2-Thiouracil deprived of thiocarbonyl function preferentially base pairs with guanine rather than adenine in RNA and DNA duplexes

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ABSTRACT

2-Thiouracil-containing nucleosides are essential modified units of natural and synthetic nucleic acids. In particular, the 5-substituted-2-thiouridines (S2Us) present in tRNA play an important role in tuning the translation process through codon–anticodon interactions. The enhanced thermodynamic stability of S2U-containing RNA duplexes and the preferred S2U-A versus S2U-G base pairing are appreciated characteristics of S2U-modified molecular probes. Recently, we have demonstrated that 2-thiouridine (alone or within an RNA chain) is predominantly transformed under oxidative stress conditions to 4-pyrimidinone riboside (H2U) and not to uridine. Due to the important biological functions and various biotechnological applications for sulfur-containing nucleic acids, we compared the thermodynamic stabilities of duplexes containing desulfured products with those of 2-thiouracil-modified RNA and DNA duplexes. Differential scanning calorimetry experiments and theoretical calculations demonstrate that upon 2-thiouracil desulfuration to 4-pyrimidinone, the preferred base pairing of S2U with adenosine is lost, with preferred base pairing with guanosine observed instead. Therefore, biological processes and in vitro assays in which oxidative desulfuration of 2-thiouracil-containing components occurs may be altered. Moreover, we propose that the H2U-G base pair is a suitable model for investigation of the preferred recognition of 3’-G-ending versus A-ending codons by tRNA wobble nucleosides, which may adopt a 4-pyrimidinone-type structural motif.

INTRODUCTION

Sulfur-containing nucleosides constitute a class of nucleosides present in natural nucleic acids (http://rna-mdb.cas.albany.edu; http://modomics.genesilico.pl) (1,2). Among these molecules is a subset of 5-substituted derivatives of 2-thiouridine (S2U) (Figure 1), which are located in the first anticodon position in transfer RNAs (tRNAs) specific for lysine, glutamic acid and glutamine. 2-Thiouridines serve as recognition elements for cognate aminoacyl tRNA synthetases (aa-RS) (10,11) and are used together with other modifications by the innate immune system to distinguish between self and pathogen RNAs (12–14). Furthermore, the thiocarbonyl function of S2U-containing human tRNA_{Lys} is involved in the formation of the initial complex with HIV-1 RNA as a prerequisite for viral RNA reverse transcription (15). Notably, 5-methyl-2-thiouridine (or 2-thioribothymidine) is also located in the TW/C loop of tRNAs of hyperthermophilic species such as Thermus thermophilus (16,17) and Pyrococcus furiosus (18). This nucleoside is thought to enhance the stability of the tertiary structure of tRNA at elevated temperatures. RNA duplexes containing S2U-A base pairs are thermodynamically more stable than those with U-A base pairs due to a preferential S2U C3′-endo sugar ring pucker, which improves base stacking in RNA chains and enhances an overall A-type RNA duplex helical structure (19–26). Furthermore, 2-thiouridines preferentially hybridize with adenosines, whereas wobble pairing with guanosine residues is restricted due to less effective hydrogen bonding between the N(1)H donor of guanine and the sulfur acceptor in 2-thiouracil.

The enhanced thermodynamic stability of S2U-containing RNA duplexes and the preference for S2U-A
versus S2U-G base pairing are appreciated characteristics of S2U-containing molecular probes used in nucleic acids biology and medicinal chemistry. For example, S2U alone or in combination with 2′-O-sugar modifications built into RNA chains increases the silencing activity of short interfering RNAs, rendering these modified duplexes promising tools for RNAi therapy (27–30). RNA probes containing 2′-O-methylated 2-thiouridine (immobilized on glass) effectively discriminate complements with complementary A versus mismatched G residues (31). Even more potent discrimination is achieved by the replacement of 2′-O-methylribose with an Locked Nucleic Acid (LNA) type bicyclic sugar analog (32). 2-Thio-5-methyluridine in combination with 2′-O-[2-(methoxy)ethyl] sugar modifications improves the thermodynamic stability and nuclease resistance of DNA/RNA heteroduplexes composed of modified DNA and complementary RNA (33). Moreover, 2-thiouridine-5′-di- and triphosphates and their analogs were found to be potent and selective agonists of the P2Y2 and P2Y14 receptors (34–36), whereas S2U-RNAs were used in Structure-Activity Relationship (SAR) studies of Toll-like receptors (12,13) and as suppressors of the interferon-inducible protein kinase PKR (14).

Additionally, 2-thio-2′-deoxyuridine (S2dU) and 2-thiopyrimidine (S2T)-labeled DNA oligomers have been widely used in molecular biology, biochemistry and biotechnology. In contrast to S2U, in S2dU and S2T, the sugar rings preferentially adopt C2′-endo puckering, which is present in the parent dU and T nucleosides (21,37). The strength of S2T-A base pairing is virtually identical to that in a T-A system (38–40). Studies on the fidelity of DNA replication conducted using an S2T-modified template confirmed the discrimination between complements containing A and those in which complementary A was substituted with a mismatched G residue (41). This result was also confirmed by hybridization studies demonstrating higher stability of the S2T-dA base pair compared with the S2T-dG base pair in DNA duplexes. Additionally, 2-thiopyrimidine 5′-O-triphosphate, which was evaluated as a substrate for the Klenow fragment of DNA Pol I, was 2.2-fold more efficient than TTP (41) and was useful in six-letter genetic alphabet PCR amplification due to increased fidelity of non-canonical iso-dC-iso-dG base pairing compared with S2T-iso-dG base pairing (42). In self-avoiding molecular recognition systems, S2T allows the discrimination between duplexes with strong S2T-dA base pairs and those with an unfavorable S2T-2-aminopurine nucleoside pattern (43).

Because of the important biological functions and various biotechnological applications of sulfur-modified nucleosides and oligonucleotides that contain them, determination of their stability under in vitro oxidative stress conditions and the influence of their possible oxidative lesions on the structure and function of S2U-containing nucleic acids are of significant importance. We have previously shown that under conditions mimicking oxidative stress in a cell (aqueous H₂O₂), S2U is effectively transformed into a mixture of uridine (U) and 4-pyrimidinone riboside (H₂U) (Figure 1), and the product ratio is pH (44) and concentration dependent (E. Sochacka et al., in preparation). Similarly, treatment of S2U-containing RNA with 100 mM H₂O₂ (in either water or a buffered solution at pH 7.5) produced corresponding desulfured products, and also in this case, the H₂U-containing RNA was a major product (45). Notably, the hydrogen bonding donor/acceptor pattern in 4-pyrimidinone differs from that in uracil/2-
thiouracil. Moreover, we demonstrated that the sugar ring in H2U predominantly adopts the C2′-endo conformation (typical for B-type DNA), which is substantially different from the C3′-endo conformation preferentially adopted by S2U (46). Undoubtedly, these two structural differences affect the Watson–Crick type interactions of H2U with adenine and the wobble interactions with guanine. Therefore, one may assume that the S2U→H2U transformation alters the thermal stability of RNA duplexes (45) and possibly their S2T/S2dU DNA congeners (47). This replacement may result in significant consequences for biological processes in which 2-thiouracil-containing nucleic acids are involved (48). Of note, anticodon-specific endonucleases are also known to induce tRNA cleavage, which is a conserved response to oxidative stress in numerous eukaryotic species ranging from Saccharomyces cerevisiae and Arabidopsis to humans (49).

To study the S2U→H2U transformation effects, we prepared a series of RNA and DNA duplexes containing uracil, 2-thiouracil or 4-pyrimidinone interacting with a complementary adenine or guanine residue (Table 1). Model-independent differential scanning calorimetry (DSC) was used to determine the thermodynamic stability of the duplexes. Moreover, we performed quantum chemical Discrete Fourier Transform (DFT) calculations and molecular mechanics modeling to assess the energies of base pairing in isolated base pairs and in pairs of nucleotides within RNA and DNA duplexes.

MATERIALS AND METHODS

Synthesis and purification of model oligonucleotides

The synthesis of non-modified DNA and RNA oligonucleotides listed in Supplementary Table S1 was done on a Gene World DNA synthesizer at the 0.2 μm scale using the phosphoramidite approach and the standard protocol, except for the synthesis of oligonucleotides containing 2-thiouracil and 4-pyrimidinone nucleobases (Figure 1), for which modified synthetic procedures described below were applied.

Synthesis of RNA oligomers

Synthesis of 14-mer RNAs containing S2U and H2U (Supplementary Table S1) was done using commercial nucleoside phosphoramidites of suitably protected nucleosides T, dC, dA and dG, and the S2U and H2U monomers, obtained in house according to previously described procedures (46). For the synthesis of H2U-oligonucleotide, the monomers with ‘mild’ Pac protecting groups (Glen Research) were used. Coupling of modified monomers was allowed to proceed for 220 s. The P(III)→P(V) oxidation step in the synthesis of the S2dU-oligonucleotide was performed with 0.25 M solution of tBuOOH in CH3CN for 5 min, while for H2dU-oligomer, standard iodine oxidation was applied. The DNA oligonucleotide containing 2-thio-2′-deoxyuridine was cleaved from the solid support by treatment with a mixture of concentrated (conc.) aqueous ammonia and ethanol (3:1) for 4 h at 55°C and deprotected by the two-step procedure (DMT-on, DMT-off) (52). The solution was evaporated under reduced pressure (Speed Vac) and loaded on the RP semipreparative column (Hamilton PRP-1 (7 mm × 305 mm, C-18, 10 μm), and eluted with 0.1 M ammonium acetate (buffer A), and 0.1 M ammonium acetate and 40% acetonitrile (buffer B) with the elution of 3 ml/min in the following program: DMT-on 0–100% buffer B in 30 min, and for DMT-off 0–100% buffer B in 15 min. The obtained eluates were desalted on C18 SepPak cartridges (Waters). The molecular mass of all synthesized oligomers was confirmed by MALDI-TOF mass spectrometry (Supplementary Table S1), and purity by polyacrylamide gel electrophoresis (PAGE) analysis (not shown).

Synthesis of DNA oligomers

Synthesis of 14-mer DNAs containing S2dU and H2dU (Supplementary Table S1) was done using commercial nucleoside phosphoramidites of suitably protected nucleosides T, dC, dA and dG, and the S2dU and H2dU monomers, obtained in house according to previously described procedures (46). For the synthesis of H2dU-oligonucleotide, the monomers with ‘mild’ Pac protecting groups (Glen Research) were used. Coupling of modified monomers was allowed to proceed for 220 s. The P(III)→P(V) oxidation step in the synthesis of the S2dU-oligonucleotide was performed with 0.25 M solution of tBuOOH in CH3CN for 5 min, while for H2dU-oligomer, standard iodine oxidation was applied. The DNA oligonucleotide containing 2-thio-2′-deoxyuridine was cleaved from the solid support by treatment with a mixture of conc. 30% aqueous ammonia and ethanol (3:1) for 4 h at 55°C and deprotected by the two-step procedure (DMT-on, DMT-off) (52). The solution was evaporated under reduced pressure (Speed Vac) and loaded on the RP semipreparative column (Hamilton PRP-1 (7 mm × 305 mm, C-18, 10 μm), and eluted with 0.1 M ammonium acetate (buffer A), and 0.1 M ammonium acetate and 40% acetonitrile (buffer B) with the elution of 3 ml/min in the following program: DMT-on 0–100% buffer B in 30 min, and for DMT-off 0–100% buffer B in 15 min. The obtained eluates were desalted on C18 SepPak cartridges (Waters). The oligomer containing 4-pyrimidinone nucleoside (H2U), because of its instability in aqueous alkali, was cleaved from the solid support and deprotected by the treatment with 0.05 M K2CO3 in anhydrous MeOH for 16 h at 30°C. The solution was neutralized with diluted acetic acid and evaporated. The resultant dry pellets were treated with the 1:1 (v/v) mixture of triethylamine tris(hydrofluoride) and of dry dimethylformamide (DMF) (120 μl) for 16 h at 25°C. The reactions were quenched by addition of the same volume of isopropyl trimethylsilylethyl ether, shaken until a precipitate appeared and centrifuged. The pellets were washed three times with diethyl ether and then dried in vacuo.

The fully deprotected RNA oligomers were purified by IE-HPLC in a gradient of buffer B (600 mM NaClO4, 10% CH3CN, 50 mM Tris-HCl, 50 μM EDTA, pH 7.6) in buffer A (10 mM NaClO4, 10% CH3CN, 50 mM Tris-HCl, 50 μM EDTA, pH 7.6) and desalted on C18 SepPak cartridges (Waters). The molecular mass of all synthesized oligomers was confirmed by MALDI-TOF mass spectrometry (Supplementary Table S1), and purity by polyacrylamide gel electrophoresis (PAGE) analysis (not shown).
Table 1. The sequences of the investigated RNA (1–6) and DNA (7–14) duplexes, their abbreviated names (in bold) and an indication of a Watson–Crick (WC) or wobble (WB) pairing mode

| No. | Duplex sequence | Name | Pairing mode |
|-----|----------------|------|--------------|
| 1   | 5′-CGGCU U UUACCAG-3′ 3′-GCCGA A AAUUGGC-5′ | U-A | WC |
| 2   | 5′-CGGCU S2U UUACCAG-3′ 3′-GCCGA A AAUUGGC-5′ | S-A | WC |
| 3   | 5′-CGGCU H2U UUACCAG-3′ 3′-GCCGA A AAUUGGC-5′ | H-A | WC |
| 4   | 5′-CGGCU U UUACCAG-3′ 3′-GCCGA G AAUUGGC-5′ | U-G | WB |
| 5   | 5′-CGGCU S2U UUACCAG-3′ 3′-GCCGA G AAUUGGC-5′ | S-G | WB |
| 6   | 5′-CGGCU H2U UUACCAG-3′ 3′-GCCGA G AAUUGGC-5′ | H-G | WB* |
| 7   | 5′-d(CGGCT T TTAACCGA)-3′ 3′-d(GCCGA A AATGGCT)-5′ | dT-dA | WC |
| 8   | 5′-d(CGGCT dU TTAACCGA)-3′ 3′-d(GCCGA A AATGGCT)-5′ | dU-dA | WC |
| 9   | 5′-d(CGGCT S2dU TTAACCGA)-3′ 3′-d(GCCGA A AATGGCT)-5′ | dS-dA | WC |
| 10  | 5′-d(CGGCT H2dU TTAACCGA)-3′ 3′-d(GCCGA A AATGGCT)-5′ | dH-dA | WC |
| 11  | 5′-d(CGGCT T TTAACCGA)-3′ 3′-d(GCCGA G AATGGCT)-5′ | dT-dG | WB |
| 12  | 5′-d(CGGCT dU TTAACCGA)-3′ 3′-d(GCCGA G AATGGCT)-5′ | dU-dG | WB |
| 13  | 5′-d(CGGCT S2dU TTAACCGA)-3′ 3′-d(GCCGA G AATGGCT)-5′ | dS-dG | WB |
| 14  | 5′-d(CGGCT H2dU TTAACCGA)-3′ 3′-d(GCCGA G AATGGCT)-5′ | dH-dG | WB* |

WB* is a wobble H2U-G-type base pair shown in Figure 7.

DSC melting measurements

DSC measurements were performed using a MicroCal VP-DSC microcalorimeter, equipped with 0.514 ml cells. Dry samples of annealed duplexes were diluted with a 10 mM phosphate buffer containing 50 mM NaCl to the final concentrations 20 mM and degassed for 10 min at 18°C. External pressure of 30 psi was applied to the sample and reference cells. Three independent dilutions of each duplex were studied. The VP-DSC was run without feedback, within temperature range 10–95°C (up scans only), at a heating rate 40°C/h. A 10-min equilibration time at 10°C was used before and between scans. Three-to-ten buffer-
buffer scans were performed initially to establish a ‘thermal history’ of the instrument, to obtain baseline and confirm reproducibility of the measurements. Then, the buffer in the sample cell was replaced by the oligonucleotide duplex solution and several scans were collected. DSC data were analyzed using ORIGIN software from MicroCal. For both, the buffer and the RNA sample, the second scans were used (all scans after the first, except few cases discussed later, were indistinguishable). Data were corrected for instrument baselines and normalized for the scan rate and the nucleic acid concentration. A cubic baseline was fitted to each profile and subtracted. To avoid controversy about the proper fitting model (which is quite common, especially for short oligonucleotide duplexes) (55) and to make the calculations model independent, enthalpies (ΔH<sub>cal</sub>) and entropies (ΔS<sub>cal</sub>) of duplex formation were calculated from the areas under the experimental ΔCp versus T and derived from ΔCp/T versus T curves, respectively, while free energy (ΔG<sub>25</sub>) values were calculated according to the standard equation ΔG<sub>25</sub> = ΔH<sub>298.15K</sub>−<sub>298.15K</sub>ΔS<sub>25</sub> (56). The results of three independent experiments were averaged. In some cases, in subsequent measurements of the same duplex, variation of pre- and post-unfolding baseline was observed. This was reflected in a higher standard deviation (SD). The errors were calculated as ±SD of the three independent measurements of each duplex.

### Circular dichroism measurements

Samples of the RNA and DNA duplexes were prepared at 2 μM concentration in a 10-mM phosphate buffer, pH 7.0, containing 50 mM NaCl. The spectra were recorded at a room temperature on a Jobin Yvon CD6 Dichrograf. Measurements were made in optically matched 0.5 cm pathlength quartz cuvettes of 1 ml capacity, 2 nm bandwidth and 1–2 s integration time. Each spectrum was smoothed with 9- and 15-point algorithm (included in the manufacturer’s software, version 2.2.1) after averaging of at least three scans.

### Computational methods

All quantum mechanical calculations were performed with the Gaussian 09 suite of programs (57). Geometries of the base pair model systems were optimized using the hybrid B3LYP functional with 6–31G(d) basis set in the gas and water phase. Methyl substituents were used in place of ribose moieties. All the stationary points have been identified to correspond to the stable minima by frequency calculations. Vibrational analysis also provided thermal enthalpy and free energy corrections to 298 K. More accurate electronic energies were obtained using the long-range and dispersion corrected ωB97XD functional with the 6–311++G(3df,2p) basis set for B3LYP/6–31G(d) geometries. This level of theory gives reasonable accuracy in thermochemical calculations (mean absolute error in test calculations was of ca. 2 kcal/mol) (58). Basis set superposition error (BSSE) error was neglected since it was found to be unimportant at this level of theory (test calculations at B3LYP/6–311+G(2d,p)/B3LYP/6–31G(d) showed BSSE of 0.3 kcal/mol and 0.4 kcal/mol for H2U-A and H2U-G pairs, respectively). Since the 6–311++G(3df,2p) basis set is larger than 6–311+G(2d,p), the BSSE is expected to be even smaller. Calculations in water solution were performed assuming polarizable conductor calculation model (CPCM) with radii = UFF option. Geometry optimizations and thermochemical corrections in solution were performed at the B3LYP/6–31+G(d) level and final energies were calculated at CPCM/ωB97 XD/6–31++G(3df,2p).

The nine base pairs (bp) RNA duplexes were constructed using the HyperChem polynucleotide builder and optimized with Amber96 force field (59) using the Hyperchem 8 software (HyperChem(TM) Professional 8.0.10). For the detailed procedure see the Supplementary Information. The model RNAs of the following sequence 5’-GCUU*UUAAC-3’, where U* is either uridine or 4-pyrimidinone riboside, together with the complementary 3’-CGAYAAUUC-5’ strand, where Y is A or G, were created to mimic the base pair interactions in the discussed original RNA duplexes 1, 3, 4 and 6, respectively (Table 1). The fragments of model isosequential DNA duplexes containing HdU-dA and HdU-dG base pairs are shown in the Supplementary Information (Supplementary Figure S3).

## RESULTS

### Preparation of RNA and DNA duplexes

All of the RNA and DNA oligonucleotides containing modified units (for sequences, see Supplementary Table S1) were synthesized according to routine phosphoramidite methodology using in-house synthesized phosphoramidite monomers of the modified nucleosides S2U, S2dU, dU, H2U and H2dU (Figure 1) (45,46). A prolonged coupling time and modified oxidation step (0.25 M solution of tBuOOH in CH3CN for 5 min) were used for the synthesis of RNA and DNA oligonucleotides containing S2U/S2dU units. A prolonged coupling time was also used for the synthesis of RNA and DNA oligomers modified with 4-pyrimidinone nucleosides. The S2U/S2dU-oligonucleotides were deprotected and cleaved from a solid support by treatment with a mixture of conc. aqueous ammonia and ethanol (3:1) due to the instability of 4-pyrimidinone nucleosides in aqueous alkali, and H2U/H2dU-oligonucleotides were cleaved from a solid support and deprotected by treatment with 0.05 M K2CO3 in anhydrous MeOH. Following neutralization and solvent evaporation, the tBDMs protecting groups were removed from the RNA oligomers using Et3Nx3HF/DMF, followed by quenching with isopropyl trimethylsilyl ether. The crude RNA and DNA products were subjected to IE-HPLC and desalted using C18 Sep-Pak cartridges. The sequences of the obtained oligonucleotides and their mass spectrometric characteristics are provided in the Supplementary Information (Supplementary Table S1).

### DSC analysis of the RNA duplexes

**Analysis of the thermodynamic stability of the H-A, S-A and U-A RNA duplexes.** DSC experiments were performed for a series of six RNA duplexes, 1–6 (summarized in Table 1), containing U, S2U or H2U interacting with an A or G in the complementary strand. The recorded DSC profiles of the thermal dissociation of the RNA duplexes are shown
in Figure 2A, and the melting temperatures, $T_m$, and other thermodynamic parameters of the transitions are summarized in Table 2.

Here, using model-independent DSC measurements (60), we obtained accurate $T_m$, $\Delta H$, $\Delta S$ and $\Delta G$ parameters for the dissociation transitions (Table 2). These results demonstrate that the most stable duplex is S-A followed by U-A, whereas the least stable duplex is H-A (containing the H2U-A pair). The $T_m$ of 53°C for H-A is 11.2°C and 7.2°C lower than that for S-A and U-A, respectively. The $\Delta G_{25}$ value (the absolute value) for H-A is the smallest of the three RNA duplexes. The DSC transition profile of the H-A duplex (Figure 2A) contains an additional shoulder at a $T_m$ of $\sim 36°C$, which can be attributed to ‘pre-melting’ of the underlined 5 bp range in 5'-CGGGCUH2UUAAACCGA-3'/3'-GCCGAAAUUGGCU-5'. Moreover, the results obtained from DSC analysis unequivocally confirm that the 2-thiouridine within the S-A RNA duplex greatly increases the specificity of the interactions with adenine compared with uridine (within U-A), with a $T_m$ value that is higher by 4°C and a $\Delta G_{25}$ value that is lower by 1.4 kcal/mol.

Analysis of the thermodynamic stability of the H-G, S-G and U-G RNA duplexes. The U-G and S-G duplexes, which contain U and S2U residues that interact with a G residue in the complementary strand, are thermally less stable than the corresponding duplexes containing a complementary A residue (Table 2 and Figure 2A), and this characteristic has the corresponding duplexes containing a complementary A in the complementary strand, are thermally less stable than RNA duplexes.

Analysis of the thermodynamic stability of the T-dA, U-dA, dS-dA and dh-dA duplexes. Analysis of the DSC melting results for duplexes 7–10 indicates that the most stable duplex is the canonical T-dA duplex, whereas for dU-dA (containing the parent thymidine residue lacking the methyl group at carbon atom C5), the $T_m$ was lower by 1.9°C ($\Delta \Delta G = -1.1$ kcal/mol). Introduction of a sulfur atom to the uracil moiety in dU resulted in a dS-dA duplex of only slightly higher $T_m$ ($\Delta \Delta T_m = 0.8°C$; the $\Delta G_{25}$ values were virtually equal). Replacement of S2dU with H2dU produced a dh-dA (10) duplex with a $T_m$ value that was lower by $\sim 13°C$ compared with the $T_m$ values for dU-dA and ds-dA. In this case, the $\Delta H$ and $\Delta G_{25}$ values of the dsDNA→ssDNA transition dramatically increased and were the least negative of all of the evaluated DNA duplexes (7–14). An additional transition at a $T_m$ of $\sim 24°C$ was also observed in the DSC profile, which, as observed for the H-A duplex, may be attributed to dissociation of the 5 bp segment of the dh-dA duplex (underlined, 5’-d(CGCGCUH2dUd(UUAACCGA)-3’/3’-dGCGAAAUUGGCU-5’).

Circular dichroism analysis of the helical structure of the RNA and DNA duplexes.

RNA duplexes 1–6 and DNA duplexes 7–14 were analyzed using circular dichroism (CD) spectroscopy. As shown in the CD spectra (Figure 3A and B, all RNA duplexes adopted an A-type overall conformation (a positive band at $\lambda_{max}$ of $\sim 265$ nm and a negative band at $\lambda_{max}$ of 210 nm) (61). Minor differences, such as a slight increase in the intensity of the positive band at $\lambda_{max}$ of 265 nm of the S2U-A and S2U-G duplexes (Figure 3A and B), were noted, which is most likely due to their improved π–π base stacking (62). The H2U residue complementary to A or G did not significantly alter the global RNA helical structure of H-A and H-G. In the CD spectra for DNA duplexes 7–14, typical positive bands at approximately $\lambda_{max}$ of 220 and 272 nm and negative bands at $\lambda_{max}$ of 245 nm were present (63), although there were some differences in the CD shape of the B-type helical structure of dS-dA and ds-dA (the green profiles in Figure 3C and D). For both duplexes, the bands at $\lambda_{max}$ of 272 nm and the isoellectric point at 257 nm were blue shifted, whereas for dS-dG, the isoellectric point at 223 nm was red shifted. For dh-dG (the red profile in Figure 3D) the long-wave positive CD band at 272 nm was significantly decreased compared with the CD profiles of T-dG and dU-dG.

Theoretical calculations.

Base pair models. Quantum chemical DFT calculations of the free base pairs were performed to determine the struc-
Figure 2. The DSC profiles of the thermal dissociation of the RNA duplexes (A) U-A (1), S-A (2), H-A (3), U-G (4), S-G (5), H-G (6) and the DNA duplexes (B) T-dA (7), dU-dA (8), dS-dA (9), dH-dA (10), T-dG (11), dU-dG (12), dS-dG (13), dH-dG (14). The sequences of duplexes are listed in Table 1.

Table 2. Thermodynamic parameters of the dissociation of the RNA duplexes determined by DSC

| No. | Name | Tm [°C] for duplex [20 μM] | -ΔH ± SD [kcal/mol] | -ΔScal ± SD [cal/K mol] | -ΔG25 for duplex formation [kcal/mol] |
|-----|------|-----------------------------|----------------------|------------------------|---------------------------------------|
| 1   | U-A  | 60.3 ± 0.3                  | 111.6 ± 5.6          | 335.8 ± 16.6           | 15.1 ± 0.8                            |
| 2   | S-A  | 64.3 ± 0.1                  | 116.4 ± 2.8          | 348.0 ± 8.6            | 16.5 ± 0.4                            |
| 3   | H-A  | 53.1 ± 0.4                  | 96.0 ± 9.2           | 296.1 ± 25.2           | 11.0 ± 1.0                            |
| 4   | U-G  | 57.1 ± 0.3                  | 106.8 ± 2.6          | 324.2 ± 7.5            | 13.7 ± 0.5                            |
| 5   | S-G  | 54.2 ± 0.2                  | 91.0 ± 0.5           | 278.0 ± 1.4            | 11.1 ± 0.1                            |
| 6   | H-G  | 54.6 ± 0.3                  | 76.1 ± 3.7           | 231.6 ± 10.7           | 9.6 ± 0.6                             |

All values are derived as averages of three independent measurements.

Table 3. Thermodynamic parameters of the dissociation transitions of the DNA duplexes determined by DSC methodology

| No. | Name | Tm [°C] for duplex [20 μM] | -ΔH ± SD [kcal/mol] | -ΔScal ± SD [cal/K mol] | -ΔG25 for duplex formation [kcal/mol] |
|-----|------|-----------------------------|----------------------|------------------------|---------------------------------------|
| 7   | T-dA | 54.6 ± 0.4                  | 118.0 ± 2.9          | 361.5 ± 9.2            | 14.2 ± 0.3                            |
| 8   | dU-dA| 52.7 ± 0.0                  | 113.6 ± 9.5          | 349.8 ± 29.5           | 13.1 ± 1.0                            |
| 9   | dS-dA| 53.5 ± 0.7                  | 103.8 ± 5.1          | 318.5 ± 16.7           | 12.3 ± 0.8                            |
| 10  | dH-dA| 40.3 ± 0.3                  | 61.4 ± 0.8           | 194.8 ± 2.6            | 5.5 ± 0.4                             |
| 11  | T-dG | 45.2 ± 0.7                  | 96.8 ± 2.9           | 304.8 ± 9.6            | 9.3 ± 0.3                             |
| 12  | dU-dG| 44.3 ± 0.2                  | 87.1 ± 24.9          | 271.4 ± 79.5           | 9.1 ± 2.2                             |
| 13  | dS-dG| 44.5 ± 0.9                  | 77.1 ± 5.7           | 242.9 ± 16.7           | 7.4 ± 1.2                             |
| 14  | dH-dG| 48.5 ± 1.1                  | 84.2 ± 0.9           | 263.2 ± 3.3            | 8.6 ± 0.1                             |

All values are derived as averages of three independent measurements.

tures of the hydrogen-bonded complexes and the bonding enthalpies between nucleobases of interest. In all of the evaluated base models, the ribose residue of the parent nucleoside was replaced with a methyl group. Atomic charges for the 4-pyrimidinone fragment of H2U were calculated at the B3LYP/6-311+G(2d,p) level using the ESP (Merz-Kollman) scheme as described in the Supplementary Information (see Supplementary Figure S1). The enthalpies of bonding for the classical base pairs have been previously calculated at various levels of theory (64–67). Structures and bonding enthalpies for the base pairs under consideration (Figure 4) demonstrate that the 4-pyrimidinone–adenine that base pairs with a single O(4)...HN(6) hydrogen bond is weak and does not reach the energy minimum upon optimization. Rather, optimization in water produces a wobble-like base pair with binding enthalpy ΔH = −4.8 kcal/mol with a weak stabilizing interaction between the H-C(2) hydrogen of the 4-pyrimidinone moiety and the endocyclic N(1) nitrogen atom of adenine (Figure 4C) (68,69). For the remaining two base pairs of adenine (U-A and S2U-A, Figure 4A and B), the binding enthalpies are similar or identical, with ΔH = −9.1 kcal/mol under the evaluated conditions (aqueous solution).
Interestingly, the most stable complex among the modeled base pairs was the H2U-G base pair shown in Figure 4F ($\Delta H = -10.0$ kcal/mol). The binding mode of this base pair differs from that of the typical U-G wobble base pair (WB, Figure 4D); therefore, we refer to this H2U-G base pair as a wobble* base pair (WB*) (Figure 4F). This base pair contains two hydrogen bonds formed between each of the HN(1) and HN(2) hydrogen atoms of G (the donors) and the O(4) and N(3) of H2U (the acceptors). The complex is not flat; the bases are twisted relative to each other by $\sim 33^\circ$, which is the result of the non-planarity of the exocyclic amine group in G involved in bonding. The bonding enthalpies (mean values) calculated for base pairs in an aqueous environment are 2–6 kcal/mol smaller than those in the gas phase (Supplementary Table S2). The observed binding destabilization in solution is due to the stronger solvation of individual bases, as previously observed (70).

Models of the duplexes. Simple molecular mechanics calculations using an Amber96 force field were performed for the model RNA duplexes to estimate the impact of the H2U modified unit on the structure of the duplex and on the bonding energy between both strands in the helix. The optimized 9-bp RNAs contained each nucleoside in the C3′-endo sugar ring conformation with the exception of the H2U-modified unit, which was adjusted to adopt the C2′-endo sugar ring conformation. The calculations demonstrate that, in contrast to the unrestricted free base pair systems, the H2U-A base pair within the RNA chain does not form a structure resembling the wobble-like complex shown in Figure 4. In contrast, due to steric restrictions imposed by the chain and $\pi$–$\pi$ stacking interactions (71), the complex adopted a Watson–Crick structure, as observed in the U-A base pair, but with only one hydrogen bond between the O(4) acceptor and the exocyclic HN(6) donor (Figure 5A).
Figure 4. DFT modeling of the possible base pairs of U, S2U and H2U with A and G in water solution. In the used models the ribose residue of nucleoside is replaced with a methyl group. For the H2U-G base pair a stable two-hydrogen bonds base pair (F) is named as wobble* base pair, WB*.

A. U-A, ΔH = -9.1 kcal/mol
Watson-Crick base pair (WC)

B. S2U-A, ΔH = -9.1 kcal/mol
Watson-Crick base pair (WC)

C. H2U-A, ΔH = -4.8 kcal/mol
wobble-like base pair (W-like)

D. U-G, ΔH = -9.5 kcal/mol
wobble base pair (WB)

E. S2U-G, ΔH = -7.5 kcal/mol
wobble base pair (WB)

F. H2U-G, ΔH = -10.0 kcal/mol
wobble* base pair (WB*)

Figure 5. Fragments of the optimized 9-bp RNA duplexes 5′-GCUH2UUAA-3′/ 3′-CGAA/GAAUUG-5′ adopting the A-type helical structures, with modified base pairs marked: H2U-A (A) and H2U-G (B). The pictures show the RNA helixes with the base pairs adopting the C3'-endo sugar ring puckering, but with the H2U-A/G base pairing positioned in the C2'-endo sugar ring puckering.
In contrast, optimization of a 9-bp RNA duplex containing the H2U-G base pair (Figure 5B) indicates that the guanosine unit shifts to form wobble* bonding as has been suggested by DFT calculations. Because the crystallographic and nuclear magnetic resonance (NMR) analyses for H2U (46) suggest that the ribose attached to the 4-pyrimidinone base preferentially adopts the C2/endo perspective with the parent 5-methyluracil, the corresponding pyrimidinone significantly reduces the thermodynamic stability of the duplex (31).

MM Amber calculations of a model 9-bp A-type RNA duplex (Figure 5A) suggest that the unstable H2U-A base pair accommodates in the double helix in the same manner as an isosteric S2U-A base pair. Adjustment of the C2-endo sugar ring conformation of the H2U residue introduces the conformational rearrangement C3-endo → C2-endo in the complementary adenosine. In addition to possible π–π stacking interactions (71), the H2U-A base pair within the RNA chain is stabilized by only one WC isosteric hydrogen bond (Figure 6A) (76). The bases are twisted to reduce the repulsive interaction between the lone electron pairs in the nitrogen atoms N(3) of 4-pyrimidinone and N(1) of adenine (47, 77). Nevertheless, these computational results are only weakly reflected in the CD spectra of H2U-A and H2dU-dA, which are nearly identical to those determined for their sulfur-containing precursors (Figure 3A and C).

When S2U in the S-G duplex is replaced with a 4-pyrimidinone riboside, the melting temperature of the resulting H-G is close to that of S-G. The observed decrease in $T_m$ is much smaller than that observed for S-A→H-A duplexes, and the $T_m$ for H-G is slightly higher than the $T_m$ value for H-A. Interestingly, the analogous dH-dG DNA duplex is much more stable than the parent dS-dG duplex. These results are in contrast to those obtained for the dH-dA duplex, which exhibited much weaker stability than that of the sulfur-containing congener. Initially, this result appears remarkable because the wobble topology (analogous to that in S-G, Figure 6B) disallows any hydrogen bonding between 4-pyrimidinone and guanine. However, as we have observed from DFT modeling, isolated 4-pyrimidinone-guanine base pairs are the most stable when arranged in the WB* mode (Figure 4F). This base pair exploits two hydrogen bonds of identical polarity that are formed between both NH donors of G (N(1) and N(2)) and the O(4) and N(3) acceptors of H2U. However, to form this H2U-G base pair, the RNA helix requires a conformational rearrangement, i.e. movement of the wobble H2U base along the y-axis to the WB* location. This shift provides two new hydrogen bonds, which are energetically equivalent to the original wobble hydrogen bonds between U and G ($\Delta H = -9.5$ kcal/mol for U-G WB versus $\Delta H = -10.0$ kcal/mol for H2U-G WB*). Our Molecular Mechanics (MM) calculations provide evidence that the WB* arrangement does not distort the RNA helix (Figure 6); thus, H-G is only slightly less stable than the parent U-G ($\Delta T_m = 2.5$ °C and $\Delta G_{298} = 4.1$ kcal/mol). Indeed, a superposition of the MM-optimized structures of U-G and H2U-G-containing duplexes (Supplementary Figure S2) reveals the translation of the 4-pyrimidinone riboside along the y-axis of the helix and the rotation of the H2U and G bases to adopt the arrangement suitable for efficient base pairing. The structures of the HdU-dA and H2dU-dG base pairs in isosequential DNA duplexes are analogous to those in the U-G- and H2U-G-containing duplexes (Supplementary Figure S3).

There is an additional and perhaps more important aspect of the discussed desulfuration. We searched for examples in the literature of preferential G (versus A) recognition by 2-oxo/thioalkylated uridine/thymidine units. In-
Figure 6. Structures of the H2U-A and H2U-G base pairs located in the topologic site of the parent Watson–Crick S2U-A and classical wobble S2U-G base pairs (with structure of the parent S2U shown in gray).

Figure 7. Proposed topology of the base pairs according to the classical mode of Watson–Crick, a wobble mode (a pyrimidine unit directed toward the major groove) and WB* (a pyrimidine unit directed toward the minor groove), according to Takai (86). The U/S2U-A, U/S2U-G and X2U-G base pairs are positioned within the RNA helix (R = H or (c)mnm and X = S−, geS or H).

Interestingly, in all identified cases, the modified pyrimidines possess a 4-pyrimidinone-type scaffold (containing a system of double bonds and hydrogen bond acceptors as in H2U), which, consistent with our hypothesis, may interact with guanine according to the WB* pairing mode. One example concerns 11-bp DNA duplexes containing O(2)-methyl thymidine, which preferentially interact with guanine rather than adenine (78). Furthermore, O(2)-alkylated thymidines are considered mutagenic lesions in DNA (79) because O(2)-alkylated thymidines in DNA templates are not accepted by DNA polymerase (80,81), which inhibits DNA replication and leads to non-coding lesions. Notably, the incorporation of thymidine lacking the 2-carbonyl function into DNA sequences destabilizes base pairing with dA (47).

A second example comes from studies on codon–anticodon interactions, which are important for the translation process in which hypermodified 2-thiouridines (located in the wobble position of anticodon of tRNAs) provide precise reading of the third letter of the codon either by Watson–Crick base pairing with A or by wobble pairing with G. Recently, new hypermodified nucleosides containing the 4-pyrimidinone motif generated by alkylation of the 2-thiocarbonyl function have been discovered. These S-geranyl-2-thiouridines, which are substituted with 5-methylaminomethyl (mnm) or 5-carboxymethylaminomethyl (cmnm) groups (Figure 7; in the structure on the right, X = geS, R = mnm or cmnm), are located in the wobble position of tRNAs specific for Lys, Glu and Gln and were identified in several bacterial strains exposed to cellular stress (82). A comparison of the translation efficiency of the glutamate codons 5′-GAA and 5′-GAG at varying degrees of geranylation revealed that the GAA/GAG translation efficiency ratio was altered from 13:1 to 6:1, indicating the preferred reading of G-ending codons (82).

This change in reading preference from A- to G-ending codons was also observed for a tRNA with a wobble mnm5S2U nucleoside (83,84). As hypothesized by Takai and Yokoyama (85), the 5-methylaminomethyl substituent of the 2-thiouracil moiety may be protonated at the amino function at physiological pH, whereas the sulfur atom carries a negative charge. An altered electronic structure of the zwitterionic mnm5S2U results in the formation of a
4-pyrimidinone-type nucleoside (R5X2U) in which the N3 atom becomes a hydrogen bond acceptor (Figure 7, R = (c)mnm, X = S−). Takai further postulated that the aforementioned wobble pyrimidine nucleosides may decode the third letter of codons in the new wobble geometry (denoted wobble*, WB*), in which the pyrimidine nucleoside is shifted toward the minor groove (Figure 7) (86). This interaction is suggested to be similarly effective as the Watson–Crick base pairing and classical wobble base pairing in which pyrimidine nucleosides are shifted toward the major groove compared with the pyrimidine position in the Watson–Crick arrangement. However, we demonstrate that this new wobble arrangement is only possible if the pyrimidine nucleoside adopts the H2U-type structure. Thus, tRNAs modified with an H2U-type wobble nucleoside (obtained either by its protonation and charge localization at the sulfur moiety or by sulfur alkylation) preferentially recognize G as the third letter of the codon, not A. We suggest that this 3′-G-ending codon reading may occur through the WB* interactions favored in the H2U-G base pair via translation of the H2U-type wobble nucleoside along the y-axis of the WB* geometry (Figure 7, right base pair), unless accommodation of this base pair is possible within the ribosomal context (87,88).

Although it is not strictly connected with our model, note that 3′-G-ending codons are also recognized by tRNAs containing a 5-methoxycarbonylmethyl-2-thiouridine (mcm5S2U) wobble nucleoside (89). However, based on crystallographic and NMR spectral studies, this characteristic is attributed to the specific binding pattern of guanine with the C(4)-keto-enol tautomer of the mcm5S2U unit (90).

Three U-G base pair spatial arrangements that are similar to our H2U-G wobble* model have emerged from crystal structures of T. thermophila 30S and archaeal Haloarcula marismortui ribosomal RNA (91). However, despite the striking similarity in the spatial orientation, both models differ in the number of identified hydrogen bonds (only one hydrogen bond between the uracil O(4) and H-N(1) of guanine is present instead of two in the H2U-G base pair). Nevertheless, the translation of the uridine base along the y-axis is observed in the crystal structures.

In conclusion, our studies demonstrate that the preferred binding of S2U with A is lost upon its desulfuration to a 4-pyrimidinone nucleoside. Moreover, the preferred binding of H2U with a guanosine partner is observed. Thus, biological processes and in vitro assays in which oxidative desulfuration of the 2-thiouracil-containing component may occur may be altered with unpredictable consequences. The proposed binding pattern of the 4-pyrimidinone–guanine base pair contains two hydrogen bonds between the O(4) and N(3) acceptors of the 4-pyrimidinone and the two NH donors of guanine and can be adopted only by proper spatial arrangement of both bases. This arrangement can be achieved by translation of the H2U nucleoside into the minor groove compared with the topology of the classical U-G wobble base pair (arranged in the major groove compared with the WC base pair). As a biological consequence, the tRNA wobble nucleosides in the anticodon, which under physiological conditions are able to adopt the 4-pyrimidinone-type double bond system, may potentially read 3′-G-ending codons rather than A-ending codons. To the best of our knowledge, this is the first thermodynamic and theoretical study suggesting the existence of WB* in an experimental system.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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