Synthesis and Pairing Properties of Oligonucleotides Containing 3-Hydroxy-4-hydroxymethyl-1-cyclohexanyl Nucleosides

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Abstract: The enantiomeric forms of cyclohexanyl adenine and thymine nucleosides were obtained by separation of their diastereomeric esters with (*R*)-(−)-methylmandelic acid. The four nucleoside analogues were appropriately protected, converted to their phosphoramidites and oligomerized. The resulting cyclohexanyl nucleic acids (CNAs) represent a new enantioselective Watson–Crick base-pairing system. Homochiral oligomers of equivalent chirality show Watson–Crick pairing, while those of opposite chirality (D-CNA and L-CNA) do not. No isochiral or heterochiral adenine–adenine or thymine–thymine base pairing is observed. Complex formation occurs only between oligomers in antiparallel orientations. D-CNA hybridizes with natural nucleic acids, and the strength of the interaction decreases in the order dsCNA > CNA:RNA > CNA:DNA. Thus, the D-cyclohexanyl nucleic acids are RNA-selective. L-CNA hybridizes either very weakly or not at all with natural nucleic acids, and the nature of this association is not clear. This study of CNAs leads us to hypothesize that a) the conformation of a single nucleoside analogue may be different from its conformation in an oligonucleotide and b) the conformational stress of a nucleotide analogue incorporated in an oligomer may contribute to the sequence-dependent thermal stability of oligonucleotide complexes.

Keywords: chirality · conformation analysis · DNA recognition · oligonucleotides · RNA

Introduction

Carbocyclic compounds are obtained by replacing the ring oxygen atom of a furanose or pyranose sugar with a methylene group. Compared with their carbohydrate congeners, these compounds have, generally, different physicochemical and biological properties. Recently, we have synthesized a series of 1,5-anhydro-2,3-dideoxy-D-*arabino*-hexitol nucleosides[1, 2] and their carbocyclic analogues.[3] At the monomeric level the anhydrohexitol nucleosides adopt a C1 conformation. Mainly owing to steric effects, this conformation is flipped when the ring oxygen atom is replaced by a methylene group.

Oligonucleotides constructed of anhydrohexitol building blocks (HNAs) hybridize strongly and sequence-selectively with RNA. Oligonucleotides with a phosphorylated cyclohexanyl backbone structure were synthesized in order to study the conformation and hybridization properties of oligomers containing 1,4-substituted six-membered carbohydrate mimics with geometries different from those of HNAs. We describe here the synthesis, studies of the hybridization, and conformational analysis of such oligonucleotides. One of the conclusions of this research is that the conformation of a nucleoside analogue when it is a monomer may be considerably different from that when it is incorporated in an oligomer.

Results and Discussion

Separation of enantiomers of cyclohexanyl nucleosides: The synthesis of racemic 3-hydroxy-4-hydroxymethyl-1-cyclohexanyl nucleosides has recently been described.[3] To continue our studies on the modified oligonucleotides, we needed to resolve the enantiomers of the thymine and adenine derivatives. As starting material, we used compounds **1** and **2**

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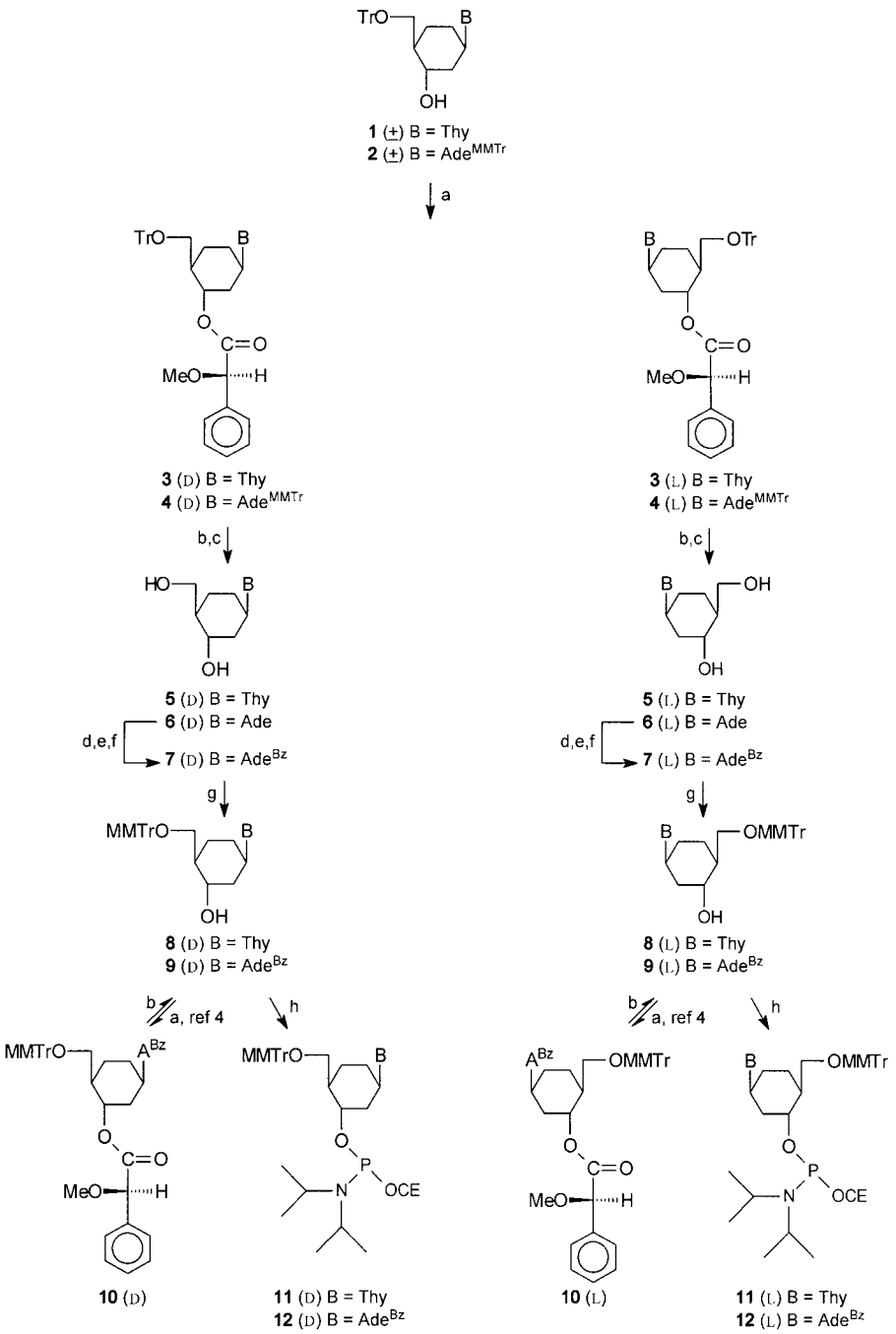
(bearing a free 3′-hydroxyl group), previously obtained as intermediates (Scheme 1).[3]

Acylation of **1** with (*R*)-(−)-methylmandelic acid[4] in the presence of DCC and DMAP, followed by separation by flash chromatography, gave the individual diastereoisomers **3(D)** and **3(L)**. The diastereomeric purity was 99% for both compounds (determined by HPLC, Figure 1). The diastereomeric purity of **3(D)** and **3(L)** was confirmed by their ^{1}H NMR spectra. The signals of H-6 and H-1′ in **3(D)** are shifted downfield 0.25 and 0.35 ppm, respectively, compared to those of **3(L)** (data not shown). Deacylation of **3(D)** with KOH/MeOH,[4] followed by treatment with 80% acetic acid to remove the trityl group, gave **5(D)**. In the same way, **5(L)** was synthesized from **3(L)**; the UV and NMR spectra of enantiomers **5(D)** and **5(L)** coincide with those reported for the racemic mixture.[3] The primary 4′-CH_2OH groups of both **5(D)** and **5(L)** were protected by monomethoxytrityl (MMTr) groups to give **8(D)** and **8(L)**, the building blocks for oligonucleotide synthesis.



Scheme 1. Synthesis of the building blocks for oligonucleotide synthesis and of the diastereomeric intermediates for the separation of the enantiomers of cyclohexanyl nucleosides. a) (*R*)-(−)-Methylmandelic acid/DCC/DMAP/CH_2Cl_2 followed by chromatographic separation; b) 0.1M KOH/MeOH; c) 80% HOAc; d) trimethylsilyl chloride (TMSCl), pyr; e) BzCl; f) 2M NH_4OH; g) MMTrCl, pyr; h) $(iPr)_2NEt$, $(iPr)_2NP(Cl)OCE$, CH_2Cl_2.

Abstract in Dutch: *De enantiomere vormen van cyclohexanyl-adenine- en -thyminenucleosiden werden verkregen door scheiding van hun diastereomere esters met (R)-(−)-methylmandelzuur. Na het invoeren van beschermgroepen werden de vier nucleoside-analogen omgezet tot hun fosforamidieten en ingebouwd in oligomeren. Cyclohexanylnucleïnezuur (CNA) vertegenwoordigt een nieuw enantioselectief Watson–Crick baseparingsysteem. Homochirale oligomeren van dezelfde chiraliteit vertonen Watson–Crick-paring, oligomeren van tegengestelde chiraliteit (*D*-CNA en *L*-CNA) niet. Er werd geen isochirale of heterochirale adenine–adenine of thymine–thymine baseparing geobserveerd. Complexvorming gebeurt in antiparallelle richting en niet parallel. D-CNA hybridizeert met natuurlijke nucleïnezuren waarbij de sterkte van de interactie daalt in de volgorde dsCNA > CNA:RNA > CNA:DNA. De D-cyclohexanylnucleïnezuren zijn RNA-selectief. L-CNA hybridizeert ofwel zeer zwak, ofwel niet met natuurlijke nucleïnezuren en de aard van deze associatie is niet duidelijk. De studie van CNA geeft aanleiding tot de hypothese dat a) de conformatie van een nucleoside-analoog verschillend kan zijn als enkelvoudig molecuul en na incorporatie in een oligonucleotide en b) conformationale spanning van een nucleotide-analoog, ingebouwd in een oligomeer, zou kunnen bijdragen tot de sequentie-afhankelijke thermische stabiliteit van oligonucleotidecomplexen.*

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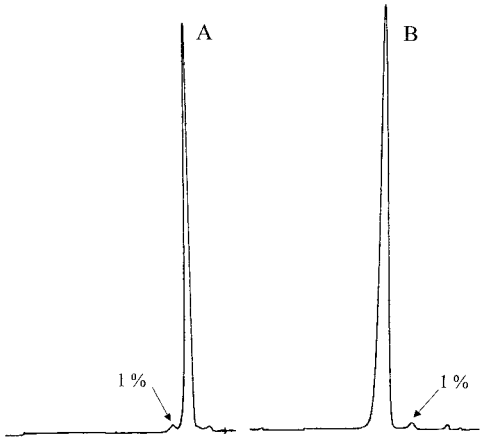


Figure 1. Diastereomeric purity of compounds **3(D)** (A) and **3(L)** (B) as determined by HPLC.

The adenine derivatives were resolved by treatment of **2** with (*R*)-(−)-methylmandelic acid in the presence of DCC and DMAP. Attempted separation of the diastereoisomers **4(D)** and **4(L)** by column chromatography was only partially successful, and a large fraction of the material remained unresolved. Likewise, HPLC was not very useful for determination of the diastereomeric purity of **4(D)** and **4(L)** because of tailing. The apparently pure fractions of **4(D)** and **4(L)** were deprotected in the same manner as the thymine derivatives to give the adenine nucleosides **6(D)** and **6(L)**. Transient protection[5] was used for **6(D)** and **6(L)** to introduce N^6-benzoyl groups, and subsequent treatment of **7(D)** and **7(L)** with monomethoxytrityl chloride gave the protected nucleosides **9(D)** and **9(L)**. Determination of the optical rotations of **9(D)** ($[\alpha]_D^{20} = +4.4$) and **9(L)** ($[\alpha]_D^{20} = -2.05$), however, showed the poor degree of separation for the adenine derivatives. Therefore, compounds **9(D)** and **9(L)** were acylated once more with (*R*)-(−)-methylmandelic acid in the presence of DCC and DMAP to give **10(D)** and **10(L)**. TLC analysis of these products showed contamination of both diastereoisomers with their congener but, because the compounds suffer from substantial tailing on silica gel chromatography, no estimate could be made of the diastereomeric excess. Products **10(D)** and **10(L)** were purified by repeated chromatotron chromatography (preparative, centrifugally accelerated, radial, thin-layer chromatography). Final confirmation of the enantiomeric purity was given by the chemical shift values of H-1′ and C-1′ in ^{1}H NMR and ^{13}C NMR spectra. In neither case could traces of the related diastereomer be detected (Figure 2). Compounds **10(D)** and **10(L)** were deprotected by selective ester hydrolysis[6] to recover enantiomers **9(D)** and **9(L)**. These compounds were also purified using chromatotron chromatography (gradient 97:3 to 95:5 of CH_2Cl_2/MeOH).

The absolute configuration of the cyclohexanyl nucleoside with an adenine base moiety was proven by chemical methods. Previously, we had obtained a cyclohexenyl adenine nucleoside by a stereospecific route starting from (*R*)-(−)-carvone (Scheme 2).[7] By hydrogenation of the double bond,[7] a compound is obtained which is identical to the compound **6(D)**. Figure 3 shows the separation of the racemic mixture of cA (a 50:50 mixture of the cyclohexanyl adenine

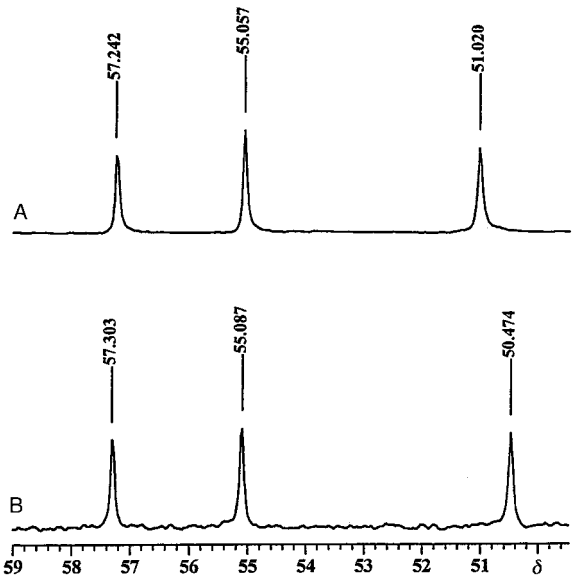
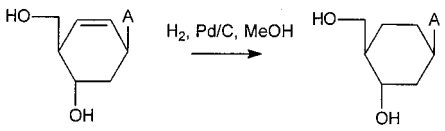


Figure 2. Confirmation of the enantiomeric purity by ^{13}C NMR. A: **10(D)**: C-1 $\delta = 51.02$; B: Compound **10(L)**: C-1 $\delta = 50.47$.



Scheme 2. Synthesis of D(A*) as described in ref. [7] and used as reference to determine the absolute configuration of the separated enantiomers D(A*) and L(A*).

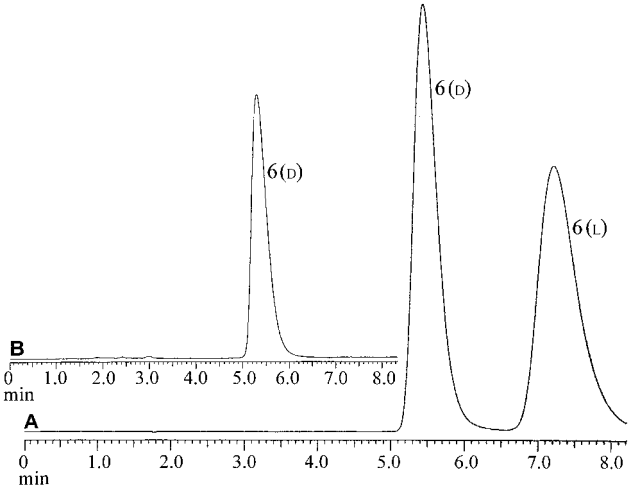


Figure 3. SFC analysis of A) the racemic mixture of cA [a 50:50 mixture of **6(D)** and **6(L)**] and B) the **6(D)** enantiomer (see Experimental Section for SFC conditions).

nucleosides **6(D)** and **6(L)**) by chiral chromatography[8] (Figure 3A), together with the identification of the fast-eluting enantiomer as the **6(D)** analogue (Figure 3B), which was obtained by stereospecific total synthesis.[7] Although not strictly correct, the sugar nomenclature D and L is used in the context of the cyclohexanyl nucleosides for clarity, so that comparison with the configuration of the anhydrohexitol nucleosides can easily be made. The cyclohexanyl thymine nucleoside has not yet been prepared by the same stereospecific strategy; this would unequivocally establish the

configuration. We therefore postulated similar chromatographic and physicochemical behaviour of monomers and oligomers of DA* and DT* to those of LA* and LT* respectively.

Hybridization properties of cyclohexanyl nucleic acids (carbocyclic pyranosyl nucleosides or CNAs): With the phosphoramidites of the building blocks **8(D)**, **8(L)**, **9(D)** and **9(L)**, several modified oligonucleotides were synthesized. All oligomers were homochiral. All-adenine, all-thymine and mixed adenine/thymine oligonucleotides were synthesized. For a preliminary insight into the pairing properties of the newly obtained oligomers, UV melting curves at 260 nm were recorded. Hybridization was studied between oligonucleotides of the same sense of chirality and between those of opposite chirality. Both self-complementary CNA complexes and complexes between CNAs and natural nucleic acids (DNA and RNA) were investigated. The samples were dissolved in a neutral buffer solution containing 0.1M NaCl, and melting curves were recorded at a heating rate of $0.2\,^{\circ}C\,min^{-1}$. To exclude the possibility of false interpretation of data due to possible self-complementarity of CNA oligomers (T–T or A–A base-pair formation), the shape of the melting curves of single-stranded oligomers was also investigated. We have previously observed the formation of thymine–thymine base pairs in the HNA series,[9] while adenine–adenine pairing has been observed for homo-DNA.[10] However, neither adenine–adenine nor thymine–thymine pairing could be observed in the CNA series, either between oligonucleotides of equivalent chirality [$D(A^*)_{13}$; $L(A^*)_{13}$; $D(T^*)_{13}$; $L(T^*)_{13}$] or between compositions of opposite chirality [$D(A^*)_{13}$ vs. $L(A^*)_{13}$]. The melting curves showed only a linear and limited increase of UV absorption as a function of temperature.

The CNAs form a homochiral, self-complementary system. Complexes between $D(A^*)_{13}$ and $D(T^*)_{13}$ (Figure 4) and between $L(A^*)_{13}$ and $L(T^*)_{13}$ gave exactly the same melting profile and were significantly more stable than the corresponding natural associations (Table 1).[11] The melting curves (upon heating) consistently showed biphasic profiles indicating multiple conformational complexes (i.e., triplex formation) for both enantiomers. The down curves of the melting profiles were broad, suggesting different association and dissociation kinetics. Surprisingly, weak quasiracemate pairing was also observed for $D(A^*)_{13}$:$L(T^*)_{13}$ (Figure 4) and for $L(A^*)_{13}$:$D(T^*)_{13}$, which has seldom been observed before.[12, 13] The stability of the association was, however, significantly less than the stability of homochiral systems and the down curve especially was rather broad. Moreover, the up melting curve revealed a clearly different T_m from the down melting curve (25.5 °C and 17.5 °C, respectively). Owing to these complexities, the nature of these associations has not been further investigated.

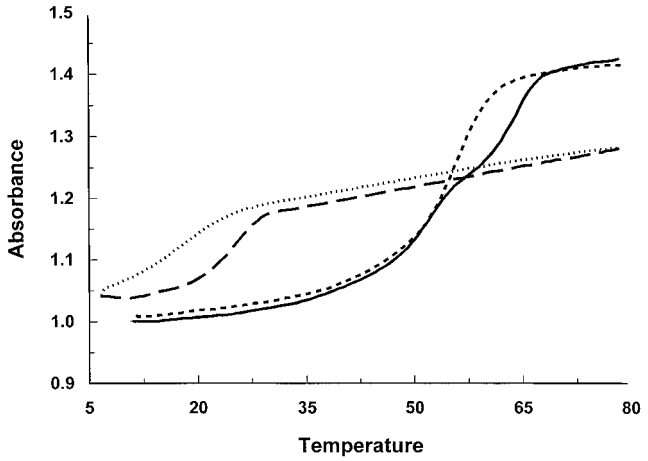


Figure 4. Melting curves for complexes between $D(A^*)_{13}$ and $D(T^*)_{13}$ (—— up, ---- down) and between $D(A^*)_{13}$ and $L(T^*)_{13}$ (––– up, •••• down).

Table 1. T_m [°C] determination of complexes formed between CNA homooligomers.

		$D(A^*)_{13}$	$L(A^*)_{13}$	$D(T^*)_{13}$	$L(T^*)_{13}$
$D(A^*)_{13}$	up	–	–	52/64[a]	25.5
	down			56	17.5
$L(A^*)_{13}$	up	–	–	25.5	52/64[a]
	down			17.5	56
$D(T^*)_{13}$	up	52/64[a]	25.5	–	–
	down	56	17.5		
$L(T^*)_{13}$	up	25.5	52/64[a]	–	–
	down	17.5	56		

[a] Upon heating, the melting curves showed biphasic profiles.

The association observed for homopurine and homopyrimidine oligonucleotides has been confirmed with mixed adenine/thymine oligomers. Three non-self-complementary mixed A–T sequences were evaluated (4′-T_2*A*T*A*-T_3*A_4*-6′, 4′-T_4*A_3*T*A*T*A_2*-6′ and 4′-A_4*T_3*A*-T*A*T_2*-6′) for both the D series and the L series. The homochiral oligonucleotides hybridized strongly (T_m: 51 °C in 0.1M NaCl) with their complements if both strands were of the same stereochemical configuration but not when the strands are of opposite chirality (Table 2, Figure 5).

The duplexes were more stable than the reference complex of complementary DNA strands (T_m: 27.3 °C in 0.1M NaCl). The synthesis of the third oligonucleotide (4′-A_4*T_3*A*-T*A*T_2*-6′) allowed us to evaluate the hybridization direction. This oligonucleotide, however, did not hybridize at all with either its parallel-D or its parallel-L complement (Table 2). These results demonstrate the requirement for an antiparallel orientation between the homochiral CNA strands in cases where thermal melting behaviour was observed.

Table 3 shows the results of T_m determinations for complexes formed between homopurine and homopyrimidine

Table 2. T_m [°C] determination of duplexes formed between CNA heterooligomers.

	D(6′-A_4*T_3*A*T*A*T_2*-4′)	L(6′-A_4*T_3*A*T*A*T_2*-4′)	L(6′-T_2*A*T*A*T_3*A_4*-4′)
D(4′-T_4*A_3*T*A*T*A_2*-6′)	51.0	–	–
L(4′-T_4*A_3*T*A*T*A_2*-6′)	–	51.0	–

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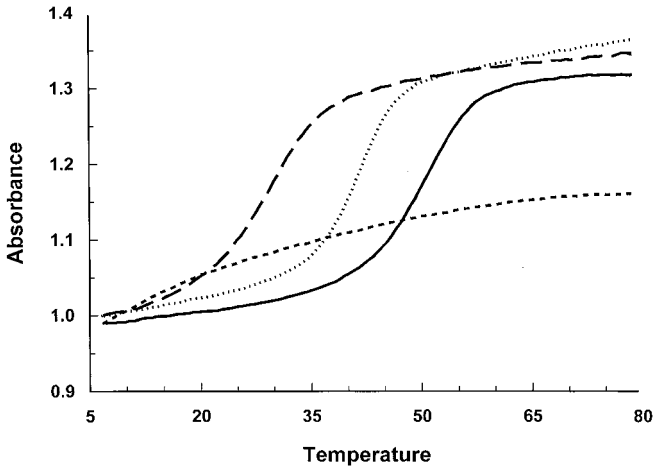

Figure 5. Melting curves for mixed A*,T* oligomers: —— D(6′-A_4*T_3*A*-T*A*T_2*-4′) mixed with D(4′-T_4*A_3*T*A*T*A*A_2*-6′); - - - - D(6′-A_4*T_3*A*-T*A*T_2*-4′) mixed with L(4′-T_4*A_3*T*A*T*A*A_2*-6′); – – – d($A_4T_3ATAT_2$) mixed with D(T_4*A_3*T*A*T*A*A_2*); •••• r($A_4U_3AUAU_2$) mixed with D(T_4*A_3*T*A*T*A*A_2*).

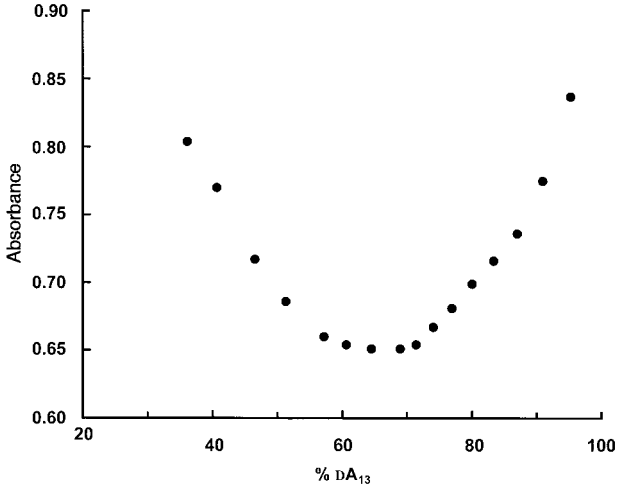

Figure 6. UV mixing curve of D(A*)$_{13}$ with dT$_{13}$, recorded at 260 nm.

Table 3. T_m [°C] determination of complexes formed between CNA homooligomers and natural oligonucleotides (DNA and RNA).

	dT$_{13}$	dA$_{13}$	r(5′-GA$_{13}$C-3′)	r(5′-GU$_{13}$C-3′)
D(T*)$_{13}$	ND[a]	37.6	48.2	ND
L(T*)$_{13}$	ND	–	–	ND
D(A*)$_{13}$	61.1	ND	ND	52.2
L(A*)$_{13}$	–	ND	ND	–
r(3′-CU$_{13}$G-5′)	ND	15.2	38.7	ND

[a] ND = not determined.

oligo-CNAs and natural nucleic acids. For the L-oligonucleotides [L(A*)$_{13}$ and L(T*)$_{13}$)], no hybridization could be detected with the complementary natural nucleic acids [dT$_{13}$ and r(GU$_{13}$C) or dA$_{13}$ and r(GA$_{13}$C), respectively]. With regard to hybridization with CNA, natural nucleic acids discriminate on the basis of chirality. Homoadenine oligomers composed of DA* hybridized strongly both with its DNA complement dT$_{13}$ (T_m 61 °C), and with its RNA complement r(GU$_{13}$C) (T_m 52 °C). Similar results were obtained with homothymine oligomers with a DT* CNA component. A T_m of 38 °C was observed for a dA$_{13}$ complement and a T_m of 48 °C for an rA$_{13}$ complement. At first sight, the fully modified oligonucleotides composed of DA* seem to be DNA-selective while the oligonucleotides composed of DT* are RNA-selective. From hypochromicity values we could already deduce that the interactions most probably had a triple-strand origin, which was confirmed by Job plots.[14] In the mixing curve of D(A*)$_{13}$ with dT$_{13}$ (Figure 6) the point of minimum absorbance corresponds to an approximate 1:2 ratio of dT$_{13}$ to D(A*)$_{13}$. Similar results were obtained for the mixtures dA$_{13}$:D(T*)$_{13}$ and D(A*)$_{13}$:r(GU$_{13}$C). For the r(GA$_{13}$C):D(T*)$_{13}$ mixture, however, the mixing curve showed no clear minimum but only a flat line between approximately 55 and 95% of D(T*)$_{13}$. This may be due to slow kinetics and/or only small differences in hypochromicity between the complexes at the wavelength applied for the Job plots.

Complexes between CNA homooligomers and natural nucleic acids are more stable than DNA:RNA and dsRNA associations. The melting temperatures of r(CU$_{13}$G):dA$_{13}$ and r(CU$_{13}$G):r(GA$_{13}$C), used as references, were 15 °C and 39 °C, respectively (Table 3). Even if the reference oligoribonucleotides are flanked by GC base pairs, the melting temperature is 10 °C lower than observed for D(T*)$_{13}$:r(GA$_{13}$C). As mentioned previously, complexes of homooligonucleotides composed of DT* units [complexed with dA$_{13}$ or r(GA$_{13}$C)] were less stable than complexes formed between DA* oligomers and dT$_{13}$ or r(GU$_{13}$C). The D(T*)$_{13}$:dA$_{13}$ association was also less stable than the comparable HNA:DNA complex, as revealed previously.[9] Besides the difference in ratio of both single-stranded oligomers (1:1 or 1:2) used to build the complexes, this difference might also result from differences in conformational stress of the DA* and DT* monomers when incorporated in oligonucleotides (see below).

To be a successful antisense therapeutic, a modified mixed-sequence oligonucleotide has to be capable of forming stable duplexes with natural nucleic acids. To assess this ability for CNAs, we tested mixed A*–T* sequences for hybridization with complementary DNA and RNA (Table 4). Two sequences were evaluated both as homochiral-D oligomers and as homochiral-L oligomers (4′-T_4*A_3*T*A*T*A_2*-6′ and 4′-T_2*A*T*A*T_3*A_4*-6′). For both sequences, the D-CNAs hybridized more strongly with RNA than with DNA (Table 4), and thus were RNA-selective, as has been observed previously with HNAs.[15] The thermal stabilities of the duplexes formed with both sequences were similar. It should be pointed out that both sequences contain six purine and six pyrimidine bases. The difference in stability may be partially explained by a difference in interstrand and intrastrand stacking within CNA:RNA and CNA:DNA (as observed in A- and B-type dsDNA).[16, 17] Neither of the L-CNA sequences was able to hybridize with either DNA or RNA complements; this supports the previous observation of chiral discrimination by natural nucleic acids for homochiral D-CNA sequences.

To rule out the possibility that L-CNA could hybridize with natural DNA or RNA in a parallel manner, we also evaluated the hybridization properties of the inverted L-CNA sequence

Table 4. T_m [°C] determination of duplexes formed between CNA heterooligomers and natural 5′→3′ oligonucleotides (DNA and RNA).

	$d(A_4T_3ATAT_2)$	$d(A_2TATA_3T_4)$	$r(A_4U_3AUAU_2)$	$r(A_2UAUA_3U_4)$
4′→6′				
D(T_4*A_3*T*A*T*A_2*)	29.6	ND[a]	41.6	ND
L(T_4*A_3*T*A*T*A_2*)	–	ND	–	ND
D(T_2*A*T*A*T_3*A_4*)	ND	30.1	ND	39.0
L(T_2*A*T*A*T_3*A_4*)	ND	–	ND	–
L(A_4*T_3*A*T*A*T_2*)	ND	–	ND	–
3′→5′				
$d(T_4A_3TATA_2)$	27.3	–	22.0	–
$d(T_2ATAT_3A_4)$	–	27.3	–	15.0

[a] ND = not determined.

4′-A_4*T_3*A*T*A*T_2*-6′. However, under no conditions was the mixed A*,T* L-CNA oligomer capable of interacting with complementary DNA or RNA.

The CNA:DNA duplex is thermally more stable than the dsDNA or the RNA:DNA hybrid of the same sequence. A T_m of 27.3 °C at 0.1M NaCl was found for dsDNA of sequence 3′-$T_2ATAT_3A_4$-5′ compared with 29.6 or 30.1 °C for the CNA:DNA duplex (depending on which DNA strand was replaced by the CNA oligomer). T_ms of only 22.0 °C and 15.0 °C were measured for the respective RNA:DNA hybrids.

Structural investigations of CNA complexes using CD spectral analysis and computational methods: Circular dichroism is a convenient technique for probing the structure of chiral polymers and polymer complexes. It may give initial information on the helix types that are involved in the complexes formed between nucleic acids.

The CD spectrum of D(A^*)$_{13}$ shows a positive Cotton effect at 219 nm and 262 nm and a negative Cotton effect at 205 nm and 243 nm (Figure 7, A). A maximum around 265 nm and a minimum around 245 nm has also been observed with HNA oligoadenylates,[9] although the intensities of the bands are different. The signal at 262 nm is stronger and the signal at 243 nm weaker for D(A^*)$_{13}$ than for HNA. The spectrum of D(A^*)$_{13}$ is very similar to the spectrum of poly(rA). The difference between the CD spectra of D(A^*)$_{13}$ and poly(dA)[18] or 2′,3′-dideoxy-β-D-glucopyranosyl(A_{10}) [ddGlc(A_{10})][10] is more pronounced than that between the spectra of D(A^*)$_{13}$ and HNA or poly(rA). This implies the absence of A–A base pairing for CNAs [as observed with ddGlc(A_{10})], and suggests similarities between the structures of single-stranded oligoadenylates with a phosphorylated hexitol backbone, of its carbocyclic congener and of RNA.

The negative and positive Cotton effects in the CD spectrum of D(T^*)$_{13}$ (Figure 7, curve B) are shifted to higher wavelengths (255 nm and 280 nm) compared to the CD spectrum of D(A^*)$_{13}$. The spectrum is of low intensity, corresponding better to the CD spectrum of poly(dT)[18] than to the spectrum of HNA oligothymidylate.[9] Likewise, similarities between the positions of the signals are greater between D(T^*)$_{13}$ and poly(dT) than between D(T^*)$_{13}$ and HNA. As the CD spectra for poly(rU) and poly(dT) are similar, further comparison cannot be made. There is very little base stacking in oligopyrimidines, and CD spectroscopy indicates similar conformations for D(T^*)$_{13}$ and for oligothymidylates of natural origin. These data further support the absence of T–T base pairing in CNAs, similar to HNAs.

When D(A^*)$_{13}$ and D(T^*)$_{13}$ were mixed, a CD spectrum was obtained with three positive bands at 222, 257 and 296 nm, and three negative bands at 209, 246 and 270 nm (Figure 7, curve C). The positive CD bands are more intense at lower wavelength while the negative bands are more intense at higher wavelengths. This corresponds roughly with the low-temperature CD spectra of poly(dA):poly(dT), of poly(rA):poly(dT) and of poly(rT):poly(dA):poly(rT),[18] though the similarities are not particularly striking. These CD experiments suggest that single-stranded D(A^*)$_{13}$ and D(T^*)$_{13}$ adopt preorganized conformations similar to those of poly(rA) and poly(rU), respectively, and that on mixing them either a double or a triple helix might be formed. T_m measurements point in the direction of triple helix formation.

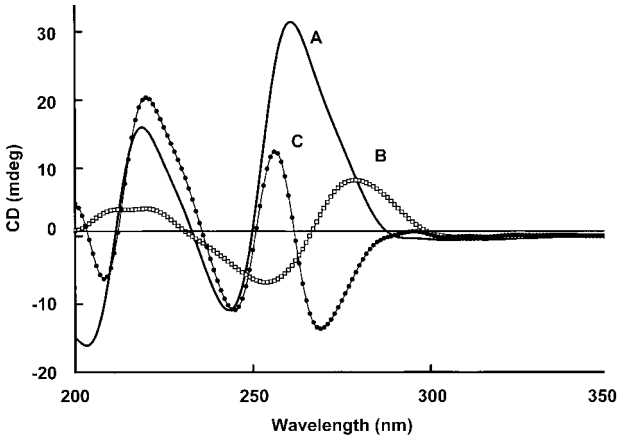

Figure 7. CD spectra of: A) D(A^*)$_{13}$; B) D(T^*)$_{13}$; C) a mixture of both strands.

To examine the structures of mixed (A*,T*) CNA sequences, either self-complexed or complexed with natural nucleic acids, we first evaluated CD spectra of dsDNA, DNA:RNA and dsRNA as reference duplexes. The sequences of these natural nucleic acids were those described before for CNAs (for the RNAs: 5′-$A_4U_3AUAU_2AU_2A$-3′ and 5′-$UA_2UA_2UAUA_3U_4$-3′; for the deoxynucleotide analogues A is replaced by dA and U by dT). The dsRNA spectrum clearly differs from the dsDNA spectrum both in the intensities of the bands and in their wavelengths. For dsDNA, positive bands at lower wavelengths are more intense than those at higher wavelengths, and the opposite is observed for dsRNA. In the spectrum of dsRNA, the negative bands are very small while an intensely negative CD at about 245 nm is observed for dsDNA. The CD spectrum for the DNA:RNA duplex is intermediate between these spectra regarding both intensity and wavelength shift.

The CD spectrum of dsCNA (Figure 8, curve A) has more similarities with the spectra of dsRNA and RNA:DNA than with the spectrum of dsDNA, although the bands are sharper

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and more intense and a shoulder appears at about 288 nm. The shapes of the curves are not completely superimposable.

The spectrum of D-CNA:RNA (sequence 5′-$A_2TATA_3T_4$-3′) (Figure 8, curve B) is practically identical with that of DNA:RNA of the same sequence. Likewise, the spectrum of D-CNA:DNA (sequence 5′-$A_2TATA_3T_4$-3′) (Figure 8, curve C) resembles that of DNA:RNA, although deviating

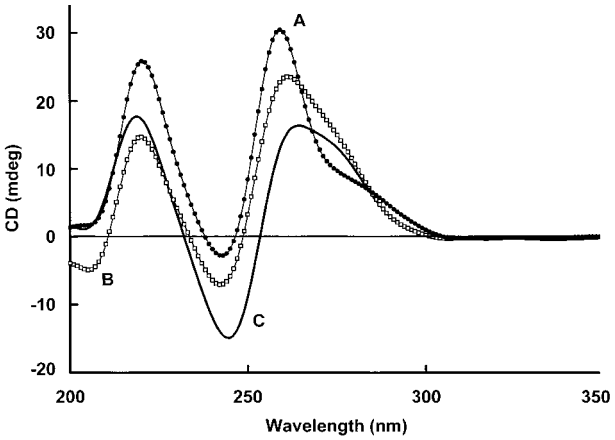


Figure 8. CD spectra of: A) dsCNA; B) D-CNA:RNA; C) D-CNA:DNA for the sequence 5′-$A_2TATA_3T_4$-3′ and its complement.

somewhat more than the CD spectrum for D-CNA:RNA. More divergent curves were obtained when the spectra were measured for L-CNA in the presence of either its DNA or its RNA complement (Figure 9). This, however, was not surprising as the mixture contained homochiral CNA with opposite sense of chirality in the presence of a natural nucleic acid with which, according to T_m results, no complex was formed. In summary, the CD spectra suggest that the helix structures obtained when D-CNA is complexed with RNA or DNA are very similar to those of RNA:DNA hybrids and of dsRNA. Likewise, similarities are observed between the structures of dsCNA and dsRNA.

At first sight, the T_m and CD results seem surprising. We have previously synthesized the carbocyclic pyranose nucleosides and determined their conformations both in solution and

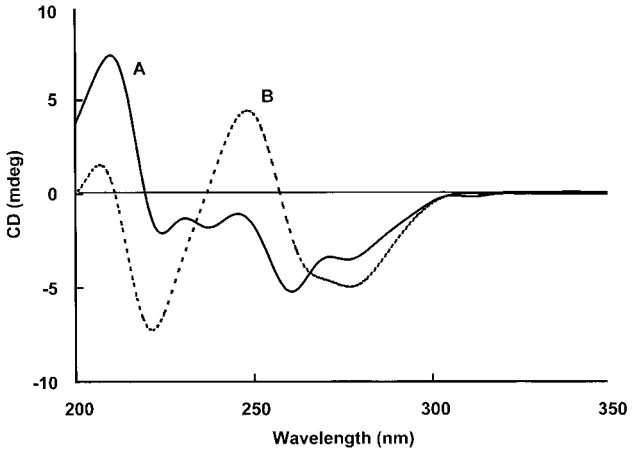
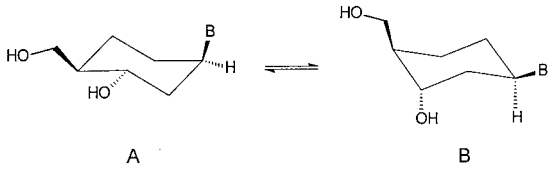


Figure 9. CD spectra of L-CNA in the presence of either its DNA (A) or its RNA (B) complement.

in the solid phase (Scheme 3).[3] Both adenine- and thymine-containing molecules adopted a conformation with an equatorially oriented base moiety and axially oriented hydroxymethyl group and hydroxyl functions (Scheme 3B). This



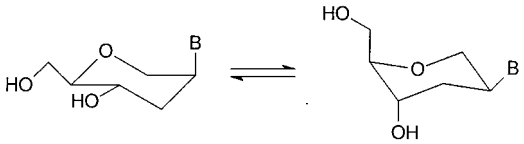
Scheme 3. Conformation of cyclohexanyl nucleosides after incorporation in an oligonucleotide (A) in comparison with the monomer conformation (B).

conformation was opposite to that found for the anhydrohexitol nucleosides,[1, 2] leading us to expect few similarities between the structures of oligonucleotides built from anhydrohexitol nucleotides (HNAs) and those built from their carbocyclic congeners (CNAs). Instead, we expected CNA to adopt a quasilinear structure, in the trend of homo-DNA,[10] and HNA to adopt a helical structure, as found in DNA and RNA. However, CNA hybridized strongly with both RNA and DNA, and the CD spectra obtained with CNA complexes resembled those of HNA complexes. This can be explained by a chair inversion of the carbocyclic nucleosides when incorporated in an oligomer and complexed with complementary nucleic acid analogues.

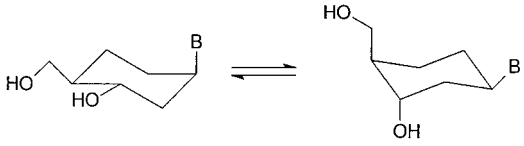
Generally, the preferred conformation of the nucleotide unit in polynucleotides is similar to that found in monomer single crystals,[19] but this was apparently not the case for oligomers built from phosphorylated cyclohexanyl nucleosides. To gain better insight into this issue, we analyzed the conformational preferences of hexitol nucleosides (hNs) and their carbocyclic congeners (cNs) using computational methods. For both series nucleosides with both a thymine base moiety and an adenine base moiety were analyzed, because we had previously observed that purine and pyrimidine bases had different effects on nucleoside conformation.[20, 21] The lowest energy conformations of hT, hA, cT and cA with the base moieties situated both equatorially and axially were calculated together with the energy differences between each pair of conformers with a simple dielectric appropriate for water solvation (Table 5). The calculations showed that hA prefers an axial base moiety and that hT prefers an equatorial base moiety, but that the energy differences between the conformers are small (1.7 and 5.9 kJ mol^{-1}, respectively). Interconversion between chair conformations is possible energetically, and this has been experimentally demonstrated for the iodouracil congener when complexed with viral thymidine kinase (TK).[22] However, both at the monomeric level[1, 2] and at the oligomeric level,[9, 15, 23] the hexitol nucleosides adopt conformations with an axially oriented base moiety.

Similar computational methods suggest that the most stable conformations of the carbocyclic nucleosides are those with equatorially oriented base moieties for both thymine- and adenine-containing nucleoside analogues; this result corresponds well with experimental data.[3] However, the energy difference between the two chair conformations is very small

Table 5. Lowest energy conformations calculated for hT, hA, cT and cA.



Base	Lowest energy of conformer with an axial base moiety	Lowest energy of conformer with an equatorial base moiety	Conformational preference and energy difference
B = T	– 288.9 kJ mol^{-1}	– 294.8 kJ mol^{-1}	Favours equatorial base by 5.9 kJ mol^{-1}
B = A	– 333.9 kJ mol^{-1}	– 332.2 kJ mol^{-1}	Favours axial base by 1.7 kJ mol^{-1}



Base	Lowest energy of conformer with an axial base moiety	Lowest energy of conformer with an equatorial base moiety	Conformational preference and energy difference
B = T	– 273.2 kJ mol^{-1}	– 291.0 kJ mol^{-1}	Favours equatorial base by 17.8 kJ mol^{-1}
B = A	– 318.8 kJ mol^{-1}	– 321.5 kJ mol^{-1}	Favours equatorial base by 2.7 kJ mol^{-1}

for the carbocyclic nucleoside with the adenine base moiety (2.7 kJ mol^{-1}) and considerably larger for the nucleoside analogue with the thymine base moiety (17.8 kJ mol^{-1}), a fact suggesting that chair inversion is easier with the adenine nucleosides than with the thymine nucleosides. The conformational preference of a nucleoside analogue is determined both by steric and by stereoelectronic effects, and it is not always easy to determine their relative contributions. However, with carbocyclic nucleoside analogues the influence of steric factors predominates. The study of the energy difference between the two chair conformations of cyclohexanyl nucleosides with either a pyrimidine or a purine base moiety confirms the considerable difference in steric influences of thymine and adenine bases.

On the basis of CD spectra, we expect that cyclohexanyl oligoadenylates will adopt a helical structure similar to those of HNA and RNA, as a result of chair inversion of the carbocyclic monomer when incorporated in an oligonucleotide. The gain in enthalpy resulting from stacking interactions between the adenine bases can easily compensate for the chair inversion and the loss in entropy resulting from the formation of a preorganized single-stranded helical structure. This is less likely for cyclohexanyl oligothymidylates, both because of the lower stacking energy for pyrimidine bases and because of the higher energy difference between the two chair conformations of the carbocyclic monomer with a thymine base moiety. It might be expected, therefore, that duplexes containing pyrimidine-rich CNA oligomers are thermodynamically less stable than duplexes containing purine-rich CNA oligomers, and such behaviour was indeed seen in the melting-temperature studies.

Conclusion

Cyclohexanyl nucleic acids (CNAs or carbocyclic pyranosyl nucleic acids) were synthesized with either thymine or adenine as the base moiety. The homochiral oligonucleotides were investigated in both the D and the L series. Oligoadenylates, oligothymidylates and mixed thymine-adenine-containing oligomers were synthesized from building blocks of the same sense of chirality. Hybridization studies revealed that hetero-complexes are formed with homochiral D-CNA but not with L-CNA. The modified oligonucleotides form isochiral Pu – Py pairing. In the oligoadenylate CNA and oligothymidylate CNA series heterochiral Pu – Py pairing is observed. However, these complexes are of lower thermal stability than the isochiral systems. No homocomplexes between CNA molecules were formed, regardless of chirality (no A – A or T – T base pairing).

For the cyclohexanyl nucleic acids comprising either homopolymeric thymine or mixed thymine/adenine sequences, self-complementary duplexes (CNA:CNA) are consistently more stable than duplexes with RNA (CNA:RNA) which, in turn, are more stable than hybrids with DNA (CNA:DNA). This order is not followed when the CNA contains only adenine bases. In this case, the CNA:DNA complex is more stable than both the self-complementary complexes and the CNA:RNA complexes. This difference may be explained by a different nature of association. Hypochromicity data suggest that oligoadenylate CNA:RNA and oligoadenylate CNA:DNA complexes are triplexes, while the corresponding oligothymidylate CNA complexes are double-helical associations.

Solution-phase analyses revealed that cyclohexanyl nucleosides with either thymine or adenine bases adopt conformations with equatorial base moieties. The energy difference between the two chair conformations is, however, considerably higher with thymine as the base than it is with adenine; this may be attributed to steric effects. Chair inversion of the adenine nucleoside analogue costs less energy than chair inversion of the thymine congener, owing to the greater steric hindrance created by a pyrimidine base relative to a purine base.

Oligonucleotides constructed of cyclohexanyl nucleosides and phosphodiester internucleoside linkages probably adopt quasilinear structures when the nucleobases are oriented equatorially (Figure 10A). This structure can be obtained with all the backbone torsion angles in staggered conformations ($\alpha = -60^\circ; \beta = 180^\circ; \gamma = 60^\circ; \delta = 180^\circ; \varepsilon = 180^\circ; \xi = 180^\circ$) and, of course, no stacking interactions and no hybridization with natural nucleic acids can be expected. Attempts to dock CNA having equatorially oriented bases with rigid templates of either A- or B-form DNA (with a Molecular Dynamics protocol which was recently used successfully for HNA[24]) produced severely strained structures. Therefore, it seems unlikely that a helical conformation with base stacking in such a manner as to allow base pairing with either A- or B-form

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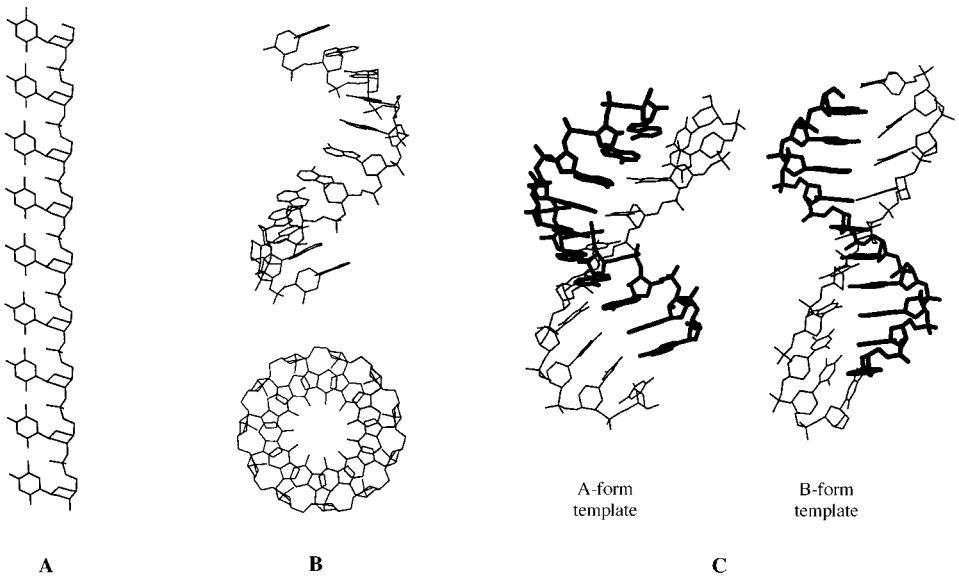

Figure 10. Structure of oligonucleotides composed of cyclohexanyl nucleosides and phosphodiester internucleoside linkages: idealized structure of a quasilinear CNA oligomer with equatorially oriented bases (A) and of a helical CNA oligomer with axially oriented bases (B); docking of CNA having equatorially oriented bases with rigid templates (in bold) of either A- or B-form DNA (C).

DNA or RNA can be obtained for CNA with equatorially oriented bases (Figure 10C). Chair inversion of the nucleosides, however, allows base stacking and hybridization with natural nucleic acids. In this way, duplexes can be formed between CNA and either DNA or RNA of similar geometries to those previously described for HNA duplexes with natural nucleic acids (Figure 10B shows the idealized CNA strand conformation). Thus, our study of cyclohexanyl nucleosides demonstrates that the preferred conformation of a single nucleoside analogue can be different from its conformation in an oligomer. Chair inversion may also partially explain the lower thermal stability of oligothymidylate CNA:DNA complexes compared to HNA:DNA and oligoadenylate CNA:DNA complexes.

The energy gain from the stacking interactions between the adenine bases of a single-stranded cyclohexanyl oligoadenylate can easily compensate for the chair inversion and the loss in entropy due to preorganization of the oligomer. This is less likely for the cyclohexanyl oligothymidylates because the energy gain resulting from stacking of the pyrimidine bases is less than that for purine bases and because the chair inversion of a cyclohexanyl thymine nucleoside costs more energy than inversion of a cyclohexanyl adenine nucleoside. Consequently, thymine-containing oligomers are expected to be less thermally stable than adenine-containing oligomers. Thus, in addition to differences in stacking interactions and hydration patterns, the sequence-dependent differences in the stability of oligonucleotide complexes may also be related to differences in conformational stress between the different nucleosides (i.e., purine and pyrimidine nucleosides) used to build the oligomer.

Experimental Section

The ^{1}H NMR and ^{13}C NMR spectra were recorded with Varian Gemini 200 MHz and Varian Unity 500 MHz spectrometers with TMS as internal standards. Liquid secondary ion mass spectra (LSIMS) were recorded on a Kratos Concept 1H mass spectrometer (Kratos Analytical, Manchester, UK) using thioglycerol (THGLY) and 3-nitrobenzylalcohol (NBA) as matrix. Mass spectra of the monomethoxytritylated compounds were obtained by doping the matrix with sodium acetate (NaOAc). The UV spectra were recorded with a Philips 8740 UV/Vis spectrophotometer. Melting points were determined with a Büchi-Tottoli apparatus and are uncorrected. Precoated Merck silica gel 60 F_{254} plates were used for TLC. Column chromatography was performed on Süd Chemie AG silica gel (0.060–0.200 nm). Solvents were dried with standard methods. Chromatotron purification was carried out by means of a Chromatotron model 8924 from Harrison Research (Palo Alto, California) equipped with a Lab Pump Jr. model RHSY from Metering Inc. (Oyster Bay, New York) on a 2 mm sorbent layer of silica gel 60 PF_{254} following the standard protocol.

1-{(1*R*,3*S*,4*R*)-4-Trityloxymethyl-3-[(*R*)-(*O*-methylmandelyl)oxy]-cyclohexanyl}-thymine [3(D)] and 1-{(1*S*,3*R*,4*S*)-4-trityloxymethyl-3-[(*R*)-(*O*-methylmandelyl)oxy]-cyclohexanyl}thymine [3(L)]: DCC (1.10 g, 5.3 mmol) was added to a solution of compound **1**[3] (2.4 g, 4.83 mmol), (*R*)-(−)-*O*-methylmandelic acid (0.88 g, 5.31 mmol) and DMAP (65 mg, 0.53 mmol) in CH_2Cl_2 (50 mL) at 0 °C. The mixture was allowed to warm to room temperature over 2 h and filtered. The filtrate was washed with aqueous H_3PO_4 (1M, 30 mL), water (30 mL) and saturated aqueous $NaHCO_3$ (15 mL), dried ($MgSO_4$) and filtered. Column chromatography (CH_2Cl_2/MeOH, 99.5:0.5 to 99:1) afforded 1.09 g (35%) of **3(D)** and 0.84 g (27%) of **3(L)** as foams, as well as 0.25 g (8%) of the mixture of **3(D)** and **3(L)**.

Compound 3(D): LSIMS (NBA): 643 $[M-H]^-$; ^{1}H NMR ($CDCl_3$): δ = 1.1–2.2 (10H), 3.18 (2H), 3.45 (3H), 4.31 (1H), 4.80 (1H), 5.30 (1H), 6.36 (1H), 7.24–7.51 (20H), 8.22 (1H); ^{13}C NMR ($CDCl_3$): δ = 12.4, 22.1, 25.6, 30.2, 36.4, 49.1, 57.4, 61.9, 71.6, 82.5, 86.6, 110.3, 127.1, 127.3, 127.5, 128.8, 128.6, 136.2, 136.5, 143.7, 150.2, 163.3, 169.5; anal. calcd for $C_{40}H_{40}N_2O_6$: C 74.51, H 6.25, N 4.34; found C 74.29, H 6.12, N 4.47.

Compound 3(L): LSIMS (NBA): 643 $[M-H]^-$; UV (MeOH): λ_{max} (ε) = 270 nm (9800 $mol^{-1}dm^3cm^{-1}$); ^{1}H NMR ($CDCl_3$): δ = 1.25–2.10 (10H), 3.15 (2H), 3.47 (3H), 4.67 (1H), 4.86 (1H), 5.32 (1H), 6.50 (1H), 7.2–7.5 (20H), 8.76 (1H); ^{13}C NMR ($CDCl_3$): δ = 12.5, 21.6, 25.7, 30.6, 35.8, 49.4, 57.4, 61.8, 71.5, 82.3, 86.7, 110.6, 127.1, 127.8, 128.7, 128.4, 135.9, 136.4, 143.6, 150.5, 163.3, 169.5; anal. calcd for $C_{40}H_{40}N_2O_6$: C 74.51, H 6.25, N 4.34; found C 74.12, H 6.24, N 4.33.

1-[(1*R*,3*S*,4*R*)-4-Hydroxymethyl-3-hydroxycyclohexanyl]thymine [5(D)]: A solution of KOH (0.1M) in MeOH (15 mL) was added to a solution of compound **3(D)** (1.24 g, 1.92 mmol) in MeOH (15 mL). The mixture was evaporated after 30 min and the residue was dissolved in CH_2Cl_2 (100 mL), washed with water (20 mL), dried ($MgSO_4$) and evaporated. The residue was dissolved in 80% HOAc and heated at 60 °C for 4 h. After removing HOAc by evaporation and coevaporation with toluene, the residue was dissolved in MeOH (10 mL), adsorbed on silica gel and put on the top of a silica gel column. Elution with CH_2Cl_2/MeOH (95:5 to 9:1) afforded, after recrystallization (MeOH/Et_2O), 0.26 g (53%) of **5(D)**: m.p. 201–202 °C; LSIMS (THGLY): 253 $[M-H]^-$, 125 $[B-H]^-$; anal. calcd for $C_{12}H_{18}N_2O_4$: C 56.68, H 7.13, N 11.02; found C 56.64, H 7.17, N 10.96.

1-[(1*S*,3*R*,4*S*)-4-Hydroxymethyl-3-hydroxycyclohexanyl]thymine [5(L)]: Compound **5(L)** was obtained by deprotection of **3(L)** with KOH/MeOH followed by treatment with 80% HOAc as described for **5(D)**. Yield of **5(L)** after crystallization (MeOH/Et_2O) 64%; m.p. 201–202 °C; LSIMS

(THGLY): 253 $[M-H]^-$, 125 $[B-H]^-$; anal. calcd for $C_{12}H_{18}N_2O_4$: C 56.68, H 7.13, N 11.02; found C 56.74, H 7.16, N 10.97.

1-[(1*R*,3*S*,4*R*)-4-(*O*-Monomethoxytrityl)oxymethyl-3-hydroxycyclohexanyl]thymine [8(D)]: A mixture of **5(D)** (0.59 g, 2.32 mmol) and MMTrCl (0.86 g, 2.78 mmol) in pyridine (30 mL) was stirred at room temperature for 2 h. The reaction was quenched with MeOH (2 mL) and evaporated. The residue was purified by column chromatography, eluted with CH_2Cl_2/MeOH (99:1 to 95:5) to give 0.79 g (65%) of **8(D)** as a foam. LSIMS: 525 $[M-H]^-$; UV (MeOH): λ_{max} (ε) = 274 nm (11 000 mol^{-1} dm^3 cm^{-1}); ^{1}H NMR ($CDCl_3$): δ = 1.27–2.25 (7H), 2.82 (1H), 3.20 (2H), 3.83 (3H), 4.24 (1H), 4.85 (1H), 6.66 (1H), 6.90 (2H), 7.21–7.52 (12H), 9.23 (1H); ^{13}C NMR ($CDCl_3$): δ = 12.5, 21.6, 26.2, 34.0, 39.0, 49.6, 55.2, 62.9, 68.0, 86.3, 110.5 113.1, 126.9, 127.5, 127.8, 128.4, 130.2, 135.6, 136.6, 144.3, 150.8, 158.5, 163.6; anal. calcd for $C_{32}H_{34}N_2O_5$: C 72.98, H 6.51, N 5.32; found C 73.02, H 6.54, N 5.32.

1-[(1*S*,3*R*,4*S*)-4-(*O*-Monomethoxytrityl)oxymethyl-3-hydroxycyclohexanyl]thymine [8(L)]: Compound **8(L)** was obtained by reaction of **5(L)** with MMTrCl as described for **8(D)** in 87% yield as a foam. LSIMS: 525 $[M-H]^-$; UV (MeOH): λ_{max} (ε) = 274 nm (11 000 mol^{-1} dm^3 cm^{-1}); ^{1}H NMR ($CDCl_3$): δ = 1.27–2.25 (7H), 2.73 (1H), 3.20 (2H), 3.83 (3H), 4.23 (1H), 4.84 (1H), 6.65 (1H), 6.88 (2H), 7.24–7.53 (12H), 9.13 (1H); ^{13}C NMR ($CDCl_3$): δ = 12.5, 21.6, 26.2, 34.0, 39.0, 49.6, 55.2, 62.9, 68.1, 86.3, 110.5 113.1, 126.9, 127.8, 128.2, 128.4, 130.2, 135.6, 136.4, 144.3, 150.8, 158.5, 163.6; anal. calcd for $C_{32}H_{34}N_2O_5$: C 72.98, H 6.51, N 5.32; found C 72.69, H 6.59, N 5.36.

***N*^{6}-Monomethoxytrityl-9-{(1*R*,3*S*,4*R*)-4-trityloxymethyl-3-[(*R*)-(*O*-methylmandelyl)oxy]-cyclohexanyl}adenine [4(D)] and *N*^{6}-monomethoxytrityl-9-{(1*S*,3*R*,4*S*)-4-trityloxymethyl-3-[(*R*)-(*O*-methylmandelyl)oxy]-cyclohexanyl}adenine [4(L)]**: DCC (1.32 g, 6.42 mmol) was added to a solution of compound **2**[3] (4.16 g, 5.35 mmol), (*R*)-(−)-*O*-methylmandelic acid (1.07 g, 6.42 mmol), and DMAP (78 mg, 0.64 mmol) in CH_2Cl_2 (100 mL) at 0 °C. The mixture was allowed to warm to room temperature over 2 h and filtered. The filtrate was washed with aqueous H_3PO_4 (1M, 50 mL), water (50 mL) and saturated aqueous $NaHCO_3$ (30 mL), dried ($MgSO_4$) and filtered. Column chromatography (CH_2Cl_2/EtOAc, 40:1 to 20:1) afforded 1.48 g (30%) of **4(D)** and 1.19 g (24%) of **4(D)** as foams, as well as 1.98 g (40%) of the mixture of **4(D)** and **4(L)**, which was chromatographed again to give a further 0.59 g (12%) of **4(D)** and 0.50 g (10%) of **4(L)**.

Compound 4(D): LSIMS (NBA): 924 $[M-H]^-$; UV (MeOH): λ_{max} (ε) = 276 nm (24 600 mol^{-1} dm^3 cm^{-1}); ^{1}H NMR ($CDCl_3$): δ = 1.58–2.10 (7H), 3.18 (2H), 3.43 (3H), 3.77 (3H), 4.54 (1H), 4.82 (1H), 5.33 (1H), 6.76–6.85 (3H), 7.19–7.50 (33H), 8.00 (1H); ^{13}C NMR ($CDCl_3$): δ = 21.9, 27.5, 31.9, 36.7, 49.7, 55.2, 57.3, 62.2, 70.9, 71.5, 82.5, 86.6, 113.1 121.1, 126.5, 126.8, 127.0, 127.4, 127.8, 128.6, 128.8, 129.2, 130.1, 136.3, 137.2, 137.6, 143.7, 145.2, 148.6, 151.9, 154.0, 158.2, 169.7; anal. calcd for $C_{60}H_{55}N_5O_5$: C 77.81, H 5.99, N 7.56; found C 77.92, H 6.03, N 7.42.

Compound 4(L): LSIMS (NBA): 924 $[M-H]^-$; UV (MeOH): λ_{max} (ε) = 276 nm (25 200 mol^{-1} dm^3 cm^{-1}); ^{1}H NMR ($CDCl_3$): δ = 1.65–2.25 (7H), 3.25 (2H), 3.43 (3H), 3.77 (3H), 4.05 (1H), 4.79 (1H), 5.33 (1H), 6.75–6.81 (3H), 7.17–7.50 (33H), 8.92 (1H); ^{13}C NMR ($CDCl_3$): δ = 22.2, 27.1, 31.2, 37.1, 50.5, 55.2, 57.3, 62.2, 70.9, 71.6, 82.4, 86.6, 113.1 121.1, 126.8, 127.0, 127.2, 127.8, 128.6, 128.7, 128.8, 129.2, 130.1, 136.5, 137.3, 138.0, 143.7, 145.2, 148.6, 151.6, 154.0, 158.2, 169.6; anal. calcd for $C_{60}H_{55}N_5O_5$: C 77.81, H 5.99, N 7.56; found C 77.62, H 6.04, N 7.53.

9-[(1*R*,3*S*,4*R*)-4-Hydroxymethyl-3-hydroxycyclohexanyl]adenine [6(D)]: Compound **6(D)** was obtained by deprotection of **4(D)** by KOH/MeOH as described for **5(D)** followed by treatment with 80% HOAc at 50 °C overnight. Following chromatographic purification as for **5(D)** and crystallization (MeOH/H_2O/Et_2O), 76% of **6(D)** was obtained: m.p. 195–197 °C; LSIMS (NBA): 262 $[M-H]^-$; anal. calcd for $C_{12}H_{17}N_5O_2$: C 54.74, H 6.51, N 26.60; found C 54.47, H 6.50, N 26.48.

9-[(1*S*,3*R*,4*S*)-4-Hydroxymethyl-3-hydroxycyclohexanyl]adenine [6(L)]: Compound **6(L)** was obtained by deprotection of **4(L)** with KOH/MeOH as described for **5(D)** followed by treatment with 80% HOAc at 50 °C overnight. Following chromatographic purification as for **5(D)** and crystallization (MeOH/H_2O/Et_2O), 85% of **6(L)** was obtained: m.p. 219–221 °C; LSIMS (NBA): 262 $[M-H]^-$; anal. calcd for $C_{12}H_{17}N_5O_2$: C 54.74, H 6.51, N 26.60; found C 54.49, H 6.54, N 26.52.

***N*^{6}-Benzoyl-9-[(1*R*,3*S*,4*R*)-4-hydroxymethyl-3-hydroxycyclohexanyl]adenine [7(D)]**: Trimethylchlorosilane (1.93 mL, 15.2 mmol) was added at 0 °C to compound **6(D)** (0.8 g, 3.04 mmol), which had been dried repeatedly by evaporation with pyridine and suspended in pyridine (15 mL). The mixture was stirred for 1 h at 0 °C, benzoyl chloride (1.76 mL, 15.2 mmol) was slowly added and stirring was continued at room temperature for 2 h. The mixture was cooled in an ice bath and water (3.5 mL) was added. After 5 min 25% NH_4OH solution (7 mL) was added; the mixture was stirred for 15 min and then evaporated. The residue was dissolved in 5 mL of MeOH, and 25% NH_4OH solution (50 mL) was added. After 30 min the solution was evaporated and purified by column chromatography (CH_2Cl_2/MeOH, 99:1 to 90:10) affording after crystallization (MeOH/Et_2O) 0.88 g (79%) of **7(D)**: m.p. 198–200 °C; LSIMS: 366 $[M-H]^-$; UV (MeOH): λ_{max} (ε) = 282 nm (19 700 mol^{-1} dm^3 cm^{-1}). ^{1}H NMR ([D$_6$]DMSO): δ = 1.58–2.38 (7H), 3.55 (2H), 4.01 (1H), 4.55 (1H), 4.79 (1H), 4.91 (1H), 7.56 (3H), 8.03 (2H), 8.63 (1H), 8.71 (1H), 11.12 (1H); ^{13}C NMR ([D$_6$]DMSO): δ = 21.4, 27.4, 34.8, 42.1, 50.0, 61.1, 65.9, 125.8, 128.6, 132.5, 133.6, 143.5, 150.1, 151.2, 152.2, 165.7; anal. calcd for $C_{19}H_{21}N_5O_3$: C 62.11, H 5.76, N 19.06; found C 62.02, H 5.79, N 19.09.

***N*^{6}-Benzoyl-9-[(1*S*,3*R*,4*S*)-4-hydroxymethyl-3-hydroxycyclohexanyl]adenine [7(L)]**: Compound **7(L)** was obtained from **6(L)** as described for **7(D)** in 84% yield. M.p. 197–198 °C; LSIMS: 366 $[M-H]^-$; UV (MeOH): λ_{max} (ε) = 282 nm (19 800 mol^{-1} dm^3 cm^{-1}); ^{1}H NMR ([D$_6$]DMSO): δ = 1.59–2.37 (7H), 3.55 (2H), 4.00 (1H), 4.55 (1H), 4.80 (1H), 4.91 (1H), 7.57 (3H), 8.03 (2H), 8.63 (1H), 8.71 (1H), 11.11 (1H); ^{13}C NMR ([D$_6$]DMSO): δ = 21.4, 27.4, 34.8, 42.1, 50.0, 61.1, 65.9, 125.8, 128.6, 132.5, 133.6, 143.5, 150.1, 151.2, 152.2, 165.7; anal. calcd for $C_{19}H_{21}N_5O_3$: C 62.11, H 5.76, N 19.06; found C 61.82, H 5.74, N 19.09.

***N*^{6}-Benzoyl-9-[(1*R*,3*S*,4*R*)-4-(monomethoxytrityl)oxymethyl-3-hydroxycyclohexanyl]adenine [9(D)]**: Compound **9(D)** was obtained from **7(D)** as described for **8(D)** in 87% yield as a foam: LSIMS: 638 $[M-H]^-$; UV (MeOH): λ_{max} (ε) = 282 nm (21 000 mol^{-1} dm^3 cm^{-1}); ^{1}H NMR ([D$_6$]DMSO): δ = 1.40–2.30 (7H), 3.09 (1H), 3.18–3.36 (2H), 3.83 (3H), 4.18 (1H), 4.97 (1H), 6.87 (2H), 7.26–7.62 (15H), 7.94 (1H), 8.05 (2H), 8.76 (1H), 9.20 (1H); ^{13}C NMR ([D$_6$]DMSO): δ = 21.8, 27.8, 35.2, 41.1, 50.8, 55.2, 64.8, 68.7, 86.7, 113.2, 123.2, 127.0, 127.3, 127.8, 128.3, 128.8, 130.1, 132.7, 133.7, 135.4, 141.3, 144.0, 144.2, 149.4, 151.9, 152.1, 158.6, 164.6; anal. calcd for $C_{39}H_{37}N_5O_4 \cdot H_2O$: C 71.21, H 5.98, N 10.65; found C 71.55, H 5.91, N 10.63.

***N*^{6}-Benzoyl-9-[(1*S*,3*R*,4*S*)-4-(monomethoxytrityl)oxymethyl-3-hydroxycyclohexanyl]adenine [9(L)]**: Compound **9(L)** was obtained from **7(L)** as described for **8(D)** in 68% yield as a foam: LSIMS: 638 $[M-H)^-$; UV (MeOH): λ_{max} (ε) = 282 nm (20 300 mol^{-1} dm^3 cm^{-1}); ^{1}H NMR ([D$_6$]DMSO): δ = 1.40–2.30 (7H), 3.07 (1H), 3.18–3.36 (2H), 3.82 (3H), 4.17 (1H), 4.97 (1H), 6.87 (2H), 7.27–7.62 (15H), 7.94 (1H), 8.04 (2H), 8.76 (1H), 9.17 (1H); ^{13}C NMR ([D$_6$]DMSO): δ = 21.8, 27.8, 35.2, 41.1, 50.8, 55.2, 64.8, 68.8, 86.7, 113.2, 123.2, 127.0, 127.3, 127.8, 128.3, 128.8, 130.1, 132.7, 133.7, 135.4, 141.3, 144.0, 144.2, 149.3, 151.9, 152.1, 158.6, 164.6; anal. calcd for $C_{39}H_{37}N_5O_4 \cdot H_2O$: C 71.21, H 5.98, N 10.65; found C 71.19, H 5.87, N 10.69.

***N*^{6}-Benzoyl-9-{(1*S*,3*R*,4*S*)-4-(monomethoxytrityl)oxymethyl-3-[(*R*)-(*O*-methylmandelyl)oxy]-cyclohexanyl}adenine [10(L)]**: DCC (0.50 g, 2.42 mmol) was added to a solution of compound **9** (a fraction with primarily **9(L)**) (1.4 g, 2.12 mol), (*R*)-(−)-*O*-methylmandelic acid (0.40 g, 2.40 mmol) and DMAP (29 mg, 0.24 mmol) in CH_2Cl_2 (20 mL) at 0 °C. The mixture was allowed to warm to room temperature over 6 h and filtered. The filtrate was washed with aqueous H_3PO_4 (1M), water and saturated aqueous $NaHCO_3$, dried ($MgSO_4$) and filtered. Repeated chromatotron purification (THF in CH_2Cl_2, 7.5% to 15%) afforded 1.2 g (70%) of **10(L)**. The yield for the conversion of **10(L)** back to **9(L)** was 94% (0.91 g).

Compound 10(L): LSIMS (THGLY/NaOAc): 810 $[M+Na]^+$; UV (MeOH): λ_{max} (ε) = 229 nm (32 500), 281 nm (23 100 mol^{-1} dm^3 cm^{-1}); ^{1}H NMR ($CDCl_3$): δ = 1.25–2.20 (m, 12H, CH_2), 3.23 (m, 2H, CH_2 on 4), 3.47 (s, 3H, CH_3O), 4.80 (s, 3H, CH_3O), 4.64 (m, 1H, H-1), 4.89 (s, 1H, H*), 5.38 (br d, 1H, H-3), 6.86 (d, 2H, ar), 7.24–7.60 (m, 20H, ar), 7.78 (s, 1H, H-8), 8.04 (d, 2H, ar), 8.74 (s, 1H, H-2), 9.30 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ = 21.9, 27.1 (C-5, C-6), 31.7 (C-2), 36.7 (C-4), 50.5 (C-1), 55.1 (OCH_3), 57.3 (OCH_3), 62.0 (CH_2), 71.4 (C-3), 82.5 (CH mand acid), 86.3 (C MMTr), 113.1 (CH MMTr), 123.3 (A-5), 126.9, 127.0, 127.8, 128.4, 128.7, 130.1 (CH ar), 132.6, 133.6, 135.4, 136.6 (C ar), 141.0 (A-8), 144.1 (C MMTr), 149.4 (A-4), 151.7 (A-2), 152.1 (A-6), 158.5 (C MMTr), 164.7 (C=O Bz), 169.6 (COO ester); HRMS: calcd $C_{48}N_{45}N_5O_6Na$ 810.3268; found 810.3288.

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N^6-Benzoyl-9-{(1*R*,3*S*,4*R*)-4-(monomethoxytrityl)oxymethyl-3-[(*R*)-(*O*-methylmandelyl)oxy]-cyclohexanyl}adenine [10(D)]: DCC (0.25 g, 1.20 mmol) was added to a solution of compound **9** (a fraction with primarily **9(D)**) (0.64 g, 0.97 mol), (*R*)-(−)-*O*-methylmandelic acid (0.20 g, 1.20 mmol), and DMAP (15 mg, 0.12 mmol) in CH_2Cl_2 (20 mL) at 0 °C. The mixture was allowed to warm to room temperature over 7 h and filtered. The filtrate was washed with 1M aqueous H_3PO_4, water and saturated aqueous $NaHCO_3$, dried (with $MgSO_4$) and filtered. Repeated chromatotron purification (THF in CH_2Cl_2, 7.5% to 15%) afforded 0.71 g (87%) of **10(D)**. The yield for the conversion of **10(D)** to **9(D)** was 91% (0.52 g).

Compound 10(D): LSIMS (THGLY/NaOAc): 810 $[M+Na]^+$; UV (MeOH): λ_{max} (ε) = 229 nm (34300), 281 nm (23900 $mol^{-1} dm^3 cm^{-1}$); ^1H NMR ($CDCl_3$): δ = 1.60–2.10 (m, 11H, CH_2), 2.30 (m, 1H, CH_2 on 4), 3.31 (d, 2H, CH_2), 3.48 (s, 3H, CH_3O), 3.79 (s, 3H, CH_3O), 4.08 (m, 1H, H-1), 4.86 (s, 1H, H*), 5.41 (br s, 1H, H-3), 6.87 (d, 2H, ar), 7.20–7.62 (m, 21H, ar, H-8), 8.03 (d, 2H, ar), 8.66 (s, 1H, H-2), 9.34 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ = 22.1, 26.6 (C-5, C-6), 30.8 (C-2), 36.9 (C-4), 51.0 (C-1), 55.1 (OCH_3), 57.2 (OCH_3), 62.0 (CH_2), 71.4 (C-3), 82.2 (CH mand acid), 86.3 (C MMTr), 113.1 (CH MMTr), 123.5 (A-5), 126.9, 127.2, 127.8, 128.4, 128.7, 128.8, 130.1, 132.6, 133.6, 135.4, 136.5 (C ar), 141.4 (A-8), 144.2 (C MMTr), 149.4 (A-4), 151.9 (A-2 + A-6), 158.6 (C MMTr), 164.8 (C=O Bz), 169.5 (COO ester); HRMS: calcd $C_{48}N_{45}N_5O_6Na$ 810.3268; found 810.3228.

Preparation of the amidite building blocks 11(D), 11(L), 12(D) and 12(L): About 1 mmol of the modified nucleoside (see Table 6), protected at the primary alcohol, was treated with dry *N,N*-diisopropylethylamine (3 equiv) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (1.5 equiv) in dry dichloromethane (10 mL) and stirred at room temperature for 30 min. The reaction was quenched by addition of water (3 mL) and stirred further for 15 min. The mixture was washed with 5% sodium bicarbonate solution (30 mL) and saturated NaCl solution (3 × 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 (2 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, starting quantity, R_f values, mass analysis and ^{31}P NMR data are given in Table 6.

Table 6. Phosphoramidite analysis.

	mmol of starting alcohol	Yield [%]	R_f value[a]	MS[b]	^{31}P NMR shift
11(D)	1.19	84	0.50	749 $[M+Na]^+$	147.38/147.13
11(L)	1.51	93	0.50	749 $[M+Na]^+$	147.38/147.13
12(D)	0.81	65	0.51	862 $[M+Na]^+$	147.96/147.59
12(L)	1.39	79	0.51	862 $[M+Na]^+$	147.96/147.59

[a] Given for *n*-hexane/acetone/triethylamine. [b] LSIMS (positive mode, thioglycerol, NaOAc).

Analysis of cA [6(D) and the racemic mixture of 6(D) and 6(L)] by subcritical fluid chromatography (SFC):[8] The racemate was derivatized into the trifluoroacyl derivatives by adding trifluoroacetic anhydride (1 mL) to cA (4 mg) and heating at 65 °C for 30 min. After evaporation the residue was redissolved in MeOH (1 mg mL^{-1}) and analyzed. The chiral separation was achieved on a Diacel Chiralpak AD column (J. T. Baker) (250 mm × 4.6 mm i.d., 10 μm particle size) at 30 °C with a mobile phase consisting of CO_2 with modifier (0.1% trifluoroacetic acid and 0.1% triethylamine in MeOH). The SFC system consisted of a Gilson Series SF3 instrument (Gilson Medical). UV detection was done at 270 nm.

Solid-phase oligonucleotide synthesis: Oligonucleotide synthesis was performed in an ABI 381A DNA synthesizer (Applied Biosystems) by the phosphoramidite approach. The classical synthesis protocol was used except for the concentration of the newly synthesized products, which was increased from 0.1 to 0.12M. The oligomers were deprotected and cleaved from the solid support by treatment with concentrated aqueous ammonia (55 °C, 16 h). After prepurification on a NAP-10® column (Sephadex G25-DNA grade, Pharmacia) with buffer A as eluent (see below), purification was achieved on a Mono-Q® HR10/10 anion exchange column (Pharmacia) with the following gradient system [A = 10 mM NaOH, pH 12.0, 0.1M NaCl; B = 10 mM NaOH, pH 12.0, 0.9M NaCl; gradient used depended on the oligonucleotide; flow rate 2 mL min^{-1}]. The low-pressure liquid chromatography system consisted of a Merck-Hitachi L 6200 A intelligent pump, a Mono Q®-HR 10/10 column (Pharmacia), a Uvicord SII2138 UV detector (Pharmacia-LKB) and a recorder. The product-containing fraction was desalted on a NAP-10® column and lyophilized.

Melting temperatures: Oligomers were dissolved in a solution containing NaCl (0.1M), potassium phosphate (0.02M, pH 7.5) and EDTA (0.1 mM). The concentration was determined by measuring the absorbance at 260 nm at 80 °C and assuming that the cyclohexanyl nucleoside analogues have the same extinction coefficients in the denatured state as the natural nucleosides. The following extinction coefficients were used: dA and A*, ε = 15000; dT and T*, ε = 8500; dG, ε = 12500; dC, ε = 7500; U, ε = 10000 $mol^{-1} dm^3 cm^{-1}$. The concentration in all experiments was ca. 4 μM for each strand unless otherwise stated. Melting curves were determined with a Uvikon 940 spectrophotometer. Cuvettes were maintained at constant temperature by means of water circulation through the cuvette holder. The temperature of the solution was measured with a thermistor directly immersed in the cuvette. The temperature was controlled and the data acquired automatically with an IBM-compatible computer. The samples were heated and cooled at a rate of 0.2 °C min^{-1}, and no difference could be observed between the heating and cooling melting curves unless otherwise stated. Melting temperatures were evaluated by plotting the first derivative of the absorbance-versus-temperature curve.

UV mixing curves: UV mixing curves were obtained by titration of a solution (6 μM) of one strand with an equimolar solution of its complement. The solutions were made of NaCl (0.1M), potassium phosphate (0.02M, pH 7.5), EDTA (0.1 mM). The mixtures were allowed to equilibrate for 10 min at 10 °C prior to measurement at 260 nm and 10 °C in a Uvikon 940 spectrophotometer.

CD experiments: CD spectra were measured with a Jasco 600 spectropolarimeter in thermostatically controlled 1 cm cuvettes connected with a Lauda RCS6 Bath. The oligomers were dissolved and analyzed in a solution of NaCl (0.1M) and K_2HPO_4 (0.02M, pH 7.5) at 25 °C and at a concentration of 4 μM for each strand.

Optical rotation: Optical rotation was measured with a Thorn NPL Automatic Polarimeter Type 243 at a temperature of 26 °C in a mixture of MeOH/H_2O 10:1.

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