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# Chemical Synthesis of LNA-2-thiouridine and Its Influence on Stability and Selectivity of Oligonucleotide Binding to RNA[†]

Marta Carlucci,[‡,§] Elzbieta Kierzek,[‡] Anna Olejnik,[‡] Douglas H. Turner,*[,∥,⊥] and Ryszard Kierzek*[,‡]

[‡]*Institute of Bioorganic Chemistry, Polish Academy of Sciences, 61-704 Poznan, Noskowskiego 12/14, Poland,* [∥]*Department of Chemistry, University of Rochester, RC Box 270216, Rochester, New York 14627-0216, and* [⊥]*Center for RNA Biology, University of Rochester, Rochester, NY 14627.* [§]*Present address: Department of Chemistry, University of Southampton, University Road Highfield, Southampton SO17 1BJ, U.K.*

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ABSTRACT: Hybridization to RNA is important for many applications, including antisense therapeutics, RNA interference, and microarray screening. Similar thermodynamic stabilities of A-U and G-U base pairs result in difficulties in selective binding to RNA. Moreover, A-U pairs are weaker than G-C pairs so that binding is sometimes weak when many A-U pairs are present. It is known, however, that replacement of uridine with 2-thiouridine significantly improves binding and selectivity. To test for additional improvement of binding and of the specificity for binding A over G, LNA-2-thiouridine was synthesized for the first time and incorporated into many LNA-2′-*O*-methyl-RNA/RNA duplexes. UV melting was used to measure the thermodynamic effect of replacing 2′-*O*-methyluridine with 2′-*O*-methyl-2-thiouridine or LNA-2-thiouridine. The 2-thiouridine usually enhances binding and selectivity. Selectivity is optimized when a single 2-thiouridine is placed at an internal position in a duplex.

Among the 107 naturally modified RNA nucleotides, 16 carry sulfur, including 9 derivatives of 2-thiouridine ($s^2U$) (*1*). Replacement of the pyrimidine oxygen at position 2 with sulfur changes biological and structural properties. Oxygen and sulfur have different radii (60 versus 100 pm) and electronegativity (3.5 versus 2.5 Pauling units). Those differences influence the ability to hydrogen bond and the ribose puckering. In general, uridine and 2-thiouridine nucleosides and nucleotides adopt ca. 50−60% and 70−100% C3′-endo conformation, respectively. In consequence, 2-thiouridine derivatives influence the overall structure of helixes (*2*, *3*).

Uridine is the least effective nucleotide to include in probes and therapeutics because it binds weakly and with low specificity to adenosine. On the basis of UV melting, CD, and NMR studies, Kumar and Davis demonstrated that substitution of uridine with 2-thiouridine but not 4-thiouridine ($s^4U$) in A-U pairs significantly enhances stabilities of RNA duplexes (*3*). CD and NMR experiments indicated that A-form helical structure is maintained. Imino proton NMR showed that proton exchange rates, chemical shift differences, and NH proton line widths indicate the following stability order in A-U pairs: $s^2U > U > s^4U$. Moreover, 2-thiouridine in oligoribonucleotides enhances the specificity for binding to complementary RNA because the difference in stabilities between A-$s^2U$ and G-$s^2U$ base pairs is larger than between A-U and G-U base pairs (*4*). Replacing U with $s^2U$ significantly enhances thermodynamic stability of A-$s^2U$ but not G-$s^2U$ containing RNA duplexes. Parallel triplexes are also significantly stabilized by 2′-*O*-methyl-2-thiouridine ($s^2U^M$) and 2-thiothymidine, which was attributed to the effect of 2-thiocarbonyl groups on stacking (*5*).

The unique features of 2-thiouridine influence RNA biological function. For example, 2-thiouridine derivatives are present very often at wobble position 34 of tRNA, and it is essential for ribosome binding (*6*, *7*). The full-length, unmodified transcript of human $tRNA^{Lys3}_{UUU}$ and unmodified $tRNA^{Lys3}_{UUU}$ anticodon stem/loop ($ASL^{Lys3}_{UUU}$) do not bind AAA- or AAG-programmed ribosomes. Single, site-specific substitution of $s^2U$ at position 34 to produce the modified $ASL^{Lys3}_{SUU}$, however, restores ribosomal binding. Moreover, investigations of thermodynamic stability and of structure by NMR demonstrated different dynamic conformations for the loop of modified $ASL^{Lys3}_{SUU}$ and unmodified $ASL^{Lys3}_{UUU}$, whereas the stems were isomorphous (*7*−*10*).

Substitution by 2-thiouridine also can affect gene silencing of short interfering RNA (*11*). Among other modified nucleotides, introducing $s^2U$ resulted in 5−10 times more effective gene silencing of pBACE1-GFP plasmid in HeLa cells.

Locked nucleic acids (LNA) are analogues of nucleic acids where C4′ and O2′ are bridged with a methylene linker which fixes the ribose ring pucker to C3′-endo conformation exclusively (*12*, *13*). Among all known modified analogues, LNA forms the most thermodynamically stable duplexes with RNA and DNA (*14*−*18*). In this paper, the chemical synthesis of LNA-2-thiouridine is described for the first time. To achieve this goal, it was necessary to change one of the protecting groups used during synthesis and to optimize the nucleoside condensation reaction to minimize S-nucleoside formation. Studies of the thermodynamic stabilities of model LNA-2′-OMeRNA/RNA duplexes containing either 2′-*O*-methyl-2-thiouridine or LNA-2-thiouridine

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*To whom correspondence should be addressed. R.K.: phone, +48-61 852-85-03; fax, +48-61 852-05-32; e-mail, rkierzek@ibch.poznan.pl. D.H.T.: phone, 585-275-3207; fax, 585-276-0205; e-mail, turner@chem.rochester.edu.

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demonstrated that both enhance selectivity of binding to A over G but that LNA-2-thiouridine has a larger effect on stability. The abbreviation, LNA-2′-OMeRNA/RNA, means that one strand of the duplex is formed by a 2′-*O*-methyl oligonucleotide with LNA nucleotide(s) (LNA-2′-OMeRNA) at selected position(s) whereas the second strand is oligoribonucleotide (RNA). The largest enhancement in selectivity occurs when $s^2U$ is at an internal position within the duplex. Similar results were observed with duplexes carrying pyrene at the 3′-side of the 2′-*O*-methyl oligonucleotide strand. This latter type of duplex mimics interactions of isoenergetic microarray probes that target RNA. In those microarrays, the probes are short 2′-*O*-methyl oligonucleotides, and they are used to study secondary structure of target RNAs (*19*−*24*). The 2′-O-methylated oligonucleotides (2′-OMeRNA) are also useful for modulating biological functions by binding to target RNA (*25*−*28*).

## MATERIALS AND METHODS

*General Methods*. Mass spectra of nucleosides and oligonucleotides were obtained on an LC MS Hewlett-Packard series 1100 MSD with an API-ES detector or a MALDI TOF MS, model Autoflex (Bruker). Thin-layer chromatography (TLC) purification of oligonucleotides was carried out on Merck 60 $F_{254}$ TLC plates with the mixture 1-propanol/aqueous ammonia/water = 55:35:10 (v/v/v). TLC analysis of reaction progress was performed on the same type of silica gel plates with various mixtures of dichloromethane and methanol [98:2 v/v (A), 95:5 v/v (B), 9:1 v/v (C)].

*Synthesis and Purification of Oligonucleotides*. Oligonucleotides were synthesized on an Applied Biosystems DNA/RNA synthesizer, using $\beta$-cyanoethyl phosphoramidite chemistry (*29*). Commercially available A, C, G, and U phosphoramidites with 2′-*O*-*tert*-butyldimethylsilyl or 2′-*O*-methyl groups were used for synthesis of RNA and 2′-*O*-methyl RNA, respectively (Glen Research, Azco, Proligo). The 3′-*O*-phosphoramidites of LNA nucleotides were synthesized according to published procedures with some minor modifications (*16*, *17*, *30*, *31*). The details of deprotection and purification of oligoribonucleotides were described previously (*32*).

*UV Melting*. Oligonucleotides were melted in buffer containing 100 mM NaCl, 20 mM sodium cacodylate, and 0.5 mM $Na_2EDTA$, pH 7.0. The relatively low NaCl concentration kept melting temperatures in the reasonable range even when there were multiple substitutions and also allowed comparison to previous experiments. Oligonucleotide single strand concentrations were calculated from absorbance above 80 °C (*33*, *34*). Absorbance vs temperature melting curves were measured at 260 nm with a heating rate of 1 °C/min from 0 to 90 °C on a Beckman DU 640 spectrophotometer with a thermoprogrammer. Melting curves were analyzed and thermodynamic parameters were calculated from a two-state model with the program MeltWin 3.5 (*35*). For most sequences, the $\triangle H°$ derived from $T_M^{-1}$ vs $\ln(C_T/4)$ plots is within 15% of that derived from averaging the fits to individual melting curves, as expected if the two-state model is reasonable.

*Synthesis of 3-O-Allyl-1,2,5,6-di-O-isopropynylene-α-D-glucose* (***3***). The derivative **2** (40.7 g, 156.3 mmol) was coevaporated twice with THF and dissolved in 163 mL of anhydrous THF. Then, to a stirred solution of **2** at room temperature, a 60% emulsion of sodium hydride (9.36g, 234 mmol) in mineral oil was added and left to dissolve the sodium hydride (ca. 30 min), whereupon 21.6 mL (250 mmol) of allyl bromide was added and left stirring for 16 h. After completion, the reaction mixture volume was reduced to half, a saturated aqueous solution of sodium bicarbonate was added, and the mixture was extracted three times with dichloromethane. The organic layers were combined, dried with anhydrous sodium sulfate, and evaporated to heavy oil. $R_f$ 0.15 (A), 0.28 (B); $^1$H NMR $\delta_H$ ($CDCl_3$) 6.01−5.90 (1H, m, All-$OCH_2$-$C\underline{H}CH_2$), 5.77 (1H, d, H-1), 5.38−5.24 (2H, 2× d, All-$OCH_2$-$CH\underline{CH_2}$), 4.65 (1H, t, H-2), 4.30−4.20 (1H, m, H-3), 4.11−4.02 (3H, m, H-5, H-1′$_a$, H-1′$_b$), 3.95−3.68 (3H, m, H-4, All-$OC\underline{H_2}CHCH_2$), 1.60 (3H, s, $CH_3$), 1.35 (3H, s, $CH_3$); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 133.97, 118.94 (All), 113.24 ($C(CH_3)_2$), 104.17 (C-1), 77.32 (C-2), 77.00 (C-3), 76.68 (C-4), 71.31 (C-5), 70.73 (All), 64.16 (C-1′), 26.74, 26.49 ($CH_3$).

*Synthesis of 3-O-Allyl-1,2-O-isopropynylene-α-D-glucose* (***4***). To derivative **3** (47.6 g, 158.7 mmol), 127 mL of glacial acetic acid, 65 mL of formic acid, and 80 mL of water were added and left stirring at room temperature for 1.5 h. After reaction completion, the reaction mixture was evaporated and coevaporated three times with toluene. $R_f$ 0.15 (A), 0.28 (B); $^1$H NMR $\delta_H$ ($CDCl_3$) 6.01−5.90 (1H, m, All-$OCH_2C\underline{H}CH_2$), 5.77 (1H, d, H-1), 5.38−5.24 (2H, 2× d, All-$OCH_2CH\underline{CH_2}$), 4.65 (1H, t, H-2), 4.30−4.20 (1H, m, H-3), 4.11−4.02 (3H, m, H-5, H-1′$_a$, H-1′$_b$), 3.95−3.68 (3H, m, H-4, All-$OC\underline{H_2}CHCH_2$), 1.60 (3H, s, $CH_3$), 1.35 (3H, s, $CH_3$); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 133.97, 118.94 (All), 113.24 ($C(CH_3)_2$), 104.17 (C-1), 77.32 (C-2), 77.00 (C-3), 76.68 (C-4), 71.31 (C-5), 70.73 (All), 64.16 (C-1′), 26.74, 26.49 ($CH_3$).

*Synthesis of 3-O-Allyl-5-aldehyde-1,2-O-isopropynylene-α-D-glucose* (***5***). To 45.5 g (175 mmol) of derivative **4** dissolved in 430 mL of ethanol, a solution of 42.2 g (192 mmol) of sodium periodite in 290 mL of water was added. The reaction mixture was stirred for 0.5 h at room temperature and volume reduced by half. Then, a saturated aqueous solution of sodium bicarbonate was added, and the mixture was extracted three times with dichloromethane. Combined organic layers were dried with anhydrous sodium sulfate and evaporated to a heavy oil. $R_f$ 0.32 (A), 0.61 (B); $^1$H NMR $\delta_H$ ($CDCl_3$) 9.70 (1H, s, CHO), 6.02−5.84 (1H, m, All-$OCH_2C\underline{H}CH_2$), 5.80 (1H, d, H-1), 5.40−5.25 (2H, m, All-$OCH_2CH\underline{CH_2}$), 4.65 (1H, t, H-2), 4.30−4.03 (3H, m, H-3, H-4, H-5), 3.95−3.82 (2H, m, All-$OC\underline{H_2}CHCH_2$), 1.60 (3H, s, $CH_3$), 1.30 (3H, s, $CH_3$); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 198.53 (CHO), 133.80, 118.89 (All), 113.94 ($C(CH_3)_2$), 104.61 (C-1), 77.32 (C-2), 77.00 (C-3), 76.68 (C-4), 71.61 (All), 26.91, 26.57 ($CH_3$).

*Synthesis of 3-O-Allyl-4-C-hydroxymethyl-1,2-O-isopropynylene-α-D-glucose* (***6***). The derivative **5** (38.3 g, 168.1 mmol) was dissolved in 170 mL of THF and 170 mL of water and cooled to 4 °C. Then, 96.3 mL (3.48 mol) of formic aldehyde and 430 mL of 1 M aqueous sodium hydroxide were added; after 10 min the mixture was left at room temperature for 16 h. The reaction mixture was extracted three times with dichloromethane. The combined organic layers were washed with saturated aqueous solution of sodium bicarbonate, dried with anhydrous sodium sulfate, and evaporated. $R_f$ 0.18 (A), 0.35 (B); $^1$H NMR $\delta_H$ ($CDCl_3$) 6.01−5.88 (1H, m, All-$OCH_2C\underline{H}CH_2$), 5.77 (1H, d, H-1), 5.37−5.21 (2H, m, All-$OCH_2CH\underline{CH_2}$), 4.67 (1H, t, H-2), 4.40−3.80 (5H, m, H-3, H-5$_a$, H-5$_b$, H-1′$_a$, H-1′$_b$), 3.92−3.80 (2H, m, All-$OC\underline{H_2}CHCH_2$), 1.61 (3H, s, $CH_3$), 1.35 (3H, s, $CH_3$); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 133.97, 118.41 (All), 113.52 ($C(CH_3)_2$), 104.34 (C-1), 86.14 (C-4), 78.51 (C-2), 78.21 (C-3), 71.90 (C-5), 70.14 (All), 64.10 (C-1′), 26.54, 25.86 ($CH_3$).

*Synthesis of 3-O-Allyl-1,2-O-isopropynylene-5-O-methanosulfonyl-4-C-methanosulfonyloxymethyl-α-D-erythro-pentafuranose* (***7***). Derivative **6** (44.5 g, 171.2 mmol) was dissolved in 140 mL of anhydrous pyridine and cooled to 4 °C, whereupon methanesulfonyl chloride (26.6 mL, 342 mmol) was added. After a few minutes, the reaction mixture was left at room temperature for 1 h. Then, the volume of the reaction mixture was reduced to half; a saturated aqueous solution of sodium bicarbonate was added, and the mixture was extracted three times with dichloromethane. The combined organic layers were dried with anhydrous sodium sulfate, and the solution was evaporated and coevaporated a few times with toluene. $R_f$ 0.56 (A), 0.85 (B); $^1$H NMR $\delta_H$ ($CDCl_3$) 5.98−5.86 (1H, m, All-$OCH_2CHCH_2$), 5.81 (1H, d, H-1), 5.38−5.25 (2H, m, All-$OCH_2CHCH_2$), 4.70 (1H, t, H-2), 4.40−4.05 (5H, m, H-3, H-$5_a$, H-$5_b$, H-$1'_a$, H-$1'_b$), 4.19 (2H, d, All-$OCH_2CHCH_2$), 3.12 (2H, s, $CH_3$-Ms), 3.10 (1H, s, $CH_3$-Ms), 3.09 (1H, s, $CH_3$-Ms), 3.05 (1H, s, $CH_3$-Ms), 1.68 (3H, s, $CH_3$), 1.35 (3H, s, $CH_3$); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 133.55, 118.95 (All), 114.03 ($C(CH_3)_2$), 104.46 (C-1), 83.22 (C-4), 78.39 (C-2), 77.93 (C-3), 72.14 (C-5), 69.47 (All), 68.75 (C-1′), 38.07, 37.56 ($CH_3$-Ms), 26.19, 25.64 ($CH_3$).

*Synthesis of 3-O-Allyl-5-O-methanosulfonyl-4-C-methanosulfonyloxymethyl-α-D-erythro-pentafuranose* (***8***). To derivative **7** (42.3 g, 101.6 mmol) was added 130 mL of 80% acetic acid, and the mixture was refluxed for 3 h at 90 °C. After completion, the reaction mixture was evaporated and coevaporated a few times with toluene and a few times with anhydrous pyridine. $R_f$ 0.05 (A), 0.21 (B); $^1$H NMR $\delta_H$ ($CDCl_3$) 5.96−5.84 (1H, m, All-$OCH_2CHCH_2$), 5.42 (1H, s, H-1), 5.39−5.25 (2H, m, All-$OCH_2CHCH_2$), 4.60 (1H, d, H-2), 4.43−4.05 (5H, m, H-3, H-$5_a$, H-$5_b$, H-$1'_a$, H-$1'_b$), 4.20 (2H, d, All-$OCH_2CHCH_2$), 3.08, 3.06 (6H, 2s, $CH_3$-Ms); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 133.16, 119.30 (All), 101.45 (C-1), 82.24 (C-4), 77.31 (C-2), 76.68 (C-3), 72.70 (C-5), 69.62 (All), 68.98 (C-1′), 37.65, 37.59 ($CH_3$-Ms).

*Synthesis of 1,2-Di-O-acetyl-3-O-allyl-5-O-methanosulfonyl-4-C-methanosulfonyloxymethyl-α-D-erythro-pentafuranose* (***9***). Derivative **8** (38.4 g, 102 mmol) was dissolved in 90 mL of anhydrous pyridine, and 38 mL (408 mmol) of acetic anhydride was added and stirred at room temperature for 16 h. After completion, the volume of the reaction mixture was reduced to half, a saturated aqueous solution of sodium bicarbonate was added, and the mixture was extracted three times with dichloromethane. The combined organic layers were dried with anhydrous sodium sulfate, and the solution was evaporated and coevaporated a few times with toluene. The remains of mineral oil were washed out with *n*-hexanes. The reaction mixture was purified by silica gel column chromatography using dichloromethane as solvent. The overall yield for nine steps was 37%, which corresponds on average to ca. 90% for each step. $R_f$ 0.66 (A), 0.78 (B); $^1$H NMR $\delta_H$ ($CDCl_3$) 6.17 (1H, s, H-1), 5.87−5.77 (1H, m, All-$OCH_2CHCH_2$), 5.38−5.30 (2H, m, All-$OCH_2CHCH_2$), 5.28−5.22 (1H, t, H-2), 4.39 (1H, d, H-3), 4.37−4.20 (4H, m, H-$5_a$, H-$5_b$, H-$1'_a$, H-$1'_b$), 4.10−3.98 (2H, m, All-$OCH_2CHCH_2$), 3.08 (3H, s, $CH_3$-Ms), 3.07 (3H, s, $CH_3$-Ms), 2.16 (3H, s, $COCH_3$), 2.11 (3H, s, $COCH_3$); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 169.15, 168.79 (C=O), 133.18, 118.55 (All), 97.33 (C-1), 82.73 (C-4), 78.46 (C-2), 76.99 (C-3), 73.47 (C-5), 72.96 (C-1′), 68.66 (All), 37.89, 37.71 ($CH_3$-Ms), 21.03, 20.65 ($CH_3$-Ac).

*Synthesis of 1-(2-O-Acetyl-3-O-allyl-5-O-methanosulfonyl-4-C-methanosulfonyloxymethyl-β-D-ribofuranosyl)-2-thiouridine* (***10***). 2-Thiouracil (12.2 g, 95 mmol) was suspended in 150 mL of hexamethyldisilazane, and 50 mg of ammonium sulfate was added and refluxed at 130 °C for 16 h. The reaction mixture was cooled to room temperature, evaporated to dryness, and coevaporated three times with 1, 2-dichloroethane. Then, 1,2-di-*O*-acetyl-3-*O*-allyl-5-*O*-methanosulfonyl-4-*C*-methanosulfonyloxymethyl-α-D-*erythro*-pentafuranose (**9**) (32.4 g, 70.4 mmol) that was previously coevaporated three times with 1,2-dichloroethane was added to silylated 2-thiouracil, and the mixture of both substrates was coevaporated once again with 1,2-dichloroethane. The residual oil was dissolved in 340 mL of anhydrous 1,2-dichloroethane and cooled to 4 °C. Then, 16.5 mL (141 mmol) of tin(IV) chloride was added dropwise and left at room temperature for 2 h. To the reaction mixture was added a saturated aqueous solution of sodium bicarbonate and sodium chloride, and the mixture was extracted three times with dichloromethane. The combined organic layers were dried with anhydrous sodium sulfate, and the solution was evaporated. The reaction mixture was purified by silica gel column chromatography using as solvent dichloromethane with gradually increasing amount of methanol (up to 2%). Yield of product (**10**) 27.3 g (51.6 mmol, 73.3%); $R_f$ 0.19 (B), 0.45 (C); $^1$H NMR $\delta_H$ ($CDCl_3$) 7.68 (1H, d, H-6), 7.26 (1H, s, H-1′), 6.05 (1H, d, H-5), 5.91−5.78 (1H, m, All-$OCH_2CHCH_2$), 5.30−5.20 (2H, m, All-$OCH_2CHCH_2$), 4.67 (1H, s, H-2′), 4.53 (1H, s, H-3′), 4.25 (2H, d, All-$OCH_2CHCH_2$), 4.15−3.96 (4H, m, H-$5'_a$, H-$5'_b$, H-$5''_a$, H-$5''_b$), 3.13 (3H, s, $CH_3$-Ms), 3.10 (3H, s, $CH_3$-Ms), 2.16 (3H, s, $COCH_3$); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 175.72 (C-2), 169.48 (C=O), 159.85 (C-4), 139.43 (C-6), 132.94, 119.10 (All), 107.18 (C-5), 91.63 (C-1′), 84.60 (C-4′), 77.21 (C-2′), 75.60 (C-3′), 73.48 (C-5′), 67.65 (All), 53.41 (C-1″), 37.92, 37.62 ($CH_3$-Ms), 20.75 ($CH_3$-Ac).

*Synthesis of (1S,3R,4R,7S)-3-(2-Thiouridin-1-yl)-7-allyloxy-1-methanosulfonyloxymethyl-2,5-dioxabicyclo[2.2.1]heptane* (***11***). Derivative **10** (24.4 g, 46.2 mmol) was dissolved in 78 mL of THF, and 138 mL of a 1 M aqueous solution of monohydrate of lithium hydroxide was added. After 1 h stirring at room temperature, the reaction was complete, and acetic acid was used to neutralize the reaction. To the reaction mixture was added a saturated aqueous solution of sodium bicarbonate, and the mixture was extracted three times with dichloromethane. The combined organic layers were dried with anhydrous sodium sulfate, and the solution was evaporated to dryness. Yield of the crude (**11**) 16.5 g (42.3 mmol, 91.5%); $R_f$ 0.39 (B), 0.60 (C); $^1$H NMR $\delta_H$ ($CDCl_3$) 7.70 (1H, d, H-6), 6.12 (1H, s, H-1′), 5.97 (1H, d, H-5), 5.86−5.70 (1H, m, All-$OCH_2CHCH_2$), 5.24−5.11 (2H, m, All-$OCH_2CHCH_2$), 4.84 (1H, s, H-2′), 4.56 (1H, s, H-3′), 4.12−3.92 (4H, m, H-$5'_a$, H-$5'_b$, H-$1''_a$, H-$1''_b$), 3.86 (2H, d, All-$OCH_2CHCH_2$), 3.07 (3H, s, $CH_3$-Ms); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 174.68 (C-2), 159.75 (C-4), 139.02 (C-6), 133.08, 118.02 (All), 106.49 (C-5), 90.43 (C-1′), 86.08 (C-4′), 77.42 (C-2′), 76.99 (C-3′), 75.84 (C-5′), 68.67 (All), 64.09 (C-1″), 37.50 ($CH_3$-Ms).

*Synthesis of (1S,3R,4R,7S)-3-(2-Thiouridin-1-yl)-7-allyloxy-1-benzoyloxymethyl-2,5-dioxabicyclo[2.2.1]heptane* (***12***). Compound **11** (16.5 g, 42.3 mmol) was coevaporated three times with anhydrous DMF, and residue was dissolved in 200 mL of anhydrous DMF. Then, 16.3 g (127 mmol) of lithium benzoate was added and stirred for 16 h at 90 °C. After completion, a saturated aqueous solution of sodium bicarbonate and sodium chloride was added to the reaction mixture, and the mixture was extracted three times with dichloromethane. The combined organic layers were dried with anhydrous sodium sulfate, and the solution was evaporated to dryness. Yield of

the crude (**12**) 15.9 g (38.1 mmol, 90.0%); $R_f$ 0.51 (B), 0.66 (C); $^1$H NMR $\delta_H$ ($CDCl_3$) 10.69 (1H, s, N-H), 8.01 (1H, d, H-6), 7.55−7.52 (5H, m, Bz), 6.14 (1H, s, H-1′), 5.90−5.79 (1H, m, All-$OCH_2\underline{C}HCH_2$), 5.76 (1H, d, H-5), 5.30−5.10 (2H, m, All-$OCH_2CHC\underline{H}_2$), 4.91 (1H, s, H-2′), 4.16 (1H, s, H-3′), 4.20−3.80 (6H, m, H-5′$_a$, H-5′$_b$, H-1″$_a$, H-1″$_b$, All-$OC\underline{H}_2CHCH_2$); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 174.58 (C-2), 162.53 (Bn), 159.69 (C-4), 139.43 (C-6), 133.82, 133.28, 129.69, 128.76 (Bn), 106.14 (C-5), 92.56 (C-1′), 86.73 (C-4′), 81.42 (C-2′), 77.32 (C-3′), 75.92 (C-5′), 71.27 (All), 58.98 (C-1″).

*Synthesis of* (*1S,3R,4R,7S*)-*3*-(*2-Thiouridin-1-yl*)-*7-allyloxy-1-hydroxymethyl-2,5-dioxabicyclo*[*2.2.1*]*heptane* (***13***). To crude derivative **12** (15.9 g, 38.1 mmol) was added 50 mL of pyridine and 32% aqueous ammonia to beginning cloudiness of solution and left at 55 °C for 16 h. After cooling to room temperature, the solution was evaporated and coevaporated several times with toluene. The reaction mixture was purified by silica gel column chromatography using as solvent dichloromethane with gradually increasing amount of methanol (up to 5%). Yield of product (**13**) 9.4 g (30.1 mmol, 79.1%); $R_f$ 0.21 (B), 0.65 (C); $^1$H NMR $\delta_H$ ($CDCl_3$) 8.04 (1H, d, H-6), 6.12 (1H, s, H-1′), 6.04 (1H, d, H-5), 5.90−5.77 (1H, m, All-$OCH_2\underline{C}HCH_2$), 5.30−5.10 (2H, 2d, All-$OCH_2CHC\underline{H}_2$), 4.79 (1H, s, H-2′), 3.99 (1H, s, H-3′), 4.10−3.70 (6H, m, H-5′$_a$, H-5′$_b$, H-1″$_a$, H-1″$_b$, All-$OC\underline{H}_2CHCH_2$); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 176.00 (C-2), 161.88 (C-4), 140.27 (C-6), 133.50, 118.59 (All), 106.19 (C-5), 90.28 (C-1′), 89.07 (C-4′), 77.32 (C-2′), 76.68 (C-3′), 75.33 (C-5′), 70.01 (All), 56.65 (C-1″).

*Synthesis of* (*1S,3R,4R,7S*)-*3*-(*2-Thiouridin-1-yl*)-*7*-(*prop-1-enyl*)-*1-hydroxymethyl-2,5-dioxabicyclo*[*2.2.1*]*heptane* (***14***). Compound **13** (8.8 g, 28.1 mmol) was dissolved in 140 mL of anhydrous DMF, and 15.8 g (140.7 mmol) of potassium *tert*-butoxide was added. The reaction mixture was refluxed for 2 h at 100 °C. After completion, a saturated aqueous solution of sodium bicarbonate was added to the reaction mixture, and the mixture was extracted three times with dichloromethane. The combined organic layers were dried with anhydrous sodium sulfate, and the solution was evaporated to dryness. Yield of the crude (**14**) 7.2 g (23.1 mmol, 82%); $R_f$ 0.24 (B), 0.72 (C); $^1$H NMR $\delta_H$ ($CDCl_3$) 7.80 (1H, d, H-6), 6.12 (1H, s, H-1′), 5.95 (1H, d, H-5), 4.80 (1H, s, H-2′), 4.10 (1H, s, H-3′), 4.04−3.82 (4H, m, H-5′$_a$, H-5′$_b$, H-1″$_a$, H-1″$_b$), 1.55−1.51 (3H, 2d, $CH_3$−All); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 174.68 (C-2), 162.62 (C-4), 140.08 (C-6), 128.78, 127.33 (All), 106.27 (C-5), 90.18 (C-1′), 89.14 (C-4′), 77.42 (C-2′), 76.31 (C-3′), 71.39 (C-5′), 56.34 (C-1″), 9.11 ($CH_3$-All).

*Synthesis of* (*1S,3R,4R,7S*)-*3*-(*2-Thiouridin-1-yl*)-*7-hydroxy-1-hydroxymethyl-2,5-dioxabicyclo*[*2.2.1*]*heptane* (***15***). To derivative **14** (7.2 g, 23.1 mmol) was added 80 mL of 80% acetic acid and then kept at 100 °C for 4 h. After reaction completion, the acetic acid was evaporated and coevaporated three times with toluene. Yield of the crude (**15**) 6.3 g (23.1 mmol, 100%); $R_f$ 0.03 (B), 0.38 (C); $^1$H NMR $\delta_H$ ($CDCl_3$) 7.84−7.79 (1H, d, H-6), 6.14 (1H, s, H-1′), 6.09 (1H, d, H-5), 4.69 (1H, s, H-2′), 4.43 (1H, s, H-3′), 4.16−3.70 (4H, m, H-5′$_a$, H-5′$_b$, H-1″$_a$, H-1″$_b$); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 176.87 (C-2), 162.69 (C-4), 140.33 (C-6), 106.04 (C-5), 90.30 (C-1′), 87.26 (C-4′), 77.42 (C-2′), 71.23 (C-3′), 71.05 (C-5′), 56.36 (C-1″).

*Synthesis of* (*1S,3R,4R,7S*)-*3*-(*2-Thiouridin-1-yl*)-*7-hydroxy-1*-(*4,4′-dimethoxytrityloxymethyl*)-*2,5-dioxabicyclo*[*2.2.1*]*heptane* (***16***). The derivative **15** (6.3 g, 23.1 mmol) was coevaporated twice with 25 mL of anhydrous pyridine. The residue was dissolved in 80 mL of anhydrous pyridine, and 4,4′-dimethoxytrityl chloride (11.7 g, 34.6 mmol) was added and left at room temperature for 2 h. After reaction completion, to the reaction mixture was added a saturated aqueous solution of sodium bicarbonate and extracted three times with dichloromethane. The combined organic layers were dried with anhydrous sodium sulfate, and the solution was evaporated and coevaporated three times with toluene. The reaction mixture was purified by silica gel column chromatography using as solvent dichloromethane with gradually increasing amount of methanol (up to 5%). Yield of product (**16**) 10.2 g (17.8 mmol, 76.9%); overall yield for the synthesis of protected LNA-2-thiouridine was 25.2%, which corresponds on average to 82% on each step; $R_f$ 0.35 (B), 0.46 (C); $^1$H NMR $\delta_H$ ($CDCl_3$) 10.40 (1H, s, N-H), 8.14 (1H, d, H-6), 7.56−7.40 (9H, m, DMTr), 7.40−7.20 (4H, m, DMTr), 6.14 (1H, s, H-1′), 5.85 (1H, d, H-5), 4.73 (1H, s, H-2′), 4.24 (1H, s, H-3′), 3.90−3.80 (2H, m, H-1″$_a$, H-1″$_b$), 3.79 (6H, s, $OCH_3$-DMTr), 3.60−3.49 (2H, m, H-5′$_a$, H-5′$_b$); $^{13}$C NMR $\delta_C$ ($CDCl_3$) 174.52 (C-2), 159.89 (C-4), 158.78 (DMTr), 144.37 (DMTr), 140.29 (C-6), 130.07, 129.99, 128.62, 128.30, 127.97, 127.33, 127.22, 113.35 (DMTr), 106.26 (C-5), 90.12 (C-1′), 88.68 (DMTr), 87.02 (C-4′), 77.42 (C-2′), 71.35 (C-3′), 70.12 (C-5′), 57.57 (C-1″), 55.26 ($OCH_3$).

*Synthesis of* (*1R,3R,4R,7S*)-*7*-(*2-Cyanoethoxy*(*diisopropylamino*)*phosphinoxy*)-*1*-(*4,4′-dimethoxytrityloxymethyl*)-*3*-(*2-thiouridin-1-yl*)-*2,5-dioxabicyclo*[*2.2.1*]*heptane* (***17***). Compound **16** (3.65 g, 6.36 mmol) and tetrazole (0.45 g, 6.36 mmol) were dried under vacuum for several hours and dissolved in 43 mL of anhydrous acetonitrile. Then, to a stirred solution was added with syringe 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (2.47 g, 8.27 mmol). The mixture was stirred at room temperature for 1.5 h. A saturated aqueous solution of sodium bicarbonate was added and extracted three times with dichloromethane containing 1% of triethylamine. The organic phase was dried with anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using hexane containing 1% of triethylamine and gradually increasing ethyl acetate up to 75%. The fractions carrying product were combined and evaporated and coevaporated three times with benzene and lyophilized to give product as a white solid material. Yield 4.28 g (5.53 mmol, 86.9%); $R_f$ 0.45 (B), 0.58 (C); $^{31}$P NMR $\delta_C$ (DMSO-$d_6$) 151.00, 148.26.

## RESULTS

*Synthesis of LNA-2-thiouridine*. The chemical synthesis of LNA-2-thiouridine is shown in Figures 1 and 2. Two major problems were found when using standard procedures for synthesis of LNA nucleosides. First, trimethylsilyl trifluoromethanesulfonate catalyzed condensation (*36*) of aglycone with silylated 2-thiouracil produced S-substituted nucleoside as the major product (K. Pasternak, Z. Gdaniec, and R. Kierzek, unpublished results). Changing temperature and solvent did not affect significantly those preferences. Tin(IV) chloride in 1,2-dichloroethane (*37*−*39*), however, provided ca. 90% N-substituted nucleosides (Figure 2). The second problem was removal of the transient protecting group from the 3′-hydroxyl. Classically, benzyl is removed by hydrogenolysis in the presence of palladium catalyst (*40*). That and palladium-free methods for deprotection of benzyl were insufficient for multigram scale of synthesis (*40*). Several alternative protecting groups, *o*-nitrobenzyl, *tert*-butyldimethylsilyl, methythiomethyl, and 2-methoxyethoxymethyl (A. Kowalska, A. Pasternak,

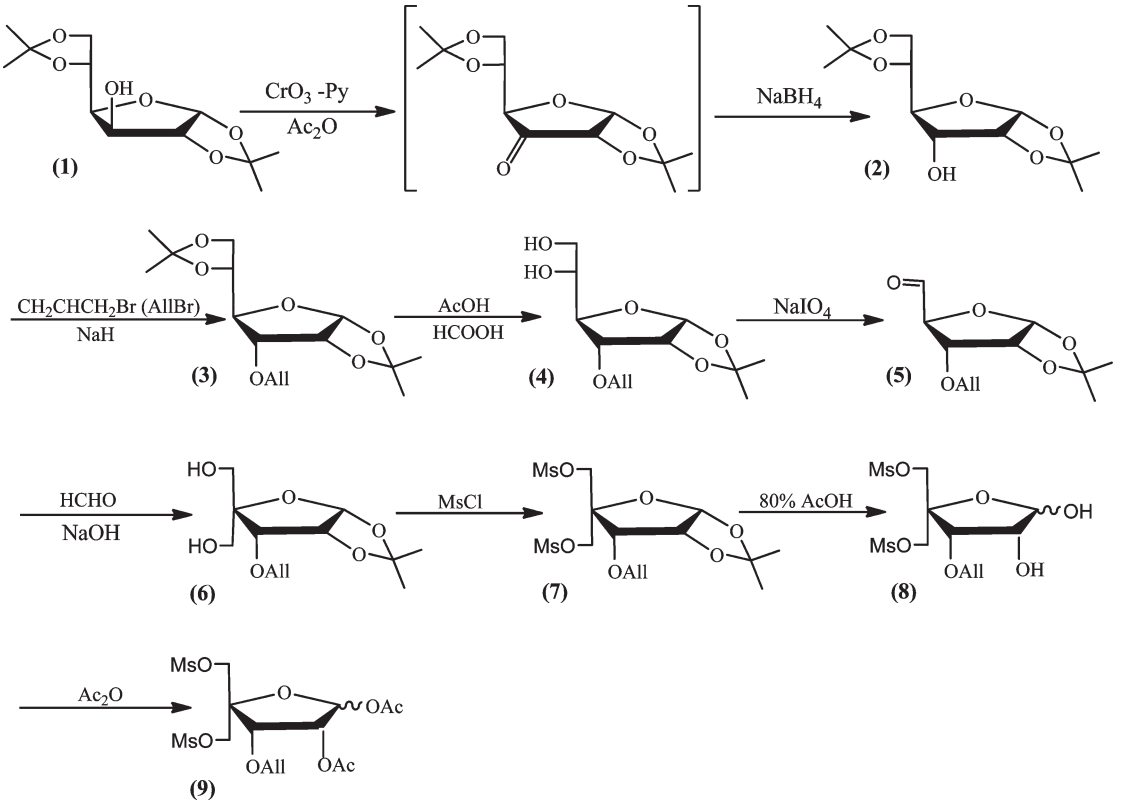


FIGURE 1: Scheme for synthesis of 1,2-di-*O*-acetyl-3-*O*-allyl-5-*O*-methanosulfonyl-4-*C*-methanosulfonyloxymethyl-α-D-*erythro*-pentafuranose, precursor of the aglycon for LNA-$s^2$U preparation.

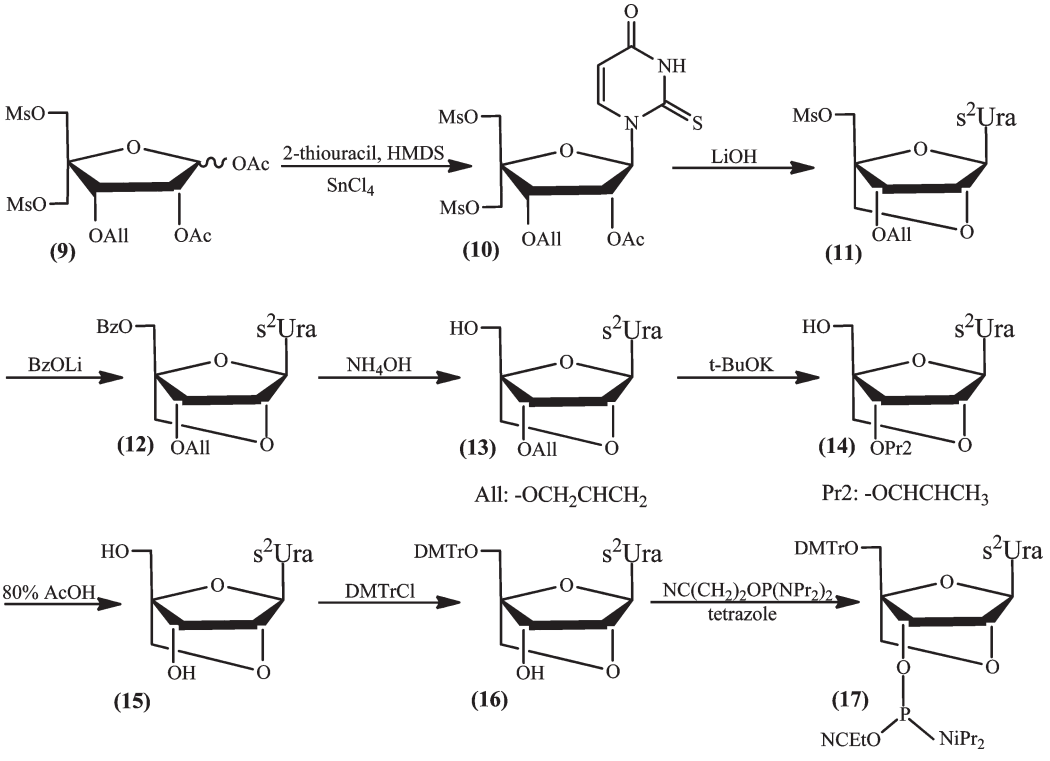


FIGURE 2: Scheme for synthesis of 5′-*O*-dimethoxytrityl-LNA-$s^2$U and it 3′-*O*-phosphoramidite.

K. Pasternak, and R. Kierzek, unpublished results) were found inapplicable for this synthesis. The allyl protection group, however, proved successful (Figure 1) (*40*−*42*).

The chemical synthesis of the precursor of the ribose analogue was performed as described for standard synthesis but with allyl bromide replacement (*12*, *30*, *40*, *41*). The allyl bromide reaction as well as all reactions toward synthesis of the precursor, 1,2-di-*O*-acetyl-3-*O*-allyl-5-*O*-methanosulfonyl-4-*C*-methanosulfonyloxymethyl-α-D-*erythro*-pentafuranose, was almost quantitative so column purification of the intermediates was omitted. The final product was purified by column chromatography, and the overall yield for nine reactions

was ca. 40%, which means that the average reaction yield was ca. 90%.

Condensation of the sugar precursor with silylated 2-thiouracil (ca. 1.3 equiv) was performed at room temperature in 1,2-dichloroethane for 2 h in the presence of tin(IV) chloride (*37*). The reaction was complete and resulted in two products, N-glycoside (ca. 90%) and S-glycoside (ca. 10%) (Figure 2). Because the character of the C−S bond in N- and S-glycosides differs, $^{13}$C NMR distinguished the character of the glycosidic bond. The chemical shifts of C2 in N-glycoside and in S-glycoside were 175.72 and 164.45 ppm, respectively (*43*). Silica gel column purified N-glycoside only was used for the next reaction. The next reactions were performed according to the original procedure, except that, in exchanging of 5′-*O*-methanosulfonyl, lithium benzoate was substituted for sodium benzoate due to solubility in DMF (*12*, *17*, *30*). A two-step deprotection was used for removal of 3′-*O*-allyl (*44*). With potassium *tert*-butoxide, 3′-*O*-allyl was converted into propenyl ether and then deprotected with acetic acid. The overall yield for synthesis of protected LNA-2-thiouridine was over 25%, which corresponds on average to 82% for each step.

*Design of Sequences*. To measure the effects of replacing 2′-*O*-methyluridine with 2′-*O*-methyl-2-thiouridine and LNA-uridine with LNA-2-thiouridine in oligonucleotides, thermodynamics was measured for duplexes containing A-U and/or G-U pairs. Moreover, those base pairs were placed in terminal and internal positions within 2′-*O*-MeRNA/RNA duplexes.

Another group of model duplexes have 2′-O-methylated oligonucleotides containing a 3′-terminal pyrene residue. Pyrene at the 3′-end of a 2′-OMeRNA oligonucleotide enhances thermodynamic stability of 2′-OMeRNA/RNA duplexes by 2.2−2.4 kcal/mol, independent of RNA strand length and nature of the RNA nucleotide opposite the pyrene (*45*). This makes pyrene very useful for preparation of probes for isoenergetic RNA microarrays. LNA-2′-OMeRNA probes rich in A and U nucleotides often form only weak hybridization duplexes with target RNA. For this reason, it was important to evaluate the influence of LNA-2-thiouridine ($s^2U^L$) on the thermodynamic stability of 3′-pyrene-terminated duplexes containing A-$s^2U^L$ and G-$s^2U^L$ pairs. To make these studies more realistic, the RNA strand (mimic of RNA target) was two nucleotides longer than the 3′-pyrene-terminated 2′-OMeRNA (mimic of microarray probe).

*Influence of 2′-O-Methyl-2-thiouridine and LNA-2-thiouridine at 5′-Terminal Positions*. The influence of 5′-terminal 2′-*O*-methyl-2-thiouridine and LNA-2-thiouridine on thermodynamic stabilities was studied in 2′-OMeRNA/RNA and chimeric LNA-2′-OMeRNA/RNA duplexes, 5′Y$C^MU^M$-$A^MC^MC^MA^M$/3′RGAUGGU. In those duplexes, the superscript M marks 2′-O-methylated nucleotides, Y means 2′-*O*-methyl-2-thiouridine ($s^2U^M$), LNA-2-thiouridine ($s^2U^L$), 2′-*O*-methyluridine ($U^M$), or LNA-uridine ($U^L$), and R means A or G. The thermodynamic stabilities ($\Delta G°_{37}$) of 5′Y$C^MU^MA^MC^MC^MA^M$/3′AGAUGGU were −7.18, −7.67, −7.64, and −8.51 kcal/mol for Y equal to $U^M$, $s^2U^M$, $U^L$, and $s^2U^L$, respectively, whereas in duplexes 5′Y$C^MU^MA^MC^MC^MA^M$/3′GGAUGGU, the thermodynamic stabilities were −6.79, −7.03, −7.01, and −7.45 kcal/mol, respectively (Table 1). Thus, 5′-terminal A-$s^2U^M$ and A-$s^2U^L$ base pairs enhance stability by 0.49 and 0.87 kcal/mol, respectively, relative to $U^M$-A and $U^L$-A, whereas 5′-terminal G-$s^2U^M$ and G-$s^2U^L$ base pairs enhance stabilities by 0.24 and 0.44 kcal/mol, respectively.

For 3′-pyrene-terminated duplexes, 5′Y$G^MU^MG^MU^M$pyrene/3′GACACAG, the thermodynamic stability was −7.35 and −8.12 kcal/mol for Y equal to $U^L$ and $s^2U^L$, respectively (Table 2). The enhancement of duplex stability ($\Delta\Delta G°_{37}$) due to replacement of 2-oxo with 2-thio analogues was 0.77 kcal/mol in an A-U pair. When $U^L$ and $s^2U^L$ pair with G in 5′Y$G^MU^MG^MU^M$pyrene/3′GGCACAG duplexes, the free energies were −5.68 and −6.12 kcal/mol for the same modified nucleotides, so the increase of the stability was 0.44 kcal/mol.

*Influence of 2′-O-Methyl-2-thiouridine and LNA-2-thiouridine at 3′-Terminal Positions*. The effects of 3′-terminal $s^2U^M$ and $s^2U^L$ were studied in duplexes, 5′$A^MC^MU^M$-$A^MC^MC^M$Y/3′UGAUGGA. The thermodynamic stabilities were −7.37, −7.20, −7.24, and −7.84 kcal/mol for Y equal to $U^M$, $s^2U^M$, $U^L$, and $s^2U^L$, respectively (Table 1). When 2-thio-modified nucleotides base paired to G in 5′$A^MC^MU^MA^M$-$C^MC^M$Y/3′UGAUGGG, the thermodynamic stabilities were −6.93, −6.78, −7.38, and −7.40 kcal/mol, respectively (Table 1). The presence of a 3′-terminal A-$s^2U^M$ base pair diminished stability by 0.17 kcal/mol whereas a 3′-terminal A-$s^2U^L$ enhanced stability by 0.60 kcal/mol relative to 3′-terminal A-$U^M$ and A-$U^L$, respectively. The 3′-terminal base pairs, G-$s^2U^M$ and G-$s^2U^L$, change the thermodynamic stability by 0.15 and −0.02 kcal/mol, respectively, relative to G-$U^M$ and G-$U^L$.

When $s^2U^L$ and 3′-terminal pyrene were placed next to each other in the duplexes 5′$U^MG^MU^MG^M$Ypyrene/3′GACACAG, the stabilities were −7.03 and −7.54 kcal/mol for Y equal to $U^L$ and $s^2U^L$, respectively (Table 2). Thus, the stability ($\Delta\Delta G°_{37}$) of the duplex increased by 0.51 kcal/mol due to the replacement of uridine with 2-thiouridine in an A-U pair. When $U^L$ and $s^2U^L$ base pair with G in 5′$U^MG^MU^MG^M$Ypyrene/3′GACACGG, the free energies were −6.42 and −6.25 kcal/mol for the same modified nucleotides, so stability was diminished by 0.17 kcal/mol (Table 2).

*Influence of 2′-O-Methyl-2-thiouridine and LNA-2-thiouridine at Internal Positions*. The $s^2U^M$ and $s^2U^L$ nucleotides were placed at two internal positions, the central and 5′-penultimate, positions within 2′-OMeRNA/RNA and LNA-2′-OMeRNA/RNA duplexes (Table 1). At the center of 5′$A^MC^MU^M$Y$G^MC^MA^M$/3′UGAACGU duplexes, the thermodynamic stabilities were −7.59, −9.00, −8.96, and −10.40 kcal/mol for Y equal to $U^M$, $s^2U^M$, $U^L$, and $s^2U^L$, respectively. When those nucleotides base paired to G, in 5′$A^MC^MU^M$Y$G^MC^MA^M$/3′UGAGCGU, the thermodynamic stabilities were −4.60, −5.17, −6.76, and −6.64 kcal/mol, respectively. Thus, replacement of $U^M$ with $s^2U^M$ or $U^L$ with $s^2U^L$ enhanced the duplex thermodynamic stability ($\Delta\Delta G°_{37}$) by 1.41 and 1.44 kcal/mol, respectively, when base pairing to A, whereas the changes when base pairing to G were −0.57 and 0.12 kcal/mol, respectively.

Two sets of LNA-2′-OMeRNA/RNA duplexes with a 3′-terminal pyrene were investigated (Table 2). For 5′$C^MU^M$-Y$G^MC^M$pyrene/3′UGAACGU, the thermodynamic stabilities were −9.52 and −10.97 kcal/mol for Y equal to $U^L$ and $s^2U^L$, respectively. The enhancement of the A-U pair stability ($\Delta\Delta G°_{37}$) was 1.45 kcal/mol. When binding of the oligonucleotides occurred via mismatch to G, the stabilities of duplexes 5′$C^MU^M$Y$G^M$-$C^M$pyrene/3′UGAGCGU were −7.18 and −7.49 kcal/mol, respectively. In that case, the replacement of $U^L$ with $s^2U^L$ increased stability by only 0.31 kcal/mol. In the second set of the duplexes, 5′$U^MG^M$Y$G^MU^M$pyrene/3′UACACAU, the stabilities were −7.90 and −8.87 kcal/mol for Y equal to $U^L$ and $s^2U^L$, respectively, so enhancement of A-U pair stability ($\Delta\Delta G°_{37}$) was

Table 1: Thermodynamic Parameters of Helix Formation with RNA and 2′-*O*-Me Oligoribonucleotides: Effect of 2′-*O*-Methyl-2-thiouridine and LNA-2-thiouridine[a]

| RNA duplexes | | average of curve fits | | | | $T_M^{-1}$ vs log $C_T$ plots | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5′−3′ | 3′−5′ | $-\Delta H°$ (kcal/mol) | $-\Delta S°$ (eu) | $-\Delta G°_{37}$ (kcal/mol) | $T_M$[b] (°C) | $-\Delta H°$ (kcal/mol) | $-\Delta S°$ (eu) | $-\Delta G°_{37}$ (kcal/mol) | $T_M$[b] (°C) | $\Delta\Delta G°_{37}$ (kcal/ mol) | $\Delta T_M$[b] (°C) | $\Delta\Delta G°_{37}$[c] (kcal/ mol) | $\Delta\Delta G°_{37}$[c] (kcal/ mol) |
| $\mathbf{U^MC^MU^MA^MC^MC^MA^M}$[d] | $\mathbf{\underline{A}}$GAUGGU[d] | 50.7 ± 5.9 | 140.0 ± 18.4 | 7.25 ± 0.19 | 41.5 | 45.2 ± 1.6 | 122.5 ± 5.3 | 7.18 ± 0.03 | 41.5 | 0 | 0 | 0 | *0* |
| $\mathbf{\underline{s^2U}^MC^MU^MA^MC^MC^MA^M}$ | $\mathbf{\underline{A}}$GAUGGU | 53.3 ± 3.5 | 146.8 ± 11.1 | 7.74 ± 0.09 | 44.2 | 51.2 ± 1.4 | 140.5 ± 4.4 | 7.67 ± 0.02 | 44.1 | −0.49 | 2.6 | −0.49 | 0 |
| $\mathbf{U^MC^MU^MA^MC^MC^MA^M}$ | $\mathbf{\underline{G}}$GAUGGU | 47.2 ± 5.1 | 130.4 ± 16.6 | 6.74 ± 0.14 | 38.4 | 42.0 ± 0.8 | 113.6 ± 2.5 | 6.79 ± 0.01 | 39.0 | 0.39 | −2.5 | *0* | *0.39* |
| $\mathbf{\underline{s^2U}^MC^MU^MA^MC^MC^MA^M}$ | $\mathbf{\underline{G}}$GAUGGU | (48.7 ± 6.7) | (134.2 ± 21.2) | (7.08 ± 0.17) | (40.5) | (40.8 ± 1.7) | (109.1 ± 5.4) | (7.03 ± 0.03) | (40.9) | (−0.24) | (−0.6) | (*−0.24*) | (0.64) |
| $\mathbf{U^LC^MU^MA^MC^MC^MA^M}$[d] | $\mathbf{\underline{A}}$GAUGGU[d] | 55.7 ± 3.3 | 154.4 ± 10.0 | 7.84 ± 0.19 | 44.4 | 47.2 ± 3.0 | 127.5 ± 9.6 | 7.64 ± 0.08 | 44.5 | 0 | 0 | 0 | *0* |
| $\mathbf{\underline{s^2U}^LC^MU^MA^MC^MC^MA^M}$ | $\mathbf{\underline{A}}$GAUGGU | 58.8 ± 3.2 | 161.7 ± 9.9 | 8.65 ± 0.13 | 48.6 | 53.9 ± 1.7 | 146.6 ± 5.2 | 8.51 ± 0.05 | 48.8 | −0.87 | 4.3 | −0.87 | 0 |
| $\mathbf{U^LC^MU^MA^MC^MC^MA^M}$ | $\mathbf{\underline{G}}$GAUGGU | 48.1 ± 4.7 | 132.5 ± 15.0 | 7.03 ± 0.10 | 40.3 | 43.3 ± 1.4 | 117.1 ± 4.7 | 7.01 ± 0.02 | 40.5 | 0.63 | −4.0 | *0* | *0.63* |
| $\mathbf{\underline{s^2U}^LC^MU^MA^MC^MC^MA^M}$ | $\mathbf{\underline{G}}$GAUGGU | 47.9 ± 8.7 | 130.2 ± 27.6 | 7.55 ± 0.16 | 43.7 | 44.2 ± 4.4 | 118.6 ± 14.2 | 7.45 ± 0.18 | 43.6 | −0.44 | −0.9 | *−0.44* | 1.06 |
| $\mathbf{A^MC^MU^MA^MC^MC^MU^M}$[d] | UGAUGG$\mathbf{\underline{A}}$[d] | 59.6 ± 5.7 | 168.4 ± 18.9 | 7.41 ± 0.28 | 41.2 | 62.2 ± 7.3 | 176.8 ± 23.6 | 7.37 ± 0.26 | 41.2 | 0 | 0 | 0 | *0* |
| $\mathbf{A^MC^MU^MA^MC^MC^M\underline{s^2U^M}}$ | UGAUGG$\mathbf{\underline{A}}$ | 49.9 ± 5.9 | 137.7 ± 18.5 | 7.28 ± 0.19 | 41.7 | 44.1 ± 0.8 | 119.1 ± 2.7 | 7.20 ± 0.01 | 41.8 | 0.17 | 0.6 | 0.17 | 0 |
| $\mathbf{A^MC^MU^MA^MC^MC^MU^M}$ | UGAUGG$\mathbf{\underline{G}}$ | 48.6 ± 4.0 | 134.2 ± 12.5 | 6.96 ± 0.11 | 39.8 | 44.4 ± 1.5 | 121.0 ± 5.0 | 6.93 ± 0.03 | 39.8 | 0.44 | −1.4 | *0* | *0.44* |
| $\mathbf{A^MC^MU^MA^MC^MC^M\underline{s^2U^M}}$ | UGAUGG$\mathbf{\underline{G}}$ | 45.2 ± 7.4 | 123.6 ± 23.3 | 6.84 ± 0.15 | 39.1 | 41.7 ± 0.8 | 112.5 ± 2.5 | 6.78 ± 0.01 | 38.9 | 0.59 | −2.3 | *0.15* | 0.42 |
| $\mathbf{A^MC^MU^MA^MC^MC^MU^L}$[d] | UGAUGG$\mathbf{\underline{A}}$[d] | 59.2 ± 5.2 | 167.7 ± 17.2 | 7.19 ± 0.17 | 40.5 | 55.6 ± 2.6 | 155.8 ± 8.5 | 7.24 ± 0.04 | 41.0 | 0 | 0 | 0 | *0* |
| $\mathbf{A^MC^MU^MA^MC^MC^M\underline{s^2U^L}}$ | UGAUGG$\mathbf{\underline{A}}$ | 54.5 ± 3.3 | 150.3 ± 10.3 | 7.92 ± 0.15 | 45.1 | 48.4 ± 1.2 | 130.9 ± 3.9 | 7.84 ± 0.02 | 45.6 | −0.60 | 4.6 | −0.60 | 0 |
| $\mathbf{A^MC^MU^MA^MC^MC^MU^L}$[d] | UGAUGG$\mathbf{\underline{G}}$[d] | 57.6 ± 11.2 | 161.3 ± 35.9 | 7.52 ± 0.29 | 42.4 | 58.4 ± 9.0 | 164.6 ± 29.0 | 7.38 ± 0.38 | 41.6 | −0.14 | 0.6 | *0* | *−0.14* |
| $\mathbf{A^MC^MU^MA^MC^MC^M\underline{s^2U^L}}$ | UGAUGG$\mathbf{\underline{G}}$ | 50.6 ± 3.1 | 139.3 ± 9.5 | 7.44 ± 0.16 | 42.7 | 44.7 ± 1.9 | 120.4 ± 6.2 | 7.40 ± 0.33 | 43.1 | −0.02 | 2.1 | *−0.02* | 0.44 |
| $\mathbf{A^MC^MU^M\underline{U^M}G^MC^MA^M}$ | UGA$\mathbf{\underline{A}}$CGU | 61.2 ± 3.3 | 172.4 ± 10.0 | 7.71 ± 0.15 | 43.1 | 52.7 ± 0.7 | 145.3 ± 2.3 | 7.59 ± 0.01 | 43.4 | 0 | 0 | 0 | *0* |
| $\mathbf{A^MC^MU^M\underline{s^2U^M}G^MC^MA^M}$ | UGA$\mathbf{\underline{A}}$CGU | 60.9 ± 5.3 | 167.4 ± 16.3 | 8.97 ± 0.22 | 50.0 | 62.4 ± 5.2 | 172.4 ± 16.3 | 9.00 ± 0.17 | 49.8 | −1.41 | 6.4 | −1.41 | 0 |
| $\mathbf{A^MC^MU^M\underline{U^M}G^MC^MA^M}$[d] | UGA$\mathbf{\underline{G}}$CGU[d] | 62.5 ± 4.1 | 186.3 ± 13.5 | 4.76 ± 0.17 | 28.4 | 66.4 ± 11.1 | 199.2 ± 37.2 | 4.60 ± 0.58 | 28.2 | 2.99 | −15.2 | *0* | *2.99* |
| $\mathbf{A^MC^MU^M\underline{s^2U^M}G^MC^MA^M}$ | UGA$\mathbf{\underline{G}}$CGU | 46.2 ± 2.8 | 132.2 ± 9.4 | 5.16 ± 0.16 | 28.1 | 46.6 ± 1.8 | 133.7 ± 6.1 | 5.17 ± 0.09 | 28.2 | 2.42 | −15.2 | *−0.57* | 3.83 |
| $\mathbf{A^MC^MU^M\underline{U^L}G^MC^MA^M}$[d] | UGA$\mathbf{\underline{A}}$CGU[d] | 61.0 ± 2.3 | 167.7 ± 7.4 | 9.00 ± 0.12 | 50.1 | 59.5 ± 1.6 | 163.1 ± 5.0 | 8.96 ± 0.05 | 50.2 | 0 | 0 | 0 | *0* |
| $\mathbf{A^MC^MU^M\underline{s^2U^L}G^MC^MA^M}$ | UGA$\mathbf{\underline{A}}$CGU | 59.6 ± 7.0 | 158.3 ± 21.1 | 10.48 ± 0.43 | 59.0 | 59.1 ± 1.4 | 157.0 ± 4.2 | 10.40 ± 0.07 | 58.7 | −1.44 | 8.5 | −1.44 | 0 |
| $\mathbf{A^MC^MU^M\underline{U^L}G^MC^MA^M}$[d] | UGA$\mathbf{\underline{G}}$CGU[d] | 60.8 ± 11.0 | 174.2 ± 35.5 | 6.78 ± 0.19 | 38.3 | 55.9 ± 4.7 | 158.3 ± 15.5 | 6.76 ± 0.13 | 38.3 | 2.20 | −11.9 | *0* | *2.20* |
| $\mathbf{A^MC^MU^M\underline{s^2U^L}G^MC^MA^M}$ | UGA$\mathbf{\underline{G}}$CGU | 35.2 ± 3.6 | 91.9 ± 11.3 | 6.71 ± 0.18 | 38.6 | 38.7 ± 5.8 | 103.3 ± 18.8 | 6.64 ± 0.31 | 37.9 | 2.32 | −12.3 | *0.12* | 3.76 |
| $\mathbf{G^M\underline{U^M}U^MA^MC^MC^MA^M}$ | C$\mathbf{\underline{A}}$AUGGU | 55.3 ± 3.5 | 156.0 ± 11.1 | 6.88 ± 0.10 | 39.0 | 49.9 ± 1.2 | 138.6 ± 3.9 | 6.85 ± 0.01 | 39.0 | 0 | 0 | 0 | *0* |
| $\mathbf{G^M\underline{s^2U^M}U^MA^MC^MC^MA^M}$ | C$\mathbf{\underline{A}}$AUGGU | 54.2 ± 3.8 | 150.4 ± 11.8 | 7.60 ± 0.13 | 43.2 | 48.8 ± 0.7 | 133.1 ± 2.3 | 7.51 ± 0.01 | 43.4 | −0.66 | 4.4 | −0.66 | 0 |
| $\mathbf{G^M\underline{U^M}U^MA^MC^MC^MA^M}$ | C$\mathbf{\underline{G}}$AUGGU | 47.4 ± 4.4 | 136.8 ± 14.4 | 4.97 ± 0.17 | 27.1 | 45.8 ± 4.8 | 131.5 ± 16.3 | 5.06 ± 0.27 | 27.3 | 1.79 | −11.7 | *0* | *1.79* |
| $\mathbf{G^M\underline{s^2U^M}U^MA^MC^MC^MA^M}$ | C$\mathbf{\underline{G}}$AUGGU | 41.8 ± 5.2 | 119.6 ± 17.3 | 4.71 ± 0.34 | 24.1 | 41.5 ± 5.3 | 118.3 ± 17.9 | 4.76 ± 0.39 | 24.3 | 2.09 | −14.7 | *0.30* | 2.75 |
| $\mathbf{G^M\underline{U^L}U^MA^MC^MC^MA^M}$ | C$\mathbf{\underline{A}}$AUGGU | 58.4 ± 3.1 | 161.6 ± 9.4 | 8.27 ± 0.16 | 46.5 | 53.7 ± 2.0 | 146.7 ± 6.4 | 8.15 ± 0.05 | 46.7 | 0 | 0 | 0 | *0* |
| $\mathbf{G^M\underline{s^2U^L}U^MA^MC^MC^MA^M}$ | C$\mathbf{\underline{A}}$AUGGU | 55.2 ± 3.7 | 149.6 ± 11.3 | 8.82 ± 0.22 | 50.4 | 50.9 ± 1.1 | 136.3 ± 3.6 | 8.67 ± 0.03 | 50.6 | −0.52 | 3.9 | −0.52 | 0 |
| $\mathbf{G^M\underline{U^L}U^MA^MC^MC^MA^M}$ | C$\mathbf{\underline{G}}$AUGGU | 51.7 ± 3.1 | 146.4 ± 9.8 | 6.29 ± 0.16 | 35.5 | 47.0 ± 2.4 | 131.1 ± 8.0 | 6.35 ± 0.06 | 35.8 | 1.80 | −10.9 | *0* | *1.80* |
| $\mathbf{G^M\underline{s^2U^L}U^MA^MC^MC^MA^M}$ | C$\mathbf{\underline{G}}$AUGGU | 41.7 ± 2.9 | 115.1 ± 9.2 | 5.98 ± 0.08 | 33.0 | 40.1 ± 1.4 | 109.9 ± 4.6 | 6.01 ± 0.05 | 33.0 | 2.14 | −13.7 | *0.34* | 2.66 |

[a]Solutions are 100 mM NaCl, 20 mM sodium cacodylate, and 0.5 mM $Na_2EDTA$, pH 7. [b]Calculated for $10^{-4}$ M total strand concentration. [c]Data with the same character of fonts belong to the same set. [d]Data from ref *16*. The data in parentheses indicate non-two-state melting.

Table 2: Thermodynamic Parameters of Helix Formation with RNA and 2′-*O*-Me Oligoribonucleotides: Effect of LNA-2-thiouridine in the Presence of 3′-Pyrene[a]

| RNA duplexes | | average of curve fits | | | | $T_M^{-1}$ vs log $C_T$ plots | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5′−3′ | 3′−5′ | $-\Delta H°$ (kcal/mol) | $-\Delta S°$ (eu) | $-\Delta G°_{37}$ (kcal/mol) | $T_M$ (°C) | $-\Delta H°$ (kcal/mol) | $-\Delta S°$ (eu) | $-\Delta G°_{37}$ (kcal/mol) | $T_M$ (°C) | $\Delta\Delta G°_{37}$ (kcal/mol) | $\Delta T_M$ (°C) | $\Delta\Delta G°_{37}$ (kcal/mol) | $\Delta\Delta G°_{37}$ (kcal/mol) |
| **U**$^{L}$G$^{M}$U$^{M}$G$^{M}$U$^{M}$pyrene | G**A**CACAG | 48.6 ± 3.1 | 133.0 ± 9.9 | 7.37 ± 0.10 | 42.4 | 43.9 ± 1.6 | 117.8 ± 5.2 | 7.35 ± 0.02 | 42.9 | 0 | 0 | 0 | *0* |
| **s$^{2}$U**$^{L}$G$^{M}$U$^{M}$G$^{M}$U$^{M}$pyrene | G**A**CACAG | 47.9 ± 2.1 | 128.0 ± 6.6 | 8.20 ± 0.08 | 48.2 | 46.0 ± 1.1 | 122.0 ± 3.5 | 8.12 ± 0.03 | 48.1 | −0.77 | 5.2 | −0.77 | 0 |
| **U**$^{L}$G$^{M}$U$^{M}$G$^{M}$U$^{M}$pyrene | G**G**CACAG | 38.2 ± 3.1 | 104.9 ± 10.3 | 5.67 ± 0.10 | 30.2 | 38.8 ± 2.2 | 106.7 ± 7.6 | 5.68 ± 0.11 | 30.3 | 1.67 | −12.6 | *0* | *1.67* |
| **s$^{2}$U**$^{L}$G$^{M}$U$^{M}$G$^{M}$U$^{M}$pyrene | G**G**CACAG | 35.7 ± 8.9 | 94.9 ± 30.3 | 6.28 ± 0.61 | 34.8 | 36.9 ± 4.2 | 99.3 ± 13.8 | 6.12 ± 0.24 | 33.6 | 1.23 | −9.3 | *−0.44* | 2.00 |
| U$^{M}$G$^{M}$U$^{M}$G$^{M}$**U**$^{L}$pyrene | GACAC**A**G | 41.6 ± 2.9 | 111.3 ± 9.1 | 7.05 ± 0.10 | 40.9 | 41.8 ± 2.3 | 112.1 ± 7.4 | 7.03 ± 0.05 | 40.7 | 0 | 0 | 0 | *0* |
| U$^{M}$G$^{M}$U$^{M}$G$^{M}$**s$^{2}$U**$^{L}$pyrene | GACAC**A**G | 44.0 ± 2.3 | 117.4 ± 7.2 | 7.60 ± 0.13 | 44.7 | 40.0 ± 0.6 | 104.7 ± 2.0 | 7.54 ± 0.01 | 45.0 | −0.51 | 4.3 | −0.51 | 0 |
| U$^{M}$G$^{M}$U$^{M}$G$^{M}$**U**$^{L}$pyrene | GACAC**G**G | 44.3 ± 2.2 | 122.4 ± 7.1 | 6.33 ± 0.09 | 35.6 | 40.3 ± 1.5 | 109.3 ± 5.1 | 6.42 ± 0.05 | 36.1 | 0.61 | −4.6 | *0* | 0.61 |
| U$^{M}$G$^{M}$U$^{M}$G$^{M}$**s$^{2}$U**$^{L}$pyrene | GACAC**G**G | 42.3 ± 5.0 | 115.8 ± 16.3 | 6.37 ± 0.17 | 35.8 | 47.7 ± 2.1 | 133.6 ± 7.0 | 6.25 ± 0.06 | 35.2 | 0.78 | −5.5 | *0.17* | 1.29 |
| C$^{M}$U$^{M}$**U**$^{L}$G$^{M}$C$^{M}$pyrene | UGA**A**CGU | 54.8 ± 5.9 | 145.4 ± 17.9 | 9.71 ± 0.32 | 56.1 | 51.1 ± 1.2 | 134.2 ± 3.7 | 9.52 ± 0.06 | 56.3 | 0 | 0 | 0 | *0* |
| C$^{M}$U$^{M}$**s$^{2}$U**$^{L}$G$^{M}$C$^{M}$pyrene | UGA**A**CGU | 57.7 ± 4.0 | 150.5 ± 11.7 | 11.05 ± 0.34 | 63.4 | 57.0 ± 3.2 | 148.5 ± 9.5 | 10.97 ± 0.23 | 63.2 | −1.45 | 6.9 | −1.45 | 0 |
| C$^{M}$U$^{M}$**U**$^{L}$G$^{M}$C$^{M}$pyrene | UGA**G**CGU | 48.9 ± 2.6 | 134.4 ± 8.3 | 7.20 ± 0.10 | 41.3 | 45.6 ± 1.1 | 124.1 ± 3.4 | 7.18 ± 0.01 | 41.5 | 2.34 | −14.8 | *0* | *2.34* |
| C$^{M}$U$^{M}$**s$^{2}$U**$^{L}$G$^{M}$C$^{M}$pyrene | UGA**G**CGU | 34.0 ± 4.0 | 85.4 ± 12.1 | 7.51 ± 0.38 | 46.2 | 34.2 ± 5.7 | 86.1 ± 18.2 | 7.49 ± 0.45 | 46.0 | 2.03 | −10.3 | *−0.31* | 3.48 |
| U$^{M}$G$^{M}$**U**$^{L}$G$^{M}$U$^{M}$pyrene | GAC**A**CAG | 47.0 ± 2.8 | 125.8 ± 8.6 | 7.97 ± 0.13 | 46.8 | 43.0 ± 1.3 | 113.2 ± 4.2 | 7.90 ± 0.02 | 47.2 | 0 | 0 | 0 | *0* |
| U$^{M}$G$^{M}$**s$^{2}$U**$^{L}$G$^{M}$U$^{M}$pyrene | GAC**A**CAG | 49.3 ± 6.1 | 130.2 ± 18.8 | 8.88 ± 0.28 | 52.5 | 49.6 ± 3.9 | 131.2 ± 12.1 | 8.87 ± 0.16 | 52.3 | −0.97 | 5.1 | −0.97 | 0 |
| U$^{M}$G$^{M}$**U**$^{L}$G$^{M}$U$^{M}$pyrene | GAC**G**CAG | 43.0 ± 5.2 | 120.7 ± 17.3 | 5.57 ± 0.21 | 30.2 | 44.4 ± 2.7 | 125.2 ± 9.0 | 5.59 ± 0.11 | 30.6 | 2.31 | −16.6 | *0* | *2.31* |
| U$^{M}$G$^{M}$**s$^{2}$U**$^{L}$G$^{M}$U$^{M}$pyrene | GAC**G**CAG | 42.3 ± 5.8 | 117.9 ± 19.9 | 5.76 ± 0.42 | 31.4 | 44.1 ± 6.5 | 124.0 ± 21.7 | 5.66 ± 0.41 | 31.0 | 2.24 | 16.2 | *−0.07* | 3.21 |
| U$^{M}$G$^{M}$**U**$^{L}$G$^{M}$**U**$^{L}$pyrene | GAC**A**C**A**G | 45.8 ± 1.4 | 119.6 ± 4.4 | 8.75 ± 0.08 | 52.8 | 45.0 ± 0.9 | 117.1 ± 2.8 | 8.71 ± 0.03 | 52.8 | 0 | 0 | 0 | *0* |
| U$^{M}$G$^{M}$**s$^{2}$U**$^{L}$G$^{M}$**s$^{2}$U**$^{L}$pyrene | GAC**A**C**A**G | 49.3 ± 4.0 | 126.3 ± 12.1 | 10.10 ± 0.29 | 61.2 | 50.1 ± 2.2 | 128.8 ± 6.7 | 10.12 ± 0.13 | 60.9 | −1.41 | 8.1 | −1.41 | 0 |
| U$^{M}$G$^{M}$**U**$^{L}$G$^{M}$**U**$^{L}$pyrene | GAC**G**C**G**G | 53.4 ± 4.8 | 152.4 ± 15.9 | 6.14 ± 0.17 | 34.7 | 50.8 ± 2.9 | 144.0 ± 9.4 | 6.21 ± 0.07 | 35.0 | 2.50 | −17.8 | *0* | *2.50* |
| U$^{M}$G$^{M}$**s$^{2}$U**$^{L}$G$^{M}$**s$^{2}$U**$^{L}$pyrene | GAC**G**C**G**G | 44.2 ± 4.9 | 115.5 ± 15.0 | 8.36 ± 0.28 | 50.4 | 43.3 ± 5.3 | 113.0 ± 16.3 | 8.28 ± 0.31 | 50.1 | 0.43 | −2.7 | *−2.07* | 1.84 |
| C$^{M}$**U**$^{L}$A$^{M}$**U**$^{L}$C$^{M}$pyrene | UGA**U**AGU | 54.9 ± 2.5 | 148.6 ± 7.6 | 8.83 ± 0.14 | 50.6 | 55.8 ± 2.8 | 151.5 ± 8.8 | 8.85 ± 0.10 | 50.5 | 0 | 0 | 0 | *0* |
| C$^{M}$**s$^{2}$U**$^{L}$A$^{M}$**s$^{2}$U**$^{L}$C$^{M}$pyrene | UGA**U**AGU | 53.1 ± 3.3 | 138.4 ± 9.9 | 10.19 ± 0.23 | 60.0 | 51.1 ± 2.0 | 132.3 ± 6.2 | 10.03 ± 0.12 | 59.8 | −1.18 | 9.3 | −1.18 | 0 |
| C$^{M}$**U**$^{L}$A$^{M}$**U**$^{L}$C$^{M}$pyrene | UG**G**U**G**GU | 48.5 ± 6.3 | 142.3 ± 21.2 | 4.37 ± 0.27 | 23.8 | 44.6 ± 5.3 | 129.2 ± 18.1 | 4.57 ± 0.36 | 24.0 | 4.28 | −26.5 | *0* | *4.28* |
| C$^{M}$**s$^{2}$U**$^{L}$A$^{M}$**s$^{2}$U**$^{L}$C$^{M}$pyrene | UG**G**U**G**GU | 25.4 ± 5.1 | 61.0 ± 17.1 | 6.51 ± 0.69 | 36.7 | 28.4 ± 11.6 | 70.6 ± 37.2 | 6.55 ± 1.76 | 37.2 | 2.30 | −13.3 | *−1.98* | 3.48 |
| C$^{M}$**U**$^{L}$A$^{M}$**U**$^{L}$C$^{M}$pyrene | UGA**U**G**G**U | 48.4 ± 4.3 | 133.8 ± 13.7 | 6.94 ± 0.12 | 39.7 | 47.5 ± 2.5 | 130.6 ± 8.3 | 6.95 ± 0.06 | 39.7 | 0 | 0 | 0 | *0* |
| C$^{M}$**s$^{2}$U**$^{L}$A$^{M}$**s$^{2}$U**$^{L}$C$^{M}$pyrene | UGA**U**G**G**U | 40.7 ± 2.5 | 109.5 ± 8.1 | 6.74 ± 0.10 | 38.6 | 41.3 ± 1.6 | 111.6 ± 5.2 | 6.70 ± 0.04 | 38.3 | 0.25 | −1.4 | 0.25 | 0 |
| C$^{M}$**U**$^{L}$A$^{M}$**U**$^{L}$C$^{M}$pyrene | UG**G**U**A**GU | 47.0 ± 4.6 | 132.1 ± 15.2 | 6.02 ± 0.19 | 33.6 | 45.5 ± 3.4 | 127.1 ± 11.2 | 6.08 ± 0.13 | 34.0 | 0.87 | −5.7 | *0* | *0.87* |
| C$^{M}$**s$^{2}$U**$^{L}$A$^{M}$**s$^{2}$U**$^{L}$**C**$^{M}$pyrene | UG**G**U**A**GU | 38.7 ± 4.8 | 105.3 ± 15.5 | 6.06 ± 0.15 | 33.3 | 41.2 ± 5.7 | 113.3 ± 18.6 | 6.03 ± 0.29 | 33.3 | 0.92 | −6.4 | *0.05* | 0.67 |

[a]Solutions are 100 mM NaCl, 20 mM sodium cacodylate, and 0.5 mM $Na_2EDTA$, pH 7. [b]Calculated for $10^{-4}$ M total strand concentration. [c]The data with the same character of fonts belong to the same set.

0.97 kcal/mol. When $U^L$ and $s^2U^L$ paired to G in 5′$U^MG^M$-$\underline{Y}G^MU^M$pyrene/3′UAC$\underline{G}$CAU, free energies of the duplexes were −5.59 and −5.66 kcal/mol, respectively, so enhancement was only 0.07 kcal/mol.

Placing of $s^2U^M$ and $s^2U^L$ at the 5′-penultimate position within 5′$G^M\underline{Y}U^MA^MC^MC^MA^M$/3′C$\underline{R}$AUGGU duplexes resulted in a similar range of thermodynamic stability changes. For 5′$G^M\underline{Y}U^MA^MC^MC^MA^M$/3′C$\underline{A}$AUGGU duplexes, the thermodynamic stabilities were −6.85, −7.51, −8.15, and −8.67 kcal/mol for Y equal to $U^M$, $s^2U^M$, $U^L$, and $s^2U^L$, respectively. When the same nucleotides paired with G in duplexes, 5′$G^M\underline{Y}U^MA^MC^MC^MA^M$/3′C$\underline{G}$AUGGU, the thermodynamic stabilities were −5.06, −4.76, −6.35, and −6.01 kcal/mol, respectively. Thus, replacing $U^M$ with $s^2U^M$ and $U^L$ with $s^2U^L$ enhanced A-U base pair interaction by 0.66 and 0.52 kcal/mol, respectively, but diminished G-U pair interaction by 0.30 and 0.34 kcal/mol, respectively.

*Influence of Multiple Substitutions of 2′-O-Methyl-2-thiouridine and LNA-2-thiouridine at Internal Positions*. It is often proposed to place more than one $s^2U^L$ within the same duplex. Thus, several 5′$U^MG^M\underline{Y}G^M\underline{Y}$pyrene/3′GAC$\underline{R}$C$\underline{R}$G and 5′$C^M\underline{Y}A^M\underline{Y}C^M$pyrene/3′UG$\underline{R}$U$\underline{R}$GU duplexes were studied (Table 2).

In the set of 5′$U^MG^M\underline{Y}G^M\underline{Y}$pyrene/3′GAC$\underline{A}$C$\underline{A}$G duplexes, the thermodynamic stabilities were −8.71 and −10.12 kcal/mol for Y equal to $U^L$ and $s^2U^L$, respectively, so the enhancement ($\Delta\Delta G°_{37}$) due to presence of 2-thio derivatives was 1.41 kcal/mol for two A-U pairs. For the duplexes, 5′$U^MG^M\underline{Y}G^M\underline{Y}$pyrene/3′GAC$\underline{G}$C$\underline{G}$G, with two G-U pairs the free energies were −6.21 and −8.28 kcal/mol, respectively, so stability was enhanced by 2.07 kcal/mol. For the set of duplexes, 5′$C^M\underline{Y}A^M\underline{Y}C^M$pyrene/3′UG$\underline{A}$U$\underline{A}$GU, the thermodynamic stabilities were −8.85 and −10.03 kcal/mol for Y equal to $U^L$ and $s^2U^L$, respectively. The increase in stability was equal to 1.18 kcal/mol for two A-U pairs. For the duplexes, 5′$C^M\underline{Y}A^M\underline{Y}C^M$pyrene/3′UG$\underline{G}$U$\underline{G}$GU, the free energies were −4.57 and −6.55 kcal/mol, respectively, so stability was increased by 1.98 kcal/mol. The $\Delta H°$ values for 5′$C^M\underline{s^2U^L}A^M\underline{s^2U^L}C^M$pyrene/3′UG$\underline{G}$U$\underline{G}$GU, however, are small, which indicates unusual melting.

Finally, the duplexes, 5′$C^M\underline{Y}A^M\underline{Y}C^M$pyrene/3′UG$\underline{R}$U$\underline{R}$GU, were designed so that one Y pairs with A and the other Y pairs with G. The thermodynamic stabilities of 5′$C^M\underline{Y}A^M\underline{Y}C^M$-pyrene/3′UG$\underline{A}$U$\underline{G}$GU were −6.95 and −6.70 kcal/mol for Y equal to $U^L$ and $s^2U^L$, respectively, so replacement of $U^L$ with $s^2U^L$ destabilized the duplex by 0.25 kcal/mol. When positions of matched and mismatched base pairs were inverted to give 5′$C^M\underline{Y}A^M\underline{Y}C^M$pyrene/3′UG$\underline{G}$U$\underline{A}$GU, the free energies were −6.08 and −6.03 kcal/mol, so there was negligible difference in the thermodynamic stability.

## DISCUSSION

The 2-thio-modified RNA nucleotides are relatively common in tRNA and important for biological functions (*1*, *6*, *7*, *9*, *10*, *46*, *47*). The presence of a sulfur instead of oxygen at position 2 of a pyrimidine residue results in predominantly C3′-endo ribose conformation (*2*, *3*) and an increase of thermodynamic stabilities of complementary duplexes (*4*, *48*−*50*).

A 2-thiouridine within an oligoribonucleotide enhances matched binding to A but diminishes mismatched binding to G in a target RNA (*4*, *48*, *49*). In RNA, the thermodynamic stabilities of unmodified A-U and G-U base pairs are very similar, so replacing U with $s^2U$ increases selectivity of binding (*4*). This is also observed with 2′-*O*-methyl-2-thiouridine

Table 3: Differences in Free Energies ($\Delta G°_{37}$ in kcal/mol) Resulting from Substitution of $U^M$ to $s^2U^M$, $U^M$ to $s^2U^L$, and $U^L$ to $s^2U^L$ at Particular Positions within 2′-OMeRNA/RNA and LNA-2′-OMeRNA/RNA Duplexes

| | 5′-terminal | | | 3′-terminal | | | central | | | 5′-penultimate | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $U^M\rightarrow s^2U^M$ ($U^M\rightarrow U^L$) | $U^M\rightarrow s^2U^L$ | $U^L\rightarrow s^2U^L$ | $U^M\rightarrow s^2U^M$ ($U^M\rightarrow U^L$) | $U^M\rightarrow s^2U^L$ | $U^L\rightarrow s^2U^L$ | $U^M\rightarrow s^2U^M$ ($U^M\rightarrow U^L$) | $U^M\rightarrow s^2U^L$ | $U^L\rightarrow s^2U^L$ | $U^M\rightarrow s^2U^M$ ($U^M\rightarrow U^L$) | $U^M\rightarrow s^2U^L$ | $U^L\rightarrow s^2U^L$ |
| $\Delta\Delta G°_{37}$ A-Y | −0.99 (−0.46) | −1.33 | −0.87 | 0.17 (0.13) | −0.47 | −0.60 | −1.41 (−1.37) | −2.81 | −1.44 | −0.66 (−1.30) | −1.82 | −0.52 |
| $\Delta\Delta G°_{37}$ G-Y | −0.24 (−0.22) | −0.66 | −0.44 | 0.59 (−0.45) | −0.47 | −0.02 | −0.57 (−2.16) | −2.04 | 0.12 | 0.30 (−1.29) | −0.95 | 0.34 |
| $\Delta\Delta\Delta G°_{37}$ (A-Y) − (G-Y) | −0.25 (−0.24) | −0.67 | −0.43 | −0.42 (0.58) | 0.00 | −0.58 | −0.84 (0.79) | −0.77 | −1.56 | −0.96 (−0.01) | −0.87 | −0.86 |

(*43*, *48*, *49*). LNA forms thermodynamically very stable duplexes with RNA, 2′-OMeRNA, and DNA strands (*12*, *16*−*18*, *51*, *52*). Thus, it is potentially useful for incorporation in probes and therapeutics (*14*, *19*−*22*, *53*−*56*). For various applications, additional modifications of LNA nucleosides have been introduced (*56*−*60*). Many applications would benefit from enhanced specificity for binding A over G, which led us to develop a chemical synthesis of LNA-2-thiouridine.

To measure the influence of $s^2U^L$ on thermodynamic stabilities, several LNA-2′-OMeRNA/RNA duplexes were studied. For comparison, isosequential duplexes carrying 2′-*O*-methyluridine, 2′-*O*-methyl-2-thiouridine, and LNA-uridine were measured to evaluate the effects of 2-thio and LNA substitution on thermodynamic stability and selectivity of base pairing.

The position of a modified nucleotide within a duplex affects its thermodynamic stability (*16*, *17*, *61*). For this reason, $s^2U^M$ and $s^2U^L$ were placed at 5′- and 3′-terminal, central internal, and 5′-penultimate positions in primarily 2′-*O*-methyl oligonucleotides. Thermodynamic properties of 2′-O-methylated oligonucleotides with 3′-terminal pyrene were also studied (*45*). Both types of oligonucleotides are useful for isoenergetic microarrays (*19*−*23*).

*Influence of LNA Rings on Enhancement of the Thermodynamic Stabilities of 2-Thiouracil Nucleotides*. LNA ribose enforces C3′-endo conformation and enhances the thermodynamic stability of RNA, 2′-OMeRNA, and DNA duplexes (*12*, *13*). Stacking and single strand preorganization are mostly responsible for this enhancement of thermodynamic stability (*52*, *62*). The increase of thermodynamic stability with $s^2U^L$ is also dependent on position within the duplex. The largest increase was found at internal positions whereas shifting the LNA nucleotide to the end of a duplex resulted in less enhancement of thermodynamic stability. For the LNA-2′-OMeRNA/RNA duplexes studied here, changing 2′-*O*-methylribose to LNA enhanced duplex stability by 0.84, 0.64, 1.40, and 1.16 kcal/mol for A-$s^2U^L$ present at 5′-terminal, 3′-terminal, central, and 5′-penultimate positions, respectively (Table 1). This compares with the average enhancements for LNA vs 2′-OMe of 0.53, 0.14, and 1.28 kcal/mol for 5′-terminal, 3′-terminal, and internal positions, respectively (*16*).

*Influence of 2-Thiouracil Nucleotide on the Selectivity of Binding to Adenosine and Guanosine in a Complementary RNA Strand*. A major goal is to enhance selectivity for binding of uridine to adenosine over guanosine. Previous studies showed that 2-thiouridine provides greater selectivity than U (*4*). For RNA/RNA duplexes with U at the central position, the difference between A-U and G-U increased by 1.45 kcal/mol when A-$s^2U$ and G-$s^2U$ substitutions were made. This corresponded to an increase of 8.0 °C in the difference between melting temperatures (*4*). Sekine et al. reported that replacing U with $s^2U$ and $s^2U^M$ in RNA duplexes increased the difference in melting temperature by 10.8 and 11.8 °C, respectively (*48*). When the same RNA strands were bound to a DNA strand, the increases in melting temperature were 7.3 and 11.2 °C due to replacement of U with $s^2U$ and $s^2U^M$, respectively. That suggested that changing 2′-*O*-methylribose to the LNA ring might further increase A-U over G-U binding selectivity (*48*).

The enhancement of the selectivity [$(\Delta\Delta\Delta G°_{37}$ (A-$s^2U^M$) − (G-$s^2U^M$)] of $s^2U^M$ over $U^M$ binding was equal to 0.25, 0.42, 0.84, and 0.96 kcal/mol for 5′-terminal, 3′-terminal, central, and 5′-penultimate positions, respectively (Table 3). Replacement of $U^M$ with $s^2U^L$ increased the selectivity of binding to A over G [$(\Delta\Delta\Delta G°_{37}$ (A-$s^2U^L$) − (G-$s^2U^L$)] by 0.67, 0.00, 0.77, and 0.87 kcal/mol, respectively (Table 3). Substitution of $s^2U^L$ for $s^2U^M$ at the central position has little effect on selectivity but a large affect on thermodynamic stability (Table 3).

For LNA-2′-OMeRNApyrene/RNA duplexes, the enhancement of selectivity for binding of $U^L$ versus $s^2U^L$ to A versus G [$\Delta\Delta\Delta G°_{37}$ (A-$s^2U^L$) − (G-$s^2U^L$)] was equal to 0.33, 0.68, 1.14, and 0.90 kcal/mol for 5′- and 3′-terminal and two central positions, respectively (Table 4). Thus, replacement of 2-oxo with 2-thio nucleotide improved selectivity most strongly at internal positions within the duplex. Surprisingly, LNA-2′-OMeRNApyrene/RNA duplexes with double substitution of $U^L$ with $s^2U^L$ provided less specificity than duplexes with $U^L$ (Table 4). It could be because an LNA nucleotide enforces its 3′-adjacent nucleotide to adopt predominantly C3′-endo conformation as well. The differences in electronegativity between sulfur and oxygen (one unit) could result in different stacking interactions of A-$U^L$ and G-$U^L$ in comparison to A-$s^2U^L$ and G-$s^2U^L$ in both single strand and duplex.

*Effects of 2-Thiouracil and LNA Substitution Are Not Always Additive*. Table 3 gives the differences in $\Delta G°_{37}$ for substituting $s^2U^M$ for $U^M$, $U^L$ for $U^M$ (in parentheses), and $s^2U^L$ for $U^M$. In most but not all cases, the sum of the effects of the first two substitutions is close to the effect of the latter substitution. This is least true at the 3′-terminal and central positions.

*Recommendations for Probe Design*. Thermodynamic stability and specificity are important considerations when designing probes and therapeutics. Uridine provides both low stability and specificity, but modifications can enhance both characteristics. Previous work has shown that $U^M$ to $U^L$ substitution has a large effect on stability when inside a duplex (*16*, *17*). Moreover, $U^M$ to $U^L$ substitutions anywhere have either negligible or negative effect on specificity for A-U vs G-U (*16*). The summaries in Tables 3 and 4 provide insight into optimal design of probes with $s^2U$. Substitution of U with $s^2U$ is most effective at enhancing both stability and specificity when a single substitution is inside the duplex. The maximum observed enhancements in binding and specificity from substituting $s^2U^L$ for

Table 4: Differences in Free Energies ($\Delta G°_{37}$ in kcal/mol) Resulting from Substitution of $U^L$ to $s^2U^L$ at Particular Positions within LNA-2′-OMeRNApyrene/RNA Duplexes

| | 5′-terminal | 3′-terminal | central 1 | central 2 | double $s^2U^L$ (1)[a] | double $s^2U^L$ (2)[b] | mixed substitutions |
|---|---|---|---|---|---|---|---|
| $\Delta\Delta G°_{37}$ A-$s^2U^L$ | −0.77 | −0.51 | −1.45 | −0.97 | −1.41 | −1.18 | 0.25[d] |
| $\Delta\Delta G°_{37}$ G-$s^2U^L$ | −0.44 | 0.17 | −0.31 | −0.07 | −2.07 | (−1.98)[c] | 0.05[e] |
| $\Delta\Delta\Delta G°_{37}$ (A-$s^2U^L$) − (G-$s^2U^L$) | −0.33 | −0.68 | −1.14 | −0.90 | 0.66 | 0.80 | |

[a]Sequences are 5′$U^M G^M s^2U^L G^M s^2U^L$pyrene/3′GACRCRG. [b]Sequences are 5′$C^M s^2U^L A^M s^2U^L C^M$pyrene/3′UGRURGU. [c]Values of $\Delta H°$ for 5′$C^M s^2U^L A^M s^2U^L C^M$pyrene/3′UGGUGGU are unusually small. [d]Sequence is 5′$C^M s^2U^L A^M s^2U^L C^M$pyrene/3′UGAUGGU. [e]Sequence is 5′$C^M s^2U^L A^M s^2U^L C^M$pyrene/3′UGGUAGU.

$U^M$ were −2.81 and −0.87 kcal/mol, respectively, which at 37 °C corresponds to more than 90- and 4-fold improvements, respectively, in binding constant and relative binding to A-U vs G-U. The maximum observed enhancements from substitution of $s^2U^M$ for $U^M$ were −1.41 and −0.96 kcal/mol, respectively. Significant enhancements in both binding (−1.33 kcal/mol) and A-U vs G-U specificity (−0.67 kcal/mol) were also observed for substituting $s^2U^L$ for $U^M$ at the 5′-terminal position. Substitution of $s^2U^L$ for $U^M$ at the 3′-terminal position, however, does not appear to be advantageous (Table 3). This could reflect the unusual stacking properties at 3′U on a CG pair, so other sequences may behave differently. Adding pyrene to the 3′-end and flipping the penultimate base pair to GC does provide large discrimination (−1.29 kcal/mol) between A-U and G-U pairs by an adjacent $s^2U^L$ (Table 2). The generalizations are based on the limited number of sequences studied but are consistent with expectation from studies of $U^M$ and $U^L$ in a larger number of sequence contexts (*16*, *17*, *45*). A surprising result, however, is that two $s^2U^L$ for $U^L$ substitutions reduce specificity for A-U vs G-U pairs.

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