α-LNA (Locked Nucleic Acid with α-D-Configuration): Synthesis and Selective Parallel Recognition of RNA

Poul Nielsen,* Nanna K. Christensen, and Jakob K. Dalskov[a]

Abstract: α-LNA is presented as a stereoisomer of LNA (locked nucleic acid) with α-D-configuration. Three different approaches towards the thymine α-LNA monomer as well as the 5-methylcytosine α-LNA monomer are presented. Different α-LNA sequences have been synthesised and their hybridisation with complementary DNA and RNA has been evaluated by means of thermal stability experiments and circular dichroism spectroscopy. In a mixed pyrimidine sequence, α-LNA displays unprecedented parallel-stranded and selective RNA binding. Furthermore, a remarkable selectivity for hybridisation with RNA over DNA is indicated.

Keywords: locked nucleic acid · nucleosides · oligonucleotides · RNA recognition

Introduction

The increasing demand for potential antisense agents and diagnostic probes has motivated an intensive search for nucleic acid analogues showing selective and high-affinity recognition of complementary nucleic acid sequences.[1, 2] Therefore, a plethora of chemically modified oligonucleotide (ON) sequences have been introduced and investigated with regard to their specific hybridisation with complementary DNA and RNA.[2] A preliminary but extensively investigated modification has been the anomeric inverted isomer of DNA, namely α-DNA.[3–5] α-DNA sequences, or α-oligodeoxynucleotides (α-ODNs), have demonstrated efficient hybridisation with complementary nucleic acids, adopting an unusual parallel strand orientation.[3, 4] Furthermore, α-ODNs are highly resistant towards degradation by nucleases.[3] Like the nucleosides of DNA and RNA, the α-2′-deoxynucleosides in α-DNA exist in an equilibrium between the two low-energy N- and S-type conformational ranges (Scheme 1).[6] In natural β-configured nucleic acids, the former is predominant in A-type duplexes and is preferred by the ribonucleosides in RNA, while the latter is predominant in B-type duplexes and is usually preferred by the 2′-deoxynucleosides in DNA.[7] The α-2′-deoxynucleosides also display a preference for S-type conformations.[6]

Chemically modified analogues of α-DNA have also been investigated.[8–12] Thus, the introduction of 5-*C*-propynylpyrimidines into α-ODNs was found to increase the thermal affinities towards both complementary DNA and RNA sequences, as similarly reported for the corresponding β-ODNs.[9] The most significant improvements of α-DNA as regards binding to RNA and DNA have been obtained with modified phosphodiester linkages.[10] Thus, with nonionic phosphoramidate or methylphosphonate linkages, the thermal affinities of α-ODNs towards complementary RNA and, especially, DNA sequences have been significantly improved with increases in thermal stability of 0–1 °C and 1–3 °C per modification, respectively, when compared to the corresponding unmodified α-ODNs.[10] In contrast, similar modifications of the β-ODNs were found to destabilise the duplexes.[10]

In general, the most successful approach towards ODNs with high-affinity recognition of complementary nucleic acid sequences has probably been the introduction of conformationally restricted analogues.[1, 13, 14] Thus, ONs in which the nucleoside monomers contain bi- or tricyclic carbohydrate moieties have been found to display high-affinity binding of, in particular, complementary RNA.[14] The most enhanced properties to date have been obtained with LNA (locked nucleic acid) (Scheme 1).[15] LNA sequences are defined as ONs that contain one or more LNA monomers, which are nucleosides locked in an N-type conformation due to a bicyclo[2.2.1]heptane carbohydrate skeleton (see the thymine monomer **1**; Scheme 1).[15] By the introduction of LNA monomers into ONs, the formation of remarkably thermodynamically favoured duplexes with both complementary DNA and RNA has been demonstrated. Thus, both fully modified LNA sequences as well as sequences that contain only a few LNA monomers in mixmers with unmodified ribo- or 2′-deoxynucleosides display very strong thermal affinities towards both DNA and RNA, with increases in thermal stability

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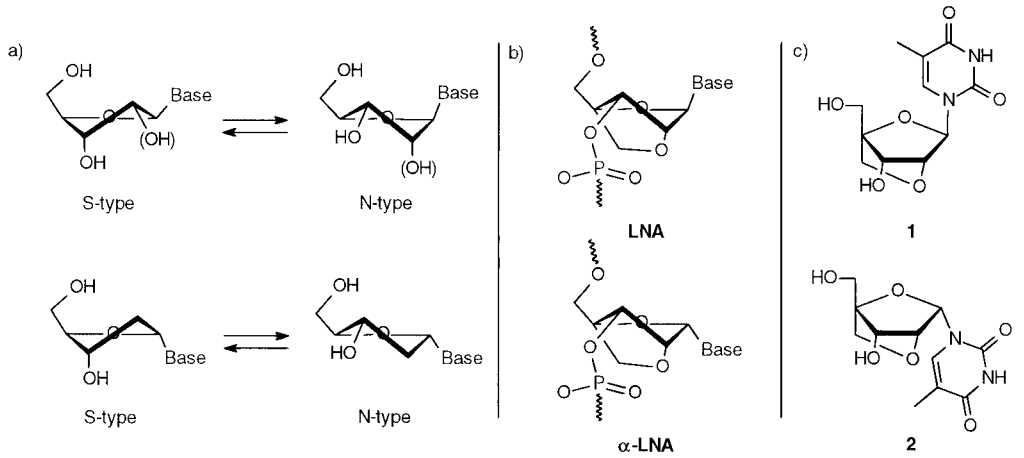


Scheme 1. a) Conformational equilibrium for nucleosides between N- and S-type conformations; top: the β-(2′-deoxy)nucleosides in DNA and RNA; bottom: the α-2′-deoxynucleosides in α-DNA; b) the structure of LNA and α-LNA; c) the α- and β-LNA thymine monomers.

of 3–8 °C per modification relative to unmodified ONs and ODNs.[15] Furthermore, LNA displays a strong resistance towards degradation by nucleases,[15] and preliminary but very promising in vivo antisense activity.[16]

Among the chemically modified analogues of α-DNA, conformationally restricted analogues that include two different α-configured bicyclic nucleoside monomers[11] have also been introduced, but no improved DNA or RNA binding was observed.[8, 11] However, these nucleoside analogues were restricted to S-type conformations.[8, 11] No analogue of α-DNA in which the α-nucleoside monomers are restricted in N-type conformations has hitherto been described. However, the N3′-P5′-phosphoramidite linkage, which has been a very successful modification of β-ODNs due to their induced preference to adopt N-type conformations,[17] has also been introduced in α-ODNs.[12] Nonetheless, the latter seem to prefer the S-type conformation, at least for pyrimidine nucleotides, and large decreases in thermal affinity of mixed pyrimidine sequences towards both DNA and RNA were observed.[12] On the other hand, purine nucleotides seem to prefer N-type conformations, and a fully N3′-P5′-modified αA_{10} displays almost the same affinity towards complementary DNA and RNA as the native αA_{10}.[12]

As a consequence of these results, a chemically modified analogue of α-DNA with a strong conformational restriction in the N-type conformational range might form the basis for high-affinity parallel recognition of DNA and RNA sequences. Evidently, this could be addressed by the preparation and oligomerisation of the anomeric inverted isomer of LNA, that is α-LNA (or α-D-LNA; see the general structure and the thymine monomer **2**, Scheme 1), in which the bicyclo[2.2.1]-heptane carbohydrate skeleton ensures a locked N-type conformation. Thus, the stereoisomers of LNA, including the enantiomer of α-LNA, that is α-L-LNA,[18] have recently been demonstrated to be a class of very efficient RNA-binding nucleic acid analogues.[19] Recently, in a preliminary form, we introduced the synthesis and hybridisation properties of α-LNA containing only the thymine monomer **2**.[20, 21] Thus, a fully modified α-LNA sequence ($\alpha\mathbf{T}^{\mathbf{L}}_{\mathbf{10}}$) was found to form a very stable duplex with complementary RNA (rA_{14}). The increase in thermal stability found was 1.2 °C per modification relative to the corresponding α-DNA sequence (αT_{10}) and 2.5 °C per modification relative to T_{10} (Table 1).[20] Thus, α-LNA was found to display an affinity towards RNA comparable to that of other stereoisomers of LNA,[19] and although not quite as high as that of the original LNA,[15, 19] this is unprecedented among α-D-configured nucleic acid analogues. However, these preliminary results could neither answer the question as to whether α-LNA prefers parallel or antiparallel strand orientation, nor explain the apparently strong selectivity for hybridisation with complementary RNA over DNA. In this paper, the synthesis of α-LNA is described in full detail, including different synthetic approaches towards two different pyrimidine monomers. Furthermore, the hybridisation properties of both oligothymidylate as well as mixed pyrimidine α-LNA sequences with DNA and RNA complements are assessed by means of thermal stability experiments and circular dichroism (CD) spectroscopy.

Table 1. Hybridisation data of α-LNA sequences.

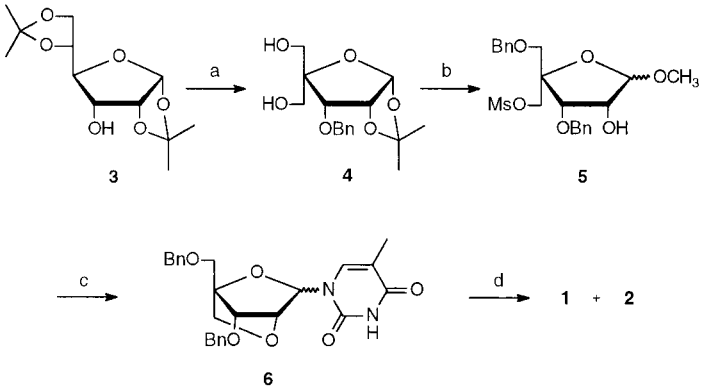
	ODN sequences	DNA (dA_{14}) T_m (ΔT_m) [°C][a]	RNA (rA_{14}) T_m (ΔT_m) [°C][a]	mm RNA (rA_6CA_7) T_m (ΔT_m) [°C][a]
21	5′-T_{14}	33.0	30.0	–
22	5′-αT_{14}	32.0	43.0	–
23	5′-$\alpha T_7\mathbf{T^L}T_6$	25.5 (−6.5)[b]	35.0 (−8.0)[b]	–
24	5′-$\alpha T_5\mathbf{T^L_4}T_5$	26.0 (−1.5)[b]	24.5 (−4.6)[b]	–
25	5′-T_{10}	22.0	20.0	–
26	5′-αT_{10}	18.0	33.5	22.0 (−11.5)[e]
27	5′-$\alpha\mathbf{T^L_{10}}$	< 10	45.0 (+1.2[c]; +2.5[d])	37.0 (−8.0)[f]

[a] Melting temperatures obtained from the maxima of the first derivatives of the melting curve (A_{260} vs. temperature) recorded in a buffer containing Na_2HPO_4 (10 mM), NaCl (100 mM), EDTA (0.1 mM), pH 7.0 with 1.5 μM concentrations of each strand. Values in brackets show the changes in T_m values per modification compared with the reference strands. [b] Relative to **22**. [c] Relative to **26**. [d] Relative to **25**. [e] Relative to **26**: rA_{14}. [f] Relative to **27**: rA_{14}.

Results

Chemical synthesis: For the synthesis of α-LNA monomers, several starting materials were considered. Thus, in a preliminary study, the coupling of thymine to precyclised bicy-

clo[2.2.1]heptane carbohydrate precursors was investigated.[22] However, this approach has not been optimised to give the target compound **2** in reasonable yields,[22] and a simpler strategy has been applied.[20, 21] Thus, the same initial steps were used and diacetone-D-allose **3** was converted to the diol **4**[23] in four standard steps. In three further known steps, including a selective benzylation,[24] **4** was converted to an anomeric mixture of methyl furanosides **5** (Scheme 2).[22, 25]

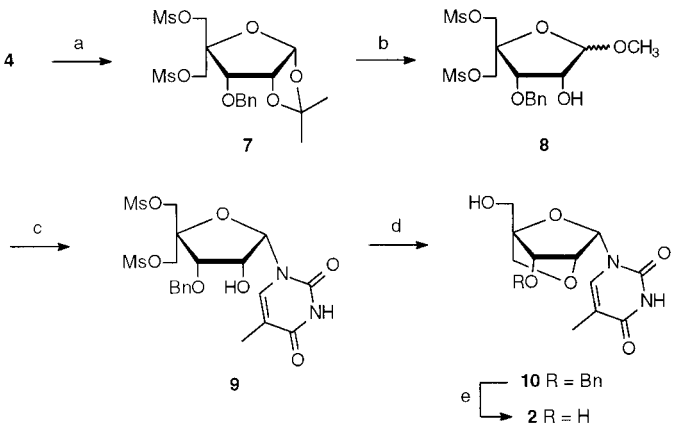


Scheme 2. Synthesis of α- and β-LNA thymine monomers **1** and **2**: a) ref. [23], 4 steps, approximately 80%; b) refs. [22, 24, 25], 3 steps, approximately 65%; c) i) TMSCl, *N*,*O*-bis(trimethylsilyl)acetamide, thymine, MeCN, then TMS triflate; ii) TBAF, THF; iii) NaH, DMF, 57% from **5**; d) H_2, $Pd(OH)_2$/C, EtOH, 97%.

Subsequently, and as an alternative to cyclisation,[22] **5** was directly used as the substrate in a modified Vorbrüggen-type coupling reaction with thymine involving in situ silylation of the 2-hydroxyl group and the nucleobase and with TMS triflate as a Lewis acid catalyst. After a long reaction time (4 days), a mixture of nucleosides was obtained which, after desilylation, was treated with sodium hydride to afford an anomeric mixture (α:$\beta \approx 1.3$:1) of bicyclic nucleosides **6** in a reasonable yield (57%) over the three steps. Other conditions were investigated for the coupling reaction, but neither the yield nor the anomeric ratio were improved. The benzyl ethers of **6** were cleaved by hydrogenation to give, after chromatographic separation, the two anomeric LNA monomers **1**[15] and **2** in high yields.[20]

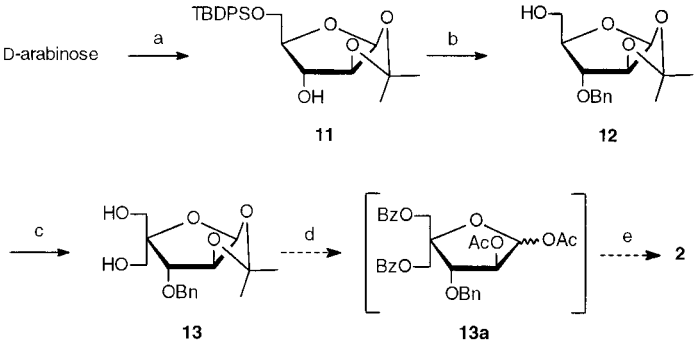
In order to avoid the selective benzylation in the preparation of **5**, another approach was investigated using a bis(methylsulfonic) ester (Scheme 3). Thus, compound **4** was esterified to give **7**,[26] which was further converted to a mixture of methyl furanosides **8**. Various conditions for the coupling of thymine to this substrate were investigated, without any significant success, and after chromatographic purification the α-nucleoside **9** could only be obtained in a relatively low yield. Nevertheless, **9** was converted to the target bicyclic nucleoside **2** by treatment with strong aqueous base to simultaneously bring about ring-closure and removal of the remaining sulfonic ester group to give **10**, followed by hydrogenation. No explanation was found for the low yields in the coupling of thymine with **8** and it was concluded that the original route via **5** (Scheme 2) was in fact superior.

Even though the first route (Scheme 2) afforded the target α-LNA monomer **2** in a reasonable overall yield, the



Scheme 3. Alternative synthesis of α-LNA thymine monomer **2**: a) MsCl, pyridine, 92%; b) HCl, MeOH, H_2O, 95%; c) i) TMSCl, *N*,*O*-bis(trimethylsilyl)acetamide, thymine, MeCN, *then* TMS triflate; ii) TBAF, THF, 24% from **8**; d) NaOH, EtOH, H_2O, 46%; e) H_2, $Pd(OH)_2$/C, EtOH, dioxane, 91%.

nucleobase coupling reaction occurred without significant stereoselectivity. Therefore, another approach was investigated in which a substrate was preconstructed for a stereoselective synthesis of an appropriate α-D-configured nucleoside (Scheme 4). Thus, the enantiomer of the target nucleoside **2** (the α-L-LNA thymine monomer)[18] has recently been



Scheme 4. Synthesis of a potential precursor for α-LNA monomers: a) ref. [31], 3 steps, approximately 50%; b) ref. [32], 2 steps, 62%; c) i) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2; ii) CH_2O, NaOH, H_2O, THF, 63% from **12**; d) ref. [29], 3 steps on enantiomers, 82%; e) refs. [27, 29], 5 steps on enantiomers, 40%.

synthesised[27] by using diacetone-D-glucose as the starting material, which was converted to the 3′-epimer of **4** (the enantiomer of **13**; Scheme 4)[28] as an intermediate product.[27, 29] Therefore, **2** should be accessible from **13**, suggesting the use of another carbohydrate precursor that may be converted to **13**. As the only reasonably cheap starting material with this potential, D-arabinose was chosen. In two[30] or three[31] steps, this was converted to the furanose derivative **11**, which was further converted to **12** (Scheme 4).[32] Subsequently, the primary alcohol function of **12** was oxidised using the Swern protocol to give an aldehyde and, after the usual aldol condensation and Cannizzarro reaction, **13** was obtained in good yield. We have not employed this material in the synthesis of **2**, but as **13**, by comparison of its NMR data, was shown to be the enantiomer

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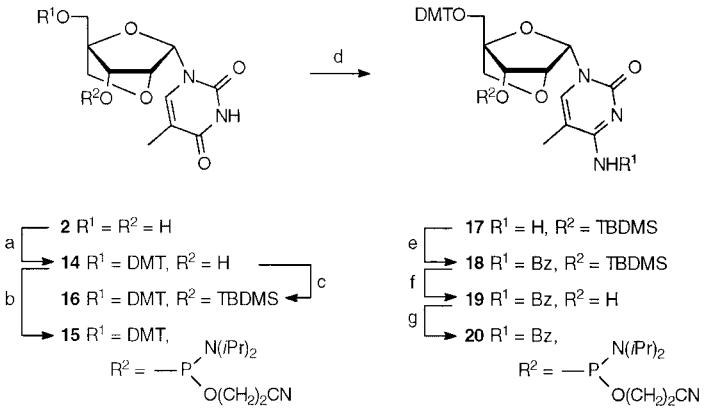
of a known compound,[28] and as that compound has been used in the stereoselective eight-step synthesis of the α-L-LNA thymine monomer,[27, 29] that is, through stereoselective coupling of thymine to the enantiomers of **13a** and without the application of any chiral reagents, we concluded that this strategy could afford **2** in the same overall yield. However, and despite the lack of stereoselectivity, the original method (Scheme 2) is still considered as superior due to the fact that it requires fewer reaction steps and the key intermediate **4**[23] is more readily available compared to its stereoisomer **13**.

To incorporate the α-LNA monomer into oligonucleotides, **2** was converted to the appropriately protected phosphoramidite derivative (Scheme 5). Thus, the primary alcohol function of **2** was easily protected with the DMT (4,4′-dimethoxytrityl) group to afford **14**, which was further converted to the phosphoramidite **15**.[20] In order to permit the preparation of mixed sequences, the well-known conversion of a thymine moiety to a 5-methyl cytosine moiety was evaluated.[33] However, the reported direct conversion of thymidine phosphoramidite derivatives to give triazole precursors of 5-methyl cytidine phosphoramidite derivatives in one step[34, 35] was found not to be successful in this case; instead a conventional multistep conversion was employed (Scheme 5).[33, 35, 36] Thus, the secondary alcohol function of **14** was converted to a silyl ether **16** and further converted to the 5-methyl cytidine derivative **17** via a triazole intermediate. Protection of the exocyclic amino group of **17** as a benzoyl amide proved surprisingly troublesome and treatment with benzoyl chloride or benzoic anhydride in combination with pyridine or DMAP did not afford **18** in more than 20% yield. However, the use of the recently reported *N*-benzoyltetrazole for this purpose[37] afforded **18** quantitatively. Subsequent desilylation to give **19** was followed by phosphitylation to give **20** as a building block for oligonucleotide synthesis.[38, 39]



Scheme 5. Synthesis α-LNA phosphoramidite building blocks **15** and **20**. a) DMTCl, $AgNO_3$, pyridine, THF, DMF, 71%; b) $NC(CH_2)_2OP(Cl)$-$N(iPr)_2$, $EtN(iPr)_2$, CH_2Cl_2, 96%; c) TBDMSCl, imidazole, DMF, 79%; d) i) 1,2,4-triazole, $POCl_3$, pyridine; ii) NH_3, H_2O, dioxane, 44% from **16**; e) *N*-benzoyltetrazole, CH_3CN, 100%; f) TBAF, THF, 83%; g) see b), 60%.

For the automated solid-phase synthesis of oligonucleotides by the phosphoramidite approach,[40] the two α-LNA monomeric building blocks **15** and **20** were used in combination with the corresponding unmodified α-2′-deoxynucleoside thymine and 5-methyl cytosine phosphoramidites, which were obtained according to literature methods.[36, 41] Hence, α-DNA and α-LNA sequences were obtained with >98% stepwise coupling yields (Tables 1 and 2) by using tetrazole activation and coupling times of 10–15 min for **15** and **20**. All modified oligonucleotides were obtained on universal CPG support (Biogenex) using the DMT-*ON* mode. This allowed the synthesis of fully modified sequences after cleavage from the solid support using LiCl in aqueous ammonia. The oligomers were purified by using disposable reversed-phase chromatography cartridges (Cruachem) yielding products with >90% purity as judged on the basis of capillary gel electrophoresis. The compositions of all the α-DNA and α-LNA sequences were verified from their MALDI mass spectra.

Thermodynamic stability: The hybridisation between α-LNA sequences and unmodified complementary DNA and RNA sequences was explored by means of thermodynamic stability examinations (Tables 1 and 2). Thus, the oligothymidylate α-LNA sequences **23**, **24** and **27**, as well as their α-DNA counterparts **22** and **26**, were mixed with the corresponding DNA and RNA complements, and the melting temperatures of the complexes were determined (Table 1).[20] As described in our preliminary communication,[20] the affinities of the α-ODNs **22** and **26** towards the corresponding 14-mer DNA sequence were, as expected,[4, 5] slightly decreased relative to those of the unmodified ODNs **21** and **25**. On the other hand, and as reported in the literature,[4, 5] the same α-DNA sequences displayed a significant increase in affinity towards complementary RNA. One α-LNA monomer incorporated in an otherwise unmodified α-DNA sequence, **23**, resulted in large decreases in affinity when compared to the unmodified sequence **22**. However, a block of α-LNA monomers, as in sequence **24**, diminished the relative decreases in affinity introduced by each modification towards both DNA and RNA.[20] As mentioned above, the fully modified decameric α-LNA sequence **27** displayed large increases in binding affinity to complementary RNA.[20, 42] On the other hand, no stable α-LNA:DNA complex was observed. The melting curves leading to these results are shown in Figure 1. The very clear sigmoidal melting transitions indicate the formation of duplexes between both α-DNA and α-LNA with complementary RNA. The observed hyperchromicity is even more pronounced with α-LNA than with α-DNA. On the other hand, the rather unstable duplex between α-DNA and RNA is only slightly indicated, whereas no transition can be observed for the mixture of α-LNA and complementary DNA. A very weak transition at around 45 °C might be suggested from the curve, but as mentioned in our former communication,[20] the presence of a duplex can, in our opinion, be excluded by the fact that this transition is not seen at a higher temperature when measured at a higher ionic strength, in contrast to the other transitions (data not shown).

Hybridisation data for the mixed decameric pyrimidine α-LNA sequences (Table 2) demonstrate the preference for parallel-stranded duplex formation for both α-DNA and α-LNA. Thus, this decameric sequence was chosen as a non-self-complementary and nonpalindromic sequence requiring only

Table 2. Hybridisation data of mixed α-LNA sequences.

	ODN sequences	DNA (p)[a] dGAGGAAGAAA T_m (ΔT_m) [°C][c]	DNA (ap)[b] dAAAGAAGGAG T_m (ΔT_m) [°C][c]	RNA (p)[a] rGAGGAAGAAA T_m (ΔT_m) [°C][c]	RNA (ap)[b] rAAAGAAGGAG T_m (ΔT_m) [°C][c]	mm RNA (p)[a] rGAGGCAGAAA T_m (ΔT_m) [°C][c]
28	5′-^{m}CT^{m}C^{m}CTT^{m}CTTT	< 10	36.0	16.0	44.5	–
29	5′-α-^{m}CT^{m}C^{m}CTT^{m}CTTT	43.0	< 10	32.0	< 10	–
30	5′-α-^{m}CT^{m}C^{m}CT**T**^{Lm}CTTT	30.5 (−12.5)[d]	< 10	26.0 (−6.0)[d]	< 10	–
31	5′-α-^{m}C**T**^{Lm}C^{m}C**T**^{L}**T**^{Lm}C**T**^{L}**T**^{L}**T**^{L}	< 10	< 10	22.0 (−1.7)[d]	< 10	–
32	5′-α-^{m}**C**^{L}**T**^{Lm}**C**^{Lm}**C**^{L}**T**^{L}**T**^{Lm}**C**^{L}**T**^{L}**T**^{L}**T**^{L}	< 10	< 10	61.5 (+2.9)[d]	< 10	47.0 (−14.5)[e]

[a] p = parallel. [b] ap = antiparallel. [c] See Table 1. Values in brackets show the changes in T_m values per modification compared with the reference strands. [d] Relative to **29**. [e] Relative to **32**: RNA(p).

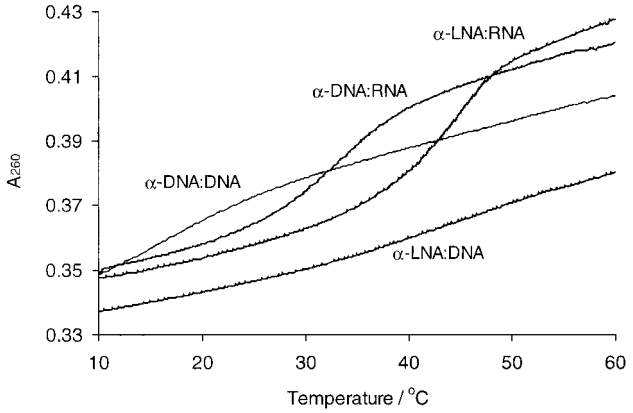


Figure 1. Absorption versus temperature curves displaying the melting profiles of homothymidine–homoadenine duplexes. α-DNA corresponds to **26**, α-LNA corresponds to **27**, DNA and RNA correspond to dA_{14} and rA_{14}, respectively.

two different building blocks, and the 5-methylcytosine monomer was chosen instead of cytosine due to its ready availability from the corresponding thymine monomer (vide supra) and as the 5-methyl groups can be expected to further stabilise the duplex structures.[1, 2] As expected,[4] the unmodified α-ODN **29** only formed stable duplexes with complementary parallel DNA and RNA sequences, whereas the corresponding unmodified ODN **28** formed the most stable duplexes with antiparallel complements. When one α-LNA thymine monomer was incorporated, the sequence **30** showed an even larger decrease in affinity towards the parallel DNA complement than that observed for the oligothymidylate sequence **23** (−12.5 °C per modification compared to −6.5 °C). For **30**, however, and in contrast to **23**, the decrease in thermal stability was smaller with complementary RNA. A mixed sequence **31** containing six α-LNA T-monomers and four conventional α-DNA 5-methylcytosine monomers displayed no recognition of either parallel or antiparallel complementary DNA, whereas a duplex was obtained with parallel RNA, for which the relative decrease in thermal stability introduced by each bicyclic monomer was smaller than that observed for **30** (−1.7 °C per modification compared to −6.0 °C).

The fully modified mixed α-LNA sequence **32** displayed, like **27**, no binding to complementary DNA, whereas a very stable parallel-stranded duplex was formed with complementary RNA. Thus, an increase in thermal stability of almost 3 °C for each bicyclic monomer was observed in comparison with the α-DNA sequence **29**. The melting transitions are displayed in Figure 2. Again, a very clear transition verifies duplex formation between the mixed α-DNA sequence and complementary parallel RNA, but in this case the corresponding α-LNA:RNA duplex, despite having a much higher

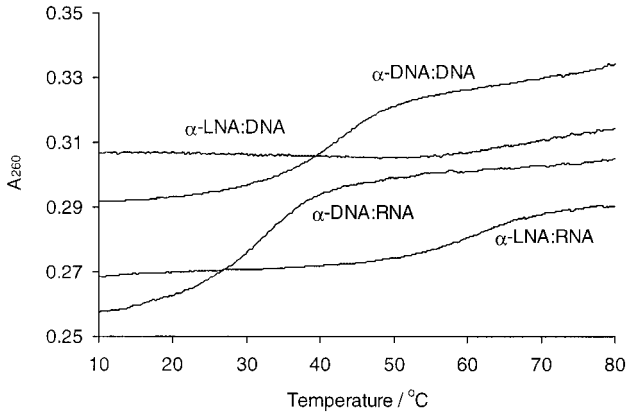


Figure 2. Absorption versus temperature curves displaying the melting profiles of duplexes formed by the mixed sequences. α-DNA corresponds to **29**, α-LNA corresponds to **32**, DNA and RNA correspond to their parallel complements (see Table 2).

thermal stability, displays a smaller hyperchromicity. A very clear transition between α-DNA and complementary parallel DNA can be observed, whereas no indication whatsoever of any complex between α-LNA and DNA can be observed. No sign of a melting transition was observed with **32** alone (not shown). As with the homothymidine sequence **27** (Table 1),[20] the presence of a duplex with RNA rather than an intramolecular complex was further verified by the observation that a stable duplex was also formed with a single mismatch RNA sequence with an expected decrease in thermal stability (−14.5 °C, Table 2). Furthermore, no significant changes in thermal stability were observed for the duplexes of **29** and **32** with RNA when measured at pH 5.6 (data not shown).

Circular dichroism: CD spectra were obtained for all the uniformly modified α-DNA, α-LNA, DNA and RNA sequences as single strands and in the different mixtures using the same buffers and strand concentrations as in the melting experiments. Selected spectra are shown in Figures 3 and 4.

As expected, the CD spectrum of the homothymidine α-LNA:RNA duplex (Figure 3) is an almost perfect mirror image of the spectrum reported earlier for the similar sequence of α-L-LNA mixed with L-RNA.[42] The correspond-

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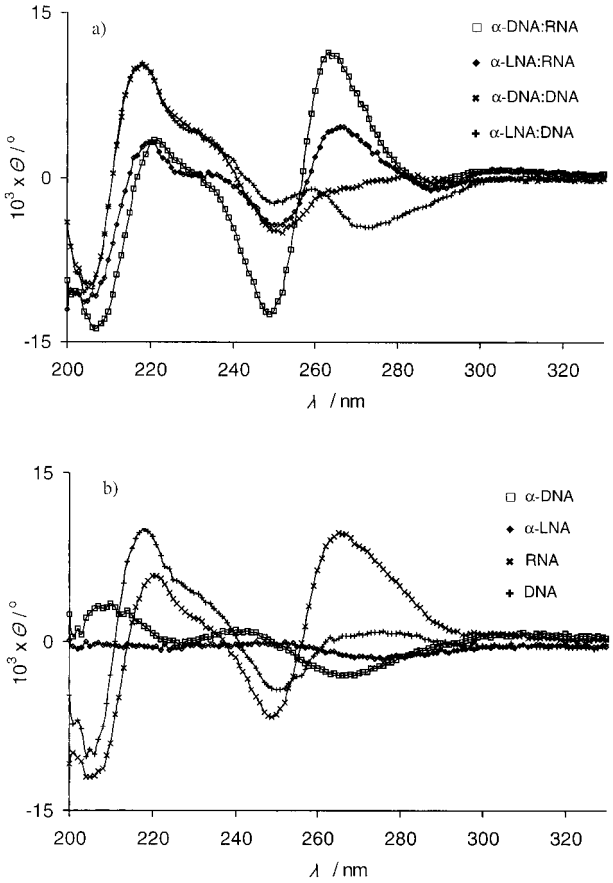

Figure 3. CD spectrocopy of homothymidine–homoadenine sequences: a) CD spectra recorded at 25 °C for the duplexes and mixtures. b) CD spectra recorded at 25 °C for the single strands. α-DNA corresponds to **26**, α-LNA corresponds to **27**, DNA and RNA correspond to dA_{14} and rA_{14}, respectively.

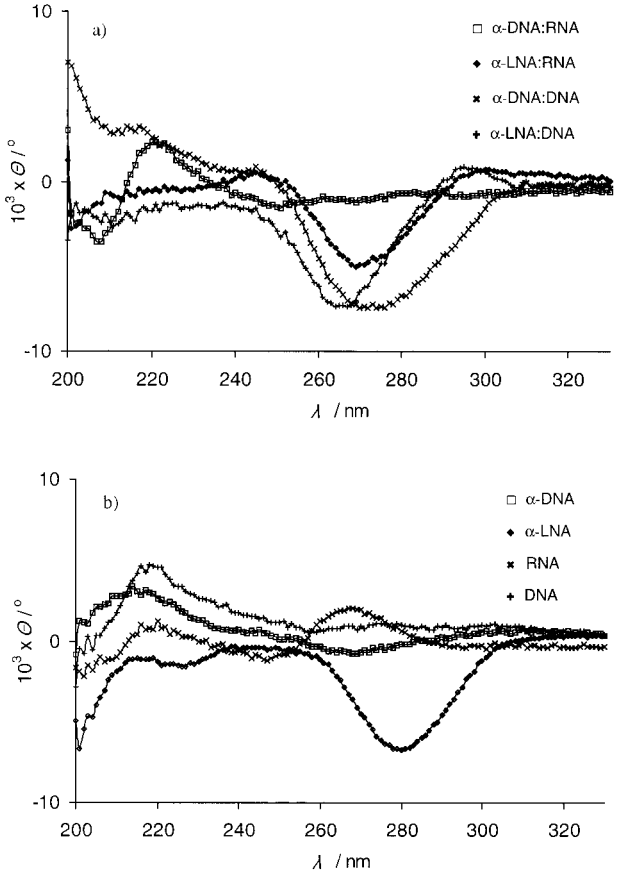

Figure 4. CD spectrocopy of the mixed sequences: a) CD spectra recorded at 25 °C for the duplexes and mixtures. b) CD spectra recorded at 25 °C for the single strands. α-DNA corresponds to **29**, α-LNA corresponds to **32**, DNA and RNA correspond to their parallel complements (see Table 2).

ing spectrum of the α-DNA:RNA duplex displays some similarities, but with a much larger negative ellipticity value at 250 nm and a much larger positive ellipticity value at 265 nm. As expected, this spectrum is very similar to that reported for a similar complex.[5] For the mixtures of homothymidine α-DNA and α-LNA with DNA, the CD spectra are relatively similar, although the latter displays a negative ellipticity value at 270 nm. As the spectra were obtained at 25 °C, no duplexes should be present in the latter two cases. For the single strands, no similarities between α-DNA and α-LNA were observed, the latter displaying a remarkably low general CD activity giving only a small band at around 270 nm (Figure 3).

For the mixed sequences (Table 2), a different picture emerges (Figure 4). Thus, for the α-LNA:RNA duplex, an unusual CD spectrum is obtained, dominated by a large negative ellipticity value at 270 nm. The spectrum of the corresponding α-DNA:RNA duplex is completely different, with a large positive ellipticity value at 220 nm. The α-LNA:DNA mixture gives rise to a CD spectrum with some similarities to that obtained with α-LNA:RNA, but it also shows a positive ellipticity value at 245 nm. The α-DNA:DNA duplex also gives rise to a somewhat similar CD spectrum. The single-stranded α-LNA has a CD spectrum dominated by the same large negative ellipticity value, but slightly shifted towards 280 nm. However, this spectrum displays no similarities with the spectra of the single-stranded α-DNA or of the complementary DNA and RNA sequences.

Discussion

Four synthetic approaches towards the α-LNA thymine monomer **2** have been investigated, three of which are described in this paper. In the first (Scheme 2), **2** was obtained in eleven steps and 15% overall yield. In the alternative approach avoiding the selective benzylation (Scheme 3), **2** was obtained in ten steps but only 7% overall yield. Following the third approach (Scheme 4), the synthesis of **2** was not accomplished but a precursor has been obtained, and, in theory, **2** could be obtained in 14 steps and 6.5% overall yield. Therefore, despite the two inconvenient isomer separations, the first approach (Scheme 2) has in general been applied for the bulk synthesis of **2**. However, the latter method (Scheme 4) might be superior in the forthcoming synthesis of the remaining pyrimidine and purine monomers of α-LNA, as the diacetylate **13a** might be much more efficient as a glycoside donor in nucleobase coupling reactions compared to the methyl furanoside **5**, when the other nucleobases are

applied. As expected, the α-LNA phosphoramidites **15** and **20**, like the parent LNA phosphoramidites,[15] can be very conveniently used in automated solid-phase synthesis of oligonucleotides.[43]

The hybridisation data (Tables 1 and 2, Figures 1 and 2) clearly demonstrate that α-LNA forms very stable complexes with complementary RNA. However, and in contrast to the behaviour of LNA in a context of 2′-deoxynucleotides,[15] ODNs with single α-LNA monomers in combination with α-2′-deoxynucleotides, that is α-LNA/α-DNA mixmers, form destabilised duplexes. Compared to the nucleosides in α-DNA, the nucleosides in α-LNA have been locked in an N-type conformation, which is unprecedented for α-DNA analogues. This conformation dictates a completely different duplex structure of the fully modified α-LNA:RNA duplexes, as indicated by the CD spectra. In general, CD spectroscopy gives a convenient but only very superficial indication of the structural behaviour of nucleic acids. However, the CD spectra of the α-LNA:RNA duplexes (Figures 3 and 4), although not very conclusive, strongly indicate a structure not resembling either the corresponding α-DNA:RNA duplexes or typical A- or B-type duplexes. Thus, the base-pairing mode followed is not necessarily the Watson–Crick type found in, for example, a parallel α-DNA:DNA duplex.[44] Nevertheless, Hoogsteen or reversed-Hoogsteen base-pairing can be excluded by the fact that in a preliminary experiment neither the mixed pyrimidine α-LNA:RNA duplex nor the α-DNA:RNA duplex displayed any increase in thermal stability when measured at a lower pH. As exemplified by a bicyclo-DNA system,[45] Hoogsteen or reversed-Hoogsteen base-pairing systems display a strong pH dependence as protonated cytidines have to be involved in the base-pairing. Moreover, the CD spectra of the two single-stranded α-LNA oligomers (Figures 3 and 4) are very peculiar, especially with regard to the very low overall ellipticities for the oligothymidylate sequence **27**. Furthermore, remarkable differences are generally observed between oligothymidylate and mixed sequences. This indicates only that the perturbation of the nucleobases by the chiral centres, especially the anomeric centre, which dictates the shape of the CD spectrum, is very different for the locked compared to the unlocked nucleotides and is highly dependent on the nucleotide sequence. Thus, the structure of the α-LNA:RNA hybrid as well as of the α-LNA single strands remains unknown and further studies on the exact duplex structure will be performed in due course.

The locked N-type conformation of the α-LNA monomer might also provide a logical explanation for the unfavourable hybridisation behaviour of α-LNA/α-DNA mixmer ODNs. Thus, for the parent LNA in LNA/DNA mixmers, NMR studies have demonstrated the ability of the LNA monomers to alter the conformational equilibrium of neighbouring 2′-deoxynucleotides in favour of N-type conformations.[46] This might be the reason for the very thermodynamically stable duplexes formed by LNA/DNA mixmers with complementary DNA and RNA (vide supra).[15] Apparently, the α-LNA monomers, which are also locked in N-type conformations, cannot induce a similar conformational shift on the neighbouring unmodified α-2′-deoxynucleotides, at least not a shift that is favourable for duplex formation. This is in agreement with the postulate that, due to stereoelectronic effects, α-2′-deoxynucleosides are more restricted towards S-type conformations than the β-2′-deoxynucleosides.[6] Thus, the anomeric effect and the O3′–O4′ *gauche* effect both push the α-2′-deoxynucleosides towards S-type conformations,[6] whereas they work in opposite directions for β-2′-deoxynucleosides.[6, 7] Therefore, the N-type conformations cannot favourably be obtained for the α-2′-deoxynucleotides, and unfavourably preorganised oligonucleotides with a mixture of N- and S-type conformations are obtained. Thus, when comparing the parallel duplexes obtained with RNA, **32** with **31**, an extraordinary decrease in thermal stability of $-10\,^{\circ}C$ for each inserted unmodified α-2′-deoxynucleotide can be observed. This further confirms the assumption that the pure α-LNA:RNA duplex has a completely different structure compared to the α-DNA:RNA duplex. This property of α-LNA diminishes the potential of tuning the parallel recognition of RNA by the design of mixmer sequences in the same way as is possible for LNA/DNA mixmers.[15, 16]

In general, α-LNA in the present sequences displays the highest affinity towards RNA obtained with any α-D-configured oligonucleotide analogue. Furthermore, the α-LNA:RNA duplex probably displays the hitherto most stable parallel-stranded duplex. Importantly, the selectivity for parallel over antiparallel complementary sequences seems to be remarkably high on the basis of our data (Table 2). Furthermore, the discrimination of single mismatches in the recognition of complementary parallel RNA, though established with only two C–T mismatches (Tables 1 and 2), seems to be comparable to the selectivity obtainable for natural ONs. For the present α-LNA sequence **32**, some duplex stabilisation is probably obtained through the complete 5-methylation of the pyrimidines. However, and even though this has to be verified experimentally, we certainly expect other mixed sequences containing the remaining natural pyrimidine and purine nucleobases to reveal similar stabilisation through the locked N-type conformations. In this way, α-LNA reveals a potential for a new concept in the design of selective nucleic acid recognising ONs for use as antisense agents or diagnostic probes. Parallel-stranded recognition is as yet a relatively unexplored and perhaps underestimated phenomenon in this context.

A strong selectivity of α-LNA for RNA over DNA has also been strongly indicated. Thus, the thermal transition curves (Figures 2 and 3) indicate no melting of a duplex in the case of the fully modified α-LNA sequences **27** and **32** with complementary DNA. The CD spectra are not very informative. Nevertheless, the CD spectrum of the α-LNA:DNA mixture (**27**:rA_{14}, Figure 4) may be interpreted as the expected sum of the spectra of the two single strands, thereby confirming that no complex between the strands is present. However, for the mixed α-LNA:DNA mixture (**32**:pDNA, Figure 5), the same conclusion cannot immediately be drawn from the CD spectra, and the alternative that complexes not detectable by UV at 260 nm might in theory be present has to be considered. Thus, a duplex structure in which the overall base-stacking is not very different to the combined base-stacking of the two single strands would give a very small and undetectable hyperchromicity in the melting transition. Nevertheless,

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we suggest on the basis of the present data that an unprecedented selectivity for RNA has been introduced. This property certainly deserves further investigation as an RNA-selectivity so pronounced is unprecedented and potentially very useful in the design of therapeutic ONs and diagnostic probes.

Conclusion

The synthesis of two α-LNA monomers and oligomerisation of these into α-LNA oligonucleotide sequences have been efficiently accomplished. In this way, the successful concept of locking the nucleoside monomers of oligonucleotides in N-type conformations by the use of the LNA-type bicyclic nucleoside structure has proven its strength in the α-DNA series as well. Thus, α-LNA displays an unprecedented parallel recognition of RNA and forms the strongest parallel orientated duplex known. A new concept for high-affinity nucleic acid recognition has thus been introduced. This extraordinary nucleic acid recognising behaviour of α-LNA, including the apparent selectivity for complementary RNA over DNA, is conducive to further investigations, as well as to the synthesis of mixed α-LNA sequences containing all the natural nucleobases.

Experimental Section

All commercial reagents were used as supplied. All reactions were performed under an atmosphere of nitrogen. Column chromatography was carried out by using glass columns packed with silica gel 60 (0.040–0.063 mm). NMR spectra were obtained on a Bruker AC250, a Varian Gemini2000, or a Varian Unity500 spectrometer. Chemical shifts are quoted relative to TMS as an internal standard. ^{1}H,^{1}H-COSY and ^{1}H NOE difference spectra were recorded for compound **2**. Assignments of NMR signals follow standard carbohydrate and nucleoside style. However, bicyclic compounds are named according to the von Baeyer nomenclature. FAB mass spectra were recorded in positive-ion mode on a Kratos MS50TC spectrometer; EI mass spectra were recorded on an SSQ710 Finnigan MAT spectrometer. High-resolution MALDI mass determinations were performed on an Ionspec Ultima Fourier transform mass spectrometer. Microanalyses were performed at The Microanalytical Laboratory, Department of Chemistry, University of Copenhagen.

(3*R*/*S*)-(1*S*,4*R*,7*S*)-7-Benzyloxy-1-benzyloxymethyl-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (6): A mixture of methyl furanosides **5**[22, 25] (6.79 g, 15.0 mmol) and thymine (3.78 g, 30.0 mmol) was dried and dissolved in anhydrous CH_3CN (55 mL). TMSCl (1.90 mL, 15.0 mmol) and *N*,*O*-bis(trimethylsilyl)acetamide (29.3 mL, 120 mmol) were added and the mixture was stirred at 60 °C for 1 h. The solution was then cooled to 0 °C and TMS triflate (13.6 mL, 75 mmol) was added dropwise. The mixture was stirred at 70 °C for 4 days and then quenched by pouring it into an ice-cold saturated aqueous solution of $NaHCO_3$ (300 mL). The resulting mixture was extracted with dichloromethane (400 mL) and the organic fraction was washed with saturated aqueous $NaHCO_3$ solution (2 × 300 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was redissolved in THF (40 mL) and to this solution was added a 1.0 M solution of TBAF in THF (15.0 mL). The resulting mixture was stirred for 1 h and then diluted with dichloromethane (250 mL). The mixture obtained was washed with saturated aqueous $NaHCO_3$ solution (2 × 250 mL) and water (2 × 250 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was redissolved in anhydrous DMF and the solution was stirred at 0 °C. A 60% dispersion of NaH in mineral oil (1.20 g, 30 mmol) was added and the mixture was stirred at room temperature for 16 h. Dichloromethane (250 mL) was then added and the mixture was washed with saturated aqueous $NaHCO_3$ solution (2 × 200 mL) and water (2 × 200 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 96:4) to afford the product as a white solid in an anomeric mixture (3.90 g; 57%, α:$\beta \approx$ 1.3:1). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 9.05 (br s), 8.98 (br s), 7.49 (d, ^3J(H,H) = 1.0 Hz), 7.46 (d, ^3J(H,H) = 1.0 Hz), 7.36–7.26 (m), 5.95 (s), 5.65 (s), 4.67–4.50 (m), 4.23 (s), 4.16 (d, ^3J(H,H) = 6.3 Hz), 4.03–3.96 (m), 3.85–3.80 (m), 1.95 (s), 1.63 (s); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C, TMS): δ = 163.83, 150.49, 149,83, 137.53, 136.99, 135.50, 134.88, 128.65, 128.59, 128.56, 128.23, 128.15, 128.03, 127.87, 127.76, 127.73, 127.71, 110.21, 109.72, 89.38, 87.40, 87.30, 87.20, 79.53, 75.61, 73.80, 73.40, 72.18, 71.99, 65.55, 64.46, 12.60, 12.15; MS FAB: *m*/*z* (%): 451(70) $[M+H]^+$; elemental analysis calcd (%) for $C_{25}H_{26}N_2O_6 \cdot 0.25\,H_2O$ (454.99): C 66.00, H 5.87, N 6.16; found: C 66.06, H 5.70, N 6.53.

(3*R*)- and (3*S*)-(1*S*,4*R*,7*S*)-7-Hydroxy-1-hydroxymethyl-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (1 and 2): A solution of **6** (520 mg, 1.15 mmol) in ethanol (3.3 mL) was stirred at room temperature and 20% palladium hydroxide on carbon (250 mg) was added. The mixture was degassed several times with argon and then placed under a hydrogen atmosphere. After stirring for 5 days, the mixture was filtered through a layer of Celite, the filtrate was concentrated and the residue was purified by column chromatography (CH_2Cl_2/MeOH, 9:1) to give three fractions as white solids: **1** (57.5 mg; 18%), a mixture of **1** and **2** (87.5 mg; 28%, ratio ≈ 7:1), and **2** (160 mg, 51%); **1**: All NMR data were in accordance with literature data.[15] Data for **2**: ^{1}H NMR (300 MHz, [D$_6$]DMSO, 25 °C, TMS): δ = 11.34 (s, 1H; NH), 7.62 (d, ^3J(H,H) = 0.9 Hz, 1H; 6-H), 5.85 (s, 1H; 1′-H), 5.84 (d, ^3J(H,H) = 4.0 Hz, 1H; 3′-OH), 4.92 (t, ^3J(H,H) = 5.5 Hz, 1H; 5′-OH), 4.25 (d, ^3J(H,H) = 4.0 Hz, 1H; 3′-H), 4.19 (s, 1H; 2′-H), 3.92 (m, 2H; 5″-H), 3.72 (d, ^3J(H,H) = 5.5 Hz, 2H; 5′-H), 1.82 (d, ^3J(H,H) = 0.9 Hz, 3H; CH_3); ^{13}C NMR (75 MHz, [D$_6$]DMSO, 25 °C, TMS): δ = 164.00, 150.43, 135.96, 107.81, 90.93, 86.36, 78.78, 72.40, 71.92, 57.33, 12.11; NMR data were in accordance with literature data on the enantiomer;[27] MS EI: *m*/*z* (%): 270 (100) $[M]^+$; elemental analysis calcd (%) for $C_{11}H_{14}N_2O_6 \cdot 0.33\,H_2O$ (276.25): C 47.78, H 5.35, N 10.14; found: C 47.96, H 4.99, N 9.73.

3-*O*-Benzyl-1,2-*O*-isopropylidene-5-*O*-methylsulfonyl-4-*C*-methylsulfonyloxymethyl-α-D-ribofuranose (7): A solution of the diol **4**[23] (9.24 g, 29.8 mmol) in anhydrous pyridine (94 mL) was cooled to 0 °C, whereupon methanesulfonyl chloride (11.5 mL, 149 mmol) was added. The mixture was stirred at room temperature for 2 h, and then the reaction was quenched by adding iced water (450 mL). The resulting mixture was extracted with dichloromethane (3 × 250 mL). The combined organic phases were washed with saturated aqueous $NaHCO_3$ solution (250 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 95:5) to give the product (12.8 g, 92%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 7.38–7.33 (m, 5H), 5.79 (d, ^3J(H,H) = 3.9 Hz, 1H), 4.88 (d, ^3J(H,H) = 11.9 Hz, 1H), 4.77 (d, ^3J(H,H) = 11.5 Hz, 1H), 4.65 (dd, ^3J(H,H) = 3.9, 4.7 Hz, 1H), 4.57 (d, ^3J(H,H) = 11.5 Hz, 1H), 4.42 (d, ^3J(H,H) = 11.9 Hz, 1H), 4.32 (d, ^3J(H,H) = 10.9 Hz, 1H), 4.20 (d, ^3J(H,H) = 4.7 Hz, 1H), 4.15 (d, ^3J(H,H) = 10.9 Hz, 1H), 3.08 (s, 3H), 2.98 (s, 3H), 1.69 (s, 3H), 1.34 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C, TMS): δ = 136.62, 128.61, 128.37, 128.10, 113.97, 104.47, 83.20, 78.32, 77.77, 72.84, 69.44, 68.62, 37.99, 37.43, 26.17, 25.61; MS FAB: *m*/*z* (%): 489 (3) $[M+Na]^+$, 451 (55) $[M-CH_3]^+$; elemental analysis calcd (%) for $C_{18}H_{26}O_{10}S_2$ (466.52): C 46.34, H 5.61; found: C 46.74, H 5.47.

Methyl (3-*O*-benzyl-5-*O*-methylsulfonyl-4-*C*-methylsulfonyloxymethyl)-D-ribofuranoside (8): A solution of the bis(sulfonic ester) **7** (1.46 g, 3.13 mmol) in water (6.0 mL) and methanol (11.5 mL) was cooled to 0 °C and a 28% solution of HCl in methanol (31 mL) was added. The mixture was stirred at room temperature for 19 h, then neutralised with $NaHCO_3$ (s), and water (60 mL) was added. The resulting mixture was extracted with dichloromethane (2 × 70 mL). The combined organic phases were washed with water (60 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 99:1) to give the product as an anomeric mixture (1.31 g; 95%, α:$\beta \approx$ 1:10) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 7.42–7.33 (m), 4.90–4.00 (m), 3.48 (s), 3.33 (s), 3.06 (s), 3.03 (s), 3.00 (s), 2.99 (s); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C, TMS): (major isomer) δ = 136.36, 128.84, 128.66, 128.25, 107.72, 81.58, 81.51, 73.88, 73.74, 69.56, 69.34, 55.42, 37.47, 37.38; MS FAB: *m*/*z* (%): 441 (32) $[M+H]^+$; elemental analysis calcd (%) for $C_{16}H_{24}O_{10}S_2$ (440.48): C 43.63, H 5.49; found: C 43.55, H 5.14.

1-(3-*O*-Benzyl-5-*O*-methylsulfonyl-4-*C*-methylsulfonyloxymethyl-α-D-ribofuranosyl)thymine (9): A mixture of methyl furanosides **8** (325 mg, 0.74 mmol) and thymine (186 mg, 1.48 mmol) were dried and dissolved in anhydrous CH_3CN (2.7 mL). TMSCl (0.094 mL, 0.74 mmol) and *N*,*O*-bis(trimethylsilyl)acetamide (1.45 mL, 5.9 mmol) were added and the mixture was stirred at 60 °C for 1 h. The solution was cooled to 0 °C, whereupon TMS triflate (0.67 mL, 3.7 mmol) was added dropwise. The mixture was stirred at 70 °C for 4 days, after which a second portion of TMS triflate (0.27 mL, 1.5 mmol) was added. After stirring for a further 2 days, a third portion of TMS triflate (0.27 mL, 1.5 mmol) was added; the mixture was stirred for a further 2 days and then finally quenched by pouring it into an ice-cold saturated aqueous $NaHCO_3$ solution (20 mL). The resulting mixture was extracted with dichloromethane (30 mL) and the organic fraction was washed with saturated aqueous $NaHCO_3$ solution (2 × 20 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was redissolved in THF (2.1 mL) and treated with a 1.0 M solution of TBAF in THF (0.74 mL). The solution was stirred for 1 h and then diluted with dichloromethane (20 mL). The resulting solution was washed with saturated aqueous $NaHCO_3$ solution (2 × 20 mL) and water (2 × 20 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 96:4) to afford the product as a white solid (93 mg; 24 %) together with other impure fractions. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 10.95 (br s, 1H), 7.85 (s, 1H), 7.42 – 7.32 (m, 5H), 6.11 (d, ^3J(H,H) = 2.3 Hz, 1H), 5.19 (d, ^3J(H,H) = 11.8 Hz, 1H), 5.08 (br s, 1H), 4.85 (d, ^3J(H,H) = 11.8 Hz, 1H), 4.60 (d, ^3J(H,H) = 11.8 Hz, 1H), 4.44 – 4.35 (m, 5H), 4.26 (d, ^3J(H,H) = 10.6 Hz, 1H), 3.07 (s, 3H), 2.90 (s, 3H), 1.83 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C, TMS): δ = 166.37, 150.69, 139.54, 136.86, 128.74, 128.47, 128.26, 108.71, 87.14, 82.33, 77.94, 72.16, 69.41, 68.55, 67.90, 37.76, 37.35, 11.81.

(1*S*,3*S*,4*R*,7*S*)-7-Benzyloxy-1-hydroxymethyl-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (10): A solution of the α-nucleoside **9** (344 mg, 0.64 mmol) in ethanol (7 mL) and water (7 mL) was treated with 2 M aqueous NaOH solution (3.2 mL) and the mixture was stirred at 75 °C for 3 days. Concentrated aqueous NaOH solution (1.0 mL) was then added and the mixture was stirred for a further 2 days. It was then neutralised with HCl and extracted with dichloromethane (3 × 15 mL). The combined organic fractions were washed with saturated aqueous $NaHCO_3$ solution (3 × 20 mL), dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 96:4) to afford the product as a white solid (107 mg; 46 %) together with its intermediate 5′-*O*-methylsulfonic ester (29 mg; 10 %); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 7.46 (d, ^3J(H,H) = 1.0 Hz, 1H), 7.38 – 7.30 (m, 5H), 5.95 (s, 1H), 4.72 (d, ^3J(H,H) = 12.0 Hz, 1H), 4.64 (d, ^3J(H,H) = 12.0 Hz, 1H), 4.57 (s, 1H), 4.25 (s, 1H), 4.19 (d, ^3J(H,H) = 8.7 Hz, 1H), 3.95 – 3.90 (m, 3H), 1.95 (s, 3H).

Alternative preparation of 2: A solution of **10** (105 mg, 0.291 mmol) in ethanol (1.0 mL) and dioxane (0.5 mL) was stirred at room temperature and 20 % palladium hydroxide on carbon (65 mg) was added. The mixture was degassed several times with argon and then placed under a hydrogen atmosphere. After stirring for 3 h, the mixture was filtered through a layer of Celite. The filter was rinsed with a mixture of dichloromethane and MeOH (1:1; 10 mL) and the combined filtrates were concentrated in vacuo to give the product (72 mg, 91 %) as a white solid.

3-*O*-Benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-α-D-arabinofuranose (13): A solution of oxalyl chloride (0.39 mL, 4.51 mmol) in anhydrous dichloromethane (11 mL) was stirred at −78 °C and a solution of DMSO (0.64 mL, 9.0 mmol) in dichloromethane (9 mL) was slowly added. A solution of the primary alcohol **12**[32] (1.012 g, 3.61 mmol) in anhydrous dichloromethane (9 mL) was then slowly added, followed by Et_3N (2.5 mL). The resulting mixture was stirred at −78 °C for 15 min. and at room temperature for 2 h. The reaction was quenched by the addition of water (20 mL) and after separation of the layers the aqueous phase was extracted with dichloromethane (3 × 20 mL). The combined organic phases were washed with 1 M aqueous HCl (20 mL), saturated aqueous $NaHCO_3$ solution (20 mL) and water (20 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was redissolved in water (5 mL) and THF (5 mL) and the solution was stirred at 0 °C. A 36 % aqueous solution of formaldehyde (1.3 mL, 15.5 mmol) was added, and then 1.0 M aqueous NaOH solution (5.2 mL) was slowly added. The mixture was stirred at room temperature for 17 h and then treated with a saturated aqueous $NaHCO_3$ solution (25 mL). The resulting mixture was extracted with dichloromethane (3 × 50 mL), and the combined organic phases were washed with saturated aqueous $NaHCO_3$ solution (50 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 97:3) to give the product (703 mg, 63 %) as a white solid. ^{1}H NMR (in accordance with literature data on the enantiomer[28]) (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 7.39 – 7.26 (m, 5H), 6.02 (d, ^3J(H,H) = 4.4 Hz, 1H), 4.79 – 4.75 (m, 2H), 4.55 (d, ^3J(H,H) = 11.6 Hz, 1H), 4.12 (d, ^3J(H,H) = 1.7 Hz, 1H), 3.77 – 3.58 (m, 4H), 2.42 – 2.31 (m, 2H), 1.54 (s, 3H), 1.35 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C, TMS): δ = 136.79, 128.65, 128.22, 127.70, 113.13, 104.89, 89.91, 85.88, 84.88, 72.63, 63.80, 63.38, 27.31, 26.77.

(1*R*,3*S*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityl)oxymethyl-7-hydroxy-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (14): A solution of **2** (152 mg, 0.56 mmol), $AgNO_3$ (191 mg, 1.1 mmol), 4,4′-dimethoxytrityl chloride (209 mg, 0.62 mmol) and anhydrous pyridine (0.45 mL, 5.6 mmol) in anhydrous DMF (0.5 mL) and THF (5.0 mL) was stirred at room temperature for 22 h. The reaction was then quenched by the addition of methanol (1.0 mL) and the mixture was concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH/pyridine, 95:4.5:0.5) to give the product (227 mg, 71 %) as a white solid, which was used in the next step without further purification; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 9.39 (br s, 1H), 7.56 (d, ^3J(H,H) = 1.2 Hz, 1H), 7.46 – 7.20 (m, 9H), 6.84 (d, ^3J(H,H) = 9.1 Hz, 4H), 5.99 (s, 1H), 4.52 (s, 1H), 4.44 (s, 1H), 4.15 (d, ^3J(H,H) = 8.9 Hz, 1H), 4.06 (d, ^3J(H,H) = 8.9 Hz, 1H), 3.79 (s, 6H), 3.56 – 3.37 (m, 2H), 1.99 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C, TMS): δ = 164.19, 158.75, 150.60, 144.50, 135.57, 135.47, 135.40, 130.09, 129.20, 129.08, 128.27, 128.06, 127.88, 127.83, 127.11, 127.07, 113.31, 113,18, 109.66, 89.63, 87.34, 86.38, 79.12, 74.19, 72.83, 59.93, 55.16, 12.71; MS FAB: *m*/*z* (%): 573 (20) $[M+H]^+$.

(1*R*,3*S*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityl)oxymethyl-7-(*O*-2-cyanoethyl-*N*,*N*-diisopropylphosphityl)oxy-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (15): Compound **14** (121 mg, 0.211 mmol) was freed of solvents by co-evaporation with anhydrous CH_3CN and redissolved in anhydrous dichloromethane (1.2 mL). The solution was stirred at room temperature and diisopropyl ethylamine (0.171 mL) and $NC(CH_2)_2OP(Cl)N(iPr)_2$ (0.081 mL, 0.36 mmol) were added. The mixture was stirred for 2 h, then diluted with EtOAc (15 mL), washed with saturated aqueous $NaHCO_3$ solution (2 × 10 mL) and brine (2 × 10 mL), dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/Et_3N, 99:1), dissolved in toluene (1.0 mL), and precipitated from petroleum ether (65 mL) at −30 °C to afford the product as a white solid (156 mg, 96 %). ^{31}P NMR (121.5 MHz, $CDCl_3$, 25 °C, 85 % H_3PO_4 external): δ = 150.9, 151.1.

(1*R*,3*S*,4*R*,7*S*)-7-(*tert*-Butyldimethylsilyl)oxy-1-(4,4′-dimethoxytrityl)oxymethyl-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (16): A solution of **14** (117 mg, 0.205 mmol) and imidazole (38 mg, 0.56 mmol) in anhydrous pyridine (2.5 mL) was treated with TBDMSCl (45 mg, 0.30 mmol) and the mixture was stirred at room temperature for 22 h. Another portion of TBDMSCl (90 mg, 0.60 mmol) was then added and the mixture was stirred at room temperature for 5 days. It was subsequently concentrated in vacuo, the residue was taken up in dichloromethane (25 mL), and the resulting solution was washed with saturated aqueous $NaHCO_3$ solution (3 × 25 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH/pyridine, 98:1.5:0.5) to give the product (110 mg, 79 %) as a white solid, which was used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 8.62 (br s, 1H), 7.59 (d, ^3J(H,H) = 1.1 Hz, 1H), 7.47 – 7.23 (m, 9H), 6.84 (d, ^3J(H,H) = 8.3 Hz, 4H), 6.04 (s, 1H), 4.34 (s, 1H), 4.33 (s, 1H), 4.01 (m, 2H), 3.79 (s, 6H), 3.41 (d, ^3J(H,H) = 10.6 Hz, 1H), 3.33 (d, ^3J(H,H) = 10.6 Hz, 1H), 2.01 (d, ^3J(H,H) = 1.1 Hz, 3H), 0.75 (s, 9H), 0.05 (s, 3H), −0.04 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C, TMS): δ = 163.78, 158.56, 150.44, 144.46, 135.65, 135.53, 135.42, 129.95, 129.81, 127.96, 127.86, 126.88, 113.16, 109.46, 90.09, 87.20, 86.11, 78.76, 74.29, 73.42, 59.83, 55.21, 25.42, 17.73, 12.90, −4.72, −5.14; MS FAB: *m*/*z* (%): 687(4) $[M+H]^+$.

(1*R*,3*S*,4*R*,7*S*)-7-(*tert*-Butyldimethylsilyl)oxy-1-(4,4′-dimethoxytrityl)oxymethyl-3-(5-methylcytosin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (17): 1,2,4-Triazole (68 mg, 0.99 mmol) was added to a solution of **16** (97 mg, 0.141 mmol) in anhydrous pyridine (1.2 mL) and the mixture was stirred at 0 °C. $POCl_3$ (0.031 mL, 0.33 mmol) was added and the mixture was stirred at room temperature for 15 h. It was subsequently diluted with dichloromethane (8 mL), washed with saturated aqueous $NaHCO_3$ solu-

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tion (2 × 5 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH/pyridine, 99:0.5:0.5) to afford the triazole intermediate (72.3 mg, 69%). This compound (113 mg, 0.153 mmol) was dissolved in dioxane (1.3 mL) and treated with 20% aqueous ammonia (0.38 mL). The mixture was stirred at room temperature for 22 h, then diluted with dichloromethane (5 mL) and washed with water (5 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH/pyridine, 98.5:1:0.5) to give the product (68 mg; 64%) as a white solid, which was used in the next step without further purification; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 7.60$ (s, 1H), 7.47–7.22 (m, 9H), 6.83 (d, ^3J(H,H) = 9.0 Hz, 4H), 6.14 (s, 1H), 4.44 (s, 1H), 4.35 (s, 1H), 4.02 (d, ^3J(H,H) = 8.4 Hz, 1H), 3.96 (d, ^3J(H,H) = 8.4 Hz, 1H), 3.79 (s, 6H), 3.42 (d, ^3J(H,H) = 10.7 Hz, 1H), 3.32 (d, ^3J(H,H) = 10.7 Hz, 1H), 2.02 (s, 3H), 0.74 (s, 9H), 0.04 (s, 3H), −0.05 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C, TMS): δ = 165.65, 158.52, 156.02, 144.55, 138.60, 135.63, 135.51, 129.97, 127.99, 127.83, 126.82, 113.13, 100.26, 89.77, 87.87, 86.03, 78.77, 74.43, 73.35, 60.14, 55.16, 25.44, 17.72, 13.60, −4.72, −5.18; MS FAB: *m*/*z* (%): 686 (3) $[M+H]^+$.

(1*R*,3*S*,4*R*,7*S*)-3-(4-*N*-Benzoyl-5-methylcytosin-1-yl)-7-(*tert*-butyldimethylsilyl)oxy-1-(4,4′-dimethoxytrityl)oxymethyl-2,5-dioxabicyclo[2.2.1]heptane (18): Compound **17** (32 mg, 0.047 mmol) was freed of solvents by co-evaporation with anhydrous CH_3CN and redissolved in anhydrous CH_3CN (0.2 mL). A solution of DMAP (5.9 mg, 0.048 mmol) in anhydrous CH_3CN (0.2 mL) and *N*-benzoyltetrazole[37] (16.5 mg, 0.095 mmol) were added to the reaction mixture. The resulting mixture was stirred at 65 °C for 15 min and then cooled to room temperature. Saturated aqueous $NaHCO_3$ solution (10 mL) was added and the mixture was extracted with dichloromethane (2 × 10 mL). The combined organic phases were dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH/pyridine, 98.5:1:0.5) to afford the product as a white solid (37 mg, 100%); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 8.34 (d, ^3J(H,H) = 6.8 Hz, 2H), 7.74 (d, ^3J(H,H) = 0.8 Hz, 1H), 7.47–7.26 (m, 12H), 6.85 (d, ^3J(H,H) = 8.6 Hz, 4H), 6.09 (s, 1H), 4.40 (s, 1H), 4.35 (s, 1H), 4.06 (d, ^3J(H,H) = 8.7 Hz, 1H), 4.00 (d, ^3J(H,H) = 8.7 Hz, 1H), 3.80 (s, 6H), 3.45 (d, ^3J(H,H) = 10.7 Hz, 1H), 3.35 (d, ^3J(H,H) = 10.7 Hz, 1H), 2.21 (d, ^3J(H,H) = 0.8 Hz, 3H), 0.76 (s, 9H), 0.06 (s, 3H), −0.03 (s, 3H); MS FAB: *m*/*z* (%): 790 (13) $[M+H]^+$, HR MALDI: calcd for $C_{45}H_{51}N_3O_8Si+Na$: 812.3338; found: 812.3338.

(1*R*,3*S*,4*R*,7*S*)-3-(4-*N*-Benzoyl-5-methylcytosin-1-yl)-1-(4,4′-dimethoxytrityl)oxymethyl-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptane (19): A solution of compound **18** (58 mg, 0.073 mmol) in THF (0.7 mL) was treated with a 1.0 M solution of TBAF in THF (0.080 mL) and the mixture was stirred at room temperature for 30 min. It was then concentrated in vacuo and the residue was redissolved in dichloromethane (10 mL). This solution was washed with saturated aqueous $NaHCO_3$ solution (2 × 10 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH/pyridine, 98:1.5:0.5) to afford the product as a white solid (41 mg, 83%). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS):[38] δ = 8.32 (d, ^3J(H,H) = 7.0 Hz, 2H), 7.70 (s, 1H), 7.47–7.26 (m, 12H), 6.86 (d, ^3J(H,H) = 8.9 Hz, 4H), 6.00 (s, 1H), 4.58 (s, 1H), 4.42 (s, 1H), 4.10 (d, ^3J(H,H) = 8.9 Hz, 1H), 4.03 (d, ^3J(H,H) = 8.9 Hz, 1H), 3.80 (s, 6H), 3.54 (m, 2H), 2.18 (s, 3H); MS FAB: *m*/*z* (%): 676 (11) $[M+H]^+$, HR MALDI: calcd for $C_{39}H_{37}N_3O_8+Na$: 698.2473; found: 698.2476.

(1*R*,3*S*,4*R*,7*S*)-3-(4-*N*-Benzoyl-5-methylcytosin-1-yl)-1-(4,4′-dimethoxytrityl)oxymethyl-7-(*O*-2-cyanoethyl-*N*,*N*-diisopropylphosphityl)oxy-2,5-dioxabicyclo[2.2.1]heptane (20): Compound **19** (40.5 mg, 0.060 mmol) was freed of solvents by co-evaporation with anhydrous CH_3CN and redissolved in anhydrous dichloromethane (0.33 mL). The solution was stirred at room temperature and diisopropyl ethylamine (0.048 mL) and $NC(CH_2)_2OP(Cl)N(iPr)_2$ (0.023 mL, 0.069 mmol) were added. The mixture was stirred for 2 h, then diluted with EtOAc (5 mL), washed with saturated aqueous $NaHCO_3$ solution (2 × 5 mL) and brine (2 × 5 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/Et_3N, 199:1), dissolved in toluene (0.5 mL) and precipitated from petroleum ether (20 mL) at −30 °C to afford the product as a white solid (31.5 mg; 60%). ^{31}P NMR (121.5 MHz, $CDCl_3$, 25 °C, 85% H_3PO_4 external): δ = 150.9, 150.7.

Synthesis of oligodeoxynucleotides: Oligonucleotide synthesis was carried out by using a Biosearch model 8750 automated DNA synthesizer following the phosphoramidite approach. Synthesis of α-oligonucleotides **22**–**24**, **26**, **27**, and **29**–**32** was performed on a 0.2 μmol scale by using α-thymidine 2-cyanoethyl phosphoramidite,[41] *N*-benzoyl-protected α-5-methylcytosine 2-cyanoethyl phosphoramidite,[36] as well as compounds **15** and **20**. The synthesis followed the regular protocol for the DNA synthesizer and a universal CPG support (Biogenex) was employed. However, for compounds **15** and **20**, a prolonged coupling time of 10 min was used. Coupling yields for all 2-cyanoethyl phosphoramidites were >98%. The 5′-*O*-DMT-*ON* oligonucleotides were removed from the universal solid support by treatment with 2% LiCl in concentrated ammonia at 55 °C for 20 h, which also removed the protecting groups. Subsequent purification using disposable reversed-phase cartridges, including 5′-*O* detritylation, afforded the pure oligonucleotides. MALDI-MS $[M+H]^+$ gave the following results (found/calcd): **22** (4196.9/4196.8); **23** (4227.1/4224.8); **24** (4309.2/4308.8); **26** (2976.4/2980.0); **27** (3261.6/3260.1); **29** (2976.6/2976.0); **30** (3001.9/3004.0); **31** (3145.0/3144.1); **32** (3257.2/3256.1). The purities of all the oligonucleotides were verified by capillary gel electrophoretic experiments.

Melting experiments: UV melting experiments were carried out on a Perkin–Elmer Lambda 2 spectrometer. Samples were dissolved in a medium salt buffer containing Na_2HPO_4 (10 mM), NaCl (100 mM) and EDTA (0.1 mM), pH 7.0 with 1.5 μM concentrations of the two complementary sequences. The extinction coefficients were calculated assuming the extinction coefficients for all thymine nucleotides to be identical, as well as those for all 5-methyl cytosine nucleotides to be identical. The increase in absorbance at 260 nm as a function of time was recorded while the temperature was increased linearly from 8 to 80 °C at a rate of 0.5 °C min^{-1} by means of a Pertier temperature programmer. The melting temperature was determined as the local maximum of the first derivatives of the absorbance versus temperature curve. All melting curves were found to be reversible.

CD experiments: CD experiments were carried out on a Jasco J710 spectropolarimeter at 25 °C using 1 cm cuvettes. The samples contained the same buffer and the same concentrations as used in the melting experiments.

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