Stabilizing contributions of sulfur-modified nucleotides: crystal structure of a DNA duplex with 2'-O-[2-(methoxy)ethyl]-2-thiothymidines

Benjamin Diop-Frimpong, Thazha P. Prakash1, Kallanthottathil G. Rajeev2, Muthiah Manoharan2 and Martin Egli3,*

Department of Chemical Engineering, School of Engineering, Vanderbilt University, Nashville, TN 37235, USA, 1Department of Medicinal Chemistry, Isis Pharmaceuticals Inc., Carlsbad, CA 92008, USA, 2Drug Discovery, Alnylam Pharmaceuticals Inc., Cambridge, MA 02142 USA and 3Department of Biochemistry, School of Medicine, Vanderbilt University, Nashville, TN 37232, USA

Received May 27, 2005; Revised and Accepted August 23, 2005

ABSTRACT
Substitution of oxygen atoms by sulfur at various locations in the nucleic acid framework has led to analogs such as the DNA phosphorothioates and 4'-thio RNA. The phosphorothioates are excellent mimics of DNA, exhibit increased resistance to nuclease degradation compared with the natural counterpart, and have been widely used as first-generation antisense nucleic acid analogs for applications in vitro and in vivo. The 4'-thio RNA analog exhibits significantly enhanced RNA affinity compared with RNA, and shows potential for incorporation into siRNAs. 2-Thiouridine (s2U) and 5-methyl-2-thiouridine (m5s2U) are natural nucleotide analogs. s2U in tRNA confers greater specificity of codon–anticodon interactions by discriminating more strongly between A and G compared with U. 2-Thio modification preorganizes the ribose and 2'-deoxyribose sugars for a C3'-endo conformation, and stabilizes heteroduplexes composed of modified DNA and complementary RNA. Combination of the 2-thio and sugar 2'-O-modifications has been demonstrated to boost both thermodynamic stability and nuclease resistance. Using the 2'-O-[2-(methoxy)ethyl]-2-thiothymidine (m5s2Umoe) analog, we have investigated the consequences of the replacement of the 2-oxygen by sulfur for base-pair geometry and duplex conformation. The crystal structure of the A-form DNA duplex with sequence GCGTAT*ACGC (T* = m5Umoe) was determined at high resolution and compared with the structure of the corresponding duplex with T* = m5Umoe. Notable changes as a result of the incorporation of sulfur concern the base-pair parameter ‘opening’, an improvement of stacking in the vicinity of modified nucleotides as measured by base overlap, and a van der Waals interaction between sulfur atoms from adjacent m5s2Umoe residues in the minor groove. The structural data indicate only minor adjustments in the water structure as a result of the presence of sulfur. The observed small structural perturbations combined with the favorable consequences for pairing stability and nuclease resistance (when combined with 2'-O-modification) render 2-thiouracil-modified RNA a promising candidate for applications in RNAi.

INTRODUCTION
Chemically modified nucleic acids have been studied extensively in the context of the development of antisense therapeutics (1,2) and more recently in the search for small interfering RNAs (siRNAs) for in vitro and in vivo applications (3,4). In both cases, increases in RNA affinity as a result of modification can be expected to play an important role in the improvement of the efficacy of putative oligonucleotide therapeutics (5). Phosphorothioate DNA (PS-DNA) (6) has undergone extensive tests in numerous clinical trials of antisense therapeutics and in recent studies for small interfering RNAs (siRNAs) for in vitro and in vivo applications (3,4). In both cases, increases in RNA affinity as a result of modification can be expected to play an important role in the improvement of the efficacy of putative oligonucleotide therapeutics (5). Phosphorothioate DNA (PS-DNA) (6) has undergone extensive tests in numerous clinical trials of antisense oligonucleotides (7). In PS-DNA, one of the non-bridging phosphate oxygen atoms is replaced by sulfur. Although this altered chemistry leads to a slight reduction in RNA affinity relative to DNA, PS-DNA was considered a promising first-generation antisense modification for a number of reasons, including ease of synthesis, increased nuclease resistance...
and binding to proteins (biodistribution) as well as degradation of RNA bound to PS-DNA by RNase H (8). Another sulfur modification, 2-thiouridine (s^2U) was found to strongly increase the melting temperature ($T_m$) of heteroduplexes between modified strands and RNA compared with the corresponding DNA:RNA hybrids (9). For example, incorporation of a single s^2U residue in the center of a nonamer DNA strand led to an increase of 9.4°C in the $T_m$ for the duplex with the complementary RNA strand compared with the unmodified hybrid duplex (10). Consecutive and interspersed replacement of several thymidines by 2′-O-[2-(methoxy)ethyl]-2-thiothymidine (m^5s^2Umoe; Figure 1) furnished an average gain in $T_m$ of 3.5 and 3°C, respectively, for heteroduplexes between modified strands and RNA relative to the native duplexes (11).

The s^2U modification occurs naturally in transfer RNAs [(12–14); see also the RNA modification database http://medstat.med.utah.edu/RNAmods/]. The modification is present at the first position of the anticodon, position 34, and affects the relative stabilities of pairing with A and the wobble pairing with G of codons, thereby stabilizing and increasing the specificity of codon–anticodon interactions. Because sulfur has a bigger atomic radius and lower electronegativity than oxygen, its H-bonding ability is weaker relative to the latter. As a result, the interaction between the sulfur in the 2-position of s^2U with the hydrogen on N1 of G (Figure 2A, right) is weakened compared with the corresponding pair between U and G (Figure 2B, right). On comparison, the hydrogen bond between N1 of A and N3-H of s^2U (Figure 2A, left) is only indirectly affected, rendering its stability similar to that of the U:A pair (Figure 2B, left). In summary, in the absence of the sulfur modification, the more electronegative oxygen in the 2-position of U will readily form a hydrogen bond with the hydrogen on N1 of G. This makes the U:G interaction more similar to the U:A interaction (Figure 2B) compared with the relative stability of the s^2U:G and s^2U:A pairs (Figure 2A) [(15) and cited references]. In addition to conferring pairing specificity, the s^2U modification also restricts uridine dynamics by locking the nucleoside in the anti, C3′-endo conformation (16,17). Moreover, the conformational effects of the modification extend into the 3′-adjacent nucleosides, causing them to assume a similar conformation (18,19). The strong conformational preorganization towards the C3′-endo pucker by the sulfur at the 2-position of the base is consistent with the aforementioned increased RNA affinity exhibited by oligonucleotides containing the s^2U modification.

To examine the potential conformational changes in a duplex as a consequence of replacement of T by m^5s^2U and to better understand the origins of the enhanced stability of s^2U-modified duplexes compared with their native counterparts, we determined the X-ray crystal structure of an A-form DNA decamer duplex with sequence 5′-GCGTA(m^5s^2Umoe)ACGC and two 2′-O-[2-(methoxy)ethyl]-2-thiothymidine residues. The structure of the duplex with the same sequence containing a single 2′-O-[2-(methoxy)ethyl]-thymidine per strand (5′-GCGTA(m^5Umoe)ACGC) had previously been determined at a similar resolution and was used as the reference (20). The 2′-O-MOE RNA analog is

Figure 1. Structure of 2′-O-[2-(methoxy)ethyl]-2-thiothymidine (s^2m^5Umoe).

Figure 2. Pairing of U and s^2U with A and G. (A) Diagram illustrating s^2U base-pairing with both A and G; s^2U favors A over G. (B) Diagram illustrating the lack in specificity with uridine; U binds with similar affinity to both A and G.
The 5′-modified oligonucleotide crystallographic data obtained for partially (20) and all-2′-modified duplexes (23).

Here, we show that the presence of sulfur at the 2-position of T results in subtle changes in the local helical parameters and goes along with a slight improvement of the stacking interactions with adjacent bases, evident from the comparison of the orientations of the m5s2U and m5U bases relative to their nearest neighbors in the m3s2U/moee-modified and reference duplex, respectively. Overall the structural data are consistent with the significant gains in thermodynamic stability afforded by the s2U modification.

MATERIALS AND METHODS

Synthesis of the 2-thio-2′-MOE-5-methyluridine modified oligonucleotide

The 5′-DMT-2′-MOE-2-thiothymidine-3′-phosphoramidite was prepared according to the published procedure (11). The oligonucleotide d(GCGTA)-m5s2Ume-d(ACGC) was synthesized on functionalized controlled pore glass (CPG) on an automated solid phase DNA synthesizer with final DMT group retained at the 5′ end. Standard phosphoramidites and solid supports were used for incorporation of A, T, G and C residues. A 0.1 M solution of the amidites in anhydrous acetonitrile was used for the synthesis of the modified oligonucleotide. For incorporation of 2-thio-2′-MOE-5-methyl U amidite, 6 equivalents of phosphoramidite solution were delivered in two portions, each followed by a 5 min coupling wait time. All other steps in the protocol supplied by the manufacturer were used without modification. Oxidation of the internucleotide phosphate to the phosphate was carried out using tert-butyl hydroperoxide/acetonitrile/water (10:87:3) with 10 min oxidation wait time. The stepwise coupling efficiencies were >97%. After completion of the synthesis, the solid support was suspended in aqueous ammonium hydroxide (30 wt%) and kept at room temperature for 2 h. The solid support was filtered and the filtrate was heated at 55°C for 6 h to complete the removal of all protecting groups. The crude oligonucleotide was purified by high performance liquid chromatography (HPLC, C-4 column, Waters, 7.8 × 300 mm, 15 μm, 300 Å, buffer A = 100 mM ammonium acetate, pH 6.5–7, buffer B = acetonitrile, 5–60% of B in 55 min, flow 2.5 ml min−1, λ 260 nm). Detritylation was achieved by adjusting the pH of the solution to 3.8 with acetic acid and keeping at room temperature until complete removal of the trityl group, as monitored by HPLC analysis. The oligonucleotide was then desalted by HPLC and characterized by ES-MS (calculated mass: 3118.35 g mol−1; found: 3119.57 g mol−1) and purity (91% full length) was assessed by capillary gel electrophoresis.

Crystallizations and data collection

Crystallization trials were performed with the Nucleic Acid Mini-screen (24) by Hampton Research (Aliso Viejo, CA), using the hanging drop vapor diffusion technique. Droplets with volume 2 μl of a 1:1 mixture of sample and mini-screen buffer were equilibrated against 1 ml of 35% 2-methyl-2,4-pentanediol (MPD). Crystals were obtained from a droplet that contained 1 mM oligonucleotide, 40 mM sodium cacodylate pH 7.0, 12 mM spermine tetrahydrochloride, 12 mM sodium chloride, 80 mM potassium chloride and 10% v/v MPD. A single crystal was mounted in a nylon loop and frozen in liquid nitrogen. Diffraction data were collected at low temperature in a cold nitrogen stream on the 5-ID beamline of the DuPont-Northwestern-Dow Collaborative Access Team at the Advanced Photon Source, Argonne, IL. Separate data sets for high and low resolution reflections were acquired. All data were processed with the program XDS (25) and a summary of selected crystal data and data collection statistics is listed in Table 1.

Crystal structure determination and refinement

Due to the close similarity between two of the unit cell constants, the diffraction data were processed both in the space groups P222 (orthorhombic) and P422 (tetragonal). The resulting values for R-merge differed only minimally (<1%) and structure determination was subsequently attempted in P222, the space group found for the vast majority of crystals of A-form DNA 10mers with isolated 2′-O-modified residues investigated in our laboratory (20), and in space group P422. Phasing was carried out by the molecular replacement method using the program AMORE (26) as part of the CCP4 suite of crystallographic programs (27). An A-form DNA decamer of the same sequence GCGTATAGCG (28) was used as the search model. Promising solutions with good R-factors, reasonable correlation coefficients, and few short lattice contacts were found in both space groups. Initial refinements of the models were performed with the program CNS (29), setting aside 5% randomly selected reflections for calculating the R-free (30). While the R-factor calculated for the model in the orthorhombic space group P222 dropped rapidly, it failed...
to drop below 35% in space group \( P4_22_12 \). Consequently, refinement was continued only in \( P2_12_12_1 \). After rigid-body refinement and simulated annealing plus multiple rounds of positional and individual B-factor refinement, the complete model, including the 2-thio and 2'-O-MOE modifications for the two modified residues, exhibited an \( R \)-factor of 30.1% (\( R \)-free of 28.5%). Refinement was then continued with the program REFMAC (31), using a bulk solvent correction and treating DNA atoms and water molecules with restraint anisotropic B-factors (32,33). The final model refined to an \( R \)-factor of 19.4%, using all reflections up to a resolution of 1.61 Å. Selected refinement parameters are listed in Table 1 and an example of the quality of the final Fourier sum \( (2F_o-F_c) \) electron density is depicted in Figure 3.

**Coordinates**

Final coordinates and structure factors have been deposited in the Protein Data Bank (http://www.rcsb.com) PDB ID code 2AXB.

**RESULTS**

**Overall structure**

Owing to the similar \( b \) and \( c \) unit cell constants (Table 1), diffraction data from a single crystal of the \( m^2s^2Umoe \)-modified DNA decamer were indexed and processed both in orthorhombic and tetragonal space groups. Following structure determination with molecular replacement using an A-form model, initial rounds of refinement in the space groups \( P2_12_12_1 \) and \( P4_22_2 \) indicated that the crystal system is orthorhombic. Thus, the crystallographic asymmetric unit consists of a single duplex and the lattice features a non-crystallographic 4-fold symmetry. Multiple rounds of coordinate and temperature factor refinements and simulated annealing led to an improved model, and electron density maps based on it revealed ordered 2'-O-MOE substituents in the minor groove. The positions of sulfur atoms were marked by spherical peaks of density that are clearly enlarged relative to those around the exocyclic O4 and O2 atoms of unmodified thymines (Figure 3). In the duplex, residues of one strand are numbered from 1 to 10, and those in the complementary strand are numbered from 11 to 20; \( m^2s^2Umoe \) residues are located at positions 6 and 16.

The modified duplex adopts an A-form conformation with an average helical rise of 2.64 Å (SD 0.69 Å) an average helical twist of 34.3° (SD 3.4°). The average inclination of base pairs relative to the helical axis is 15.6°. Except for residues at the 5’-terminal ends, the conformations of all sugars fall into the C3'-endo range. The deoxypyrimidines of residues G1 and C2 adopt C2'-exo and C4'-exo puckers, respectively, and G11 is flipped into the C2'-endo conformation. Similarly, most of the backbone torsion angle conformations observed in the decamer duplex are those typically associated with an A-form geometry (-sclap/sclap/sclap/sclap: \( \alpha \) to \( \zeta \)). Exceptions are constituted by A5 in strand 1 and G13 in strand 2 that both assume an extended backbone variant with \( \alpha \), \( \beta \) and \( \gamma \) in the \( ap \) conformation. The conformation is brought about by a crankshaft motion of the backbone and is accompanied by elongated distances between adjacent intra-strand phosphorus atoms (6.9 Å between A5 and \( m^2s^2Umoe \)6 and 6.4 Å between G13 and T14; avg P'·P distance: 6.1 Å).

To analyze the geometry of the \( m^2s^2Umoe \)-modified duplex further and to determine potential changes as a result of the presence of sulfur atoms in the central \( A;m^2s^2Umoe \) base pairs, we used the previously determined structure of the duplex with identical sequence and \( m^2Umoe \) residues at positions 6 and 16 as a reference (20). The resolution of that structure is similar (1.93 Å) to that of the \( m^2s^2Umoe \)-modified duplex described here. Both crystals belong to space group \( P4_22_12 \) and have very similar cell constants \([a = 24.93, b = 44.59, c = 45.38 \text{ Å} \text{ (reference)}; a = 25.25, b = 45.00, c = 44.97 \text{ Å}]\). We will refer to the reference...
and the 2-thio-modified duplexes as the \(5^\circ \text{Umoe} \) and \(m^{5}s^2\text{Umoe} \) structures, respectively.

**Crystal packing effects**

Because the conformational perturbations caused by the 2-thio modification can be expected to be quite subtle, it is important to identify and quantify conformational differences between the \(m^{5}s^2\text{Umoe}-\text{modified and reference duplexes that are due to crystal packing effects. Interactions between neighboring duplexes in the crystal lattices of } m^{5}\text{Umoe} \) and \(m^{5}s^2\text{Umoe} \) decamers involve stacking of terminal base pairs into the outer portions of the minor groove (34). This particular mode of interaction triggers various degrees of kinking into the major groove. Individual crystallization conditions, the chemistry and spatial properties of substituents of \(2'-O\)-modified residues and their location in the decamer strand, as well as data collection temperature all appear to play a role in the relative degree of kinking (35). Superimposition of the two duplexes reveals that the \(5^\circ \text{Umoe} \) decamer exhibits a slightly stronger kink than the \(m^{5}s^2\text{Umoe} \) decamer (Figure 4).

The kink compresses the major groove and occurs between the tetramer \(5'-\text{G1pC2pG3pT4} \) and the hexamer \(p\text{A5p(}m^{5}\text{Umoe6)pA7pC8pG9pC10} \). It is accompanied by significant changes in the local base-pair (stagger), base-pair step (rise, twist and roll) and base-pair helical parameters (inclination). The absolute values of these parameters at that site constitute either the minimum (stagger \(m^{5}\text{Umoe} \), rise, twist) or maximum (stagger \(m^{5}s\text{Umoe} \), roll, inclination) among the individual observations with both duplexes. Most probably, these changes are only weakly affected by the oxygen—sulfur substitution in residue 16, although the kink occurs between the \(T4:A17 \) and \(A5:m^{5}s\text{Umoe16} \) pairs. Rather, they are a reflection of the intrinsic conformational properties (‘bendability’) of the decamer sequence and the packing forces in the orthorhombic crystal lattice. Regarding the possible origins of the observed difference in kinking in the two duplexes, it is noteworthy that the \(m^{5}\text{Umoe} \) structure was determined at room temperature (20) whereas the \(m^{5}s\text{Umoe} \) structure is derived from diffraction data collected at \(-170^\circ C \). Thus, slightly divergent packing forces due to the temperature change are the most likely cause of the observed differences between the global conformations of the \(m^{5}s^2\text{Umoe} \) and \(m^{5}\text{Umoe} \) duplexes. Because of the kink adjacent to the \(A5:m^{5}s\text{Umoe16} \) base pair and the ensuing perturbations in its immediate vicinity, one would expect that careful analysis of the geometry of the other modified base pair, \(m^{5}s\text{Umoe6:A15} \), might yield a more reliable estimate of the changes as a result of the 2-thio modification.

**Effects of the 2-thio modification on the local conformation**

Both structures were analyzed with the program 3DNA that allows calculation of geometric parameters for nucleic acid molecules (36). Among the parameters, one would expect those that concern the local geometry of base pairs and the stacking interactions to be the most informative in terms of potential conformational changes due to 2-thio modification. Figure 5 depicts graphic representations of selected geometric parameters for base pairs in the \(m^{5}s^2\text{Umoe} \) and reference duplexes. A significant change (11°) is observed in the parameter opening for base pair \(m^{5}s^2\text{Umoe6:A15} \) compared with the corresponding pair in the reference structure (Figure 5A). This change in opening results in a larger separation between the C2 atoms of the two bases in the minor groove with the 2-thio modified pair [4.23 Å versus 3.89 Å (ref. structure); Figure 6A]. Comparison of the stretch parameters also indicates a small change (0.15 Å; not shown) for this base pair that is related to accommodating the larger sulfur atom. Compared with the above change in opening, the differences in the parameters shear, stagger and propeller twist (Figure 5B–D, respectively) are relatively minor for base pair \(m^{5}s\text{Umoe6:A15} \). Likewise, it does not appear that buckling of bases is affected by the sulfur substitution. The calculated values for both the \(m^{5}s^2\text{Umoe6:A15} \) and the \(A5:m^{5}s\text{Umoe16} \) pairs differ by <1° from those for the corresponding pairs in the reference structure. Interestingly, the latter base pair does not exhibit an altered opening angle relative to the reference duplex (Figure 5A). The difference amounts to less, ~2° although the separation between C2 atoms of bases (4.11 Å) is comparable with that seen in

---

**Figure 4.** Overall conformation of the \(m^{5}s^2\text{Umoe}-\text{modified decamer (atoms are colored green, blue, red, orange and yellow for carbon, nitrogen, oxygen, phosphorus and sulfur, respectively) compared with the } m^{5}\text{Umoe}-\text{modified reference duplex (thin blue lines)). The view of the A-form duplexes is across the major (top) and minor grooves (bottom) and the 5'-terminal G1 and G11 residues are located at the top left and bottom left, respectively. 2'-O-Substituents are jutting into the minor groove and 2-sulfur atoms highlighted in yellow. The reduced kink into the major groove in the \(m^{5}s^{2}\text{Umoe}-\text{modified decamer between the tetramer } 5'-\text{G1pC2pG3pT4} \) and the hexamer \(p\text{A5p(}m^{5}\text{Umoe6)pA7pC8pG9pC10} \). It is accompanied by significant changes in the local base-pair (stagger), base-pair step (rise, twist and roll) and base-pair helical parameters (inclination). The absolute values of these parameters at that site constitute either the minimum (stagger } m^{5}\text{Umoe} \), rise, twist) or maximum (stagger } m^{5}s\text{Umoe} \), roll, inclination) among the individual observations with both duplexes. Most probably, these changes are only weakly affected by the oxygen—sulfur substitution in residue 16, although the kink occurs between the \(T4:A17 \) and \(A5:m^{5}s\text{Umoe16} \) pairs. Rather, they are a reflection of the intrinsic conformational properties (‘bendability’) of the decamer sequence and the packing forces in the orthorhombic crystal lattice. Regarding the possible origins of the observed difference in kinking in the two duplexes, it is noteworthy that the \(m^{5}\text{Umoe} \) structure was determined at room temperature (20) whereas the \(m^{5}s\text{Umoe} \) structure is derived from diffraction data collected at \(-170^\circ C \). Thus, slightly divergent packing forces due to the temperature change are the most likely cause of the observed differences between the global conformations of the \(m^{5}s^2\text{Umoe} \) and \(m^{5}\text{Umoe} \) duplexes. Because of the kink adjacent to the \(A5:m^{5}s\text{Umoe16} \) base pair and the ensuing perturbations in its immediate vicinity, one would expect that careful analysis of the geometry of the other modified base pair, \(m^{5}s\text{Umoe6:A15} \), might yield a more reliable estimate of the changes as a result of the 2-thio modification.
the m$^5$s$^2$Umoe6:A15 pair (see above). Similarly, other parameters, including stretch, shear, stagger and propeller twist change subtly, but none of them appears to be making the major contribution such as opening in case of the m$^5$s$^2$Umoe- (filled circles, thin solid lines) and m$^1$Umoe-modified decamer duplexes (filled squares, dashed lines). All parameters were calculated with the program 3DNA (36) and a cartoon of the particular parameter is shown at the upper right in each panel.

In order to examine whether presence of the sulfur atoms in the m$^5$s$^2$Umoe structure led to changes in the stacking interactions involving the four central base pairs, we calculated the overlap areas between adjacent pairs in Å$^2$ units (36). The 3DNA program allows computation of the surface areas of polygons projected in the mean plane of a base pair at a particular step, including exocyclic ring atoms. Stacking interactions at selected base-pair steps are shown in Figure 7.
Inspection of the plotted diagrams for the m5s2Umoe and reference duplexes reveals minor alterations in stacking at most base-pair steps. An exception is found at the (A5)p(m5s2Umoe6):(A15)p(m5s2Umoe16) step (Figure 7C) that displays a clearly visible shift between base pairs relative to the reference structure. Indeed, the calculated y-displacement at that site shows a difference of 2.3 Å between the m5s2Umoe and m5Umoe structures (1.1 versus −1.2 Å, respectively; Figure 5E). The difference in x-displacement is somewhat smaller, −1.3 Å (−5.3 versus −4.0 Å in m5s2Umoe and m5Umoe, respectively; Figure 5F), but constitutes the largest change for that parameter in the entire duplex. The two neighboring steps also show considerable changes in y-displacement that amount to −1.6 Å (Figure 7B) and −1.7 Å (Figure 7D). At the central step, it is readily apparent that the two sulfur atoms have moved toward one another. Indeed, the distance between the sulfur atoms (3.48 Å) is shorter than the one between the corresponding O2 atoms in the reference structure (3.73 Å). The former is slightly shorter than the one between the corresponding O2 atoms in the distance between the sulfur atoms (3.48 Å) is shorter than the one between the corresponding O2 atoms in the reference structure (3.73 Å).

DISCUSSION

The primary goal of the present study was an assessment of the structural perturbations induced by the 2-thio modification in an A-form duplex environment. The m5s2Umoe nucleoside does not occur naturally; the choice to focus on this modification rather than the natural, biologically active s2U and m5s2U pyrimidines (13,38) is based on the expectation that the 2-thio modification alone will most probably not provide sufficient nuclease resistance for in vivo and cell-based RNAi or antisense applications. A facile synthesis of the m5s2Umoe phosphorimidate building block for solid-phase synthesis has been reported, and, indeed, the combination of the 2-thio and 2’-OMOE modifications confers increased nuclease resistance based on in vitro measurements of resistance against snake venom phosphodiesterase degradation (half-lives of oligos with m5s2Umoe modifications >24 h) (11).

Replacement of the 2-oxygen of U or T by sulfur was shown to greatly increase RNA affinity of base-modified oligonucleotides (9–11). The combination of the 2-thio and 2’-OMOE modifications also resulted in a higher thermodynamic stability of duplexes between modified oligonucleotides and complementary DNA (11), albeit to a lesser extent compared with the corresponding RNAs. One would not expect a modification
that brings about a considerable increase in the stability of duplexes to significantly alter the geometry of the nucleobase, sugar or phosphate portions, and/or the local and global conformations of a secondary or tertiary nucleic acid structural motif. This assumption is based on observations from crystallographic data, indicating that stabilizing modifications commonly mimic the native conformation, whereas those that lead to a loss of stability often display significant deviations.

**Figure 7.** Improvement of stacking interactions as a result of the 2-thio modification. Panels on the left show stacking interactions in the m5s2Umod-modified duplex and panels on the right show stacking interactions at the corresponding steps in the m5Umod-modified reference duplex. Base-pair steps (A) (G3)p(T4):(A17)p(C18), (B) (T4)p(A5):(m5s2Umod16)p(A17), (C) (A5)p(m5s2Umod6):(A15)p(m5s2Umod16), (D) (m5s2Umod6)p(A7):(T14)p(A15) and (E) (A7)p(C8):(G13)p(T14). G, C, A and T are colored green, yellow, red and blue, respectively, and sulfur atoms are highlighted as yellow spheres. The figures were generated with the program 3DNA (36).
including sterically unfavorable interactions (20,39). Right from the outset of the work described here, we were aware that effects triggered by 2-thio modification would be rather subtle and hence their detection would require relatively precise structural data. The resolution of the structure of the A-form decamer duplex with m5s2Umoe modifications, although not extremely high, should be sufficient to ferret out some of the consequences of the 2-thio and sugar modifications for base-pair geometry and duplex conformation. Likewise, availability of a structure as a reference molecule that differs from the duplex investigated here only by the absence of the sulfur atom in the m5Umoe residues renders the analysis more meaningful as it allows for a separation of various overlaid effects.

Key features of the m5s2Umoe residues in the crystal structure of the modified DNA duplex are the C3'-endo pucker and various subtle changes in the base pair, base-pair step and base-pair helical parameters. In addition, the sulfur atoms are in van der Waals contact across the minor groove as a result of γ- and x-displacements at the central base-pair step (Figures 5 and 7C) and, judging from base overlaps in the vicinity of m5s2Umoe residues, 2-thio modification leads to small but measurable improvements in the stacking interactions compared with the reference structure. Among the changes in base-pair geometry, the altered opening between m5s2Umoe6 and A15 is readily apparent (Figures 5A and 6A). In fact, this is perhaps the most straightforward way to accommodate the larger sulfur atom opposite the C2–H2 function of adenine. Increased propeller twist, stagger or shear relative to a canonical geometry of the native T:A pair in principle could each be used separately to relieve the steric challenge posed by the larger sulfur. However, the opening seen in the m5s2Umoe6:A15 pair is unique in the sense that this adjustment seems sufficient alone to accommodate the sulfur atom. In contrast, it appears that, rather than relying on a single parameter to adjust to the bulkier sulfur, a combination of adjustments including all of the above parameters is responsible for generating the particular arrangement seen in the A5:m5s2Ume16 pair.

Sugar puckering, nucleoside conformation and base-pair geometry are the result of a host of factors, including stereo electronic effects. However, unlike improvements in stacking or extensive hydration networks (the structure reveals both), the structure does not allow any conclusions per se regarding either a favorable or unfavorable contribution by some of these factors to the net increase in stability observed with the m5s2Umoe modification. In other words, although 2-thio modification will probably cause a stronger preference of the sugar for the C3'-endo conformation, all we see in the structure is the product of the various stereo electronic effects that bear on the conformation and we can only speculate as to the relative magnitude of the individual contributions toward the net gain in stability. One of these (hidden) contributions is the strengthening of the O4'-C1'-N1 anomic effect in m5s2U compared with thymine (see reference (40), pp. 43–46, for a discussion of the relation between sugar pucker type and molecular orbital overlap and hyperconjugation). Because oxygen is a more electronegative element than sulfur, it may appear counter-intuitive to consider sulfur a better acceptor of negative charge than oxygen. Indeed, oxygen has a greater ability to attract electron density per unit surface area. But if the greater surface area of sulfur compared with oxygen is taken into account (factor 2.5), and the potentials for each atom are summed over their respective spherical surfaces, sulfur may well end up having an equal or greater potential than oxygen. Another effect that could contribute favorably to the higher stability afforded by the m5s2Umoe modification is a change in Watson–Crick hydrogen bonding strength compared with RNA A:U or DNA A:T pairs. The N1'-N3 hydrogen bond between A:U pairs in RNA was recently shown to be stronger than the corresponding interaction in DNA (41).

With the m5s2Umoe nucleoside, the modulation of stereo electronic effects extends into the 2'-O- substituent. For example, the gauche effect between O4' and O2' that drives the sugar conformational equilibrium toward the C3'-endo side is enhanced by the presence of electronegative atoms or groups in the substituent, as recently demonstrated by extensive analyses of the influence of the chemistry of RNA 2'-O-substituents on the stability of duplexes (37,39,42–44). Combining the 2-thio and 2'-O-MOE modifications generates synergistic stereoelectronic effects that are absent, e.g. in oligonucleotides modified by 2'-O-methyl-2-thiouridine (10). These additive effects are the basis for the high RNA affinity and increased nuclease resistance of the m5s2Umoe modification.

Nature’s use of the s2U and m52U pyrimidines (i.e. in tRNA) is clearly related to the conformational rigidity of their ribose moiety as a result of the substitution of the 2'-oxygen by sulfur. The influence of the sulfur substitution is demonstrated by the altered binding affinity and cleavage activity of RNase H, an enzyme that is exquisitely sensitive to conformational or steric changes in DNA:RNA substrates (45). Although the enzyme binds RNA duplexes they are not recognized as substrates (46), presumably because a canonical A-form geometry precludes endonucleolytic cleavage of the RNA strand. Consistent with this observation, incorporation of m5s2U into the DNA strand of heteroduplexes dramatically reduced the cleavage rate at ribonucleotides opposite modified residues (47). Although this puts limitations on the use of this modification in antisense applications, where RNase H-mediated degradation of the target mRNA is considered important for efficacy, the properties of the s2U, m52s2U and m5s2Umoe modifications may render them of interest for use in siRNAs. Together with DNA phosphorothioates (8) and 4'-thio-RNA (3,48.49), they constitute the third class of sulfur-modified nucleic acid building blocks with promising features for the generation of nucleic acid therapeutics.

**ACKNOWLEDGEMENTS**

We are grateful to Dr Zdzislaw Wawrzak for help with data collection and processing, and to Dr Pradeep S. Pallan for comments on the manuscript. Use of the Advanced Photon Source was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38. The DuPont-Northwestern-Dow Collaborative Access Team (DND-CAT) Synchrotron Research Center at the Advanced Photon Source (Sector 5) is supported by E. I. DuPont de Nemours & Co., The Dow Chemical Company, the National Science Foundation, and the State of Illinois. This work was supported by the US National Institutes of Health (grant GM55237 to M.E.).
Funding to pay the Open Access publication charges for this article was provided by grant NIH R01 GM55237.

Conflict of interest statement. None declared.

REFERENCES

1. Crooke,S.T. (1998) Basic principles of antisense therapeutics. In Crooke,S.T. (ed.), Antisense Research and Application, Vol. 131, Springer, Berlin, pp. 1–50.
2. Manoharan,M. (1999) 2'-Carbohydrate modifications in antisense oligonucleotide therapy: importance of conformation, configuration and conjugation. Biochim. Biophys. Acta., 1489, 117–130.
3. Manoharan,M. (2004) RNA interference and chemically modified small interfering RNAs. Curr. Opin. Chem. Biol., 8, 570–579.
4. Soutchek,J., Akinc,A., Bramlage,B., Charisse,K., Constien,R., Donoghue,M., Elbashir,S., Geick,A., Hadwiger,P., Harborth,J. et al. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature, 432, 173–178.
5. Egli,M. (1998) Towards the structure-based design of nucleic acid therapeutics. In Weber,G. (ed.), Advan. Enzyme Regul., Vol. 38, Elsevier Science Ltd., Oxford, UK, pp. 181–203.
6. Levin,A.A. (1999) A review of issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. Biochim. Biophys. Acta., 1489, 69–84.
7. Filimore,D. (2004) Assessing antisense. Modern Drug Discovery June, 49–50.
8. Crooke,S.T. (1995) Phosphorothioate oligonucleotides. In Crooke,S.T. (ed.), Therapeutic Applications of Oligonucleotides. R. G. Landes, Austin, pp. 63–79.
9. Kumar,R.K. and Davis,D.R. (1997) Synthesis and studies on the effect of 2-thiouridine and 4-thiouridine on sugar conformation and RNA duplex stability. Nucleic Acids Res., 25, 1272–1280.
10. Shohda,K., Okamoto,I., Wada,T., Seio,K. and Sekine,M. (2000) Synthesis and properties of 2'-O-methyl-2-thiouridine and oligoribonucleotides containing 2'-O-methyl-2-thiouridine. Bioorg. Med. Chem. Lett., 10, 1795–1798.
11. Rajeev,K.G., Prakash,T.P. and Manoharan,M. (2003) 2'-Modified-2-thiothymidine oligonucleotides. Org. Lett., 5, 3005–3008.
12. Grosjean,H. and Benne,R. (eds) (1998) Modification and Editing of RNA. ASM Press, Washington, DC.
13. Rozenski,J., Crain,P.F. and McCloskey,J.A. (1999) The RNA modification database: 1999 update. Nucleic Acids Res., 27, 196–197.
14. Agris,P. (2004) Decoding the genome: a modified view. Nucleic Acids Res., 32, 223–238.
15. Testa,S.M., Disney,M.D., Turner,D.H. and Kierzek,R. (1999) Total synthesis of a pair of 2'-O-thiouridine-containing oligoribonucleotides and their hybridization and nuclease resistance properties. J. Org. Chem., 64, 1272–1280.
16. Yokoyama,S., Watanabe,T., Murao,K., Ishikura,H., Yamaizumi,Z., Tereshko,V., Portmann,S., Tay,E.C., Martin,P., Altman,K.-H. and Egli,M. (1999) Correlating structure and stability of DNA duplexes with incorporated 2'-O-modified RNA analogues. Biochemistry, 38, 10626–10634.
17. De Mesmaeker,A., Hûner,R., Martin,P. and Moser,H.E. (1995) Antisense oligonucleotides. Acc. Chem. Res., 28, 366–374.
oligonucleotides: symbiosis of charge interaction factors and stereoelectronic effects. *Org. Lett.*, 5, 2017–2020.

44. Prakash, T.P., Pueschl, A., Lesnik, E., Tereshko, V., Egli, M. and Manoharan, M. (2004) 2'-O-[2-(guanidinium)ethyl]-5-methyluridine modified oligonucleotides: synthesis and stabilizing effect on duplex and triplex nucleic acid structures. *Org. Lett.*, 6, 1971–1974.

45. Minasov, G., Teplova, M., Nielsen, P., Wengel, J. and Egli, M. (2000) Structural basis of cleavage by RNase H of hybrids of arabinonucleic acids and RNA. *Biochemistry*, 39, 3525–3532.

46. Lima, W.F. and Crooke, S.T. (1997) Binding affinity and specificity of *Escherichia coli* RNase H1: impact on the kinetics of catalysis of antisense oligonucleotide-RNA hybrids. *Biochemistry*, 36, 5004–5019.

47. Lima, W.F., Nichols, J.G., Wu, H., Prakash, T.P., Migawa, M.T., Wyrzykiewicz, T.K., Bhat, B. and Crooke, S.T. (2004) Structural requirements at the catalytic site of the heteroduplex substrate for human RNase H1 catalysis. *J. Biol. Chem.*, 279, 36317–36326.

48. Hoshika, S., Minakawa, N. and Matsuda, A. (2004) Synthesis and physical and physiological properties of 4'-thioRNA: application to post-modification of RNA aptamer toward NF-κB. *Nucleic Acids Res.*, 32, 3815–3825.

49. Hauberli, P., Berger, I., Pallan, P.S. and Egli, M. (2005) Syntheses of 4'-thioribonucleosides and thermodynamic stability and crystal structure of RNA oligomers with incorporated 4'-thiocytosine. *Nucleic Acids Res.*, 33, 3965–3975.