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An efficient approach for conversion of 5-substituted 2-thiouridines built in RNA oligomers into corresponding desulfured 4-pyrimidinone products

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ABSTRACT

An efficient approach for the desulfuration of C5-substituted 2-thiouridines (R5S2U) bound in the RNA chain exclusively to 4-pyrimidinone nucleoside (R5H2U)-containing RNA products is proposed. This post-synthetic transformation avoids the preparation of a suitably protected H2U phosphoramidite, which otherwise would be necessary for solid-phase synthesis of the modified RNA. Optimization of the desulfuration, which included reaction stoichiometry, time and temperature, allowed to transform a set of ten R5S2U-RNAs into their R5H2U-RNA congeners in ca. 90% yield.

Transfer RNAs are unique among natural RNAs due to the presence of modified nucleosides.[1,2] Those located in the tRNA anticodon tune the translation process through highly accurate codon–anticodon recognition. Among them there are C5-substituted 2-thiouridines (R5S2U) present in the first (wobble) position of the anticodon in transfer RNAs specific for lysine ($tRNA^{Lys3}$), glutamic acid ($tRNA^{Glu}$) and glutamine ($tRNA^{Gln}$). Nucleosides containing thiocarbonyl function may be sensitive to oxidizing conditions. It was reported that thiouridines in tRNA chain get desulfured in the presence of oxidative agents, although detailed structures of the products were not determined.[3] Other studies reported that in 100 mM H_2O_2 2-thiouridines were oxidized to uridines and converted into another, unidentified product, which retained the ring structure and did not have a disulfide bond.[4] However, when S2U nucleoside is oxidized in 'organic' non-aqueous conditions, for example, in the presence of *trans*-2-phenylsulfonyl-3-phenyloxaziridine (PSO)[5] or *m*-chloroperbenzoic acid (*m*CPBA),[6] it undergoes selective transformation to the 4-pyrimidinone nucleoside (H2U), which is a uridine analog deprived of the C2 oxygen and N3 hydrogen atoms (Scheme 1). The loss of the sulfur atom was also observed in aqueous solutions. First effective desulfuration of 2-thiouridine located in a DNA pentamer 5′-TdA(S2U)dGdC-3′ was carried out with PSO in water/acetonitrile solution.[5] The product contained a H2U unit (identified by MALDI-TOF mass spectrometry), so at the nucleoside and oligonucleotide levels the desulfuration of 2-thiouridine predominantly yielded the H2U analog. In further studies we applied conditions mimicking an oxidative stress in a cell (100 mM H_2O_2) and found that, either alone or in an RNA chain, S2U is desulfured to uridine and 4-pyrimidinone nucleoside (Scheme 1).[7] The products ratio depended on pH,[8] C5-substituent and concentration of the oxidant (Bartos et al., will be published elsewhere). Importantly, the reactions carried out either in aqueous H_2O_2 or $KHSO_5$ (oxone) at pH 6.6 furnished mainly H2U, while uridine was a major product at pH 7.6.

Conformational and crystallographic studies demonstrate that S2U predominantly exists in the C3′-*endo* sugar ring conformation,[9] typical for ribonucleosides/ribonucleotides, while H2U preferentially adopts the C2′-*endo* sugar puckering, characteristic for the 2′-deoxyribo congeners.[10] Moreover, due to the altered pattern of hydrogen bonding, the H2U nucleoside exerts different base pairing. Thus, oxidative modification of S2U-tRNA would substantially affect the codon–anticodon interactions, as well as recognition of tRNA by proteins involved in cellular processes. Data obtained from UV-melting[7] and DSC experiments[11] indicate that the affinity of H2U-RNA to its Watson–Crick complement is much

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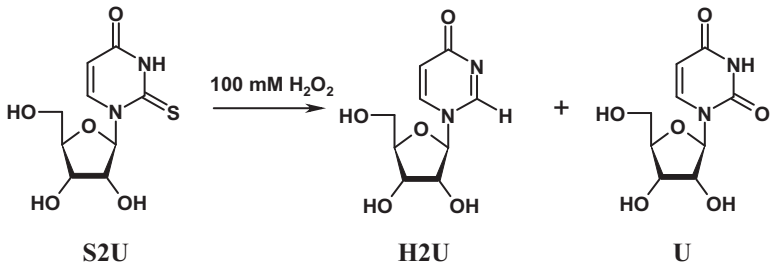

Scheme 1. Transformation of 2-thiouridine (S2U) to 4-pyrimidinone nucleoside (H2U) and uridine (U).

smaller than that of S2U-RNA. Additionally, H2U-RNA under basic conditions decomposes to the abasic form, with eventual strand scission.[7] Therefore, it should be clarified whether the S2U→H2U transformation occurs in vivo resulting in the tRNA chain cleavage within the damaged anticodon wobble site. For that purpose, a reliable access to H2U-RNA models is urgently needed.

In our earlier reports we have described the synthesis of a suitably protected H2U phosphoramidite monomer[5] and its incorporation into an RNA chain.[7,11] That approach is not straightforward because H2U-RNA is unstable in basic environment, even during routine RP-HPLC purification employing only slightly alkaline triethylammonium bicarbonate eluent (pH 7.5). Here we report an optimized method for the S2U-RNA→H2U-RNA transformation after the model RNA oligomers containing different hypermodified 5-substituted 2-thiouridines (R5S2U) were already assembled and deprotected.

For optimization of the transformation we selected ten RNAs (Table 1) containing 2-thiouridine or its C5-substituted derivatives (Fig. 1), naturally occurring in tRNAs. The modified nucleosides (generally denoted X) were built into the RNA hairpin structure (with the R5S2U modification in the position corresponding to the wobble position, Table 1, oligomers **1**–**6**, **8**), into a U_{11} oligomer (**7**), or into the RNA chain coding the passenger strands of siRNA (oligomers **9**, **10**). These oligomers were synthesized according to already described procedures.[11–14] Illustrative procedures for synthesis of the R5S2U-RNA oligomers are given in Supplementary material.

To prepare all solutions intended to contact with R5S2U-RNAs, sterile water (0.1% DEPC-treated, and then autoclaved) was used. Desulfuration was done with aqueous solutions of $KHSO_5$ (oxone®), as this oxidizing agent is easy to handle and one can precisely control its ratio (5–20-fold molar excess, 2 mM solution) to the R5S2U-

Table 1
Sequences, calculated molecular weights (M.W.) and mass spectrometry data for RNA oligonucleotides used

No.	X	Oligonucleotide sequence	M.W. (g/mol)	MALDI-TOF MS (*m*/*z*)
1	S2U	5′-CGGCU**X**UUAACCGA-3′	4423	4418.9
2	mcm5S2U	5′-CGGCU**X**UUAACCGA-3′	4495	4490.9
3	τm5S2U	5′-UUAACCU**X**UUAAGUUAA-3′	5472	5468.0
4	mcm5S2U	5′-CCAGUUGACU**X**UUAAUCAAUUGG-3′	7354	7352.9
5	mnm5S2U	5′-CCAGUUGACU**X**UUAAUCAAUUGG-3′	7325	7324.6
6	S2U	5′-CCAGUUGACU**X**UUAAUCAAUUGG-3′	7282	7280.0
7	mnm5S2U	5′-UUUUUU**X**UUUU-3′	3366	3361.4
8	S2U	5′-UCACGCU**X**UCACCGUGG-3′	5355	5350.0
9	m5S2U	5′-UUACCAACCACAACACUG**X**TT-3′	6583	6583.3
10	S2Um	5′-UUACCAACCACAACACUG**X**TT-3′	6583	6583.5

The structures of nucleosides denoted X are given in Figure 1. The *m*/*z* values correspond to $[M-H]^-$ ions.

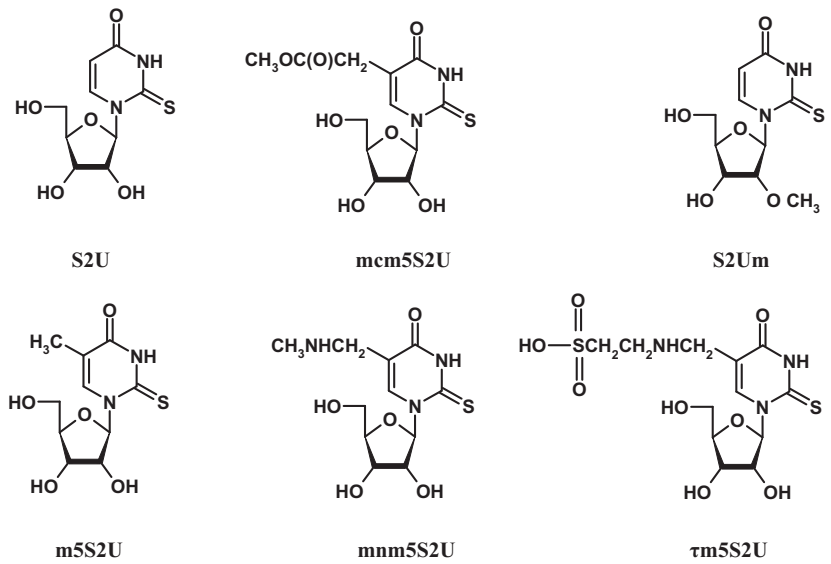

Figure 1. Modified C5-substituted 2-thiouridines used in these studies.

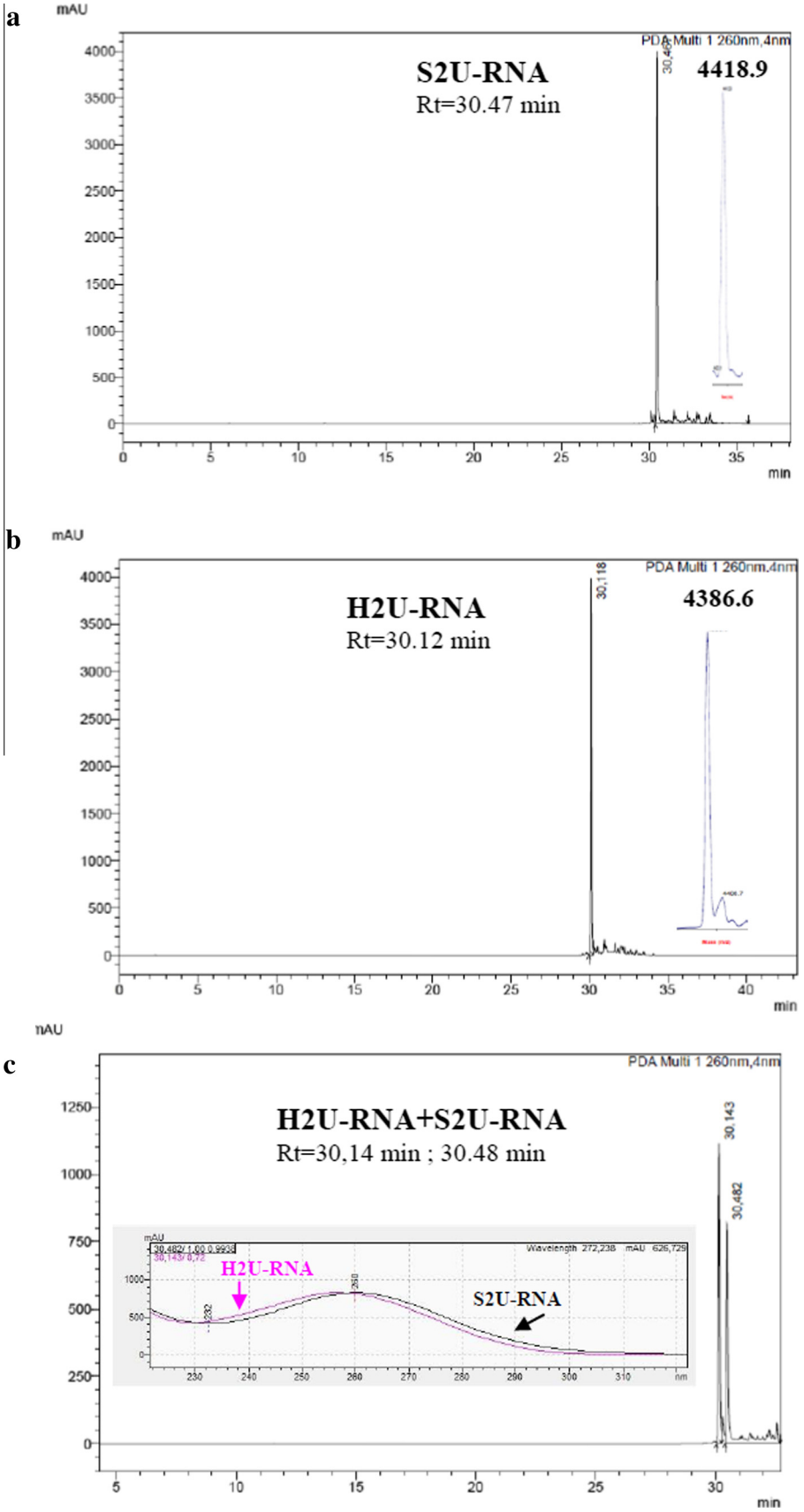

Figure 2. Desulfuration of S2U-RNA **1** monitored by RP-HPLC; (a) the profile of S2U-RNA substrate and (b) its desulfured H2U-RNA product; insets are the fragments of the MALDI-TOF MS spectra of the corresponding analyte; (c) the profile of the co-injected S2U-RNA and H2U-RNA, and inset shows the UV spectra of both oligomers.

RNA substrate (prepared at 0.4–0.8 μM concentration, reaction volumes ca. 1 mL). The reactions were carried out either at room temperature or at 0 °C for 5–180 min, and were terminated by loading on a C18 Sep-Pack cartridge (Waters) and washing with sterile water. The RNA products were released from the column by elution with 50% aq acetonitrile, and quantified spectrophotometrically. The products (obtained in the yields close to quantitative) were analyzed by MALDI-TOF mass spectrometry in a negative ions mode. The effectiveness of the S2U→H2U transformation was calculated from the MALDI-TOF mass spectra, where the intensity of a band at the *m*/*z* attributed to the starting R5S2U-RNA was compared with that at *m*/*z*-32. We have earlier demonstrated that molecular ions for RNA oligonucleotides containing S2U or H2U are of similar intensities, what indicates their similar ionization properties.[7] The product of desulfuration of oligomer **1** was positively identified by IE-HPLC co-injection with corresponding synthetically prepared H2U-RNA.[7]

The example of the desulfuration of S2U-RNA **1** monitored by RP-HPLC is shown at Figure 2. The profile of H2U-RNA clearly indicates that the process of S2U-RNA desulfuration undergoes selectively. Co-injection of both compounds shows that the H2U-RNA product is less hydrophobic, and its UV spectrum is shifted towards lower wavelength due to the presence of H2U component (λ_{max} = 242 nm, see Fig. S2).

The H2U-RNA product was also digested with nuclease P1 and alkaline phosphatase (Sigma–Aldrich), and the resultant mixture of nucleosides was analyzed by RP-HPLC. The identity of the released H2U nucleoside was confirmed by co-injection with the genuine H2U nucleoside, and by UV analysis (see Figs. S1–S3, Supporting information).[10]

The results indicate that low excess of oxone is beneficial for yield and purity of the desired desulfured R5H2U-RNAs (Table 2). For the oxidation with a 10-fold molar excess of oxone at room temperature, the reaction time was optimized in the range of 5–120 min (Table 2). The mass spectra for substrates **1**–**10** and for crude mixtures containing their R5H2U-RNA desulfured products are shown in the Supplementary material (Fig. S4–S13). In the spectra, the bands expected for R5H2U-RNA were accompanied by those at *m*/*z*+22, +38 or +60, which can be assigned to ions which acquired Na^+, K^+, or Na^+ and K^+ cations, respectively. It was found that the reactions lasting for not more than 30 min furnished the expected products in the yields >90%, except for **5** containing mnm5S2U located in the loop region (87% yield), for which certain amount of uridine-RNA was observed. Possible residual amounts of R5U-RNA products obtained during the desulfuration reaction or R5S2U-RNA contaminations introduced into the reaction mixture with the R5S2U substrate exhibit signals of *m*/*z* bigger by 16 amu comparing R5H2U-RNA signals.

All R5H2U-RNA products but these obtained from m5S2U (**9**) and τm5S2U (**3**) were stable in the oxidation conditions for 180 min. The two less stable were prone for further oxidation, giving in MALDI-TOF MS signals at higher *m*/*z*. Recently, we have reported that in ASL RNA models the guanosine units located in a single stranded regions of the loop are primary sites for action of the manganese porphyrin/oxone oxidizing system, resulting in dehydroguanidinohydantoin and spiroiminodihydantoin derivatives of molecular weight increased by 4 and 32 amu, respectively.[15] Also other guanosine lesions having higher M.W. than the starting RNA were reported.[16] Therefore, it is possible that guanosine residues in oligomers **3** and **9** might get oxidized, although detailed explanation cannot be offered, especially because in the remaining examples we have observed only the expected S2U→H2U desulfuration. Selected mass spectra recorded after desulfuration of **9** over different time and oxidant concentration conditions are presented in Figure S14 (annotations m5H2U-RNA^{ox} indicate the unidentified oxidized products).

To check whether H2U product can be obtained from S2U located in the double-stranded oligonucleotide, **1** was hybridized with an RNA complement (T_m = 64.3 °C for the duplex, in 10 mM phosphate buffer, 50 mM NaCl)[11] and treated with aqueous oxone under conditions earlier established for single-stranded **1** (10× molar excess of oxone, rt, 5 min.). Standard work-up delivered the product identified by MALDI-TOF MS (Fig. 3) as the expected H2U-RNA/RNA product (*m*/*z* 4386.6, M.W. 4392).

In conclusion, we found that several 2-thiouridine nucleosides present in an RNA chain (in single stranded and double stranded models), can be post-synthetically transformed to corresponding desulfured 4-pyrimidinone riboside products. The reactions efficiently undergo with 10-fold molar excess of aqueous oxone, at room temperature, but the reaction time has to be controlled and optimized. Longer exposition of the R5H2U-RNA products to the

Table 2
Optimized time and yield of R5S2U-RNA transformation to R5H2U-RNA for the reaction carried out with 10-fold molar excess of oxone, at 25 °C

No. of oligomer	Time (min)	Yield of the R5H2U-RNA (%)
1	10	>95
2	10	93
3	5[a]	>95
4	30	>95
5	10	87
6	30	>95
7	10	94
8	10	>95
9	5	>95
10	10	>95

[a] 15-Fold excess.

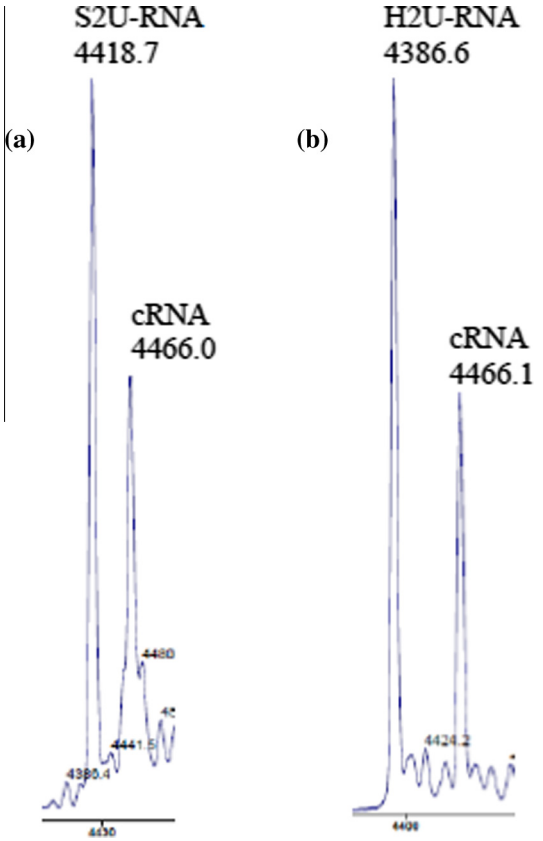

Figure 3. MALDI-TOF mass spectrometry analysis: (a) S2U-RNA **1** (*m*/*z* 4418.7, MW 4423) hybridized with the RNA complement 3′-GCCGAAAAUUGGCU-5′, (cRNA; *m*/*z* 4466.0, M.W. 4470); (b) the product of desulfuration, H2U-RNA (*m*/*z* 4386.6) and the unchanged RNA complement (*m*/*z* 4466.1).

oxidizing agent leads to further unidentified oxidative lesions. The R5H2U-RNA model oligomers may be used for biological/biophysical studies directly as crude desalted products.

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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.bmcl.2015.06.019.

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