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The influence of the C5 substituent on the 2-thiouridine desulfuration pathway and the conformational analysis of the resulting 4-pyrimidinone products

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ABSTRACT

In recent years, increasing attention has been focused on the posttranscriptional modifications present in transfer RNAs (tRNAs), which have been suggested to constitute another level of regulation of gene expression. The most representative among them are the 5-substituted 2-thiouridines (R5S2U), which are located in the wobble position of the anticodon and play a fundamental role in the tuning of the translation process. On the other hand, sulfur-containing biomolecules are the primary site for the attack of reactive oxygen species (ROS). We have previously demonstrated that under in vitro conditions that mimic oxidative stress in the cell, the S2U alone or bound to an RNA chain undergoes desulfuration to yield uridine and 4-pyrimidinone nucleoside (H2U) products. The reaction is pH- and concentration-dependent. In this study, for the first time, we demonstrate that the substituent at the C5 position of the 2-thiouracil ring of R5S2Us influences the desulfuration pathway, and thus the products ratio. As the substituent R changes, the amount of R5H2U increases in the order H– > CH_3O– > $CH_3OC(O)CH_2$– > $HOC(O)CH_2NHCH_2$– ≈ CH_3NHCH_2–, and this effect is more pronounced at lower pH. The conformational analysis of the resulting R5H2U products indicates that independent of the nature of the R5 substituent, the R5H2U nucleosides predominantly adopt a C2′-*endo* sugar ring conformation, as opposed to the preferred C3′-*endo* conformation of the parent R5S2Us.

1. Introduction

5-Substituted 2-thiouridines (R5S2U) are modified nucleosides located in the wobble position of the anticodon (position 34) in transfer RNAs (tRNAs).[1] Their structural features play a fundamental role in the codon reading and efficiency of the translation process. These features are related to the presence of a sulfur atom at position 2 as well as the presence of a substituent at the C5 atom of the uracil moiety. Replacement of the oxygen atom at C2 with a sulfur atom shifts the puckering equilibrium of the ribose ring towards the C3′-*endo* form, which is present in A-type RNA helices and enhances the base stacking in the RNA chain.[2] In this conformation, 2-thiouridines predominantly recognize adenosine as the third letter of the codon via a classical Watson–Crick base pairing, while the wobble base pairing with a G residue at the third position of the codon is limited due to the less preferred formation of a hydrogen bond between the sulfur acceptor of S2U and the N1H donor of G.[3] The influence of wobble R5S2U nucleosides on codon–anticodon recognition is strictly dependent on the characteristics of the substituent at the C5 position of the heterobase.[4,5] The role of 5-substituents in tRNA wobble 2-thiouridines/uridines has been widely studied and has recently been excellently summarized by Takai and Yokoyama,[4a] who noted that the electronic characteristics of the 5-substituent play a role in 5′-NNA/G-3′ codon reading. All substituents present in wobble uridines/2-thiouridines which contain X-CH_2– type groups[4a] exhibit low electron-donating characteristics; thus, their contribution to the electron density of the pyrimidine ring is limited. However, the situation becomes more complicated when the X residue contains an amino group, such as in a methylaminomethyl (CH_3NHCH_2–, mnm) substituent. According to the suggestion of Takai and Yokoyama, such a substituent can be protonated under physiological pH. Therefore, the preferred formation of the tautomeric structures of 5-substituted uridines/2-thiouridines is observed in which the positively charged protonated 5-$CH_3NH(H^+)CH_2$ group is accompanied by the negatively

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charged sulfur in the thiolate residue of the heterobase (Fig. 1). In this case, the heterobase moiety adopts a double bond system, which provides two acceptors, one at the O(4) atom and one at the N(3) atom, that are available for the formation of the two hydrogen bonds with a complementary unit. Interestingly, the same nucleobase double bond pattern is present in the recently discovered S-geranyl 2-thiouridines (R5geS2Us, where R = mnm or carboxymethylaminomethyl ($HOC(O)CH_2NHCH_2$–, cmnm))[6] in which the alkylated sulfur atom of 2-thiouridine leads to the replacement of the N(3)H donor with the N(3) acceptor center (Fig. 1). These two types of wobble nucleosides, R5S2U and R5geS2U, are present in the first letter of the anticodon of the tRNA specific for glutamine, glutamic acid and leucine and they preferentially recognize the 3′-G-ending codons. In contrast, 3′-A-ending codons are preferentially read by R5S2U wobble units with R = H, methylcarboxymethyl ($CH_3OC(O)CH_2$–, mcm) or carboxymethyl ($HOC(O)CH_2$–, cm) substituents. We have recently published data on the preferred pathway of the desulfuration reaction performed on 2-thiouridine, either alone or assembled into the RNA chain, demonstrating that the 4-pyrimidinone riboside (H2U, **3a**, R = H, Fig. 1) is the main product of this transformation.[7,8] This nucleoside contains a double bond system similar to the zwitterionic form of R5S2U as well as R5geS2U units, where R = (c)mnm, and forms more stable RNA duplexes when base-paired with G, and not with A.[9]

An efficient transformation of S2U to H2U occurs under in vitro conditions that mimic oxidative stress in the cell (100 mM H_2O_2) and is pH- and concentration-dependent.[8,10] The preliminary data indicate that the RNA chain containing 4-pyrimidinone riboside is prone to depyrimidination and sugar–phosphate backbone cleavage.[7,11] We hypothesize that the sulfur atom in S2U-tRNAs may be a primary site for ROS attack in the cells. The resulting major H2U-tRNA lesion, in addition to changing the A to G codon reading preference, might be a site of RNA chain cleavage via abasic RNA intermediate.[12,13] This hypothesis, although already confirmed by in vitro experiments, has not been proven in the cell, and studies focused on the effect of S2U-tRNA damage on cellular oxidative stress are currently being performed in our laboratory. One cannot exclude the fact that the 'chemically driven' tRNA damage is accompanied by anticodon-specific nuclease-assisted cleavage induced by oxidative stress, as has been demonstrated in the literature.[9,14]

In this study, we discuss the influence of the C5 substituent on the R5S2U desulfuration pathway performed in the presence of aqueous H_2O_2, which are conditions that mimic oxidative stress in the cell (Scheme 1). For the studies, the 5-substituents differently affecting the decoding properties of the wobble 2-thiouridines were selected, as mcm and H, which preferentially recognize the 3′-A-ending codons, and mnm and cmnm which recognize also the 3′-G-ending codons. In addition, a 2-thioanalog of the wobble 5-methoxyuridine (mo5U) was also selected (non-identified in the natural tRNA) to study the influence of the X-O- type substituent on the evaluated process. The ratio of the R5U/R5H2U reaction products was determined by ^1H NMR measurements and was found to be strongly dependent on the characteristics of the C5 substituent and the reaction conditions (water or buffered aqueous solutions of pH 6.6–7.6). The availability of detailed spectral and conformational data (evaluated based on the values of the ^1H–^1H and ^1H–^{13}C coupling constants) of a set of 4-pyrimidinone nucleosides **3a**–**e** obtained independently (under 'organic conditions'), as well as the respective spectral and conformational data for the parent 5-substituted 2-thiouridines **1a**–**e** and uridines **2a**–**e** (obtained *in house*) enabled a complete comparable conformational analysis. The results aided in the elucidation of how the loss of the 2-thiocarbonyl/carbonyl function changes the structural and electronic features of the resulting 4-pyrimidinone nucleosides in solution and how these features may influence the hybridization properties of RNA duplexes containing these units.

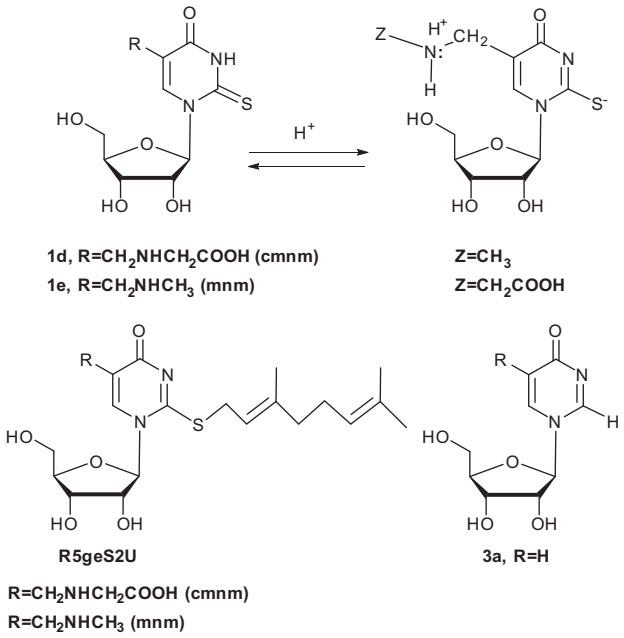
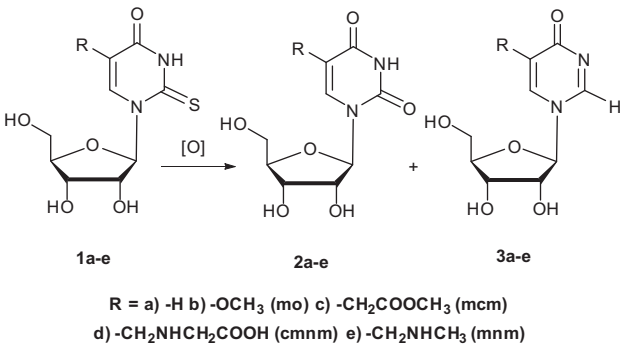


Figure 1. Structure of modified nucleosides S2U, geS2U and H2U.



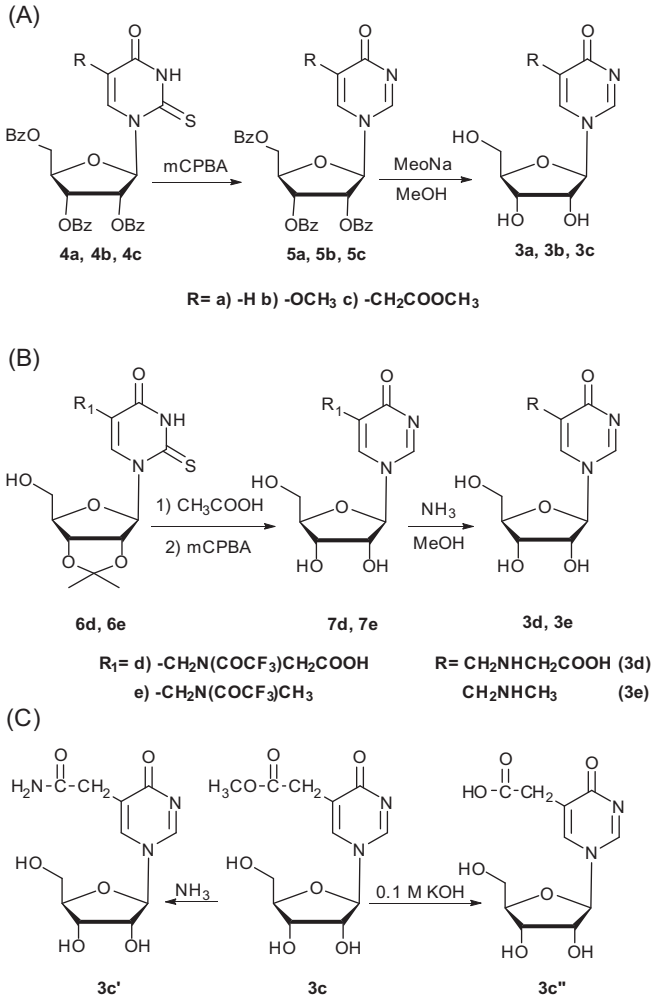
Scheme 1. General scheme of desulfuration of 2-thiouridines **1a**–**e** with hydrogen peroxide in aqueous solution.

2. Results and discussion

2.1. Chemistry

In our previous studies, we have shown that the 5′,3′,2′-*O*-benzoylated derivative of 2-thiouridine could be exclusively transformed into H2U (**3a**, R = H) under 'organic conditions' by treating the starting 2-thiouridine derivative with *trans*-2-phenylsulfonyl-3-phenyloxaziridine (PSO) in pyridine or with *meta*-chloroperbenzoic acid (*m*CPBA) in a mixture of dichloromethane/pyridine at room temperature.[15] *m*-Chloroperbenzoic acid was also successfully applied to the selective oxidative desulfuration of 5′-DMT (4,4′-dimethoxytrityl)-protected 2′-deoxy-5-methyl-2-thiouridine.[16]

In this study, we successfully applied this 'organic' methodology to the oxidative desulfuration of the *per*-*O*-benzoylated derivatives of 5-methoxy-2-thiouridine (mo5S2U) **4b** and 5-methylcarboxymethyl-2-thiouridine (mcm5S2U) **4c** (Scheme 2A) as well as to the 2′,3′-*O*-isopropylidene derivative of N-$COCF_3$-protected cmnm5S2U **6d** and the 2′,3′-*O*-isopropylidene derivative of



Scheme 2. Synthetic pathways for the preparation of 5-substituted 4-pyrimidinone nucleosides **3b**–**e**, starting either from perbenzoylated derivatives (A) or *N*-protected 2′,3′-*O*-isopropylidene derivatives of 5-substituted-2-thiouridines **b**–**e** (B) (the descriptions are in Scheme 1). The synthesis of amido- and carboxy-derivatives of **3c** (**3c**′ and **3c**″, respectively) is shown in Scheme C.

N-$COCF_3$-protected mnm5S2U **6e** (Scheme 2B). Further deprotection procedures under alkaline conditions afforded the final 5-substituted 4-pyrimidinone nucleosides **3b**–**e**, where R = mo (b), mcm (c), cmnm (d) or mnm (e). Additionally, 4-pyrimidinone nucleoside **3c** with a methyl ester functionality in the 5-substituent was transformed into the corresponding amide and carboxylic derivatives **3c**′ and **3c**″, respectively (Scheme 2C). All of the 4-pyrimidinone nucleosides were purified by silica gel column chromatography and were obtained as white foams that were characterized by spectral analysis (^{1}H and ^{13}C NMR and fast atom bombardment mass spectrometry (FAB-MS)). The obtained data are included either in Experimental section or in Supplementary material. Note that that **3a**–**c**, **3c**′ and **3c**″ were sufficiently stable in aqueous solution, while **3d** and **3e** underwent decomposition under the same conditions. Due to the instability of **3d** and **3e**, only limited spectral analysis of these nucleosides could be performed.

The required substrates, 2-thiouridines **1a**–**e,** as well as their parent uridine derivatives **2a**–**e**, are known compounds, and their syntheses were previously established either in our laboratory or elsewhere using either the methodology of *N*-glycosidic bond formation (nucleosides with R = H, mo, mcm)[2a,17] or by the introduction of a 5-substituent into the appropriate derivative of 2-thiouridine/uridine (nucleosides with R = mnm and cmnm).[18] These syntheses were repeated, the sets of **1a**–**e** and **2a**–**e** were obtained, and their structures were confirmed. The respective ^{1}H NMR spectra of compounds used in these studies are given in Supplementary material.

2.2. Conformational analysis of 5-substituted 4-pyrimidinone nucleosides

Generally, nucleoside conformation is described relative to three different aspects, namely (i) the dominant sugar ring pucker, (ii) the preferred glycosidic bond arrangement and (iii) the orientation of the 5′-hydroxyl group against the furanose ring.[19] In the present studies, a conformational analysis of 5-substituted 4-pyrimidinone nucleosides **3**, as well as their parent 2-thiouridines **1** and uridines **2** was performed based on the NMR data collected in the aqueous solutions.

2.2.1. Conformation of the sugar moiety

It is assumed that the sugar ring of the nucleoside in solution exists as an equilibrium mixture of the two puckered forms: C2′-*endo* (S conformer) or C3′-*endo* (N conformer).[20] The percentage of S and N conformers can be estimated based on the values of the $^3J_{H1'-H2'}$ and $^3J_{H3'-H4'}$ coupling constants according to the following equations:% C2′-*endo* = 100 $J_{H1'-H2'}/(J_{H1'-H2'} + J_{H3'-H4'})$ and % C3′-*endo* = 100 − % C2′-*endo*.[21] Thus, ^{1}H NMR data were collected, and experimental $^3J_{H-H}$ coupling constants were extracted for all of the screened compounds. These data were used for the calculation of a population of C3′-*endo* conformers of 4-pyrimidinone nucleosides **3** and their parent 2-thiouridines **1** and uridines **2** for the R substituents abbreviated as **a**–**e** as well as **c**′ and **c**″ (Table 1).

Previous studies have shown that the population of the C3′-*endo* conformer of 2-thiouridines is markedly higher than that of the analogous uridines.[2b,c,22] The rigidity of the C3′-*endo* form of S2U is caused by the steric effect of the large 2-thiocarbonyl group of the heterobase and the 2′-hydroxyl group of the ribose ring.[23] This intrinsic conformational rigidity strongly contributes to the stability of the duplexes containing S2U-modified strands, thereby influencing the decoding process performed by 2-thiouridine-modified tRNA.[24] Deprivation of the sulfur moiety in 2-thiouridine **1** leads to a dramatic change in the sugar ring puckering in the resulting H2U nucleosides **3**. This is observed for all seven cases analyzed (Table 1) for which 2-thiouridines **1a**–**e** adopt the C3′-*endo* conformation in 71–82%, while their desulfuration products **3a**–**e** adopt the C3′-*endo* conformation only in 37–43%. This result indicates that the C2′-*endo* conformation is dominant in the equilibrium of the sugar conformers of 4-pyrimidinone nucleosides in solution. Interestingly, the substituent at the C5 position alters the sugar ring puckering to a lesser extent, although it is clearly observed that in the case of mo5H2U, the more electron-donating characteristics of the methoxyl substituent shifts the sugar ring puckering equilibrium towards the C3′-*endo* conformation (43% of **3b** compared with 37–39% of the remaining R5H2Us studied).

2.2.2. Conformation around the glycosidic bond

The *syn*/*anti* conformation around the *N*-glycosidic bond was investigated using vicinal carbon–proton coupling. Proton-coupled ^{13}C spectra reveal carbon–proton couplings that indicate the *syn*/*anti* conformation according to the following relationships: $J_{C6-H1'} > J_{C2-H1'}$ for the *anti* conformation and $J_{C2-H1'} > J_{C6-H1'}$ for the *syn* conformation. The data presented in Table 2 indicate that the **3a**–**c** H2U units adopt a slightly prevailing *syn* conformation around the *N*-glycosidic bond, in contrast to the predominant *anti* conformation of the parent R5S2Us and R5Us.[25] This observed feature originates from the lack of the sulfur/oxygen moiety at C2 of the 2-thiouracil/uracil ring, which ensures fast rotation around

Table 1
Ribose ring puckering calculated from the coupling constants (J in Hz)

Nucleoside	Number	$J_{H1'-H2'}$	$J_{H3'-H4'}$	C3′-*endo* (%)
S2U	**1a**	2.5	6.0	71
U	**2a**	4.8	5.4	53
H2U	**3a**	5.7	3.5	38
mo5S2U	**1b**	1.8	8.1	82
mo5U	**2b**	4.1	5.6	58
mo5H2U	**3b**	5.3	4.0	43
mcm5S2U	**1c**	2.3	7.8	77
mcm5U	**2c**	4.5	5.7	56
mcm5H2U	**3c**	5.8	3.6	38
ncm5S2U	**1c′**	2.0	7.4	79
ncm5U	**2c′**	4.4	5.5	56
ncm5H2U	**3c′**	5.8	3.7	39
cm5S2U	**1c″**	2.3	7.5	77
cm5U	**2c″**	4.4	5.6	56
cm5H2U	**3c″**	5.8	3.7	39
cmnm5S2U	**1d**	1.7	7.7	82
cmnm5U	**2d**	4.2	5.7	58
cmnm5H2U	**3d**	5.7	3.6	39
mnm5S2U	**1e**	2.4	7.7	76
mnm5U	**2e**	4.2	5.6	57
mnm5H2U	**3e**	5.9	3.4	37

the *N*-glycosidic bond; thus, similar populations of both of the possible conformers are feasible. Therefore, there is no significant preference of one, neither *syn* nor *anti*, conformation. Again, the 5-methoxyl substituent that has more electron-donating characteristics than hydrogen drives the nucleobase towards the *syn* conformation (34% of the *anti* conformation of mo5H2U compared with 48% of H2U).

2.2.3. Conformation around the C4′–C5′ bond

The local conformation around the C4′–C5′ bond was examined by analyzing the contribution of each of the three distinct forms, *gauche* (+), *trans*, and *gauche* (−), using the coupling constants of the H5′ and H5″ protons with H4′.[25a,26] The absolute assignment of the H5′ and H5″ protons in the ^{1}H NMR spectra was evaluated using the rules elaborated based on the deshielding effect of the phosphate group on H5′ and H5″ in the 3′-monophosphates of the uridines.[27] It was shown that the H5′ and H5″ spectral regions show similar characteristic signal patterns, δ(H5′) > δ(H5″) and $J_{H4'-H5'} < J_{H4'-H5''}$, to a number of other nucleosides and nucleotides.[25a,28] Therefore, it appears reasonable to assume that for all of these nucleosides, where this pattern is observed, the more shielded proton is assigned as H5″. The specific assignments of the H5′ and H5″ methylene protons in the ^{1}H NMR spectra of 4-pyrimidinone nucleosides **3a–c** and the measurements of the $J_{H4'-H5'}$ and $J_{H4'-H5''}$ proton–proton coupling constants enabled the determination of the populations of the three exocyclic C4′–C5′ rotamers *gauche* (+) (g$^+$), *trans* (t) and *gauche* (−) (g$^-$) (Table 3). The obtained data demonstrate that for the R5H2U nucleosides, the prevailing conformation around the C4′–C5′ bond is the one described as *gauche* (+). This feature is similar to the prevailing structure of R5S2Us and R5Us in aqueous solutions.

Table 2
Population of the conformers of R5H2U nucleosides around the *N*-glycosidic bond

Nucleoside	Number	$J_{C2-H1'}$	$J_{C6-H1'}$	% *anti*/*syn*
H2U	**3a**	3.4	3.5	48:52
mo5H2U	**3b**	3.8	4.0	34:66
mcm5H2U	**3c**	3.6	3.7	42:58
ncm5H2U	**3c′**	3.5	3.6	45:55
cm5H2U	**3c″**	3.5	3.8	43:57

Table 3
Population of the conformers of R5H2U nucleosides around the C4′–C5′ bond

Nucleoside	$J_{H4'-H5'}$	$J_{H4'-H5''}$	% g$^+$	% t	% g$^-$
H2U (**3a**)	3.1	4.0	65	27	8
mo5H2U (**3b**)	2.9	3.3	74	20	6
mcm5H2U (**3c**)	3.1	4.0	65	27	8
ncm5H2U (**3c′**)	3.0	4.1	65	28	7
cm5H2U (**3c″**)	3.0	3.9	67	26	7

The obtained data for the sugar ring conformation of H2U in solution are in agreement with those obtained for the H2U crystal structure.[29] In the crystal, the H2U nucleoside exclusively adopts the C2′-*endo* sugar ring conformation. The above conformational analysis of the R5H2U units clearly demonstrates that the substituents at C5 on the 4-pyrimidinone ring have only a slight effect on their structure. The only substituent that influences the conformational parameters slightly more than the others is the 5-methoxyl group in mo5H2U for which the highest population of the C3′-*endo*, *syn* and g$^+$ conformer is observed compared with the other R substituents.

To summarize this conformational analysis, we demonstrate for the first time that independent of the nature of the R5 substituent, the R5H2U nucleosides predominantly adopt a C2′-*endo* sugar ring conformation. This feature, along with the restructured hydrogen bond donor/acceptor pattern, has dramatic consequences on the codon–anticodon interactions if these H2U-type nucleosides are located in a wobble position of the anticodon, as in the case for those mentioned above, that is, (c)mnm5geS2U. NMR data for the geS2U nucleoside obtained by Dumelin et al.[6] and by our group[30] have shown that this nucleoside preferentially adopts a C2′-*endo* sugar ring conformation. The respective coupling constants $J_{1',2'}$ are 5.4[6] and 5.7 Hz,[8] respectively, in a similar range to those assigned in the reported studies ($J_{1',2'}$ = 5.3–5.9 Hz for R5H2U units, see Table 1). Note that in the Yokoyama model of wobble recognition,[4a] the protonated mnm5S2U nucleoside is suggested to preferentially read 3′-G-ending codons via a H2U-type structure in the wobble base pair, assuming that the wobble nucleoside is constrained in the C3′-*endo* conformation. Our results, however, show that the H2U-type units preferentially adopt the C2′-*endo* sugar ring puckering, suggesting that in this conformation, H2U-type thiouridines are more flexible and prefer the decoding of guanosine at the 3′-end of the codon according to the wobble mode.

2.3. Desulfuration of 5-substituted 2-thiouridines in aqueous buffered solutions

To understand the influence of the C5 substituent on the desulfuration pathway of the R5S2U units under conditions that may occur during oxidative stress in the cell, desulfuration reactions were performed in the presence of aqueous H_2O_2, either in water or in phosphate buffer (pH 6.6, 7.2 and 7.6), at 25 °C (Scheme 1). The progress of the reaction was monitored by ^{1}H NMR spectroscopy. For this reaction, 10 mM solutions of R5S2U substrate **1** and 100 mM H_2O_2 were used to achieve sufficient substrate transformation and to ensure the proper ^{1}H signal integration required to monitor the ratio of the reaction mixture components. Figure 2 represents an example of a set of three spectra for S2U **1a**, U **1b** and H2U **1c** and the signals (preferably H6) that were used to evaluate the ratio of the reaction mixture components (description of the spectra are provided in Supplementary material, Fig. S1). The remaining sets of spectra for R5S2U, R5U and R5H2U, where R = mcm, mnm, cmnm and mo, are given in Figures S2–S5.

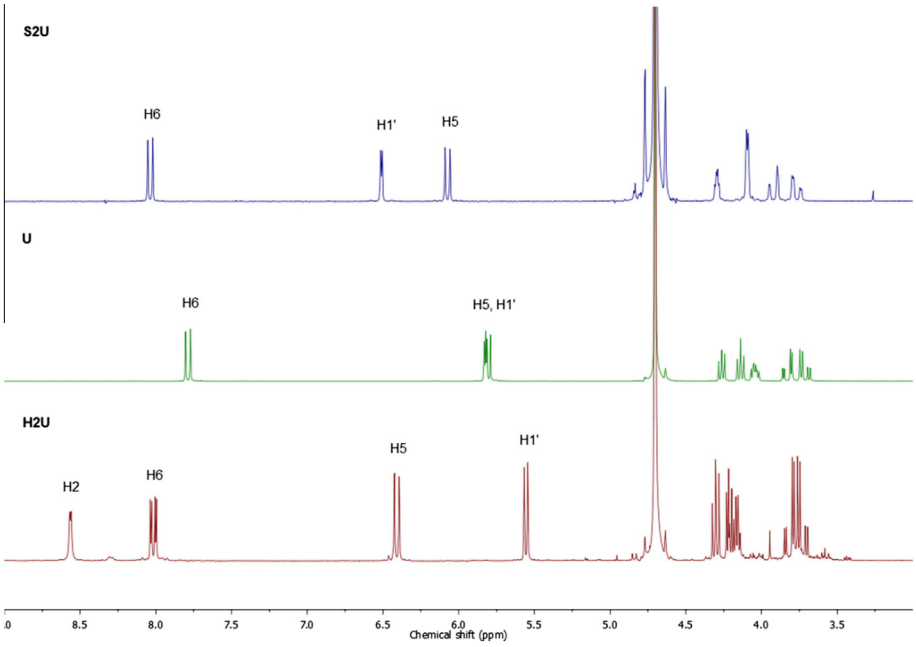


Figure 2. ^{1}H NMR spectra of S2U, U and H2U collected in D_2O.

First, we monitored the progress of the desulfuration reaction under three different pH conditions, at 6.6, 7.2 and 7.6. At given time intervals, the ^{1}H NMR spectra were collected (Figs. S6–S20), and the k_{obs} values were calculated from the pseudo first-order equation $k_{obs} = A_0e^{-kx} + y_0$. The values of k_{obs} and $t_{1/2}$ for the desulfuration reactions of **1a**–**e** in pH 6.6, 7.2 and 7.6 are given in Table 4. The lowest reaction rate at each monitored pH is observed for the desulfuration of S2U itself, with R being a hydrogen atom. This is also confirmed by the longest half-life ($t_{1/2}$) for S2U disappearance, 78.41, 23.74 and 9.11 min at pH values of 6.6, 7.2 and 7.6, respectively. Interestingly, the highest desulfuration rates and the smallest respective half-lives are observed for the reactions performed under the most basic conditions (pH 7.6). At the lowest pH (6.6), the most efficient reaction was the desulfuration of mo5S2U for which the $t_{1/2}$ value was 20.49 min, and this value decreased ca. 2.5-fold at the highest pH (at pH 7.6, $t_{1/2}$ was 8.8 min). The rate of this reaction was less dependent on the pH, while the other desulfuration reactions were more sensitive to pH, as demonstrated by a shortening of their $t_{1/2}$ values at pH 7.6 by ca. 6–9 times relative to those at pH 6.6. The reactions required 10–160 min for completion. The results given in Table S1 and presented in Figure 3 indicate that the desulfuration reaction of R5S2U is both pH- and C5-substituent dependent.

At a lower pH (6.6), each of the screened substrates delivered a 4-pyrimidinone derivative, and when the C5 substituents were hydrogen, methoxyl or methylcarboxymethyl groups, the R5H2Us were the prevailing reaction products (**3a**–**c**, R = H, mo and mcm). In contrast, mnm5H2U (**3e**) and cmnm5H2U (**3d**) comprised only ca. 30% of the reaction products. An increase in pH for the desulfuration reaction resulted in the lowering of the contribution of the 4-pyrimidinone nucleosides in the reaction mixture, and this effect was the most visible for desulfuration of mnm5S2U (**1e**) and cmnm5S2U (**1d**). The remaining substrates were still partially transformed to products **3** at pH 7.2 and 7.6.

Our results clearly demonstrate that the replacement of the hydrogen atom at the 5 position of 2-thiouridine by a substituent with different electronic properties changes the reactivity towards oxidation agents. This is due to the different electron distributions within the π system of the heterobase ring and the different electron densities on the C2 sulfur atom (causing the different reactivity of C2 sulfur atom). The electron-donating 5-methoxy substituent (mo) influences the heterobase (pyrimidine) electron density via a mesomeric effect that directs the reaction pathway to the more preferable H2U nucleoside formation under the physiological pH range. Note, that 2-thiouridine with a 5-methoxy substituent at the pyrimidine 5 position has not been found in nature, and only 5-methoxyuridine was identified at the wobble position of *Bacillus subtilis* tRNAs specific for Thr, Ala and Pro.[31]

In the case of the remaining substituents connected to the pyrimidine ring by the methylene group, the resonance effect is relatively small, and the inductive effect may play a dominant role, especially for substituents that can be protonated, thus altering their electron-withdrawing properties. The partial protonation of the amine function of the cmnm and mnm substituents of **1d** and **1e** at physiological pH causes the localization of a partial negative charge on the sulfur atom, which promotes faster oxidation to 5-cmnm- and 5-mnm-uridine products **2d** and **2e**, respectively. This transformation should be less dangerous for cell metabolism. Interestingly, the H2U lesion is observed only for the transformation that occurs at a lower pH (6.6), which may appear in cancer-changed cells. One may conclude that oxidative stress in cancer cells induces further lesions, leading to further alterations of cell metabolism.

2.3.1. Desulfuration of 5-substituted 2-thiouridines in water

It is important to note that the release of the sulfate oxygen during the reaction causes an acidification of the reaction mixture to pH 3–4.[7] Therefore, we monitored the direction of the desulfuration pathway for the reactions performed also in water. Substrates **1a**–**e** in water solutions were treated with H_2O_2, and after the reaction completion, the ratio of the products was determined by ^{1}H NMR integration. At a low pH, all of the reactions yielded the prevailing 4-pyrimidinone products (Table 5), except of desulfuration of mnm5S2U **1e**, which resulted in the prevailing mnm5U product (59%) against mnm5H2U **3e** (41%). Also it was visible, that **1d** (cmnm5S2U) gave more cmnm5U (**2d**) product than **1a**,**b** and **c**, but less than latter one, although the 4-pyrimidinone congener **3d** was still the major one (69%).

Table 4
Values of k_{obs} ($min^{-1} \times 10^{-3}$) and $t_{1/2}$ (min) for the desulfuration reactions of **1a–e** at pH 6.6, 7.2 and 7.6

Substrate	pH 6.6		pH 7.2		pH 7.6	
	k_{obs} [$min^{-1} \times 10^{-3}$]	$t_{1/2}$ (min)	k_{obs} [$min^{-1} \times 10^{-3}$]	$t_{1/2}$ (min)	k_{obs} [$min^{-1} \times 10^{-3}$]	$t_{1/2}$ (min)
S2U (**1a**)	8.84 ± 0.38	78.41	29.20 ± 5.00	23.74	76.06 ± 8.17	9.11
mo5S2U (**1b**)	33.83 ± 5.00	20.49	61.24 ± 7.99	11.32	78.77 ± 2.20	8.80
mcm5S2U (**1c**)	15.95 ± 1.61	43.46	52.24 ± 5.35	13.27	118.37 ± 13.70	5.96
cmnm5S2U (**1d**)	21.85 ± 2.98	31.72	55.55 ± 4.86	12.48	132.99 ± 12.50	5.21
mnm5S2U (**1e**)	22.01 ± 2.73	31.49	80.78 ± 6.13	8.58	156.98 ± 6.37	4.42

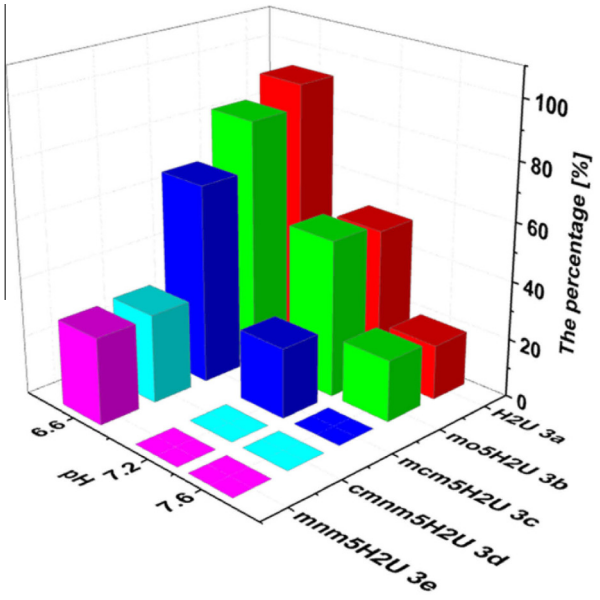


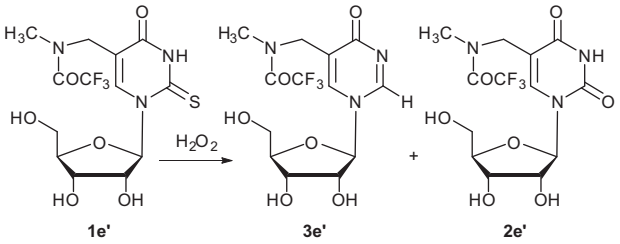
Figure 3. The yields of the R5H2U products for the reactions performed under three different pH conditions, at 6.6, 7.2 and 7.6. R = H, mo, mcm, cmnm, mnm.

Probably, in such a low pH, the amino function of **1e** is almost fully protonated and this electronic feature of the nucleobase influences the reaction pathway. To prove this assumption, that is the contribution of the side chain in the desulfuration pathway of mnm5S2U we blocked its amine function with a trifluoroacetic group (CF_3CO) (Scheme 3, compound **1e′**).

The desulfuration of **1e′** in water was performed under analogous conditions and delivered the respective 4-pyrimidinone product **3e′** with 88% yield. This result clearly indicates that the blocking of the amine function of the mnm substituent prevents its protonation and changes the pathway of the desulfuration reaction. Thus, an earlier suggestion by Yokoyama and Takai[4a] that NH protonation of **1e** may contribute to charge distribution in the mnm5S2U nucleoside is proved here, by the results shown in this study. This phenomenon most likely contributes to the fact that only 2-thiouridines substituted with mnm or cmnm are shown to be geranylated in a natural system, and this confirms also that the sulfur atom in these two nucleosides is more electronegative, comparing other 5-substituted 2-thiouridines.

Table 5
The ratio of R5H2U to R5U products of the desulfuration reaction of R5S2Us performed in water

S2U (**3a**/**2a**)	mo5S2U (**3b**/**2b**)	mcm5S2U (**3c**/**2c**)
88:12	87:13	87:13
mnm5s2U (**3e**/**2e**)	mnm5($COCF_3$)S2U (**3′e**/**2e′**)	cmnm5S2U (**3d**/**2d**)
41:59	88:12	69:31



Scheme 3. Transformation of N-protected mnm5S2U to its desulfured mnm5U and mnm5H2U products.

3. Conclusions

Although 5-substituents of 2-thiouridines do not directly participate in reactions with oxidizers, they strongly influence the oxidation process, and the differences in product distribution (H2U-type nucleosides versus uridines). The amount of R5H2U increases in the order H > mo > mcm > cmnm ≈ mnm, and this effect is observed within the physiological range of one pH unit between pH 6.6 and 7.6. Protonation of the (c)mnm substituent in (c)mnm5S2U is a decisive parameter of the transformation pathway. The conformational analysis of the resulting R5H2U products indicates that independent of the nature of the R5 substituent, the R5H2U nucleosides predominantly adopt a C2′-*endo* sugar ring conformation, as opposed to the preferred C3′-*endo* conformation of the parent R5S2Us. Our results contribute to the extension of knowledge on the function of 5-substituted 2-thiouridines in reading the genetic information.

4. Experimental

4.1. General procedure for the synthesis of 5-substituted 4-pyrimidinone nucleosides (3a–e)

4.1.1. Procedure (A) for 3b and 3c

Sugar-benzoylated 2-thionucleoside (0.5 mmol) (**4b**, R = $-OCH_3$ or **4c**, R = $-CH_2COOCH_3$, according to Scheme 2) was dissolved in anhydrous CH_2Cl_2 (4 ml), and then 0.2 M *m*CPBA in CH_2Cl_2 (4 ml) was added. The solution was stirred at room temperature, and after 3 h, the reaction was judged to be complete (TLC, 2% MeOH in $CHCl_3$). Then, the reaction mixture was washed with 10% Na_2SO_3 (15 ml), washed with 5% $NaHCO_3$ (15 ml), dried over $MgSO_4$ and evaporated. The final solution was filtered off, evaporated in vacuo and purified by silica gel column chromatography (2% MeOH in $CHCl_3$) to give **5b** in 79% yield and **5c** in 77% yield.

Removal of the protecting groups

The benzoylated derivative **5b** or **5c** (0.4 mmol) was suspended in 1 M MeONa in MeOH (5 ml). The mixture was stirred at room temperature for 2 h. Then, TLC analysis (20% MeOH in $CHCl_3$) showed that the starting material was completely consumed. The mixture was evaporated in vacuo, and the oily residue was purified by silica gel column chromatography with $CHCl_3$/MeOH (a gradient

from 20% to 30% MeOH) to give mo5H2U (**3b**) in 83% yield or mcm5H2U (**3c**) in 77% yield.

mo5H2U (3b). UV λ_{max} (H_2O)/nm 267.0 (ε/dm^3 mol^{-1} cm^{-1} 16 630); ^{1}H NMR (700 MHz, D_2O): δ 3.79 (s, 3H, OCH_3), 3.82 (dd, 1H, $^3J_{H5''-H4'}$ = 3.3 Hz, $^2J_{H5''-H5'}$ = 12.8 Hz, H5″), 3.90 (dd, 1H, $^3J_{H5'-H4'}$ = 2.9 Hz, $^2J_{H5'-H5''}$ = 12.8 Hz, H5′), 4.23 (m, 1H, $^3J_{H4'-H5'}$ = 2.9 Hz, $^3J_{H4'-H5''}$ = 3.3 Hz, $^3J_{H4'-H3'}$ = 4.0 Hz, H4′), 4.31 (pt, 1H, $^3J_{H3'-H4'}$ = 4.0 Hz, $^3J_{H3'-H2'}$ = 5.1 Hz, H3′), 4.39 (pt, 1H, $^3J_{H2'-H3'}$ = 5.1 Hz, $^3J_{H2'-H1'}$ = 5.3 Hz, H2′), 5.32 (d, 1H, $^3J_{H1'-H2'}$ = 5.3 Hz, H1′), 7.78 (d, 1H, $^4J_{H6-H2}$ = 1.9 Hz, H6), 8.40 (d, $^4J_{H2-H6}$ = 1.9 Hz, H2); ^{13}C NMR (176 MHz, D_2O): 56.09 (CH_3O), 60.69 (C5′), 70.05 (C3′), 75.17 (C2′), 86.11 (C4′), 95.38 (C1′), 117.74 (C6), 145.19 (C5), 146.81 (C2), 168.13 (C4); MS (CI, [M+H$^+$]) for $C_{10}H_{16}O_6N_2$ calcd 259.2, found: *m*/*z* 259.1.

mcm5H2U (3c). UV λ_{max} (H_2O)/nm 246.8 (ε/dm^3 mol^{-1} cm^{-1} 14 008); ^{1}H NMR (700 MHz, D_2O): 3.50 (s, 2H, CH_2COOCH_3), 3.67 (s, 3H, CH_2COOCH_3), 3.77 (dd, 1H, $^3J_{H5''-H4'}$ = 4.0 Hz, $^2J_{H5''-H5'}$ = 12.8 Hz, H5″), 3.84 (dd, 1H, $^3J_{H5'-H4'}$ = 3.1 Hz, $^2J_{H5'-H5''}$ = 12.8 Hz, H5′), 4.19 (pq, 1H, $^3J_{H4'-H5'}$ = 3.1 Hz, $^3J_{H4'-H5''}$ = 4.0 Hz, $^3J_{H4'-H3'}$ = 3.6 Hz, H4′), 4.25 (dd, 1H, $^3J_{H3'-H4'}$ = 3.6 Hz, $^3J_{H3'-H2'}$ = 5.4 Hz, H3′), 4.34 (pt, 1H, $^3J_{H2'-H3'}$ = 5.4 Hz, $^3J_{H2'-H1'}$ = 5.8 Hz, H2′), 5.59 (d, 1H, $^3J_{H1'-H2'}$ = 5.8 Hz, H1′), 8.06 (d, 1H, $^4J_{H6-H2}$ = 2.4 Hz, H6), 8.58 (d, $^4J_{H2-H6}$ = 2.4 Hz, H2); ^{13}C NMR (176 MHz, D_2O): 33.51 (CH_2COOCH_3), 52.81 (CH_2COOCH_3), 60.87 (C5′), 70.05 (C3′), 75.04 (C2′), 86.10 (C4′), 94.93 (C1′), 119.31 (C5), 139.00 (C6), 151.6 (C2), 172.76 (C4), 173.24 (CH_2COOCH_3); MS (CI, [M+H$^+$]) for $C_{12}H_{17}O_6N_2$ calcd 301.3, found: *m*/*z* 301.2.

4.1.2. Procedure (B) for 3d and 3e

2′,3′-*O*-Isopropylidene *N*-trifluoroacetyl-protected nucleoside (0.5 mmol) (**6d** or **6e**) was dissolved in 25% aqueous acetic acid (10 ml) and heated at 90 °C for 1 h. Then, the reaction mixture was frozen and lyophilized to give the crude *N*-protected nucleoside. This product was dissolved in pyridine (2 ml) and anhydrous CH_2Cl_2 (2 ml), and then 0.2 M *m*CPBA in CH_2Cl_2 (4 ml) was added dropwise. The solution was stirred at room temperature, and after 2.5 h, the reaction was judged to be complete by TLC (20% MeOH in $CHCl_3$). Then, the reaction mixture was washed with 10% Na_2SO_3 (15 ml), washed with 5% $NaHCO_3$ (15 ml), dried over $MgSO_4$ and evaporated. The residue was subjected to chromatographic purification. The elution of a silica gel column with $CHCl_3$/MeOH gave pure product **7d** (35% yield) or **7e** (47% yield).

Removal of the protecting groups

Compound 7d or 7e (0.1 mmol) was dissolved in ethanol (2 ml). Then, 4 ml of 8.6 M NH3 in ethanol was added dropwise, and the reaction mixture was stirred for 1 h at room temperature. Then, TLC analysis (isopropanol/ammonia/water 7:2:1) showed that the starting material was completely consumed. The mixture was slowly evaporated in vacuo, and the remaining residue was coevaporated with ethanol (2 × 10 ml), toluene (3 × 10 ml) and ethanol (3 × 10 ml) to give cmnm5H2U 3d in 92% yield or mnm5H2U 3e in 95% yield.

cmnm5H2U (3d). UV λ_{max} (H_2O)/nm 245.3 (ε/dm^3 mol^{-1} cm^{-1} 12 790); ^{1}H NMR (700 MHz, D_2O) δ 3.83 (dd, $^2J_{H5''-H5'}$ = 12.8 Hz, $^3J_{H5''-H4'}$ = 3.9 Hz, 1H, H5″), 3.90 (dd, $^2J_{H5''-H5'}$ = 12.8 Hz, $^3J_{H5'-H4'}$ = 3.0, 1H, H5′), 3.94 (s, 2H, CH_2COOH), 4.19 (s, 2H, CH_2-R), 4.26 (pq, $^3J_{H4'-H3'}$ = 3.6 Hz, 1H, H4′), 4.30 (dd, $^3J_{H3'-H2'}$ = 5.4 Hz, $^3J_{H3'-H4'}$ = 3.6 Hz, 1H, H3′), 4.39 (pt, $^3J_{H2'-H3'}$ = 5.4 Hz, $^3J_{H2'-H1'}$ = 5.7 Hz, 1H, H2′), 5.66 (d, $^3J_{H1'-H2'}$ = 5.7 Hz, 1H, H1′), 8.33 (d, $^4J_{H6-H2}$ = 2.5 Hz, 1H, H6), 8.72 (d, $^4J_{H2-H6}$ = 2.5 Hz, 1H, H2); ^{13}C NMR (176 MHz, D_2O) δ 48.10 (CH_2-R), 51.86 (CH_2COOH), 63.83 (C5′), 73.05 (C3′), 78.15 (C2′), 89.30 (C4′), 97.92 (C1′), 118.12 (C5), 144.16 (C6), 172 (C4), 174.98 (CO), 151 (C2); MS (CI, [M+H$^+$]) for $C_{12}H_{18}O_7N_3$ calcd 316.1, found: *m*/*z* 316.0.

mnm5H2U (3e). UV λ_{max} (EtOH)/nm 247.8 (ε/dm^3 mol^{-1} cm^{-1} 14 682); ^{1}H NMR (700 MHz, D_2O): 2.37 (s, 3H, CH_2NHCH_3), 3.62 (s, 2H, CH_2NHCH_3), 3.72 (dd, 1H, $^3J_{H5''-H4'}$ = 3.9 Hz, $^2J_{H5''-H5'}$ = 12.8 Hz, H5″), 3.80 (dd, 1H, $^3J_{H5'-H4'}$ = 3.0 Hz, $^2J_{H5'-H5''}$ = 12.8 Hz, H5′), 4.15 (pq, 1H, $^3J_{H4'-H5'}$ = 3.0 Hz, $^3J_{H4'-H5''}$ = 3.9 Hz, $^3J_{H4'-H3'}$ = 3.4 Hz, H4′), 4.20 (dd, 1H, $^3J_{H3'-H4'}$ = 3.4 Hz, $^3J_{H3'-H2'}$ = 5.20 Hz, H3′), 4.27 (pt, 1H, $^3J_{H2'-H3'}$ = 5.2 Hz, $^3J_{H2'-H1'}$ = 5.8 Hz, H2′), 5.54 (d, 1H, $^3J_{H1'-H2'}$ = 5.8 Hz, H1′), 8.04 (d, 1H, $^4J_{H6-H2}$ = 2.3 Hz, H6), 8.53 (d, $^4J_{H2-H6}$ = 2.3 Hz, H2); ^{13}C NMR (176 MHz, D_2O): 33.45 (CH_3NHCH_2), 46.99 (CH_3NHCH_2) 60.81 (C5′), 70.07 (C3′), 75.06 (C2′), 86.17 (C4′), 94.86 (C1′), 119.00 (C5), 138.63 (C6), 151.74 (C2), 172.53 (C4); MS (CI, [M+H$^+$]) for $C_{11}H_{18}O_5N_3$ calcd 272.3, found: *m*/*z* 272.2.

4.1.3. Transformation of mcm5H2U (3c) to ncm5H2U (3c′) or to cm5H2U (3c″)

5-Methoxycarbonylmethyl-4-pyrimidinone nucleoside **3c** (0.074 g, 0.25 mmol) was dissolved in saturated methanolic ammonia (4 ml). The mixture was stirred at room temperature for 48 h. Then, the reaction was judged to be complete (TLC, isopropanol/ammonia/water 8:1:1). The reaction mixture was evaporated in vacuo to give pure ncm5H2U **3c′** (0.067 g) in 95% yield.

ncm5H2U (3c′). UV λ_{max} (H_2O)/nm 247.2 (ε/dm^3 mol^{-1} cm^{-1} 12 485); ^{1}H NMR (700 MHz, D_2O): 3.39 (s, 2H, CH_2CONH_2), 3.77 (dd, 1H, $^3J_{H5''-H4'}$ = 4.1 Hz, $^2J_{H5''-H5'}$ = 12.8 Hz, H5″), 3.84 (dd, 1H, $^3J_{H5'-H4'}$ = 3.0 Hz, $^2J_{H5'-H5''}$ = 12.8 Hz, H5′), 4.19 (pq, 1H, $^3J_{H4'-H5'}$ = 3.0 Hz, $^3J_{H4'-H5''}$ = 4.1 Hz, $^3J_{H4'-H3'}$ = 3.6 Hz, H4′), 4.25 (dd, 1H, $^3J_{H3'-H4'}$ = 3.6 Hz, $^3J_{H3'-H2'}$ = 5.3 Hz, H3′), 4.34 (pt, 1H, $^3J_{H2'-H3'}$ = 5.3 Hz, $^3J_{H2'-H1'}$ = 5.80 Hz, H2′), 5.59 (d, 1H, $^3J_{H1'-H2'}$ = 5.8 Hz, H1′), 8.03 (d, 1H, $^4J_{H6-H2}$ = 2.3 Hz, H6), 8.56 (d, $^4J_{H2-H6}$ = 2.3 Hz, H2); ^{13}C NMR (176 MHz, D_2O): 34.47 (CH_2CONH_2), 60.88 (C5′), 70.03 (C3′), 75.00 (C2′), 86.07 (C4′), 94.91 (C1′), 119.79 (C5), 139.05 (C6), 151.52 (C2), 172.77 (C4), 175.08 (CH_2CONH_2); MS (CI, [M+H$^+$]) for $C_{11}H_{16}O_6N_3$ calcd 286.1, found: *m*/*z* 286.2.

5-Methoxycarbonylmethyl-4-pyrimidinone nucleoside **3c** (0.125 g, 0.4 mmol) was dissolved in 0.1 M KOH (6 ml). Then, 3 ml of water and 3 ml of methanol was added, and the reaction mixture was stirred at room temperature for 2 h. Then, TLC analysis (isopropanol/ammonia/water 8:1:1) showed that the starting material was completely consumed. The mixture was worked up with Dowex 50 (H^+ form), and after filtering off the resin, the remaining solution was evaporated in vacuo to give cm5H2U **3c″** (0.046 g) in 42% yield.

cm5H2U (3c″). UV λ_{max} (H_2O)/nm 249.8 (ε/dm^3 mol^{-1} cm^{-1} 11 358); ^{1}H NMR (700 MHz, D_2O): 3.32 (s, 2H, CH_2COOH), 3.82 (dd, 1H, $^3J_{H5''-H4'}$ = 4.3 Hz, $^2J_{H5''-H5'}$ = 12.8 Hz, H5″), 3.90 (dd, 1H, $^3J_{H5'-H4'}$ = 3.1 Hz, $^2J_{H5'-H5''}$ = 12.8 Hz, H5′), 4.23 (pq, 1H, $^3J_{H4'-H5'}$ = 3.1 Hz, $^3J_{H4'-H5''}$ = 4.3 Hz, $^3J_{H4'-H3'}$ = 3.7 Hz, H4′), 4.30 (dd, 1H, $^3J_{H3'-H4'}$ = 3.7 Hz, $^3J_{H3'-H2'}$ = 5.4 Hz, H3′), 4.40 (pt, 1H, $^3J_{H2'-H3'}$ = 5.4 Hz, $^3J_{H2'-H1'}$ = 5.8 Hz, H2′), 5.63 (d, 1H, $^3J_{H1'-H2'}$ = 5.8 Hz, H1′), 7.97 (d, 1H, $^4J_{H6-H2}$ = 2.3 Hz, H6), 8.57 (d, $^4J_{H2-H6}$ = 2.3 Hz, H2); ^{13}C NMR (176 MHz, D_2O): 36.53 (CH_2COOH), 60.92 (C5′), 70.01 (C3′), 74.87 (C2′), 85.95 (C4′), 94.89 (C1′), 122.12 (C5), 137.92 (C6), 150.98 (C2), 172 (C4), 174 (CH_2COOH); MS (CI, [M+H$^+$]) for $C_{11}H_{15}O_7N_2$ calcd 287.1, found: *m*/*z* 287.1.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.07.030.

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