5')-terminals of the sugar residue have been modified[9] to attach various conjugate groups. This brings about distortion of ionic and/or tautomeric properties of the structural units and, thereby affecting the behaviour of the entire modified oligomer toward the target. Therefore, nucleoside analogues which possess the ionic and tautomeric properties and all functional groups of the natural 2'-deoxyribonucleosides, but display additional functionalities for further derivatisation, are of considerable interest. Hence, 3'-deoxypsicofuranosyl nucleosides which fulfil these requirements have been used for derivatization and synthesis of different oligonucleotide conjugates, which have so far been used only for studying the thermal stability of their duplexes with DNA and RNA targets.[10-16]

In this regard, Azhaev *et al.*[10] reported on the preparation of branched oligonucleotides starting from 3'-deoxy-β-D-psicothymidine. Phosphoramidite of 3'-deoxy-β-D-psicouridine carrying a covalently bound terpyridine at 1'-position has been synthesised by Bashkin *et al.*[11], which could be used for automated solid phase DNA synthesis. The tethering of intercalators such as anthraquinone and pyrene through C1' of psicofuranose to synthesize different oligonucleotide conjugates resulted in enhanced stability in some cases of DNA-DNA and DNA-RNA duplexes.[12,13]

However, no oligonucleotide analogue conjugated to psicofuranose residue has been hithertofore reported for use as a triplexing agent in the antigene approach. In the present study, we mainly examine the triplex-binding behaviour of phenazine tethered to the oligo-DNA through the C1' of psicofuranose sugar moiety, and compare this triplex binding behaviour with those of the corresponding duplexes. We also report on a comparative study of the thermal stability and spectroscopic behaviour of C1'-tethered psicofuranose oligonucleotide **24** with those oligonucleotides that are tethered at the 5'-end of the thymidine block **24'**.[22] Additionally, we have addressed the issue of the mode of chromophore - nucleotide interaction that takes place both in double strand and triple strand structures by fluorescence spectroscopy.

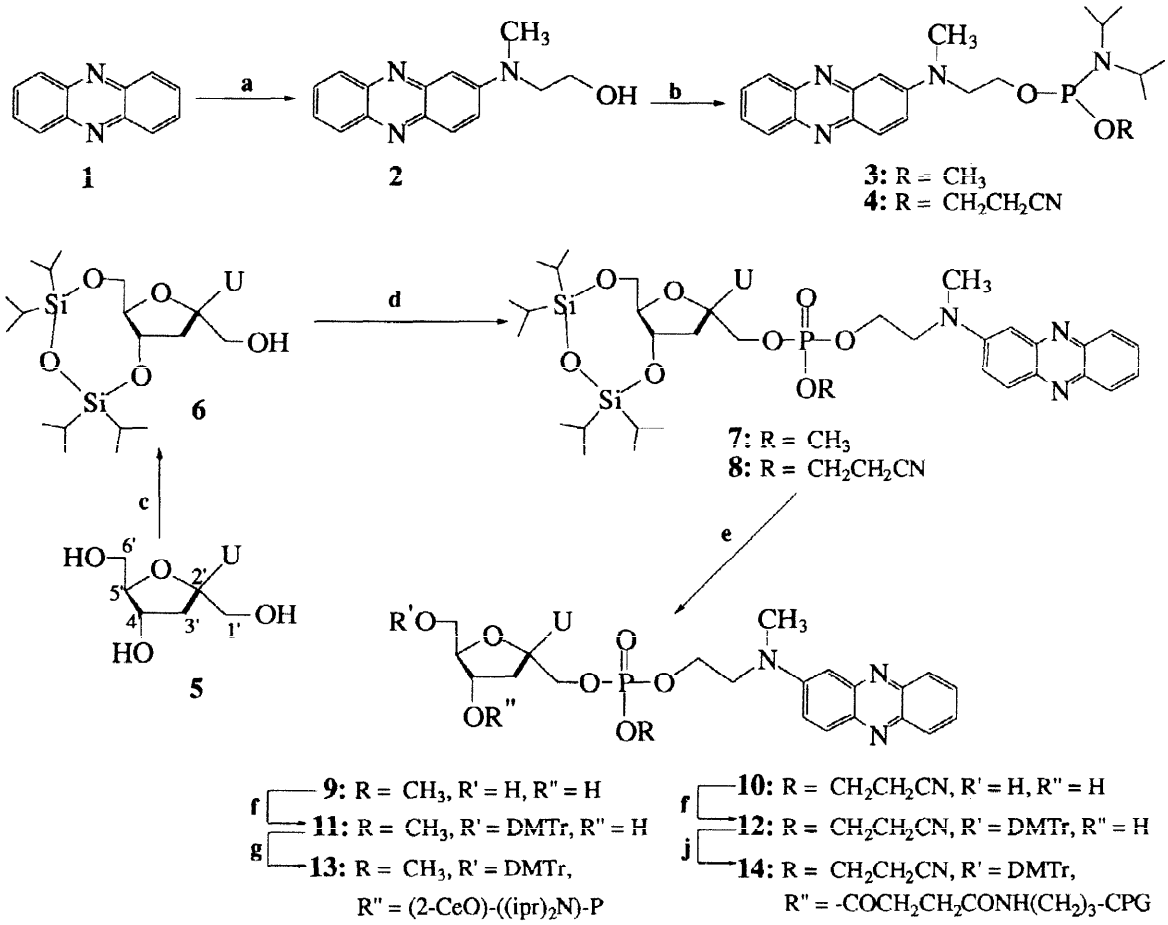
Results and Discussion

(I) Synthesis of 6'-*O*-DMTr-1'-*O*-Pzn-tethered 1-(3'-deoxypsicofuranosyl)uracil phosphor amidite (13) and its 3'-succinamido-anchored CPG (14).

The starting psicofuranosyl nucleoside **5** was synthesised in six steps starting from D-fructose.[17] Selective protection of the primary 1'- and 6'-hydroxy functions in **5** was an arduous task due to their similar reactivity.[14] The silylation of **5** with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane ($TIPDSCl_2$) under the same conditions used by Ono *et al.*[12] proceeded with poor selectivity (26%) in comparison with the reported yield (57%). However, it was possible to recover 45% of **5** via deprotection of the undesired silylated products.

The protected nucleoside **6** was then coupled to the 2-(*N*-(2-hydroxyethyl)-*N*-methyl)aminophenazine phosphoramidite **3** or **4** in a tetrazole mediated reaction[20] in acetonitrile, followed by iodine oxidation of the

intermediary phosphite triester to give the phosphotriester block **7** (62%) or **8** (70%). The phosphoramidite blocks **3** (61%) and **4** (64%) were prepared in the standard way[19] from 2-(*N*-(2-hydroxyethyl)-*N*-methyl)aminophenazine **2**, which was obtained from phenazine **1** by 9-*N*-methylation[18] followed by coupling with 2-(*N*-methylamino)ethanol and removal of 9-*N*-methyl group in an overall yield of 15% (Experimental section). Compounds **9** (83%) and **10** (28%) were obtained from phosphotriesters **7** and **8** after the



(a) (i) Me_2SO_4 / o-nitrotoluene, 3 min, 140 °C, (ii) $MeNHCH_2CH_2OH$ / MeOH, 140 h, 20 °C; (b) (MeO)-((ipr)$_2$N)-PCl or (2-CeO)-((ipr)$_2$N)-PCl / (ipr)$_2$EtN / THF, 1.5 h, 20 °C; (c) TIPDSCl$_2$ / Imidazole / DMF, 2 h, -30 °C, overnight, 20 °C; (d) (i) 3 or 4 (0.9 eq.) / tetrazole / MeCN, 1.5 h, 20 °C, (ii) I_2 / THF / H_2O / pyridine, 1 h, 20 °C; (e) TBAF·H_2O / THF, 5 min, 0 °C; (f) DMTrCl / pyridine, 15 h, 20 °C; (g) (2-CeO)-((ipr)$_2$N)-PCl / (ipr)$_2$EtN / THF, 1 h, 20 °C; (j) (i) succinic anhydride / DMAP / CH_2Cl_2, 4 h, 20 °C, (ii) (ipr)CH_2OCOCl / (ipr)$_2$EtN / THF, 2 h, 20 °C, (iii) THF / (ipr)$_2$EtN / aminopropyl-CPG, 2 h, 20 °C.

deprotection of the silyl group with *n*-tetrabutylammonium fluoride (TBAF) in dry THF at 0°C for 5 min, which were then converted into the corresponding 6'-*O*-DMTr-blocks **11** (83%) and **12** (70%). The 2-cyanoethyl group in **8** has turned out to be very unstable in presence of TBAF even at 0°C for 5 min, thereby explaining the poor yield obtained for the conversion of **8**→**10**. Compound **12** was treated with succinic anhydride and DMAP in CH_2Cl_2 to give the corresponding 3'-succinate block (82%), which was then immobilised onto aminopropyl-CPG support by reaction with isopropyl chloroformate in presence of diisopropylethylamine.[21]

(A) Duplex targets for triplex formation

15: 3'-d(CGCAGAAAAAAGAAAAAAGA^{4}A^{3}C^{1}G)-5' 24-mer)

16: 5'-d(^{1}GCGTCTTTTTTCTTTTTTCT^{21}T^{22}GGC)-3' (24-mer)

17: 3'-d(CGCAGAAAAAAGAAAAAAGA^{10}A^{9}AAAAACC^{1}G)-5' (29-mer)

18: 5'-d(^{1}GCGTCTTTTTTCTTTTTTCT^{21}T^{22}TTTTTTGGC)-3' (29-mer)

(B) Single strand targets for duplex formation

19: 5'-d(CATGTTTGGAC)-3' (11-mer)

20: 5'-d(CATGTATGGAC)-3' (11-mer)

(C) Non-tethered and modified 18-mers: 3'-d(^{18}XCTTTTTTC^{9}YTTTTTCT^{1}Z)-5'

Modifications	Oligonucleotides					
	21	**22**	**23**	**24**	**25**	**24'**
X =	T	**U***	T	T	**U***	T
Y =	T	T	**U***	T	T	T
Z =	T	T	T	**U***	**U***	**T***

(D) Non-tethered and modified 9-mers: 3'-d(XACAYACCZ)-5'

Modifications	Oligonucleotides					
	26	**27**	**28**	**29**	**30**	**31**
X =	T	T	**U***	T	T	**U***
Y =	A	T	A	**U***	A	A
Z =	T	T	T	T	**U***	**U***

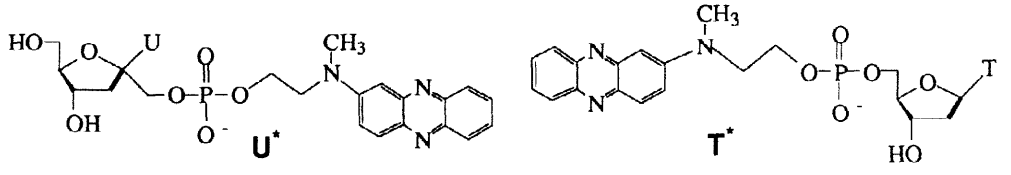


Fig. 1

Subsequently, capping with acetic anhydride gave 19.9 mmol of bound modified nucleoside per gram of CPG **14**, which was used for incorporation of the phenazine-tethered psicofuranosyl nucleoside at the 3'-end of the oligonucleotides. Compound **11** was also converted in the usual way[19] into the corresponding phosphoramidite block **13** (83%) for incorporation of the phenazine tethered derivative into oligodeoxynucleotides at the 5'-end

or in the middle of the chain. The oligonucleotide analogues used (Fig. 1) in this study were synthesized by the phosphoramidite methodology[21] on a commercially available DNA/RNA synthesiser.

(II) Synthesis of 1'-Pzn tethered 9-mers 28 - 31 and 18-mers 22 - 25

In this study, we prepared four phenazine-tethered 9-mer oligo-DNA sequences **28 - 31** for the duplex studies, and four pyrimidine-rich phenazine-tethered 18-mers **22 - 25** for the triplex studies. The model nucleotide sequences chosen in the above oligos were identical to the ones used in our previous study with

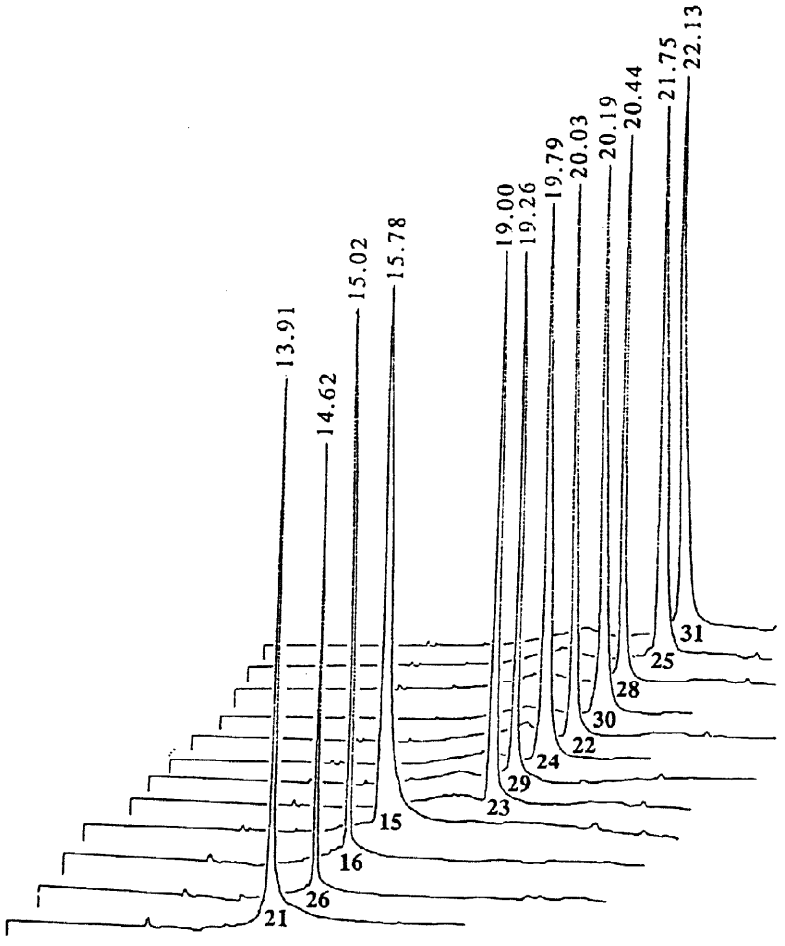


Figure 2. The HPLC profiles of purified oligonucleotides **15 - 16, 21 - 26** and **29 - 31** using the gradient 0 - 100% B in 30 min at 1 ml/min. Buffer A: 0.1 M triethylammonium acetate (TEAA), 5% MeCN; Buffer B: 0.1 M TEAA, 50% MeCN. Retention times (R_t) shown at the top of the peaks are given in minutes.

5'-phenazine-tethered thymidine nucleotide[22], which gave us a nice possibility for comparison of the present data with those previously published[22]. Synthesis, deprotection and purification of oligonucleotides **15 - 31** are described in the experimental section. Examination of the HPLC profiles of the purified sodium exchanged oligomers reveals (Fig. 2) that the phenazine residue attached either to the 3'-, 5' or both in 3' and 5'-ends or to the middle part of the 9-mer and 18-mer oligonucleotides gives strongly retarded retention times (R_t) compared to the corresponding underivatized oligomers.

(III) Thermal Denaturation Studies of Triplexes

Triplexes were generated by hybridisation of modified 18-mers **22** - **25** with target 24-mer duplex **15•16**[23] in a 1:1:1 ratio containing 1 mM of each strand in buffers at pH 7.3, 6.5, and 6.0 containing 20 mM PO_4^{3-} and 0.1 M NaCl. Three similar transition curves were observed for all melting profiles. The transition occurring in the temperature range 12 - 35°C was attributed to the thermal dissociation of the third strand, the transition observed in the temperature range 42 - 50°C corresponded to the dissociation of the mismatch duplex between the 18-mer and the GA-rich 24-mer oligo, while the transition observed in the temperature range 60 - 63°C corresponded to the dissociation of the 24-mer duplex, **15•16**. All T_ms are shown in Table 1. The effect of stabilisation of the triple-helical form (**24** + **15** + **16**) was only moderate (ΔT_m = 4°C at pH 7.3, 2.2°C at pH 6.5, 5.2°C at pH 6.0) when the modified site is located at the 5'-end of the third strand, as in **24**. Attachment of phenazine to the 3'-end nucleotide of the third strand, as in **22**, resulted in the destabilisation of the triplex (**22** + **15** + **16**) at pH 6.5 (ΔT_m = -4.9°C) and pH 6.0 (ΔT_m = -4.7°C) while no triplex formation was detected at pH 7.3. Nevertheless, the co-operative modification of both 3'- and 5'-ends of the triplex forming strand, as in **25**, showed the best stability of triplex formed with 24-mer target duplex **15•16** (ΔT_m = 5.2°C at pH 7.3, 3.7°C at pH 6.5, 6.4°C at pH 6.0). Finally, the middle-modified oligonucleotide **23**, carrying the linker group in the center, shows no triplex formation at pH 7.3 and 6.5, but at pH 6.0 a triplex structure with considerably poor stability (ΔT_m = -9.9°C) was observed.

The above data thus clearly confirmed that *stability of the triplexes indeed depends upon the location of the phenazine-tethered psicofuranosyl moiety* in the triplex-forming oligonucleotide strand.

Table 1. Thermal Stability (T_m in °C) and Fluorescence Properties of Triplexes resulting from a 1:1:1 mixture of octadecamer conjugates **21** - **25** with the duplexes **15 • 16 & 17 • 18.**

Triplexes	pH 7.3			pH 6.5			pH 6.0		
	T_m	ΔT_m	ΔF	T_m	ΔT_m	ΔF	T_m	ΔT_m	ΔF
21 -15•16	13.5	-	-	21.5	-	-	25.6	-	-
22 -15•16	n.d.	n.d.	1.45	16.6	-4.9	1.42	20.9	-4.7	1.69
23 -15•16	n.d.	n.d.	a	n.d.	n.d.	a	15.7	-9.9	a
24 -15•16	17.5	4.0	2.21	23.7	2.2	2.18	30.9	5.3	2.31
24'-15•16[b]	17.4	3.9	a	24.3	2.8	a	30.3	4.7	1.30
25 -15•16	18.7	5.2	4.33	25.2	3.7	4.04	32.0	6.4	4.49
21 -17•18	13.7	-	-	16.8	-	-	19.3	-	-
24 -17•18	20.4	6.7	2.09	25.4	8.6	2.08	30.0	10.7	2.37

[a] Values were not measured.
[b] This data had been obtained in our previous work (see ref. 22)
n.d. implies could not be determined.

Comparison of T_ms of triplexes composed of the third strand carrying 5'-phenazine-tethered psicouridine nucleotide, as in **24**, with the corresponding triplex carrying 5'-phenazine-tethered thymidine nucleotide as in **24'**[22], showed the following: (i) Stability of the triplex **24-15•16** was only slightly more than the triplex **24'-15•16** (Table 1) ($\Delta\Delta T_m$ = +0.1- 0.6°C between pH 7.3 - 6.0). (ii) In both cases, a decrease of pH decreased

ΔT_ms from pH 7.3 to pH 6.5, and then it sharply increased from pH 6.5 to 6.0. This comparative study of T_m values in general showed that *the triplex stability depends, as expected, upon the nature of sugar-linker residue.* Moreover, the data on the phenazine tether at 1'-position of 3'-deoxypsicouridine shows its slightly greater interactive ability with the neighbouring nucleobases than that of the 5'-tethered thymidine moldified oligos.

Furthermore, we have investigated the binding capacity of oligonucleotide **24** to form the triplex with a larger target duplex **17•18**, which is more A-T rich (by five basepairs) than the duplex **15•16**. The enhanced ΔT_m values ($\Delta\Delta T_m$ = 2.7°C at pH 7.3, 5.4°C at pH 6.0) for triplex **24-17•18** in comparison with **24-15•16** testify that the most probably the phenazine ring is situated in the vicinity of the last base triplet (*i.e.* $^{21}T•^{4}A\text{-}^{1}U^{*}$ in the triplex **24-15•16** or $^{21}T•^{10}A\text{-}^{1}U^{*}$ in the triplex **24-17•18**), and at the first base pair of the duplex at the duplex-triplex junction (*i.e.* $^{22}G•^{3}C$ in triplex **24-15•16** or $^{22}T•^{9}A$ in triplex **24-17•18**).

It is noteworthy that the T_m of the natural triplex **21-17•18** with extra five A-T basepairs in the target duplex **17•18** is considerably reduced at pH 6.5 and 6.0 compared to **21-15•16**, showing the destabilizing influence of the extra five A-T basepairs in the target duplex **17•18**.

This situation however is reversed in the triplexes with 5'-phenazine-tethered oligo **24** and the target duplexes **17•18** and **15•16**. The increased T_ms of triplex **24-17•18** compared to **24-15•16** show at the first glance that the global thermodynamic stability of the former probably enahnces simply because of the fact that phenazine is someway interacting with some of the additional five A-T basepairs in the former. We argued that although none of these A-T basepairs in the target duplex is involved in the triplex formation, but their interaction with the 5'-tethered phenazine in **24-17•18** results presumably in the stabilization of the Hoogsteen basepairing for the terminal basepairs with the third strand (*i.e.* $^{21}T•^{4}A\text{-}^{1}U^{*}$). This was neatly resolved by carefully looking at the fluorescence properties of the both **24-17•18** and **24-15•16** triplexes, which showed

Table 2. Thermal Stability (T_m in °C) and Fluorescence Properties of Duplexes resulting from a 1:1 mixture of nonamer conjugates **26 - 31** with the targets **19 & 20.**

Duplexes	pH 7.3		
	T_m	ΔT_m	ΔF
26•19	25.9	-	-
27•20	22.4	-	-
28•19	25.0	-0.9	1.16
29•20	9.5	-12.9	1.49
30•19	32.0	6.1	-1.22
31•19	28.4	2.5	4.96

that their fluorescence intensities remain unchanged (*i.e.* $\Delta F \approx 0$, see below). This means that the stacking properties of the 5'-tethered phenazine moiety with the neighbouring basepairs remains the same in both triplexes. Hence, the most likely reason for the enahnced stability of **24-17•18** over **24-15•16** is that the phenazine in the former stabilizes the hydrogen bonds of the terminal triplet hydrophobically[24,32,33] rather than by dipole-induced dipole interaction. This is consistent with our earlier comparative hydration studies[24,32,33] of natural duplexes with those of the 5'-phenazine-tethered duplexes, in which we showed that the terminal

phenazine indeed enhances the thermodynamic stability of the duplxes by stabilizing the terminal hydrogen bond simply by reducing the hydration level in the first spine of hydration (the "oil effect").[34]

(IV) Thermal Denaturation Study of the Duplexes

Duplexes were generated by hybridisation of the phenazine-tethered 9-mers **28, 30, 31** with target 11-mer oligo **19**, and the phenazine-tethered 9-mer **29** with the target 11-mer oligo **20** in a 1:1 ratio with 1 mM of each strand in 20 mM PO_4^{3-}, 0.1 M NaCl buffer at pH 7.3. All the melting curves of these duplexes showed a monophasic dissociation and Table 2 shows the melting temperatures. Following comparisons are noteworthy: (i) The 5'-Phenazine tethered oligo **30** with **19** formed a duplex with a higher T_m value (ΔT_m = 6.1°C) than the duplex containing phenazine conjugate in the center, as in **29•20** (ΔT_m = -12.9°C) or with the nonconjugated duplexes **26•19** (T_m = 25.9°C) and **27•20** (T_m = 22.4°C). (ii) When the 3'-phenazine-tethered oligonucleotide was used as the complementary strand, the T_m was slightly less (ΔT_m = -0.9°C) than that of the nonconjugated duplex **26•19** . (iii) The 3',5'-bis-phenazine tethered oligonucleotide **31** with **19** had somewhat poorer stabilisation (ΔT_m = 2.5°C) compared to that of the duplex formed by oligo **30** and **19** because of destabilising influence of tethering the chromophore through the 3'-nucleotide (Table 2), which is consistent with the result obtained by Ono *et al.*[12]. It is likely that the 5'-end tethered phenazine stabilises the duplex by stabilising hydrogen bond of the terminal basepair[24,25], whereas the middle-modified phenazine is bulky for the minor groove to accommodate and thereby destabilizes the duplex[12].

(V) Fluorescence Studies on Triplexes

The fluorescence properties of the phenazine tethered oligonucleotides were used to investigate triple- and double-helix formation. For triplex study the change of fluorescence intensity (ΔF) values were calculated from the fluorescence intensity of state **B** [*i.e.* 18-mer+24-mer+24-mer triplex / 18-mer+24-mer duplex mixtures of a phenazine-tethered 18-mers mixed with the 24-mers **15** and **16** or 18-mer+29-mer+29-mer triplex / 18-mer+29-mer duplex mixtures of a phenazine-tethered 18-mer **24** mixed with the 29-mers **17** and **18**] and state **A** (phenazine-tethered 18-mer single strand). Correspondingly, for duplex study, the ΔF values were estimated as difference of the fluorescence intensity between state **C** (*i.e.* phenazine-tethered 9-mer single strand) and **D** (*i.e.* 9+11 double strand). The fluorescence measurements of solutions of state **A** and **B** were carried out in 20 mM PO_4^{3-}, 1.0 M NaCl at pH 7.3, 6.5 and 6.0 at 6°C. The measurements of solutions of state **C** and **D** were carried out at 16°C in 20 mM PO_4^{3-}, 1.0 M NaCl at pH 7.3. ΔF values were calculated[26] using the fluorescence intensity value of 1.0 as a reference for phenazine residue in state **A** or **C** since they are single stranded states.

The following fluorescence characteristics of phenazine-conjugated DNA triplexes were observed (Table 1): (i) All selected triplexes for fluorescence measurements showed an enhanced fluorescence of phenazine and a blue shift of the emission maximum upon triplex formation. (ii) There was a straight correlation between T_ms presented above and the fluorescence data: the most stable triplex formed with 3',5'-di-phenazine-conjugated third strand (as in **25**) gave a 4 - 4.5 fold increase in ΔF, while the fluorescence of the triplexes with 5'-phenazine-tethered third strand (as in **24**) was only enhanced 2.2 - 2.3 times and, finally, the least enhancement in ΔF (1.5 - 1.7) was observed with 3'-phenazine-tethered third strand **22** mixed with the 24-mer targets **15** and **16**. (iii) Changing of pH led to a very insignificant alteration in ΔF but they had the same effect as found for the ΔT_m: under decreasing pH (7.3 → 6.5 → 6.0), ΔF decreased first from pH 7.3 → 6.5, and then increased when the pH was changed from pH 6.5 → 6.0. (iv) The stability of triplex with modified 24-mer third strand **22**

(**22-15•16**) was weaker than the unmodified counterpart at pH 6.0; at pH 7.3 however no triplex formation was detectable. Despite this weaker stability of **22-15•16** triplex over the native counterpart, we still observed an enhancement of the phenazine fluorescence intensity for the former, which means that small changes of phenazine ring's fluorescence intensity (ΔF = 1.5 - 1.7) does not necessarily reflect the thermodynamic stability of the whole triplex DNA structure. This small changes in ΔF perhaps only indicate the microenvironment alterations around the phenazine chromophore. (v) Tethering of phenazine residue to the 1'-position psicofuranosyl nucleotide (which is 4'→ 5' linked as in **24**) compared to the tethering of phenazine at the 5'-terminal thymidine nucleotide (which is 3'→ 5' linked as in **24'**)[22] showed a larger enhancement (ΔF = 2.3-fold increase) in the phenazine fluorescence in the former compared to the latter (ΔF = 1.3-fold increase) at pH 6.0. It has been shown previously[22] that in case of tethering of phenazine at the 5'-phosphate of pentofuranosyl nucleotide (as in oligo **24'**), the fluorescence of phenazine was enhanced upon weak exterior binding of the chromophore to the nucleobases.

Thus, our above comparative data for **24** and **24'** shows that tethering of the phenazine residue at C1' of psicofuranosyl sugar moiety (as in **24**) is perhaps potentially more useful as an antigene agent because it provides slightly stronger interaction of the chromophore with one or more neighbouring nucleobases, despite the fact that the stabilities of both the triplexes **24-15•16** and **24'-15•16** are approximately equal (Table 1).[27,28]

We also examined the fluorescence characteristics of the triplex **24-17•18** in which the A-T rich binding site was present in the duplex region adjacent to the DNA triplex. This conjugate did not exhibit any changes in fluorescence intensity compared to the triplex **24-15•16** (ΔF was within the range 2.08 and 2.37), suggesting no considerable changes in the interaction between phenazine residue and surrounding base pairs.

(VI) Fluorescence Studies on Duplexes

In studies with duplexes with monomodified oligomers **28 - 30**, the fluorescence intensity changed very little (ΔF = -1.2 - +1.5) compared with the corresponding triplexes **22 - 24** (ΔF = 1.5 - 2.3). Since the three bases in the triple helix formed by Watson-Crick and Hoogsteen base pairing occupy more space in the helical structure than the basepair in the duplex, the intramolecular interaction between phenazine and the nucleobases is statistically more likely to happen in triplexes than in duplexes. Although the 3'-modified, 5'-modified and middle-modified oligonucleotides **28 - 30** form the corresponding duplexes with varying stabilities, their fluorescence properties however change very little, thereby suggesting[29] that the fluorescence emission observed reflects only the change of the microenvironment around the chromophore and poor intramolecular interactions between chromophore and the surrounding nucleobases.

In general, the fluorescence data suggest that the intercalation by the covalently bound phenazine residue between heterocyclic bases is unlikely in duplexes compared to the triplexes under the condition of the fluorescence measurement. The small quenching/enhancement effects observed with the double-stranded 9-mers **28 - 30** are consistent with the weak interaction between the phenazine and the nucleotides in targets **19 & 20**.

CONCLUSION

Oligodeoxynucleiotide conjugates have been prepared with 1'-phenazine-tethered 1-(3'-deoxypsicofuranosyl)uracil, in which 2-(phenazin-2-ylmethylamino)ethyl moiety is covalently bound to the 1'-hydroxy group through a phosphate linkage. Conversion of 1'-phenazine-tethered 1-(3'-

deoxypsicofuranosyl)uracil into the appropriate phosphoramidite or attachment to a solid support allowed the preparation of site-specifically modified oligodeoxynucleotides with this polycyclic phenazine chromophore at 3', 5' or in both 3',5'-terminals or in the middle part. Our melting studies have shown that the thermal stability of duplexes and triplexes decreased when the modified unit was incorporated into the middle of the oligonucleotide probe. In contrast, tethering the phenazine fluorophore to the 5'-terminus of the pyrimidine third strand of parallel-stranded DNA triplexes or to the 5'-terminus of the second strand of DNA duplexes results in enhanced stability. Flurorescence studies on the phenazine-tethered triplexes showed that the mechanism of the triplex stabilization by intramolecularly linked phenazine is *sequence-specific*, and involves stacking by dipole-induced dipole interaction with the neighbouring basepairs as well as through stabilization of the hydrogen bonds of the terminal triplet hydrophobically. This observation of hydrophobic stabilization of the triplex is consistent with our earlier comparative hydration studies of natural duplexes with those of the 5'-phenazine-tethered duplexes, in which we showed that the terminal phenazine indeed enhances the thermodynamic stability of the duplxes by stabilizing the terminal hydrogen bond simply by reducing the hydration level in the first spine of hydration (the "oil effect").

EXPERIMENTALS

^{1}H-NMR spectra were obtained at 270 MHz on a JNM-GX 270 spectrometer with tetramethylsilane as an internal standard. ^{31}P-NMR spectra were obtained at 36 MHz in the same solvent using 85% phosphoric acid as an external standard. TLC was performed on pre-coated silica gel F_{254} plates with fluorescent indicator in the following dichloromethane - ethanol mixtures: (A) 95:5 (v/v), (B) 90:10 (v/v), (C) 80:20 (v/v). Dry pyridine was obtained by distillation over 4-toluenesulphonyl chloride. Acetonitrile and dichloromethane were distilled from P_2O_5 under argon. Dimethylformamide and tetrahydrofuran were distilled over CaH_2. The silica gel Merck G60 was used for column chromatographic separations of all the protected intermediates. Semipreparative RP-HPLC was carried out on Spherisorb 5ODS2 using a Gilson equipment with Pump Model 303, Manometric Module Model 802C and Dynamic Mixer 811B connected to a Dynamax computer program for gradient control. Thermal denaturation experiments were performed on a PC-computer interfaced Perkin Elmer UV/VIS spectrophotometer Lambda 40 with PTP-6 peltier temperature controller. Fluorescence emission spectra were collected on an Aminco SPF-500 Corrected Spectra Spectrofluorometer with a Xenon lamp power supply.

2-[*N*-[2-Hydroxyethyl]-*N*-methyl]aminophenazine (**2**) was synthesised as previously described.[22]

[2-[Phenazin-2-ylmethylamino]ethyl] methyl *N,N*-diisopropyl phosphoramidite (3). 2-(*N*-(2-Hydroxyethyl)-*N*-methyl)aminophenazine **2** (418 mg, 1.65 mmol) was coevaporated with dry tetrahydrofuran twice and dissolved in the same solvent (23 ml). Then dry diisopropylethylamine (1.43 ml, 8.23 mmol) was added followed by *O*-methyl-*N,N*-diisopropylphosphoramidic chloride (0.48 ml, 2.7 mmol) under vigorous stirring for 1.5 h under argon. Then the reaction was quenched by addition of dry MeOH (0.5 ml) and kept stirring for 30 min. The crude material obtained after aqueous saturated $NaHCO_3/CH_2Cl_2$ work up and drying by filtration through $MgSO_4$ was then silica gel column chromatographed (CH_2Cl_2 : Et_3N, 95 : 5 v/v). Yield 519 mg, 61%. R_f: 0.52 (B); ^{1}H-NMR ($CDCl_3$): 8.14-8.05 (2H, m, arom), 8.03 (1H, d, arom), 7.78-7.60 (3H, m, arom), 7.06 (1H, d, arom, J = 2.7 Hz), 3.91-3.62 (4H, m, $POCH_2CH_2CN$), 3.57, 3.54 (3H, 2 x s,

CH_3OP), 3.56-3.42 (2H, m, $CH(CH_3)_2$), 3.22 (3H, s, $-NCH_3$), 1.21, 1.17 (12H, 2 x d, $CH(CH_3)_2$, J = 6.93 Hz); ^{31}P-NMR ($CDCl_3$): +150.7 ppm.

[2-[Phenazin-2-ylmethylamino]ethyl] 2-cyanoethyl *N,N*-diisopropyl phosphoramidite (4). The procedure was the same as for synthesis of **3**. Compound **2** (235 mg, 0.39 mmol), diisopropylethylamine (0.81 ml, 4.63 mmol), *O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidic chloride (0.31 ml, 1.39 mmol) were used. A mixture of CH_2Cl_2:Et_3N, 98:2 v/v was used for silica gel column chromatography. Yield 265 mg, 64.8%. R_f: 0.70 (B); ^{1}H-NMR ($CDCl_3$): 8.15-8.06 (2H, m, arom), 8.03 (1H, d, arom), 7.77-7.61 (3H, m, arom), 7.1 (1H, d, arom, J = 2.7 Hz), 4.00-3.68 (6H, m, OCH_2CH_2N, $POCH_2CH_2CN$), 3.63-3.49 (2H, m, $CH(CH_3)_2$), 3.23 (3H, s, $-NCH_3$), 2.57 (2H, t, $POCH_2CH_2CN$, J = 6.75 Hz), 1.36, 1.32 (12H, 2 x d, $CH(CH_3)_2$, J = 7.0 Hz); ^{31}P-NMR ($CDCl_3$): +147.9 ppm.

1-[3'-Deoxy-4',6'-*O*-[1,1,3,3-tetraisopropyldisiloxan-1,3-diyl]-β-D-psicofuranosyl]uracil (6). 1-[3'-deoxy-β-D-psicofuranosyl]uracil **5** (2.05 g, 7.93 mmol) was repeatedly coevaporated with dry DMF and dissolved in the same solvent (51 ml). Imidazole (1.29 g, 19.03 mmol) was added to the solution and the mixture was stirred at -30°C under an argon atmosphere for 15 min. $TIPDSCl_2$ (2.54 ml, 7.93 mmol) was added dropwise over 10 min to the solution and the mixture was stirred for 2 h at -30°C, then overnight at room temperature. Dry MeOH (2.6 ml) was added to the reaction mixture and the solvents were concentrated. The crude material obtained after aqueous saturated $NaHCO_3$/CH_2Cl_2 work up and drying over $MgSO_4$ was then silica gel column chromatographed (0-8% EtOH in CH_2Cl_2, product was eluted with 4% EtOH in CH_2Cl_2).Yield 1.03 g, 26%. R_f = 0.33 (B); ^{1}H-NMR ($CDCl_3$): 9.29 (1H, br s, 3-NH), 7.96 (1H, d, H-6, $J_{5,6}$ = 8.16 Hz), 5.65 (1H, d, H-5), 4.33 (1H, ddd, H-4', $J_{4'5'}$ = 8.16 Hz, $J_{3'a,4'}$ = 6.9 Hz, $J_{3'b,4'}$ = 10.4 Hz), 4.19-3.88 (4H, m, H-6'a,b and H-1'a,b, $J_{6'gem}$ = 13.24, $J_{5',6'}$ = 2.6 Hz, $J_{1'gem}$ = 11.8 Hz), 3.84 (1H, dt, H-5', $J_{5',6'}$ = 2.72 Hz, $J_{4',5'}$ = 8.16 Hz), 2.95 (1H, dd, H-3'a, $J_{3'gem}$ = 13.6 Hz, $J_{3'a,4'}$ = 6.9 Hz), 2.38 (1H, dd, H-3'b, $J_{3'b,4'}$ = 10.4 Hz), 1.04 (28H, m, isopropyl).

1-[4',6'-*O*-[1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl]-3'-deoxypsicofuranosyl]uracil-1'-[2-[phenazin-2-ylmethylamino]ethyl] methyl phosphate (7).
Phosphoramidite **3** (169 mg, 0.40 mmol) and 1-[3'-deoxy-4',6'-*O*-[1,1,3,3-tetraisopropyldisiloxan-1,3-diyl]-β-D-psicofuranosyl]uracil **6** (218 mg, 0.44 mmol) were coevaporated together with dry acetonitrile twice and then dissolved in dry acetonitrile (4 ml). Then tetrazole (139 mg, 1.99 mmol) was added and the resulting solution was stirred under argon for 1.5 h. Then the reaction was quenched by addition of 5.2 ml of 0.1 M iodine in THF/pyridine/water (7:2:1 v/v/v) and stirred for 1.25 h. The crude residue obtained after aqueous saturated $NaHCO_3$/0.1 M $Na_2S_2O_3$/dichloromethane work up and drying by filtration through $MgSO_4$ was then silica gel column chromatographed (0-5% EtOH in CH_2Cl_2). Yield 225 mg, 62%. R_f: 0.47 (B); ^{1}H-NMR ($CDCl_3$): 8.16-8.05 (3H, m, arom.), 7.89, 7.85 (1H, 2 x d, H-6, $J_{5,6}$ = 8.29 Hz), 7.78-7.57 (3H, m, arom), 7.15, 7.14 (1H, 2 x d, arom), 5.67, 5.64 (1H, 2 x d, H-5), 4.58 (1H, dt, H-4', $J_{4',5'}$ = $J_{3'b,4'}$ = 10.64 Hz, $J_{3'a,4'}$ = 6.68Hz), 4.35-4.24 (4H, m, OCH_2CH_2N-, H-1'a,b), 4.07-3.74 (5H, m, OCH_2CH_2N-, H-5', H-6'a,b), 3.71, 3.67 (3H, 2 x d, CH_3OP), 3.24, 3.23 (3H, 2 x s, N-CH_3), 2.92, 2.91 (1H, 2 x dd, H-3'a, $J_{3'a,4'}$ = 6.68 Hz, $J_{3'gem}$ = 13.61 Hz), 2.27, 2.26 (1H, 2 x dd, H-3'b, $J_{3'b,4'}$ = 10.64 Hz), 0.97 (28H, m, isopropyl).

1-[4',6'-*O*-[1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl]-3'-deoxypsicofuranosyl]uracil-1'-[2-[phenazin-2-ylmethylamino]ethyl] 2-cyanoethyl phosphate (8).
The procedure was the same as for synthesis of **7**. Compound **4** (260 mg, 0.59 mmol), **6** (324 mg, 0.65 mmol), tetrazole (206 mg, 2.94 mmol) were used. A mixture of 0-4% EtOH in CH_2Cl_2 was used for silica gel column chromatography . Yield 319 mg, 70.6%. R_f: 0.43 (B); ^{1}H-NMR ($CDCl_3$): 8.16-8.06 (3H, m, arom), 7.89, 7.86 (1H, 2 x d, $J_{5,6}$ = 8.4 Hz), 7.79-7.59 (3H, m, arom), 7.18, 7.17 (1H, 2 x d, arom), 5.67, 5.65 (1H, 2 x d, H-5), 4.68-4.61 (1H, ddd, H-4', $J_{4',5'}$ = 8.91 Hz, $J_{3'a,4'}$ = 6.93 Hz, $J_{3'b,4'}$ = 10.64 Hz), 4.39-4.23 (4H, m, H-1'a,b, OCH_2CH_2N-), 4.20-4.12 (2H, m, H-6'a,b, $J_{6'gem}$ = 8.16 Hz), 4.06-3.75 (5H, m, H-5', OCH_2CH_2N-, $POCH_2CH_2CN$), 3.25, 3.24 (3H, 2 x s, N-CH_3), 2.94-2.85 (1H, 2 x dd, H-3'a, $J_{3'a,4'}$ = 6.93 Hz, $J_{3'gem}$ = 13.61 Hz), 2.67 (2H, t, $POCH_2CH_2CN$, J = 6.19 Hz), 2.27-2.19 (1H, dd, H-3'b, $J_{3'b,4'}$ = 10.64 Hz), 1.00 (28H, m, isopropyl), ^{31}P-NMR ($CDCl_3$): -1.95 ppm.

1-[3'-Deoxypsicofuranosyl]uracil-1'-[2-[phenazin-2-ylmethylamino]ethyl] methyl phosphate (9). Tetrahydrofuran coevaporated **7** (244 mg, 0.29 mmol) was dissolved in dry tetrahydrofuran (2.25 ml) and the solution was cooled to 0°C. The solution of TBAF x H_2O (100 mg, 0.38 mmol) in dry THF (2.25 ml) was also cooled to 0°C, and then added to the cooled nucleoside solution under vigorous stirring. The resulting mixture was stirred for 5 min at 0°C, and then immediately precipitated by pouring into cyclohexane (~30 ml). After decantation the solid residue was dissolved in CH_2Cl_2 (6 ml) and the solution was loaded to a silica gel column (0-20% EtOH in CH_2Cl_2, product was eluted with 20% EtOH in CH_2Cl_2). Yield 143 mg, 83%. R_f: 0.27 (C); ^{1}H-NMR (CD_3OD with $CDCl_3$): 8.16-8.05 (3H, m, arom), 7.96 (1H, 2 x d, H-6, $J_{5,6}$ = 8.16 Hz), 7.81-7.67 (3H, m, arom), 7.06 (1H, 2 x d, arom), 5.59, 5.55 (1H, 2 x d, H-5), 4.56-4.47 (1H, ddd, H-4'), 4.44-4.31 (3H, m, H-5', OCH_2CH_2N-), 4.23-4.12 (2H, m, OCH_2CH_2N-), 3.96-3.91 (2H, m, H-1'a,b), 3.74-3.51 (5H, m, CH_3OP, H-6'a,b), 3.27, 3.26 (3H, 2 x d, N-CH_3), 2.78-2.71 (1H, m, H-3'a), 2.51-2.41 (1H, m, H-3'b).

1-[3'-Deoxypsicofuranosyl]uracil-1'-[2-[phenazin-2-ylmethylamino]ethyl] 2-cyanoethyl phosphate (10). The procedure was the same as for synthesis of **9**. Compound **8** (310 mg, 0.36 mmol), TBAF x H_2O (122 mg, 0.47 mmol). Silica gel column chromatography (0-20% EtOH in CH_2Cl_2). Yield 62 mg, 27.8%. R_f: 0.07 (B); ^{1}H-NMR (CD_3OD with $CDCl_3$): 8.15-8.05 (3H, m, arom), 7.97 (1H, 2 x d, H-6, $J_{5,6}$ = 8.17Hz), 7.83-7.68 (3H, m, arom), 7.09 (1H, 2 x d, arom, J = 2.97 Hz), 5.59, 5.54 (1H, 2 x d, H-5), 4.60-4.53 (1H, ddd, H-4'), 4.45-4.32 (3H, m, H-5', OCH_2CH_2N-), 4.26-4.12 (4H, m, OCH_2CH_2N-, $POCH_2CH_2CN$), 3.95-3.90 (2H, m, H-1'a,b, $J_{1'gem}$ = 4.7 Hz), 3.69-3.51 (2H, m, H-6'a,b, $J_{6'gem}$ = 13.24 Hz, $J_{5',6'}$ = 5.32 Hz), 3.28, 3.27 (3H, 2 x s, N-CH_3), 2.76-2.68 (3H, m, H-3'a, $POCH_2CH_2CN$, J = 6.19 Hz), 2.48-2.40 (1H, m, H-3'b).

1-[6'-*O*-[4,4'-Dimethoxytrityl]-3'-deoxypsicofuranosyl]uracil-1'-[2-[phenazin-2-ylmethyl amino]ethyl] methyl phosphate (11). Pyridine coevaporated dihydroxy block **9** (140 mg, 0.24 mmol) was dissolved in dry pyridine (1.8 ml) and then DMTr-Cl (105 mg, 0.31 mmol) was added to the solution. Reaction mixture was then left stirring overnight at room temperature. Mixture was poured into aqueous saturated $NaHCO_3$ and extracted with dichloromethane. The residue obtained from evaporation of organic phase was then coevaporated with mixture of toluene and dichloromethane, and subjected to silica gel column chromatography (0-10% EtOH in CH_2Cl_2, product was eluted with 10% EtOH in CH_2Cl_2). Yield 176 mg,

83%. R_f: 0.43 (B); ^{1}H-NMR ($CDCl_3$): 8.15-8.03 (3H, m, arom (Pzn)), 7.77, 7.75 (1H, 2 x d, H-6, $J_{5,6}$ = 8.29 Hz), 7.73-7.55 (3H, m, arom (Pzn)), 7.24-7.09 (10H, m, arom (Pzn and DMTr)), 6.76-6.71 (4H, m, arom (DMTr)), 5.48, 5.46 (1H, 2 x d, H-5), 4.52-4.28 (7H, m, H-4', H-1'a,b, H-6'a,b, OCH_2CH_2N-), 3.93-3.75 (3H, m, H-5', OCH_2CH_2N-), 3.75 (6H, s, OCH_3), 3.71, 3.69 (3H, 2 x d, CH_3OP), 3.22, 3.21 (3H, 2 x s, N-CH_3), 3.10-3.02 (1H, m, H-3'a), 2.79-2.63 (1H, m, H-3'b).

1-[6'-*O*-[4,4'-Dimethoxytrityl]-3'-deoxypsicofuranosyl]uracil-1'-[2-[phenazin-2-ylmethyl amino]ethyl] 2-cyanoethyl phosphate (12). The procedure was the same as for the synthesis of **11**. **10** (62 mg, 99 mmol), DMTr-Cl (44 mg, 0.129 mmol) were used. A mixture of 0-10% EtOH in CH_2Cl_2 was used for silica gel column chromatography . Yield 64 mg, 69.9%. R_f: 0.34 (B); ^{1}H-NMR ($CDCl_3$): 8.16-8.04 (3H, m, arom (Pzn)), 7.76, 7.75 (1H, 2 x d, H-6, $J_{5,6}$ = 8.16 Hz), 7.71-7.57 (3H, m, arom (Pzn)), 7.23-7.09 (10H, m, arom (Pzn and DMTr)), 6.76-6.72 (4H, m, arom (DMTr)), 5.49, 5.47 (1H, 2 x d, H-5), 4.51-4.46 (1H, m, H-4'), 4.49-4.30 (4H, m, H-1'a,b, OCH_2CH_2N-), 4.21-4.13 (2H, m, H-6'a,b, $J_{6'gem}$ = 8.16 Hz), 3.95-3.66 (4H, m, H-5', OCH_2CH_2N-, $POCH_2CH_2CN$), 3.75 (6H, s, OCH_3), 3.23 (3H, s, N-CH_3), 3.12-2.99 (1H, m, H-3'a), 2.73-2.53 (1H, m, H-3'b), 2.65 (2H, t, $POCH_2CH_2CN$, J = 6.19Hz).

1-[6'-*O*-[4,4'-Dimethoxytrityl]-3'-deoxypsicofuranosyl]uracil-1'-[2-[phenazin-2-ylmethyl amino]ethyl] methyl phosphate-4'-2-cynoethyl *N*,*N*-diisopropylphosphoramidite (13). 3'-Hydroxy block **11** (170 mg, 0.19 mmol) was coevaporated with dry THF twice and dissolved in 3 ml of dry THF. Then dry diisopropylethylamine (0.17 ml, 0.95 mmol) was added, followed by addition of *O*-(2-cyanoethyl)-N,N-diisopropylphosphoramidic chloride (64 ml, 0.29 mmol) under vigorous stirring and the stirring was continued for a further period of 1 h under argon. The reaction was quenched by addition of dry MeOH (0.5 ml) and continued stirring for 30 min. The crude material obtained after aqueous saturated $NaHCO_3$/CH_2Cl_2 work up and drying over $MgSO_4$ was then coevaporated with toluene, CH_2Cl_2 twice and silica gel column chromatographed (EtOH:CH_2Cl_2:Et_3N, 0:98:2 to 4:94:2 v/v/v, product eluted at 4% EtOH). Yield 174 mg, 83%. R_f: 0.53 (B); ^{1}H-NMR ($CDCl_3$): 8.16-8.03 (3H, m, arom (Pzn)), 7.82, 7.81, 7.77, 7.76 (1H, 4 x d, H-6, $J_{5,6}$ = 8.29 Hz), 7.75-7.54 (3H, m, arom (Pzn)), 7.33-7.19 (10H, m, arom (Pzn and DMTr)), 6.82-6.78 (4H, m, arom (DMTr)), 5.53, 5.52, 5.50, 5.49 (1H, 4 x d, H-5), 4.62 (1H, ddd, H-4', $J_{4',5'}$ = 10.89 Hz, $J_{3'a,4'}$ = 7.67 Hz, $J_{3'b,4'}$ = 5.44 Hz), 4.46-4.20 (6H, m, H-1'a,b, H-6'a,b, OCH_2CH_2N-), 3.84-3.50 (7H, m, H-5', $POCH_2CH_2CN$, OCH_2CH_2N-, $CH(CH_3)_2$), 3.77-3.76 (6H, m, OCH_3), 3.67, 3.62 (3H, 2 x d, CH_3OP), 3.20 (3H, br s, N-CH_3), 3.04 (1H, q, H-3'a, $J_{3'a,4'}$ = 7.67 Hz), 2.90-2.79 (1H, m, H-3'b, $J_{3'gem}$ = 14.85 Hz), 2.60, 2.46 (2H, 2 x t, $POCH_2CH_2CN$, J = 6.43 Hz), 1.17-1.08 (12H, m, $CH(CH_3)_2$).

1-[6'-*O*-[4,4'-Dimethoxytrityl]-3'-deoxypsicofuranosyl]uracil-1'-[2-[phenazin-2-ylmethyl amino]ethyl] 2-cyanoethyl phosphate-4'-succinamido-N^3-propyl-CPG (14). 3'-Hydroxy block **12** (64 mg, 69 μmol) and 4-(*N*,*N*-dimethylamino)pyridine (18 mg, 0.145 mmol) were dissolved in dry CH_2Cl_2 (1 ml). Then succinic anhydride (14 mg, 0.140 mmol) was added and the solution was stirred at room temperature for 4 h. Then the reaction mixture was extracted first with 0.1 M citric acid and then aqueous saturated $NaHCO_3$. The organic phase was dried over $MgSO_4$ and the residue consisting of 3'-succinate block was used directly for coupling to CPG support without further purification in the following way: To a solution of diisopropylethylamine (13.8 mg, 106.9 μmol) and isobutyl chloroformate (7.7 mg, 56.7 μmol) in THF (0.85

ml) was added 3'-succinate block (59 mg, 56.7 μmol). Then the reaction mixture was shaken for 2 h and a solution of DIPEA (0.24 ml) in dry THF (0.63 ml) and aminopropyl-CPG (406 mg) were added. The suspension was shaken for 2 h, then filtered and thoroughly washed with THF, CH_2Cl_2 and diethyl ether. The support was then suspended in dry pyridine (3.2 ml) and then 4-(*N*,*N*-dimethylamino)pyridine (67 mg) and acetic anhydride (0.31 ml) were added and the suspension was shaken for 2 h, after which the suspension was filtered and thoroughly washed with pyridine, CH_2Cl_2 and diethyl ether and then vacuum dried over P_2O_5. DMTr release with acid and measurement at 498 nm showed a loading of 19.94 mmol/g CPG.

Synthesis, deprotection and purification of oligonucleotides 15 - 31

Modified ligonucleotides **23, 24, 29, 30** and the native oligonucleotides were synthesised on standard CPG support. The preparation of the oligonucleotides **22, 25, 28, 31** involved the use of modified support **14**. These syntheses were performed on 1.0 μmol scale with an 8-channel Applied Biosystems 392 DNA/RNA synthesiser using conventional 2-cyanoethyl phosphoramidite chemistry. The tethered uridine amidite block **13** was dissolved in dry acetonitrile with a final concentration of 0.1 M and used for solid-phase synthesis with a coupling time of 20 min (25 sec for standard nucleoside amidites). An estimation of the efficiency of the tethered amidite and modified solid support was determined from RP-HPLC after deprotection. This efficiency of coupling varied between 90 and 99%. The oligomers **15 - 18, 21 - 23, 28, 29** were synthesised with "5'-*O*-trityl on".

After each synthesis of the protected oligomers, the solid support was transferred directly out from the cassette to a 50 ml RB flask containing 20 ml of concentrated aq. NH_3 and was shaken for 2 h at 20°C. After removal of CPG by filtration and evaporation of the filtrate, the residue was redissolved in concentrated aq. NH_3 and stirred at 55°C for 16 h. The crude detritylated and 5'-*O*-tritylated oligomers were purified by reverse-phase HPLC eluting with the following gradient systems: **A** (0.1 M triethylammonium acetate, 5% MeCN, pH 7.0) and **B** (0.1 M triethylammonium acetate, 50% MeCN, pH 7.0). A linear gradient of 0-100% buffer **B** over 30 min at a flow rate of 1 ml / min was used. Retention times (R_t in minutes) of fully deprotected oligomers **15 - 16, 21 - 26** and **28 - 31** were as follows: **15** (15.78'), **16** (15.02'), **21** (13.91'), **22** (20.03'), **23** (19.0'), **24** (19.79'), **25** (21.75'), **26** (14.62'), **28** (20.44'), **29** (19.26'), **30** (20.19'), **31** (22.13'). Thus the purified solutions of the 5'-O-tritylated oligomers were evaporated and coevaporated with water 5 times and then dissolved in 2-3 ml 80% AcOH and shaken for 10 min, and then evaporated and neutralised with water and triethylamine, followed by evaporation. The residue was dissolved in water and extracted with diethylether. The water phase was then evaporated and residue lyophilised (5 x 1 ml H_2O). The detritylated oligomers were evaporated and coevaporated with water 5 times and then directly lyophilised (6 x 1 ml H_2O) to dryness. All oligonucleotides were subsequently sodium exchanged through a column of Dowex-50 Na^+-form. The absobance of the eluent was measured in A_{260} units (OD): 9-mers **26 - 31:** 8.5-21.8 OD; Target 11-mers **19 & 20:** 24.5 & 27.0 OD; 18-mers **21 - 25:** 20.4-53.8 OD; Target 24-mers **15 & 16:** 59.2 & 40.0 OD; Target 29-mers **17 & 18:** 14.7 & 17.0 OD.

Physico-chemical measurements

(i) Melting measurements.[30] UV melting profiles were obtained by scanning A_{260} absorbance versus time at a heating rate of 0.5°C/min from 5°C to 70°C for triplexes and 1.0°C/min from 5°C to 55°C for duplexes. The melting temperature T_m (±0.5°C) was determined as the maximum of the first derivative of melting curves. The triplex melting experiments were performed in 20 mM Na_2HPO_4/NaH_2PO_4, 0.1 M NaCl at pH 7.3, 6.5, 6.0

and the duplex melting experiments in the same buffer at pH 7.3. The approximate extinction coefficients for oligonucleotides **15 - 21, 26** were calculated as previously described.[30,31] In cases of the tethered oligomers (**22 - 25, 28 - 31**), the extinction coefficients were corrected for the absorbance contribution of the phenazine moiety at 260 nm by subtraction. After preparation, the solutions consisting of three components (for forming of triplexes) were heated to 70°C for 3 min and then allowed to cool down to 20°C for 30 min and then kept at 0°C overnight. The appropriate solutions consisting of two components (for forming of duplexes) were heated to 70°C for 3 min, and then allowed to cool down to 20°C for 30 min under shaking. During the melting measurements at temperatures below ~15°C, nitrogen gas was continuously passed through the sample compartment to prevent moisture condensation.

(ii) Static fluorescence measurements. Fluorescence measurements were made in 20 mM Na_2HPO_4/NaH_2PO_4, 1.0 M NaCl at pH 7.3 for duplexes and pH 7.3, 6.5, 6.0 for triplexes. Temperature of the cuvette holder was controlled with a recirculating water bath thermostated at 6°C for triplexes and 16°C for duplexes. For the fluorescence measurements, the concentration of each oligomer mixture (1.3 ml) was set relative to 0.02 absorbance units at the phenazine excitation wavelength. The ratio of the single strands in the mixtures of duplexes and triplexes were always 1:1 and 1:1:1 respectively. Emission spectra were recorded with λ_{ex} = 503 nm. Relative fluorescence intensities and Stoke's shifts were determined for each sample at the same excitation/emission bandpass width.

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