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Synthesis of Phenazine-tethered Arabino and Xylofuranosyl Oligonucleotide Conjugates: The Thermal Stability and Fluorescence Properties of Their Duplexes (DNA-DNA & DNA-RNA) & Triplexes

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Abstract: The synthesis of phenazine (Pzn) tethered ara-U and xylo-U incorporated oligonucleotides, and their properties as DNA-DNA, DNA-RNA duplexes and as triplexes are reported. 2'-O-Pzn-tethered ara-U amidite (11) and Pzn-linked amidite block (4) as well as CPG solid supports functionalised with 2'-O-Pzn-tethered ara-U (10a -10c) and 3'-O-Pzn-tethered xylo-U succinates (22a - 22c) were used in the solid-phase DNA synthesis to prepare modified 9mers 25 - 29, 31, 41 - 45 (for DNA-DNA & DNA-DRA duplex studies) and 18mers 35 - 40, 46 - 50 (for the DNA triplex studies). Thermal melting experiments with the resulting duplexes and triplexes showed that all oligo-DNAs, except for the middle-modified ones (31 & 40), have enhanced affinity to the DNA and RNA targets (23&52) as well as for the DNA duplex target 32.03. Pzn-tethered ara-U block was more efficient at the 3'-terminal of oligonucleotides than at the 5'-end in the duplexes and especially in triplexes, where it provided a dramatic improvement in the stability ($\Delta T_m = 16.1^{\circ}C$). Employment of Pzn-tethered *ara*-U block at the 3'-end together with the Pzn-tethered dT block at the 5'-end of the oligo-DNAs (29, 38) provided the best duplex and triplex stabilisation to give a highest ΔT_m of 14.4°C for DNA-DNA duplexes, ΔT_m of 11.7°C for DNA-RNA duplexes and ΔT_m of 19.6°C for triplexes. All DNA-DNA and DNA-RNA duplexes as well as DNA triplexes, formed by the oligos modified with 2'-O-Pzn-tethered ara-U blocks showed geater stability than those formed by the oligos modified with xylo-analogs with the same length of the linker arms. For both ara- and xylo-configurations, the best DNA-DNA & DNA-RNA duplex stabilisation was provided by the short ethyl linkers, and increasing the length of the linker led to considerable destabilisation of the duplexes. In case of triplexes, longer linker arms were required to obtain better stabilisation. Hexyl linker provided the highest triplex stabilisation for the oligonucleotides modified with Pzn-tethered ara-U block $(\Delta T_m = 16.5^{\circ}C)$ and butyl linker was found to be most suitable for the oligo-DNAs modified with Pzn-tehtered xylo-U block ($\Delta T_m = 12.3^{\circ}C$). Fluorescence studies showed that Pzn behaves as a weak exterior binder upon DNA-DNA or DNA-RNA duplex or DNA triplex formation which accounts for moderate changes in the fluorescent properties of the Pzn moiety (ΔF for DNA-DNA and DNA-RNA duplexes = ±0.2, ΔF for triplexes = 1.4 - 2.5). Employment of Pzn at both 3' and 5' ends of the oligonucleotides (as in 27, 29, 38 & 39) provided the greatest duplex and triplex stabilisation so far, and led to more effective interaction between the Pzn moieties and the double and triple helixes (ΔF for DNA-DNA and DNA-RNA duplexes = 4, ΔF for triplexes = 5). © 1998 Published by Elsevier Science Ltd. All rights reserved.

Design and synthesis of the antisense or antigene oligonucleotides have recently attracted much attention owing to their potential importance in the treatment of pathogen infection and genetic diseases. Some of the limitations to the development of antisense and antigene strategies include poor stability of short

0040-4020/98/\$19.00 © 1998 Published by Elsevier Science Ltd. All rights reserved. *PII:* S0040-4020(98)00457-8 double and triple helix structures under physiological conditions at low salt concentration and neutral pH, their cellular instability as well as their poor penetration properties through the cell wall.

In order to improve the aspect of poor stability of short double and triple helix structures, covalent attachment of polyaromatic fragments to the antisense or antigene oligonucleotides have been introduced¹⁻¹⁵. The advantage of such covalently linked polyaromatic tether is that they do not interfere with the ionic and tautomeric properties of natural nucleotides, but they allow further derivatization for engineering additional functionalities such as possibilities of oxidative or free-radical promoted cleavage or alkylation of the opposite strand either in the antisense or antigene approach. A number of heterocyclic aromatic compounds¹⁻¹⁵ have shown to be capable of stabilising duplexes and triplexes by intercalating between adjacent basepairs of a double-stranded nucleic acid or binding within the minor groove. The choice of intercalator is not the only important variable in the design of oligonucleotide, the nature and the length of the linker chain and position used for its attachment to the oligo-DNA (3'- or 5'-terminal or in the middle, nucleobase, phosphate backbone or apurinic spacers) are also of central importance.

Intercalating agents such as N-hydroxyethyl-phenazinium¹, 2-methoxy-6-chloro-9-aminoacridine², ethidium³, daunomycine⁴ have been conjugated to the oligonucleotides through terminal phosphates using post-synthetic coupling technique, while acridine^{5a} and psoralen^{5b} have been conjugated via the solid-phase synthesis. Groove-binding agent such as Hoechst 33258 has been introduced at the 5'-end^{5c} and in the middle⁶ of the oligo-DNAs, and showed to enhance the stability of duplexes and triplexes. Intercalating derivatives have been also attached to the N-2 of purine and C-5 of pyrimidine bases in the oligonucleotides^{7a,b,8a,b}. Binding properties of the resulting oligonucleotide conjugates have shown^{8b} to be dependant on the location of the modified blocks within the oligonucleotide chain. Internucleotidic phosphate has also been used⁹ for incorporating acridine into the middle of the deoxyoligonucleotide. Intercalating agents such as pyrene^{10a}, anthracene^{10a} and phenazine^{10b} have been linked to the C-1' position of the 3'-deoxypsicofuranosyluridine located at the interior^{10a,b} or at the ends^{10b} of a oligonucleotide sequence to study the stabilities of duplexes^{10a,b} and triplexes^{10b}. The 2'-hydroxy group of ribonucleosides has also been used for tethering different polyaromatic systems, e.g. 2'-O-(1-anthraquinonylmethyl)uridine¹¹ and 2'-O-(1-pyrenylmethyl) uridine¹², which were introduced at different positions of oligonucleotides by solid-phase synthesis. Acridine has been incorporated at the 5'-end of the oligonucleotide chain using an abasic phosphoramidite¹³ and to the internal abasic spacers by post-synthetic¹⁴ tethering to the depurination site of a deoxyoligonucleotide.

All these studies show that the stabilising properties of intercalators is sequence-specific, and they also strongly depend on factors such as (i) the type of the intercalator, (ii) the chemical character and length of the linker, and (iii) the site of tethering to the monomeric block (*i.e.* at the sugar, aglycone or phosphate) as well as site of this tethered block into the oligonucleotide chain.

To the best of our knowledge, very little systematic study has been performed to elucidate how the configuration of the 2'- and 3'-OH in the pentofuranoside moiety in *ara*- or *xylo*-configuration, as the site of the tether attachment in a modified oligonucleotide, may have control on the relative stability of the DNA-DNA, DNA-RNA duplexes as well as for the DNA triplxes. The only study has so far appeared concerns the 2'-hydroxy group of ribonucleosides^{11,12} or 1'-OH of psicofuranosyl nucleosides^{10a,b} as the position of attachment of a tethered intercalator, which have shown some encouraging results enhncing the stabilization of both duplexes^{10a,b} and triplexes^{10b}.

We herein report our results on the improvement of DNA-DNA and DNA-RNA duplex and DNA triplex stabilities by incorporating *ara*-U and *xylo*-U nucleotides with covalently attached phenazine (Pzn) intercalator at the 3' or/and 5'-end or in the middle of the antisense DNA strand or in the triplex forming oligonucleotide strand. Detailed fluorescence studies have been also undertaken to address the mechanism of stabilization by the Pzn intercalator.

The 2'-O-Pzn-tethered *ara*-U and 3'-O-Pzn-tethered *xylo*-U nucleotides with the $-(CH_2)_2$ -, $-(CH_2)_4$ - and $-(CH_2)_6$ - linkers (**8a** - **8c**, **20a** - **20c**) were synthesised to explore the stabilising influence of suitable orientations of the Pzn intercalator for the DNA-DNA and DNA-RNA duplexes resulting from modified 9mer oligodeoxynucleotides (**25** - **29**, **31**, **41** - **45**) and 11mer oligo-DNA **23** or **51** or 11mer oligo RNA **52** or **53** targets. We have also similarly examined the stability of triplexes resulting from the Pzn-modified 18mer third strands (**35** - **40**, **46** - **50**) with the target duplex (**32** • **33**). Subsequently, the relative stabilities of the resulting DNA-DNA duplexes **23** • (**25** - **27**, **31**, **41** - **45**) and DNA-RNA duplexes **52** • (**25** - **27**, **31**, **41** - **45**) and the triplexes (**32**•**33**)-(**35**, **36**, **39**, **40**, **48** - **50**) were also compared with the results obtained with the 5'-Pzn tethered oligo-DNAs¹⁵.

RESULTS AND DISCUSSIONS

(A) Preparation of the Pzn-tethered blocks

The 2-(N-(2-hydroxyethyl)-N-methyl)aminophenazine (1) was synthesized using an earlier procedure from 9methyl phenazinium methylsulphate^{15,16}. A minor modification of this procedure gave Pzn derivatives with butyl and hexyl linkers, as in 2a and 2b, which were converted to the the corresponding phenoxyacetyl (Pac) derivatives 3a and 3b (Scheme 1) (experimental section).



Reaction conditions: (a) $H_2N(CH_2)_nOH / MeOH$, 25°C, 72 h; (b) chlorotrimethylsilane / pyridine, 25°C, 30 min then PacCl, 1 h, followed by aq. NH₃ treatment for 30 min; (c) (2-(CeO)-((ipr)_2N)PCl / (ipr)_2EtN / THF, 1 h, 20°C

Scheme 1

(i) Preparation of Pzn-linked amidite block 4 for the synthesis of 5'-Pzn tethered oligonucleotides 28, 29, 37 & 38.

For the attachment of Pzn to the 5'-end of the oligonucleotides through the terminal phosphate, phosphoramidite 4 (Scheme 1) was prepared from 2-(N-(2-hydroxyethyl)-N-methyl)aminophenazine 1 in the usual way¹⁵ and used directly in the solid-phase synthesis (experimental).

(ii) Preparation of 2'-Pzn-tethered ara-U blocks 10a-c & 11 for the synthesis of the Pzn-tethered oligonucleotides 25-27, 29, 31, 35, 36, 38-40, 41, 42, 46 & 47.

In order to attach phenazine to the 3'-ends of the oligonucleotides, we functionalised 3-aminopropyl-CPG with phenazine-tethered *ara*-U succinates 10a - 10c (Scheme 2). Preparation of the *ara*-succinates was



Reaction conditions: (a) $CH_3O((ipr)_2N)PCl / (ipr)_2EtN / THF, 1 h, 20°C;$ (b) Tetrazole / MeCN / PnzN(R₃)(CH₂)_nOH, 1 h, 20°C then I₂ / THF / H₂O / pyridine, 30 min, 20°C; (c) TBAF.H₂O / THF, 30 min, 0°C; (d) DMTrCl / pyridine, 4 h, 20°C; (e) succinic anhydride / DMAP / CH₂Cl₂, 1.5 h, 20°C; (f) (2-CeO)-((ipr)₂N)PCl / (ipr)₂EtN / THF, 1 h, 20°C.

Scheme 2

carried out starting from (1,1,3,3-tetraisopropyl-1,3-yl)ara-uridine 5^{17} which was converted to the corresponding 2'-phosphoramidite block 6^{17} . The amidite 6 was then condensed with 2-(*N*-(2-hydroxyethyl)-*N*-methyl)aminophenazine 1, with 2-(*N*-(4-hydroxybutyl)-*N*-phenoxyacetyl) aminophenazine 3a and with 2-(*N*-(6-hydroxyhexyl)-*N*-phenoxyacetyl)aminophenazine 3b (Scheme 1) through tetrazole activation¹⁸,



Reaction conditions: (a) p-TolCl / pyridine, 2 h, 20°C; (b) $Ac_2O / AcOH / H_2SO_4 / CH_2Cl_2 dry$, 0°C, 15 min; (c) silylated uracil / $C_2H_4Cl_2 dry$ / trimethylsilyl trifluoromethanesulfonate, 45°C, overnight; (d) NH₃/MeOH, 50°C, 24 h; (e) DMTrCl / pyridine, 20°C, 4 h than DMAP / more DMTrCl, 60°C, overnight; (f) CH₃O((ipr)₂N)PCl / (ipr)₂EtN / THF, 3 h, 25°C; (g) tetrazole / CH₃CN / PznN(R₃)(CH₂)_nOH, 1 h, 20°C then $I_2 / THF / H_2O / pyridine, 30 min, 20°C;$ (h) CCl₃COOH / MeOH / CH₂Cl₂, 0°C, overnight; (i) DMTrCl / pyridine, 4 h, 20°C; (j) succinic anhydride / DMAP / CH₂Cl₂ dry, 1.5 h, 20°C.

Scheme 3

followed by oxidation by iodine to give the phosphotriester blocks 7a - 7c (70 - 82%). Compounds 7a - 7c were treated with n-tetrabutytlammonium fluoride (TBAF) in THF to give the corresponding stable 2'-*ara* phosphotriester blocks 8a - 8c (72-83%). Blocks 8a - 8c were converted to their corresponding 5'-O-DMTr-derivatives 9a - 9c (78-83%), which were 3'-O-succinylated to give the corresponding succinates 10a - 10c.

They were coupled to the 3-aminopropyl-CPG support to give loading of 24.0 μ m / g for 10a, 24.5 μ m / g for 10b and 22.9 μ m / g for 10c, after capping with acetic anhydride (see experimental).

An aliquot of 5'-O-DMTr-derivative 9a was converted in a usual way¹⁵ into the corresponding phosphoamidie block 11, which was used in the solid-phase synthesis for its incorporation at the 5'-end and in the middle of oligo-DNA chain (Scheme 2).



Fig. 1: The RP-HPLC profiles of the pure modified 9-mers 25 - 29, 41 - 45, modified 18-mers 35 - 39, 46 - 50 and blank 9- and 18-mer 23 & 34. Gradient 0 - 100% B in 30 min at 1ml/min was used. Buffer A: 0.1M TEAA, 5% MeCN; Buffer B: 0.1M TEAA, 50% MeCN. Retention times are given in minutes.

(iii) Preparation of 3'-Pzn tethered xylo-U blocks 22a-c for the synthesis of the Pzn-tethered oligonucleotides 43-45 & 48-50.

The succinates of Pzn-tethered xylo-U 22a-c were obtained from a mixture of α - and β -methyl-Dxylofuranosides 12¹⁹. The anomeric mixture 12 was p-toluoylated to give 13 which was converted to the



Fig. 2: Oligonucleotide conjugates 25 - 29, 31 modified with phenazine-tethered *ara*-U and phenazine-tethered dT blocks, blank 9-mers 24, 51 and their targets 23, 30 for duplex study.

Table 1. Melting temperatures $(T_m \text{ in }^{\circ}C)$ of duplexes formed by mixing target 23 and 9-mers 25 - 35 with Pzn-tethered *ara*-U and dT blocks conjugated at different positions of the oligonucleotides.

Entry #	9-mer conjugates	T _m	ΔT _m *
1	25	34.6	8.7
2	26	34.1	8.2
3	27	37.3	11.4
4	28	35.9	10.0
5	29	40.3	14.4
6	31	а	•
7	24	25.9	-

Table 2. Melting temperatures $(T_m \text{ in }^{\circ}C)$ of duplexes formed by mixing target 23 and 3'-end-modified 9-mers 41 - 45 containing Pzn- tethered *ara*-U and *xylo*-U blocks through linkers of different lengths.

Entry #	9-mer conjugates	Tm	ΔT _m
1	25	34.6	8.7
2	41	30.0	4.1
3	42	31.6	5.7
4	43	31.8	5.9
5	44	29.9	4.0
6	45	28.2	2.3

a : not detected

* $\Delta T_m = T_m \text{ of } (23 \cdot (25 - 29)) - T_m \text{ of } (23 \cdot 24) \text{ in }^{\circ}C$

corresponding 1'-O-acetyl derivative 14, and was directly used for glycosylation with silylated uracil to give 1-(2', 3', 5'-tri-O-(p-toluyl)- β -D-xylofuranosyl)uracil 15 (76%). Compound 15 was deprotected with NH₃/MeOH to give fully deprotected xylo-uridine 16 (94%), which was converted to an isomeric mixture of 2', 5'-di-O-DMTr-xylo-U and 3', 5'-di-O-DMTr-xylo-U in 8:1 ratio, from which pure 2', 5'-di-O-DMTr-derivative 17 (79%) was isolated. Compound 17 was converted to the corresponding amidite 18 (91%) using methyl N,N-diisopropylchlorophosphoramidate ¹⁵. The amidite 18 was coupled to Pzn-linkers 1, 3a or 3b in a similar way as described for the compounds 7a-7c, to give the corresponding triester-blocks 19a - 19c (80 -

87%). Deprotection of these derivatives with trichloroacetic acid in dichloromethane-methanol mixture (9:1, v/v) gave 3'-O-Pzn tethered xylo-U-blocks 20a - 20c in 79 - 86% yields. The 5'-O-DMTr-derivatives 21a - 21c and their 2'-O-succinates 22a - 22c were made in the same way as for compounds 9a - 9c and 10a - 10c respectively (Scheme 3). Succinates 22a, 22b and 22c were coupled to the 3-aminopropyl-CPG with loadings of 23.1, 21.4 and 22.6 μ m / g respectively.

32: 5'-d(GCGTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	CTTGGC)-3'	(24-mer) -	target
	AGAACCG)-5'	(24-mer)	duplex
34 : 3'-d(TCTTTTTTTTTTTTTTTTTTTTTT)-5'	nonmodified	18-mer	
35 : 3'-d(<u>UCTTTTTTTTTTTTTTTTTTTTTT</u>)-5'	36 : 3'-d(TCTTT	TTTCTTT	ГТТСТ <u>U</u>)-5'
37 : 3'-d(TCTTTTTTTTTTTTTTTTTTTTTTTTTTT	38 : 3'-d(<u>U</u> CTTT	TTTCTTT	ГТТСТ <u>Т</u>)-5'
39 : 3'-d(<u>UCTTTTTTTTTTTTTTTTTTTTTTTTTT</u>)-5'	40 : 3'-d(TCTTT	TTTC <u>U</u> TT	ГТТСТТ)-5'

Fig. 3: Oligonucleotide conjugates 35 - 40 modified with phenazine-tethered *ara*-U and phenazine-tethered dT blocks and their duplex target for triplex formation $32 \cdot 33$.

Table 3. Melting temperatures (ΔT_m in °C) of triplexes and 18-mer +24-mer duplexes formed by target duplex 32•33 and 18-mers 35 - 40 with *ara*-U and dT blocks tethered phenazine through the ethyl lihker, conjugated at different positions of the oligonucleotides.

Entry	18-mer	Triplex		18+24 duplex	
#	conjugates	T _m	ΔT_m	Tm	ΔT _m
1	35	29.6	16.1	48.1	5.3
2	36	16.8	3.3	50.2	7.4
3	37	17.4	3.9	46.6	3.8
4	38	33.1	19.6	50.5	7.7
5	39	24.8	11.3	52.5	9.7
6	40	а	-	37.6	-5.2
7	34	13.5	-	42.8	-

a : not detected

(B) Preparation of Pzn-tethered arabino and xylofuranosyl oligonucleotide conjugates.

All modified oligoconjugates 25 - 29, 31, 35 - 40, 41 - 50 as well as natural oligos 23, 24, 30, 32 - 34, 51, 52 and 53 were synthesised in 1.0 μ mol scale (see experimental section for details) using an automated DNA / RNA synthesiser using conventional β -cyanoethyl phosphoramidite chemistry^{18,24a,b}. oligonucleotides were deprotected with concentrated aq. NH₃ at 55°C in 17 h. Crude oligomers were purified on reverse-phase HPLC. The purities and HPLC retention times of all modified oligos are shown in Fig. 1, showing that the modified oligos have higher retention times than the natural counterparts. All the oligomers were subsequently sodium exchanged through a column of Dowex-50 Na⁺-form. (C) Melting experiments. The T_m for all oligonucleotides containing 2'-O-Pzn-tethered *ara*-U, 3'-O-Pzn-tethered *xylo*-U and 5'-O-Pzn-tethered dT were measured in the same buffer system, and the specific conditions for the melting experiments are described in details in the experimental section.

(i) The T_m study of DNA-DNA duplexes with the modified oligonucleotides containing Pzn-tethered ara-U or dT blocks with the ethyl [-(CH₂)₂-] linker

The effect of conjugating Pzn-tethered *ara*-U block at 3' or/and 5'-terminal ends of the oligo-DNAs on duplex stability has been investigated by hybridising the modified 9-mers 25 - 27 with the target 11-mer oligo-DNA 23. In order to compare this effect, oligonucleotides with the Pzn tethered to the 5'-end of the terminal thymidine, as in 28 and 29 (Fig. 2), were also made because this has proven to be an efficient means for the duplex stabilisation¹⁵. Table 1 shows the T_ms of the resulting duplexes from each of modified oligos 25 - 29 with target 23, as well as the blank experiment with the duplex formed by mixing the non-modified 9-mer 24 and target 23.

All oligonucleotides modified with Pzn-tethered *ara*-U block (**25-29**) showed enhanced affinity to the target **23**. The Pzn-tethered *ara*-U moiety at the 5'-end of the oligonucleotide **26** (entry #2 in Table 1) formed slightly less stable duplex ($\Delta T_m = +8.2^{\circ}C$) compared to the 5'-Pzn-tethered dT block in oligonucleotide **28** (entry #4, $\Delta T_m = +10.0^{\circ}C$, Table 1)¹⁵. It was also observed that the effect of the 3'-Pzn-tethered *ara*-U block as in oligonucleotide **25** (entry #1) is slightly higher ($\Delta T_m = +8.7^{\circ}C$) than that at the 5'-end in **26** (entry #2, $\Delta T_m = +8.2^{\circ}C$). It is noteworthy that the employment of the Pzn-tethered *ara*-U block at both 3'- and 5'-terminals of oligo-DNA, as in **27**, enhanced the duplex stability with target **23** (entry #3, $\Delta T_m = +11.4^{\circ}C$), which even increased more remarkably with a bis-modified oligonucleotide with the phenazine-tethered *ara*-U block at 3'-end and phenazine-tethered dT block at the 5'-end, as in **29** (entry #5, $\Delta T_m = +14.4^{\circ}C$). When the Pzn-tethered *ara*-U block was placed in the middle of the oligonucleotide, as in **31**, it failed to form a duplex with the target **30**, compared to the blank **30** • **51** ($T_m = 22.4^{\circ}C$), presumably because of steric reasons.

(ii) The T_m study of DNA triplexes with the modified oligonucleotides containing Pzn-tethered ara-U or dT blocks with the ethyl [-(CH₂)₂-] linker

The ability of the oligos modified with the Pzn-tethered ara-U block to stabilise the triple helix was tested by preparing different 18-mer analogues 35 - 40 as the triplex forming strands. These were modified in the same way as the 9-mers used for the duplex study. Each of these oligos containing Pzn-tethered ara-U was mixed with 24-mer duplex target (32•33) in equimolar ratio (Fig. 3).

All melting curves obtained from samples **34-39** showed a triphasic dissociation behaviour. Table 3 shows the T_ms of the triplexes as well as of 18+24 duplexes (resulting from hybridization of the modified 18-mers with the A-rich strand of the target duplex (**33**)).

It can be seen that the triplex [35-32•33] formed with Pzn-tethered *ara*-U conjugated at the 3'-end of 18-mer 35 shows remarkable stabilisation (entry #1, Table 3, $\Delta T_m = +16.1^{\circ}$ C) compared to oligo 36 in which Pzn-tethered *ara*-U is conjugated at the 5'-end of 18-mer (entry #2, $\Delta T_m = +3.3^{\circ}$ C) or oligo 37 in which phenazine-tethered dT is conjugated at the 5'-end of 18-mer (entry #3, $\Delta T_m = +3.9^{\circ}$ C). Consistent with our above observation on duplex studies, phenazine-tethered *ara*-U block at the 3'-end together with dT block at the 5'-end of oligonucleotide, as in 38, gave considerably improved stability of the triplexs [38•35-32] (entry #4, $\Delta T_m = +19.6^{\circ}$ C) than the triplex [39-32•33] containing 3', 5'-bis-Pzn-*ara*-U tethered oligonucleotide **39** (entry #4, $\Delta T_m = +11.3^{\circ}$ C). No triplex formation was detectable with the Pzn-tethered *ara*-U block in the

middle of the oligonucleotide as in 40. Even the duplex formed from $33 \cdot 40$ was considerable destabilised (entry #6, $T_m = 37.6^{\circ}$ C) compared to the blank duplex $33 \cdot 34$ (entry #7, $T_m = 42.8^{\circ}$ C).

(iii) The T_m study of DNA/RNA conjugates formed by modified oligonucleotides, containing Pzn-tethered ara-U or dT blocks with the ethyl [-(CH₂)₂-] linker (25 - 29, 31), and target RNAs 52, 53.

It has been previously shown that oligonucleotide-dye conjugates can be used for inhibiting mRNA translation and viral expression.^{20 - 22} We decided to test the ability of phenazine-tethered oligo-DNAs to enhance the thermal stability of DNA/RNA duplexes to explore their usefulness in the antisense research. Target 11-mer RNAs **52** and **53** (Fig. 4) were synthesised and mixed with phenazine-tethered oligonucleotides at the same concentrations and buffer conditions as described above for the DNA/DNA duplexes. The results of these melting experiments are presented in the Table 4.

52: 5'-CAUGUUUGGAC-3' 53: 5'-CAUGUAUGGAC-3'

Fig. 4: Target 11-mer RNAs used in antisense approach.

Table 4. Melting temperatures (T_m in °C) of duplexes formed by target 11-mer RNAs 52, 53 and 9-mer DNAs 25 - 35, 41 - 45 containing 2'-phenazine-tethered *ara*-U, 3'-phenazine-tethered *xylo*-U and 5'-phenazine-tethered dT blocks conjugated to different positions of the oligonucleotide.

Entry #	DNA/RN	Tm	ΔT _m	Entry #	DNA/RN	T _m	ΔT _m
1	52 + 24	20.4	-	8	52 + 41	25.0	4.6
2	53 + 51	25.7	-	9	52 + 42	27.1	6.7
3	52 + 25	29.1	8.7	10	52 + 43	25.6	5.2
4	52 + 26	25.8	5.4	11	52 + 44	25.1	4.7
5	52 + 27	31.3	10.9	12	52 + 45	21.8	1.4
6	52 + 28	29.3	8.9	13	53 + 31	а	-
7	52 + 29	32.6	11.7				

a : not detected

As in the case of DNA/DNA duplexes (Table 1), all modified oligonucleotides 25 - 29 (entries #3-7, Table 4), showed enhanced affinity to the RNA strand as compared to the blank 9-mer 24 (entry #1, Table 4). For the middle-modified 9-mer 31 no duplex formation with target 53 was observed within the temperature range studied (entry #13, Table 4) when blank 9-mer 51 formed duplex with target 53 with T_m of 25.7°C (entry #2, Table 4). Modified 9-mer containing the Pzn-tethered *ara*-U block at the 3'-end of the oligonucleotide 25 again showed higher duplex (52•25) stabilisation ($\Delta T_m = 8.7^{\circ}C$, entry #3) than the oligo having 5'- Pzn-tethered *ara*-U block as in 26 (*i.e.* 52•26) ($\Delta T_m = 5.4^{\circ}C$, entry #4), and gave almost the same increase of T_m as oligo-DNA 28 (*i.e.* 52•28), modified with 5'-phenazine-tethered dT block at the 5'-end ($\Delta T_m = 8.9^{\circ}C$, entry #6). Oligonucleotides tethered with phenazine at both 3' and 5' terminals as in 27 and 29 formed the most stable duplexes (*i.e.* 52•27 & 52•29) as was expected (entry #5, $\Delta T_m = 10.9^{\circ}C$, entry #7, $\Delta T_m = 11.7^{\circ}C$ in Table 4).

It is seen from the melting experiments with DNA/DNA and DNA/RNA duplexes as well as DNA triplexes that the oligo-DNAs modified with the Pzn-tethered ara-U block, conjugated to the 3'-terminal form the most stable double and triple helixes. Having obtained these data we decided to explore the posibility to improve our results by the following two ways: (i) optimisation of the chain length of the tether conjugating the phenazine moiety with the nucleotide, and/or (ii) changing the configuration of the secondary hydroxyl group in a nucleoside, which is used for anchoring the tethered intercalator, *i.e.* substitution of the arabinouridine by the xylouridine.

Hence, *ara*-U nucleosides bearing Pzn conjugated through the butyl [-(CH_2)₄-] linker (**10b**) and hexyl [-(CH_2)₆-] linker (**10c**) were preapared (Scheme 2). Scheme 3 shows the preparation of the *xylo*-U analogues where phenazine is tethered at the 3'-O-position of sugar moiety through the ethyl (**22a**), butyl (**22b**) and hexyl (**22c**) linkers. All these modified blocks were incorporated at the 3'-ends of the corresponding 9 and 18-mers (Fig. 5) and their abilities to stabilise duplexes and triplexes were tested on the same targets, *i.e.* **23** for probing DNA-DNA coconjugates, **52** for DNA-RNA coconjugates, and the duplex **32**•**33** for studying DNA triplexes.

3'-d(<u>U</u> ACAAACCT)-5'	3'-d(<u>UCTTTTTTCTTTTTCTT</u>)-5			
41: $\underline{U} = 8b$ 42: $\underline{U} = 8c$ 43: $\underline{U} = 20a$ 44: $\underline{U} = 20b$ 45: $\underline{U} = 20c$	46: $\underline{U} = 8b$ 47: $\underline{U} = 8c$ 48: $\underline{U} = 20a$ 49: $\underline{U} = 20b$ 50: $U = 20c$			

Fig. 5: Oligonucleotide conjugates modified at 3'-terminal with phenazine attached to the *ara*- and *xylo*-uridines through linkers of different length.

(iv) The T_m study of DNA-DNA and DNA-RNA duplexes formed by the 3'-end modified 9-mer oligonucleotides containing Pzn-tethered ara-U blocks with the linkers of different sizes (25, 41 & 42) (Fig. 2 & 5).

Table 2 shows the results of the thermal melting experiments for the DNA-DNA duplexes formed with target 11-mer DNA 23 (entries #1-3). Increasing the length of the linker chain from $-(CH_2)_2$ - as in 25 ($\Delta T_m = +8.7^{\circ}C$) to $-(CH_2)_4$ - as in 41 ($\Delta T_m = +4.1^{\circ}C$) decreased duplex stability, but further chain elongation to $-(CH_2)_6$ - as in 42 gave a slight recovery of stabilization (entry #3, $\Delta T_m = +5.7^{\circ}C$). The same tendency was found for the DNA-RNA duplexes formed by modified 9-mers 25, 41 & 42 and target 11-mer RNA 53 (Table 4, entries #3,8&9), where ethyl linker (as in 23) provided the best duplex stabilisation (entry #3, $\Delta T_m = +8.7^{\circ}C$), followed by a drop of T_m for the butyl linker (41) (entry #8, $\Delta T_m = +4.6^{\circ}C$) and slight recovery of stabilization for the hexyl linker (42) (entry #9, $\Delta T_m = +6.7^{\circ}C$).

(v) The T_m study of DNA triplexes formed by the 3'-end modified 18-mer oligonucleotides containing Pzntethered ara-U blocks with the linkers of different sizes (35, 46 & 47) (Fig. 3 & 5).

Table 5 shows the data from the melting experiments of triplexes formed by mixing target duplex 32•33 and 3'-modified 18-mers 35, 46 or 47. An increase of the linker chain length from ethyl (entry #1) to butyl (entry #2) had a negative effect on triplex stability (ΔT_m dropped from 16.1°C for 35 to 12.7°C for 46), but further chain elongation to a hexyl linker as in 47 dramaticly improved the affinity of the third strend to the duplex and gave even a slightly higher stabilisation than in case of the ethyl linker (entry #3, $\Delta T_m = +16.5^{\circ}$ C).

(vi) The T_m study of the DNA-DNA and DNA-RNA duplexes formed by the 3'-end modified 9-mer oligo-DNAs containing Pzn-tethered xylo-U blocks with the linkers of different sizes (43 - 45) (Fig. 5).

The results of the thermal melting experiments for the duplexes formed with target 11-mer DNA 23

are presented in Table 2 (entries #4-6). It is clear that, in modified 9-mers with phenazine-tethered xylo-U block at the 3'-ends, a stepwise increase of the linker chain length brings about a steady drop in the duplex stabilisation (ΔT_m) from 5.9°C for ethyl linker (as in 43) to 2.3°C for hexyl linker (as in 45). Similar results were obtained when oligo-DNAs 43 - 45 were conjugated with target RNA strand 52 (Table 4, entries #10-12), where oligonucleotides containing butyl linker as in 44 ($\Delta T_m = +4.7$ °C) and hexyl linker as in 45 ($\Delta T_m = +1.4$ °C) showed pure stabilisation compered to oligo 43 containing short ethyl linker ($\Delta T_m = +8.7$ °C).

(vii) The T_m study of DNA triplexes formed by the 3'-end modified 18-mer oligonucleotides containing Pzntethered xylo-U blocks with the linkers of different sizes (48 - 50) (Fig. 5).

In case of triplexes formed by modified oligo-DNAs 48 - 50 and target duplex 32•33 an increase in the linker size from ethyl as in 48 (Table 5, entry #4, $\Delta T_m = 8.0^{\circ}$ C) to butyl as in 49 (Table 5, entry #5) improved the triplex stability providing the highest T_m for this group of oligonucleotides ($\Delta T_m = 12.3^{\circ}$ C). Further chain elongation to the hexyl linker as in 50 decreased the triplex stability (entry #6, $\Delta T_m = +10.9^{\circ}$ C). Comparison of ΔT_m s for duplexes formed by modified 18-mers 48 - 50 (entries #4-6, Table 4) and 24-mer purine-rich strand 33 from the target duplex shows that shortest linker, as in 48, (entry #4, $\Delta T_m = 5.2^{\circ}$ C) provides the best duplex stabilisation. This observation fits with the data obtained from melting experiments of the duplexes formed by 3'-end modified 9-mers 25, 41 - 45 and target 11-mer 23 (Table 2), where blocks with ethyl linker as in 25 (entry #1, $\Delta T_m = 8.7^{\circ}$ C) and 43 (entry #4, $\Delta T_m = 5.9^{\circ}$ C) showed the highest stabilisation of duplexes among the phenazine-tethered xylo-U and ara-U modified oligo-DNAs.

Table 5. Melting temperatures (T_m in °C) of triplexes and 18mer + 24mer duplexes formed by target duplex 32 + 33 and 3'-end-modified 18-mers 35, 46 - 50 containing *ara*-U and *xylo*-U blocks tethered phenazine through the linkers of different sizes.

Entry	18-mer	Triplex		18+24 duplex	
#	conjugates	T _m	ΔT_{m}	T _m	ΔT_m
1	35	29.6	16.1	48.1	5.3
2	46	26.2	12.7	45.3	2.5
3	47	30.0	16.5	45.6	2.8
4	48	21.5	8.0	48.0	5.2
5	49	25.8	12.3	45.5	2.7
6	50	24.4	10.9	45.7	2.9
7	34	13.5	-	42.8	-

(D) Fluorescence experiments.

We examined the fluorescence properties of each phenazine-tethered oligonucleotide in the single strand state (conditions are described in experimental part) and as 1:1 complexes with complementary DNA target (23), RNA target (52) and 24-mer duplex DNA target (32 + 33) at the same concentration of the modified strand.

(i) Fluorescence experiments with DNA-DNA and DNA-RNA duplexes formed by Pzn-modified 9-mers 25 - 29, 41 - 45 and targets 23 (DNA) and 52 (RNA).

Upon hybridisation to the target DNA or RNA sequence no significant changes in flourescence intensities ($\Delta F \approx \pm 0.2$) and no significant shift of the emission maximum were detected for any oligos bearing one phenazine moiety (25, 26, 28, 41 - 45). The moderate changes found do not suggest intercalative mode of binding of phenazine to the double helix or any cosiderable binding to the minor or major groove of the duplexes. Enhancement of thermal stability of duplexes by phenazine without any significant change in its fluorescent character (no changes in its microenviroment upon the duplex formation) suggests that it is binding weakly to the exterior of the heterocyclic bases of DNA/DNA and DNA/RNA duplexes.

Oligonucleotides containing two phenazines as in 27 and 29 gave increase in the flourescence intensities by 4-fold when conjugated to DNA or RNA targets. Strong fluorescence signals observed for these complexes suggest that phenazine moiety is capable of more efficient stacking (probably within the minor grove of the duplex) or is intercalating more efficiently if the appropriate binding sites are available. Here, one of the phenazine moieties maybe assisting in the formation of a more stable duplex where the minor grove is more defined and can more easily accommodate another phenazine than in the case of the native duplex. Also possible is that a more ordered interstrand stacking of terminal base-pairs, provided by one of the phenazines is giving the other better conditions for terminal stacking or intercalation.

(ii) Fluorescence studies with triplexes formed by Pzn-modified 18-mers 35 - 40, 46 - 50 and target duplex 32•33.

When modified 18-mers were conjugated with 24-mer DNA duplex target a slight blue shift (6-11 nm) of the emission maximum was observed for the all samples. The enhancement in the flourescence intensities by 1.4 - 2.5-folds detected for these complexes was also moderate and suggests a weak exterior interactions between phenazine and resulting triplexes. No correlation between thermal stability and the fluorescent properties of the triplexes was found. Exceptional fluorescent behaviour upon the triplex formation ($\Delta F \approx 5$ -fold) in this group of oligos was observed for 18-mers tethered with phenazine at both the terminals (**38**, **39**). That can probably be explained in a similar way as done for duplexes formed by double-modified 9-mers (**27**, **29**) (see above).

CONCLUSIONS

In the present work phenazine was tethered to the 2'-O-position of *ara*-uridine and 3'-O-position of *xylo*uridine through linkers of different sizes. These modified blocks were conjugated to the 9mer and 18mer oligo-DNAs, and the thermal stability as well as fluorescence properties of their duplexes (DNA-DNA & DNA-RNA) and triplexes were investigated. For comparative studes oligonucleotides containing phenazinetethered dT block attached at the 5'-terminale (Figs. 2 & 3) were also made. The main objectives of this work were the followings: (i) Is there any significant influence of length of the linker chain connecting phenazine to the oligonucleotide on thermal stability of double and triple helixes? (ii) Can we affect the duplex and triplex stability by changing the site of the phenazine-tethered nucleotide in oligo-DNA? (iii) Are these parameters same for both duplex and triplex stabilities ? The following conclusions are noteworthy: (i) For both duplexes and triplexes, oligonucleotides containing 5'-Pzn-tethered dT block at the 5'-end as in 28, 29 (entries #4&5 in Table 1; entries #6&7 in Table 4) and 37, 38 (entries #3&4 in Table 3) showed better stability than the oligos modified with Pzn-tethered *ara*-U block at the 5'-end as in 26, 27 (entries #2 & 3 in Table 1; entries #4 & 5 in Table 4) and 36, 39 (entries #2 & 5 in Table 3).

(ii) Pzn-tethered *ara*-U block was more efficient at the 3'-terminal of oligonucleotides than at the 5'-end in the duplexes (entries #1 & 2 in Table 1; entries #3 & 4 in Table 4), and especially triplexes (entries #1 & 2 in Table 3), where it provided a dramatic improvement in stability ($\Delta T_m = 16.1^{\circ}$ C).

(iii) Employment of Pzn-tethered *ara*-U block at the 3'-end together with dT block at the 5'-end of the oligo-DNAs (**29**, **38**) provided the best duplex (entry #5 in Table 1 and entry #7 in Table 4) and triplexes stabilisation (entries #4 in Table 3) to give a highest ΔT_m of 14.4°C for DNA-DNA duplexes, ΔT_m of 11.7°C for DNA-RNA duplexes and ΔT_m of 19.6°C for triplexes.

(iv) Incorporation of Pzn-tethered *ara*-U block in the middle of the oligonucleotides as in **31** and **40** had a negative effect on the formation of DNA-DNAduplexes (entry #6 in Table 1), DNA-RNA duplexes (entry #13 in Table 4) and triplexes (entry #6 in Table 3).

(v) In all cases, oligos modified with phenazine-tethered *ara*-U blocks **25**, **41**, **42&35**, **46**, **47** (entries #1-3 in Table 2, entries #1-3 in Table 4 and entries #3, 8, 9 in Table 4) showed better stabilisation as compared to the ones modified with Pzn-tethered *xylo*-U analogues **43** - **45**, **48** - **50** (entries #4-6 in Table 2, entries #4-6 in Table 5 and entries #10-12 in Table 4).

(vi) For both *ara*- and *xylo*-configurations, the best DNA-DNA & DNA-RNA duplex stabilisation was provided by the short ethyl linkers (entries #1 & 4 in Table 2 and entries #3 & 10 in Table 4) and increasing the length of the linker led to considerable destabilisation of the duplexes.

(vii) In triplexes, elongation of the linker chain had a different effect on oligos modified with Pzntethered *ara*-U and Pzn-tethered *xylo*-U blocks. For *ara*-U conjugates the best stabilisation was found with ethyl (as in **35**) and hexyl (as in **47**) linkers with $\Delta T_m = 16.1^{\circ}$ C and 16.5° C respectively (entries #1 & 3 in Table 5), while for *xylo*-U modified oligos, butyl linker (as in **49**) proved to be most effective ($\Delta T_m = 12.3^{\circ}$ C, entry # 5 in Table 5).

(viii) All DNA-DNA duplexes, DNA-RNA duplexes as well as DNA triplexes, formed by oligos modified with $2^{-}O$ -Pzn-tethered *ara*-U blocks showed geater stability than those formed by oligos modified with *xylo*-analogs with the same length of the linker arms (Tables 2, 4 & 5).

(ix) All DNA-DNA duplexes were thermally more stable than corresponding DNA-RNA duplexes. It is noteworthy that all tendencies found for the DNA-RNA duplexes are consistent with the results obtained from the melting experiments with DNA-DNA duplexes.

(x) Fluorescence studies showed that phenazine does not behave as an intercalator or a strong minor groove binder upon DNA-DNA or DNA-RNA duplex or DNA triplex formation, but appears to be a weak exterior binder which accounts for moderate changes in fluorescent properties of phenazine (ΔF for DNA-DNA and DNA-RNA duplexes = ±0.2, ΔF for triplexes = 1.4 - 2.5). No difference in fluorescent properties between DNA-DNA and DNA-RNA duplexes was noted for all modified oligonucleotides.

(xi) Employment of phenazine at both ends of the oligonucleotides (as in 27, 29, 38 & 39) provided the greatest duplex and triplex stabilisation and led to more effective interaction between the phenazine moieties and the double and triple helixes (ΔF for DNA-DNA and DNA-RNA duplexes = 4, ΔF for triplexes = 5).

EXPERIMENTAL

TLC was carried out using silica gel F_{254} plates in the following dichloromethane-methanol mixtures: (A) 95: 5 (v/v), (B) 90: 10 (v/v), (C) 80: 20 (v/v). Pyridine was dried by distillation over 4-toluenesulphonyl chloride followed by distillation over CaH₂. Acetonitrile and dichloromethane were distilled over P₂O₅. ¹H-NMR spectra were recorded (δ scale) on a Jeol JNM-GX 270 spectrometer at 270 MHz, using TMS as an internal standard. ³¹P-NMR spectra were recorded at 36 MHz in the same solvent using 85% phosphoric acid as external standard. The column chromatographic separations were performed using Merck G 60 silica gel. A Gilson equipment with Pump Model 303, Manometric Module 802C and Dynamic Mixer 811B connected to a Dynamax computer program for gradient control were used for semi-preparative RP-HPLC separations on Spherisorb 50DS2. Melting experiments were carried out using Perkin Elmer UV/VIS spectrofotometer Lambda 40 with PTP-6 peltier temperature controller.

2-N-hydroxyalkylaminophenazines (2a, 2b).

General procedure. Erliar procedure¹⁵ with minor changes was peformed. 3.06g (10 mmols) of the crude N-methyl phenazinium methylsulphate^{15, 16} was dissolved in 10 ml of methanol and 50 mmol of aminoalcohol was added. Mixture was stirred for 72 h, evaporated and the residue subjected to silica gel chromatography.

Compound 2a. 4-amino-1-butanol (4.6 ml. Silica gel chromatography (5% EtOH / CH₂Cl₂). (680 mg, 32%). R_f: 0.6 (B); ¹H-NMR (CDCl₃): 8.25-8.06 (*m*, 2H) arom.; 7.95 (*d*, 1H) arom.; 7.80-7.61 (*m*, 2H) arom.; 7.25-7.20 (*m*, 1H) arom.; 6.94 (*m*, 1H) arom.; 4.56 (br, 1H) NH; 3.76 (*m*, 2H) -CH₂OH; 3.41-3.31 (*m*, 2H) - NCH₂CH₂OH; 1.90-1.62 (*m*, 5H) -(CH₂)₂- from butyl, OH. ¹³C-NMR (CD₃OD): 152.5, 147.6, 144.3, 141.8, 141.2, 131.9, 130.6, 130.1, 128.9, 128.8, 128.3, 98.1 (arom); 62.9 (C4); 44.4 (C1); 31.5, 26.3.

Compound 2b. 6-amino-1-hexanol (5.85 g). Silica gel chromatography (4% EtOH / CH₂Cl₂). (720 mg, 30%). R_f: 0.64 (B). ¹H-NMR (CDCl₃): 8.15-8.09 (*m*, 2H) arom.; 7.95 (*d*, 1H) arom.; 7.77-7.62 (*m*, 2H) arom.; 7.22 (*m*, 1H) arom.; 6.96 (*m*, 1H) arom.; 4.49 (br, 1H) NH; 3.68 (*m*, 2H) -CH₂OH; 3.36 (*m*, 2H) NCH₂CH₂OH; 1.79-1.43 (*m*, 9H) -(CH₂)₄- from hexyl, OH. ¹³C-NMR (CD₃OD): 152.3, 147.4, 144.0, 141.6, 140.9, 131.7, 130.4, 129.8, 128.6, 128.4, 127.9, 97.7 (arom.); 62.8 (C6); 44.2 (C1); 33.6, 29.5, 28.2, 26.7.

2-(N-hydroxyalkyl-N-phenoxyacetyl)aminophenazines (3a, 3b). General procedure. 2-Nhydroxyaikylaminophenazines were disolved in pyridine and 10 eq. of chlorotrimethylsilane was added. Mixture was stirred at room temperature for 30 min followed by addition of 2 eq. of phenoxyacetyl chloride. After 1 h watter and aq. NH₃ were added. The mixture was stirred for 30 min and pured into the 150 ml of saturated watter bicarbonate solution. Product was extracted with dichloromethane and after removal of the solvent, the residue was silica gel column chromatographed to give **3a** and **3b**.

Compound 3a. 2a (420 mg, 1.57 mmol), pyridine (10 ml), TMS-Cl (2 ml, 15.7 mmol), Pac-Cl (450 μ l, 3.14 mmol). Silica gel column chromatography (2% - 4% EtOH / CH₂Cl₂). Yield 611 mg (97%). R_f: 0.65 (B). ¹H-NMR (CDCl₃): 8.34-8.20 (*m*, 3H) arom.; 8.10 (*d*, 1H) arom.; 7.92-7.88 (*m*, 2H) arom.;7.66 (*m*, 1H) arom.; 7.26-6.69 (*m*, 5H) arom.; 4.60 (*s*, 2H) PhO-CH₂; 3.95 (*m*, 2H) -CH₂OH; 3.68 (*t*, J = 6.2 Hz, 2H) NCH₂CH₂OH; 2.05 (br, 1H) OH; 1.80-1.60 -(CH₂)₂-.

Compound 3b. **2b** (965 mg, 3.27 mmol), pyridine (15 ml), TMS-Cl (4.2 ml, 32.7 mmol), Pac-Cl (950 μl, 6.54 mmol). Silica gel column chromatography (2% - 4% EtOH / CH₂Cl₂). Yield 1.24 g (89%). R_f: 0.7 (B). ¹H-

NMR (CDCl₃): 8.33-8.22 (*m*, 3H) arom.; 8.07 (*d*, 1H) arom.; 7.90-7.87 (*m*, 2H) arom.; 7.64 (*m*, 1H) arom.; 7.22-6.69 (*m*, 5H) arom.; 4.59 (*s*, 2H) PhO-CH₂; 3.89 (*m*, 2H) -CH₂OH; 3.59 (*t*, J = 6.4 Hz, 2H) - NCH₂CH₂OH; 2.55 (br, 1H) OH; 1.63-1.24 -(CH₂)₄-.

2-(N-(2-hydroxyethyl)-N-methyl)aminophenazine 2-O-(2-Cyanoethyl)-(N,N-diisopropylphosphorami-dite) (4). Compound 1 (100 mg, 0.39 mmol) was dissolved in 6 ml of dry THF, and 350 μ l of diisopropylethyl amine (1.97 mmol, 5 eq.) was added under argon followed by addition of 140 μ l of 2-Cyanoethyl N, N-diisopropylchlorophosphoramidite (0.59 mmol, 1.5 eq). Mixture was stirred for 1 h and quenched with 200 μ l of methanol. It was stirred for another 15 min and poured into the 50 ml of saturated NaHCO₃ solution. Product was extracted twice with dichloromethane. oganic extracts were combined, and was washed with saturated NaCl solution and dried over Na₂SO₄. Residue obtained after removal of solvent was silica gel column chromatographed (CH₂Cl₂ : Et₃N, 98:2 v/v) to give after precipitation from hexane 113 mg of 4 (0.25 mmol, 65%). R_f: 0.66. ¹H-NMR (CDCl₃): 8.18-8.01 (m, 3H) arom; 7.79-7.61 (m, 3H) arom; 7.08 (d, 1H) arom; 3.77-3.45 (m, 8H) N-(CH₂)₂-OP, PO-CH₂-, 2 x CH; 3.23 (s, 3H) N-CH₃; 2.56 (m, 2H) -CH₂CN; 1.15-1.04 (m, 12H) 4 x CH₃. ³¹P-NMR (CDCl₃): 147.92 ppm.

3',5'-O-1,1,3,3-tetraisopropyl-1,3-disiloxyl-arabinouridine 2'-(O-methyl)-(O-2-(N-methyl)amino phenazine-2-N-ethyl) phosphate (7a). Common procedure for the compounds 7b and 7c. Phosphoramidate 6 (500 mg, 0.8 mmol) together with 2-(N-(2-hydroxyethyl)-N-methyl)aminophenazine (165 mg, 0.65 mmol) were coevaporated with dry acetonitrile and redissolved in 6 ml of dry acetonitrile. Tetrazole (225 mg, 3.25 mmol) was added and mixture was stirred for 1 h at room temperaturte under argon. Then the reaction was quenched with 6 ml of 0.1M iodine in THF / pyridine / watter (7:2:1 v/v/v) and stirred for further 30 min. The residue obtained after aqueous sodium bicarbonate / sodium thiosulphate / dichloromethane work up was silica gel column chromatographed (2% - 4% EtOH / CH₂Cl₂) to give 7a. Yield 430 mg (81%). Rf: 0.73 (B). ¹H-NMR (CDCl₃): 10.84 (br, 0.75 H) NH; 10.12 (br, 0.25 H) NH; 8.19-8.01 (m, 3H) arom; 7.78-7.45 (m, 4H) arom; 7.13 (m, 1H) arom; 6.23-6.16 (m, 1H) H1'; 5.75-5.69 (m, 1H) H5; 5.07 (m, 0.75H) H2'; 4.89 (m, 0.25H) H2'; 4.45 (m, 1H) H3'; 4.29 (m, 2H) PO-CH₂-; 4.06-3.90 (m, 2H) H5', H5''; 3.82-3.69 (m, 3H) H4', N-CH₂-; 3.63 (d, J = 11.3 Hz, 3H) PO-CH₃; 3.16 (m, 3H) N-CH₃; 1.15-0.94 (m, 28H) TIPSI. ³¹P-NMR: 7.8, 6.6, 0.25, -1.44 ppm.

Compound 7b. 6 (485 mg, 0.75 mmol), **3b** (200 mg, 0.5 mmol), CH₃CN (5 ml), tetrazole (260 mg, 3.75 mmol), oxidation solution 10 ml. Silica gel column chromatography (2% - 4% EtOH / CH₂Cl₂). Yield 350 mg (70%). R_f: 0.75 (B). ¹H-NMR (CDCl₃): 10.79 (br, 0.5H) NH; 10.08 (br, 0.5H) NH; 8.21-8.05 (*m*, 3H) arom; 7.69-7.43 (*m*, 4H) arom; 7.26-6.71 (*m*, 6H) arom; 6.19-6.15 (*m*, 1H) H1'; 5.76-5.71 (*m*, 1H) H5; 5.02-4.90 (*m*, 1H) H2'; 4.48-4.39 (*m*, 3H) H3', PhO-CH₂; 4.30 (*m*, 2H) PO-CH₂-; 4.08-3.91 (*m*, 2H) H5', H5"; 3.79-3.63 (*m*, 3H) H4', N-CH₂-; 3.59 (*m*, 3H) PO-CH₃; 1.70 (*m*, 4H) -(CH₂)₂-; 1.12-0.88 (*m*, 28H) TIPSI. ³¹P-NMR: 8.20, 7.93, 0.12, -1.23 ppm.

Compound 7c. 6 (485 mg, 0.75 mmol), **3c** (214 mg, 0.5 mmol), CH₃CN (5 ml), tetrazole (260 mg, 3.75 mmol), oxidation solution 10 ml. Silica gel column chromatography (2% - 4% EtOH / CH₂Cl₂). Yield 405 mg (82%). R_f: 0.75 (B). ¹H-NMR (CDCl₃): 10.83 (br, 0.5H) NH; 10.12 (br, 0.5H) NH; 8.22-8.01 (*m*, 3H) arom; 7.72-7.49 (*m*, 4H) arom; 7.30-6.72 (*m*, 6H) arom; 6.21-6.17 (*m*, 1H) H1'; 5.78-5.70 (*m*, 1H) H5; 5.06-4.89 (*m*, 1H) H2'; 4.49-4.40 (*m*, 3H) H3', PhO-CH₂; 4.34 (*m*, 2H) PO-CH₂-; 4.10-3.92 (*m*, 2H) H5', H5"; 3.78-3.65 (*m*, 3H) H4', N-CH₂-; 3.60 (*m*, 3H) PO-CH₃; 1.61 (*m*, 8H) -(CH₂)₄-; 1.12-0.86 (*m*, 28H) TIPSI. ³¹P-NMR: 8.13, 7.36, 0.21, -1.09 ppm.

Arabinouridine 2'-(O-methyl)-(O-2-(N-methyl)aminophenazine-2-N-ethyl) phosphate (8a). Common procedure for the compounds 8b and 8c. 410 mg of 7a (0.48 mmol) was dissolved in 3 ml of dry THF and solution was cooled down to 0°C. Than 2 ml of dry THF containing 230 mg of TBAF x $3H_2O$ (0.72 mmol, 1.5 eq.) was added and the mixture was stirred at 0°C for 30 min. Dry hexane (20 ml) was added and the resulting gum, after decanting, was subjected to silica gel chromatography (6 - 7% EtOH / CH₂Cl₂). Yield 223 mg (83%). R_f: 0.3 (B). ¹H-NMR (CDCl₃): 8.26-8.06 (m, 3H) arom; 7.91-7.50 (m, 5H) arom; 6.22 (m, 1H) H1'; 5.72-5.50 (m, 1H) H5; 4.91 (m, 1H) H2'; 4.51 (m, 1H) H3'; 4.32 (m, 2H) PO-CH₂-; 4.08-3.77 (m, 5H) *N*-CH₂-, H5', H5'', H4'; 3.66 (m, 3H) P-OCH₃; 3.24 (m, 3H) *N*-CH₃. ³¹P-NMR: -0.55, -1.22 ppm.

Compound 8b. 7 b (313 mg, 0.33 mmol), dry THF (5 ml), TBAF (135 mg, 0.42 mmol). Silica gel chromatography (6 - 7% EtOH / CH₂Cl₂). Yield 188 mg (80%). R_f: 0.31 (B). ¹H-NMR (CDCl₃): 10.10 (*br*, 1H) NH; 8.35-8.26 (*m*, 4H) arom., 7.88 (*m*, 2H) arom., 7.68 (*m*, 2H) arom., 7.13 (*m*, 2H) arom., 7.87-7.67 (*m*, 3H) arom., 6.24 (*m*, 1H) H1', 5.68 (*m*, 1H) H5; 4.96 (*m*, 1H) H2'; 4.64 (*s*, 2H) PhCH₂CO; 4.54 (*m*, 1H) H3'; 4.06-3.83 (*m*, 7H) H4', H5', H5'', PO-CH₂-, N-CH₂-; 3.66 (*m*, 3H) PO-CH₃; 1.71 (*m*, 4H) -(CH₂)₂-. ³¹P-NMR: -1.95, -1.34 ppm.

Compound 7c. 7 b (300 mg, 0.3 mmol), dry THF (5 ml), TBAF (125 mg, 0.39 mmol). Silica gel chromatography (6 - 7% EtOH / CH₂Cl₂). Yield 160 mg (72%). R_f: 0.31 (B). ¹H-NMR (CDCl₃): 10.11 (br, 1H) NH; 8.38-8.27 (*m*, 4H) arom., 7.87 (*m*, 2H) arom., 7.62 (*m*, 2H) arom., 7.13 (*m*, 2H) arom., 7.86-7.65 (*m*, 3H) arom., 6.24 (*m*, 1H) H1', 5.63 (*m*, 1H) H5; 4.99 (*m*, 1H) H2'; 4.67 (*s*, 2H) PhCH₂CO; 4.52 (*m*, 1H) H3'; 4.01-3.80 (*m*, 7H) H4', H5', H5'', PO-CH₂-, N-CH₂-; 3.67 (*m*, 3H) PO-CH₃; 1.64 (*m*, 6H) -(CH₂)₄-. ³¹P-NMR: -1.82, -1.23 ppm.

5'-O-(4,4'-dimethoxytrityl)-arabinouridine-2'-(O-methyl)-(O-2-(N-methyl)aminophenazine-2-N-ethyl)

phosphate (9a). Common procedure for the compounds 9b and 9c. Compound 8a (200 mg, 0.35 mmol) was coevaporated with dry pyridine and redissolved in 3 ml of dry pyridine. To this solution, 170 mg of DMTr-Cl was added (0.5 mmol, 1.5 eq) and mixture was stirred for 4 h. The residue obtained after usual aqueous sodium bicarbonate / dichloromethane work up was silica gel column chromatographed (3 - 4% EtOH / CH₂Cl₂). Yield 242 mg (79%). R_f: 0.8 (B). ¹H-NMR (CDCl₃): 8.63 (br, 1H) NH; 8.23-8.54 (m, 8H) arom, H6; 7.38-7.16 (m, 5H) arom; 6.29 (m, 1H) H1'; 5.39 (m, 1H) H5; 5.00-4.84 (m, 1H) H2'; 4.68-4.55 (m, 1H) H4'; 4.31 (m, 2H) PO-CH₂; 4.04-3.92 (m, 1H) H4'; 3.83 (m, 2H) N-CH₂; 3.79-3.72 (m, 6H) 2 x OCH₃; 3.71-3.60 (m, 3H) PO-CH₃; 3.54-3.41 (m, 2H) H5'; H5''; 3.21 (m, 3H) N-CH₃. ³¹P-NMR: 0.37, 0.01 ppm.

Compound 9b. **8b** (180 mg, 0.25 mmol), dry pyridine (3 ml), DMTr-Cl (100 mg, 0.3 mmol). Silica gel chromatography (3 % EtOH / CH₂Cl₂). Yield 200 mg (78%). R_f: 0.8 (B). ¹H-NMR (CDCl₃): 9.77 (*s*, 1H) NH; 8.36-8.24 (*m*, 4H) arom.; 7.87 (*m*, 2H) arom.; 7.77 (*d*, 1H) H6; 7.64 (*m*, 1H) arom.; 7.42-7.09 (*m*, 10H) arom.; 6.83 (*m*, 6H) arom.; 6.67 (*m*, 2H) arom.; 6.29 (*d*, 1H) H1'; 5.45 (*m*, 1H) H5; 4.88 (*m*, 1H) H2'; 4.69 (*m*, 1H) H4'; 4.63 (*s*, 2H) PhOCH₂CO; 4.52 (*m*, 1H) H3'; 4.10-3.83 (*m*, 4H) N-CH₂-, PO-CH₂-; 3.77 (*s*, 6H) 2 x OCH₃; 3.64-3.58 (*m*, 3H) PO-CH₃; 3.50-3.39 (*m*, 2H) H5', H5''; 1.72 (*m*, 4H) -(CH₂)₂-. ³¹P-NMR: 0.52, 0.08 ppm.

Compound 9c. 8c (160 mg, 0.21 mmol), dry pyridine (2 ml), DMTr-Cl (90 mg, 0.25 mmol). Silica gel chromatography (3 % EtOH / CH₂Cl₂). Yield 185 mg (83%). R_f: 0.81 (B). ¹H-NMR (CDCl₃): 9.71 (*s*, 1H) NH; 8.34-8.23 (*m*, 4H) arom.; 7.86 (*m*, 2H) arom.; 7.78 (*d*, 1H) H6; 7.66 (*m*, 1H) arom.; 7.40-7.10 (*m*, 10H) arom.; 6.81 (*m*, 6H) arom.; 6.66 (*m*, 2H) arom.; 6.29 (*d*, 1H) H1'; 5.43 (*m*, 1H) H5; 4.90 (*m*, 1H) H2'; 4.67 (*m*, 1H) H4'; 4.65 (*s*, 2H) PhOCH₂CO; 4.51 (*m*, 1H) H3'; 4.12-3.81 (*m*, 4H) *N*-CH₂-, PO-CH₂-; 3.77 (*s*, 6H) 2 x

OCH₃; 3.68-3.59 (*m*, 3H) PO-CH₃; 3.55-3.41 (*m*, 2H) H5', H5"; 1.70 (*m*, 8H) -(CH₂)₄-. ³¹P-NMR: 0.43, 0.07 ppm.

5'-O-(4,4'-dimethoxytrityl)-arabinouridine-2'-[(O-methyl)-(O-2-(N-methyl)aminophenazine-2-N-ethyl)

phosphate]-3'-sodium succinate (10a). Common procedure for the compounds 10b and 10c. 70 mg of compound **9a** (80 µmol) was dissolved in 1 ml of dry dichloromethane followed by addition of 21 mg (170 µmol) of DMAP. Then 16 mg of succinic anhydride was added and mixture was stirred for 1.5 h. Reaction mixture was extracted first with 0.1M citric acid and then with aqueous sodium bicarbonate. After evaporation of solvent, the residue consisting of **10a** was silica gel column chromatographed (6 - 8% EtOH / CH₂Cl₂). Yield 71 mg (88%). R_f: 0.3 (B). ¹H-NMR (CDCl₃): 8.60 (m, 1H) NH; 8.20-7.96 (m, 3H) arom; 7.91-7.15 (m, 13H) arom; 6.83 (m, 5H) arom; 6.20 (m, 1H) H1'; 5.56 (m, 1H) H5; 5.43 (m, 1H) H2'; 4.95 (m, 1H) H3'; 4.40-4.01 (m, 3H) H4', PO-CH₂-; 3.97-3.65 (m, 8H) *N*-CH₂-, 2 x OCH₃; 3.62-3.37 (m, 5H) PO-CH₃, H5', H5''; 3.18 (m, 3H) N-CH₃; 2.76-2.52 (m, 4H) CH₂CH₂ of succinyl. ³¹P-NMR: -0.41, -1.97 ppm.

Compound 10b. 9 b (180 mg, 0.17 mmol), dry CH₂Cl₂ (2 ml), DMAP (50 mg, 0.37 mmol), succinic anhydride (35 mg, 0.35 mmol). Silica gel chromatography (6 - 8 % EtOH / CH₂Cl₂). Yield 185 mg (93%). R_f: 0.31 (B). ¹H-NMR (CDCl₃): 8.66-8.25 (*m*, 4H) arom.; 7.89 (*m*, 2H) arom; 7.66 (*m*, 1H) arom.; 7.47-7.10 (*m*, 13H) arom.; 6.88-6.68 (*m*, 8H) arom.; 6.18 (*m*, 1H) H1'; 5.63 (*m*, 1H) H5; 5.34 (*m*, 1H) H2'; 5.01-4.55 (*m*, 3H) H3', PO-CH₂-; 4.30-3.84 (*m*, 5H) H4', *N*-CH₂-, PhOCH₂CO; 3.78 (*m*, 6H) 2 x OCH₃; 3.75-3.51 (*m*, 3H) PO-CH₃; 3.46 (*m*, 2H) H5', H5''; 2.71-2.52 (*m*, 4H) CH₂CH₂ of succinyl; 1.78-1.56 (*m*, 8H) -(CH₂)₄-. ³¹P-NMR (CDCl₃): -0.24, -1.53 ppm.

Compound 10c. **9b** (189 mg, 0.17 mmol), dry CH₂Cl₂ (2 ml), DMAP (50 mg, 0.37 mmol), succinic anhydride (35 mg, 0.35 mmol). Silica gel chromatography (6 - 8 % EtOH / CH₂Cl₂). Yield 170 mg (86%). R_f: 0.33 (B). ¹H-NMR (CDCl₃): 8.64-8.20 (*m*, 4H) arom.; 7.89 (*m*, 2H) arom; 7.64 (*m*, 1H) arom.; 7.51-7.10 (*m*, 13H) arom.; 6.87-6.67 (*m*, 8H) arom.; 6.18 (*m*, 1H) H1'; 5.59 (*m*, 1H) H5; 5.33 (*m*, 1H) H2'; 5.01-4.55 (*m*, 3H) H3', PO-CH₂-; 4.26-3.83 (*m*, 5H) H4', N-CH₂-, PhOCH₂CO; 3.78 (*m*, 6H) 2 x OCH₃; 3.75-3.48 (*m*, 3H) PO-CH₃; 3.40 (*m*, 2H) H5', H5''; 2.75-2.51 (*m*, 4H) CH₂CH₂ of succinyl; 1.81-1.58 (*m*, 8H) -(CH₂)₄-. ³¹P-NMR (CDCl₃): -0.37, -1.95 ppm.

$\label{eq:constraint} 5'-O-(4,4'-dimethoxytrityl)-arabinouridine-2'-[(O-methyl)-(O-2-(N-methyl)aminophenazine-2-N-ethyl)]$

phosphate]-3'-O-(2-cyanoethyl)-(N, N-diisopropyl)phosphoramidite (11). The 3'-hydroxy block 9a (183 mg, 0.21 mmol) was dissolved in dry THF (3 ml). Dry diisopropylethylamine (200 μ l, 1.1 mmol) was added, followed by addition of O-(cyanoethyl)-N,N-diisopropylphosphoramidic chloride (80 μ l, 0.34 mmol). Reaction mixture was stirred under argon for 1 h. Then reaction was quenched by addition of 100 μ l of methanol and continued stirring for 15 min. The material obtained after aqueous sodium bicarbonate / saturated NaCl / ethyl acetate work up was then silica gel column chromatographed (20 - 30% CH₂Cl₂/ cyclohexane). Yield 150 mg (70%). R_f: 0.83 (A). ¹H-NMR (CDCl₃): 8.21-7.98 (*m*, 4H) arom.; 7.80-7.11 (*m*, 12H) arom.; 6.82 (*m*, 5H) arom.; 6.21 (*m*, 1H) H1'; 5.56 (*m*, 1H) H5; 4.95 (*m*, 1H) H2'; 4.54 (*m*, 1H) H3'; 4.19 (*m*, 3H) H4', PO-CH₂-; 3.88-3.34 (*m*, 17H) 2 x OCH₃, N-CH₂-, OCH₂CH₂CN, PO-CH₃, H5', H5'', 2 x CH; 3.17 (*m*, 3H) N-CH₃; 2.79-2.34 (*m*, 2H) OCH₂CH₂CN; 1.47-1.03 (*m*, 12H) 4 x CH₃ of isopropyl. ³¹P-NMR (CDCl₃): 150.8, -0.73, -1.71.

1-O-Acetyl-2, 3, 5-tri-O-(4-toluoyl)- α , β -D-arabinofuranoside (14). 1-O-methyl- α , β -D-arabinofuranoside (12) (10.2 g, 62.2 mmol) was coevaporated with dry pyridine and dissolved in 100 ml of this solvent. Then 27.1 ml of toluoyl chloride (205.3 mmol) was added under vigorous stirring. After 2 h reaction mixture was

poured into 500 ml of saturated aqoeous solution of sodium bicarbonate. The oil obtained after dichloromethane extraction, drying over MgSO₄, evaporation of solvents and coevaporation with toluene was dissolved in mixture of 37 ml of acetic anhydride and 30 ml of acetic acid in 150 ml of dry dichloromethane. Resulting solution was cooled down to 0°C on the ice bath and 6.3 ml of H₂SO₄ was added under vigirous stirring. Mixture was stirred at 0°C for 12 min after which it was carefully added to a pre-cooled saturated solution of NaHCO₃ in watter. After extraction with dichloromethane organic phases were dried over MgSO₄, filtered and evaporated to give 14 as yellow oil (31.2 g, 92%). R_f: 0.9 (A). Resulting material was used was taken to the next step without any further purification. ¹H-NMR (CDCl₃): 7.99-7.85 (*m*, 6H) arom; 7.40-7.16 (*m*, 6H) arom; 6.67 (*d*, 0.4H) α H1; 6.38 (*s*, 0.6H) β H1; 6.05 (*m*, 0.4H) α H3; 5.85 (*m*, 0.6H) β H3; 5.77 (*m*, 0.4H) α H2; 5.63 (*s*, 0.6H) β H2; 4.95 (*m*, 1H) α , β H4; 4.67-4.50 (*m*, 2H) α , β H5, α , β H5', 2.47-2.34 (*m*, 9H) 3 x CH₃; 2.12 (*s*, 1.8H) β CH₃CO; 2.04 (*s*, 1.2H) α CH₃CO.

I-(2', 3', 5'-tri-O-(4-toluoyl)-\beta-D-xylofuranosyl)uracil (15). Uracil (4.8 g, 42.8 mmol) was suspended in mixture of HMDS (66 ml) and TMS-Cl (8 ml). Suspension was refluxed under argon for 1.5 h to give a clear solution, which was evaporated and coevaporated with dry toluene. The residue was dissolved in dry dichloroethane (100 ml) and added to the solution of **14** (18 g, 32 mmol) in dry dichloroethane (250 ml). Then trimethylsilyl trifluoromethane sulphonate (9 ml) were added and mixture was kept at 45°C overnight, under argon. After usual aq. NaHCO₃ / dichloromethane work up, drying over MgSO₄ and evaporation of solvent the residue was silica gel column chromatographed (2 - 3% EtOH / CH₂Cl₂). Yield 15.4 g (76%). R_f: 0.6 (A). ¹H-NMR (CDCl₃): 8.38 (br, 1H) NH; 7.97-7.81 (*m*, 6H) arom; 7.77 (*d*, J = 8.12 Hz, 1H) H6; 7.28-7.17 (*m*, 6H) arom; 6.23 (*d*, J = 2.2 Hz, 1H) H1'; 5.83-5.80 (*m*, 1H) H3'; 5.71 (*m*, 1H) H5; 5.64-5.30 (*m*, 1H) H2'; 4.88-4.83 (*m*, 1H) H4'; 4.72 (*m*, 2H) H5', H5"; 2.43-2.35 (*m*, 9H) 3 x Ph-CH₃.

I-(\beta-D-xylofuranosyl)uracil (16). **15** (4.3 g) was dissolved in 25% NH₃ in methanol and kept for 10 h at room temperature and overnight at 50°C. Then mixture was evaporated and product was silica gel column chromatographed (15% EtOH / CH₂Cl₂). Yield 1.49g (94%). R_f: 0.12 (C). ¹H-NMR (D₂O): 7.83 (*d*, J = 8.2 Hz, 1H) H6; 5.78 (*d*, J = 8.2 Hz, 1H) H5; 5.73 (*s*, 1H) H1'; 4.34 (*m*, 1H) H4'; 4.21 (*m*, 2H) H2', H3'; 3.90 (*m*, 2H) H5', H5''.

1-(2', 5'-di-O-(4,4'-dimethoxytrityl)- β -D-xylofuranosyl)uracil (17). Compound 16 (1.04 g, 4.67 mmol) was coevaporated with dry pyridine and dissolved in dry pyridine (25 ml). Then DMTr-Cl (2 g, 6.1 mmol, 1.3 eq.) was added. After 4 h, formation of 5'-O-DMTr derivative was complete (judging by TLC) and more DMTr-Cl (3.16 mmol) together with DMAP (1.15 g, 9 mmol) were added. Reaction mixture was kept overnight at 60°C. After aqueous NaHCO₃ / dichloromethane work up, drying over MgSO₄ and evaporation of solvent the residue was silica gel column chromatographed (1% EtOH / CH₂Cl₂). Yield 3.1 g (79%). R_f: 0.44 (A). ¹H-NMR (CDCl₃): 8.24 (br, 1H) NH; 7.48-7.14 (m, 17H) arom, H6; 6.82-6.74 (m, 10H) arom; 6.01 (d, 1H) H1'; 5.56 (m, 1H) H5; 4.11 (m, 2H) H2', H4'; 3.77-3.75 (m, 12H) 4 x CH₃O-Ph; 3.40 (m, 3H) H3', H5', H5''; 2.85 (br, 1H) 3' OH.

$1-(2',5'-di-O-(4,4'-dimethoxytrityl)-\beta-D-xylofuranosyl)uracil-3'-(O-methyl)-(N,N-diisopropyl)-$

phosphoramidite (18). 600 mg (0.71 mmol) of compound 16 was dissolved in dry THF (5 ml) and DIPEA (3650 μ l, 55 mmol) was added under argon, followed by addition of) of *O*-methyl-*N*,*N*-diisopropylphosphoramidic chloride (200 μ l, 1 mmol. Mixture was stirred for 2 h, then quenched by addition of dry methanol (150 μ l) and kept stirring for 15 min. The crude material obtained after aqueous NaHCO₃ / saturated NaCl / ethyl acetate work up was silica gel column chromatographed (Hexane:CH₂Cl₂:Et₃N,

80:20:2 to 70:30:2 v/v/v). Yield 630 mg (91%). R_f: 0.63 (A). ¹H-NMR (CDCl₃): 8.09 (br, 1H) NH; 7.61 (*m*, 1H) H6; 7.48-7.18 (*m*, 16H) arom; 6.80 (*m*, 10H) arom; 6.22 (*m*, 0.64H) H1'; 5.73 (*m*, 0.25H) H1'; 5.43 (*m*, 1H) H5; 4.45-4.07 (*m*, 2H) H2', H4'; 3.77 (*m*, 12H) 4 x Ph-O*CH*₃; 3.73-3.19 (*m*, 5H) H3', H5', H5'', 2 x CH; 3.03-2.83 (*m*, 3H) PO-*CH*₃; 1.03 (*m*, 6H) 2 x CH₃ of isopropyl; 0.82 (*m*, 6H) 2 x CH₃ of isopropyl. ³¹P-NMR: 153.60, 147.13 ppm.

Synthesis of 2',5'-di-O-(4,4'-dimethoxytrityl)xylouridine-3'-(O-methyl)-(O-aminophenazine-alkyl)phosphates 19a - 19c. (Procedure described for the preparation of the phosphates 7a - 7c has been used.)

Compound 19a. **18** (420 mg, 0.4 mmol), **3a** (76 mg, 0.3 mmol), dry CH₃CN (3 ml), tetrazole (157 mg, 2.25 mmol). Silica gel column chromatography (3 - 4% EtOH / CH₂Cl₂). Yield 312 mg (80%). R_f: 0.7 (B). ¹H-NMR (CDCl₃): 8.20-8.01 (*m*, 3H) arom.; 7.82-7.64 (*m*, 3H) arom.; 7.52-7.18 (*m*, 17H) arom.; 7.01 (*m*, 1H) H6; 6.87-6.48 (*m*, 10H) arom.; 5.67 (*m*, 1H) H1'; 5.51 (*m*, 1H) H5; 4.95-4.36 (*m*, 4H) H2', H3', PO-CH₂-; 3.98-3.34 (*m*, 17H) 4 x Ph-OCH₃, H4', H5', H5'', N-CH₂-; 3.31 (*m*, 3H) PO-CH₃; 3.08 (*m*, 3H) N-CH₃. ³¹P-NMR: 0.11 ppm.

Compound 19b. **18** (800 mg, 0.75 mmol), **3a** (200 mg, 0.5 mmol), dry CH₃CN (6 ml), tetrazole (260 mg, 3.75 mmol). Silica gel column chromatography (3 - 4% EtOH / CH₂Cl₂). Yield 613 mg (87%). R_f: 0.7 (B). ¹H-NMR (CDCl₃): 8.45-8.23 (*m*, 4H) arom.; 8.07 (*m*, 1H) arom.; 7.92-7.88 (*m*, 2H) arom.; 7.64-7.57 (*m*, 1H) H6; 7.43-7.05 (*m*, 18H) arom.; 6.90-6.67 (*m*, 13H) arom.; 5.81 (*s*, 1H) H1'; 5.46 (*m*, 1H) H5; 4.65-4.39 (*m*, 6H) H2', H3', PhOCH₂CO, PO-CH₂-; 3.82-3.35 (*m*, 17H) 4 x Ph-OCH₃, H4', H5', H5'', N-CH₂-; 3.32 (*m*, 3H) PO-CH₃; 1.51-1.43 (*m*, 4H) -(CH₂)₂-. ³¹P-NMR: 0.00 ppm.

Compound 19c. **18** (800 mg, 0.75 mmol), **3a** (214 mg, 0.5 mmol), dry CH₃CN (6 ml), tetrazole (260 mg, 3.75 mmol). Silica gel column chromatography (3 - 4% EtOH / CH₂Cl₂). Yield 590 mg (82%). R_f: 0.71 (B). ¹H-NMR (CDCl₃): 8.48-8.24 (*m*, 4H) arom.; 8.08 (*m*, 1H) arom.; 7.93-7.87 (*m*, 2H) arom.; 7.63-7.57 (*m*, 1H) H6; 7.41-7.10 (*m*, 18H) arom.; 6.90-6.61 (*m*, 13H) arom.; 5.80 (*s*, 1H) H1'; 5.48 (*m*, 1H) H5; 4.64-4.41 (*m*, 6H) H2', H3', PhO*CH*₂CO, PO-*CH*₂-; 3.80-3.31 (*m*, 17H) 4 x Ph-O*CH*₃, H4', H5', H5'', N-*CH*₂-; 3.31 (*m*, 3H) PO-*CH*₃; 1.49-1.35 (*m*, 8H) -(CH₂)₄-. ³¹P-NMR: 0.00 ppm.

Synthesis of xylouridine-3'-(O-methyl)-(O-aminophenazine-alkyl)phosphates 20a - 20c. General procedure. DMTr-protected phosphates 19a - 19c were dissolved in 0.15M solution of CCl₃COOH (10 eq) in dry CH₂Cl₂, containing 10% of methanol (v/v) at 0°C. Mixture was kept at this temperature overnight. Then pyridine was added followed by removal of all solvents in vacuum. Crude materials were silica gel column chromatographed.

Compound 20a 19a (175 mg, 0.14 mmol), 0.15M CCl₃COOH in CH₂Cl₂ (18 ml), 1.8 ml of methanol. Silica gel column chromatography (9 - 13% EtOH / CH₂Cl₂). Yield 75 mg (86%). R_f: 0.14 (B). ¹H-NMR (CDCl₃): 9.23-9.03 (*m*, 1H) NH; 8.17-7.90 (*m*, 3H) arom; 7.80-7.63 (*m*, 3H) arom; 7.27-7.01 (*m*, 2H) arom; '5.79-5.65 (*m*, 2H) H1', H5; 4.80-4.48 (*m*, 4H) H2', H3', PO-*CH*₂-; 4.17-3.83 (*m*, 5H) H4', H5', H5'', N-*CH*₂-; 3.68 (*m*, 3H) PO-*CH*₃; 3.21 (*s*, 3H) N-*CH*₃. ³¹P-NMR: 0.29, -0.93 ppm.

Compound 20b. 19b (550 mg, 0.41 mmol), 50 ml of 0.15M CCl₃COOH in CH₂Cl₂, 5 ml of methanol. Silica gel column chromatography (8 - 12% EtOH / CH₂Cl₂). Yield 244 mg (81%). R_f: 0.34 (B). ¹H-NMR (CDCl₃): 9.53 (br, 0.5H) NH; 9.30 (br, 0.5H) NH; 8.27 (m, 4H) arom; 7.91 (m, 2H) arom; 7.62 (m, 2H) arom; 7.14 (m, 2H) Pac; 6.86 (m, 1H) Pac; 6.69 (m, 2H) Pac; 5.83-5.69 (m, 2H) H1', H5; 4.89-4.55 (m, 6H) H2', H3', PO-CH₂-, PhOCH₂O; 4.14-3.85 (m, 5H) H4', H5', H5'', N-CH₂-; 3.74 (m, 3H) PO-CH₃; 1.95-1.59 (m, 4H) - (CH₂)₂-. ³¹P-NMR: 0.18, -0.86 ppm.

Compound 20c. 19c (200 mg, 0.15 mmol), 0.15M CCl₃COOH in CH₂Cl₂ (19 ml), methanol (2 ml). Silica gel column chromatography (8 - 12% EtOH / CH₂Cl₂). Yield 90 mg (79%). R_f: 0.38 (B). ¹H-NMR (CDCl₃): 9.50 (br, 0.5H) NH; 9.31 (br, 0.5H) NH; 8.22 (m, 4H) arom; 7.91 (m, 2H) arom; 7.63 (m, 2H) arom; 7.15 (m, 2H) Pac; 6.88 (m, 1H) Pac; 6.71 (m, 2H) Pac; 5.82-5.70 (m, 2H) H1', H5; 4.83-4.48 (m, 6H) H2', H3', PO-*CH*₂-, PhO*CH*₂O; 4.11-3.80 (m, 5H) H4', H5', H5'', N-*CH*₂-; 3.74 (m, 3H) PO-*CH*₃; 1.82-1.51 (m, 8H) - (CH₂)₄-. ³¹P-NMR: 0.20, -0.72 ppm.

Synthesis of 5'-O-(4,4'-dimethoxytrityl)xylouridine-3'-(O-methyl)-(O-aminophenazine-alkyl)phos-phates 21a - 21c. (Procedure described for preparation of the DMTr-blocks 9a - 9c was used.) Compound 21a. 20a (75 mg, 0.12 mmol), dry pyridine (1.5 ml), DMTr-Cl (53 mg, 0.15 mmol). Silica gel column chromatography (5 - 6% EtOH / CH₂Cl₂). Yield 88 mg (80%). R_f: 0.57 (B). ¹H-NMR (CDCl₃): 9.24-8.92 (m, 1H) NH; 8.16-7.95 (m, 3H) arom., 7.81-7.60 (m, 3H) arom., 7.54-7.13 (m, 10H) arom., 6.88-6.72 (m, 5H) arom., 5.79 (m, 1H) H1'; 5.38 (m, 1H) H5; 4.90-4.57 (m, 6H) H2', H3', H4'; 4.27 (m, 2H) PO-CH₂-; 4.02-3.30 (m, 13H) H5', H5", N-CH₂-, 2 x Ph-OCH₃; 3.16 (s, 3H) N-CH₃. ³¹P-NMR: 1.18, 0.20 ppm.

Compound 21b. 20b (270 mg, 0.37 mmol), dry pyridine (5 ml), DMTr-Cl (150 mg, 0.44 mmol). Silica gel column chromatography (5 - 6% EtOH / CH₂Cl₂). Yield 292 mg (76%). R_f: 0.67 (B). ¹H-NMR (CDCl₃): 9.42 (*br*, 0.5H) NH; 9.23 (br, 0.5H) NH; 8.41-8.26 (*m*, 4H) arom.; 7.89 (*m*, 2H) arom.; 7.62 (*m*, 1H) H6; 7.48-7.10 (*m*, 12H) arom.; 6.95-6.66 (*m*, 7H) arom.; 5.81 (*m*, 1H) H1'; 5.54 (*m*, 1H) H5; 4.83-4.63 (*m*, 7H) H2', H3', H4', PO-CH₂-, PhOCH₂CO; 4.14-3.33 (*m*, 13H) H5', H5'', N-CH₂-, 2 x Ph-OCH₃, PO-CH₃; 1.80-1.51 (*m*, 4H) -(CH₂)₂-. ³¹P-NMR: 1.01, 0.34 ppm.

Compound 21c. **20**c (90 mg, 0.12 mmol), dry pyridine (1.5 ml), DMTr-Cl (48 mg, 0.14 mmol). Silica gel column chromatography (5 - 6% EtOH / CH₂Cl₂). Yield 106 mg (83%). R_f: 0.69 (B). ¹H-NMR (CDCl₃): 9.39 (br, 0.5H) NH; 9.27 (br, 0.5H) NH; 8.40-8.24 (*m*, 4H) arom.; 7.87 (*m*, 2H) arom.; 7.61 (*m*, 1H) H6; 7.51-7.07 (*m*, 12H) arom.; 6.92-6.61 (*m*, 7H) arom.; 5.80 (*m*, 1H) H1'; 5.52 (*m*, 1H) H5; 4.79-4.61 (*m*, 7H) H2', H3', H4', PO-CH₂-, PhOCH₂CO; 4.12-3.29 (*m*, 13H) H5', H5'', N-CH₂-, 2 x Ph-OCH₃, PO-CH₃; 1.79-1.48 (*m*, 8H) -(CH₂)₄-. ³¹P-NMR: 1.08, 0.29 ppm.

Synthesis of 5'-O-(4,4'-dimethoxytrityl)xylouridine-3'-(O-methyl)-(O-aminophenazine-alkyl)phos-phate-3'sodium succinates 22a - 22c. (Procedure described for preparation of the succinates 10a - 10c was used.) Compound 22a. 21a (70 mg, 80 μmol), dry CH₂Cl₂ (1 ml), DMAP (21 mg, 170 μmol), succinic anhydride (16 mg, 160 μmol). Silica gel column chromatography (6 - 10% EtOH / CH₂Cl₂). Yield 67 mg (86%). R_f: 0.3 (B). ¹H-NMR (CDCl₃): 9.26-9.00 (*m*, 1H) NH; 8.15-7.98 (*m*, 3H) arom., 7.82-7.64 (*m*, 3H) arom., 7.55-7.13 (*m*, 10H) arom., 6.88-6.72 (*m*, 4H) arom., 5.96 (*m*, 1H) H1'; 5.59-5.02 (*m*, 2H) H2', H5; 4.97-4.43 (*m*, 4H) H3', H4'; PO-CH₂-; 4.00-3.33 (*m*, 13H) H5', H5'', N-CH₂-, 2 x Ph-OCH₃, PO-CH₃; 3.16 (*m*, 3H) N-CH₃, 2.61 (*m*, 4H) -(CH₂)₂- from succinyl. ³¹P-NMR: 0.14, -0.98 ppm.

Compound 22b. 21b (240 mg, 0.23 mmol), dry CH₂Cl₂ (3 ml), DMAP (60 mg, 0.49 mmol), succinic anhydride (46 mg, 0.46 mmol). Silica gel column chromatography (6 - 10% EtOH / CH₂Cl₂). Yield 211 mg (81%). R_f: 0.35 (B). ¹H-NMR (CDCl₃): 8.32-8.26 (*m*, 4H) arom.; 7.89 (*m*, 2H) arom.; 7.61 (*m*, 1H) H6; 7.47-7.11 (*m*, 12H) arom.; 6.89-6.66 (*m*, 7H) arom.; 5.97 (*m*, 1H) H1'; 5.62-5.49 (*m*, 2H) H5, H2'; 4.83-4.41 (*m*, 5H) H3', PO-*CH*₂-, PhO*CH*₂CO; 4.04-3.34 (*m*, 14H) H4', H5', H5", N-*CH*₂-, 2 x Ph-O*CH*₃, PO-*CH*₃; 2.69 (*m*, 4H) CH₂CH₂ from succinyl, 1.64 (*m*, 4H) -(CH₂)₂-. ³¹P-NMR: 0.11, -1.12 ppm.

Compound 22c. 21c (106 mg, 0.1 mmol), dry CH₂Cl₂ (1.5 ml), DMAP (30 mg, 0.21 mmol), succinic anhydride (20 mg, 0.2 mmol). Silica gel column chromatography (6 - 10% EtOH / CH₂Cl₂). Yield 102 mg

(87%). R_f: 0.35 (B). ¹H-NMR (CDCl₃): 8.35-8.25 (*m*, 4H) arom.; 7.87 (*m*, 2H) arom.; 7.60 (*m*, 1H) H6; 7.46-7.09 (*m*, 12H) arom.; 6.87-6.64 (*m*, 7H) arom.; 5.96 (*m*, 1H) H1'; 5.62-5.47 (*m*, 2H) H5, H2'; 4.84-4.39 (*m*, 5H) H3', POCH₂-, PhOCH₂CO; 4.01-3.32 (*m*, 14H) H4', H5', H5'', NCH₂-, 2 x OCH₃, POCH₃; 2.67 (*m*, 4H) CH₂CH₂ from succinyl, 1.61 (*m*, 8H) -(CH₂)₄-. ³¹P-NMR: 0.16, -1.11 ppm.

General procedure for derivatisation of 3-aminopropyl-CPG with phenazine-tethered ara-U-succinates (10a - 10c) and xylo-U succinates (21a - 21c). Loading procedure. 50 μ mol of phenazine-tethered ara- or xylo-U succinate was dissolved in dry THF (1.3 ml). 18 μ l (104 μ mol, 2 eq) of dry DIPEA was added, followed by addition of of isobutyl chloroformate (6.3 μ l, 49 μ mol). Resulting solution was shaken for 2 h and then 400 μ l of dry DIPEA and 200 mg of 3-aminopropyl-CPG were added. Mixture was shaken for 3 h. Then CPG was filtered off, washed twice with dichloromethane and ether and dryed.

Capping procedure. Dry CPG was placed into 4 ml of dry pyridine and 55 mg of DMAP and 0.3 ml of aceic anhydride were added. Mixture was shaken for 1 h. Then CPG was filtered off and washed twice with dichloromethane and ether. Loading was determined by measuring UV absorbance of DMTr cation at 498 nm. Loadings found for the CPGs tethered with phenazine-containing succinates are as follows: 24.0 μ mol / g for 10b; 22.9 μ mol / g for 10c; 23.1 μ mol / g for 22a; 21.4 μ mol / g for 22b; 22.6 μ mol / g for 22c.

Synthesis, deprotection and purification of the oligonucleotides \cdot All oligonucleotides were synthesised in 1.0 µmol scale with an 8-channel Applied Biosystems 392 DNA / RNA synthesiser using conventional β cyanoethyl phosphoramidite chemistry. Phenazine containing amidites 4 and 11 were dissolved in dry
acetonitrile with a final concentration of 0.1M. Coupling time of 1 min was used for amidite 4. Coupling
efficiency of this amidite, judging from HPLC profiles of deprotected oligoconjugates, was higher than 95%.
For the amidite 11, coupling time of 10 min was used to give a coupling yields of 96%. Solid supports
derivatised with phenazine-tethered nucleotides were used in 1.0 µmol scale synyhesis employing standard
1.0 µmol cycles with coupling time of 25 sec, which provided average coupling efficiency of 98-99%.

Synthesised oligonucleotides were deprotected with concentrated aq. NH₃ at 55°C in 17 h. Crude oligomers were purified on reverse-phase HPLC (semi-preparative column Spherisorb 5ODS2) using following gradient buffers: A (0.1M triethylammonium acetate, 5% MeCN, pH 7.0); B (0.1M triethylammonium acetate , 50% MeCN, pH 7.0). After collection of appropriate fractions solutions of the oligoconjugates 23, 24, 26 - 30, 32 - 34, 36 - 39 were evaporated and lyophilized (5 x 1 ml H₂O).

3'-end and middle modified oligonucleotides (25, 31, 35, 40 - 50) were synthesised and RP-HPLC purified as "5'-O-trityl on". Obtained samples were deprotected using 80% AcOH^{15, 17}, repurified by RP-HPLC and lyophilized (5 x 1 ml H₂O). All the oligomers were subsequently sodium exchanged through a column of Dowex-50 Na⁺-form.

All RNAs were synthesised on the same equipment using conventional 3'-O-TBDMS β -cyanoethyl phosphoramidites with average coupling yields greater then 98%. Resulted oligonucleotides were deprotected using 30% NH₃ in MeOH, desylilated with triethylamine trihydrofluoride and desalted on Sephadex G-25 column. Crude materials were purified on reverse-phase HPLC using same **A** and **B** buffer systems as for DNA separations and than sodium exchanged.

Melting measurements. UV melting profiles were obtained by scanning A_{260} absorbance versus time at a heating rate of 1.0°C / min for duplexes and 0.5°C / min for triplexes. The T_ms were calculated from the maximum point of the first derivative of the melting curves with an accuracy of ±0.1°C. The duplex and

triplex melting experiments were carried out in the same buffer: 20 mM Na₂HPO₄ / NaH₂PO₄, 0.1M NaCl at pH 7.3. The approximate extinction cofficientes were calculated as reported earlier^{15,17,23}. No corrections for the extinction coefficientes of phenazine-tethered oligonucleotides was nesessary, judging from the UV spectra of the single-strand oligoconjugates and phenazine-bearing linkers.

In a duplex melting measurements, 1 µM concentration of each single strand was used. The solutions were heated to 70°C for 3 min and then allowed to cool down to 20°C for 30 min. The melting curves and and dissociating T_ms were measured with a temperature gradient 10 - 60°C (50 min) (entries #1 - 7, Table 1; entries #1 - 6, Table 2; entries #1 - 13, Table 4).

In a triplex melting measurements 1 μ M concentration of each single strand was also used. After heating to 70°C for 3 min, followed by cooling to 20°C for 30 min samples were kept at 0°C overnight. The melting curves were measured with a temperature gradient 5 - 70°C (130 min) (entries #1 - 7, Table 3; entries #1 - 7, Table 5).

Fluorescence measurements were carried out in 20 mM PO4³⁻, 1M NaCl, pH 7.3 at 15°C for duplexes and 5°C for triplexes. Concentration of 2µM of each single strend was used. Excitation wavelength of 483 nm was chosen for the oligonucleotides 25-29, 31, 35 - 40 and 501 nm for the oligonucleotides 41 - 50. For each modified oligonucleotide fluorescence intensity was measured for single strand state and duplex or triplex state and ΔF values were calculated, using the ΔF value of the single strend state as 1.0.

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