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ARTICLES

Synthesis of 3′-*C*- and 4′-*C*-Branched Oligodeoxynucleotides and the Development of Locked Nucleic Acid (LNA)

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Introduction

During the past decade a large number of novel chemically modified analogues of the natural oligonucleotides (ONs) have been synthesized.[1−5] Introduction of one or several modified monomeric or dimeric building blocks into an otherwise unmodified ON or synthesis of completely modified ON analogues has been performed. The impetus for this research has been the important therapeutic (e.g., antisense drugs) and diagnostic (e.g., DNA-array technologies) implications, the need for novel biochemical research tools, and sheer intellectual curiosity. When designing DNA/RNA mimics, a number of characteristics are generally recognized as desirable, e.g., (1) efficient oligomerization of the modified building blocks following standard DNA chemistry on automated DNA synthesizers, (2) high-affinity *and* selective recognition of complementary DNA and/or RNA, (3) stability toward degradation by nucleases, and (4) good aqueous solubility of oligomers.

High affinity and selective recognition of complementary ONs are considered especially important for most applications. This property is most conveniently estimated by comparing the melting temperature (T_m value) of a duplex involving a partly or fully modified ON and an unmodified complement with the T_m value of the corresponding reference duplex involving two unmodified complementary ON strands.[6] Experimentally, equimolar amounts of the two complementary strands are mixed and annealed (by cooling) in an aqueous buffer. Subsequently, the UV absorption at 260 nm is followed while the temperature is increased linearly from, e.g., 10 to 90 °C resulting in duplex dissociation with concomitant decreased base stacking and UV hyperchromicity. An S-shaped T_m curve (A_{260} vs temperature) is obtained,[3] and from its midpoint the melting temperature (T_m), and thus the change in melting temperature per modification (ΔT_m), can be extracted and used as an estimate of duplex stability.[7]

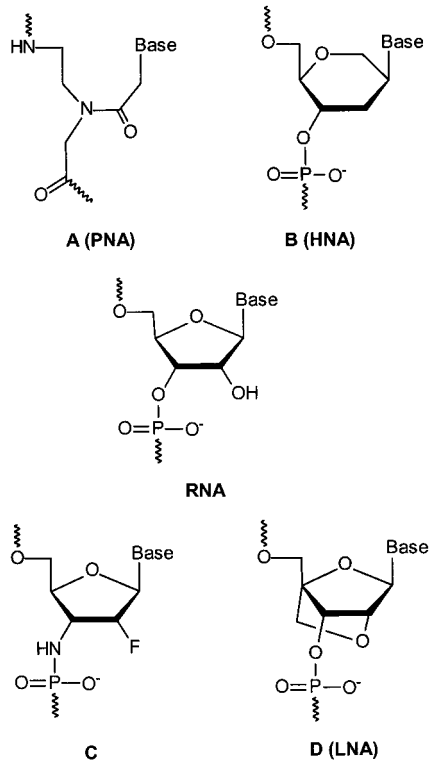

FIGURE 1. Structures of selected high-affinity nucleic acid analogues and RNA (depicted as monomeric units): PNA (**A**, $\Delta T_m \approx +1-2$ °C for DNA and RNA complements in duplex mode);[11] HNA (**B**, $\Delta T_m \approx +3-5$ °C for RNA complements;[13,14] 2′-fluoro N3′−P5′ phosphoramidates (**C**, pyrimidine derivatives reported, $\Delta T_m \approx +4-5$ °C for DNA and RNA complements);[15] LNA (**D**) and RNA (in DNA the 2′-OH group is exchanged with a 2′-H). ΔT_m = change in melting temperature per modification. Base = nucleobase.

Different strategies have been adopted for synthesis of high-affinity nucleic acid mimics. For example, several interesting dimeric dephospho dinucleoside building blocks have been developed displaying ΔT_m values of up to +4 °C *per dinucleoside*.[8−10] However, e.g., the fact that 16 dimers have to be synthesized for sequence independent complementary strand recognition, instead of only 4 when synthesizing monomeric building blocks, renders the dimer strategy less attractive. Below (Figure 1), three of the most interesting monomer units for high-affinity ON analogues, namely, PNA[11,12] (peptide nucleic acid, **A**), HNA[13,14] (anhydrohexitol nucleic acid, **B**), 2′-fluoro N3′−

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P5′ phosphoramidates[15] (**C**), are shown together with the structure of LNA (locked nucleic acid, **D**) and RNA. Though very attractive, analogues **A**−**C** suffer from a number of shortcomings, e.g., low aqueous solubility of parent PNA, less convincing recognition of complementary DNA by HNA, and the necessity of introducing a 2′-fluoro substituent and an alternative coupling chemistry in the case of the 2′-fluoro N3′−P5′ phosphoramidates.

In this account I will describe the central parts of our efforts of the past few years to synthesize novel *C*-branched and bicyclic nucleosides and ONs with the overall aim of developing DNA/RNA mimics capable of recognizing complementary ONs with high affinity and selectivity. We rationalized that the probability of obtaining a mimic with superior properties would be maximized if the natural pentofuranose 3′-*O*- to 5′-*O*-linked phosphordiester backbone remained intact, and as organic chemists we anticipated that much chemistry at the pentofuranose scaffold remained to be explored. We have synthesized and evaluated the most easily obtainable thymine (or uracil) building block for all analogues, and only when justified by the extraordinary properties of LNA (vide infra) continued with synthesis of the other nucleobase analogues.[16] For synthetic and comparative reasons, we have in several projects evaluated only modified oligothymidylate sequences which are known to form (oligo-T:oligo-dA) duplexes with structures deviating from the standard A- or B-type duplexes.[17] Therefore, the predictive value on mixed base sequences from hybridization data obtained for modified oligothymidylate sequences can be questioned, but we have in general observed a fair correlation when both oligothymidylate and mixed base sequences were evaluated (e.g., monomers **J** and **N**, vide infra).

The ribofuranose rings of the natural DNA (2′-deoxy-β-D-ribofuranosyl) and RNA (β-D-ribofuranosyl) monomers are flexible and the energy of interconversion between the different conformers is low. On the contrary, the conformational flexibility of the monomeric nucleotides when present in a double helix is strongly restricted into, generally, either an S-type conformation (2′-endo conformation, B-form duplexes) or an N-type conformation (3′-endo conformation, A-form duplexes) (Figure 2). DNA/DNA hybrids adopt either the A- or B-form, RNA/RNA hybrids exclusively the A-form, whereas RNA/DNA duplexes adopt either the A-form or an alternative intermediate form.[4,18−21] Thus, though the exact structure of a given duplex depends on its sequence, these conformational relations indicate that a universal nucleic acid mimic should preferably consist of monomers adopting an N-type (3′-endo) conformation.

3′-*C*- and 4′-*C*-Branched Nucleoside and Oligonucleotide Analogues

In addition to a possible positive effect on the nuclease resistance and thermal affinity, our work in this area was stimulated by the possibility of linking, e.g., reporter groups, intercalators, lipophilic carriers, or a third ON strand at an additional *C*-alkyl branch facing either the major groove (3′-*C*-branched analogues) or the minor groove (4′-*C*-branched analogues) of a B-DNA helix.

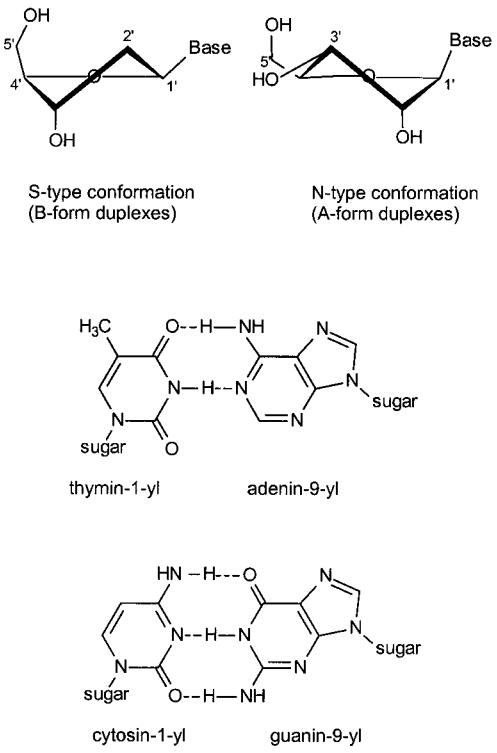


FIGURE 2. Predominant conformations of the pentofuranose monomers in nucleic acid duplexes. Base = nucleobase. Also shown are the Watson−Crick hydrogen bonds between thymin-1-yl and adenin-9-yl and between cytosin-1-yl and guanin-9-yl.

3′-*C*-Branched Nucleoside and Oligonucleotide Analogues. Our preparation of 3′-*C*-hydroxymethylthymidine[22,23] involved osmium tetroxide catalyzed dihydroxylation of 3′-*C*-methylene nucleoside **1**, affording stereoselectively 3′-*C*-hydroxymethyl 2′-deoxynucleoside **2** in 70% yield. Silylation of **2** to give nucleoside **3** in 81% yield followed by phosphitylation afforded the nucleoside phosphoramidite **4** in 90% yield (Figure 3).[22,23] Using the phosphoramidite approach[24] on an automated DNA synthesizer, 17-mer mixed oligodeoxynucleotide (ODN) sequences containing one or two 3′-*C*-hydroxymethyl nucleotides (monomer **E**) were obtained using amidite **4** and phosphoramidite derivatives of the parent 2′-deoxynucleosides. The stepwise coupling yield for amidite **4** (12 min couplings) was approximately 60% compared to >99% for unmodified 2′-deoxynucleoside phosphoramidites (2 min couplings).[25] Incorporation of monomer **E** in the middle of a sequence led to unchanged T_m values whereas minor decreases in T_m were observed for end-modified sequences. The satisfactory T_m values obtained for mid-modified ODNs and the short synthesis of amidite **4** suggest monomer **E** to be convenient for introduction of functionalities facing the major groove.

Our initial attempts to derivatize (using, e.g., Mitsunobu type reactions) the 3′-*C*-hydroxymethyl functionality of nucleoside **2** were unsuccessful, probably due to steric hindrance from the thymine moiety. Recently, conversion

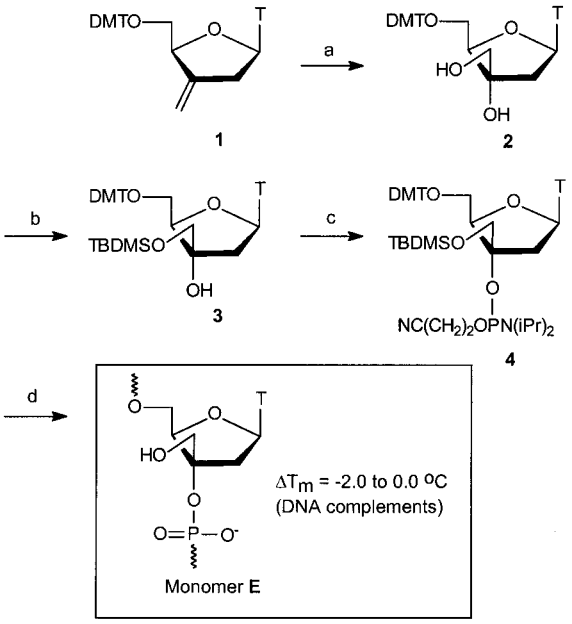


FIGURE 3. (a) OsO_4/*N*-methylmorpholine *N*-oxide/pyridine/H_2O/*tert*-butyl alcohol (79%); (b) TBDMSCl/imidazole/DMF (81%); (c) $NC(CH_2)_2$-$OP(Cl)N(^iPr)_2$/DIPEA/CH_2Cl_2 (90%); (d) DNA synthesizer. T = thymin-1-yl. DMT = 4,4′-dimethoxytrityl. TBDMS = *tert*-butyldimethylsilyl.

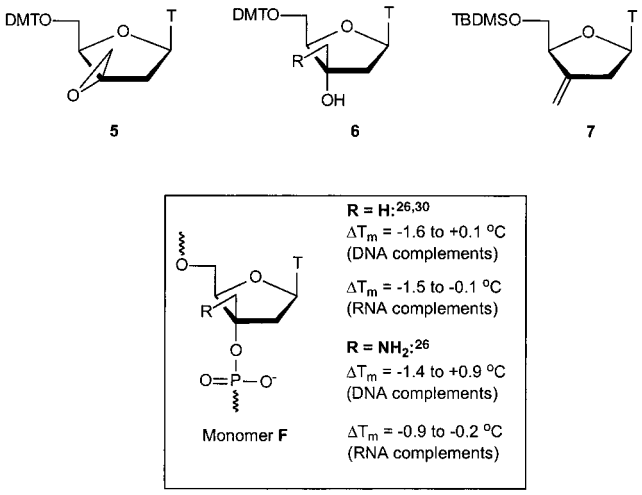


FIGURE 4.

of **2** via a 3′-*C*-tosyloxymethyl derivative into 3′-spiro epoxide **5** has been reported as an intermediate step for introduction of a 3′-*C*-methyl and a 3′-*C*-aminomethyl group (Figure 4, **6**, R = H, R = NH_2).[26] Incorporation of these modified building blocks (monomers **F**) into mixed sequence ODNs induced only minor changes in the thermal stability (toward both DNA and RNA complements; data shown in Figure 4) in line with the results obtained by us for monomer **E**. We have independently developed an analogous route to 3′-*C*-methyl- and 3′-*C*-azidomethyl thymidines embarking on ring-opening of the corresponding 5′-*O*-*tert*-butyldimethylsilyl 3′-spiro epoxide nucleoside which was synthesized in 70% yield directly from 3′-*C*-methylene nucleoside **7** using 3-chloroperoxybenzoic acid (Figure 4).[27]

Elongation of the 3′-*C*-branch beyond a one-carbon unit would be favorable for several reasons. Thus, derivatizations, e.g., of a 3′-*C*-hydroxypropyl branch, should be straightforward, and the potential problem of 3′-*O*-phosphate group migration (or cleavage) in an ON due to attack from a 3′-*C*-hydroxymethyl or 3′-*C*-aminomethyl substituent should be ruled out. As a consequence, we initiated a program for synthesis of 3′-*C*-propyl derivatized nucleosides and ONs. Stimulated by a report on stereoselective synthesis of 3′-*C*-alkyl nucleosides from 3′-keto nucleosides using organocerium reagents,[28] we reacted differently protected 3′-ketouridines with allylmagnesium bromide and $CeCl_3$.[29] Preferential attack from the α-face of the furanose ring was observed, and we were unable to obtain the desired ribonucleosides as main products. For the synthesis of ODNs containing 3′-*C*-(3-hydroxypropyl)thymidine, the alternative convergent synthetic strategy depicted in Figure 5, comparable to the one reported for synthesis of 5-methyl-3′-*C*-methyluridine and 3′-*C*-methylthymidine,[30] was used. Starting from 3-ketofuranoside **8**,[31] stereoselective Grignard addition of an allyl group, desilylation, dibenzylation, acetolysis, and acetylation afforded key furanose intermediate **9**. Due to anchimeric assistance from the 2′-*O*-acetyl group, coupling with thymine yielded exclusively the β-nucleoside (86%). Removal of the 2′-*O*-acetyl group afforded nucleoside **10** in 47% yield (from **8**), which by hydroboration/oxidation gave 3′,5′-di-*O*-benzyl-3′-*C*-(3-hydroxypropyl) nucleoside **11** in 54% yield.[32,33] Nucleoside **11** was converted into the 3′-*C*-benzoyloxypropyl intermediate in 62% yield and subsequently deoxygenated[30] via the corresponding 2′-*O*-pentafluorophenoxythiocarbonyl nucleoside (synthesized in 52% yield) using Bu_3SnH and 2,2′-azobis-2-methylpropionitrile (AIBN) to give the desired 2′-deoxynucleoside **12** in 52% yield. Debenzylation, dimethoxytritylation, and phosphitylation afforded in 41% yield the phosphoramidite building block **13**.[27] Using amidite **13** (stepwise coupling yield approximately 20%, varying coupling times), monomer **G** was incorporated once or twice in 14-mer oligothymidylates leading to minor decreases in T_m against complementary DNA and moderate decreases against complementary RNA (Figure 5).[27] Analogously, incorporation of the two anomeric 3′-*C*-allyl modified monomeric units **H** and **I** (Figure 5) once or twice into 14-mer β- and α-oligothymidylates, respectively, induced minor decreases in T_m toward complementary DNA and moderate decreases when RNA was used as complementary strand (the absolute T_m values were higher for the α-ODNs than for the β-ODNs).[34] The 3′-*C*-(3-aminopropyl) monomer corresponding to monomer **I** has been incorporated once in a 14-mer oligothymidylate. On the basis of the measured T_m values, the effect on the thermal stability of introducing a 3′-*C*-aminopropyl group instead of a 3′-*C*-allyl group in an α-ODN seems to be, if anything, slightly favorable (decrease in T_m values approximately 1 °C less for the 3′-*C*-aminopropyl than for the 3′-*C*-allyl α-monomer in a singly mid-modified oligothymidylate sequence).[34]

Generally, the incorporation of 3′-*C*-branched monomers induced decreased thermal stability of duplexes compared to the corresponding references.[22,26,27,30,34] The

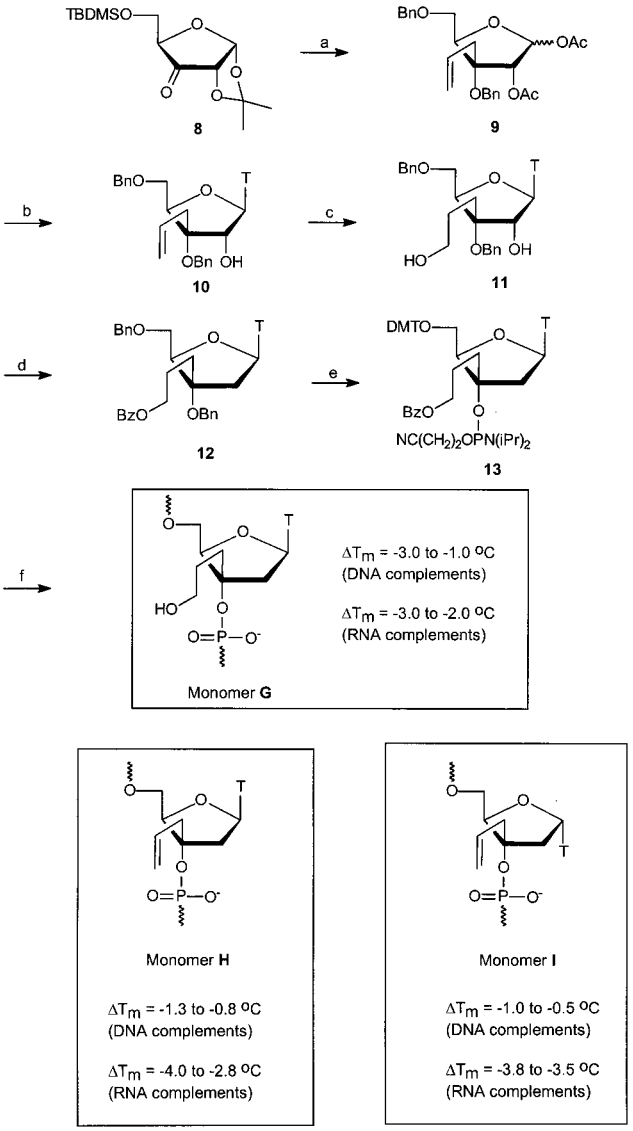


FIGURE 5. (a) (i) AllylMgBr, Et_2O, THF, (ii) TBAF, THF, (iii) BnBr, NaH, DMF, (iv) (1) 80% AcOH, (2) Ac_2O, pyridine (58%); (b) (i) thymine, BSA, CH_3CN, TMS-triflate, (ii) CH_3ONa, CH_3OH (82%); (c) (1) BH_3:1,4-oxathiane, THF, (2) NaOH, H_2O_2, H_2O (54%); (d) (i) BzCl, 2,6-lutidine, CH_2Cl_2, (ii) $C_6F_5OC(S)Cl$, DMAP, CH_2Cl_2—pyridine (1:1, v/v), (iii) Bu_3-SnH, AIBN, benzene (17%); (e) (i) H_2, 20% $Pd(OH)_2$/C, EtOH, (ii) DMTCl, pyridine, (iii) $NC(CH_2)_2OP(Cl)N(^iPr)_2$, DIPEA, CH_2Cl_2 (41%); (f) DNA synthesizer.

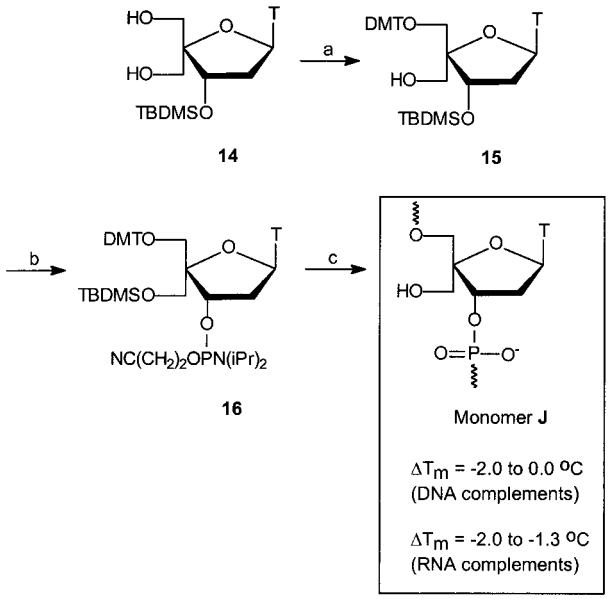


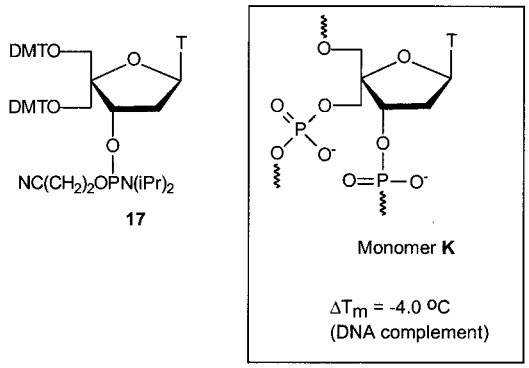
FIGURE 6. (a) (i) BzCl, pyridine, (ii) DMTCl, pyridine, (iii) NH_3, NaOH, MeOH (22%); (b) (i) TBAF, THF, (ii) TBDMSCl, imidazole, DMF, (iii) $NC(CH_2)_2OP(Cl)N(^iPr)_2$, DIPEA, CH_2Cl_2 (59%); (c) DNA synthesizer.

destabilizing effect is very limited toward complementary DNA but more pronounced toward RNA. The probable structural basis for this weak DNA selectivity is the induction of a 2′-endo type pentofuranose conformation (as in B-form DNA/DNA duplexes; equatorial preference of the 3′-*C*-alkyl branch) as reported earlier for structurally closely related nucleosides.[35]

4′-*C*-Branched Nucleoside and Oligonucleotide Analogues. Aldol condensations of a variety of nucleoside 5′-aldehydes with formaldehyde followed by sodium borohydride reduction or in situ Cannizzaro reduction have been central steps in reported syntheses of 4′-*C*-hydroxymethyl nucleosides.[36,37] Using analogous procedures, we obtained 4′-*C*-(hydroxymethyl)thymidine nucleoside **14** in 52% yield starting from 3′-*O*-(*tert*-butyldimethylsilyl)thymidine. Selective benzoylation of the more reactive 4′-*C*-hydroxymethyl group, dimethoxytritylation, and debenzoylation afforded nucleoside **15** in 22% yield. Desilylation, silylation of the primary hydroxy group, and phosphitylation gave the phosphoramidite building block **16** (59% yield) which was used on a DNA synthesizer (12 min coupling time, 90% stepwise yield) to give oligothymidylate and mixed sequence ODNs containing monomer **J** (Figure 6).[38,39] Promising properties were obtained as only minor decreases in T_m toward both complementary DNA and RNA were induced by one to three incorporations of monomer **J**.

The ability of the 4′-*C*-hydroxymethyl branch to function as an attachment site was evaluated by incorporation of amidite **17** as a branching point (monomer **K**) in a Y-shaped ODN (Figure 7).[39] Remarkably, the exchange of a natural thymidine monomer with monomer **K** containing an additional 4′-*C*-(5′-$dC_{10}T_2$-PO_2)oxymethyl branch only resulted in a moderate decrease in T_m of −4 °C when evaluated in an oligopyrimidine sequence. This result, in combination with the recent reports[40−42] that mixed sequence ODNs containing derivatized 4′-*C*-branched thymidine building blocks (monomers **L**, Figure 7) form stable duplexes with complementary nucleic acids (data shown in Figure 7), indicates 4′-*C*-alkyl branches to be versatile attachment sites facing the minor groove.

By applying chemistry similar to that described in Figure 6, uridine was converted into 4′-*C*-hydroxymethyl uridine derivatives, and the ribonucleotide monomer **M** (Figure 8) was incorporated into ODNs.[43] The destabilizing effect of monomer **M** varied from substantial against complementary DNA (ΔT_m = −7 and −9 °C for mid-modified sequences) to only minor against complementary RNA (ΔT_m = −1 and −3 °C for mid-modified sequences).[43] A possible explanation for this trend is that



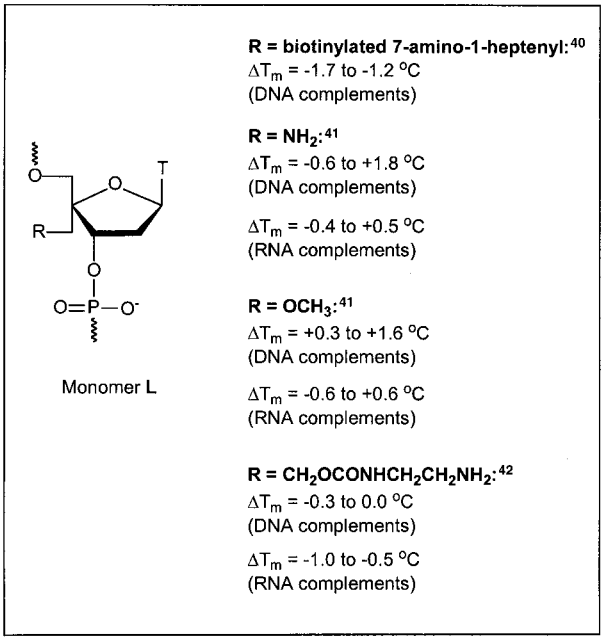


FIGURE 7.

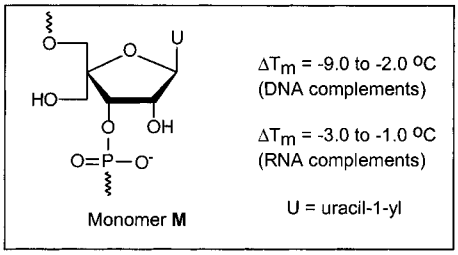


FIGURE 8.

monomer **M**, like the natural ribonucleotide monomers, adopts a 3′-endo type conformation expected to be favorable for DNA/RNA (but not DNA/DNA) duplexes.

Summary: Key Characteristics of 3′-*C*- and 4′-*C*-Branched ONs. (1) Short and versatile synthetic methods have been developed for synthesis of the 3′-*C*- and 4′-*C*-hydroxymethyl derivatives (monomers **E** and **J**). (2) The thermal stability of duplexes toward complementary nucleic acids is only weakly reduced by incorporation of a limited number of 3′-*C*- and 4′-*C*-branched monomeric nucleotides. (3) Derivatizations of both 3′-*C*- and 4′-*C*-alkyl functionalities have been successfully accomplished without compromising the ability to recognize complementary ONs.

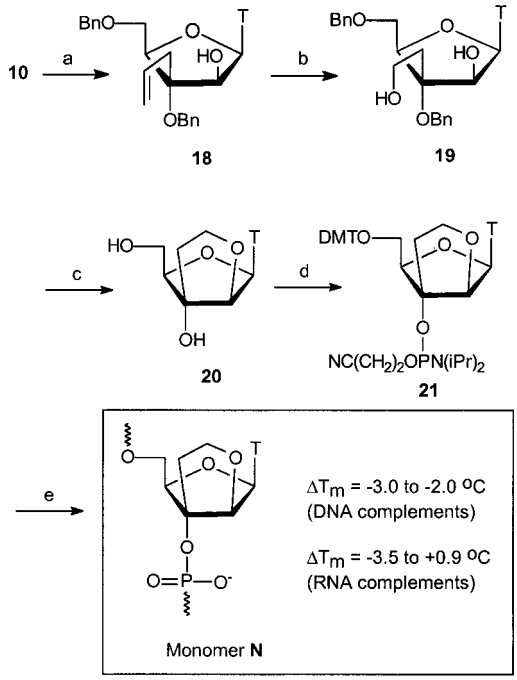


FIGURE 9. (a) (i) MsCl, pyridine, (ii) NaOH, H_2O, ethanol (66%); (b) (i) $NaIO_4$, catalytic OsO_4, *tert*-butyl alcohol, THF, H_2O, (ii) $NaBH_4$, THF, H_2O (49%); (c) (i) TsCl, pyridine, (ii) NaH, DMF, (iii) H_2, 20% Pd-$(OH)_2$/C, EtOH (68%); (d) (i) DMTCl, pyridine, (ii) $NC(CH_2)_2OP(Cl)N(^iPr)_2$, DIPEA, CH_2Cl_2 (83%); (e) DNA synthesizer.

2′-*O*,3′-*C*-Bicyclofuranose Nucleoside and Oligonucleotide Analogues

Having established synthetic methods for 3′- and 4′-*C*-branched ON analogues, the idea of utilizing the branches in syntheses of novel bicyclopentofuranose nucleosides and ONs occurred to us. Especially appealing seemed the possibility of enhancing duplex stability by the incorporation of such conformationally preorganized monomeric nucleosides (entropic advantage). A number of conformationally restricted bi- and tricyclic ON analogues have been reported containing, among others, bicyclopentofuranose nucleosides with an additional 3′,5′-ethylene bridge,[44] bicyclocarbocyclic nucleosides,[45−47] and tricyclopentofuranose nucleosides.[48] On the basis of our continued interest in modified ODNs containing derivatized pentofuranose nucleosides linked through the natural 3′-*O*- to 5′-*O*-phosphordiester linkages, we chose monomer **N** as initial target (Figure 9).

Synthesis of bicyclo[3.3.0]nucleoside **20** was accomplished using a linear strategy. Inversion of the configuration at the 2′-carbon of nucleoside **10** via a 2,2′-anhydro intermediate afforded nucleoside **18** in 66% yield. Oxidative cleavage of the double bond using catalytic osmium tetroxide and sodium periodate followed by reduction gave the corresponding 3′-*C*-hydroxyethyl nucleoside **19** in 49% yield. Selective tosylation of the primary hydroxyl group, cyclization, and debenzylation proceeded efficiently to give the bicyclo[3.3.0]nucleoside **20** in 68% yield. The overall yield of the target nucleoside **20** was 17% from furanose **8**.[33] Standard conversion of **20** into the corresponding phosphoramidite building block **21** was ac-

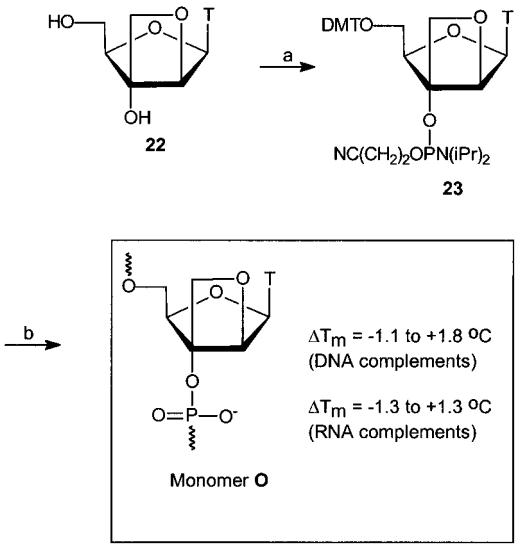


FIGURE 10. (a) (i) DMTCl, pyridine, (ii) $NC(CH_2)_2OP(Cl)N(^iPr)_2$, DIPEA, CH_2Cl_2 (61%); (b) DNA synthesizer.

complished in 83% yield. Phosphoramidite **21** (stepwise coupling yield 95%, 2 × 12 min coupling time) was applied for synthesis of 14-mer oligothymidylates containing from 1 to 13 bicyclic monomer **N** units (Figure 9). Interestingly, consecutive incorporations of monomer **N** 4 or 13 times lead to RNA-selective binding with increased thermal affinity compared to the unmodified reference ODN. However, incorporation of monomer **N** into partly modified mixed sequences or alternating with thymidine monomers in oligothymidylates resulted in reduced affinity toward DNA and RNA.[33,49] Molecular modeling indicated the furanose ring of the bicyclo[3.3.0]nucleoside **20** to be locked in a 1′-exo conformation, deviating from the standard furanose conformations in A- or B-type duplexes (see Figure 2).[49,50] This explains the need for consecutive incorporations in order to obtain enhanced thermal affinities. The corresponding *ribo*- and *arabino*-configurated bicyclo[4.3.0]pentofuranose nucleosides induced strongly decreased affinities toward complementary DNA and RNA ($\Delta T_m = -10.5$ to -6.0 °C/modification).[33]

The bicyclo[3.2.0]nucleoside **22** was synthesized in 12 steps from ulose **8** in an overall yield of 8.5% using a synthetic route closely resembling the one shown in Figure 9 but involving a stereoselective Grignard addition of a vinyl group as the initial step (Figure 10).[49] Standard incorporation of nucleoside **22** (using amidite **23**, stepwise coupling yield 95%, 12 min coupling time) as monomer **O** in the same sequences as synthesized with monomer **N** was accomplished. Remarkably, a 14-mer oligothymidylate containing 13 monomer **O** units exhibited strongly increased thermal affinities toward both the DNA and the RNA complement when compared to the unmodified control.[49] In addition, incorporation of monomer **O** three times in a mixed sequence 9-mer induced increased thermal stabilities.[49] A pronounced conformational restriction of the furanose ring of bicyclo[3.2.0]nucleoside **22** into an O4′-endo conformation was suggested by molecular modeling. Thus, with the results obtained for monomers **N** and **O** the use of modified ONs containing

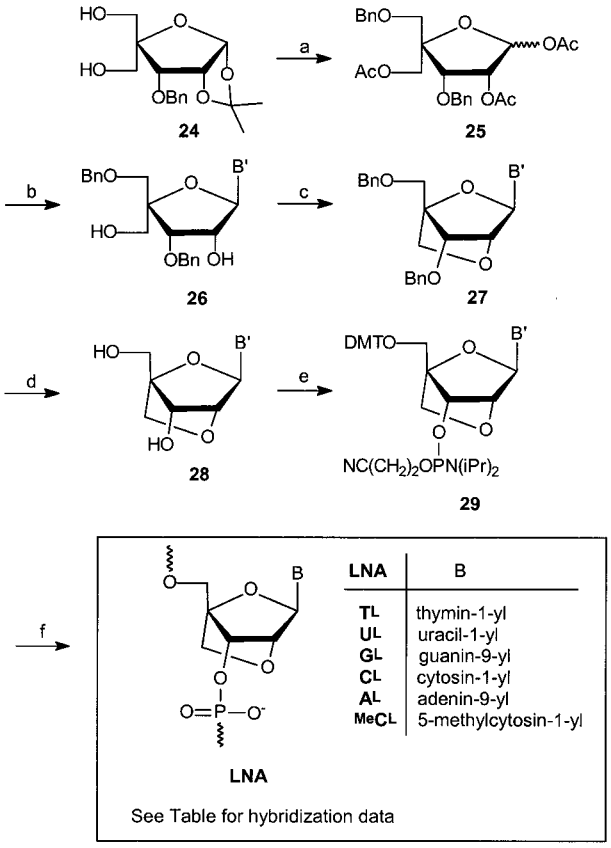


FIGURE 11. (a) (i) BnBr, NaH, DMF, (ii) Ac_2O, pyridine, (iii) 80% AcOH, (iv) Ac_2O, pyridine (55%); (b) (i) nucleobase, BSA, TMS-triflate, CH_3CN or CH_2ClCH_2Cl, (ii) $NaOCH_3$, CH_3OH (38−74%); (c) (i) TsCl, pyridine, (ii) NaH, DMF (30−51%); (d) $Pd(OH)_2/C$, ethanol, H_2; or 1,4-cyclohexadiene, 10% $Pd(OH)_2/C$, methanol; or BCl_3, CH_2Cl_2, hexane (36−98%); (e) (i) DMTCl, pyridine, (ii) $NC(CH_2)_2OP(Cl)N(^iPr)_2$, DIPEA, CH_2Cl_2 (37−65%); (f) DNA synthesizer. B′ = thymin-1-yl, uracil-1-yl, 6-*N*-isobutyrylguanin-9-yl, 4-*N*-benzoylcytosin-1-yl, 2-*N*-benzoyladenin-9-yl, and 4-*N*-benzoyl-5-methylcytosin-1-yl.

monomers preorganized into an unnatural furanose conformation has proven a viable strategy for high-affinity targeting of both DNA and RNA.[49,51]

Summary: Key Characteristics of 2′-*O*,3′-*C*-Bicyclofuranose ONs. (1) The bicyclo[3.3.0] and bicyclo[3.2.0] pentofuranose monomeric nucleosides **20** and **22** are preorganized in conformations deviating from the standard 3′-endo and 2′-endo type conformations generally found in A- or B-form duplexes. (2) Efficient oligomerizations with stepwise coupling yields of 95% for amidites **21** and **23** were accomplished. (3) Moderately increased thermal stability of duplexes toward complementary nucleic acids was obtained for some sequences containing monomers **N** or **O**.

LNA (Locked Nucleic Acid)

Synthesis of LNA. To further evaluate the potential of bicyclic pentofuranose nucleoside monomers, we have introduced LNA (locked nucleic acid, Figure 11).[52−54] It should be mentioned that Imanishi et al. independently were the first to report synthesis of the uracil and cytosine LNA nucleosides and to suggest their usefulness as build-

ing blocks for modified ONs[55] and that the same authors very recently have published some properties of uracil- and cytosine-containing LNA.[56] Molecular modeling and simple model building indicated 2′-*O*,4′-*C*-methylene bicyclonucleoside LNA monomers to be strongly locked in an N-type conformation (^{3}E conformation) which has subsequently been confirmed by X-ray crystallography[56] and NMR.[52,53] For synthesis of the LNA monomers containing six different nucleobases, namely, adenine, cytosine, guanine, 5-methylcytosine, thymine, and uracil, we used a strategy starting from 4′-*C*-hydroxymethyl pentofuranose derivative **24**[36] (Figure 11). Regioselective 5-*O*-benzylation followed by acetylation, acetolysis, and repeated acetylation yielded tri-*O*-acetyl furanose **25** (55% yield) as an intermediate for coupling reactions with silylated nucleobases. Stereoselective reaction with silylated thymine followed by deacetylation afforded in 74% yield nucleoside diol **26** (B′ = thymin-1-yl). Cyclization (4′-*O*-tosylation followed by treatment with base) afforded the 2′-*O*,4′-*C*-linked bicyclo[2.2.1]nucleoside derivative **27** (B′ = thymin-1-yl) in 42% yield. By debenzylation, (*1S*,*3R*,*4R*,*7S*)-7-hydroxy-1-hydroxymethyl-2,5-dioxabicyclo-[2.2.1]heptane thymine nucleoside analogue **28** (B′ = thymin-1-yl) was obtained. The phosphoramidite derivative **29** (B′ = thymin-1-yl) was eventually obtained in 65% yield from nucleoside **28** (B′ = thymin-1-yl) using standard procedures. Stimulated by the appealing properties of LNA containing LNA thymine monomer $\mathbf{T^L}$ (vide infra), synthesis of other nucleobase derivatives was accomplished using similar glycosylations (Figure 11).[52,53] We have developed an alternative strategy for convergent synthesis of LNA nucleosides based on glycosylation reactions on a 4-*C*-tosyloxymethyl furanose derivative.[57] Oligomerizations using LNA amidites **29** proceeded efficiently on a DNA synthesizer (stepwise coupling yields for **29** were 95−99%, 2−12 min couplings). Thus, all modified LNA, deoxy-LNA (a combination of LNA and 2′-deoxynucleotide monomers), and ribo-LNA (a combination of LNA and ribonucleotide monomers) have been synthesized.[52−54,58]

Properties of LNA. The ability of LNA to recognize complementary nucleic acids has been evaluated mainly in a 9-mer mixed sequence context (Table 1; results for the reference strands are depicted in entries 1−4). It can be concluded from the representative data shown in the table that LNA-mediated high-affinity recognition of single-stranded nucleic acids is universal:[53,58,59] (1) Deoxy-LNA (Table 1, entries 5−7) hybridizes strongly with complementary DNA and RNA (ΔT_m = +4.0−7.3 °C per LNA monomer). (2) All-modified LNA (Table 1, entries 8 and 9) hybridizes strongly with complementary DNA and RNA (ΔT_m = +4.0−5.1 °C per LNA monomer). (3) Ribo-LNA (Table 1, entries 10 and 11) hybridizes strongly with complementary DNA and RNA (ΔT_m = +8.3 and +9.3 °C per LNA monomer). (4) LNA:LNA duplexes (Table 1, entries 12−14) display strongly increased thermal affinities and constitute the most stable duplex-type nucleic acid system yet discovered.

We have reported that LNA-mediated nucleic acid recognition (for LNA monomers containing all the major natural bases) obeys the Watson−Crick base-pairing rules with selectivities generally better than those obtained for the corresponding unmodified reference strands.[53,58,59] This, in combination with the unprecedented increases in the thermal stability obtained per LNA monomer incorporated (Table 1), the universality of LNA recognition, and the chemical similarity between LNA and RNA (and DNA), establishes LNA as a unique molecule in the nucleic acid field. CD curves recorded for the various duplex types shown in the table involving fully modified LNA are evidence for an A-form character (especially for the duplex of entry 9), indicating that LNA has a strong influence on the conformation of its complementary strands.[59,60]

Table 1. Hybridization Data of LNA and Reference Strands[a]

entry	duplex	T_m/ °C	ΔT_m/ °C
	References		
1	5′-d(GTGATATGC)/3′-d(CACTATACG)	28	ref
2	5′-d(GTGATATGC)/3′-r(CACUAUACG)	28	ref
3	5′-r(GUGAUAUGC)/3′-d(CACTATACG)	27	ref
4	5′-r(GUGAUAUGC)/3′-r(CACUAUACG)	38	ref
	Deoxy-LNA		
5	5′-d(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)/3′-d(CACTATACG)	44	+5.3[b]
6	5′-d(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)/3′-r(CACUAUACG)	50	+7.3[c]
7	5′-d(GTGATATGC)/3′-d(C$\mathbf{A^L}$CT$\mathbf{A^L}$T$\mathbf{A^L}$CG)	40	+4.0[b]
	All-Modified LNA		
8	5′-(G^{L}T^{L}G^{L}A^{L}T^{L}A^{L}T^{L}G^L ^{Me}C^L)/ 3′-d(CACTATACG)	64	+4.0[b]/ +4.1[d]
9	5′-(G^{L}T^{L}G^{L}A^{L}T^{L}A^{L}T^{L}G^L ^{Me}C^L)/ 3′-r(CACUAUACG)	74	+5.1[c]/ +4.0[e]
	Ribo-LNA		
10	5′-r(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)/3′-d(CACTATACG)	55	9.3[d]
11	5′-r(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)/3′-r(CACUAUACG)	63	8.3[e]
	LNA vs LNA		
12	5′-d(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)/3′-d(C$\mathbf{A^L}$CT$\mathbf{A^L}$T$\mathbf{A^L}$CG)	63[f]	
13	5′-r(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)/3′-d(C$\mathbf{A^L}$CT$\mathbf{A^L}$T$\mathbf{A^L}$CG)	74[f]	
14	5′-G^{L}T^{L}G^{L}A^{L}T^{L}A^{L}T^{L}G^L ^{Me}C^L/ 3′-d(C$\mathbf{A^L}$CT$\mathbf{A^L}$T$\mathbf{A^L}$CG)	85[f]	

[a] A = adenosine monomer, C = cytidine monomer, G = guanosine monomer, U = uridine monomner, T = thymidine monomer, ^{Me}C = 5-methylcytidine monomer, $\mathbf{X^L}$ = LNA monomer. Oligo-2′-deoxynucleotide sequences are depicted as d(sequence) and oligoribonucleotide sequences as r(sequence). The melting temperatures (T_m values) were recorded as described earlier[53] using a medium salt buffer (10 mM sodium phosphate, pH 7.0, 100 mM sodium chloride, 0.1 mM EDTA). ΔT_m values are *the increases in T_m per LNA monomer incorporated* compared to the corresponding reference duplex (ref). [b] Compared to entry 1. [c] Compared to entry 2. [d] Compared to entry 3. [e] Compared to entry 4. [f] Recorded in a buffer without EDTA.

Summary: Key Characteristics of LNA. (1) The bicyclo-[2.2.1]pentofuranose monomeric LNA nucleosides are strongly preorganized in a 3′-endo conformation. (2) Efficient oligomerizations with stepwise coupling yields of 95−99% for amidites **29** were accomplished using standard phosphoramidite chemistry. (3) Unprecedented increases in the thermal affinity of LNA:DNA, LNA:RNA, and LNA:LNA duplexes were obtained without compromising base pairing selectivities.

Conclusion

The work described herein resulted in the development of *C*-branched monomers allowing attachment of ligands to predefined positions in ONs. In addition, by selecting

a 3′-*C*-branched monomer or a 4′-*C*-branched monomer, ligands can be introduced facing either the major or the minor groove of a B-form duplex. The initial promising result obtained by incorporation of the 2′-*O*,3′-*C*-branched bicyclic monomers prompted the synthesis of LNA (locked nucleic acid). Regarding key characteristics, e.g., ease of automated oligomerization and binding properties of both partly and all-modified sequences, LNA certainly is the most promising of the *C*-branched and bicyclic ON analogues described herein. In fact, the universal high-affinity nucleic acid recognition obtained for LNA is unprecedented, also in comparison with PNA, HNA, or N3′−P5′-phosphoramidates, and at present it appears possible to upgrade the affinity of virtually any ON by incorporating a selected number of LNA monomers. On the basis of the developments described herein, the full potential of LNA is presently being evaluated in a biotechnological context. Thus, the reported stability against 3′-exonucleolytic degradation[52] is being further evaluated, the prospect of recognizing nucleic acid duplexes by LNA is being pursued, the properties of LNA and LNA monomers as substrates for a variety of enzymes are being probed, and the function of LNA as an antisense agent is being examined. However, the results already obtained establish the development of LNA as an illustrative example of biomimetic chemistry.

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