# Thermodynamics of RNA−RNA Duplexes with 2- or 4-Thiouridines: Implications for Antisense Design and Targeting a Group I Intron†

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ABSTRACT: Antisense compounds are designed to optimize selective hybridization of an exogenous oligonucleotide to a cellular target. Typically, Watson−Crick base pairing between the antisense compound and target provides the key recognition element. Uridine (U), however, not only stably base pairs with adenosine (A) but also with guanosine (G), thus reducing specificity. Studies of duplex formation by oligonucleotides with either an internal or a terminal 2- or 4-thiouridine ($s^2U$ or $s^4U$) show that $s^2U$ can increase the stability of base pairing with A more than with G, while $s^4U$ can increase the stability of base pairing with G more than with A. The latter may be useful when binding can be enhanced by tertiary interactions with a $s^4U$-G pair. To test the effects of $s^2U$ and $s^4U$ substitutions on tertiary interactions, binding to a group I intron ribozyme from mouse-derived *Pneumocystis carinii* was measured for the hexamers, r(AUGACU), r(AUGAC$s^2$U), and r(AUGAC$s^4$U), which mimic the 3′ end of the 5′ exon. The results suggest that at least one of the carbonyl groups of the 3′ terminal U of r(AUGACU) is involved in tertiary interactions with the catalytic core of the ribozyme and/or thio groups change the orientation of a terminal U-G base pair. Thus thio substitutions may affect tertiary interactions. Studies of trans-splicing of 5′ exon mimics to a truncated rRNA precursor, however, indicate that thio substitutions have negligible effects on overall reactivity. Therefore, modified bases can enhance the specificity of base pairing while retaining other activities and, thus, increase the specificity of antisense compounds targeting cellular RNA.

Antisense oligonucleotides on the order of 15−20 nucleotides long are typically used to target a single site in cellular RNA. Due to the intrinsic high stability of base pairs, these oligonucleotides may also bind strongly to partially complementary sequences, thus reducing specificity (*1*−*4*). Sequences that can hybridize to form one or more uridine-guanosine (U-G) or thymidine-guanosine (T-G) wobble base pairs are particularly problematic because U-G and T-G wobble base pairs can be thermodynamically (*5*, *6*) and sterically (*7*) similar to uridine-adenosine (U-A) and thymidine-adenosine (T-A) Watson−Crick base pairs.

A related specificity problem is presented by codon-anticodon recognition in translation. Many tRNAs alleviate this problem by having anticodons containing 2-thiouridine ($s^2U$)[1] instead of U (*8*, *9*). It is thought that because of the reduced hydrogen-bonding potential of sulfur compared to oxygen (*10*), $s^2$U-G wobble base pairs are destabilized relative to U-G wobble base pairs, but $s^2$U-A Watson−Crick base pairs are as stable as U-A pairs (see Figure 1 for hydrogen-bonding schemes). In addition, $s^2$U favors an anti, C3′ endo, gauche+ conformation for nucleotides in the anticodon loop, which is thought to impart thermodynamic stability to the anticodon−codon interaction (*11*−*14*). This allows tRNA anticodons containing $s^2$U to at least partially

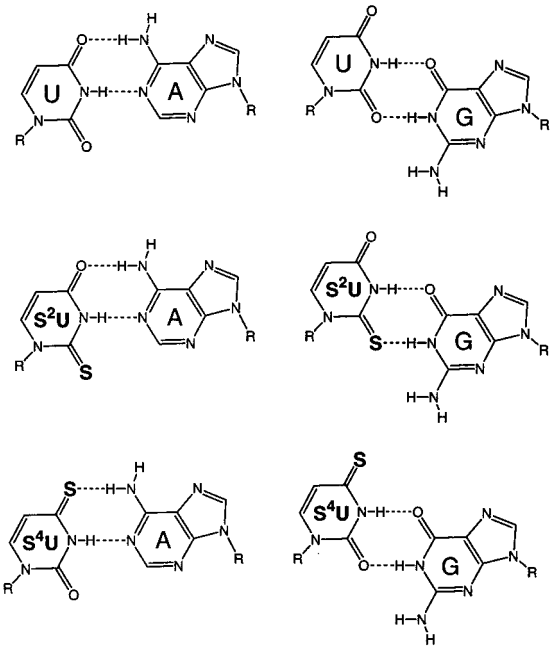


FIGURE 1: Hydrogen-bonding schemes of U-A Watson−Crick base pairs and U-G wobble base pairs. $s^2$U and $s^4$U represent 2-thiouridine and 4-thiouridine, respectively. Modified functional groups are in bold.

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[1] Abbreviations: $s^2$U, 2-thiouridine; $s^4$U, 4-thiouridine.

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discriminate between codons that contain A and those that contain G (*9*, *15*). Presumably, antisense compounds that contain $s^2U$ instead of U could also help discriminate between targets that contain A and those that contain G. This could enhance the specificity of antisense oligonucleotides for their intended target.

Another way to increase the specificity of antisense compounds for their target is to take advantage of binding enhancement by tertiary interactions (BETI) (*4*, *16*), which depends on the three-dimensional folding of the target. This strategy will sometimes rely on the presence of U-G wobble base pairs, since U-G wobble base pairs are often involved in tertiary interactions (*17*−*20*). Specificity can be further enhanced by exploiting the catalytic activity of target RNAs for the design of oligonucleotide suicide inhibitors (*21*). This approach has been used to target a group I intron in *Pneumocystis carinii* (*21*). In this case, suicide inhibition is facilitated by formation of a terminal U-G wobble base pair. Thus, there are situations where specificity is favored by the formation of U-G rather than U-A base pairs. On the basis of the pairing schemes in Figure 1, U-G should be more stable than U-A when U is replaced by 4-thiouridine ($s^4U$).

To determine the effects on specificity from substituting U with $s^2U$ or $s^4U$ at an internal position of a helix, the thermodynamics of duplex formation were measured for 5′GAG**U**GAG3′ with either 5′CUCACUC3′ or 5′CUCGCUC3′, where the bold, underlined U is either U, $s^2U$, or $s^4U$. To determine the effects on specificity from substituting U with $s^2U$ or $s^4U$ at a terminal position, the thermodynamics of duplex formation were measured for 5′AUGAC**U**3′ with either 5′GGUCAU3′ or 5′AGUCAU3′, where the bold, underlined U is either U, $s^2U$, or $s^4U$. The 5′AUGACU3′/5′GGUCAU3′ duplex mimics the helix formed during self-splicing by the *P. carinii* group I intron (*4*, *21*). The effect of thio substitution on tertiary interactions for this case was tested by measuring the binding of 5′AUGAC**U**3′ to the group I intron ribozyme. The effect on reactivity was tested by measuring reaction of 5′AUGAC**U**3′ with a truncated rRNA precursor containing the group I intron.

## MATERIALS AND METHODS

Unmodified oligoribonucleotides and the P-8/4x ribozyme were synthesized and purified essentially as described (*4*).

*5′AUGAC($s^2$U)3′ and 5′GAG($s^2$U)GAG3′*. The 2-thiouridine was obtained by condensation of the silylated derivative of 2-thiouracil with 1-O-acetyl-2,3,5-tri-*O*-$\beta$-D-ribofuranose in the presence of $SnCl_4$ (*22*). The 2-thiouridine was converted to 5′-*O*-dimethoxytrityl-2′-*O*-tetrahydropyranyl-2-thiouridine (*23*) and then into a 3′-phosphoramidite (*24*). For unmodified bases, phosphoramidites with the 2′−O protected by *tert*-butyl-dimethylsilyl were obtained from Glen Research. The synthesis of oligoribonucleotides was performed on an Applied Biosystems 392 solid-phase synthesizer using the manufacturer's suggested protocol for RNA synthesis, except *tert*-butyl hydroperoxide was used instead of the standard iodine treatment in the oxidation step to avoid oxidation of 2-thiouridine (*25*). The oligonucleotides were deprotected as previously described (*26*−*29*), desalted using Sep-Pak C18 cartridges (Waters), and then treated for 6 h with 0.01 M HCl (pH 2) to remove the tetrahydropyranyl group. The solutions were neutralized with dilute ammonia and purified by thin-layer chromatography on Baker Si500F TLC silica gel plates (*30*).

*5′AUGAC($s^4$U)3′ and 5′GAG($s^4$U)GAG3′*. The 4-thiouridine was obtained by conversion of uridine into 4-thiouridine (*31*). Solid-phase synthesis, deprotection, and purification were conducted as described above for $s^2U$-containing oligonucleotides except that standard iodine treatment was used in the oxidation step.

*Optical Melting Curves.* The thermodynamics of base pairing for each duplex was measured by thermal denaturation experiments with a Gilford 250 UV−vis spectrophotometer equipped with a Gilford 2527 thermoprogrammer. Denaturation was followed by monitoring the absorbance at 280 nm while increasing the temperature of the oligomer solution by 1 °C/min. The buffer for measurements with GAG**U**GAG sequences was 1.0 M NaCl, 20 mM sodium cacodylate, and 0.5 mM $Na_2$ EDTA, pH 7.0. The buffers for measurements with AUGAC**U** sequences were H15Mg buffer consisting of 50 mM Hepes (25 mM NaHepes), 15 mM $MgCl_2$, and 135 mM KCl at pH 7.5, a buffer that optimizes the extent of self-splicing of the *P. carinii* group I intron (*4*), and H12Mg3Mn buffer, which is the same as H15Mg buffer, except that the 15 mM $MgCl_2$ is replaced by 12 mM $MgCl_2$ and 3 mM $MnCl_2$. Melting curves were fit to a two-state non-self-complementary model to quantify the thermodynamic parameters of each duplex (*32*, *33*). Each molecule was analyzed over a concentration range of approximately 70-fold (typically 15−1000 $\mu$M), and thermodynamic parameters were also obtained from plots of $T_m^{-1}$ vs $\log(C_T/4)$, where $T_m$ is the melting temperature and $C_T$ is the total oligonucleotide concentration (*34*). In most cases, the enthalpy change determined from the two analysis methods agreed within 15%, consistent with the two-state assumption. Some transitions are not quite two-state by this criterion. In such cases, however, free-energy changes derived from $T_m^{-1}$ vs $\log(C_T/4)$ plots are still reliable (*35*). Thus, comparisons are restricted to free-energy changes.

The sequences 5′GGUCAU3′ and 5′GAG($s^4$U)GAG3′ exhibit self-complementary duplex formation in the same temperature range, but at less than 10% of the total duplexes formed (*4*, *16*, *32*). Therefore, the contribution of the homoduplexes to the thermodynamic stability of the heteroduplex is negligible. Nevertheless, the reported duplex stabilities are considered upper limits.

*Band-Shift Gel Electrophoresis.* Competitive band shift assays were conducted by competition with 5′ end labeled 5′ exon mimic 5′AUGACU3′ for binding to the P-8/4x ribozyme in H15Mg and H12Mg3Mn buffers essentially as described (*16*). Direct band-shift assays with 5′AUGACU3′ were conducted in H12Mg3Mn buffer essentially as described (*4*).

*Synthesis of 3′ End and Internally Labeled Precursor*. Precursor was internally labeled as described (*4*). After transcription, the unincorporated $\alpha$-[$^{32}$P]ATP was removed by a Chromaspin P-10 column (Clon-Tech) and the transcripts purified on a 5% polyacrylamide, 8 M urea gel. The full-length precursor was removed from the gel via the crush and soak method, ethanol precipitated twice to remove any salts, and stored at −20 °C in sterile water.

The 3′ end labeled precursor was synthesized via addition of $\alpha$-[$^{32}$P]3′deoxyadenosine (cordycepin) to the precursor's 3′ end by yeast poly(A) polymerase. In a typical reaction, a

Table 1: Thermodynamic Parameters for Base Pairing[a]

| | $1/T_M$ vs $\log(C_T/4)$ Parameters | | | | curve fit parameters | | | |
|---|---|---|---|---|---|---|---|---|
| oligonucleotide | $-\Delta G^\circ_{37}$ (kcal/mol)[b] | $-\Delta H^\circ$ (kcal/mol)[b] | $-\Delta S^\circ$ (eu)[b] | $T_M$ (°C)[c] | $-\Delta G^\circ_{37}$ (kcal/mol)[b] | $-\Delta H^\circ$ (kcal/mol)[b] | $-\Delta S^\circ$ (eu)[b] | $T_M$ (°C)[c] |
| **5′AUGAC(U)/UACUG(R)5′** | | | | | | | | |
| U-A | 4.92 ± 0.21 | 45.1 ± 4.9 | 129.6 ± 16.2 | 26.3 | 5.02 ± 0.18 | 43.1 ± 4.0 | 122.8 ± 12.8 | 26.5 |
| | (4.89) | (43.1) | (123.2) | (25.6) | | | | |
| $s^2$U-A | 5.12 ± 0.03 | 45.3 ± 1.5 | 129.4 ± 4.9 | 27.7 | 5.25 ± 0.15 | 42.8 ± 4.2 | 121.1 ± 13.8 | 28.0 |
| $s^4$U-A | 4.63 ± 0.67 | 38.3 ± 7.3 | 108.5 ± 25.1 | 22.3 | 5.09 ± 0.38 | 32.5 ± 7.2 | 88.5 ± 24.4 | 23.8 |
| U-G[d] | 4.96 ± 0.07 | 44.6 ± 1.8 | 127.8 ± 6.0 | 26.4 | 4.91 ± 0.07 | 46.4 ± 2.3 | 133.8 ± 7.6 | 26.5 |
| | (4.92) | (44.7) | (128.3) | (26.3) | | | | |
| $s^2$U-G | 5.06 ± 0.03 | 44.4 ± 1.1 | 126.7 ± 3.8 | 27.0 | 5.08 ± 0.07 | 42.5 ± 2.4 | 120.7 ± 7.7 | 26.8 |
| $s^4$U-G | 6.61 ± 0.04 | 52.2 ± 2.6 | 147.0 ± 8.4 | 37.5 | 6.58 ± 0.16 | 49.6 ± 2.8 | 138.8 ± 8.9 | 37.3 |
| U-G[e] | 5.16 ± 0.13 | 46.13 ± 4.1 | 132.1 ± 13.4 | 28.1 | 5.22 ± 0.18 | 48.7 ± 7.8 | 140.4 ± 25.2 | 28.9 |
| $s^2$U-G[e] | 5.01 ± 0.10 | 37.7 ± 2.4 | 105.4 ± 8.1 | 25.0 | 4.87 ± 0.14 | 42.7 ± 3.8 | 122.1 ± 12.1 | 25.4 |
| $s^4$U-G[e] | 6.40 ± 0.13 | 45.3 ± 5.4 | 125.5 ± 17.3 | 36.1 | 6.50 ± 0.08 | 45.5 ± 8.0 | 125.8 ± 25.6 | 36.8 |
| **5′GAG(U)GAG/CUC(R)CUC5′** | | | | | | | | |
| U-A | 9.71 ± 0.05 | 70.5 ± 1.5 | 196.0 ± 4.7 | 51.6 | 10.03 ± 0.19 | 80.1 ± 4.7 | 226.1 ± 14.8 | 51.2 |
| | (9.12) | (64.1) | (177.1) | (50.2) | | | | |
| $s^2$U-A | 10.82 ± 0.09 | 69.5 ± 1.6 | 189.2 ± 4.9 | 57.4 | 11.60 ± 0.43 | 82.3 ± 3.7 | 228.1 ± 10.7 | 57.3 |
| $s^4$U-A | 10.18 ± 0.07 | 66.5 ± 1.3 | 181.6 ± 3.8 | 55.0 | 11.01 ± 0.47 | 81.0 ± 4.6 | 225.7 ± 14.4 | 55.1 |
| U-G | 7.78 ± 0.05 | 64.2 ± 1.9 | 181.8 ± 6.1 | 43.1 | 8.00 ± 0.28 | 70.3 ± 4.9 | 200.8 ± 14.9 | 43.6 |
| | (8.69) | (60.4) | (166.7) | (48.7) | | | | |
| $s^2$U-G | 7.44 ± 0.01 | 63.9 ± 1.4 | 181.9 ± 4.5 | 41.5 | 7.46 ± 0.08 | 61.8 ± 3.7 | 175.1 ± 11.8 | 41.7 |
| $s^4$U-G | 10.80 ± 0.18 | 71.3 ± 2.9 | 195.0 ± 8.9 | 56.8 | 10.47 ± 0.25 | 64.9 ± 3.3 | 175.4 ± 9.8 | 57.0 |

[a] Unless noted, AUGAC**U** sequences were in H15Mg buffer consisting of 50 mM Hepes (25 mM $Na^+$), 15 mM $MgCl_2$, and 135 mM KCl at pH 7.5. GAG**U**GAG sequences were in 1.0 M NaCl, 20 mM sodium cacodylate, and 0.5 mM $Na_2$ EDTA, pH 7.0. R represents the purines A or G. U represents U or thio derivatives of U. Values in parentheses are predicted for 1 M NaCl based on a nearest neighbor model (*36*, *37*). [b] The error was calculated as described (*52*−*54*). Significant figures are given beyond error estimates to allow accurate calculation of $T_m$ and other parameters. [c] Calculated for $10^{-4}$ M oligonucleotide concentration. [d] Ref *4*. [e] Measured in H12Mg3Mn buffer, which is the same as H15Mg except that the 15 mM $MgCl_2$ is replaced by 12 mM $MgCl_2$ and 3 mM $MnCl_2$.

0.2 $\mu$M precursor solution was incubated at 30 °C for 20 min in 3′ end reaction buffer (20 mM Tris-HCl, pH 7.0, 50 mM KCl, 700 $\mu$M $MnCl_2$, 200 $\mu$M EDTA, 100 $\mu$g/mL BSA, and 10% glycerol) with 70 $\mu$Ci α-[$^{32}$P]cordycepin (3′ deoxyadenosine) and 500 units of yeast poly(A) polymerase. After the reaction, the unincorporated α-[$^{32}$P]cordycepin was removed via a Chromaspin P-10 column (Clon-Tech). The radiolabeled product was purified on a 5% polyacrylamide, 8 M urea gel. The 3′ end labeled precursor was eluted from the gel via the crush and soak method, ethanol precipitated twice, and stored at −20 °C in sterile water.

*Inhibition of Self-Splicing*. A solution containing internally labeled precursor in HXMg buffer [135 mM KCl, 50 mM Hepes (25 mM NaHepes), and *X* mM $MgCl_2$ adjusted to pH 6.5 with HCl] was reannealed by heating at 55 °C for 5 min, cooled at room temperature for 2 min, then incubated at 37 °C. Another solution containing 60 $\mu$M of 5′ exon mimic and 2 mM pG in HXMg buffer was incubated at 37 °C for 5 min and added to an equal volume of the above precursor solution. The reaction was incubated at 37 °C for 1 h and stopped by addition of a 2/3 volume of stop buffer (16 M urea, 16 mM EDTA). The reaction products were separated on a 5% polyacrylamide, 8 M urea gel. The gel was dried and radioactivity in bands quantified on a Molecular Dynamics Phosphorimager using ImagQuaNT v. 4.2a software. The radioactivity of each band was corrected for the number of adenines in each product.

The dependence of self-splicing inhibition on oligonucleotide concentration was tested with 3′ end labeled precursor in H3Mg buffer at pH 6.5. The precursor was reannealed in H3Mg buffer as described above. A solution containing 2 mM pG and serially diluted 5′ exon mimic in H3Mg buffer was preequilibrated at 37 °C for 5 min and added to an equal volume of the 3′ end labeled precursor solution. The reaction was incubated at 37 °C for 1 h and stopped via the addition of a 2/3 vol of stop buffer. Reaction products were separated on a 5% acrylamide, 8 M urea gel. The gel was dried, and the radioactivity in bands quantified on a Molecular Dynamics phosphorimager using ImagQuaNT v. 4.2a software.

## RESULTS

*Effects of Thio-Derivatives of U on Base-Pairing Stability.* The stabilities of duplexes formed by modified and unmodified 5′AUGAC**U**3′ with 5′GGUCAU3′ or 5′AGUCAU3′, and by modified and unmodified 5′GAG**U**GAG3′ with 5′CUCACUC3′ or 5′CUCGCUC3′ were quantified by UV-detected thermal denaturation. The results are listed in Table 1.

The thermodynamic stabilities of the duplexes containing the unmodified sequence 5′AUGACU3′ are the same regardless of whether the 3′ terminal base pair is U-A or U-G. For duplexes containing the unmodified sequence 5′GAGUGAG3′, however, the duplex with a central U-A base pair is 1.9 kcal/mol more stable at 37 °C than the duplex with a central U-G base pair. This difference is 1.5 kcal/mol larger than expected based on predictions (*36*, *37*).

The thermodynamic stabilities of the duplexes containing the sequence 5′AUGAC($s^2$U)3′ are essentially the same regardless of whether the 3′ terminal base pair is $s^2$U-A or $s^2$U-G. This is similar to the results for terminal unmodified U-A and U-G base pairs. For duplexes containing the sequence 5′GAG($s^2$U)GAG3′, however, the duplex with an $s^2$U-A base pair is 3.4 kcal/mol more stable than the duplex with an $s^2$U-G base pair. The duplex with an $s^2$U-G base pair is 0.3 kcal/mol less stable than that with a U-G base pair, but the duplex with an $s^2$U-A base pair is approximately 1 kcal/mol more stable than that with a U-A base pair.

Table 2: Thermodynamic Parameters for Binding to the P-8/4x Ribozyme in H15Mg and H12Mg3Mn Buffers[a]

| | binding to P-8/4x | | binding to r(GGUCAU) | | tertiary stability | |
|---|---|---|---|---|---|---|
| oligonucleotide | $K_{d,TOTAL}$ (nM)[b] | $-\Delta G^{\circ}_{37,TOTAL}$ (kcal/mol)[c] | $K_{d,BP}$ ($\mu$M)[b] | $-\Delta G^{\circ}_{37,BP}$ (kcal/mol)[c] | $-\Delta G^{\circ}_{37,BETI}$ (kcal/mol)[d] | $K_2$[e] |
| AUGACU[f] | 5.21± 1.4 (13.9 ± 2.6)[h] | 11.75 ± 0.19 | 318 ± 38 | 4.96 ± 0.07 | 6.79 ± 0.20 | 61 000 |
| AUGAC($s^2$U) | 43.6 ± 0.2 | 10.44 ± 0.01 | 271 ±13 | 5.06 ± 0.03 | 5.38 ± 0.03 | 6200 |
| AUGAC($s^4$U) | 31.0 ± 4.6 | 10.65 ± 0.10 | 21.9 ±1.4 | 6.61 ± 0.04 | 4.04 ± 0.10 | 700 |
| AUGACU[g] | 22.5 ± 2.1 (89.4 ± 3.5)[h] | 10.85 ± 0.06 | 230 ± 54 | 5.16 ± 0.13 | 5.69 ± 0.13 | 10 200 |
| AUGAC($s^2$U)[g] | 70.6 ± 23.2 | 10.14 ± 0.25 | 294 ± 52 | 5.01 ± 0.10 | 5.13 ± 0.27 | 4200 |
| AUGAC($s^4$U)[g] | 44.7 ± 16.5 | 10.42 ± 0.29 | 30.7 ±7.2 | 6.40 ± 0.13 | 4.02 ± 0.32 | 700 |

[a] H15Mg buffer consists of 50 mM Hepes (25 mM $Na^+$), 15 mM $MgCl_2$, and 135 mM KCl at pH 7.5. H12Mg3Mn is the same as H15Mg except that 15 mM $MgCl_2$ is replaced by 12 mM $MgCl_2$ and 3 mM $MnCl_2$. All measurements were done in H15Mg buffer, unless otherwise noted. [b] The error is the standard deviation of the measurements. [c] Calculated from $\Delta G^{\circ}_{37} = RT \ln(K_d)$ where $R = 0.001\,987$ kcal mol$^{-1}$ K$^{-1}$ and $T = 310$ K, using more significant digits than listed in this table. [d] Calculated from the difference in $\Delta G^{\circ}_{37}$ from binding to P−8/4x and r(GGUCAU). This is the free-energy increment from tertiary interactions. The error is the square root of the sum of the squares of each individual error. [e] Calculated by dividing the $K_d$ value for binding to r(GGUCAU) by the $K_d$ for binding to P−8/4x. The $K_2$ value was calculated using $K_d$ values containing more significant digits than those listed in this table. [f] Ref *4*. [g] Measured in H12Mg3Mn buffer. [h] Measured in a direct band shift assay.

Evidently, $s^2$U selectively increases the stability of this internal U-A pair and marginally decreases the stability of the corresponding U-G base pair, thus increasing the difference in stability between the two pairings.

In contrast to the duplexes containing the sequences 5′AUGACU3′ and 5′AUGAC($s^2$U)3′, the thermodynamic stabilities of the duplexes containing the sequence 5′AUGAC($s^4$U)3′ differ depending on whether the terminal $s^4$U is forming a $s^4$U-A or $s^4$U-G base pair. The duplex with the $s^4$U-G base pair is 2 kcal/mol more stable than the duplex with the $s^4$U-A base pair and 1.6 kcal/mol more stable than the duplex with the U-G pair. For the internal substitutions, the duplex with the $s^4$U-G base pair has similar stability as the duplex with the $s^4$U-A base pair but is 3 kcal/mol more stable than the duplex with the internal U-G base pair. The duplex with an internal $s^4$U-A base pair is 0.5 kcal/mol more stable than the corresponding duplex with an internal U-A base pair. Evidently, $s^4$U greatly increases the stability of the internal U-G wobble base pair, but only marginally increases the stability of the internal U-A base pair, thus largely eliminating the difference in stability between these U-A and U-G base pairs.

*Effects on Binding 5′ Exon Mimics to the P-8/4x Ribozyme.* The 5′ exon mimic 5′AUGACU3′ binds to the *P. carinii* group I intron ribozyme through base pairing and tertiary interactions (*4*). To determine whether thio substitutions affect tertiary interactions with the ribozyme's catalytic core, we measured the binding of 5′AUGACU3′, 5′AUGAC($s^2$U)3′, and 5′AUGAC($s^4$U)3′ to the ribozyme in H15Mg buffer. The stability of tertiary interactions was quantified by subtracting the free energy of base pairing, estimated from the results in Table 1, from the free energy of the complex with ribozyme (*16*). The results are listed in Table 2. They show that 5′AUGAC($s^2$U)3′ and 5′AUGAC($s^4$U)3′ bind to the ribozyme 8- and 6-fold less tightly, respectively, than 5′AUGACU3′.

Effects of oxygen to sulfur substitutions are sometimes due to disruption of $Mg^{2+}$ coordination to oxygen (*38*−*40*). Since $Mn^{2+}$ coordinates more strongly than $Mg^{2+}$ to sulfur, any reduction in stability due to the loss of $Mg^{2+}$−oxygen contacts for the thio-U derivatives should be at least partially restored by adding $Mn^{2+}$ (*38*−*40*). Thus, competitive band-shift binding assays and UV-detected thermal denaturation experiments were conducted with 3 mM $Mn^{2+}$ in a background of 12 mM $Mg^{2+}$. The results in Tables 1 and 2 show that, within experimental error, the presence of $Mn^{2+}$ has little effect on the thermodynamic stability of base pairing and tertiary interactions when compared to the results in 15 mM $Mg^{2+}$. Thus, there is no evidence for $Mn^{2+}$ rescue of the loss of tertiary interactions that occurs with the thio substitutions. This suggests that the carbonyl oxygens of the terminal U are not involved in $Mg^{2+}$-mediated tertiary interactions with the ribozyme's catalytic core.

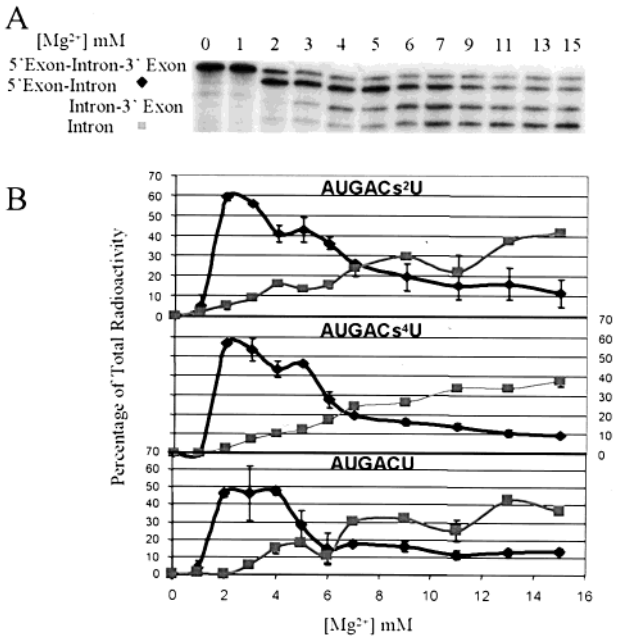


FIGURE 2: The splicing of internally labeled precursor measured in the presence of the 5′ exon mimics, AUGACs$^2$U, AUGACs$^4$U, and AUGACU, as a function of [$Mg^{2+}$]. All reactions were completed with 1 mM pG and 30 $\mu$M 5′ exon mimic in HXMg buffer. (A) An autoradiogram of a typical gel with AUGACs$^2$U. (B) Plots of trans (◆) and cis (■) splicing products as a function of [$Mg^{2+}$] in the presence of the indicated 5′ exon mimic.

*Reactivity of 5′ Exon Mimics with the rRNA Precursor.* Short oligonucleotides can splice in trans to rRNA precursors containing group I introns (*21*, *41*−*43*). The effects of thio substitutions on the reactivity of r(AUGAC**U**) were determined by measuring the dependence of trans-splicing on $Mg^{2+}$ concentration at 30 $\mu$M r(AUGAC**U**) (Figure 2) and on oligonucleotide concentration at 3 mM $Mg^{2+}$ (Figure 3).

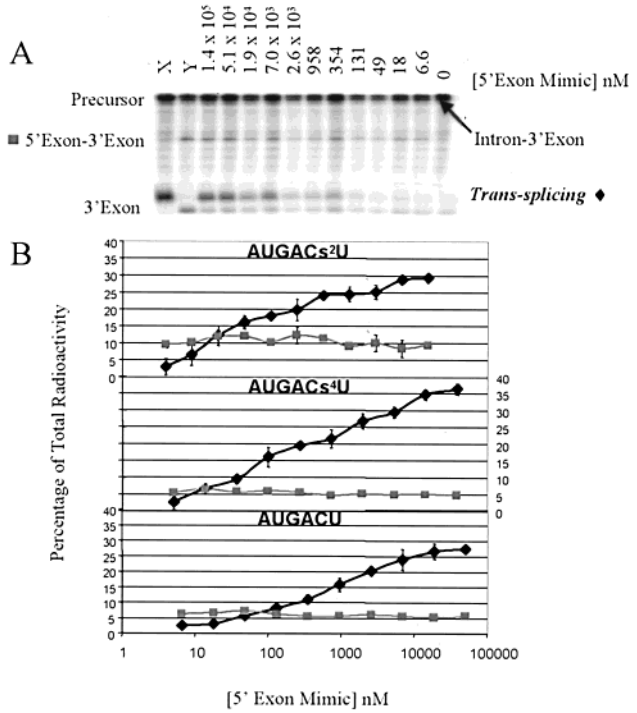


FIGURE 3: The dependence on oligonucleotide concentration of splicing in H3Mg buffer measured in the presence of the various 5′ exon mimics. (A) An autoradiogram of a typical gel with AUGACU. X and Y are markers for trans-spliced and 3′ exon products, respectively. (B) Plots of trans (◆) and cis (■) splicing products as a function of concentration of the indicated 5′ exon mimic.

All the reactions were carried out in the presence of 1 mM pG so that normal cis-splicing was also possible. All three oligonucleotides exhibit trans-splicing of similar magnitude.

## DISCUSSION

The effects of 2- and 4-thio substitutions of uridine (Figure 1) on the stabilities of internal and terminal U-A Watson−Crick and U-G wobble base pairs were studied. The effects of these substitutions on binding and reactivity with a group I intron from *P. carinii* were also measured.

*Sequence Effects on the Stability of a Terminal Base Pair.* For duplexes formed between 5′AUGAC**U**3′ and 5′AGUCAU3′ or 5′GGUCAU3′, the identity of the terminal base pair has little effect on stability, except that the $s^4$U-G duplex is roughly 1.5 kcal/mol more stable than the others. The lack of an effect for the other terminal modifications is probably due in part to the high flexibility inherent at the end of a duplex. Therefore, the discussion of duplex stabilities will focus mainly on internal modifications. The relevant comparisons are outlined in Table 3, the expected base pairing structures are shown in Figure 1, and some of the factors that may affect the stabilities of duplexes with thiolated bases are listed in Table 4. Note that the difference between oxygen and sulfur hydrogen-bonding strength is only one of eight factors listed in Table 4. Hydrogen bonding to the substituted atom cannot explain most of the observed changes in stability.

*Effects of $s^2$U and $s^4$U Modifications on the Stability of an Internal U−A Base Pair.* The 2-carbonyl group of U is not hydrogen bonded with A in a Watson−Crick U-A base pair (Figure 1). Therefore, it was expected that replacing the oxygen with a less electronegative sulfur would have little effect on the overall stability of a duplex with a U-A base pair. It was determined, however, that the stability of the 5′GAG**U**GAG3′/5′CUCACUC3′ duplex containing the $s^2$U-A base pair is 1.1 kcal/mol, or 6-fold, more stable than the corresponding duplex with a U-A base pair (Table 3). An increase in thermodynamic stability of $s^2$U-A over U-A base pairs has been reported and has been attributed to effects 1−4 in Table 4 (*10*−*13*, *44*−*46*).

Table 3: Relative Thermodynamic Stability of Base Pairing[a]

| oligonucleotide | modified vs unmodified: $\Delta\Delta G^\circ_{37}$ (kcal/mol)[b] | modified vs unmodified: relative $K_d$ of binding[c] | **U**-G vs **U**-A: $\Delta\Delta G^\circ_{37}$ (kcal/mol)[d] | **U**-G vs **U**-A: relative $K_d$ of binding[e] |
|---|---|---|---|---|
| 5′AUGAC(**U**)/UACUG(R)5′ | | | | |
| U-A | 0 | 1.0 | | |
| $s^2$U-A | −0.20 | 0.72 | | |
| $s^4$U-A | +0.29 | 1.61 | | |
| U-G | 0 | 1.0 | −0.04 | 0.94 |
| $s^2$U-G | −0.10 | 0.85 | +0.06 | 1.10 |
| $s^4$U-G | −1.65 | 0.07 | −2.0 | 0.034 |
| $s^2$U-G[f] | +0.15 | 1.28 | | |
| $s^4$U-G[f] | −1.24 | 0.13 | | |
| 5′GAG(**U**)GAG/CUC(R)CUC5′ | | | | |
| U-A | 0 | 1.0 | | |
| $s^2$U-A | −1.11 | 0.17 | | |
| $s^4$U-A | −0.47 | 0.46 | | |
| U-G | 0 | 1.0 | +1.93 | 23 |
| $s^2$U-G | +0.34 | 1.45 | +3.38 | 242 |
| $s^4$U-G | −3.02 | 0.007 | −0.62 | 0.37 |

[a] R represents the purines A or G. **U** represents U or thio derivatives of U. All comparisons are based on $1/T_M$ vs $\log(C_T/4)$ parameters. [b] Free-energy difference between the modified duplex and the corresponding unmodified duplex. Minus (−) means the modification increases the stability of the duplex. [c] Calculated by dividing the $K_d$ of the duplex listed to the left by the $K_d$ for the unmodified duplex. A number less than 1.0 indicates that the listed duplex is more stable than the unmodified reference duplex. [d] Free-energy difference between the U-A and U-G containing duplexes, both modified and unmodified. Minus (−) means the U-G base pair is more stable than the U−A base pair. [e] Calculated by dividing the $K_d$ of the duplex listed to the left by the $K_d$ of the **U**−A containing duplex (where **U** is U, $s^2$U or $s^4$U). A number less than 1.0 indicates that the listed duplex is more stable than the **U**−A containing duplex. [f] Measured in H12Mg3Mn buffer.

The 4-carbonyl group of U is hydrogen bonded with A in a Watson−Crick U-A base pair (Figure 1). Therefore, it was expected that replacing the oxygen with a less electronegative sulfur would destabilize a duplex having a U-A base pair (7 in Table 4). It was determined, however, that a $s^4$U substitution increases the stability of the 5′GAG**U**GAG3′/5′CUCACUC3′ duplex by almost 0.5 kcal/mol, or 2-fold (Table 3). This is surprising and could be due to effects 1, 2, 5, and 6 in Table 4. A-form helix modeling suggests that the 4-thio group is stacked upon the carbonyl group of a neighboring G in 5′GAG$s^4$UGAG3′/5′CUCACUC3′. Thus, the thio substitution could be partially relieving the destabilizing effects of electrostatic repulsion (5 in Table 4).

*Effects of $s^2$U and $s^4$U Modifications on the Stability of an Internal U-G Base Pair.* The 2-carbonyl group of U is hydrogen bonded with G in a U-G wobble base pair (Figure 1). Therefore, it was expected that replacing the 2-oxygen with a less electronegative sulfur would have a destabilizing effect on a duplex with a U-G base pair (7 in Table 4). As expected, an $s^2$U substitution decreases the stability of the 5′GAG**U**GAG3′/5′CUCGCUC3′ duplex by 0.3 kcal/mol, or almost 2-fold (Table 3). It is interesting that this destabilizing effect is slightly dominant over all the potential stabilizing effects listed in Table 4.

Table 4: Some Factors that Affect the Stability of Thio Substituted RNA Duplexes

| | stability increase | ref |
|---|---|---|
| 1 | more polarizable sulfur increases stacking | *44* |
| 2 | less electronegative sulfur increases the acidity of the N-3 proton, thus increasing the strength of its hydrogen bond | *13*, *45* |
| 3 | $s^2U$ prefers the 3′ endo conformation which increases the rigidity of the backbone preorganizing it for duplex formation | *11*, *12* |
| 4 | $s^2U$ results in a stable S−N1 stacking interaction | *44*, *46* |
| 5 | in cases where carbonyl groups overlap, decreasing the electronegativity and increasing the polarizability of one of the substituents with a thio substitution reduces electrostatic repulsion | |
| 6 | thio substitution results in a conformational rearrangement with a more stable stacking orientation | *44* |
| | **stability decrease** | **ref** |
| 7 | the less electronegative sulfur forms weaker hydrogen bonds | *10* |
| 8 | decreasing the electronegativity of a group involved in a favorable electrostatic interaction reduces stability | |

The 4-carbonyl group of U is not hydrogen bonded with G in a U-G wobble base pair (Figure 1). Therefore, it was expected that replacing the 4-oxygen with a less electronegative sulfur would have little effect on the overall stability of a duplex with a U-G base pair. Surprisingly, however, it was determined that a $s^4U$ substitution increases the stability of the 5′GAG**U**GAG3′/5′CUCGCUC3′ duplex by more than 3 kcal/mol or more than 100-fold (Table 3). This large stability increase could be due to effect 1 in Table 4, except that the increased polarizability did not have such a large effect on the other base pairings. That $s^4U$-G base pairs are substantially more stable than U-G and $s^2U$-G base pairs can, at least partially, be explained by effect 2 in Table 4. The N-3 imino proton of $s^4U$ is more acidic ($pK_a = 8.2$) than that of U ($pK_a = 9.3$) or $s^2U$ ($pK_a = 8.8$) (*44*) and, therefore, forms stronger hydrogen bonds in a U-G base pair.

*Effects of Substitutions at Non-Hydrogen-Bonded Positions.* It is interesting that the two internal thio substitutions resulting in the largest increase in stability over the unmodified base pairs ($s^2U$-A and $s^4U$-G) are to the non-hydrogen-bonded carbonyl groups (Figure 1). In both cases, the thio substitution increases the acidity and therefore hydrogen-bonding strength of the imino proton. If the thio substitutions do not significantly alter the conformations, then these substitutions also reduce the electronegativity and increase the polarizability of the substituent in the minor ($s^2U$-A) or major ($s^4U$-G) groove of the resultant duplex. These results suggest that non-hydrogen-bonded carbonyl groups of uridine, in the major and minor grooves, may be destabilizing to duplexes. Alternatively, or in addition, the thio substitution may increase the partial negative charge on the remaining carbonyl group thus increasing the strength of the hydrogen bond it forms.

*Differential Effects of Modified Uridines on U-A and U-G Base Pairs*. The stabilities of terminal U-A and U-G base pairs are essentially the same, as expected (*5*). This is also true if the terminal U is replaced with $s^2U$. If the terminal U is replaced with $s^4U$, however, the $s^4U$-G base pair is 2 kcal/mol, or 30-fold, more stable than the duplex with a $s^4U$-A base pair (see right side of Table 3). Apparently, $s^4U$ specifically enhances the stability of this terminal U-G base pair, while leaving the stability of the corresponding terminal U-A base pair essentially unchanged.

The $s^4U$ substitution also specifically enhances the stability of the 5′GAG**U**GAG3′/5′CUCGCUC3′ duplex with a central U-G base pair relative to the 5′GAG**U**GAG3′/5′CUCACUC3′ duplex with a central U-A base pair. With unmodified U's, the duplex with the central U-A pair is more stable by 2 kcal/mol. With $s^4U$, however, the duplexes with central $s^4U$-G and $s^4U$-A pairs have similar stability (see right side of Table 3). Thus, a $s^4U$ substitution can be used to favor $s^4U$-G pairings at both terminal and internal positions.

If the U of 5′GAGUGAG3′ is replaced with $s^2U$, then the duplex with a central $s^2U$-G base pair is about 3.4 kcal/mol, or more than 200-fold, less stable than the duplex with a central $s^2U$-A base pair (see right side of Table 3). This increases the specificity for pairing with A by 10-fold.

*Comparison with Previous Reports*. It has been reported in a different system that $s^2U$ increases the stability of an internal U-Am (where Am is 2′-O methyl A) base pair in a duplex by 2 kcal/mol, while $s^4U$ decreases the stability of an internal U-Am base pair by approximately 0.5 kcal/mol (*13*). For our results, $s^2U$ increases the stability of an internal U-A base pair in a duplex by 1 kcal/mol, while $s^4U$ increases the stability of an internal U-A base pair by approximately 0.5 kcal/mol. The small differences in results could stem from buffer conditions, sequence dependencies, the difference in U-A and U-Am base pairs, or other factors. Thus thio substitutions may alter specificities in a context dependent manner, suggesting they should be tested on a case-by-case basis.

*Thermodynamics of Binding to the P-8/4x Ribozyme*. The results on simple helices suggest that $s^4U$ can be employed to favor U-G over U-A base pairing. Since U-G base pairs are known to sometimes form tertiary interactions, the enhanced specificity for $s^4U$-G base pairing could be helpful in using binding enhancement by tertiary interactions (BETI) to target RNA. For example, the 5′ exon mimic, 5′AUGACU3′, of a *P. carinii* group I intron binds to a derived ribozyme through base pairing and tertiary interactions (*4*). On the basis of studies of other group I introns (*17*−*20*, *47*−*50*), one important feature of the helix formed is a terminal U-G wobble base pair because the exocyclic amino group of this wobble base pair makes tertiary contacts with the catalytic core. To see if thio substitutions could enhance the specificity of a 5′ exon mimic binding to the ribozyme, the binding of 5′AUGAC($s^2U$)3′ and 5′AUGAC($s^4U$)3′ to the ribozyme was measured. From the base pairing results in Table 1, we would expect 5′AUGAC($s^2U$)3′ to have a dissociation constant similar to 5′AUGACU3′. Binding to the ribozyme, however, is less favorable by about 1.3 kcal/mol, or 8-fold. Considering the stronger base pairing of this mimic with its complement in the ribozyme (5′GGUCAU3′), the tertiary interactions are reduced by 1.4 kcal/mol, or 10-fold (see Table 2). Similarly, 5′AUGAC($s^4U$)3′ binding to the ribozyme is less favorable by 1.1 kcal/mol, or 6-fold, compared with 5′AUGACU3′. Considering the stronger base pairing of this mimic with its complement, the tertiary interactions are reduced by almost

3 kcal/mol, or nearly 100-fold.

There are at least two possible ways that thio substitutions can interfere with tertiary interactions. The 2- and/or 4-carbonyl oxygens may be involved in tertiary interactions with the ribozyme's catalytic core. If so, the failure of the addition of $Mn^{2+}$ to clearly restore tertiary interactions lost with the sulfur substitutions (see Table 2) suggests that the tertiary interactions do not involve carbonyl oxygen−$Mg^{2+}$ contacts. Hydrogen-bonding contacts, however, are possible. Indeed, the Strobel model of the G−U wobble base pair in the catalytic core of the *Tetrahymena thermophila* group I intron suggests that the 2-carbonyl oxygen of U is in the proximity of other functional groups with which it could hydrogen bond (*50*). Substitution with $s^2U$ could directly decrease the strength of such a hydrogen bond. Alternatively, or in addition, formation of tertiary interactions, including those of the exocyclic amino group of G (*20*), may be dependent on exact positioning of functional groups from the U-G base pair. This positioning could be very dependent on interactions between the U-G base pair and its C-G neighbor. Such nearest-neighbor steering has been suggested to explain the different hydrogen-bonding patterns for tandem G-A mismatch base pairs adjacent to G-C or C-G base pairs (*51*). Sulfur substitutions could have a major effect on such interactions. For example, sulfur substitutions are associated with particular stacking patterns in crystal structures (*44*). Thus, substitution of either $s^2U$ or $s^4U$ could make tertiary interactions less favorable by changing the most favorable orientation of the U-G wobble base pair.

While the tertiary interactions are less favorable, both $s^2U$ and $s^4U$ substituted oligonucleotides trans-splice with a truncated rRNA precursor containing the group I intron. The extent of trans-splicing is similar to that observed with 5′AUGACU3′ (Figures 2 and 3). Thus, perturbations made by thio substitutions affect binding but not overall reactivity.

*Implications for Antisense Design*. Antisense compounds are designed to base pair with biologically important nucleic acids. The building blocks for antisense compounds have traditionally been the commonly occurring nucleic acid bases A, C, G, and U (T). This takes advantage of the simple Watson−Crick base pairing rules for design of a sequence that will hybridize to one particular nucleic acid target. Unfortunately, U base pairs stably with A and G. This promiscuity reduces the specificity of an antisense compound by increasing the number of sites that allow stable hybridization. There is no reason, however, to use only A, C, G, and U (T), when other bases might have the important advantage of preferentially base pairing with only one of the four canonical bases.

The results reported here show that $s^2U$ can favor formation of an internal $s^2U$-A base pair relative to an $s^2U$-G base pair more than U favors an internal U-A relative to a U-G base pair. Thus, substituting $s^2U$ for U in antisense compounds can result in more specific binding of the antisense with its target. Conversely, $s^4U$ can increase the stability of U-G base pairs relative to U-A base pairs. Thus, when targeting Gs to form U-G base pairs, U could be replaced with $s^4U$ to achieve a heightened level of specificity. This will be beneficial when binding is enhanced by tertiary interactions that are not compromised by the thio substitution. Of course, in practical antisense applications, the backbone must be modified to inhibit oligonucleotide degradation, and the context will likely differ in additional ways from those studied here. Thus, it will be necessary to determine the effects of thio substitutions for each case.

These results suggest that there is a thermodynamic basis for enhancing specificity of antisense-target interactions by using certain modified bases in the antisense compound. This is likely not only the case with U, but also with G (which can base pair with U, A, and C) and A (which can base pair with U and G). Sophisticated nucleic acid synthesis methods have opened up the possibility of designing any number of base derivatives that are better suited for their intended application than the canonical bases.

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