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Desulfurization of 2-thiouracil nucleosides: Conformational studies of 4-pyrimidinone nucleosides

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ABSTRACT

4-Pyrimidinone ribofuranoside (**H^{2}o^{4}U**) and 4-pyrimidinone 2′-deoxyribofuranoside (**dH^{2}o^{4}U**) were synthesized by the oxidative desulfurization of parent 2–thiouracil nucleosides with *m*-chloroperbenzoic acid. The crystal structures of **H^{2}o^{4}U** and **dH^{2}o^{4}U** and their conformations in solution were determined and compared with corresponding 2-thiouracil and uracil nucleosides. The absence of a large 2-thiocarbonyl/2-carbonyl group in the nucleobase moiety results in C2′-*endo* puckering of the ribofuranose ring (S conformer) in the crystal structure of **H^{2}o^{4}U**, which is not typical of RNA nucleosides. Interestingly, the hydrogen bonding network in the crystals of **dH^{2}o^{4}U** stabilizes the sugar moiety conformation in the C3′-*endo* form (N conformer), rarely found in DNA nucleosides. In aqueous solution, **dH^{2}o^{4}U** reveals a similar population of the C2′-*endo* conformation (65%) to that of 2′-deoxy-2-thiouridine (62%), while the 62% population of the S conformer for **H^{2}o^{4}U** is significantly different from that of the parent 2-thiouridine, for which the N conformer is dominant (71%). Such a difference may be of biological importance, as the desulfurization process of natural tRNA 2-thiouridines may occur under conditions of oxidative stress in the cell and may influence the decoding process.

1. Introduction

Sulfur modification of pyrimidine nucleosides by the replacement of the natural carbonyl group with thiocarbonyl is of great importance for their biological functions[1–8] and for practical applications.[9–13] It has been recognized that the 2-thiocarbonyl group of 2-thiouracil nucleosides strongly influences their conformation properties and plays a key role in the modulation of base pair recognition.[1,2] These properties are significant for the decoding ability of natural 2-thiouracil ribonucleosides located in the anticodon wobble positions of many tRNAs.[3,4] 2-Thiouridines preferentially adopt a rigid C3′-*endo* sugar ring conformation,[5,6] and therefore the modified s^{2}U-A base pair in RNA duplexes is more stabilized than an unmodified one.[7,8] Furthermore, due to steric hindrance and the weaker H-bonding ability of sulfur relative to oxygen, 2-thiouracil ribonucleosides make the U–G wobble base pairs less stable than base pair containing uridine.[7,8] The specific hybridization properties of 2-thiouracil ribonucleosides led to their practical use in the antisense strategy and SNPs analysis,[9,10] while 2′-deoxy analogs of 2-thiouracil nucleosides found successful application as useful tools for enhancing base pairing and replication efficiency in a synthetic biological system.[11,12] In this context, the stability of the 2-thiocarbonyl functionality of nucleoside units during the synthesis of modified oligonucleotide tools as well as their processing in cells is of great importance.

However, recently it has been recognized that under different oxidation conditions 2-thiouracil nucleosides easily undergo oxidative desulfurization to corresponding 4-pyrimidinone nucleosides and oxidation to natural uridines (Fig. 1). Oxidative desulfurization of the 2-thiopyrimidine moiety has been observed under treatment with aqueous iodine,[14] *m*-chloroperbenzoic acid,[15] dimethyldioxirane[16] and *trans*-2-phenylsulfonyl-3-phenyloxaziridine (PSO).[17] The reactivity of the 2-thiocarbonyl function towards oxidizing agents was found to be the main cause of the low efficiency of 2-thionucleoside incorporation into the oligonucleotide chain via the standard phosphoramidite method, as desulfurization occurred at the iodine oxidation step.[18–21] Loss of the sulfur atom from natural thiomodified nucleosides was also reported in the studies on tRNA probing with different oxidizing agents,[22–24] indicating the probability of this process occurring under the conditions of oxidative stress in the cells.

The loss of 2-thiocarbonyl/2-carbonyl function may significantly influence the conformational preferences of nucleosides and, as a consequence, be important for their biological roles. Additionally, it is worth noting that 4-pyrimidinone nucleosides **3** and **4** are 'truncated' nucleoside analogs lacking both the N3-amide

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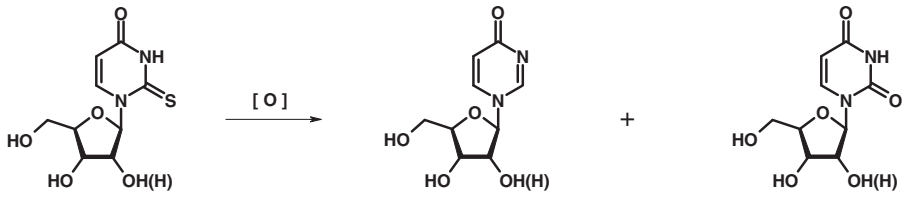


Figure 1. Oxidative desulfurization and oxidation of 2-thiouridine and 2′-deoxy-2-thiouridine. (The abbreviations **H²o⁴U** for **3** means that the hydrogen atom is at C-2 and the carbonyl function at C-4 of the pyrimidine ring in the structure of 4-pyrimidinone ribofuranoside; **dH²o⁴U** denotes the modified nucleoside in 2′-deoxy series).

hydrogen and the thiocarbonyl/carbonyl function at C-2 position in the heterobase moiety characteristic of 2-thiouridines or natural uridines and thus they offer entirely different possibilities for base pairing within RNA and DNA duplex complexes.

In the present work, we describe the synthesis and conformational studies of 4-pyrimidinone nucleosides **3** (**H²o⁴U**) and **4** (**dH²o⁴U**) in the solid state and in aqueous solution. The crystal structures of **3** and **4** are compared with the structures of corresponding 2-thiouracil nucleosides: 2-thiouridine (**1**, **s²U**),[25] 2′-deoxy-2-thiouridine (**2**, **ds²U**) and uracil nucleosides: uridine (**5**, **U**),[26] 2′-deoxyuridine (**6**, **dU**),[27] respectively. Since the crystallographic data of **ds²U** have not been published yet, its crystal structure is also resolved and reported. The conformational study of **3** (**H²o⁴U**) and **4** (**dH²o⁴U**) in solution was performed by means of high-resolution NMR methods. Detailed conformational analysis of **H²o⁴U** and **dH²o⁴U**, and a comparison of their conformational properties with the related nucleosides (**s²U**,[28] **U**,[28,29] **ds²U**,[30] **dU**[31]) make it possible to determine how the loss of the 2-thiocarbonyl functionality influences the sugar/base conformation of pyrimidine nucleosides in solution and to gain some insight into the consequences of the desulfurization process on the hybridization properties of double helical sulfur-modified DNA and RNA.

2. Results and discussion

2.1. Synthesis

4-Pyrimidinone ribonucleoside (**H²o⁴U**, **3**) and 4-pyrimidinone 2′-deoxyribofuranoside (**dH²o⁴U**, **4**) were synthesized according to the reactions presented in Scheme 1.

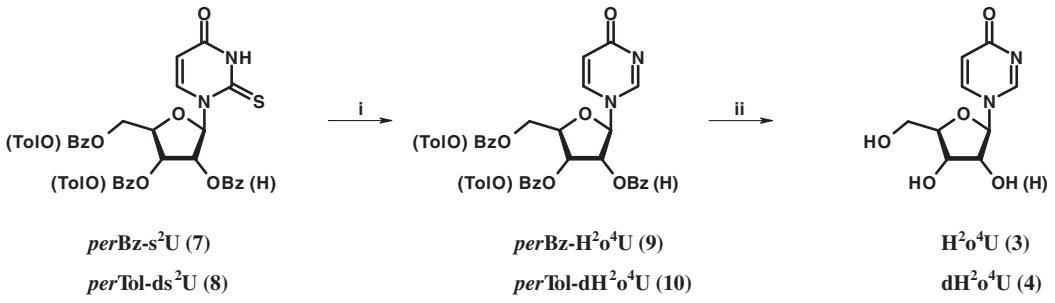
Sugar protected 2-thionucleosides **7** and **8** were obtained by the silyl method of N-glycoside bond formation from the silylated derivative of 2-thiouracil and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-ribofuranose[32] or 2-deoxy-3,5-di-*O*-toluyl-ribofuranosyl chloride.[14] Desulfurization of **7** or **8** by the treatment with 0.1 M *m*-chloroperbenzoic acid in dichloromethane/pyridine[15] for 3 h at rt afforded 4-pyrimidinone nucleosides **9** (yield 81%) and **10** (yield 83%), respectively. Removal of the sugar protecting groups of **9** and **10** by transestrification with 0.1 M NaOMe in MeOH, following work-up with Dowex (in pyridinium salt form), quantitatively gave title nucleosides **3** and **4**. The purity and structures of **H²o⁴U** (**3**) and **dH²o⁴U** (**4**) were fully confirmed by chromatography (TLC, HPLC) and spectral data (UV, MS and NMR).

Conformational analysis of nucleosides **3** and **4** was carried out based on parameters defined by Altona and Sundaralingam.[33] Three parameters describe the primary features of the conformation of a pyrimidine nucleoside: the glycosidic bond torsion angle χ (O4′–C1′–N1–C2) describes the orientation of the base relative to the furanose ring; the C4′–C5′ torsion angle γ determines the orientation of the 5′-hydroxyl group relative to the furanose ring (angle O5′–C5′–C4′–C3′); and the furanose ring puckering is specified by the pseudorotational phase angle *P*. Two major furanose ring conformations are strongly preferred for the nucleosides: C3′-*endo* (North, N-type conformation) and C2′-*endo* (South, S-type conformation).[33,34]

2.2. X-ray structure analysis

The ORTHEP drawings of the 4-pyrimidinone ribofuranoside **H²o⁴U** (**3**) and 4-pyrimidinone 2′-deoxyribofuranoside **dH²o⁴U** (**4**) are presented in Figure 2 in projections perpendicular to the plane passing through atoms C1′, C4′ and O4′, showing conformational details of the molecules. Selected geometrical parameters indispensable for conformation discussion are listed in Table 1 for ribonucleosides **1**, **3**, **5** and in Table 2 for 2′-deoxy analogs **2**, **4**, **6**. The remaining crystallographic data are given in Supplementary data. The corresponding ORTHEP for one of four independent molecules of **ds²U** (**2**) structure, is also given in Figure 2, while geometrical details are included in Table 2.

The geometry of the 4-pyrimidinone ring, a heterobase moiety in both studied nucleosides, ribonucleoside **H²o⁴U** (**3**) and 2′-deoxyribonucleoside **dH²o⁴U** (**4**), is not remarkably changed in



Scheme 1. Synthetic route for the preparation of **H²o⁴U** (**3**) and **dH²o⁴U** (**4**). Reagents and conditions: (i) 0.1 M *m*-chloroperbenzoic acid in CH_2Cl_2/pyridine 9:1, rt, 3 h; (ii) 0.1 M NaOMe/MeOH, rt, 30 min.

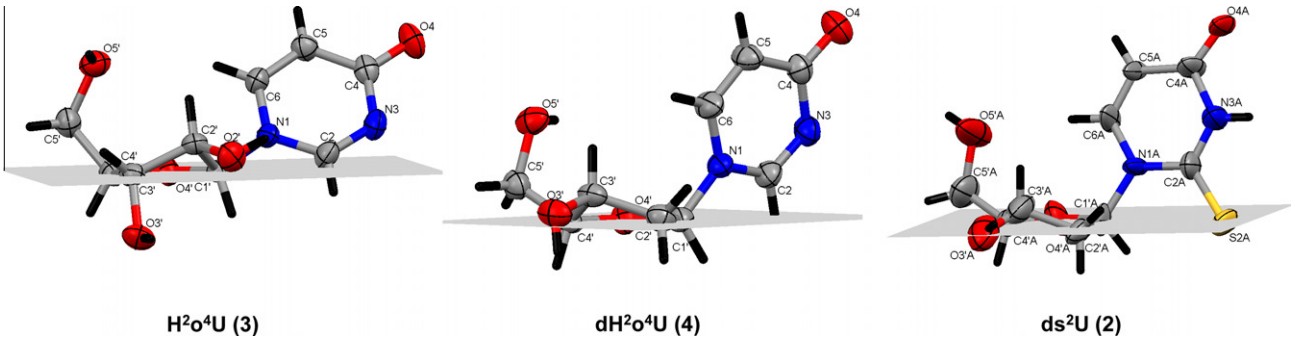


Figure 2. ORTHEP drawing of **H²o⁴U** (**3**), **dH²o⁴U** (**4**) and **ds²U** (**2**) with respect to the plane passing through atoms C1′, C4′ and O4′.

Table 1
Selected torsion angles and conformational parameters for **H²o⁴U** (**3**) in comparison with **s²U** (**1**)[25] and **U** (**5**)[26]

	H²o⁴U (**3**)	**s²U** (**1**)[25]	**U** (**5**)[26]	
			A	**B**
(a) Torsion angels [°]				
χ = O4′–C1′–N1–C2	**−142.1** (**1**)	−163.0	−161.7	−155.7
ν_0 = C4′–O4′–C1′–C2′	**−26.8** (**1**)	6.0	10.5	3.4
ν_1 = O4′–C1′–C2′–C3′	**41.2** (**1**)	−27.0	−31.4	−27.9
ν_2 = C1′–C2′–C3′–C4′	**−38.9** (**1**)	36.0	39.5	40.4
ν_3 = C2′–C3′–C4′–O4′	**24.6** (**1**)	−34.0	−34.6	−39.5
ν_4 = C3′–C4′–O4′–C1′	**1.3** (**1**)	18.0	15.3	22.8
γ = O5′–C5′–C4′–C3′	**57.6** (**1**)	−169.0	45.9	39.6
(b) Conformational parameters				
P [°]	**160.6** (**S**)	9.7 (N)	3.8 (N)	13.9 (N)
ψ_m [°]	**42.6**	36.5	39.6	41.6
Sugar moiety	**C2′-*endo***	C3′-*endo*	C3′-*endo*	C3′-*endo*
C4′–C5′ bond	***gauche*** (+)	*trans*	*gauche* (+)	*gauche* (+)
C1′–N1 bond	***anti***	*anti*	*anti*	*anti*

Table 3
Hydrogen bond geometry in **H²o⁴U** (**3**) and **dH²o⁴U** (**4**)

	D–H··A	(sym. code)	D···A (Å)	D–H··A (°)
H²o⁴U (**3**)	O2′–H2′O···O4	−1/2+*x*,3/2−*y*,1−*z*	2.745 (2)	169
	O5′–H5′O···O4	5/2−*x*,1−*y*,−1/2+*z*	2.818 (2)	163
	C3′–H3···N3	3/2−*x*,1−*y*,−1/2+*z*	3.490 (2)	168
	O3′–H3′O···O5′	−1+*x*,*y*,*z*	2.856 (1)	156
	C5–H5···O2′	1−*x*,*y*,*z*	3.132 (2)	127
	C6–H6···O2′	1−*x*,*y*,*z*	3.220 (2)	119
dH²o⁴U (**4**)	O3′–H3′···O4	(3/2−*x*,2−*y*,1/2+*z*)	2.786 (1)	174
	O5′–H5′···N3	(1/2+*x*,5/2−*y*,1−*z*)	2.860 (1)	175
	C6–H6···O5′	(−1/2+*x*,3/2−*y*,1−*z*)	3.053 (2)	133
	C3′–H3′···O5′	(−1/2+*x*,3/2−*y*,1−*z*)	3.340 (2)	124
	C2–H2···O3′	(*x*,1+*y*,*z*)	3.281 (2)	152

comparison with those of 2-thiouracil or uracil residues in **s²U**,[25] **ds²U** and **U**,[26] **dU**[27] structures (for details see Supplementary data). However, the absence of the bulky group at C-2 position and hydrogen at N-3 position in **H²o⁴U** and **dH²o⁴U** heterobases reveals a significant impact on the conformation of the modified nucleosides treated as a whole (Tables 1 and 2), as well as on hydrogen bond pattern in their crystals (Table 3).

In the crystal structure of **H²o⁴U** (**3**) the sugar residue adopts C2′-*endo* (S-type) conformation (phase angle of pseudorotation *P* = 160.6°, pseudorotation amplitude 42.6°), which is not often observed in the structure of RNA nucleosides.[34] Usually the pyrimidine ribonucleosides in the solid state are fixed in the C3′-*endo* (N-type) form of their ribose puckering, characteristic for nucleotide units in double helical RNA, as it was present in the crystal structures of reference **s²U** and **U** nucleosides.[25,26] Nevertheless, modification of the **H²o⁴U** heterobase does not influence the conformation around the C4′–C5′ ribose bond and the nucleoside adopts a typical *gauche*(+) arrangement.[34] The orientation of the heterocyclic base relative to the sugar moiety in **H²o⁴U** molecules, (χ = −142.0°) is in the typical *anti* range similar to **s²U** and **U** structures, although the small hydrogen atom at C-2 position of **H²o⁴U** heterobase instead of the bulky 2-thiocarbonyl group of **s²U** or the carbonyl of **U** does not sterically favor this arrangement around the N-glycosidic bond. The preference of the *anti* conformer of **3** is caused by hydrogen bonding network in the crystal state.

Analogous conformational parameters derived from crystallographic studies of **dH²o⁴U** (**4**) (Fig. 2 and Table 2) show that modified 2′-deoxynucleoside **4** crystallizes in the C3′-*endo*, *gauche(+)*, *anti* conformation. The *anti* orientation around the N-glycosidic

Table 2
Selected torsion angles and conformational parameters for **dH²o⁴U** (**4**) in comparison with d**s²U** (**2**) and d**U** (**6**)[27]

	dH²o⁴U (**4**)	**ds²U** (**2**)				**dU** (**6**)[27]	
		A	**B**	**C**	**D**	**A**	**B**
(a) Torsion angles [°]							
χ	**−99.3** (**1**)	−157.3 (7)	−150.0 (7)	−155.3 (7)	−146.6 (7)	−154.0	−155.0
ν_0	**−4.1** (**1**)	4.0 (1)	−0.1 (7)	1.9 (8)	−0.5 (8)	−16.8	−13.0
ν_1	**−17.4** (**1**)	−26.4 (8)	−17.5 (8)	−24.3 (8)	−21.6 (8)	33.9	33.1
ν_2	**30.7** (**1**)	37.6 (8)	27.5 (8)	34.8 (8)	34.0 (8)	−37.5	−35.3
ν_3	**−33.7** (**1**)	−36.6 (8)	−29.1 (8)	−34.4 (8)	−33.5 (8)	28.8	28.8
ν_4	**24.1** (**1**)	20.6 (8)	18.4 (7)	21.3 (8)	22.7 (8)	−7.6	−10.9
γ	**57.9** (**1**)	54.1 (9)	56.1 (9)	60.8 (9)	49.3 (9)	186.0	192.0
(b) Conformational parameters							
P [°]	**24.9** (**N**)	12.6 (N)	19.2 (N)	15.1 (N)	18.8 (N)	173.5 (S)	177.7 (S)
ψ_m [°]	**34.5**	35.8	35.1	39.3	34.4	37.7	35.3
Sugar	**C3′-*endo***	C3′-*endo*	C3′-*endo*	C3′-*endo*	C3′-*endo*	C2′-*endo*	C2′-*endo*
C4′–C5′	***gauche*** (+)	*gauche* (+)	*gauche* (+)	*gauche* (+)	*gauche* (+)	*gauche* (−)	*gauche* (−)
C1′–N1	***anti***	*anti*	*anti*	*anti*	*anti*	*anti*	*anti*

bond and *gauche*(+) around C4′–C5′ of **dH^{2}o^{4}U** (**4**) is the same as in the structure of ribonucleoside **H^{2}o^{4}U** (**3**), as well as in **ds^{2}U** (**2**) and **dU** (**6**) reference 2′-deoxynucleosides. Unexpectedly, the sugar moiety of **dH^{2}o^{4}U** (**4**) adopts the C3′-*endo* conformation, which is rarely found in 2′-deoxynucleosides in the solid state.[34] The C3′-*endo* folding of 2′-deoxyribose residue was also observed in the resolved **ds^{2}U** structure (Fig. 2, Table 2). In the case of **ds^{2}U** structure it is mainly caused by the steric interactions of the bulky 2-tiocarbonyl group in heterobase residue with sugar moiety, while the non-typical folding of the 2′-deoxyribose moiety in **dH^{2}o^{4}U** is maintained by the specific hydrogen bond system in the crystal.

It is important to underline that the absence of an N-3 hydrogen donor and a 2-tiocarbonyl/2-carbonyl acceptor function in the heterobase of the modified nucleosides **3** and **4** has a crucial impact on molecular packing in their crystals and causes a different hydrogen bond system as compared to the related pyrimidine nucleosides **s^{2}U**,[25] **U**[26] and **ds^{2}U**, **dU**.[27] The architecture of crystals of **H^{2}o^{4}U** and **dH^{2}o^{4}U** nucleosides (both in the $P2_12_12_1$ space group) is based on loops of helical and columnar molecular arrangements forming infinitive three dimensional structures. Comprehensive descriptions of the observed hydrogen bonding interactions for both structures are summarized in Table 3. The corresponding illustrations are placed in Supplementary data (Figs. S8 and S9 for **H^{2}o^{4}U** and **dH^{2}o^{4}U**, respectively). A description of molecular packing for the **ds^{2}U** structure is also enclosed (Fig. S10, Supplementary data). As in the molecules of **H^{2}o^{4}U** (**3**) and **dH^{2}o^{4}U** (**4**) there is no strong H-bond donor in the heterobase, the primary molecular bonding in the crystals is based on the strong hydrogen bonds of O2′-H···O4 in **H^{2}o^{4}U** and O3′-H···O4 in **dH^{2}o^{4}U** leading to the main motif connecting the heterobase with the sugar moiety ('base-to-sugar' motif), whereas the molecules in the crystals of reference nucleosides **s^{2}U**,[25] **U**,[26] **dU**[27] and **ds^{2}U** are mainly joined by strong N3-H···O4 bonds, providing a 'base-to-base' dominated pattern. Thus, the different molecular packing of modified nucleosides **H^{2}o^{4}U** and **dH^{2}o^{4}U** as compared to unmodified pyrimidine nucleosides resulted in the observed atypical C2′-*endo* folding of the sugar residue in the structure of **H^{2}o^{4}U** and C3′-*endo* folding for 2′-deoxynucleoside **dH^{2}o^{4}U** in their crystal structures.

2.3. NMR conformational analysis

1D and 2D NMR techniques were used to determine the solution conformations of **H^{2}o^{4}U** (**3**) and **dH^{2}o^{4}U** (**4**). Nucleoside conformation in solution is characterized by the dominant sugar pucker and preferred glycosidic bond arrangement.[35–38]

2.3.1. Conformation of the sugar moiety

It was assumed that the sugar ring of a nucleoside in solution exists as an equilibrium mixture of the two puckered forms C2′-*endo* (S conformer) or C3′-*endo* (N conformer). The percentage of S and N conformers can be estimated based on $^3J_{H1'-H2'}$ and $^3J_{H3'-H4'}$ coupling constants applying the equations: % C2′-*endo* = 100 $J_{H1'-H2'}/(J_{H1'-H2'} + J_{H3'-H4'})$ and % C3′-*endo* = 100 − % C2′-*endo*.[35,36] The experimental $^3J_{H-H}$ coupling constants and the calculated populations of C2′-*endo* and C3′-*endo* conformers are listed in Table 4 and Table 5, respectively.

Complete pseudorotation analysis of **H^{2}o^{4}U** (**3**), **dH^{2}o^{4}U** (**4**) was performed using the PSEUROT software (version 6.2).[39] In this program, minimization of the differences between the experimental and calculated couplings is accomplished by non-linear Newton–Raphson minimization, while the quality of the fit is expressed by root–mean–square (rms) differences. The input of five coupling constants: *J*(H1′, H2′), *J*(H1′, H2″), *J*(H2′, H3′), *J*(H2″, H3′) and *J*(H3′, H4′) made it possible to define the ratio of two conformers with rms <0.01 for 2′-deoxynucleoside **dH^{2}o^{4}U**. The S conformer is more populated (60%) with $P_S = 135.3°$, $\psi_m(S) = 33.6°$, while the minor N conformer (40%) is characterized by $P_N = 340.8°$, $\psi_m(N) = 30.3°$. PSEUROT calculations performed for the modified ribonucleoside **H^{2}o^{4}U**, based on three coupling constants *J*(H1′,H2′), *J*(H2′,H3′), and *J*(H3′,H4′), lead to a varied family of conformations within the range of *P* = 135°–155° for the S conformer and *P* = 330°–350° for the N conformer. Puckering amplitudes for both conformers were fixed and equal to 37°. For 2′-deoxyribonucleosides, **dH^{2}o^{4}U** (**4**), **ds^{2}U** (**2**) and **dU** (**6**), the conformations of the deoxyribose ring (the population of C2′-*endo*/C3′-*endo*) are nearly the same (Table 5). However, the 2-thiolation in ribonucleosides significantly affects the conformational characteristics of the ribose moiety. The population of the C3′-*endo* conformation of 2-thiouridine (**1**) is much higher than that of the canonical uridine (**5**).[5,40,41] The rigidity of the C3′-*endo* form of **s^{2}U** is caused by the large steric effect of the 2-thiocarbonyl group of the heterobase and the 2′-hydroxyl group of the ribose ring. This inherent conformational rigidity of the 2-thiopyrimidine ribonucleosides strongly contributes to the stability of **s^{2}U**-modified polyribonucleotides[7] and influences the decoding process by 2-thiomodified tRNAs.[40,41] Recently, hybrid DFT and MP2 calculations showed that it is distant electrostatic effects rather than the hydrogen bond between 2′-OH and the 2-tiocarbonyl function that enhance the selectivity of the C3′-*endo* conformation of ribose

Table 5
Population of sugar S and N conformers (%) calculated on the basis of $^3J_{H-H}$ coupling constants

Nucleoside	X_S	X_N	Nucleoside	X_S	X_N
H^{2}o^{4}U (**3**)	62	38	**dH^{2}o^{4}U** (**4**)	65	35
s^{2}U[35] (**1**)	29	71	**ds^{2}U** (**2**)	62	38
U[35] (**5**)	47	53	**dU** (**6**)	64	36

Table 4
Proton–proton vicinal (*n* = 3) and geminal (*n* = 2) coupling constants (Hz) for **H^{2}o^{4}U** (**3**) and **dH^{2}o^{4}U** (**4**) (25 °C in D_2O) in comparison with data for **s^{2}U** (**1**), **U** (**5**) and **ds^{2}U** (**2**), **dU** (**6**)

^nJ (H,H)	**H^{2}o^{4}U** (**3**)	**s^{2}U** (**1**)[28]	**U** (**5**)[29]	**dH^{2}o^{4}U** (**4**)	**ds^{2}U** (**2**)[31]	**dU** (**6**)[30]
2–6	2.6	–	–	2.5	–	–
5–6	7.7	8.3	8.1	7.8	–[a]	–[a]
1′–2′	5.7	2.5	4.8	6.8	6.6	6.7
1′–2″	–	–	–	6.4	6.3	6.6
2′–2″	–	–	–	14.4	–[a]	14.3
2′–3′	5.4	4.0	5.2	6.5	6.6	6.8
2″–3′	–	–	–	3.9	4.9	4.1
3′–4′	3.5	6.0	5.4	3.7	4.1	3.7
4′–5′	3.2	1.6	2.9	3.6	4.0	3.5
4′–5″	4.1	3.0	4.4	4.9	5.0	5.1
5′–5″	12.7	13.5	12.7	12.6	–[a]	12.5

[a] Not reported in cited publications.

in the 2-thiouridine molecule.[42] The desulfurization of **s^{2}U** (**1**) leading to **H^{2}o^{4}U** (**3**) dramatically changes the conformation of the sugar residue. In solution, the dominating conformer of **1** is C3′-*endo* (71%), while the conformer C2′-*endo* (62%) dominates in the case of **3**, similarly as in the desulfurized DNA nucleoside **dH^{2}o^{4}U** (65%).

2.3.2. Conformation around the glycosidic bond

The *syn*/*anti* conformation around the N-glycosidic bond was probed by means of vicinal carbon-proton couplings and NOE effects. Proton-coupled ^{13}C spectra reveal carbon-proton couplings that indicate the *syn*/*anti* conformation according to the following relationships $J_{C6-H1'} > J_{C2-H1'}$ = *anti*; $J_{C2-H1'} > J_{C6-H1'}$ = *syn*.[37,38,43,44] For example, the magnitudes of the corresponding J values $J_{C6-H1'}$ = 3.6 Hz and $J_{C2-H1'}$ = 2.4 Hz for uridine, and $J_{C6-H1'}$ = 3.6 Hz and $J_{C2-H1'}$ = 2.1 Hz for 2′-deoxyuridine, clearly confirm the preference of *anti* conformation for both nucleosides in solution.[44] The population of the *anti* conformer can be estimated from the equation% *anti* = [10-($J_{C2-H1'} + J_{C6-H1'}$)/6.4].[44] In the case of **H^{2}o^{4}U** and **dH^{2}o^{4}U**, $J_{C6-H1'}$ and $J_{C2-H1'}$ are similar (3.3–3.5 Hz), and the populations of *syn* and *anti* conformers are practically equal (Table 6).

In order to further substantiate this observation, 1D ^{1}H NOE experiments were performed[45] (Table 7). Irradiation of the H2 proton in **H^{2}o^{4}U** (**3**) gave a NOE of 19.1% for H1′ and 2.7% for H2′ and H3′. Such a relationship of NOEs is observed when the *anti* conformer is dominating. To confirm this, the H6 proton was irradiated and almost equal NOEs of 9.8% at H1′ and 8.8% at H2′ and H3′ were observed. Additionally, the signal of H5 was enhanced by 16.1%. The irradiation of H1′ gave a NOE of 17.3% at H2 and of 3.2% at H6. These results indicate that the H6 proton is situated closer to sugar H2′ and H3′ protons than H2. The opposite relationship applies to distances between H2, H6 and H1′.

Analogous experiments were performed for **dH^{2}o^{4}U** (**4**), and again NOEs were consistent with the dominating *anti* conformer. Irradiation of H2 led to NOEs of 16.0% at H1′ and 2.0% at H2′ and H3′, an analogous experiment with H6 led to NOEs of 15.4% at H5, 7.3% at H1′ and 6.0% at H2′ and H3′. The experiment with H1′ produced uneven enhancement of H2 (16.7%) and H6 (1.4%) signals. Moreover, in the last experiment the assignments of the 2.50 ppm signal as the H2″ proton was confirmed by a NOE of 6.0%.

Although measurements of vicinal proton–carbon coupling constants suggested almost equal populations of *syn* and *anti* conformers, the results of 1D NOE experiments are consistent with the dominating *anti* conformation around the N-glycosidic bond of the desulfurized nucleosides **H^{2}o^{4}U** and **dH^{2}o^{4}U**.

Table 6
Population of *anti* and *syn* conformers of **H^{2}o^{4}U** and **dH^{2}o^{4}U** calculated on the basis of $^3J_{C-H1'}$ coupling constants (Hz) in D_2O at 25 °C (in%)

Nucleoside	$^3J_{H1'-C2}$	$^3J_{H1'-C6}$	*anti*	*syn*
H^{2}o^{4}U (**3**)	3.4	3.5	48	52
dH^{2}o^{4}U (**4**)	3.3	3.4	51	49

Table 8
Population (%) of *gauche*(+), *trans* and *gauche*(−) conformers at 25 °C in D_2O

Nucleoside	$^3J_{H4'-H5'}$ [Hz]	$^3J_{H4'-H5''}$ [Hz]	*gauche*(+)	*trans*	*gauche*(−)
H^{2}o^{4}U (**3**)	3.2	4.2	62	29	9
s^{2}U (**1**)[28]	1.6	3.0	94	5	1
U (**5**)[29]	3.1	4.4	61	32	7
dH^{2}o^{4}U (**4**)	3.5	4.8	55	33	12
ds^{2}U (**2**)[31]	4.0	5.0	52	37	11
dU (**6**)[30]	3.5	5.1	49	39	12

2.3.3. Conformation around the C4′–C5′ bond

The local conformation around the C4′–C5′ bond was examined by analysis with three staggered forms: *gauche(+), trans*, and *gauche*(−), using coupling constants H5′ and H5″ protons with H4′.[37,46] The absolute assignment of the H5′ and H5″ proton in ^{1}H NMR spectra was based on the deshielding effect of the phosphate group on H5′ and H5″ in 3′-monophosphates of uridines.[47] It was shown that the H5′ and H5″ spectral region shows a similar characteristic spectral pattern: δ(H5′) > δ(H5″) and $J_{4'-5'} < J_{4'-5''}$ in a number of other nucleosides and nucleotides.[35] Therefore, it appears reasonable to assume that for all these nucleosides where this pattern is observed the more shielded proton is assigned as H5″.

The specific assignments of H5′ and H5″ methylene protons in the ^{1}H NMR spectra of **H^{2}o^{4}U** and **dH^{2}o^{4}U** and measurements of the $J_{4'-5'}$ and $J_{4'-5''}$ proton–proton coupling constants, enabled the unequivocal determination of the population of three exocyclic C4′–C5′ rotamers *gauche(+), trans* and *gauche*(−). The results are given in Table 8. All of the presented nucleosides exhibit preferentially *gauche*(+) conformation. An appreciably higher population of the *gauche*(+) rotamer in **s^{2}U** (94%) than in the other listed nucleosides (50–60%) is due to the dominant C3′-*endo*, *anti* conformation of 2-thioribonucleoside.[5,40]

3. Conclusions

The modified nucleosides **H^{2}o^{4}U** (**3**) and **dH^{2}o^{4}U** (**4**) were synthesized in good overall yields by desulfurization of appropriate 2-thiouracil nucleosides with *m*-chloroperbenzoic acid. X-ray crystallography and NMR spectroscopy made it possible to determine the conformational parameters of **H^{2}o^{4}U** and **dH^{2}o^{4}U** nucleosides, and compare them to reference ribonucleosides **s^{2}U**, **U** and 2′-deoxynucleosides **ds^{2}U**, **dU**. In the DNA nucleoside series, desulfurization of 2′-deoxy-2-thiouridine does not remarkably influence the sugar moiety conformation in solution. The **dH^{2}o^{4}U** nucleoside exhibits a slightly higher proportion of C2′-*endo* sugar pucker than **ds^{2}U** or **dU**. However, in the solid state **dH^{2}o^{4}U** adopts C3′-*endo*

Table 7
Results of 1D NOE experiments for **H^{2}o^{4}U** (**3**) and **dH^{2}o^{4}U** (**4**) (25 °C in D_2O at 700 MHz)

Compound	Irradiated proton	NOE (%)	Compound	Irradiated proton	NOE (%)
H^{2}o^{4}U	H2	H1′ (19.1)	**dH^{2}o^{4}U**	H2	H1′ (16.0)
		H2′ + H3′ (2.7)			H2′ + H3′ (2.0)
	H6	H1′ (9.8)		H6	H1′ (7.3)
		H2′ + H3′ (8.8)			H2′ + H3′ (6.0)
		H5 (16.1)			H5 (15.4)
	H1′	H2 (17.3)		H1′	H2 (16.7)
		H6 (3.2)			H6 (1.4)
					H2″ (6.0)
					H4′ (2.3)

conformation, rarely found in the crystals of 2′-deoxynucleosides, resulting from a specific hydrogen bond network. By contrast, in the RNA nucleoside series the conformational characteristics of the ribofuranose ring are dramatically affected by desulfurization, and the 4-pyrimidinone nucleoside **3** predominantly takes the C2′-*endo* form (S conformer) in aqueous solution. This ribose pucker is also fixed for **H^{2}o^{4}U** molecules in the crystal state. The observed difference between the **s^{2}U** and **H^{2}o^{4}U** ribose folding (71% of N conformer for **s^{2}U**, 62% of S conformer for **H^{2}o^{4}U**) may have important biological consequences as desulfurization of natural 2-thiouridines may occur under conditions of oxidative stress in the cell.

Additionally, the absence of a 2-thiocarbonyl/2-carbonyl acceptor and a N3-H donor in the structure of **H^{2}o^{4}U** and **dH^{2}o^{4}U** may play a significant role in modifying hydrogen bonding specificity with regard to the pair with purine nucleosides. The development of an efficient synthesis of modified nucleosides allows for the generation of phosphoramidites or nucleotide triphosphates, followed by incorporation into oligonucleotides for further biophysical studies of modified sequences. It remains to be determined how these modified nucleosides behave within the context of the oligonucleotide. We are currently assessing the conformational impact of **H^{2}o^{4}U** (**3**) and **dH^{2}o^{4}U** (**4**) within the context of model DNA and RNA oligonucleotides.

4. Experimental

4.1. Synthesis of modified nucleosides

4.1.1. General methods

All reagents were commercially available. CH_2Cl_2 was predried with K_2CO_3 and distilled from K_2CO_3/P_2O_5; pyridine was stirred with NaOH, refluxed over ninhydrin for several hours, distilled once from ninhydrin and once from CaH_2; MeOH was distilled from Mg; toluene was distilled from Na. Silica gel column chromatography was performed using Merck silica gel 60 (230–400 mesh). TLC was performed on analytical silica plates (Kieselgel 60 F_{254}/0.2 mm thickness). NMR spectra were obtained on Bruker Avance 250 ^{1}H and ^{13}C NMR chemical shifts are in ppm, and *J* values are in Hz. Mass spectra were obtained on Finnigan MAT 95 spectrometer.

4.1.2. General procedure for oxidative desulfurization

Nucleoside **7** or **8** (3 mmol) was dissolved in the mixture of anhydrous CH_2Cl_2 (25 mL) and anhydrous pyridine (5 ml) and then 0.2 M *m*CPBA in CH_2Cl_2 (30 ml) was added. The solution was stirred at room temperature and after 3 h reaction was judged to be complete by TLC (2% MeOH in $CHCl_3$). The mixture was washed with 10% Na_2SO_3 (50 ml), 5% $NaHCO_3$ (50 ml), dried over $MgSO_4$ and evaporated. The crude product was coevaporated with toluene (3×10 ml) and purified by silica gel column chromatography (0–5% MeOH in $CHCl_3$) to give **9** or **10**, respectively.

4.1.2.1. 2′,3′,5′-Tri-*O*-benzoyl-1-(β-D-ribofuranosyl)-4-pyrimidinone (9). Yield = 81%; TLC R_f: 0.26 (CH_3Cl/CH_3OH, 95:5); 0.23 (benzene/EtOAc, 7:3); ^{1}H NMR ($CDCl_3$) δ 4.73 (dd, 1H, *J* = 2.6 Hz, 12.0 Hz, H5″), 4.84 (m, 1H, H4′), 4.88 (dd, 1H, *J* = 2.5 Hz, 12.0 Hz, H5′), 5.67 (pt, 1H, *J* = 5.8 Hz, H3′), 5.83 (m, 2H, H2′, H5), 6.13 (d, 1H, *J* = 7.9 Hz, H1′), 7.36–7.68 (m, 10H, H6, Bz), 7.90–8.12 (m, 6H, Bz), 8.35 (d, 1H, *J* = 2.6 Hz, H2).

4.1.2.2. 3′,5′-Di-*O* -toluyl-1-(β-D-2′-deoxyribofuranosyl)-4-pyrimidinone (10). Yield = 83%; TLC R_f: 0.23 (CH_3Cl/CH_3OH, 95:5); ^{1}H NMR ($CDCl_3$) δ 2.43 (d, 6H, *J* = 2.1 Hz, CH_3), 2.50 (m, 1H, H2″), 2.77 (ddd, 1H, *J* = 1.8 Hz, 5.5 Hz, 14.3 Hz, H2′), 4.60 (m, 1H, H4′), 4.71 (m, 2H, H5′ and H5″), 5.66 (m, 1H, H3′), 5.95 (dd, 1H, *J* = 5.5 Hz, 8.5 Hz, H1′), 6.14 (d, 1H, *J* = 7.9 Hz, H5), 7.27 (m, 4H, Ar), 7.56 (dd, 1H, *J* = 2.5 Hz, 7.9 Hz, H6), 7.91 (m, 4H, Ar), 8.61 (d, 1H, *J* = 2.5 Hz, H2).

4.1.3. General procedure for sugar deprotection

Compound **9** or **10** (2.9 mmol) was suspended in 0.1 M solution of MeONa in MeOH. The mixture was stirred at room temperature for 30 min. After this time TLC analysis (20% MeOH in $CHCl_3$) showed that the starting material was completely consumed. The mixture was worked up with Dowex (pyridine salt form) and after filtered off the resin, the remaining solution was evaporated in vacuo and coevaporated with toluene. The oily residue was dissolved in water (15 ml) and washed with diethyl ether (3×10 ml). The aqueous layer was frozen and lyophilized to give **3** or **4**, respectively.

4.1.3.1. 1-(β-D-Ribofuranosyl)-4-pyrimidinone (3). Yield = 92%, TLC R_f: 0.23 (CH_3Cl/CH_3OH, 80:20), 0.42 (isopropanol/aq 25% NH_3/H_2O, 7:1:2), 0.50 (*n*-butanol/ethanol/H_2O, 40:11:19); ^{1}H NMR (D_2O) δ 3.75 (dd, 1H, *J* = 4.1 Hz, 12.7 Hz, H5″), 3.82 (dd, 1H, *J* = 3.2 Hz, 12.7 Hz, H5′), 4.19 (ddd, 1H, *J* = 3.2 Hz, 3.5 Hz, 4.1 Hz, H4′), 4.24 (dd, 1H, *J* = 3.5 Hz, 5.4 Hz, H3′), 4.32 (dd, 1H, *J* = 5.4 Hz, 5.7 Hz, H2′), 5.58 (d, 1H, *J* = 5.7 Hz, H1′), 6.43 (d, 1H, *J* = 7.7 Hz, H5), 8.07 (dd, 1H, *J* = 2.6 Hz, 7.7 Hz, H6), 8.58 (d, 1H, *J* = 2.6 Hz, H2); ^{13}C NMR (D_2O) δ 60.9 (C5′), 70.1 (C3′), 75.0 (C4′), 86.1 (C2′), 94.8 (C1′), 112.5 (C5), 140.7 (C6), 152.2 (C2), 173.8 (C4); FAB MS *m/z* 229 $[M+H]^+$; 227 $[M-H]^-$; HRMS calculated for $C_9H_{13}O_5N_2$ ($[M+H]^+$) 229.0835, found *m/z* 229.0813; UV (H_2O) λ_{max} 243 nm; λ_{min} 210 nm; ε = 7200 $M^{-1}cm^{-1}$.

4.1.3.2. 1-(β-D-2′-Deoxyribofuranosyl)-4-pyrimidinone (4). Yield = 91%, TLC R_f: 0.23 (CH_3Cl/CH_3OH, 80:20), 0.45 (isopropanol/aq 25% NH_3/H_2O, 7:1:2); ^{1}H NMR (D_2O) δ 2.44 (ddd, 1H, *J* = 6.5 Hz, 6.8 Hz, 14.4 Hz, H2′), 2.50 (ddd, 1H, J = 3.9 Hz, 6.4 Hz, 14.4 Hz, H2″), 3.64 (dd, 1H, *J* = 4.9 Hz, 12.6, H5″), 3.72 (dd, 1H, *J* = 3.6 Hz, 12.6, H5′), 4.00 (ddd, 1H, *J* = 3.6 Hz, 3.7 Hz, 4.9 Hz, H4′), 4.40 (ddd, 1H, *J* = 3.7 Hz, 3.9 Hz, 6.5 Hz, H3′), 5.93 (dd, 1H, *J* = 6.4 Hz, 6.8 Hz, H1′), 6.33 (d, 1H, *J* = 7.8 Hz, H5), 7.95 (dd, 1H, *J* = 2.5 Hz, 7.8 Hz, H6), 8.49 (d, 1H, *J* = 2.5 Hz, H2), ^{13}C NMR (D_2O) δ 38.28 (C2′), 59.22 (C5′), 68.59 (C3′), 85.77 (C4′), 90.04 (C1′), 110.45 (C5), 139.24 (C6), 150.49 (C2), 171.85 (C4); FAB MS *m/z* 213 $[M+H]^+$; HRMS calculated for $C_9H_{13}O_4N_2$ ($[M+H]^+$) 213.0834, found *m/z* 213.0815; UV (H_2O) λ_{max} = 241 nm, ε = 16,200 M^{-1} cm^{-1}.

4.2. X-ray structure analysis

Crystal data for **dH^{2}o^{4}U** (**4**): $C_9H_{12}N_2O_4$, *M* = 212.21, orthorombic, space group $P2_12_12_1$, *a* = 7.6490(2) Å, *b* = 8.1401(2) Å, *c* = 15.2382(4) Å, *V* = 948.79(4) Å^3, *Z* = 4, D_x = 1.486 g cm^{-3}, *T* = 293 K, μ = 0.118 mm^{-1}, λ = 0.71073 Å, data/parameters = 1669/139; Flack *x* = 0.0(9), final R_1 = 0.0226.

Crystal data for **H^{2}o^{4}U** (**3**): $C_9H_{12}N_2O_5$, *M* = 228.21, orthorombic, space group $P2_12_12_1$, *a* = 6.5028(2) Å, *b* = 9.4577(3) Å, *c* = 15.7834(4) Å, *V* = 970.70(5) Å^3, *Z* = 4, D_x = 1.561 g cm^{-3}, *T* = 293 K, μ = 0.129 mm^{-1}, λ = 0.71073 Å, data/parameters = 1726/147; Flack *x* = 0.2(8), final R_1 = 0.0221.

Crystal data for **ds^{2}U** (**2**): $C_9H_{12}N_2O_4$ S, *M* = 244.28, triclinic, space group P1, *a* = 10.377(3) Å, *b* = 11.784(3) Å, *c* = 13.434(4) Å, α = 100.2(1)°, β = 112.6(1)°, γ = 103.9(1)°, *V* = 1403.4(7) Å^3, *Z* = 4, D_x = 1.156 g cm^{-3}, *T* = 293 K, μ = 0.231 mm^{-1}, λ = 0.71073 Å, data/parameters = 4410/580; Flack *x* = 0.3 (1), final R_1 = 0.0631.

Crystals of **H^{2}o^{4}U** (**3**) and **dH^{2}o^{4}U** (**4**) as well as **ds^{2}U** (**2**) were obtained by slow evaporation from their aqueous methanolic solutions. The measurements of the crystals were performed on a SMART diffractometer with graphite-monochromated MoKα radiation (λ = 0.71073 Å) at room temperature. The structures were

solved by direct method and refine with SHELXTL.[48] E-maps provided positions for all non-H-atoms. The full-matrix least-squares refinement was carried out on F^2's using anisotropic temperature factors for all non-H-atoms. All C-bound H atoms were placed in idealized locations and refined using a riding model, with C−H = 0.93 Å and $U_{iso}(H) = 1.2U_{eq}(C)$.

Special comments to structure **ds^{2}U** (**2**): The structure was solved and refined in P1 non-centrosymmetric space group with four, insignificantly differing, molecules in independent crystallographic unit. In brought channel shaped by four molecules down a-axis numbers of disordered solvents (water and ethanol) are localized. The final refinement of that structure was based on SQUEEZE procedure from PLATON packet.[49]

Crystallographic data (excluding structural factors) for the structure reported in this paper have been deposited at the Cambridge Crystallographic Data Center and allocated with the deposition numbers: CCDC 798540–798542 for compounds **2**,**3**,**4** Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EW, UK (Fax: Int code + (1223)336 033; E-mail: deposit@ccdc.cam.ac.uk).

4.3. NMR studies of modified nucleosides

The one- and two-dimensional NMR experiments were recorded on 700.2 MHz spectrometer at 25 °C in D_2O with DSS as the internal standards. The samples for the NMR measurements were prepared by dissolving 6–8 mg of the nucleosides in 0.6 mL of D_2O. Spectra were processed by means of TopSpin 2.1 software (Bruker BioSpin). In the case of overlapping signals in ^{1}D, ^{1}H NMR spectra the DAISY (Bruker BioSpin) deconvolution procedure was applied. ^{1}H–^{13}C vicinal coupling constants were derived from the coupled ^{13}C NMR spectra and verified by J-HMBC experiments.[50]

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Supplementary data

Supplementary data (^{1}H, ^{13}C, ^{1}D NOE NMR spectra for **H^{2}o^{4}U** (**3**), **dH^{2}o^{4}U** (**4**) and crystallographic data for compounds **3**, **4**, **2** (**ds^{2}U**)) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.02.008.

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