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Insights from Crystal Structures into the Opposite Effects on RNA Affinity by the *S*- and *R*-6′-Methyl Backbone Modifications of 3′-Fluoro Hexitol Nucleic Acid (FHNA)†

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Abstract

Locked nucleic acid (LNA) analogs with 2′,4′-bridged sugars show promise in antisense applications. *S*-5′-Me-LNA has high RNA affinity and modified oligonucleotides show reduced immune stimulation *in vivo*. Conversely, an *R*-5′-methyl group dramatically lowers RNA affinity. To test the effects of *S*- and *R*-6′-methyl groups on 3′-fluoro hexitol nucleic acid (FHNA) stability, we synthesized *S*- and *R*-6′-Me-FHNA thymidine and incorporated them into oligo-2′-deoxynucleotides. As with LNA, *S*-6′-Me is stabilizing whereas *R*-6′-Me is destabilizing. Crystal structures of 6′-Me-FHNA-modified DNAs explain the divergent consequences for stability and suggest convergent origins of these effects by *S*- and *R*-6′-Me (FHNA) [-5′-Me (LNA, RNA)] substituents.

Second generation antisense oligonucleotides (ASOs) are being evaluated for their therapeutic potential in the clinic.[1,2] The most advanced ASOs are gapmers that combine the 2′-(2-methoxy)-ethyl (MOE) RNA modification[3] in their flanks with a central DNA window and a fully modified phosphorothioate (PS[4]) backbone. Additional ASO modifications with enhanced RNA affinity and a signature 2′,4′-bridged nucleic acid (BNA) sugar framework have been found to exhibit promising properties for antisense applications (Figure 1). Among them, locked nucleic acid (LNA **1**[5,6]) constitutes the basic representative and recent research has demonstrated that ASOs carrying locked nucleotides allow modulation of gene expression via a variety of mechanisms.[7–9]

As part of a comprehensive program aimed at elucidating the structure activity relationships (SAR) of gapmer ASOs containing high affinity modifications,[10–15] we combined the LNA modification with a methyl substitution at the 5′-position of the bicyclic sugar.[14] Introduction of *S*-5′-Me-LNA **2** residues into ASOs furnished high affinity recognition comparable to that seen with native LNA. Conversely, introduction of *R*-5′-Me-LNA **3** residues neutralized the gains afforded by the LNA modification and resulted in an

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COORDINATE DEPOSITION

The PDB ID codes (http://www.rcsb.org) for the *S*- and *R*- 6′-Me decamers are 3V06 and 3V07, respectively.

SUPPORTING INFORMATION

Materials and methods, Schemes S1 and S2, Tables S1 and S2, Figures S1–S4. This material is available free of charge via the Internet at http://pubs.acs.org.

unfavorable RNA affinity relative to native DNA. In animal experiments, gapmers with central DNA windows and *S*-5′-Me-LNA in their wings exhibited lower drug-induced increases in spleen weights, indicative of reduced immune stimulation, as compared to their LNA counterparts.

Recently, we also evaluated the effect of introducing a methyl group in the (*R*) and (*S*) configuration at the 5′-position of α-L-LNA **4**, which also shows LNA-like high affinity recognition of complementary RNA. However, the consequences on RNA affinity were different from those observed in the β-D-LNA series with the *R*-5′-Me isomer **6** now displaying enhanced affinity as compared to the *S*-5′-Me analog **5**.[16]

In view of the attractive antisense properties displayed by the *S*-5′-Me-LNA modification, and the configuration-dependent divergent effects on RNA affinity in the α-L versus the β-D series, we decided to evaluate the consequences for stability and structure of the methyl backbone modification in the context of a hexitol nucleic acid (HNA[17]) analog, 3′-fluoro hexitol nucleic acid (FHNA) **7**. FHNA-modified ASOs (unlike those containing Ara-FHNA **8**) showed comparable potency to LNA in animal trials without producing hepatotoxicity.[15] Interestingly, the excellent *in vivo* activity observed with FHNA was achieved in the absence of elaborate fomulations to improve delivery and despite the lower RNA affinity of this modification relative to LNA.

Here we report the synthesis, biophysical evaluation and crystal structures of oligonucleotides containing *S*-6′-Me-FHNA **9** or *R*-6′-Me-FHNA **10** residues (Figure 1). The phosphoramidite T building blocks of **9** and **10** and the modified oligonucleotides were synthesized as outlined in Schemes S1 and S2 and Figure S1 (supporting information file). To establish the consequences of the two analogs for the stability of hybrids between modified DNA and RNA, we conducted UV melting experiments with duplexes containing either one or two modified nucleotides (Table 1). The *S*-6′-Me-FHNA-T enhances duplex thermal stability similar to FHNA-T (Figure 1). On the other hand, incorporation of *R*-6′-Me-FHNA-T has a destabilizing effect, amounting to ca. 4°C relative to FHNA-T.[15]

To understand the opposite effects on stability triggered by a methyl substituent at C6′ with *R* or *S* configuration, we studied the crystal structures of A-form decamer duplexes $[d(GCGTA\mathbf{T^*}ACGC)]_2$ (**T***=*S*-6′-Me-FHNA-T **13** or *R*-6′-Me-FHNA-T **14**) with a single modified nucleotide per strand. Both crystallize in the same space group ($P2_12_12_1$) and are isomorphous. The structure of the duplex with *S*-6′-Me-FHNA Ts (*S*-6′-Me decamer) was refined to 1.55 Å resolution and that of the duplex with *R*-6′-Me-FHNA Ts (*R*-6′-Me decamer) was refined to 1.24 Å resolution. Experimental procedures are summarized in the supporting information, selected crystal data and refinement parameters are listed in Table S1, and examples of the quality of the final electron density are depicted in Figure S2 (si file).

In both duplexes all 2′-deoxyribose sugars adopt the C3′-*endo* conformation, consistent with the overall RNA-like A-form conformation (Figure S3; si file). In the region of the modified residues **T*6** and **T*16** (nucleotides in strands 1 and 2 are numbered 1–10 and 11–20, resp.), paired strands exhibit only minimal conformational deviations (Figure S4; si file).

Inspection of the helical parameters and backbone torsion angles in the *S*- and *R*-6′-Me duplexes and comparing them to the structure of the decamer with FHNA T residues at positions 6 and 16 (Figure 2),[15] reveals subtle changes in the torsion angles α (wider in *S*-6′-Me-FHNA and compressed in *R*-6′-Me-FHNA), β (expanded to pure *ap* in *S*-6′-Me-FHNA and compressed in *R*-6′-Me-FHNA), as well as in torsion angle ε of the preceding residues (A5 and A15; Figure 2C, arrow). However, in both 6′-Me-FHNA structures, the sugar-phosphate backbone geometries of modified residues conform to the standard sc^-, *ap*, sc^+,

sc^{+} (60° in HNAs[15,17]), *ap*, *sc*$^{-}$ (α to ζ) genus of A-form duplexes. In both the *S*- and *R*-6′-Me decamers, residue A5 exhibits an extended backbone variant with α, β and γ in the *ap* conformations. In the latter duplex, this conformation is also observed for residue G13.

The most obvious difference between the methyl group in the *S* and *R* configurations at C6′ (note the different atom numberings in FHNA and LNA) in the two structures is a short 1···5 intra-nucleoside contact between C7′ (Me) and O4′ in *R*-6′-Me-FHNA (Figure 2B). In the *S*-6′-Me decamer the spacing between the methyl group and O4′ is considerably larger (Figure 2A). Apart from the aforementioned minor deviations in the torsion angles α, β and ε in the region of the modified residue (Figure 2C), there are no obvious deviations between the conformations of the *R*- and *S*-6′-Me-FHNA nucleotides and the backbone of the latter appears unable to avoid the 1···5 contact.

Because of the conformational similarities between FHNA, HNA, LNA and RNA,[15] the above energetically unfavorable interaction involving O4′ (O3′ in LNA and RNA) as a result of an *R*-6′-Me (*R*-5′-Me in LNA and RNA) substituent will persist in all of these analogs as well as in A-form DNA duplexes. Even when alternative backbone conformations of DNA are considered,[14] such as the above extended backbone variant with α, β and γ all in the *ap* range, or the tricyclo-DNA *ac*$^{-}$ (α), *sc*$^{+}$ (β), *sc*$^{+}$ (γ) backbone,[18] an *R*-configured methyl group will cause energetically unfavorable interactions (Figure 3).

In addition to causing an unfavorable 1···5 backbone, contact, a 6′-methyl group (5′ in LNA and RNA) in the *R* configuration can also be expected to perturb the water structure around O2P phosphate oxygens (Figure 4). By comparison, the *S*-6′-methyl group is directed toward the minor groove (Figures 2, **S3**) and away from the negatively polarized environment around phosphates.

In summary, the structural data provide insight into the opposite effects on RNA affinity seen with the two 6′-Me-FHNA modifications described here and help rationalize the previous observations regarding the modulation of β-D-LNA's duplex stability as a function of the configuration of the 5′-methyl substituent.[14]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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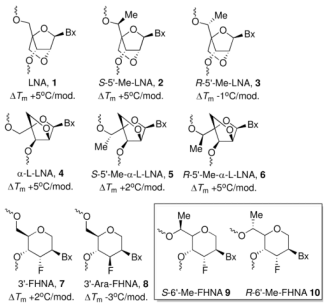

Figure 1.
Structures and duplex thermal stability properties of LNA, α-L-LNA, FHNA and 6′-Me-modified FHNAs.

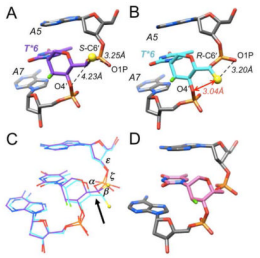

Figure 2.

Conformations of (A) *S*- and (B) *R*-6′-Me-FHNA (purple and cyan carbon atoms, resp.), (C) superimposition of the two, and (D) the conformation of FHNA (pink carbon atoms) for comparison. The methyl carbon is shown as a yellow sphere, F3′ is green, residues are labeled and the short 1···5 contact in *R*-6′-Me-FHNA T is highlighted with a red arrow.

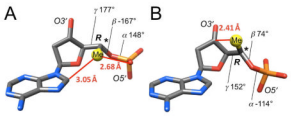

Figure 3.

An *R*-5′-methyl substituent (yellow) in RNA or A-DNA will cause energetically unfavorable, short contacts (red lines) even when sugar-phosphate backbone conformations other than the standard *sc*$^{-}$, *ap*, *sc*$^{+}$, *sc*$^{+}$, *ap*, *sc*$^{-}$ (α to ζ) geometry are considered. (A) Extended backbone variant with α, β and γ in the *ap* range (seen for residue A5 in the *S*-6′-Me decamer structure). (B) Backbone conformation in tricyclo-DNA[18] with a compensatory change in β and γ.

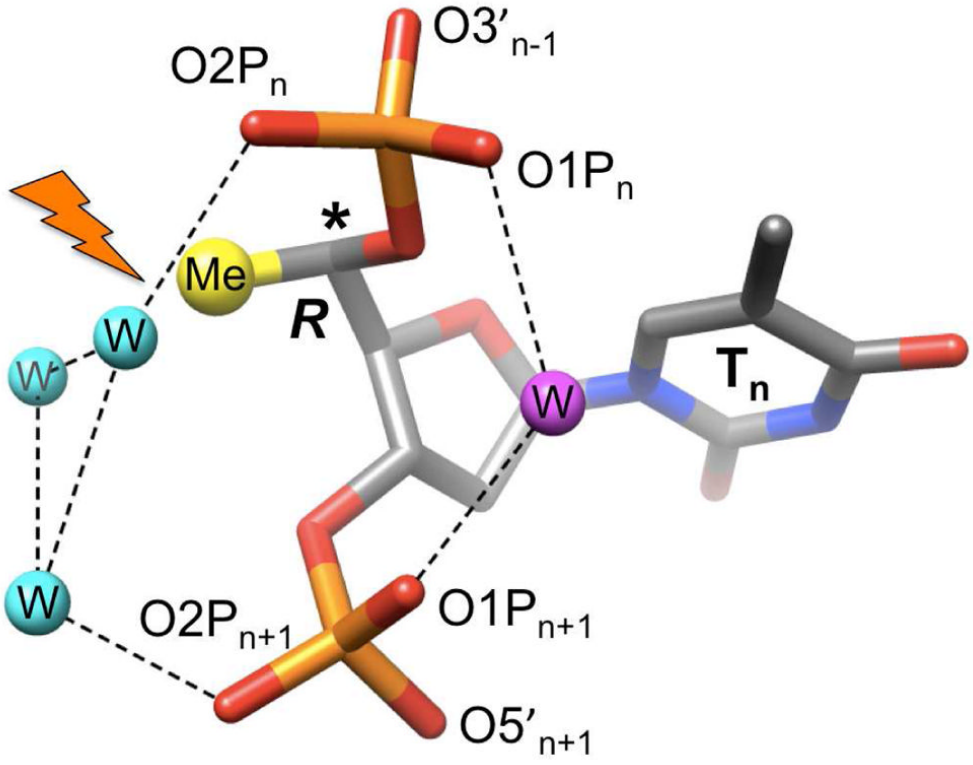

Figure 4.

The *R*-5′-methyl group (modeled) juts into a hydrophilic environment and will interfere (flash) with phosphate hydration (water molecules are cyan and purple spheres) as observed in the 0.83 Å crystal structure of an A-form DNA.[19] The shorter distance between O1P oxygens on the edge of the major groove can typically be bridged by a single water (purple), whereas the wider spacing between O2P oxygens requires 2-water bridges (cyan).

TABLE 1

Thermal stabilities of the duplexes between *S*-and *R*-6′-Me-FHNA modified DNA and RNA.

Oligonucleotidea	**FHNA-T***	**T_m[b][°C]**	**ΔT_m/mod[°C]**
GCGTTTTTTGCT	DNA	45.6	—
GCGTT-**T***-TTTGCT	*S*-6′-Me **9**	46.5	+ 0.9
GCGTT-**T*T***-TTTGCT	*S*-6′-Me **9**	48.2	+ 1.3
GCGTT-**T***-TTTGCT	*R*-6′-Me **10**	42.3	− 3.3
GCGTT-**T*T***-TTTGCT	*R*-6′-Me **10**	40.7	− 2.4

[a] **T*** indicates a modified nucleotide.

[b] T_m values (error ± 0.5°C) were measured at 4 μM oligo concentration in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 0.1 mM EDTA. The RNA complement was 5′-r(AGCAAAAAACGC)-3′.