Oligonucleotide Analogues with 4-Hydroxy-*N*-acetylprolinol as Sugar Substitute

Griet Ceulemans, Arthur Van Aerschot, Berthold Wroblowski, Jef Rozenski, Chris Hendrix and Piet Herdewijn*

Abstract: Modified oligonucleotides incorporating *trans*-4-hydroxy-*N*-acetyl-L-prolinol (*trans*-4-HO-L-NAP) or its D-analogue as sugar substitute were synthesised with adenine and thymine as nucleobases. All-adenine oligonucleotides built from (2*S*,4*S*) or (2*R*,4*R*)-*cis*-4-hydroxy-*N*-acetylprolinol were likewise prepared. Hybridisation studies revealed that heterocomplexes formed between polyU and homochiral *trans*-4-hydroxy-*N*-acetylprolinol-based oligomers of the same as well as of opposite chirality (polyU/*trans*-DA$^*_{13}$ and polyU/*trans*-LA$^*_{13}$). The former, however, were triple-stranded. Other complexes with ribonucleic acids were polyA/*trans*-LT$^*_{13}$ and polyU/*cis*-LA$^*_{13}$. Heteroduplexes with deoxynucleic acids were formed between *trans*-LA$^*_{13}$ and oligothymidylate. Interaction was also observed for *cis*-LA$^*_{13}$ and oligothymidylate, but not with the D-hydroxyprolinol analogues. Microcalorimetry proved this interaction to be the formation of a triple-stranded complex. Two heteroduplexes, *trans*-LA$^*_{13}$/dT$_{13}$ and *trans*-LA$^*_{13}$/polyU, had similar or slightly increased stability when compared to the natural dA$_{13}$/dT$_{13}$ or dA$_{13}$/polyU systems. Microcalorimetry clearly indicated the formation of a duplex, in contrast to interactions with *N*-acetylprolinol oligonucleotides of different stereochemistry. Moreover, the enthalpy change was of the same magnitude but the association constant was slightly lower. Natural nucleic acids thus clearly prefer hybridisation with L-hydroxyprolinol oligomers over D-hydroxyprolinol oligomers. For the series investigated, the L-*trans* oligomers (Figure 1) seem best to mimic natural oligonucleotides. These modified oligonucleotides formed homocomplexes if both strands were of the same chirality, that is, homocomplexes formed between *trans*-LA* and *trans*-LT* and between *trans*-DA* and *trans*-DT*, reflecting the isochiral pu–py pairing found in natural nucleic acids. Once more, however, calorimetry proved these to be triplex interactions. Heterochiral pairing was not observed between modified oligonucleotides, but only between modified oligonucleotides and natural polyU. The thermal stabilities of these heterochiral complexes differed clearly.

Keywords
antisense agents · chiral recognition · oligonucleotides

Introduction

Nucleosides derived from 4-hydroxyprolinol (Figure 1) give rise to oligomeric nucleotides with the same number of atoms between their repeating unit as the RNA-selective 2′-5′-oligonucleotides.[1, 2] Because of this resemblance, and because of the presence of a five-membered-ring–phosphate backbone similar to the backbone of natural nucleic acids, they were selected as the first representatives of a new series of modified oligonucleotides.

We synthesised oligonucleotides derived from all possible stereomers [(2*S*,4*R*); (2*S*,4*S*); (2*R*,4*S*) and (2*R*,4*R*), Figure 2] in order to investigate the dependence of hybridisation on *cis*/*trans*

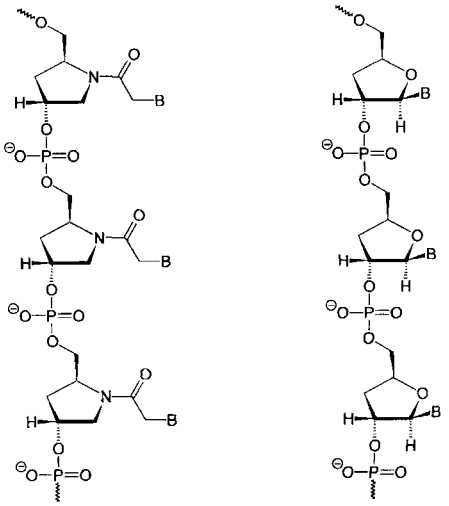

Figure 1. Structures of oligonucleotides with 4-hydroxy-*N*-acetylprolinol as sugar substitute ((2*S*,4*R*)-*trans*-4-HO-L-prolinol oligonucleotides, left) and of 2′-5′ linked 3′-deoxyoligonucleotides (right).

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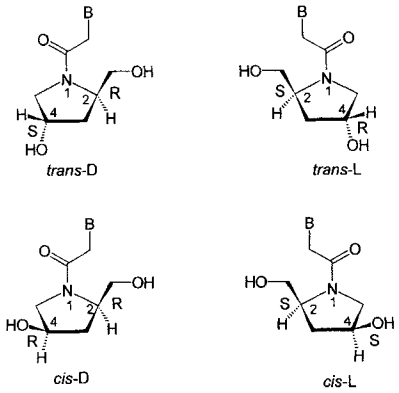


Figure 2. Hydroxyprolinol nucleosides used for the synthesis of modified oligonucleotides.

configuration and also the possibility of finding pairing systems between optical antipoles. The pairing system that dominates in natural nucleic acids is an isochiral purine–pyrimidine system and this is also found in p-RNA.[3]

Abstract in Flemish: *Oligonucleotiden, waarbij de suikerring werd vervangen door ofwel cis-4-hydroxy-N-acetylprolinol, (2S,4S) of (2R,4R) ofwel trans-4-hydroxy-N-acetyl-L-prolinol (trans-4-HO-L-NAP) of zijn D-analoog, werden gesynthetiseerd. Hybridizatiestudies toonden de vorming aan van heterocomplexen tussen polyU en homochirale oligomeren bestaande uit trans-4-hydroxy-N-acetylprolinol, van beide chirale vormen (polyU/trans-DA*$_{13}$ en polyU/trans-LA*$_{13}$). De eerstvermelde vormt echter een tripel helix structuur. Andere associaties met ribonucleinezuren zijn polyA/trans-LT*$_{13}$ en polyU/cis-LA*$_{13}$. Heteroduplexen met deoxynucleinezuren werden gevormd tussen trans-LA*$_{13}$ en oligothymidylaat en tussen cis-LA*$_{13}$ en oligothymidylaat, maar niet voor de D-hydroxyprolinol analogen. Microcalorimetrie toonde aan dat dit complex bestaat uit drie strengen. Twee heteroduplexen, trans-LA*$_{13}$/dT$_{13}$ en trans-LA*$_{13}$/polyU, vertonen gelijkaardige of licht verbeterde stabiliteit in vergelijking met de natuurlijke dA$_{13}$/dT$_{13}$- en dA$_{13}$/polyU-systemen. Microcalorimetrie wees duidelijk in de richting van duplexvorming, in tegenstelling tot interacties met de D-N-acetylprolinol-oligonucleotiden. De enthalpieverandering is van dezelfde grootte-orde maar de associatieconstante is lichtjes gedaald. De natuurlijke nucleinezuren vertonen dus een duidelijke voorkeur voor hybridizatie met de L-hydroxyprolinol-oligomeren boven de D-hydroxyprolinol-oligomeren. Van de onderzochte structuren blijken de L-trans oligomeren, zoals voorgesteld in Figuur 1, het best de natuurlijke oligonucleotiden na te bootsen. Deze gewijzigde oligonucleotiden zijn in staat om homocomplexen te vormen indien beide strengen van dezelfde chirale vorm zijn. Homocomplexen worden gevormd tussen trans-LA* en trans-LT* en tussen trans-DA* en trans-DT*, hetgeen analoog is aan de isochirale pu–py baseparing, voorkomend in natuurlijke nucleinezuren. Calorimetrische metingen tonen aan dat deze interactie te wijten is aan triplex vorming. Heterochirale hybridizatie werd niet waargenomen tussen gewijzigde oligonucleotiden onderling maar enkel tussen gewijzigde oligonucleotiden en natuurlijk polyU.*

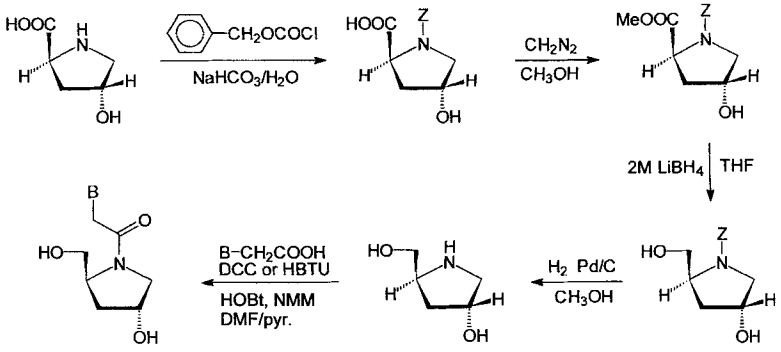
From the antisense point of view, this series of compounds may display the constrained flexibility that can be found in DNA and RNA. The presence of the five-membered ring is crucial for the retention of this characteristic. The amide bond attaching the nucleobase to the backbone also has limited freedom of rotation. The balance between flexibility and rigidity is considered to be an advantage for effective hybridisation with natural nucleic acids.[4, 5] Rigidity is necessary to ensure some preorganisation in order to prevent a huge entropy loss upon duplexation. Flexibility is essential to allow for the adaptation of the conformation to the natural nucleic acids, on the sequence of which the structure depends.

During the course of this work, the interest of several other research groups in the *trans*-4-HO-L-NAP (2*S*,4*R*) structure (NAP stands for *N*-acetylprolinol) as a sugar substitute in oligonucleotides has become apparent. Prokhorenko et al. used the compound as carrier of a fluorescence label in DNA,[6] Hebert et al. synthesised the phosphoramidites[7] (identical to the thyminyl building block **1.1**, except for the use of a dimethoxytrityl group instead of a monomethoxytrityl group) and Wilson-Lingardo et al. included the modified nucleotide in the synthesis of a chemical library.[8] Chemical concern Bayer patented the peptide analogue (both enantiomers and their epimer) in view of its potential antisense properties.[9] Most recently Gangamani et al. reported synthesis of two peptide dimers with the L-*cis*/L-*cis* and D-*trans*/L-*cis* conformations.[10]

Results and Discussion

Synthesis: The synthesis of the 2(*S*)-stereomers was started from commercially available *trans*-4-hydroxy-L-proline (Scheme 1). The procedure essentially follows the method previously described by Reed et al.[11, 12] Only the reduction of the carboxyl function is not carried out on the free acid with $BH_3 \cdot THF$ but on the methyl ester instead with $LiBH_4$. This small synthetic detour causes a substantial increase of the overall yield of the reduction process from 40% ($BH_3 \cdot THF$ reduction) to 90% (diazomethane and $LiBH_4$). After removal of the phenylmethoxycarbonyl (Z) protecting group, the amino diol is condensed with (thymin-1-yl)acetic acid[13] or (N^6-benzoyladenin-9-yl)acetic acid (Scheme 1).

(Thymin-1-yl)acetic acid was prepared as reported by Kosynkina et al.;[13] we found this to be the most efficient method.[14, 15] The preparation of the adenine acetic acid



Scheme 1. Synthesis of *trans*-4-HO-*N*-acetyl-L-prolinol nucleosides (B: adenin-9-yl; thymin-1-yl).

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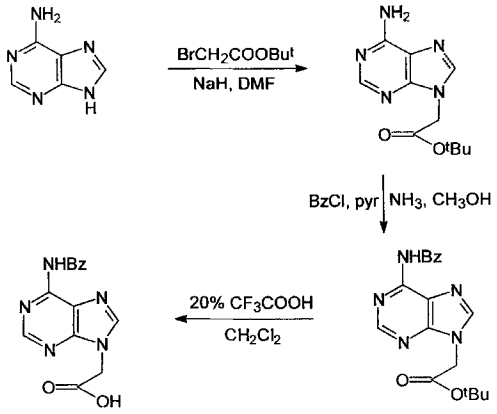
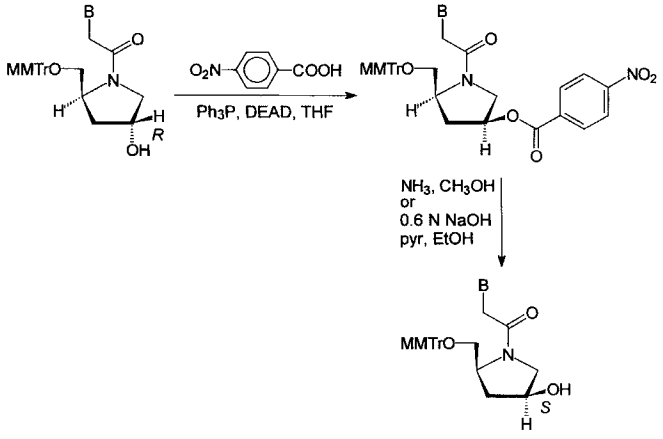
derivative required first reaction of adenine with *tert*-butyl bromoacetate, followed by benzoylation at N 6 and deprotection by acid treatment (Scheme 2). The *trans*-4-hydroxy-*N*-



Scheme 2. Synthesis of (N^6-benzoyladenin-9-yl)acetic acid.

[(thymin-1-yl)acetyl]-L-prolinol obtained and its adenine congener were then monomethoxytritylated at the primary alcohol, converted to the 4-*O*-phosphoramidites and oligomerised by standard phosphoramidite chemistry.[16] The *cis* compounds (2*S*,4*S*) were prepared analogously, by a Mitsunobu reaction with *p*-nitrobenzoic acid for the Walden inversion of the secondary alcohol in a final reaction step. This reaction was carried out at the level of the monomethoxytritylated derivative. Deprotection of the *p*-nitrobenzoate (with MeOH/NH_3 for the thymine derivative or 0.6 N NaOH in a pyridine/ethanol mixture[17] for the adenine derivative) yielded the desired compounds (Scheme 3) which were again appropriately derivatised



Scheme 3. Synthesis of the monomethoxytritylated *cis*-4-hydroxy-*N*-acetyl-L-prolinol nucleosides (B: thymin-1-yl; N^6-benzoyladenin-9-yl).

and submitted to standard phosphoramidite oligomerisation. The difference between the deprotection of the adenine derivatives and of the thymine compounds was necessary to prevent debenzoylation of the adenine moiety. All synthesised nucleoside analogues are included in Figure 3 and Table 1.

The synthesis of the 2(*R*) stereomers started from commercial *cis*-4-hydroxy-D-proline. The synthesis followed the same

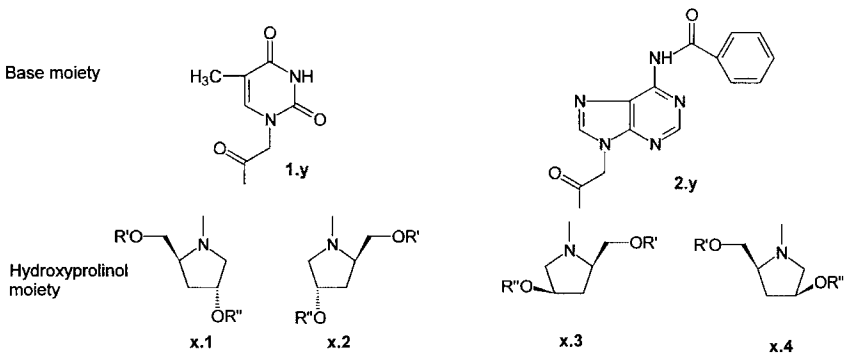


Figure 3. Schematic overview of the synthesised nucleoside analogues. Products are categorised according to the nucleobase present (first number: **1** is thymin-1-yl; **2** is N^6-benzoyladenin-9-yl), their stereochemistry (second number: **1** is 2(*S*),4(*R*); **2** is 2(*R*),4(*S*); **3** is 2(*R*),4(*R*); **4** is 2(*S*),4(*S*)) and the protecting group and its position (final character).

Table 1. Identity of the synthesised nucleoside analogues.

	2	4	R′	R″		2	4	R′	R″
1.1a	*S*	*R*	H	H	**2.1a**	*S*	*R*	H	H
1.1b	*S*	*R*	MMTr	H	**2.1b**	*S*	*R*	MMTr	H
1.1c	*S*	*R*	*t*BDMS	H	**2.1**	*S*	*R*	MMTr	P
1.1d	*S*	*R*	*t*BDMS	MMTr	**2.4a**	*S*	*S*	H	H
1.1e	*S*	*R*	H	MMTr	**2.4b**	*S*	*S*	MMTr	H
1.1	*S*	*R*	MMTr	P	**2.4**	*S*	*S*	MMTr	P
1.1i	*S*	*R*	P	MMTr	**2.2a**	*R*	*S*	H	H
1.2a	*R*	*S*	H	H	**2.2b**	*R*	*S*	MMTr	H
1.2b	*R*	*S*	MMTr	H	**2.2**	*R*	*S*	MMTr	P
1.2c	*R*	*S*	*t*BDMS	H					
1.2d	*R*	*S*	*t*BDMS	MMTr					
1.2e	*R*	*S*	H	MMTr					
1.2	*R*	*S*	MMTr	P					
1.2i	*R*	*S*	P	MMTr					
1.3a	*R*	*R*	H	H	**2.3a**	*R*	*R*	H	H
1.3b	*R*	*R*	MMTr	H	**2.3b**	*R*	*R*	MMTr	H
1.3c	*R*	*R*	*t*BDMS	H	**2.3**	*R*	*R*	MMTr	P

[a] MMTr stands for monomethoxytrityl ether protection. [b] P indicates β-cyanoethoxy diisopropylamine phosphoramidite derivative.

scheme as presented for the 2(*S*) stereomers, but in this case the *cis* compounds (2*R*,4*R*) are directly available, whereas the (2*R*,4*S*) products require an inversion of configuration at position 4. The reaction conditions are the same as described for the 2(*S*) series.

The reaction scheme presented here did not result in racemisations. Diastereomeric products, if any, would have been detected by chromatographic mobility or NMR analysis. The pair of *cis* and *trans* monomethoxytritylated 4-hydroxy-*N*-[(thymin-1-yl)acetyl]prolinol diastereomers, as well as the deprotected diastereomeric compounds, can be detected as separate spots in TLC; the *cis* compound is the more slowly migrating product. The NMR spectra of the two are clearly distinct. This holds also for the adenine derivatives.

Analysis of the Monomeric Units: The spectrum arising from a heteronuclear correlation experiment was used to determine the position of most protons. The ^{13}C NMR spectra showed doubled signals for virtually all nuclei in the compounds studied. This phenomenon is most likely due to the presence of two slowly exchanging conformers.

For monomethoxytritylated compounds, the trityl group probably sterically slows the conformational transition. This hypothesis was verified from the deprotected derivatives. The

fully protected compound with a monomethoxytrityl group on the primary and a *tert*-butyldimethylsilyl group on the secondary alcohol displayed a ^{13}C NMR spectrum with all signals doubled. When the trityl group was removed, the NMR analysis, run without specific purification of the compound, showed only two peaks doubled. Products bearing no protecting groups on the alcohol functions also occur as two distinct conformers. Their interconversion is probably slowed down by hydrogen bonding to the nucleobase (Figure 4). In fact, molecular modelling, a Monte Carlo search through all rotatable bonds[18] with an OPLS forcefield and GB/SH solvent treatment,[19] confirmed the possibility of the primary as well as the secondary alcohol forming a hydrogen bond with N3 of adenine. These two conformations are both of minimal energy with energy differences less than 4 kJ mol^{-1} and relate to two different molecular shapes (Figure 4A and A'). For the thymine derivative too two conformations could be found with low energy and less than 4 kJ mol^{-1} difference in potential energy (Figure 4B and B'). However, the overall shape differences between the two thymine analogue conformations are small. The origin of the two forms does not reside in hydrogen bonds from the alcoholic functions to the nucleobase, as was found for the adenine derivatives. In this case, only the primary alcohol function engages in hydrogen bonding. In the thymine derivative, the primary alcohol function has a preference for interacting with the carbonyl of the methylene carbonyl linker. Beside this conformation of lowest energy, the primary alcohol is also found to interact with O2 of thymine. In molecular dynamics simulations, interconversion between the low-energy conformations of the adenine derivative occurred, and on a much shorter timescale (500 ps) than expected from the NMR results. However, the modelling experiment was performed as if the molecule were in a vacuum environment, whereas water is known to slow down conformational transitions. It should be mentioned that the existence of several rotamers has also been observed for the PNA dimer H-AC-NH_2.[20]

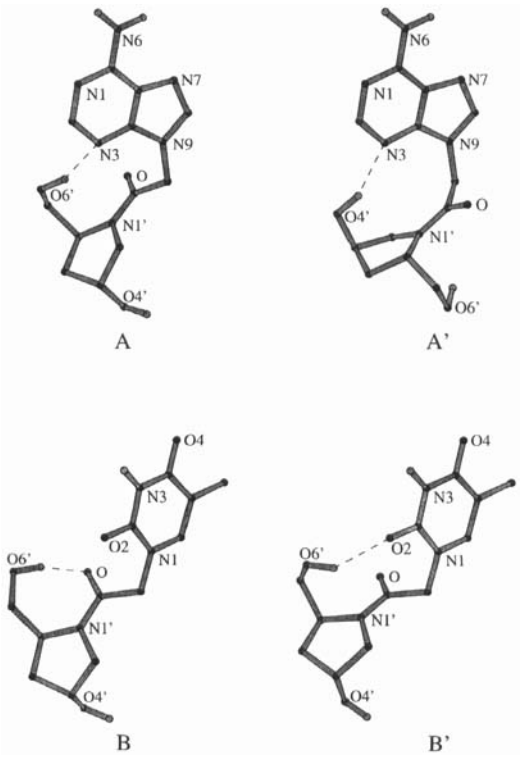


Figure 4. The two conformers of *trans*-4-hydroxy-*N*-(N^6-benzoyladenin-9-yl)acetyl)-L-prolinol (A and A') and *trans*-4-hydroxy-*N*-(thymin-1-yl)acetyl)-L-prolinol (B and B').

Our differentiation between *cis* and *trans* geometrical stereomers was confirmed by analysis of their proton spectra. The commercial *trans*-4-HO-L-proline and *cis*-4-HO-D-proline were analysed as model compounds and the obtained spectra were compared with those of the prolinol derivatives. From these data a similar configuration for both the *trans*-proline or *cis*-proline starting material and the isolated end-products *trans*-prolinol or *cis*-prolinol, respectively, could be deduced (^1H NMR and ^{13}C NMR data are given in the experimental section).

Synthesis of Oligonucleotides and Mass Spectrometric Analysis: Standard phosphoramidite chemistry was used for assembly of the modified oligonucleotides; a slightly higher amidite concentration (0.13 M) and prolonged coupling time were used to ensure adequate coupling of the modified building blocks. Deprotonation and purification by ion-exchange chromatography were carried out following published procedures.[21] No deleterious effect for the amide was noticed upon prolonged (16 h at 55 °C) treatment with ammonia.

Electrospray ionisation mass spectrometric analysis showed that the obtained oligonucleotides were pure and the correct molecular weight was found in all cases (Table 2). The purification method proved to be very efficient for the removal of interfering sodium ions; only slight cation adducts are seen in the spectra (Figure 5).

Table 2. Electrospray ionisation mass spectrometric analysis of fully modified *N*-acetylprolinol oligonucleotides.

	Sequence [a]	Calcd.	Found
4-HO-L-*trans*	T^*_{13}-prop	4564.2	4564.5
	A^*_{13}-prop	4681.4	4682.0
	T^*_5-prop	3264.4	3264.5
	$T_4A^*_5$-prop	4481.2	4481.0
	$T^*_4A^*_5$-prop	4645.4	4644.0
4-HO-L-*cis*	CCA^*_{13}-prop	5259.8	5259.4

[a] "prop" denotes the 3'-terminal propanediol phosphate moiety.

Hybridisation of Modified Oligonucleotides: UV melting curves of all-thymine and all-adenine 4-HO-NAP oligomers with complementary DNA and RNA sequences were recorded at 260 nm to assess the degree of duplex and triplex formation. The samples were dissolved in neutral buffer solutions containing 0.1 or 1 M NaCl and curves were run at a heating rate of 0.2 °C min^{-1}. The degree of intrastrand base stacking and the shape of the melting curves of the single-stranded oligomers were investigated to exclude ambiguous melting temperatures with complementary nucleic acid sequences. A hyperchromicity of about 6% for the *trans*-adenine oligomer was detected in the 5–55 °C range, indicating that no self-aggregation occurs. Intramolecular stacking was more substantial for the *cis*-4-HO-NAP

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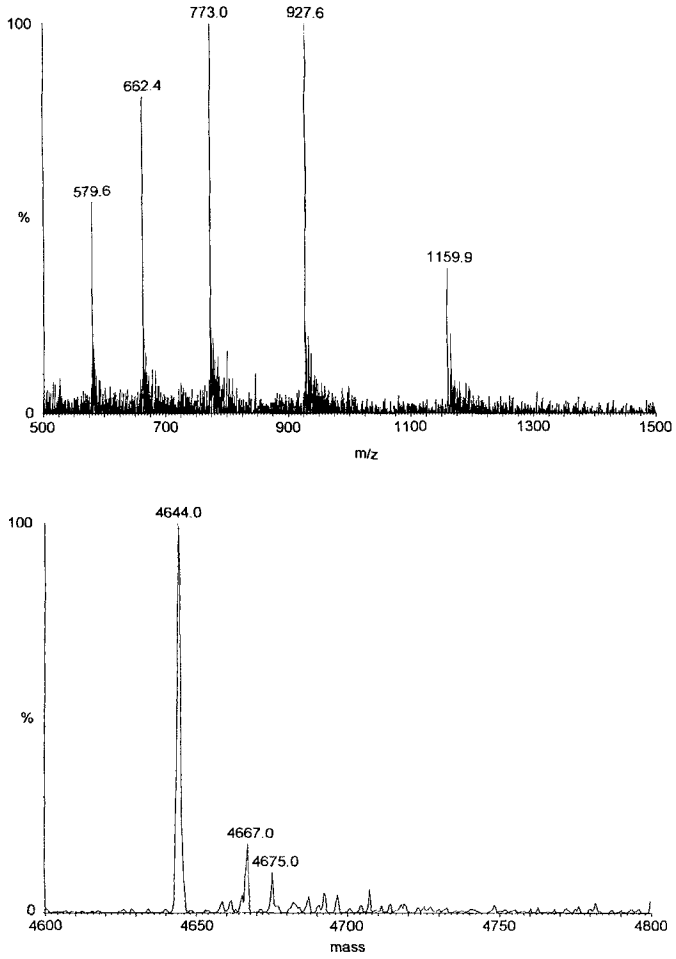

Figure 5. Electrospray mass spectrum of a fully modified $T_4^*A_9^*$ 3′-propanediol phosphate 4-HO-L-*trans*-*N*-acetylprolinol oligonucleotide (top) and the deconvolution of the spectrum in the molecular region (bottom).

Table 3. T_m values of oligonucleotides containing *trans*-4-HO-*N*-acetylprolinol as carbohydrate substitute, with complementary DNA sequences.

	trans-L	T_m [a] 0.1 M NaCl	1 M NaCl	*trans*-D	T_m [a] 0.1 M NaCl	1 M NaCl
1	T_{13}/A_{13} [b]	33	48	T_{13}/A_{13}	33	48
2	$T_6LT^*T_6/A_{13}$	23	38	$T_6DT^*T_6/A_{13}$	16	31
3	$T_5LT_3^*T_5/A_{13}$	9	23	$T_5DT_3^*T_5/A_{13}$	[c]	20
4	LT_{13}^*/A_{13}	[c]	[c]	DT_{13}^*/A_{13}	[c]	[c]
5	$T_6LiT^*T_6/A_{13}$	22	37	$T_6DiT^*T_6/A_{13}$	19	33
6	$T_5LiT_3^*T_5/A_{13}$	[c]	17	$T_5DiT_3^*T_5/A_{13}$	[c]	17.5
7	$A_6LA^*A_6/T_{13}$	25	40	$A_6DA^*A_6/T_{13}$	25	39
8	$A_5LA_3^*A_5/T_{13}$	21	37	$A_5DA_3^*A_5/T_{13}$	11	32.5
9	LA_{13}^*/T_{13}	35	48	DA_{13}^*/T_{13}	[c]	[c]
10	LA_{13}^*/LT_{13}^*	15	28.5	DA_{13}^*/DT_{13}^*	9	25
11	$(LA^*LT^*)_6$	[c]	[c]	$(DA^*DT^*)_6$	[c]	[c]
12	LT_{13}^*/DA_{13}^*	[c]	[c]			
13				DT_{13}^*/LA_{13}^*	[c]	[c]

[a] T_m (°C) was measured in a buffer containing 0.02 M KH_2PO_4, pH 7.5, 0.1 mM EDTA with 0.1 or 1 M NaCl and 4 μM of each ON. [b] T stands for thymidine and A for deoxyadenosine. [c] No hypochromicity detectable.

oligonucleotides (15%). However, no adenine–adenine or thymine–thymine pairing could be observed in the hydroxyprolinol oligonucleotides.

Trans Compounds

T_m with complementary DNA: Table 3 shows that the fully modified oligonucleotide composed of *trans*-4-hydroxy-*N*-[(adenin-9-yl)acetyl]-L-prolinol [*trans*-4-HO-L-NAP-adenine (LA*)] units is capable of forming a complex with its DNA complement, with a stability that equals that of the common DNA polyA/polyT duplex (compare entries 9 and 1, left). Single and triple substitutions of an artificial unit in the middle of a natural sequence induce substantial decreases in duplex stability (entries 7 and 8). While the destabilisation of the duplex upon incorporation of LA* units seems to level off after multiple incorporations, the thymine analogue LT* seems not to contribute to binding. The melting points recorded are in the same range as for a natural T_{13}/A_{13} duplex with a single mismatch (T_m's of 18–21 °C for all possible different mismatches at 0.1 M NaCl; see also Table 6). Introduced D-compounds cause a larger destabilisation than L-derived structures (compare entries 2, 3, 5, 8, left and right) and the fully modified oligonucleotide composed of DA* units does not hybridise with an oligothymidine complement. Stereochemically, the L-HO-prolinol ring is the congener of the natural D-ribose sugar, which might explain this experimental observation.

Modified thymine units (DT* and LT*) were also inserted in an "inverted" manner (DiT* and LiT*), where the primary alcohol takes the 3′ position in D-ribose. In the L series, T_m's were slightly decreased compared to those for normal insertion, suggesting more distortion of base pairing than when the primary alcohol position is equivalent to the 5′-(primary) alcohol in (deoxy)riboses and the secondary alcohol substitutes for the 3′-hydroxyl group (entries 2, 3 and 5, 6).

Under no circumstances is the fully modified thymine 13-mer capable of interacting with complementary DNA (entry 4, left and right), and single and triple substitution of a modified thymine in the middle of a natural sequence gives a larger drop in T_m than does adenine (entries 2, 3 and 7, 8). Homocomplexes do form with the modified thymine oligonucleotides (entry 10) (although the nature of this complex is different, as will be further discussed later). The lack of hybridisation of the fully modified thymine 13-mer cannot be solely attributed to lower stacking interactions between pyrimidine bases. It has been observed for natural nucleic acids that the pyrimidine bases have less rotational freedom than the purines, which has been attributed to the fact that they are more bulky in the region of the sugar–phosphate backbone.[22] Although the spacer would seem to allow sufficient rotational freedom, it might be possible that such a factor would be responsible for the inability of the modified oligothymidylate to adopt a conformation for duplexation with DNA. It has also been noticed that tritylated monomers (thymine as well as adenine) are locked in two different conformations (all carbon atom signals are doubled in NMR analysis), indicating an unexpected rigidity of these compounds. Alternatively, the difference in stability of LT_{13}^*/A_{13}, LA_{13}^*/T_{13}, LA_{13}^*/LT_{13}^* and A_{13}/T_{13} associations might be a reflection of a different orientation (parallel/antiparallel) of the strand and/or a different base pairing system (Hoogsteen or reverse-Hoogsteen instead of Watson–Crick pairing). Which factor exactly is responsible for the lack of complexation of the modified T oligomers with DNA is not clear yet (see also "Strand orientation").

Homocomplexes: As mentioned before, *trans*-4-HO-NAP oligonucleotides are capable of forming homocomplexes, at least if both strands are of the same stereochemical configuration (homochiral strands with the same sense of chirality). Such complexes are of reduced stability compared with the natural systems. The recorded T_m's indicate slightly lower stability for the D-homocomplex than the L-complex (compare entry 10, left and right), but this could be an artefact, since the DT* oligonucleotide was built from 13 DiT* units (instead of DT*) and one natural thymidine unit was attached to it (for labelling experiments) giving T–DiT*$_{13}$. Alternate homochiral self-complementary sequences (entry 11) and homochiral strands with opposite chirality (entries 12, 13) do not display melting behaviour. The former especially was surprising in view of the pairing of homochiral strands (entry 10).

T_m with Complementary RNA: Melting profiles of all modified *trans*-oligomers with complementary RNA revealed stable complexes at 1 M salt concentration for all structures, L as well as D, except for the D-derived thymine oligomer (entry 4, right, in Table 4). These results are in agreement with previous observations as to RNA's lower discrimination concerning the stereochemistry of its associate.[23–28] It is probably no coincidence that again a thymine oligomer does not hybridise. Also, the stability of the complex formed between the *trans*-4-HO-L-NAP all-thymine oligonucleotide and complementary RNA is weak (Table 4, entry 4, left). The melting profiles gathered with RNA further confirm the potential of the *trans*-4-HO-L-NAP all-adenine oligonucleotide, which forms the most stable complex, one that equals the stability of its natural counterpart the A-DNA:U-RNA hybrid (Table 4, entries 1 and 3, left). Here too, the orientation of the hybridising strands and the pairing system itself cannot be deduced from the experiment.

Table 4. T_m values recorded for complexation of *trans*-4-HO-NAP oligonucleotides with complementary RNA.

	trans-L	T_m [a] 0.1 M NaCl	1 M NaCl	*trans*-D	T_m [a] 0.1 M NaCl	1 M NaCl
1	polyU/dA_{13}	29	61	polyU/dA_{13}	29	61
2	polyA/dT_{13}	29	47	polyA/dT_{13}	29	47
3	polyU/LA^*_{13}	33	57	polyU/$\mathbf{D}A^*_{13}$	[b]	27/23[c]
4	polyA/LT^*_{13}	13/17 [c]	30	polyA/$\mathbf{D}T^*_{13}$	n.a.	[b]

[a] T_m (°C) was measured in a buffer containing 0.02 M KH_2PO_4, pH 7.5, 0.1 mM EDTA with 0.1 or 1 M NaCl and 4 μM of each ON. [b] No hypochromicity detectable. [c] The first number applies to the T_m obtained upon heating, the second one to the cooling experiment. n.a.: not assessed.

Table 5. T_m values of oligonucleotides with *N*-acetyl-*cis*-HO-prolinol as sugar substitute (entries 1 and 2 with DNA complement, entries 3 and 4 with RNA complement).

	cis-L	T_m [a] 0.1 M NaCl	1 M NaCl	*cis*-D	T_m [a] 0.1 M NaCl	1 M NaCl
1	LA^*_{13}/dT_{13}	[b]	20/21 [c]	DA^*_{13}/dT_{13}	n.a.	[b]
2	dA_{13}/dT_{13}	35	48	dA_{13}/dT_{13}	35	48
3	LA^*_{13}/polyU	7	33	DA^*_{13}/polyU	n.a.	[b]
4	dA_{13}/poly U	29	61	dA_{13}/poly U	29	61

[a] T_m (°C) was measured in a buffer containing 0.02 M KH_2PO_4, pH 7.5, 0.1 mM EDTA with 0.1 or 1 M NaCl and 4 μM of each ON. [b] No hypochromicity detectable. [c] The first number applies to the T_m obtained upon heating, the second one to the cooling experiment. n.a.: not assessed.

In an effort to obtain information about the helix types that are involved in the complexes formed, CD spectral analysis was used. Complexes with RNA in particular, which can include modified oligonucleotides built from either L- or D-monomers, are of interest. The CD spectra obtained are depicted in Figure 6. The shape of the curves allows the spectra to be categorised with respect to the prevailing helix type. There is a relationship between the nature of the strands in the complex and the conformation detected. Hybrids with polyU (RNA) adopt an A-type conformation unless the associating strand is dA_{13} (which induces B-type). The curves of LA^*_{13}/T_{13} (spectrum A) and dA_{13}/polyU (spectrum F) are very similar. The modified single strands give rise to barely any signal (data not shown). Also, the fully artificial system composed of two complementary homochiral *trans*-4-HO-NAP strands of the same sense of chirality hardly gives any CD signal (spectrum E). Insofar as a pattern can be detected, no relationship with a known

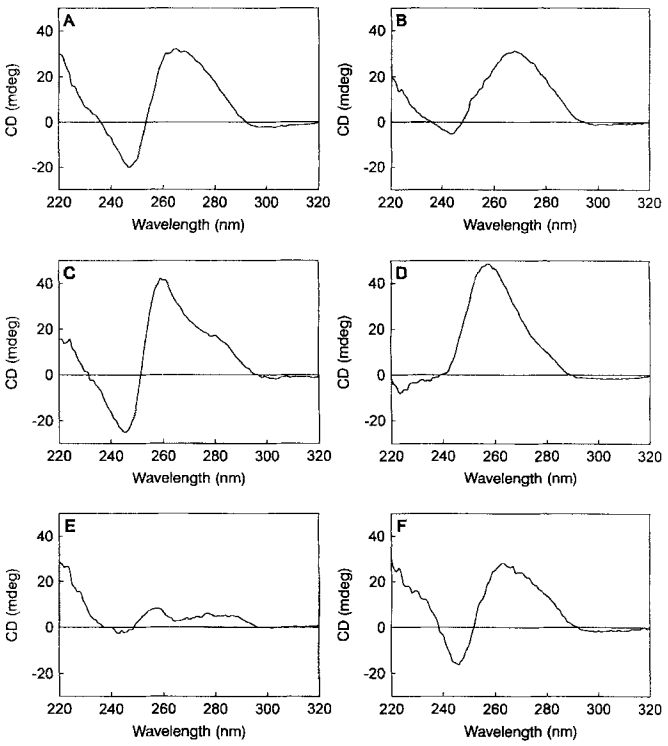

Figure 6. CD spectra obtained with *trans* oligomers in a 1 M NaCl solution at 5 °C. A: LA^*_{13}/T_{13}; B: LA^*_{13}/polyU; C: LT^*_{13}/polyrA; D: DA^*_{13}/polyU; E: homocomplex (DA^*_{13}/dT^*_{13}); F: dA_{13}/polyU.

Cis Compounds

Cis-4-HO-L-NAP adenine oligomers are capable of hybridisation with their RNA as well as their DNA complement (Table 5, entry 1 and 3, left), whereas the D-compounds are not at all (Table 5, entries 1 and 3, right), as demonstrated by UV melting experiments. The complexes formed were of low stability.

CD Spectral Analysis

The sensitivity of circular dichroism to conformational alterations together with its ease of use (compared to NMR and X-ray analysis) forms the basis of its application in the structural investigation of chiral polymers and polymer complexes.

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conformation of natural nucleic acids can be recognised for the artificial system. The fact that no distinctive patterns arise might be due to the fact that the chiral centre is rather far removed from the UV-active nucleus that is co-responsible for the observed signal. The bridge between the ring inducing the feature of chirality and the nucleobases also contains an sp^3 carbon, allowing the base to rotate. The *trans*-4-HO-D-NAP-A oligomer that interacts with polyU (spectrum D) displays the conformational pattern of a normal A-helix. Substantial differences can be found between this spectrum and the one obtained with its L-congener (spectrum B). The differences might also be due to the formation of triplexes instead of duplexes. A similarity of the *trans*-4-HO-L-NAP-T_{13}/polyrA (spectrum C) with the α-bicyclo-A/β-bicyclo-T system became evident upon comparison with known CD structures of modified oligonucleotides.[29]

For the complexes formed with *trans*-HO-NAP compounds, the complementary natural nucleic acids were always capable of imposing their own helical preferences to some extent. For the *cis* compounds, investigation by circular dichroism reveals that the complexes no longer resemble the classical nucleic acid helices. All curves of the complexes more closely resemble the ones obtained with the single-stranded modified oligonucleotides than those obtained for the single-stranded natural sequences (spectra not shown). This indicates conformational rigidity of the *cis*-HO-NAP oligonucleotides that forces the natural oligonucleotide strands to adapt. No analogy could be seen between any of our structures and the CD spectra obtained by Gangamani et al. for their peptide dimers.[10]

Investigation of trans-4-HO-L-NAP Oligonucleotide Hybridisation

UV Mixing Curves: The *trans*-4-HO-L-NAP structure is the most promising modification of the series of products investigated here. Complexes formed with complementary DNA or RNA have similar stability to the natural duplexes if modified all-adenine oligomers are considered. The exact identity of the complex (triplex↔duplex) between the *trans*-4-HO-L-NAP all-adenine oligonucleotides with complementary DNA and RNA is of great interest for the determination of the real potential of such modified oligonucleotides as antisense agents. Hypochromicity for the LA^*_{13}/T_{13} complex was calculated as 33%, where 25% was registered for the natural A_{13}/T_{13} system. UV melting experiments performed at 284 nm generate a melting profile for the LA^*_{13}/T_{13} complex as well as for the modified homocomplex, hinting at triplex formation (UV melting curves not shown). Considering the homocomplex, it was also noted for both chirality series (D or L) that self-complementary alternate sequences $[(A^*,T^*)_n]$ *do not* display hybridisation, an observation that also fits the triplex formation hypothesis. On the other hand, alternating purine–pyrimidine sequences are known to form less stable duplexes, due to diminished stacking as compared to (all-purine)–(all-pyrimidine) hybridisation.[30] In view of the relatively low stability already encountered for the LA^*_{13}/LT^*_{13} complex, it is therefore equally likely that the duplex with the alternating sequence is simply too unstable to be detected.

UV mixing curves obtained according to the method of Job[31] at wavelengths of 260 and 284 nm for the T_{13}/*trans*-LA^*_{13} show a single discontinuity in the latter case (UV min at 1:1) and an apparent double discontinuity at 260 nm (Figure 7). The

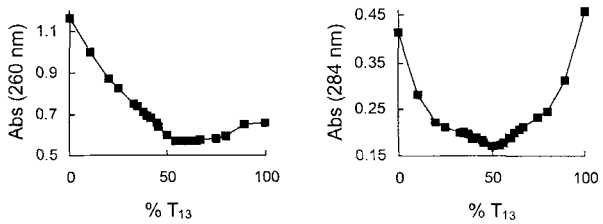

Figure 7. UV mixing curves of *trans*-4-HO-L-NAP-A_{13} with T_{13} recorded at 260 (left) and 284 nm (right).

interpretation of these results seems untrustworthy, considering the apparent contradiction: UV hypochromicity at 284 nm is commonly associated with TAT triplex formation,[32] whereas a UV minimum at 1:1 indicates duplexation. However, hypochromicity at 284 nm is obviously conformation-dependent, considering the fact that RNA triplexes do not display this feature.[33] UV mixing data are more reliable in this respect, and therefore the available data indicate the formation of a double helix. Moreover, the CD spectra clearly indicate conformational deviations from the normal DNA·DNA or RNA·DNA helical systems, which might provide an explanation for the melting profile observable at 284 nm.

The assessment of the nature of the complex with RNA is even more critical than the one with DNA in view of antisense potential. The UV mixing curves clearly indicate duplex formation in this case too (curves not shown). These results are not in agreement with the different phenomena that apparently occur upon melting of the complex as evidenced in the UV melting profile recorded at 280 nm. However, it is possible that the nonequivalence in chain length[34] between the modified oligonucleotide and its RNA complement is at the base of these strange observations.

Gel Shift Assays and CD Melting Profiles: Our modified all-adenine oligonucleotides were not recognised by the T4 polynucleotide kinase, the enzyme used to radiolabel the oligonucleotides. Therefore we attached two natural cytidine units (C) at the 5′ end in the synthesis of a second batch of modified all-adenine oligonucleotides to give CCA^*_{13}; this allowed very efficient radioactive labelling.

Gel mobility shift assays were run as described in the experimental section. The autoradiograph in Figure 8 shows the hy-

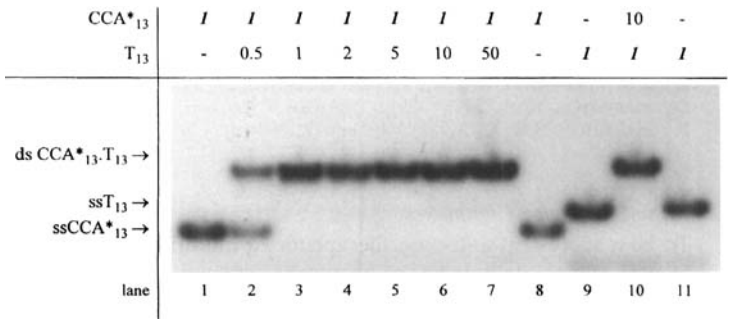

Figure 8. Autoradiograph of the gel mobility shift assay on a low-temperature (10 °C) native 15% polyacrylamide gel. The radiolabeled oligonucleotides are indicated in bold italic characters (CCA^*_{13}: modified *trans*-LA^*_{13} oligoadenylate with two natural cytidine nucleotides at the 5′ end). Mixing ratios of the strands are indicated along the top. See experimental for other conditions.

bridisation characteristics of CCA^*_{13} with natural T_{13}. The ratios of the oligonucleotides are shown on top. Both oligonucleotides were subsequently radiolabelled and mixed with their respective cold complements. Lanes 1 to 8 show radiolabelled CCA^*_{13} mixed with cold T_{13} in increasing ratios. Even a CCA^*_{13}:T_{13} ratio of 1:0.5 results in complex formation (lane 2); at a ratio of 1:1 (lane 3) the band of single-stranded CCA^*_{13} has completely shifted to the position of a CCA^*_{13}:T_{13} hybrid. The position of the band does not change upon increasing the T_{13} concentration (up to a CCA^*_{13}:T_{13} ratio of 1:50 in lane 7). The hybrid observed in lanes 2 to 7 shows the same electrophoretic mobility as the complex in lane 10. However, this is not conclusive for duplex or triplex formation as both TA*T and A*A*T complexes are theoretically possible. Therefore, microcalorimetric titration was carried out on the related A^*_{13}/T_{13} complex (see below). The experiment was repeated with natural U_{13} as the complementary strand, but here neither duplex nor triplex formation could be observed.

The CD melting profiles showed no indication of any other, transient complexes (Figure 9). This observation is, however, less reliable than a UV titration, since small changes will not be visible easily.

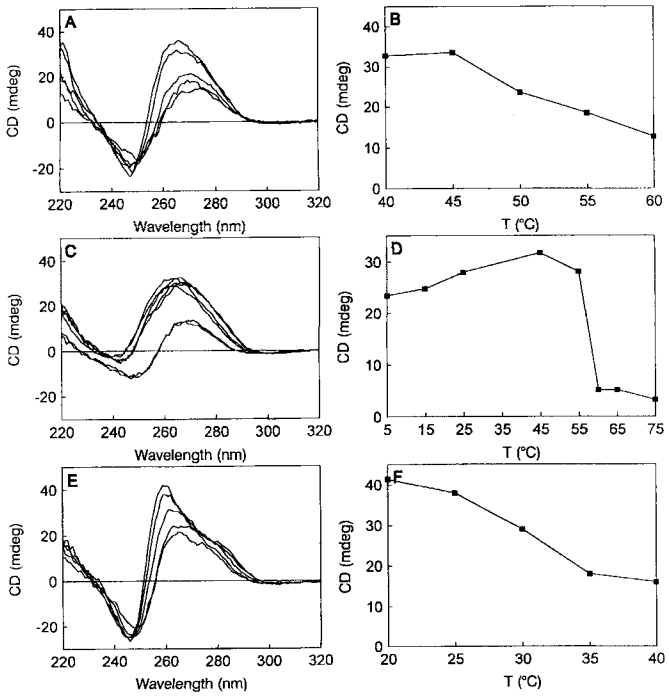

Figure 9. CD melting profiles and temperature-dependent CD measurements. A and B: *trans*-4-HO-L-NAP-A_{13}/T_{13} (B at 220 nm) [T_m(260 nm) = 48 °C]; C and D: *trans*-4-HO-L-NAP-A_{13}/U_{13} (D at 260 nm) [T_m(260 nm) = 57 °C]; E and F: *trans*-4-HO-L-NAP-T_{13}/A_{13} (F at 260 nm) [T_m(260 nm) = 29 °C].

Mixed-Sequence Complexes

To be a successful antisense therapeutic, a modified mixed-sequence oligonucleotide has to be capable of forming stable duplexes. To assess this quality for our oligonucleotide, we prepared non-self-complementary mixed A,T sequences (5′-T*A*A*T*A*A*T*A*T*A*T*A*A*A*T*T*T*T*T*-3′), where A* and T* represent the *trans*-L-nucleotide, and tested their hybridisation with complementary DNA and RNA, antiparallel as well as parallel. Neither with DNA nor with RNA we could detect hybridisation, whereas the reference complex of complementary DNA strands has a T_m of 48 °C in 1 M NaCl.

Specificity of Hybridisation

The sequence specificity of the interaction was probed by running UV melting experiments with single mismatch systems (Table 6). The stability of complexes formed by *trans*-4-HO-L-NAP-A_{13} and DNA T_6XT_6 oligonucleotides is indicated in Table 6, where X represents 2′-deoxycytidine, 2′-deoxyguanosine or 2′-deoxyadenosine. These data indicate that the specificity of the modified oligonucleotides is lower than that of natural DNA.

Table 6. T_m (°C) of single mismatch sequences of 4-HO-L-NAP-A_{13} oligonucleotides with DNA.

	T_6AT_6	T_6CT_6	T_6GT_6	T_{13}
LA^*_{13}	26.5	26.2	28.0	35.4
A_{13}	18.5	17.9	20.0	33.4

Strand Orientation

Intrigued by the question of the orientation of the homocomplexes obtained, we assembled two more oligonucleotides in the *trans*-4-HO-L-NAP series. It was hoped that addition of four modified thymidine units to a nonamer stretch of adenosine NAP monomers would increase the association strength with a complementary natural oligonucleotide, either in a parallel (p) or antiparallel (ap) orientation. The results in Table 7 seem to indicate the orientation probably to be antiparallel, but these results are not fully convincing, with only 2° difference for either a parallel or antiparallel orientation (entry 1, 2). However, the discrimination remains small upon exchanging the thymine NAP's for a natural thymidine sequence (entry 3, 4). Therefore, no clear-cut conclusion can be drawn, but an ap pairing seems more likely. Obviously, within the natural system the ap orientation is clearly favoured.

Table 7. T_m values for mixed oligonucleotides with *trans*-4-HO-L-NAP as sugar substitute in a quest for strand orientation.

		T_m [a] (hypochromicity)	Orientation [b]
1	$T^*_4A^*_5/d(A_4T_9)$	36.9 (37%)	p
2	$T^*_4A^*_5/d(T_9A_4)$	38.8 (24%)	ap
3	$dT_4A^*_5/d(A_4T_9)$	36.9 (36%)	p
4	$dT_4A^*_5/d(T_9A_4)$	39.2 (45%)	ap
5	$A^*_5/d(A_4T_9)$	37.1 (42%)	5′-dA_4 overhang
6	$A^*_5/d(T_9A_4)$	38.4 (30%)	3′-dA_4 overhang
7	$d(T_4A_9)/d(A_4T_9)$	14.6 (32%)[c]	p
8	$d(T_4A_9)/d(T_9A_4)$	40.4 (40%)	ap

[a] T_m (°C) was measured in a 1 M NaCl buffer containing 0.02 M KH_2PO_4, pH 7.5 and 0.1 mM EDTA and 4 μM of each ON. [b] Either a parallel (p) or antiparallel (ap) orientation would allow full base pairing. [c] A small second transition is noticed at 32 °C.

Microcalorimetry

An alternative option for stoichiometric determination of the different interactions was microcalorimetric titration. The calorimetric cell was filled with 5 μM solution of the modified

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A_{13}-oligomer and titrated with a 100 μM solution of tridecathymidine (or its congener). As can be deduced from the titrations in 1 M NaCl (Figure 10), the 4-HO-L-*trans* A* oligonucleotide clearly forms a 1:1 complex with the complementary tridecathymidine (panel A) with a ΔH comparable to the ΔH obtained for the natural duplex (panels E and F). In contrast, however, all other homooligonucleotide interactions consist of triple-stranded complexes with only one transition from single strand to triple-stranded complex (panels B, C, D). In the natural system, the thymidine strand can interact only with the preformed duplex, and does so by the well-known Hoogsteen base pairing. Accordingly, a double transition (with clearly different ΔH and K, panel E) can be obtained when titrating at a temperature well below the melting temperature obtained under these conditions (1 M NaCl, 48 °C). At higher temperature, only duplex formation is seen, while the triple-stranded complex only contributes marginally to the heat produced above the 1:1 ratio (panel F). T_m determination (by UV absorption changes at 260 nm) for the triplex at 4 μM in 1 M NaCl (1:2 ratio of A_{13}:T_{13}) revealed only a very broad and weak transition with a maximum between 25 and 30 °C. Analogously a second (very weak) interaction could be seen for the 4-HO-L-*trans* A* oligonucleotide, but only by microcalorimetric titration. The formation of a triplex or no complex at all has previously been described within the 2′,5′-isoDNA series,[35] as well as for homooligomers of acyclic adenosine analogues.[36]

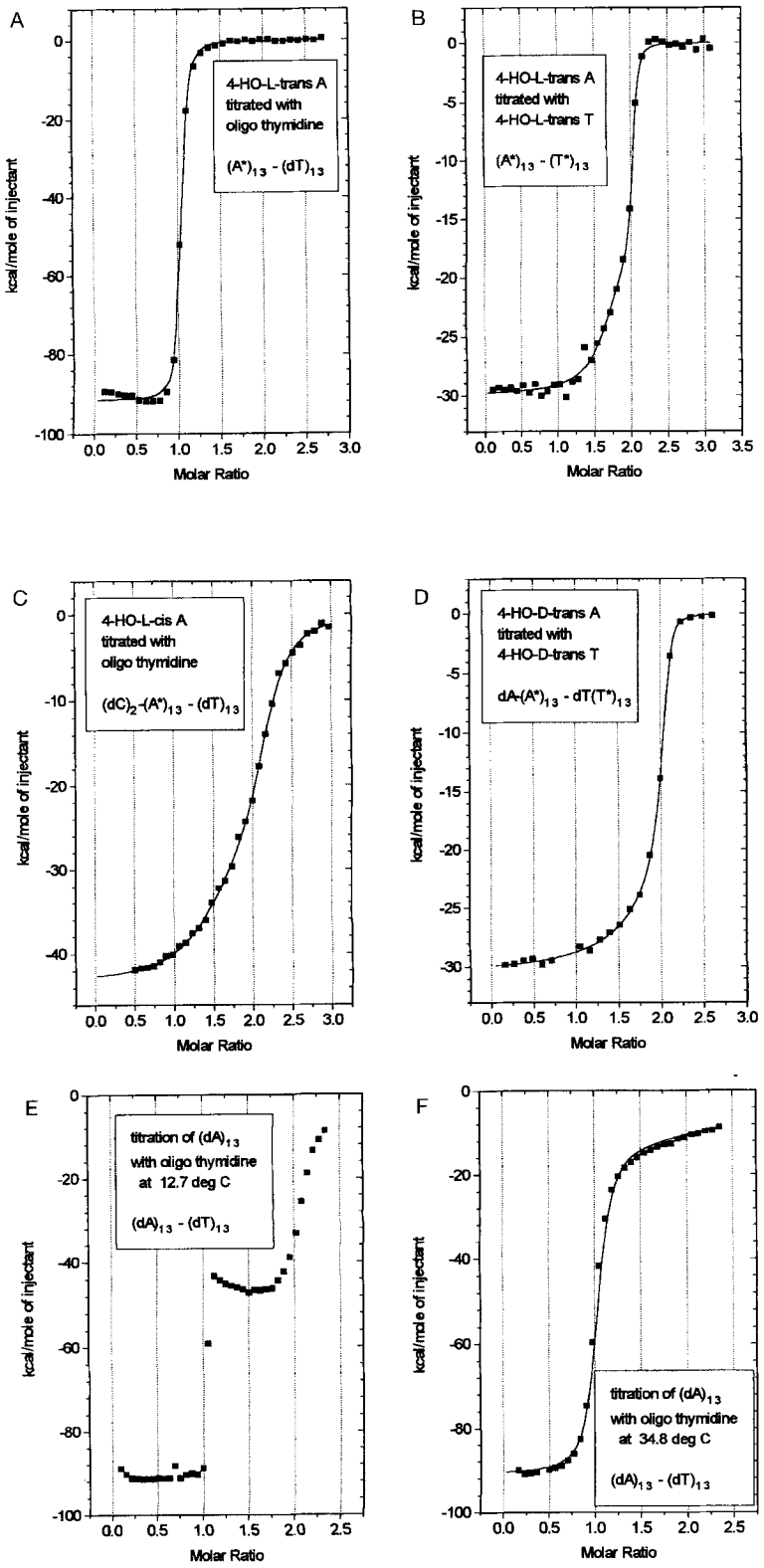

Figure 10. Microcalorimetric titrations for the different homocomplexes.

Stability: The enzymatic stability of a modified *trans*-4-HO-L-NAP-A_{13} (CCA^*_{13}) was probed by exposure to snake venom phosphodiesterase (SVPDE). This enzyme is one of the 3′-exonucleases, which are generally responsible for the degradation of natural nucleic acids.[37] As a reference, radiolabelled natural dA_{13} was also subjected to degradation by SVPDE. After 25 minutes of incubation, no more intact dA_{13} could be observed (Figure 11 A). In contrast, even after 24 h incubation of CCA^*_{13} under the same conditions, no degradation could be observed (Figure 11 B).

Conclusions

The synthesis of *trans*- and *cis*-4-HO-NAP-modified monomeric nucleosides and oligonucleotides is straightforward and gives high yields. Modified oligonucleotides incorporating *trans*-4-HO-L-NAP as sugar substitute display interesting hybridisation features with complementary sequences of natural nucleic acids. The modified A^*_{13} oligomer forms heteroduplexes with T_{13} as well as U_{13}. The stability of the duplexes equals that of the fully natural systems, and microcalorimetry records about the same enthalpy change upon duplex formation. The conformations too are comparable. The T^*_{13} oligomer forms a duplex only with complementary RNA. The stability of this duplex is low and, in contrast to natural duplexes as well as to the A^*_{13}/T_{13} or A^*_{13}/U_{13} systems, cannot be improved by the addition of Mg^{2+} ions. The conformation of the duplex is, however, still similar to the natural T_{13}/rA_{13} duplex. RNA selectivity has also been observed with 2′-5′-linked 3′-deoxyoligonucleotides,[1] of which the present oligomers can be con-

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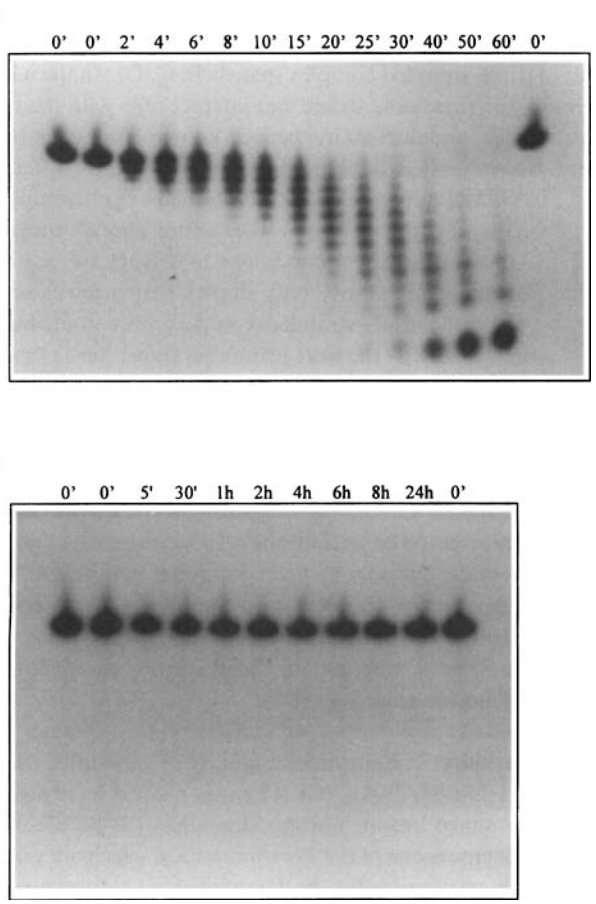

Figure 11. Autoradiograph of the enzymatic stability of natural dA_{13} (A) and the *trans*-4-HO-NAP-A_{13} oligonucleotides (B) against SVPDE, analysed on a 20% denaturing polyacrylamide gel. Oligonucleotides were incubated with SVPDE for the times indicated on top, as described in the experimental section.

sidered to be analogues. It is clear that the natural nucleic acids preferentially hybridise with L-hydroxyprolinol nucleic acids over the D-hydroxyprolinol oligomers. PolyU seems to be the most versatile acceptor of hydroxyprolinol oligonucleotides hybridising with *cis*-LA$^*_{13}$, *trans*-LA$^*_{13}$ and *trans*-DA$^*_{13}$. These interactions, however, are probably of a different nature, with formation of triple-stranded complexes as proven for DNA as the counterpart. When further characteristics important for antisense potential are investigated, the A$^*_{13}$-mer proves to be stable against SVPDE degradation. On the other hand it shows a diminished sequence-specific interaction as compared to natural DNA. However, a mixed, non-self-complementary A*,T* sequence accepts neither complementary DNA nor complementary RNA as hybridisation partner in either parallel or in antiparallel orientation. This seems to indicate a conformational incompatibility between the A*- and T*-nucleotides, although hybridisation by Hoogsteen or reverse-Hoogsteen pairing may also be involved. In this case the absence of hybridisation with a mixed A*T* sequence might be due to the instability of a duplex composed of a W–C or reverse W–C oriented T* and a Hoogsteen or reverse-Hoogsteen oriented A*.

All-adenine oligonucleotides built from *cis*-4-hydroxy-*N*-acetyl-L-prolinol, (2*S*,4*S*), hybridise with their RNA complement as well as their DNA complement. The stability of the complexes is low. Microcalorimetric titration proves the interaction with DNA as the complement to be triple-stranded in nature: a direct transition of the single-stranded A to a triple-stranded TAT complex is observed. By analogy, we believe the interactions with RNA as the complement to be of the same nature. *cis*-D-Compounds do not hybridise. Other pairing systems with low stability are the *trans*-DA*:polyU heteroduplexes and the homocomplexes *trans*-LA*:*trans*-LT* and *trans*-DA*:*trans*-DT*. The latter two interactions are again of a TAT-like nature with only a single transition. These are new examples of isochiral py–pu–py pairing systems as found in natural nucleic acids.

Experimental Section

Ultraviolet spectra were recorded with a Philips PU 8740 UV/Vis spectrophotometer. The ^{1}H NMR and ^{13}C spectra were recorded with a Varian Gemini 200 spectrometer, the ^{31}P spectra with a Varian Unity 500 spectrometer. Tetramethylsilane was used as internal standard for the ^{1}H NMR spectra (s = singlet, d = doublet, t = triplet, br = broad signal, m = multiplet), [D_6]DMSO (δ = 39.6), $CDCl_3$ (δ = 76.9) or CD_3OD (δ = 49.0) for the ^{13}C NMR spectra and 85% H_3PO_4 in H_2O for the ^{31}P NMR spectra. Mass spectrometry measurements were obtained using a Kratos Concept 1 H mass spectrometer. Elemental analyses were performed by Prof. Dr. W. Pfleiderer, Universität Konstanz (Germany). Anhydrous solvents were obtained as follows: DMF was stored on molecular sieves (3 Å) for 48 h prior to use. Pyridine and triethylamine were refluxed overnight on potassium hydroxide and distilled. Methanol was refluxed with magnesium/iodine overnight and distilled. Tetrahydrofuran (THF) was refluxed overnight on lithium aluminium hydride and distilled immediately prior to use. Dichloromethane was stored on calcium hydride, refluxed and distilled. *n*-Hexane and acetone, used in the purification of phosphoramidites, were purified by distillation. Precoated Machery–Nagel Alugram® silG/UV 254 plates were used for TLC and the products were visualised with UV light and sulfuric acid/anisaldehyde spray or $KMnO_4$ solution. Column chromatography was performed on Janssen Chimica silica gel (0.2–0.5 mm, pore size 4 nm), flash chromatography on Janssen Chimica silica gel (0.035–0.07 mm, pore size 6 nm).

9-(*tert*-Butyloxycarbonylmethyl)adenine: A 60% NaH oil dispersion (1.36 g, 34 mmol) was added to adenine (4 g, 29.6 mmol), suspended in DMF (60 mL). After stirring for 1 h at RT, *tert*-butyl bromoacetate (8.6 mL, 45 mmol) was added dropwise and the mixture was stirred further overnight. After agitation with water, a precipitate formed which was filtered off and washed with hexane to remove excess bromoacetate. The filtrate was evaporated and the residue was triturated with water, filtered off under suction and dried to give the product. An analytical sample was obtained by crystallisation from ethyl acetate. Total yield: 4.1 g (16.5 mmol, 55%). ^{1}H NMR ([D_6]DMSO): δ = 1.43 (s, 9H, 3 Me), 4.96 (s, 2H, CH_2), 7.27 (brs, 2H, NH_2), 8.11 (s, 1H, H-8), 8.15 (s, 1H, H-2); ^{13}C NMR ([D_6]DMSO): δ = 27.8 (3 Me), 44.6 (CMe_3), 82.1 (CH_2), 118.4 (C-5), 141.5 (C-8), 149.8 (C-4), 152.7 (C-2), 156.0 (C-6), 167.1 (CO); LSIMS (Thgly): *m/z* = 250 ([*M*+H]$^+$, 40), 194 ([*M*H − C_4H_8]$^+$, 100); HRMS $C_{11}H_{16}N_5O_2$: calcd 250.1304, found 250.1299; elem anal. $C_{11}H_{15}N_5O_2$ (249.3): calcd C 53.00, H 6.07, N 28.10; found C 52.95, H 6.05, N 27.85.

N^6-Benzoyl-9-(*tert*-butyloxycarbonylmethyl)adenine: 9-(*tert*-butyloxycarbonylmethyl)adenine (9.67 g, 38.8 mmol) was evaporated with pyridine and dissolved in anhydrous pyridine (200 mL), to which benzoyl chloride (9 mL, 77 mmol) was added. The reaction mixture was stirred overnight and was quenched by addition of methanol. Evaporation left an oil which was partitioned between dichloromethane and an aqueous saturated sodium bicarbonate solution. The organic layer was evaporated and the residue treated with a 2M ammonia/methanol mixture (1:1) from which the desired product precipitated. Further product was obtained upon chromatographic purification of the filtrate to yield a total of 9.17 g (26 mmol, 67%). ^{1}H NMR ([D_6]DMSO): δ = 1.44 (s, 9H, 3 Me), 5.11 (s, 2H, CH_2), 7.50–7.70 (m, 3H, aryl), 8.02–8.09 (m, 2H, aryl), 8.45 (s, 1H, H-8), 8.74 (s, 1H, H-2), 11.10 (brs, NH); ^{13}C NMR ([D_6]DMSO): δ = 27.7 (3 Me), 44.9 (CMe_3), 82.4 (CH_2), 125.0 (C-5), 128.5 (Co, c_m-aryl), 132.5 (c_p-aryl), 133.5 (c_x-aryl), 145.2

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(C-8), 150.2 (C-4), 151.7 (C-2), 152.7 (C-6), 165.6 (NHCO), 166.8 (COO); LSIMS (Thgly): $m/z = 354$ ($[M+H]^+$, 75), 298 ($[MH - C_4H_8]^+$, 100), 105 ($C_6H_5CO^+$, 80); HRMS $C_{18}H_{20}N_5O_3$: calcd 354.1566, found 354.1562; elem anal. $C_{18}H_{19}N_5O_3 \cdot 0.5H_2O$ (362.39): calcd C 59.66, H 5.56, N 19.33; found C 59.60, H 5.75, N 19.30.

N^6-Benzoyl-9-(carboxymethyl)adenine: The material obtained in the previous preparation (8.28 g, 23.4 mmol) was dissolved in dichloromethane (100 mL) to which trifluoroacetic acid (20 mL) was added. The mixture was stirred for 3 days, evaporated and coevaporated with toluene to obtain an oil from which the desired compound was crystallised from water. Yield: 5.56 g (18.7 mmol, 80%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 4.54$ (s, 2H, CH_2), 7.50–7.70 (m, 3H, *m*-, *p*-aryl), 8.0 8.1 (m, 2H, *o*-aryl), 8.33 (s, 1H, H-8), 8.65 (s, 1H, H-2), 11.05 (brs, 1H, NH); ^{13}C NMR ([D$_6$]DMSO): $\delta = 47.2$ (CH_2), 125.2 (C-5), 128.6, 132.3, 133.8 (aryl), 146.2 (C-8), 149.5 (C-4), 150.9 (C-2), 152.0 (C-6), 165.8 (amide), 168.2 (COOH); LSIMS (Thgly): $m/z = 298$ ($[M+H]^+$, 100), 105 ($C_6H_5Co^+$, 60); HRMS $C_{14}H_{12}N_5O_3$: calcd 298.0940, found 298.0933.

Condensation Reactions, Procedure 1 (1.1a, 1.2a and 1.3a):

4(*R*)-Hydroxy-2(*S*)-hydroxymethyl-*N*-[(thymin-1-yl)acetyl]pyrrolidine (1.1a): Thymin-1-yl acetic acid (2.513 g, 13.66 mmol) was preactivated for 5 min with benzotriazol-1-yl-*N*-tetramethyluronium hexafluorophosphate (HBTU, 6.2 g, 1.2 equiv) and 1-hydroxy-1 H-benzotriazole (HOBt, 2.2 g, 1.2 equiv) in the presence of *N*-methylmorpholine (2 equiv) in a mixture of DMF/pyridine. The suspension was added to a solution of *trans*-4-HO-L-prolinol (1.60 g, 13.66 mmol) in DMF. The mixture became a clear solution after a few minutes and was left to react at room temperature for 2 h. After addition of water, the solvent was evaporated and the mixture was purified by column chromatography (90/10 CH_2Cl_2/MeOH). Compound **1.1a** was obtained as a foam in 65% yield (2.53 g).

4(*R*)-Hydroxy-2(*R*)-hydroxymethyl-*N*-[(thymin-1-yl)acetyl]pyrrolidine (1.3a): By the same procedure as described above, the reaction of *cis*-4-HO-prolinol (0.75 g) with thymin-1-yl acetic acid (1.2 equiv) gave 1.16 g (64%) of **1.3a**.

Condensation Reactions, Procedure 2 (2.1a, 2.2a, 2.3a, 2.4a):

4(*R*)-Hydroxy-2(*S*)-hydroxymethyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]-pyrrolidine (2.1a): N^6-benzoyladenin-9-yl acetic acid (1.90 g, 6.4 mmol) was preactivated for 5 min with 1,3-dicyclohexylcarbodiimide (DCC, 1.60 g, 7.68 mmol) and HOBt (1.04 g, 7.68 mmol) in the presence of *N*-methylmorpholine (2 equiv) in DMF. This solution was added to *trans*-4-HO-L-prolinol (0.75 g, 6.4 mmol) in DMF and left to react at room temperature overnight. After addition of H_2O, evaporation of the solvent and purification by column chromatography (95/5 CH_2Cl_2/MeOH with 0.1% triethylamine), 0.99 g (40%) of **2.1a** was obtained as a foam.

4(*R*)-Hydroxy-2(*R*)-hydroxymethyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]-pyrrolidine (2.3a): Reaction of *cis*-D-prolinol (0.93 g) with N^6-benzoyladenin-9-yl acetic acid (1 equiv) as described for **2.1a** gave 2 g (64%) of **2.3a**. This product could not be purified at this stage to allow meaningful analysis.

Monomethoxytritylation Reactions (1.1b, 1.2b, 1.3b, 2.1b, 2.2b, 2.3b, 2.4b, 1.1d, 1.2d): Monomethoxytritylation was carried out in anhydrous pyridine and under heating (40–50 °C). Reactions were monitored by TLC and worked up when the amount of tritylated product seemed to have reached a maximum. The amount of *p*-anisylchlorodiphenylmethane used and the reaction times necessary ranged from 1.2 to 5 equiv and 1 night to 7 days. Reaction outcome also met with variable success, yields ranging from 50 to 90% (averaging 60%).

4(*R*)-Hydroxy-2(*S*)-monomethoxytrityloxymethyl-*N*-[(thymin-1-yl)acetyl]-pyrrolidine (1.1b): Starting from **1.1a** (0.342 g) with *p*-anisylchlorodiphenylmethane (3 equiv), 62% of **1.1b** was obtained.

4(*R*)-Hydroxy-2(*R*)-monomethoxytrityloxymethyl-*N*-[(thymin-1-yl)acetyl]-pyrrolidine (1.3b): Compound **1.3a** (0.30 g) with *p*-anisylchlorodiphenylmethane (4 equiv) gave 90% **1.3b**.

4(*R*)-Hydroxy-2(*S*)-monomethoxytrityloxymethyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]pyrrolidine (2.1b): Compound **2.1a** (0.69 g) with *p*-anisylchlorodiphenylmethane (2 equiv) gave 84% of **2.1b**.

4(*S*)-Monomethoxytrityloxy-2(*R*)-*tert*-butyldimethylsilyloxymethyl-*N*-[(thymin-1-yl)acetyl]pyrrolidine (1.2d): From **1.2c** (0.99 g) and *p*-anisylchlorodiphenylmethane (2.5 equiv), 85% of **1.2d** was obtained. According to TLC analysis, this product was identical to **1.1d**.

4(*R*)-Monomethoxytrityloxy-2(*S*)-(*tert*-butyldimethylsilyloxymethyl-*N*-[(thymin-1-yl)acetyl]pyrrolidine (1.1d): Compound **1.1c** (0.56 g) with *p*-anisylchlorodiphenylmethane (2 equiv) gave 75% of **1.1d**.

4(*R*)-Hydroxy-2(*R*)-monomethoxytrityloxymethyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]pyrrolidine (2.3b): Starting materials **2.3a** (0.77 g) and *p*-anisylchlorodiphenylmethane (2.5 equiv) yielded 64% of **2.3b**.

Silylation (1.1c, 1.2c, 1.3c):

4(*R*)-Hydroxy-2(*S*)-*tert*-butyldimethylsilyloxymethyl-*N*-[(thymin-1-yl)acetyl]-pyrrolidine (1.1c): Compound **1.1a** (0.67 g, 2.36 mmol) was silylated at the primary alcohol using *tert*-butyldimethylsilylchloride (0.426 g, 2.83 mmol, 1.2 equiv) and imidazole (1.3 equiv) in DMF. The reaction was allowed to proceed for 48 h. After evaporation and column chromatography 0.64 g (68%) of **1.1c** was obtained as a foam.

Compound 1.3c was analogously prepared from **1.3a** (1.51 g) and *tert*-butyldimethylsilylchloride (1.4 equiv), which gave 53% of **1.3c**.

Desilylation (1.1e, 1.2e):

2(*S*)-Hydroxymethyl-4(*R*)-monomethoxytrityloxy-*N*-[(thymin-1-yl)acetyl]-pyrrolidine (1.1e): Compound **1.1d** (700 mg, 1.46 mmol) in anhydrous THF was desilylated using TBAF (1 M solution in THF, 1.15 mL, 1.15 mmol). After 45 min the mixture was evaporated and purified by column chromatography (95/5 CH_2Cl_2/MeOH) providing 443 mg (76%) of **1.1e**.

Compound **1.2e** was analogously prepared in 90% yield from **1.2d** (1.35 g).

Mitsunobu Reactions (1.2b, 2.2b, 2.4b, 1.2c): The protected compound together with triphenylphosphine (1.6 equiv) and *p*-NO_2-benzoic acid (1.6 equiv) was suspended in THF and treated dropwise with a solution of diethylazodicarboxylate (DEAD, 1.6 equiv) diluted in THF. The reaction was left overnight and evaporated to yield an oil. The work-up to cleave the *p*-NO_2-benzoic acid ester used MeOH/NH_3 for thymine nucleosides and 0.6 N NaOH in EtOH/pyridine (5 min, RT) for adenine compounds to safeguard the benzoyl protecting group on N6. Evaporation, extraction and silica gel purification (98/2 CH_2Cl_2/MeOH → 95/5 CH_2Cl_2/MeOH) provided the required stereomers. This procedure was used for the conversion of **1.3b** (0.50 g) to **1.2b** (53%), **2.3b** (1.92 g) to **2.2b** (84%), **2.1b** (1.57 g) to **2.4b** (65%) and **1.3c** (1.13 g) to **1.2c** (85%). The latter compound was identical to **1.1c** according to TLC analysis.

Preparation of the Amidite Building Blocks (1.1, 1.1i, 1.2, 1.2i, 2.1, 2.2, 2.3, 2.4): About 1 mmol of the modified nucleoside, protected at either the primary or the secondary alcohol, was treated with dry *N*,*N*-diisopropylethylamine (3 equiv) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (1.5 equiv) in dry dichloromethane (2.5 mL) and stirred at room temperature for 30 min. The reaction was quenched by adding ethanol (3 mL) and stirred further for 15 min. The mixture was washed with 5% sodium bicarbonate solution (30 mL) and saturated NaCl solution (three times 30 mL), dried and evaporated. Column chromatography with *n*-hexane/acetone/triethylamine as eluent afforded the amidite. The product thus obtained was dissolved in dry dichloromethane (1 mL) and precipitated by dropwise addition to cold (−70 °C) *n*-hexane (100 mL). The product was isolated, washed with *n*-hexane, dried and used as such for DNA synthesis. Yields are listed in Table 8; R_f values, mass analysis and ^{31}P NMR data are given in Table 9.

Table 8. Yields obtained for the amidite building blocks.

	Yield (%)		Yield (%)
1.1	83	**2.1**	87
1.1i	94	**2.2**	97
1.2	92	**2.3**	97
1.2i	92	**2.4**	86

Preparation of the Solid Support: Long chain allylamine-controlled pore glass (LCAA-CPG) was derivatised with a 1,3-propanediol linker.[38, 39] A mixture of LCAA-CPG (5 g), 4-dimethylaminopyridine (DMAP, 0.5 equiv), Et_3N (2.88 equiv), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide·HCl (0.9 equiv), and DIC (4.1 equiv) in anhydrous pyridine (40 mL) with 1-*O*-dimethoxytritylated 1,3-propanediol succinate was first sonicated for 5 min and then shaken at room temperature for 24 hours. After shaking,

Table 9. Phosphoramidite analyses.

	TLC system [a]	R_f	MS	^{31}P NMR	
1.1	49/49/2	0.47	756 $(M+H)^-$	[b]	[b]
1.1i	49/49/2	0.45	756 $(M+H)^-$	[b]	[b]
1.2	49/49/2	0.44	754 $(M-H)^-$	149.79 (149.25) [c]	148.57 (148.60) [c]
1.2i	49/49/2	0.38	754 $(M-H)^-$	149.96 (150.36) [c]	149.49 (149.92) [c]
2.1	28/70/2	0.66	867 $(M-H)^-$	149.97 (149.72) [c]	148.69 (149.09) [c]
2.2	49/49/2	0.30	867 $(M-H)^-$	149.97 (149.72) [c]	148.69 (149.09) [c]
2.3	28/70/2	0.50	867$(M-H)^-$	150.09 (149.62) [c]	149.86 (149.51) [c]
2.4	28/70/2	0.50	867$(M-H)^-$	150.11 (149.64) [c]	149.87 (149.53) [c]

[a] The ratios given are those for the system *n*-hexane/acetone/triethylamine. [b] Not assessed. [c] The numbers in brackets are the minor peaks.

the CPG was filtered off and washed successively with pyridine, methanol and chloroform and then dried in vacuo. The unreacted sites were capped using 1-methylimidazole in THF (Applied Biosystems) and acetic anhydride/lutidine/THF 1:1:8 (Applied Biosystems). After shaking for 2 hours the CPG was filtered off again, washed with chloroform and dried in vacuo. Colorimetric dimethoxytrityl analysis indicated a loading of 64 $\mu mol\,g^{-1}$.

Oligonucleotide Synthesis: Oligonucleotide synthesis was carried out on an automated DNA synthesiser using the phosphoramidite approach, model ABI 381A (Applied Biosystems). Condensations were run at 0.13 M of the respective modified building block for 3 min to ensure adequate coupling yields. Intermediate deprotection of the monomethoxytrityl protecting group was achieved by prolonged acid treatment (90 s). The obtained sequences were deprotected and cleaved from the solid support by treatment with concentrated ammonia at 55 °C for 16 h. After a first purification on a NAP-10® column (Sephadex G 25-DNA grade), a Mono-Q® HR 10/10 anion exchange column (Pharmacia) was used with the following gradient systems: A: NaOH, pH 12.0 (10 mM), NaCl (0.1 M); B: NaOH, pH 12.0 (10 mM), NaCl (0.9 M). The gradient used depended on the oligonucleotide. Flow rate 2 $mL\,min^{-1}$. The low-pressure liquid chromatography system consisted of a Merck–Hitachi L6200A Intelligent pump, a Mono-Q HR 10/10 column, a Uvicord SII 2138 UV detector (Pharmacia–LKB) and a recorder. Product-containing fractions were immediately neutralised by addition of aqueous ammonium acetate. Following concentrations, the eluent was desalted on a NAP10 column and lyophilised.

UV Melting Experiments and Wavelength Scans: UV melting experiments and wavelength scans were recorded using a Uvikon 940 spectrophotometer. Samples were dissolved in a buffer solution containing 0.1 or 1 M NaCl, 0.02 M potassium phosphate, pH 7.5, 0.1 mM EDTA. The oligomer concentration was determined by measuring the absorbance at 80 °C and assuming extinction coefficients in the denatured state of 8500 for T, 15000 for A and 10000 for U. The concentration in all experiments was 4 μM of each strand. Cuvettes were kept at constant temperature with water circulating through the cuvette holder and with a thermistor immersed directly in the cuvette. For the melting experiments, temperature control and data acquisition were carried out automatically with an IBM/PC AT compatible computer. The samples were heated and cooled at a rate of 0.2 $°C\,min^{-1}$ with data sampling every 30 seconds. T_m values were determined from the maximum of the first derivative curve. If a substantial difference could be detected between heating and cooling melting curves, both recorded T_ms are given (first figure relates to heating experiment).

UV Mixing Curves: UV mixing curves were obtained by titration of a 6 μM solution of one strand with a 6 μM solution of its complement. Experiments were run in a buffer containing 1 M NaCl, 0.02 M potassium phosphate pH 7.5 and 0.1 μM EDTA at a temperature of 10 °C. Data were recorded with a Uvikon 940 spectrophotometer after stabilisation of the absorbance.

CD Spectra: CD spectra were recorded on a JASCO-600 spectropolarimeter in thermostatically controlled (5 °C) 1 cm cuvettes. Samples were dissolved in the 1 M NaCl buffer used for the UV melting experiments, which was also run as baseline.

Gel Mobility Shift Assays: Oligo(ribo)nucleotides were radiolabelled (^{32}P) at the 5′ end by means of T4 polynucleotide kinase (Gibco BRL) and [γ-^{32}P]ATP (4500 Ci $mmol^{-1}$, ICN) by standard procedures[40] and purified on a NAP-5 column (Pharmacia). The radiolabelled oligo(ribo)nucleotides were dissolved in a buffer containing KCl (140 mM), Na_2HPO_4 (5 mM), $MgCl_2$ (10 mM), spermine (1 mM) and glycerol (5%). Samples were mixed and heated at 80 °C for 3 min, slowly cooled, and stored at 10 °C for 4 h. The concentration of the ^{32}P-labelled oligo(ribo)nucleotide was held constant at 0.33 μM throughout. The concentration of the complementary unlabelled strand was 0.16, 0.33, 0.67, 1.67, 3.33 or 16.67 μM (0.5, 1, 2, 5, 10 and 50 equiv respectively). The samples were analysed on a 15% nondenaturing polyacrylamide gel (19:1 acrylamide:*N*,*N*′-methylenebisacrylamide). Electrophoresis was performed at 10 °C (Haake K 20 and DC 3 cooling unit at 2 °C) with TBM buffer (89 mM trisborate, 5 mM $MgCl_2$, pH 8) at 2 W over 3 h. The gels were visualised by autoradiography.

Enzymatic Degradation: Oligonucleotides were radiolabelled (^{32}P) at the 5′ end as described above and dissolved in H_2O. In a total reaction volume of 100 μL, radiolabelled oligonucleotide (50 pmol) was incubated for 5 min at 37 °C in a buffer containing Tris-HCl pH 8.6 (100 mM), NaCl (100 mM) and $MgCl_2$ (14 mM). Degradation was started by addition of 4×10^{-3} U of snake-venom phosphodiesterase (*Crotalus adamanteus* venom, Pharmacia). The mixture was further incubated at 37 °C. At appropriate time intervals 4 μL aliquots were withdrawn, mixed with an equal volume of stop mix (50 mM EDTA, 0.1% xylene cyanol FF and 0.1% bromophenol blue in 90% formamide) and chilled on ice. Samples were analysed by denaturing 20% PAGE (19:1 acrylamide:*N*,*N*′-methylenebisacrylamide) containing urea (8.3 M) with TBE buffer[40] at 400 V over 4.5 h, followed by autoradiography and scanning laser densitometry. Densitometry was performed with a DeskTop Densitometer (pdi, NY, USA) equipped with The Discovery Series® (Diversity One®) software.

Mass Spectrometric Analysis of Oligonucleotides: The appropriate oligonucleotide (5 nmol) was taken up in 0.2 M triethylammonium bicarbonate (TEAB), pH 8 (0.5 mL) and loaded on a C_{18} cartridge (Waters) preequilibrated with aqueous TEAB. After washing the cartridge with TEAB solution (2 mL), the oligomer was eluted with MeOH:TEAB 0.2 M 1:1 and the eluate was lyophilised. Immediately before use, the samples were dissolved in a mixture of acetonitrile:0.01 M ammonium acetate 1:1 (300 μL) (final concentration of the oligonucleotide about 16 $pmol\,\mu L^{-1}$). Electrospray ionisation mass spectra were recorded in negative mode on a VG Quattro II mass spectrometer (Micromass, Manchester, UK) equipped with a Mass Lynx data system. The sample spray flow was set to 10 $\mu L\,min^{-1}$. Five spectra were acquired and summed in MCA mode in the *m*/*z* range 500 to 1500. The molecular weights were determined by application of the maximum entropy algorithm.

Analytical Data

^{13}C NMR: The first number indicates the shift of the major signals, except for signals assigned to aromatics.

4(*R*)-Hydroxy-2(*S*)-hydroxymethyl-*N*-[(thymin-1-yl)acetyl]pyrrolidine (1.1a): ^{1}H NMR (D_2O): δ = 1.8 (s, 3H, CH_3), 2.0–2.2 (m, 2H, H-3), 3.5–3.9 (m, 5H, CH_2OH, H-5, H-2), 4.1–4.3 (br, 1H, H-4), 4.6 (d, 2H, CH_2), 7.3 (s, 1H, H-6 of thymine); ^{13}C NMR (D_2O): δ = 11.7 (CH_3), 35.2 and 36.6 (C-3), 50.6 and 50.5 (CH_2), 54.9 and 54.0 (C-5), 58.9 and 58.2 (C-2), 61.5 and 63.5 (CH_2OH), 69.9 and 68.8 (C-4), 111.5 (C-5 of thymine), 143.6 and 143.8 (C-6 of thymine), 152.8 (C-2 of thymine), 167.7 (C-4 of thymine), 168.0 (amide); UV (MeOH): λ_{max} (ε) = 268 nm (10100); LSIMS (Thgly): *m*/*z* = 284 $[M+H]^+$, 167 $[BCH_2CO]^+$, 118 [aminodiol + 2H]$^+$; HRMS $C_{12}H_{18}N_3O_5$: calcd 284.1246, found 284.1236; elem. anal. $C_{12}H_{17}N_3O_5$: calcd C 50.88, H 6.05, N 14.83; found C 50.62, H 5.82, N 14.57.

4(*S*)-Hydroxy-2(*R*)-hydroxymethyl-*N*-[(thymin-1-yl)acetyl]pyrrolidine (1.2a): ^{1}H NMR (D_2O): δ = 1.8 (s, 3H, CH_3), 2.0–2.2 (m, 2H, H-3), 3.4–3.9 (m, 5H, CH_2OH, H-5, H-2), 4.1–4.3 (br, 1H, H-4), 4.6 (d, 2H, CH_2), 7.3 (s, 1H, H-6 of thymine); ^{13}C NMR (D_2O): δ = 11.8 (CH_3), 35.2 and 36.6 (C-3), 50.6 (CH_2), 54.9 and 54.0 (C-5), 58.9 and 58.2 (C-2), 61.5 and 63.5 (CH_2OH), 69.9 and 68.8 (C-4), 111.4 (C-5 of thymine), 143.7 (C-6 of thymine), 153.0 (C-2 of thymine), 168.0 (C-4 of thymine), 168.0 (amide); UV (MeOH): λ_{max} (ε) = 269 nm (10300); LSIMS (Thgly): *m*/*z* = 284 $[M+H]^+$, 167 $[BCH_2CO]^+$, 118 [aminodiol + 2H]$^+$; HRMS (LSIMS) $C_{12}H_{18}N_3O_5$: calcd 284.1246, found 284.1254; elem. anal. $C_{12}H_{17}N_3O_7$: calcd C 50.88, H 6.05, N 14.83; found C 50.71, H 6.17, N 14.67.

4(*R*)-Hydroxy-2(*R*)-hydroxymethyl-*N*-[(thymin-1-yl)acetyl]pyrrolidine or *cis*-4-hydroxy-*N*-[(thymin-1-yl)acetyl]-D-prolinol (1.3a): ^{1}H NMR (CD_3OD):

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δ = 1.9 (s, 3H, CH_3), 2.2–2.4 (m, 2H, H-3), 3.2–4.0 (m, 5H, CH_2OH, H-5, H-2), 4.1–4.3 (br, 1H, H-4), 4.5 (s, 2H, CH_2), 7.3 (s, 1H, H-6 of thymine); ^{13}C NMR (CD_3OD): δ = 12.2 (CH_3), 36.9 and 38.6 (C-3), 50.3 (CH_2), 56.4 (C-5), 60.5 (C-2), 63.1 and 65.6 (CH_2OH), 71.0 and 69.3 (C-4), 111.0 (C-5 of thymine), 143.7 and 144.0 (C-6 of thymine), 153.1 (C-2 of thymine), 166.8 (C-4 of thymine), 168.2 (amide); UV (MeOH): λ_{max} (ε) = 268 nm (9600); LSIMS (Thgly): m/z = 284 $[M+H]^+$, 167 $[BCH_2CO]^+$, 118 [aminodiol + 2H]$^+$; HRMS $C_{12}H_{18}N_3O_5$: calcd 284.1246, found 284.1229; elem. anal. $C_{12}H_{17}N_3O_7$: calcd C 50.88, H 6.05, N 14.83; found C 50.53, H 6.21, N 14.66.

4(*R*)-Hydroxy-2(*S*)-monomethoxytrityloxymethyl-*N*-[(thymin-1-yl)acetyl]-pyrrolidine (1.1b): ^{13}C NMR (CD_3OD): δ = 11.4 (CH_3), 36.3 and 37.7 (C-3), 49.4 (CH_2), 54.3 and 54.1 (C-5), 54.8 (CH_3O), 57.3 and 56.9 (C-2), 36.2 and 65.8 (CH_2OH), 69.9 and 68.7 (C-4), 110.9 (C-5 of thymine), 86.7, 113.0, 127.7–130.6, 135.7, 144.9, 159.2 (MMTr), 142.7 (C-6 of thymine), 152.1 (C-2 of thymine), 166.2 (C-4 of thymine), 166.7 (amide); UV (MeOH): λ_{max} (ε) = 231 nm (16700), 270 nm (11600); LSIMS (Thgly, NaOAc): m/z = 578 $[M+Na]^+$, 600 $[M+2Na]^+$, 273 $[MMTr]^+$; HRMS $C_{32}H_{33}N_3O_6Na$: calcd 578.2267, found 578.2281; elem. anal. $C_{32}H_{34}N_3O_6 \cdot 0.3H_2O$: calcd C 68.51, H 6.04, N 7.49; found C 68.41, H 6.01, N 7.51.

4(*S*)-Hydroxy-2(*R*)-monomethoxytrityloxymethyl-*N*-[(thymin-1-yl)acetyl]-pyrrolidine (1.2b): ^{13}C NMR ($CDCl_3$): δ = 12.3 (CH_3), 36.0 and 37.8 (C-3), 48.9 and 49.1 (CH_2), 53.7 and 55.2 (C-5), 55.2 (CH_3O), 56.5 and 55.8 (C-2), 62.9 and 65.6 (CH_2OH), 7.03 and 68.9 (C-4), 110.6 (C-5 of thymine), 86.1, 87.6, 113.1, 126.8–130.5, 135.5, 144.5, 158.8 (MMTr), 141.3 (C-6 of thymine), 151.6 (C-2 of thymine), 164.5 (C-4 of thymine), 165.3 and 166.2 (amide); UV (MeOH): λ_{max} (ε) = 231 nm (17300), 269 nm (11700); LSIMS (Thgly, NaOAc): m/z = 578 $[M+Na]^+$, 600 $[M+2Na]^+$, 273 $[MMTr]^+$; HRMS $C_{32}H_{33}N_3O_6Na$: calcd 578.2267, found 578.2247; elem. anal. $C_{32}H_{34}N_3O_6 \cdot 0.5H_2O$: calcd C 68.07, H 6.07, N 7.44; found C 68.40, H 6.13, N 7.40.

4(*R*)-Hydroxy-2(*R*)-monomethoxytrityloxymethyl-*N*-[(thymin-1-yl)acetyl]-pyrrolidine (1.3b): ^{13}C NMR ($CDCl_3$): δ = 12.1 (CH_3), 36.5 and 38.1 (C-3), 48.7 and 47.5 (CH_2), 55.2 and 55.6 (C-5), 56.6 (CH_3O), 57.5 and 57.1 (C-2), 63.6 (CH_2OH), 70.6 and 68.8 (C-4), 110.5 (C-5 of thymine), 87.0, 113.3, 127.3–130.5, 134.4 (MMTr), 140.9 (C-6 of thymine), 150.6 (C-2 of thymine), 165.4 (C-4 of thymine), 165.4 (amide); UV (MeOH): λ_{max} (ε) = 231 nm (16800), 269 nm (11600); LSIMS (Thgly, NaOAc): m/z = 578 $[M+Na]^+$, 600 $[M+2Na]^+$, 273 $[MMTr]^+$; HRMS $C_{32}H_{33}N_3O_6Na$: calcd 578.2267, found 578.2292; elem. anal. $C_{32}H_{34}N_3O_6 \cdot 0.3H_2O$: calcd C 68.51, H 6.04, N 7.49; found C 68.57, H 6.07, N 7.43.

4(*R*)-Hydroxy-2(*R*)-*tert*-butyldimethylsilyloxymethyl-*N*-[(thymin-1-yl)acetyl]-pyrrolidine (1.3c): ^{13}C NMR ($CDCl_3$): δ = 12.4 (CH_3), 18.3 (C-Si), 25.8 (CH_3-Si), 36.9 (C-3), 48.7 (CH_2), 57.1 (C-5), 59.1 (C-2), 63.2 (CH_2OH), 70.0 (C-4), 110.6 (C-5 of thymine), 140.8 (C-6 of thymine), 151.0 (C-2 of thymine), 164.2 (C-4 of thymine), 165.6 (amide); elem. anal. $C_{18}H_{31}N_3O_5Si$: calcd C 54.38, H 7.86, N 10.57; found C 54.12, H 7.94, N 10.44.

4(*R*)-Hydroxy-2(*S*)-*tert*-butyldimethylsilyloxymethyl-*N*-[(thymin-1-yl)acetyl]-pyrrolidine (1.1c) and **4(*S*)-hydroxy-2(*R*)-*tert*-butyldimethylsiloxymethyl-*N*-[(thymin-1-yl)acetyl]pyrrolidine (1.2c)**: ^{13}C NMR ($CDCl_3$): δ = 12.4 (CH_3), 18.1 (C-Si), 25.8 (CH_3-Si), 35.6 (C-3), 49.0 (CH_2), 55.6 (C-5), 58.2 (C-2), 62.2 (CH_2OH), 70.3 (C-4), 110.8 (C-5 of thymine), 141.2 (C-6 of thymine), 151.5 (C-2 of thymine), 164.8 (C-4 of thymine), 165.2 (amide); UV (MeOH): λ_{max} (ε) = 270 nm (9700); LSIMS (Thgly): m/z = 398 $[M+H]^+$, 167 $[BCH_2CO]^+$, 232 [aminodiol + 2H]$^+$; HRMS $C_{18}H_{32}N_3O_5Si$: calcd 398.2111, found 398.2121; elem. anal. $C_{18}H_{31}N_3O_5Si$: calcd C 54.38, H 7.86, N 10.57; found C 54.20, H 7.77, N 10.25. ^{13}C NMR spectra are the same as for **1.1c**, elem. anal. $C_{18}H_{31}N_3O_5Si$: found C 54.32, H 7.95, N 10.21.

4(*R*)-Monomethoxytrityloxy-2(*S*)-(*tert*-butyldimethylsilyloxymethyl-*N*-[(thymin-1-yl)acetyl]pyrrolidine (1.1d) and **4(*S*)-monomethoxytrityloxy-2(*R*)-*tert*-butyldimethylsilyloxymethyl-*N*-[(thymin-1-yl)acetyl]pyrrolidine (1.2d)**: ^{13}C NMR ($CDCl_3$): δ = 12.2 (CH_3), 17.8 and 25.6 (C-Si and CH_3-Si); 34.7 (C-3), 47.7 and 47.8 (CH_2), 51.8 (C-5), 55.2 (OCH_3), 57.9 and 57.7 (C-2), 63.0 and 65.6 (CH_2OH), 72.5 and 71.0 (C-4), 110.2 (C-5 of thymine), 96.9, 113.2, 127.0–130.0, 136.0, 144.7, 158.7 (MMTr), 140.8 (C-6 of thymine), 150.9 (C-2 of thymine), 164.2 (C-4 of thymine), 164.3 (amide); UV (MeOH): λ_{max} (ε) = 231 nm (17000), 270 nm (12200); LSIMS (Thgly, NaOAc): m/z = 692 $[M+Na]+$, 273 $[MMTr]^+$; HRMS $C_{38}H_{47}N_3O_6NaSi$: calcd 692.3131, found 692.3156; elem. anal. $C_{38}H_{48}N_3O_6Si$: calcd C 68.13, H 7.07, N 6.27; found C 68.29, H 7.08, N 6.51. ^{13}C NMR data for **1.2d** are the same as for **1.1d**. Elem. anal. $C_{38}H_{48}N_3O_6Si$: found C 68.10, H 5.93, N 6.42.

2(*S*)-Hydroxymethyl-4(*R*)-monomethoxytrityloxy-*N*-[(thymin-1-yl)acetyl]-pyrrolidine (1.1e) and **2(*R*)-hydroxymethyl-4(*S*)-monomethoxytrityloxy-*N*-[(thymin-1-yl)acetyl]pyrrolidine (1.2e)**: ^{13}C NMR ($CDCl_3$): δ = 12.2 (CH_3), 35.0 (C-3), 48.7 (CH_2), 52.6 (C-5), 55.2 (OCH_3), 60.0 (C-2), 64.1 (CH_2OH), 72.0 (C-4), 110.6 (C-5 of thymine), 87.1, 113.4, 127.2–130.0, 135.8, 144.5, 158.8 (MMTr), 140.8 (C-6 of thymine), 151.2 (C-2 of thymine), 164.2 (C-4 of thymine), 165.9 (amide); UV (MeOH): λ_{max} (ε) = 231 nm (17500); 268 nm (12200); LSIMS (Thgly, NaOAc): m/z = 578 $[M+Na]^+$, 600 $[M+2Na]^+$, 273 $[MMTr]^+$, HRMS $C_{32}H_{33}N_3O_6Na$: calcd 578.2267, found 578.2283; elem. anal. $C_{32}H_{34}N_3O_6 \cdot 0.5H_2O$): calcd C 68.07, H 6.07, N 7.44; found C 68.27, H 6.01, N 7.69. ^{13}C NMR and LSIMS data for **1.2e** are the same as for **1.1e**. UV (MeOH): λ_{max} (ε) = 231 nm (17700), 268 nm (12600); HRMS $C_{32}H_{33}N_3O_6Na$: calcd 578.2267, found 578.2263; elem. anal. $C_{32}H_{34}N_3O_6.0.5H_2O$): found C 67.93, H 6.12, N 7.66.

4(*R*)-Hydroxy-2(*S*)-hydroxymethyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]-pyrrolidine (2.1a) and **4(*S*)-hydroxy-2(*S*)-hydroxymethyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]pyrrolidine (2.2a)**: ^{1}H NMR ([D$_6$]DMSO): δ = 1.8–2.1 (m, 2H, H-3), 3.3–3.8 (m, 4H, CH_2OH, H-5), 4.0–4.1 (br, 4H, H-2), 4.2–4.5 (m, 1H, H-4), 5.2–5.3 (d, 2H, CH_2), 7.5–7.7 (m, 3H, aryl), 8.0–8.2 (d, 2H, aryl), 8.4 (s, 1H, H-8), 8.7 (s, 1H, H-2 of adenine); ^{13}C NMR ([D$_6$]DMSO): δ = 35.9 and 37.4 (C-3), 45.2 and 44.7 (CH_2), 54.3 and 53.8 (C-5), 54.3 and 55.0 (CH_3O), 58.5 and 57.7 (C-2), 61.2 and 63.7 (CH_2OH), 68.5 and 67.4 (C-4), 125.0 (C-5 of adenine), 132.6 and 133.7 (benzoyl), 145.8 (C-8), 150.0 (C-4 of adenine), 151.4 (C-2 of adenine), 152.9 (C-6 of adenine), 164.2 (Co-bz), 164.9 (amide); UV (MeOH): λ_{max} (ε) = 281 nm (22200); LSIMS (Thgly): m/z = 397 $[M+H]^+$, 240 $[BCH_2CO]^+$; HRMS $C_{19}H_{21}N_6O_4$: calcd 397.1624, found 397.1622; elem. anal. $C_{19}H_{20}N_6O_4$: calcd C 57.57, H 5.09, N 21.20; found C 57.51, H 5.02, N 21.03. ^{1}H NMR and ^{13}C NMR data for **2.2a** are the same as for **2.1a**. Elem. anal. $C_{19}H_{20}N_6O_4$: found C 57.75, H 4.85, N 20.96.

4(*R*)-Hydroxy-2(*R*)-hydroxymethyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]-pyrrolidine (2.3a) and **4(*S*)-hydroxy-2(*S*)-hydroxymethyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]pyrrolidine (2.4a)**: As both compounds proved very difficult to purify at this stage into an analytical pure form, they were directly monomethoxytritylated and identified as the respective derivatised compounds (**2.3b** and **2.4b**).

4(*R*)-Hydroxy-2(*S*)-monomethoxytrityloxymethyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]pyrrolidine (2.1b) and **4(*S*)-hydroxy-2(*R*)-monomethoxytrityloxymethyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]pyrrolidine (2.2b)**:

For **2.1b**: ^{13}C NMR ($CDCl_3$): δ = 36.1 and 37.9 (C-3), 44.9 and 44.6 (CH_2), 55.2 and 54.1 (C-5), 55.2 (OCH_3), 56.7 and 56.2 (C-2), 62.8 and 65.7 (CH_2OH), 70.2 and 68.7 (C-4), 86.2, 87.6, 113.0, 113.2, 126.9–130.3, 135.3, 144.3, 158.4 (MMTr), 121.9 and 121.8 (C-5 of adenine), 128.5, 132.5, 133.6 (benzoyl), 144.1 and 143.7 (C-8 of adenine), 149.3 (C-4 of adenine), 152.4 and 151.1 (C-2 of adenine), 152.4 (C-6 of adenine), 165.0 and 165.3 (CO), 165.2 and 165.7 (amide); UV (MeOH): λ_{max} (ε) = 232 nm (28000), 282 nm (22200); LSIMS (Thgly, NaOAc): m/z = 691 $[M+H]^+$, 105 [benzoyl]$^+$, 273 $[MMTr]^+$; HRMS $C_{39}H_{36}N_6O_5Na$: calcd 691.2645, found 691.2662; elem. anal. $C_{39}H_{37}N_6O_5.H_2O$: calcd C 68.21, H 5.58, N 12.24; found C 68.07, H 5.48, N 12.20.

For **2.2b**: ^{13}C NMR ($CDCl_3$): δ = 36.2 and 38.0 (C-3), 44.9 and 44.6 (CH_2), 54.1 and 55.2 (C-5), 55.2 (OCH_3), 56.7 and 56.5 (C-2), 62.8 and 65.8 (CH_2OH), 70.3 and 68.8 (C-4), 86.2, 113.0, 113.2, 126.9–130.3, 135.4, 144.1, 158.5 (MMTr), 122.1 (C-5 of adenine), 132.7 and 133.7 (benzoyl), 144.2 and 144.3 (C-8 of adenine), 149.3 (C-4 of adenine), 152.5 (C-2 of adenine), 151.9 (C-6 of thymine), 164.1 (CO), 165.2 and 164.8 (amide); UV (MeOH): λ_{max} (ε) = 232 nm (28600), 282 nm (22200); LSIMS (as **2.1b**); HRMS $C_{39}H_{36}N_6O_5Na$: calcd 691.2645, found 691.2639; elem. anal. $C_{39}H_{37}N_6O_5 \cdot 1.5H_2O$: calcd C 67.32, H 5.65, N 12.08; found C 67.35, H 5.31, N 12.03.

4(*R*)-Hydroxy-2(*R*)-monomethoxytrityloxymethyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]pyrrolidine (2.3b) and **4(*S*)-hydroxy-2(*S*)-monomethoxytrityloxy-**

methyl-*N*-[(N^6-benzoyladenin-9-yl)acetyl]pyrrolidine (2.4b):

For **2.3b:** ^{13}C NMR ($CDCl_3$): δ = 36.5 and 38.0 (C-3), 44.9 and 44.7 (CH_2), 56.7 and 55.6 (C-5), 55.2 (OCH_3), 57.7 and 57.5 (C-2), 63.4 and 66.2 (CH_2OH), 70.4 and 68.4 (C-4), 88.2, 113.2, 127.2–130.4, 134.5, 144.2, 158.5 (MMTr), 122.1 (C-5 of adenine), 132.7 and 133.6 (benzoyl), 143.6 and 143.2 (C-8 of adenine), 149.4 (C-4 of adenine), 152.6 and 152.5 (C-2 of adenine), 152.0 (C-6 of adenine), 164.5 (CO), 164.5 (amide); UV (MeOH): λ_{max} (ε) = 232 (27900), 281 nm (22300); LSIMS (Thgly, NaOAc): m/z = 691 $[M+H]^+$, 105 [benzoyl]$^+$, 273 [MMTr]$^+$; HRMS $C_{39}H_{36}N_6O_5Na$: calcd 691.2645, found 691.2660; elem. anal. $C_{39}H_{37}N_6O_5$: calcd C 70.05, H 5.43, N 12.57; found C 69.96, H 5.62, N 12.67.

For **2.4b:** ^{13}C NMR ($CDCl_3$) spectra are exactly the same as for **2.3b** except for signals at δ = 55.2 and 55.3 (C-5), 56.7 (OCH_3), 144.0 and 143.6 (C-8 of adenine), 149.4 and 149.3 (C-4 of adenine); UV (MeOH): λ_{max} (ε) = 233 nm (27900), 282 nm (22500); LSIMS: data are the same as for **2.3b**; HRMS $C_{39}H_{36}N_6O_5Na$: calcd 691.2645, found 691.2660; elem. anal. $C_{39}H_{37}N_6O_5 \cdot 0.7\ H_2O$: calcd C 68.75, H 5.53, N 12.33; found C 68.69, H 5.49, N 12.34.

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