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

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Supporting Information

**ABSTRACT:** Locked nucleic acid (LNA) analogues with 2′,4′-bridged sugars show promise in antisense applications. *S*-5′-Me-LNA has high RNA affinity, and modified oligonucleotides show weakened 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 and 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 unfavorable RNA affinity relative to native DNA. In animal experiments, gapmers with central DNA windows and *S*-5′-Me-LNA in their wings exhibited smaller drug-induced increases in spleen weights, indicative of weakened immune stimulation, than their LNA counterparts.

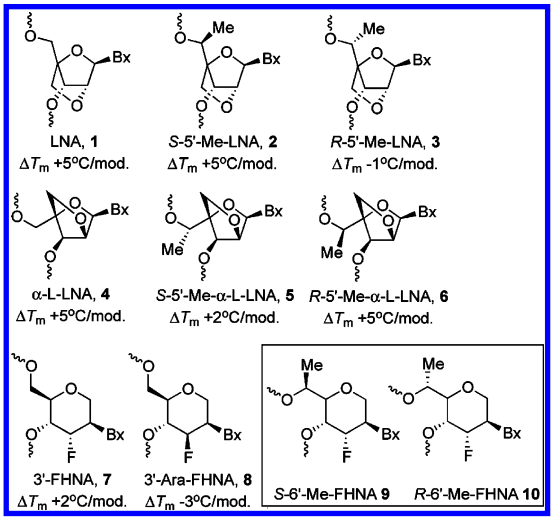


**Figure 1.** Structures and duplex thermal stability properties of LNA, α-L-LNA, FHNA, and 6′-Me-modified FHNAs.

**Table 1. Thermal Stabilities of the Duplexes between *S*- and *R*-6′-Me-FHNA-Modified DNA and RNA**

| oligonucleotide[a] | FHNA-T* | $T_m$[b] (°C) | $\Delta T_m$/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 of ±0.5 °C) were measured at 4 μM oligo 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′.

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 for RNA affinity were different from those observed in the β-D-LNA series with the *R*-5′-Me isomer **6** now displaying enhanced affinity compared to that of *S*-5′-Me analogue **5**.[16]

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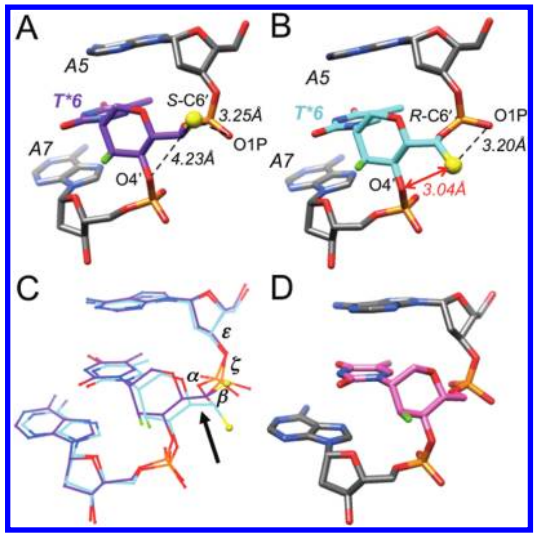


**Figure 2.** Conformations of (A) *S*- and (B) *R*-6′-Me-FHNA (purple and cyan carbon atoms, respectively), (C) superimposition of the two, and (D) conformation of FHNA (pink carbon atoms) for comparison. The methyl carbon is shown as a yellow sphere. F3′ is colored green. Residues are labeled. The short 1···5 contact in *R*-6′-Me-FHNA T is highlighted with a red arrow.

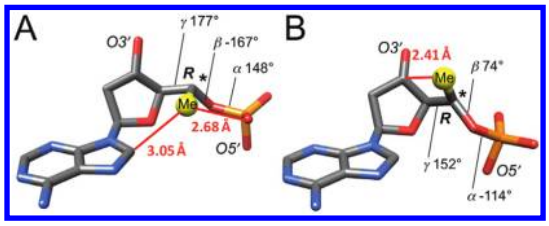


**Figure 3.** An *R*-5′-methyl substituent (yellow sphere) in RNA or A-DNA causes energetically unfavorable, short contacts (red lines) even when sugar−phosphate backbone conformations other than the standard *sc*$^-$, *ap*, *sc*$^+$, *sc*$^+$, *ap*, *sc*$^-$ (*α* to *ζ*) geometry are considered. (A) 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*.

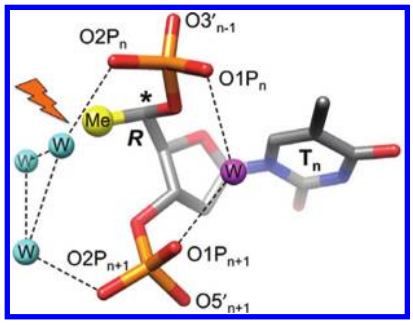


**Figure 4.** An *R*-5′-methyl group (modeled) juts into a hydrophilic environment and will interfere (flash) with phosphate hydration (water molecules are shown as cyan and purple spheres) as observed in the 0.83 Å crystal structure of an A-form DNA.[19] The shorter distance between O1P atoms on the edge of the major groove can typically be bridged by a single water (purple), whereas the wider spacing between O2P atoms requires two-water bridges (cyan).

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 series versus the *β*-D series, we decided to evaluate the consequences for the stability and structure of the methyl backbone modification in the context of a hexitol nucleic acid (HNA[17]) analogue, 3′-fluoro hexitol nucleic acid (FHNA) **7**. FHNA-modified ASOs (unlike those containing Ara-FHNA **8**) showed potency comparable to that of 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 that of LNA.

Here we report the synthesis, biophysical evaluation, and crystal structures of oligonucleotides containing an *S*-6′-Me-FHNA **9** or *R*-6′-Me-FHNA **10** residue (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. To establish the consequences of the two analogues 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 like FHNA-T does (Figure 1). On the other hand, incorporation of *R*-6′-Me-FHNA-T has a destabilizing effect, amounting to ∼4 °C relative to FHNA-T.[15]

To understand the opposite effects on stability triggered by a methyl substituent at C6′ with the *R* or *S* configuration, we studied the crystal structures of A-form decamer duplexes [d(GCGTA**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) 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.

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

Inspection of the helical parameters and backbone torsion angles in the *S*- and *R*-6′-Me duplexes and comparison of them to the structure of the decamer with FHNA T residues at positions 6 and 16 (Figure 2)[15] reveal subtle changes in torsion angles *α* (wider in *S*-6′-Me-FHNA and compressed in *R*-6′-Me-FHNA) and *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 types of atom numbering in FHNA and LNA) in the two structures is a short 1···5 intranucleoside contact between C7′ (Me) and O4′ in *R*-6′-Me-FHNA (Figure 2B). In the *S*-6′-Me decamer, the


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space between the methyl group and O4′ is considerably larger (Figure 2A). Apart from the aforementioned minor deviations in torsion angles $\alpha$, $\beta$, and $\varepsilon$ 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 of FHNA, HNA, LNA, and RNA,[15] the energetically unfavorable interaction described above 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 analogues 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 $\alpha$, $\beta$, and $\gamma$ all in the *ap* range, or the tricyclo-DNA $ac^{-}$ ($\alpha$), $sc^{+}$ ($\beta$), $sc^{+}$ ($\gamma$) 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 atoms (Figure 4). By comparison, the *S*-6′-methyl group is directed toward the minor groove (Figure 2 and Figure 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 $\beta$-D-LNA's duplex stability as a function of the configuration of the 5′-methyl substituent.[14]

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

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

### Accession Codes

The Protein Data Bank entries for the *S*- and *R*-6′-Me decamers are 3V06 and 3V07, respectively.

## ■ AUTHOR INFORMATION

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