

S-Geranyl-2-thiouridine wobble nucleosides of bacterial tRNAs; chemical and enzymatic synthesis of S-geranylated-RNAs and their physicochemical characterization

Malgorzata Sierant^{1,†}, Grazyna Leszczynska^{2,†}, Klaudia Sadowska²,
Agnieszka Dziergowska², Michal Rozanski¹, Elzbieta Sochacka² and Barbara Nawrot^{1,*}

¹Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Department of Bioorganic Chemistry, Sienkiewicza 112, 90-363 Lodz, Poland and ²Institute of Organic Chemistry, Faculty of Chemistry, Lodz University of Technology, Zeromskiego 116, 90-924 Lodz, Poland

Received June 29, 2016; Revised July 30, 2016; Accepted August 06, 2016

ABSTRACT

Recently, highly lipophilic S-geranylated derivatives of 5-methylaminomethyl-2-thiouridine (mnm5geS2U) and 5-carboxymethylaminomethyl-2-thiouridine (cmnm5geS2U) were found at the first (wobble) anticodon position in bacterial tRNAs specific for Lys, Glu and Gln. The function and cellular biogenesis of these unique tRNAs remain poorly understood. Here, we present one direct and two post-synthetic chemical routes for preparing model geS2U-RNAs. Our experimental data demonstrate that geS2U-RNAs are more lipophilic than their parent S2U-RNAs as well as non-modified U-RNAs. Thermodynamic studies revealed that the S-geranyl-2-thiouridine-containing RNA has higher affinity toward complementary RNA strand with G opposite the modified unit than with A. Recombinant tRNA selenouridine synthase (SelU) exhibits sulfur-specific geranylation activity toward model S2U-RNA, which is composed of the anticodon-stem-loop (ASL) from the human tRNA^{Lys3} sequence. In addition, the presence of magnesium ions is required to achieve appreciable geranylation efficiencies.

INTRODUCTION

A majority of the RNA species in cells undergo post-transcriptional modifications, and the chemical groups introduced by these modifications have distinct effects not only at the site of modification but also on the global structure of the modified RNA. In particular, transfer RNAs (tRNAs) acquire chemically diverse posttranscrip-

tional modifications important for their structure and function. According to available online databases of RNA modifications (<http://rna-mdb.cas.albany.edu>; <http://modomics.genesilico.pl>) (1,2), 91 of the 109 currently known RNA nucleoside modifications have been found in tRNA molecules. The most frequently modified sites in tRNAs are nucleoside 34 (the anticodon wobble position) and the purine nucleoside 37 (adjacent to the anticodon). These modifications increase the fidelity of codon recognition, allow for efficient recognition of several codons by the same tRNA (3) and prevent frame-shift reading. Modifications in other tRNA domains mainly contribute to tRNA folding and stability (4,5). Certain stress-induced changes in tRNA modification patterns are postulated to function as another level of regulating gene expression (6).

At the wobble position, 5-substituted uridines (R5U) and 2-thiouridines (R5S2U) are present in the tRNAs for lysine (tRNA^{Lys}), glutamic acid (tRNA^{Glu}) and glutamine (tRNA^{Gln}). These modified bases promote the reading of both NNA and NNG codons, tending to favor the recognition of adenosine-containing codons (7,8). These modifications are essential for the viability of eukaryotic and prokaryotic cells. The absence of a side chain in the C5 position or the lack of a sulfur atom in the C2 position leads to detrimental frame-shift reading (9).

Most of the RNA modifications reported to-date involve the introduction of small and hydrophilic groups. However, several years ago an unusual, large hydrophobic group was found in bacterial tRNAs (10). This hydrophobic group was later identified as a geranyl group (11) and represented the first isoprenylated nucleoside observed in natural ribonucleic acids. Previously, a similar modification was found in proteins, where geranylgeranyl group (introduced by geranyl transferases) facilitate at-

*To whom correspondence should be addressed. Tel: +48 42 6803248; Fax: +48 42 6815483; Email: bnawrot@cbmm.lodz.pl

†Equal contribution.

tachment to cellular membranes (similar to lipid anchors) and is important for protein-protein interactions (12). The geranyl group in tRNA is attached to the sulfur atom in 5-methylaminomethyl-2-thiouridine (mnm5S2U) and 5-carboxymethylaminomethyl-2-thiouridine (cmnm5S2U) (Figure 1). The resulting *S*-geranylated-2-thiouridines (mnm5geS2U and cmnm5geS2U) account for up to 6.7% of the (c)mnm5S2Us (~400 geranylated nucleosides) within a cell (11).

The bacterial tRNA selenouridine synthase (SelU) has been recognized not only as the biocatalyst for the *S*-geranylation of tRNAs in cells (9–11) but also as the catalyst for S→Se replacement (13). Liu *et al.* (11) claimed that the *S*-geranylation of tRNAs is an alternative to the selenation of tRNA, with *S*-geranylation being executed at low selenium concentrations. Thus, S→Se replacement and *S*-geranylation are catalyzed by the same enzyme operating on the same substrate (S2U). However, this dual *modus operandi* leads to an awkward (and perhaps truly conflicting) situation: for selenation, the sulfur atom must be converted into a fairly good leaving group (the C2 atom to be attacked by an Se⁻ nucleophile). In contrast, during *S*-geranylation, the sulfur atom (preferably within an RS⁻-like moiety) is expected to act as the nucleophile. Recently, we proposed a ‘linear’ model wherein the *S*-geranyl-2-thiouridine (geS2U) is not a final product but is rather an intermediate in the S2U→Se2U conversion (14). This hypothesis was supported significantly by our success in performing the two-step chemical transformation S2U→geS2U→Se2U and was further supported by evidence from Jager *et al.* (9). Several mutations introduced into the SelU (MnmH) polypeptide chain allowed the amino acids responsible for interactions within the tRNA molecule and for binding of modified nucleosides (the Walker’s motif in the P-loop domain) to be identified. In addition, two amino acids important for *S*-geranylation (G67) and selenation (C97) have been identified in the rhodanese domain.

It has been suggested that geranyl hydrophobic group confers unique physicochemical and conformational features to the whole tRNA molecule. Such modified tRNA favorably recognizes 5'-NNG-3' codons over 5'-NNA-3' codons (11). This enhancement in specificity was recently reported by Wang *et al.*, who found that the thermal stability of a DNA duplex containing a geS2T-dG base pair was higher than a duplex containing a geS2T-dA base pair (15). The enhanced discrimination between G- and A-ended codons conferred by *S*-geranylation has been attributed to the conversion of the N3H donor center into an N3 acceptor center.

Although the *S*-geranylation of tRNAs has been found in several bacterial strains (e.g. *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa* and *Salmonella Typhimurium*), detailed data on the properties and cellular functions of these tRNAs have not been reported, with the exception of data on the hydrophobicity and structural characteristics of mnm5geS2U and cmnm5geS2U at the nucleoside level (16).

Here, we report three original and effective methods for the chemical synthesis of model RNA oligonucleotides containing *S*-geranyl-2-thiouridine. We also demonstrate

that an S2U-RNA homologous in sequence to the human tRNA^{Lys3} anticodon stem-loop (ASL) domain is geranylated by the recombinant SelU enzyme, albeit only in the presence of Mg²⁺. The efficiency of enzymatic *S*-geranylation of the S2U-RNA model (lacking the 5-(c)mnm-substituent and trimmed to the ASL domain only) and the demonstrated substrate tolerance suggest promising approach for future research based on the enzymatic preparation of *S*-geranylated-ASL oligoribonucleotides. The geranylated RNA models produced in this study were characterized in terms of their lipophilicity and binding preferences in comparison with their parent S2U-RNAs as well as non-modified U-RNAs.

MATERIALS AND METHODS

Chemical synthesis of geS2U-RNA

Synthesis of S-geranyl-2-thiouridine phosphoramidite 1. 2'-O-(tert-Butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-S-geranyl-2-thiouridine (6). 5'-O-DMTr-geS2U (5) (14) (650 mg, 0.93 mmol) was dissolved in anhydrous pyridine (10 ml). Then imidazole (190 mg, 2.79 mmol, 3 equiv.) and TBDMSCl (209 mg, 1.40 mmol, 1.5 equiv.) were added and the mixture was stirred at room temperature (rt) for 20 h. The reaction was quenched with water (20 ml) and the mixture was extracted with CHCl₃ (3 × 30 ml). The combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. The residue was co-evaporated with anhydrous toluene and purified on a silica gel column (MeOH/CH₂Cl₂ 4:96, v/v) to yield a 2'-O-silylated isomer **6** as a white foam (302 mg, 0.37 mmol, 40%) and an undesired 3'-O-silylated isomer in 45%. To get additional quantities of **6**, the 3'-isomer was partially isomerized in methanol with a trace amount of triethylamine (TEA) and additional 114 mg of **6** were isolated rising the overall yield to 55%. TLC R_f = 0.75 (CH₂Cl₂/acetone 90:10, v/v). NMR (δ [ppm], DMSO-d₆): ¹H (250 MHz) 0.02 (s, 3H), 0.06 (s, 3H), 0.86 (s, 9H), 1.56 (s, 3H), 1.63 (s, 3H), 1.71 (s, 3H), 1.98–2.00 (m, 2H), 2.03–2.06 (m, 2H), 3.25 (dd, *J* = 2.8 Hz, *J* = 11.2 Hz, 1H), 3.38 (dd, *J* = 4.2 Hz, *J* = 11.2 Hz, 1H), 3.75 (s, 6H), 3.79–3.82 (m, 2H), 4.07–4.12 (m, 2H), 4.30 (t, *J* = 7.5 Hz, 1H), 5.04–5.06 (m, 1H), 5.29–5.32 (m, 1H), 5.38 (d, *J* = 7.5 Hz, 1H), 5.64 (d, *J* = 7.5 Hz, 1H), 5.73 (d, *J* = 4.9 Hz, 1H), 6.91–7.38 (m, 13H), 7.90 (d, *J* = 7.5 Hz, 1H); ¹³C (176 MHz) -4.76, -4.22, 16.59, 18.01, 18.32, 25.92, 26.08, 26.37, 29.93, 39.05, 55.56, 63.24, 70.16, 76.88, 84.85, 86.67, 91.77, 109.35, 113.81, 117.63, 124.09, 127.39, 128.08, 128.39, 130.24, 131.52, 135.31, 135.54, 139.23, 141.79, 145.04, 158.69, 162.04, 166.67. HRMS (FAB) calc. for C₄₆H₆₀N₂O₇SSi [M+H]⁺ 813.3969, found 813.3999.

2'-O-(tert-Butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-S-geranyl-2-thiouridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite (1). To a solution of 5'-O-DMTr-2'-O-TBDMS-*S*-geranyl-2-thiouridine **6** (256 mg, 0.32 mmol) in anhydrous THF (3 ml, under argon atmosphere), 4-dimethylaminopyridine (DMAP) (7.7 mg, 0.06 mmol, 0.2 equiv.), *N,N*-diisopropylethylamine (DIEA) (221 μl, 1.27 mmol, 4 equiv.) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (141 μl, 0.63 mmol, 2 equiv.) were added. The reaction mixture (protected from

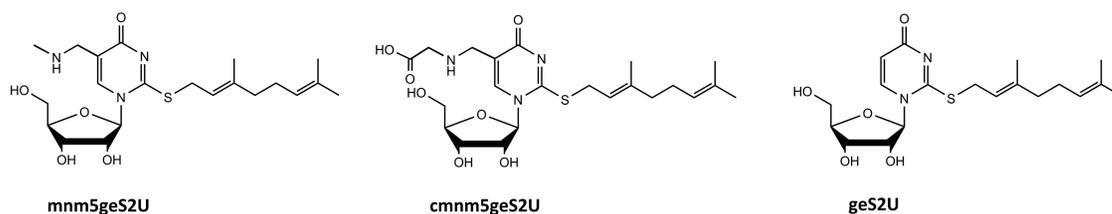


Figure 1. Chemical structures of *S*-geranylated nucleosides.

the daylight) was stirred at rt. After reaction completion (2 h, TLC control) the mixture was diluted with AcOEt (30 ml), treated with 5% aq. NaHCO₃ (10 ml) and washed with water (10 ml). The combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. The oil residue was co-evaporated with anhydrous toluene and purified on a silica gel flash column (petroleum ether/AcOEt 1:1, v/v) to yield **1** as a white foam (214 mg, 0.21 mmol, 66%). TLC: R_f = 0.83 (CH₂Cl₂/AcOEt/TEA, 10:10:1, v/v). NMR (δ [ppm], C₆D₆): ¹H (250 MHz) -0.07–0.17 (m, 6H), 0.66–0.96 (m, 7H), 0.99–1.01 (m, 9H), 1.13–1.19 (m, 5H), 1.54–1.67 (m, 10H), 1.90–2.08 (m, 5H), 2.98–3.77 (m, 12H), 3.86–3.94 (m, 2H), 4.10–4.51 (m, 2H), 4.65–4.77 (m, 1H), 5.30–5.40 (m, 1H), 5.97–6.05 (m, 1H), 6.19–6.27 (m, 1H), 6.76–6.82 (m, 4H), 7.05–7.08 (m, 1H), 7.18–7.60 (m, 8H), 8.03–8.13 (m, 1H); ³¹P (101 MHz) 149.07, 151.91; HRMS (ESI): calc. for C₅₅H₇₇N₄O₈PSSi [M+H]⁺ 1013.5047, found 1013.5042.

Chemical synthesis of geS2U-RNA via direct incorporation of the geS2U unit (method I). A geS2U-RNA oligomer of the 5'-GUUGACUgeS2UUUAAUCAAC-3' sequence was synthesized automatically on a 0.2 μmole scale with the use of an H6 GeneWorld DNA/RNA automated synthesizer (K&A, Laborgeraete GbR, Schaafheim, Germany). The commercially available phosphoramidites of A, C, U and G protected on the 5'- and 2'-hydroxy functions with DMTr and TBDMS groups, respectively, were used. The exocyclic amine functions were masked with phenoxyacetyl (A and G units), or acetyl (C) groups (Proligo). The rC(tac)-succinyl-CPG (Proligo) support and 0.07 M acetonitrile (ACN) solutions of the monomers were used. The geranyl-modified S2U-phosphoramidite **1** was dissolved in a mixture of THF/ACN (1:9, v/v). All amidities were used in a 10-fold molar excess and the coupling in the presence of a BMT activator (0.25 M in ACN) was executed for 10 min. For the oxidation step, a solution of I₂ (0.02 M in THF/H₂O/pyridine 90.54:9.05:0.41, v/v/v) was used for 5 min. Capping was performed with TAC₂O in THF using a mixture of Fast Deprotection Cap A (Proligo) and Cap B (Proligo) (1.1:1, v/v) for 2 min. After the last coupling the DMTr group was removed and the support was washed, dried and transferred to a screw cap glass vial. Then a TEA/ACN mixture (265 μl, 1:1, v/v) was added and the suspension was stirred for 20 min, and then the volatile components were removed. The support-bound RNA was washed with ACN (3 × 100 μl), dried *in vacuo* for 30 min (a checkpoint A), and treated with ethanolic ammonia (8 M, 340 μl) at rt for 8 h. The supernatant was removed and the support was washed with anhydrous ethanol (3 × 150

μl). The combined washings were evaporated on a Speed-Vac concentrator and the solid residue was treated with Et₃N·3HF in NMP (1:1 v/v, 120 μl) for 24 h at rt (the checkpoint B). The reaction was quenched by addition of 240 μl of ethoxytrimethylsilane and the crude RNA was precipitated using 600 μl of *tert*-butyl methyl ether. The fully deprotected RNA was purified by anion-exchange (IE) HPLC (Source 15Q 4.6/100PE) at a constant flow rate of 1 ml/min. The column was eluted with a linear gradient 50–500 mM NaBr in a 20 mM Na₂HPO₄–NaH₂PO₄ buffer solution pH 7.5, containing 50 μM EDTA and 10% ACN. Fractions containing the desired product were collected, concentrated and desalted on a C-18 cartridge (Sep-Pak, Waters). The desalted RNA was lyophilized (19 OD₂₆₀ units, ca. 55% yield) and analyzed by MALDI-TOF mass spectrometry (*m/z* 5481, MW 5483) (Supplementary Figure S1).

Chemical synthesis of geS2U-RNA via the post-synthetic geranylation of support-linked S2U-RNA (method II). The S2U-RNA of the 5'-GUUGACUS2UUUAAUCAAC-3' sequence was synthesized automatically on a 0.2 μmole scale analogously to the synthesis of geS2U-RNA with the exception of the use of S2U-phosphoramidite **2** (17), and conditions for the oxidation steps, where a solution of *t*-BuOOH in toluene (0.25 M, 8 equiv.) was applied for 2 min. The support carrying detritylated S2U-RNA was worked-up until the checkpoint A (*vide supra*) was reached. Now the sample was treated with a mixture of geranyl bromide (4.2 μl, 213 equiv.), triethylamine (3 μl, 213 equiv.) and ethanol (45 μl) and the suspension was shaken vigorously for 3 h at rt. The supernatant was removed and the support was washed with anhydrous ethanol (3 × 150 μl) and dried *in vacuo* for 30 min, followed by treatment with ethanolic ammonia (8 M, 340 μl) for 8 h at rt. The supernatant was removed and the support was washed with ethanol (3 × 150 μl). The combined supernatant and washings were evaporated on a Speed-Vac concentrator. To the solid residue a solution of Et₃N·3HF in NMP (1:1, v/v, 120 μl) was added and the reaction was left for 24 h at rt. Further RNA isolation and purification was analogous to the procedure after the checkpoint B (*vide supra*) and 13 OD₂₆₀ units of geS2U-RNA were obtained (ca. 40% yield). Taking into account that the average yield of the synthesis of S2U-RNA is 71% (24 OD from 0.2 μmole scale), the efficacy of the CPG-S2U-RNA → CPG-geS2U-RNA conversion could be estimated at 55%.

The selectivity of the post-synthetic geranylation of S2U-RNA was confirmed by enzymatic hydrolysis of the final RNA with nuclease P1 and alkaline phosphatase (18,19). The resulting mixture of nucleosides was analyzed by RP-

HPLC on a C18 column (Ascentis, 4.6 × 250 mm) using a mixture of buffer A (0.1 M CH₃COONH₄) and buffer B (40% ACN in 0.1 M CH₃COONH₄) at a constant flow rate of 1 ml/min (details are given in Supplementary Figure S2A). The retention time (Rt) of the geS2U was compared to the Rt of the reference samples in a separate control experiment (Supplementary Figure S2B).

Chemical synthesis of geS2U-RNA via the post-synthetic geranylation of fully deprotected S2U-RNA (method III). The S2U-RNA was synthesized automatically in a 0.2 μmole scale according to the procedure described above. The support carrying detritylated S2U-RNA was treated with aq. NH₃/EtOH (3 : 1, v/v, 1.5 ml) for 16 h at 40°C. The supernatant was collected and the support was washed with ethanol/H₂O (1 : 1, v/v, 3 × 150 μl). The combined fractions were evaporated on a Speed-Vac concentrator. To the solid residue a solution of Et₃N·3HF in NMP (1:1 v/v, 120 μl) was added and the reaction mixture was left for 24 h at rt. Further isolation and purification steps were performed analogously as described in Method II. The desalted S2U-RNA was lyophilized and analyzed by mass spectrometry (MALDI-TOF, *m/z* 5342, MW 5349). For the geranylation, to a solution of S2U-RNA (24 OD) in an EtOH/H₂O mixture (1/1, v/v, 90 μl), TEA (6 μl, 213 equiv.) and geranyl bromide (8.4 μl, 213 equiv.) were added. The mixture was shaken vigorously for 3 h at rt. The resultant solution was diluted with 20% aq. EtOH and the RNA oligonucleotide was isolated using a NAP-25 column (GE Healthcare). The eluate was evaporated in a Speed-Vac concentrator and purified by IE HPLC according to the procedure described in method I to furnish the geS2U-RNA product in 67% yield. The relevant MALDI-TOF mass spectrum is shown in Supplementary Figure S3.

Enzymatic geranylation of S2U-RNA with a recombinant SelU enzyme

Overexpression and purification of the SelU enzyme. A 1095 bp gene for selenouridine tRNA synthase (SelU, EC: 2.9.1- EAL2_c13120) was amplified from total bacterial RNA (*E. coli*, TOP10 strain) using a Transcriptor One-Step RT-PCR kit (Roche) with the appropriate forward (Fow) and reverse (Rev) primers: selU-For (5'-AAAAAaccatggATGCAAGAGAGACACGGA-3') and selU-Rev (5'-ATATATctcagCCGCGCCTTAACCCATTCC-3'). An insert coding the *selU* gene was cloned into a pET28c expression vector (Novagen) between the T7 promoter and the region encoding a His6 affinity tag. The recombinant protein was overexpressed in BL21 Star (DE3) cells (Invitrogen) in 3 l of 2xYT medium supplemented with kanamycin (50 μg/ml). After growth at 37°C to an OD₆₀₀ = 0.6, the SelU synthesis was induced by addition of isopropyl-β-D-thiogalactopyranoside (1 mM) and a culture was continued to grow at 20°C for 4 h. After that, the cells were harvested by centrifugation and the cell pellet (~15 g of the wet mass) was resuspended in 20 ml of a 50 mM sodium phosphate buffer, pH 8.0, with the EDTA-free Protease Inhibitor cocktail (Roche) and protamine sulfate (0.5 mg/ml) (Sigma-Aldrich). Cells were disrupted by sonication. All subsequent steps were performed at 4°C. After

centrifugation for 0.5 h at 15 000 ×g, the clarified extract was loaded on a column with ~3 ml of HisPur Cobalt Resin (ThermoScientific) pre-equilibrated in a buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl and 10 mM imidazole). After washing with 30 ml of buffer A, the protein was eluted with 20 ml buffer A containing 100 mM imidazole, followed by two consecutive dialyses against 1 l of a 50 mM sodium phosphate buffer, pH 8.0. Purification of the dialyzed protein was repeated by affinity chromatography and the solution of the protein was dialyzed twice against 1 l of 20 mM Tris HCl, pH 8.0. After dialysis, the protein was concentrated (using an Amicon Ultra centrifugal filter unit 30K, Merck-Millipore), and the final concentration was determined spectrophotometrically. The protein was preferably used immediately after purification. The surplus protein was frozen in liquid nitrogen and stored at -70°C.

Synthesis of geS2U-RNA by enzymatic geranylation with recombinant SelU enzyme. The 17-mer S2U-RNA (assigned as S^b) of the 5'-UCAGACUS2UUUUAUCUGA-3' sequence, isosequential to the anticodon-stem-loop (ASL) of human tRNA^{Lys3}_{UUU} (1 OD, 40 μg) was incubated with the purified enzyme SelU-His6 (10 μg) in the presence of 5-fold molar excess of ammonium geranyl pyrophosphate (Sigma-Aldrich). The reaction was performed in the buffer composed of 10 mM Tris-HCl pH 8.0, 0.2 mM dithiothreitol and 2% glycerol without or with addition of MgSO₄ salt (up to 100 mM), in total reaction volume 50 μl, at 25°C. A reaction with the thermally inactivated enzyme served as the negative control. After 24 h incubation the mixture was analyzed by RP-HPLC (a Prominence chromatograph, Shimadzu, Japan) using a Kinetex 5μ C-18 column (100Å; 250 × 4.60 mm, Phenomenex) eluted with a mixture of two buffers (A: 0.1 M CH₃COONH₄; B: 0.1 M CH₃COONH₄/40% ACN) according to the following gradient: 0% B for 20 min.; 0→100 % B over 30 min.; then isocratically 100% B for 10 min. The fractions collected at Rt = 39 min were desalted using a standard SepPak C-18 (Waters) procedure and the identity of the geranylated product was confirmed by MALDI-TOF MS (*m/z* 5477, MW 5483, Supplementary Figure S4).

Physicochemical characterization

UV melting temperature measurements. All UV absorption measurements were carried out in a 1-cm path length cells using a Cintra 4040 spectrophotometer, equipped with a Peltier Thermocell (GBC, Dandenong, Australia), with a detector set at 260 nm. Complementary RNA/RNA or RNA/DNA oligonucleotide strands (detailed sequences are listed in Table 1) were mixed in a phosphate buffer (10 mM sodium phosphate pH 7.4 containing 100 mM NaCl) at the final 2 μM concentration. For the experiments with the Ag⁺ ions mediated base-pairing three equivalents of AgNO₃ (6 μM final concentration) were added. The samples were then heated to 85°C, and cooled to 15°C with a temperature gradient of 1.5°C/min. The melting profiles were recorded from 15 to 85°C, with the temperature gradient of 0.5°C/min. The melting temperatures were calculated using the first derivative method implemented in the Cintra

Table 1. UV melting temperatures and Gibbs free energy values for RNA/RNA and RNA/DNA duplexes → ss RNA/DNA transitions

No.	Name	Sequence	T _m (°C)	T _m after addition of Ag ⁺ (°C)	ΔG° (kcal/mol)
1.	U/A	5'-GUUGACU U UAAUCAAC-3' 3'-CAACUGA A AAUUAGUUG-5'	55.9 ± 0.3	55.7 ± 0.1	-15.1 ± 0.1
2.	S/A	5'-GUUGACU S UAAUCAAC-3' 3'-CAACUGA A AAUUAGUUG-5'	59.5 ± 0.2	58.7 ± 0.2	-15.8 ± 0.1
3.	geS/A	5'-GUUGACU geS UAAUCAAC-3' 3'-CAACUGA A AAUUAGUUG-5'	46.9 ± 1.0	49.9 ± 0.3	-10.7 ± 0.2
4.	U/G	5'-GUUGACU U UAAUCAAC-3' 3'-CAACUGA G AAUUAGUUG-5'	53.5 ± 0.3	53.5 ± 0.1	-13.9 ± 0.3
5.	S/G	5'-GUUGACU S UAAUCAAC-3' 3'-CAACUGA G AAUUAGUUG-5'	51.6 ± 0.5	53.7 ± 0.5	-12.9 ± 0.2
6.	geS/G	5'-GUUGACU geS UAAUCAAC-3' 3'-CAACUGA G AAUUAGUUG-5'	50.1 ± 0.2	50.0 ± 1.0	-12.2 ± 0.2
7.	U/dA	5'-GUUGACU U UAAUCAAC-3' 3'-d(CAACTGA dA AATTAGTTG)-5'	43.4 ± 0.4	44.3 ± 0.1	-9.4 ± 0.1
8.	S/dA	5'-GUUGACU S UAAUCAAC-3' 3'-d(CAACTGA dA AATTAGTTG)-5'	46.6 ± 0.2	44.8 ± 0.2	-11.1 ± 0.1
9.	geS/dA	5'-GUUGACU geS UAAUCAAC-3' 3'-d(CAACTGA dA AATTAGTTG)-5'	44.3 ± 0.4	45.1 ± 1.7	-10.1 ± 0.2
10.	U/dG	5'-GUUGACU U UAAUCAAC-3' 3'-d(CAACTGA dG AATTAGTTG)-5'	44.1 ± 0.5	47.4 ± 0.1	-9.7 ± 0.1
11.	S/dG	5'-GUUGACU S UAAUCAAC-3' 3'-d(CAACTGA dG AATTAGTTG)-5'	44.7 ± 0.2	43.4 ± 0.2	-9.8 ± 0.1
12.	geS/dG	5'-GUUGACU geS UAAUCAAC-3' 3'-d(CAACTGA dG AATTAGTTG)-5'	43.3 ± 0.8	45.1 ± 0.2	-9.3 ± 0.1

Duplex abbreviated names and their sequences are given in columns 2 and 3. T_m values were determined by calculating the first derivative of the melting curve functions generated by the melting software Cintra 4040. Duplexes (2 μM) were dissolved in 10 mM sodium phosphate (pH 7.4) containing 100 mM NaCl. For sealing measurements, duplexes were incubated with 3 equiv. of AgNO₃.

4040 software. The calculation of thermodynamic parameters (ΔG°, ΔH° and ΔS°) was done by numerical fitting of a given melting curve using a two-state model algorithm provided by a MeltWin v.3.5 software. Each result was taken as an averaged one from three independent experiments.

Circular Dichroism Measurements. Circular dichroism measurements were performed using a J-815 circular dichroism (CD) spectrometer (Jasco, Japan). The duplex samples were prepared at a 2 μM concentration in a 10 mM sodium phosphate buffer, pH 7.4 containing 100 mM NaCl. Equimolar amounts of complementary oligonucleotides were mixed in a buffer, heated to 95°C and slowly cooled down to room temperature. CD spectra were obtained using a 0.5 cm path length quartz cell (Helma). The recording parameters were: scan speed 50 nm/min, response time 2 s, band width 1.0 nm and step resolution 1.0 nm. The spectra were recorded at 24°C, in the wavelength range from 200 to 360 nm. The spectrum recorded for the buffer was numerically subtracted from the spectrum for each sample (recorded in triplicate), and the resultant averaged spectra were smoothed with a Savitzky–Golay algorithm (convolution width 7).

Determination of Log P of geS2U–RNA. The partition coefficient (P) was determined in a water/1-octanol immiscible bi-phase system. The samples of single stranded RNAs (1 OD₂₆₀ unit of U, S or geS) were suspended in a mixture of 1 ml of RNase free water and 1 ml of 1-octanol reciprocally saturated with 1-octanol or RNase free water, respectively. The samples were shaken vigorously at room temperature for at least 2 h to ensure that the partitioning of the oligonucleotide reached the equilibrium. The aqueous

and organic phases were separated by centrifugation (10 min, 15 000 *xg*, rt). Approximately 900 μl aliquots of the aqueous and 1-octanol layers were taken, and the concentrations of dissolved oligonucleotides were assessed by UV absorption measurements at 260 nm (Cintra 4040 spectrophotometer). The partition coefficient P, defined as the ratio of the oligonucleotide concentrations in the organic and aqueous phases, was determined and its log P-value was calculated according to the equation: $\text{Log } P_{o/w} = \text{Log } [c_{\text{RNA}}]_o - \text{Log } [c_{\text{RNA}}]_w$.

RESULTS

Design of model RNAs

We designed a model 17-mer geS2U–RNA oligomer (abbreviated as geS) with the following sequence: 5'-GUUGACUgeS2UUAAUCAAC-3'. This sequence is homologous to the sequence of the ASL of *E. coli* tRNA^{Lys}_{UUU}. For the enzymatic geranylation, an analogous ASL model of S2U–RNA with the sequence 5'-UCAGACUS2UUAAUCUGA-3' was used; this sequence is identical to that of the anticodon-stem-loop of human tRNA^{Lys}_{UUU}. The S2U–RNA was abbreviated as S^h, while the geranylated product was abbreviated as geS^h. The modified unit in both RNA models was inserted in the location corresponding to the wobble position in the intact tRNAs. For UV melting experiments, CD measurements and lipophilicity measurements, the corresponding analogs of geS were prepared, namely U–RNA (abbreviated as U) and S2U–RNA (abbreviated as S) as well as their complementary strands with A or G nucleotides (abbreviated as A or G, respectively) located opposite of the modified unit.

Chemical synthesis of geS2U–RNA

Chemical syntheses of model oligoribonucleotides containing *S*-geranyl-2-thiouridine were performed using three different chemical methods, numbered I–III (Scheme 1). These approaches included direct incorporation of the geS2U unit into the RNA chain by standard phosphoramidite chemistry using the monomer **1** (I), the post-synthetic geranylation of CPG-linked S2U–RNA (II) or the post-synthetic geranylation of fully deprotected S2U–RNA (III).

Optimization of conditions of the geS2U-RNA synthesis. To determine an effective protocol for the synthesis of geS2U-containing oligoribonucleotides via phosphoramidite chemistry (Scheme 1, method I), the stability of *S*-geranyl-2-thiouridine was first tested under typical conditions for RNA synthesis: geS2U was incubated in a 3% trichloroacetic acid (TCA) in DCM, a 0.02 M iodine solution, a mixture of Cap A and Cap B or in 0.25 M 5-(benzylmercapto)-1*H*-tetrazole (BMT) in ACN. Subsequent TLC analysis indicated that geS2U is sufficiently stable under these conditions. The stability of geS2U was further analyzed under conditions commonly used for the removal of base-labile protecting groups and for the hydrolysis of succinyl linker. TLC analysis indicated that an aq. ammonia/ethanol (3:1, v/v) solution is not suitable, as the *S*-geranyl moiety is lost within 3 h of incubation at room temperature. However, an alternative treatment with anhydrous 8 M NH₃/EtOH for 8 h at room temperature did not result in any visible degradation products. Furthermore, the *S*-geranyl group remained intact after geS2U was treated with Et₃N·3HF/NMP or 1 M TBAF/NMP (desilylating conditions) for 24 h at room temperature.

Synthesis of S-geranyl-2-thiouridine phosphoramidite 1. *S*-geranylated 5'-*O*-DMTr-2-thiouridine (**5**), obtained according to a previously described procedure (14) and as shown in Scheme 2, was further protected at the 2'-hydroxyl group with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) under standard conditions (20,21), producing an equimolar mixture of 2'- and 3'-*O*-TBDMS regioisomers. These regioisomers were separated by column chromatography and isomerization of the 3'-*O*-TBDMS regioisomer (in a triethylamine/methanol solution), raised the overall yield of **6** to 55%. The structure of **6** was confirmed by ¹H and ¹³C NMR (Supplementary Figures S5 and S6) and by HRMS (FAB) mass spectrometry. A 2D COSY NMR experiment (Supplementary Figure S7) confirmed the identity of the 2'-isomer via correlation of its H3' with 3'-OH protons.

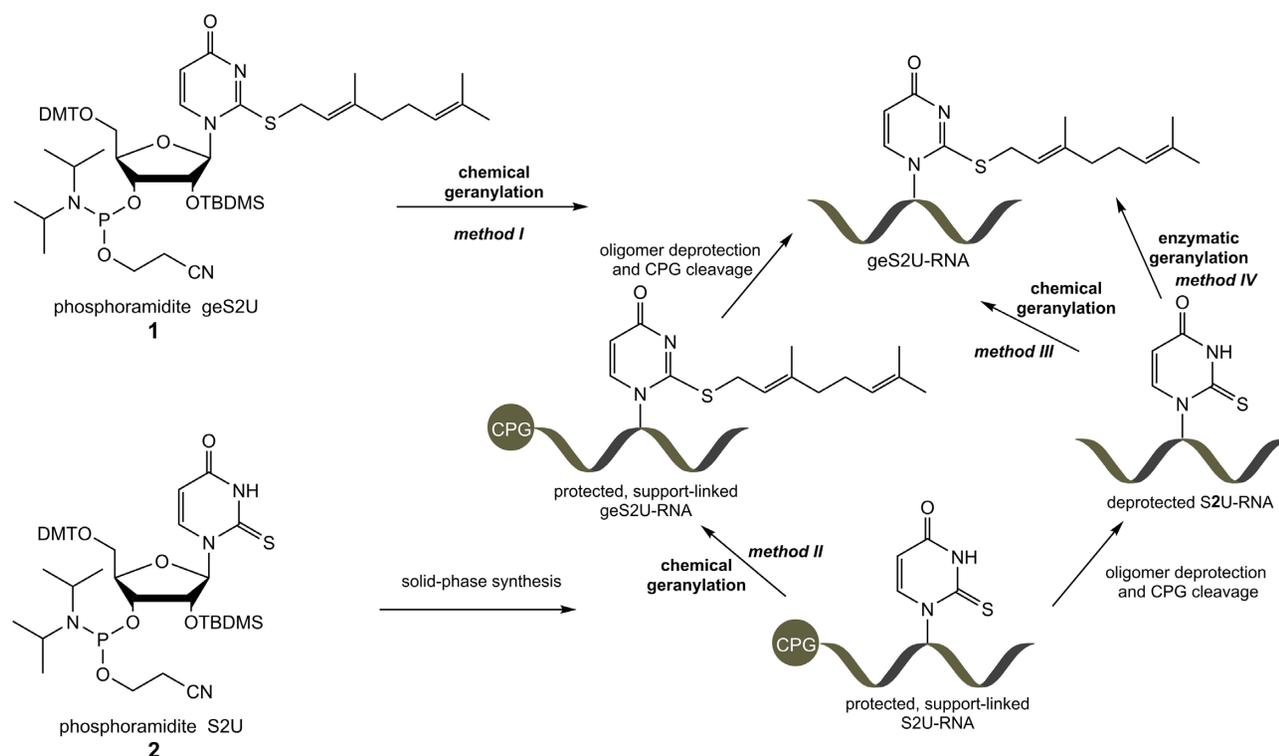
Phosphitylation of **6** was performed in anhydrous THF using 2-cyanoethyl *N,N*-diisopropylaminochlorophosphoramidite in the presence of DMAP and DIEA (21,22). The product **1** was isolated by silica gel flash column chromatography in 66% yield and characterized by ¹H and ³¹P NMR (Supplementary Figures S8 and S9) as well as HRMS FAB.

Synthesis of geS via direct incorporation of geS2U unit into the RNA chain (method I). The phosphoramidite monomer **1** was used for the synthesis of geS RNA. Because of the monomer's modest solubility in ACN, a THF:ACN mixture was used to prepare a monomer solution suitable

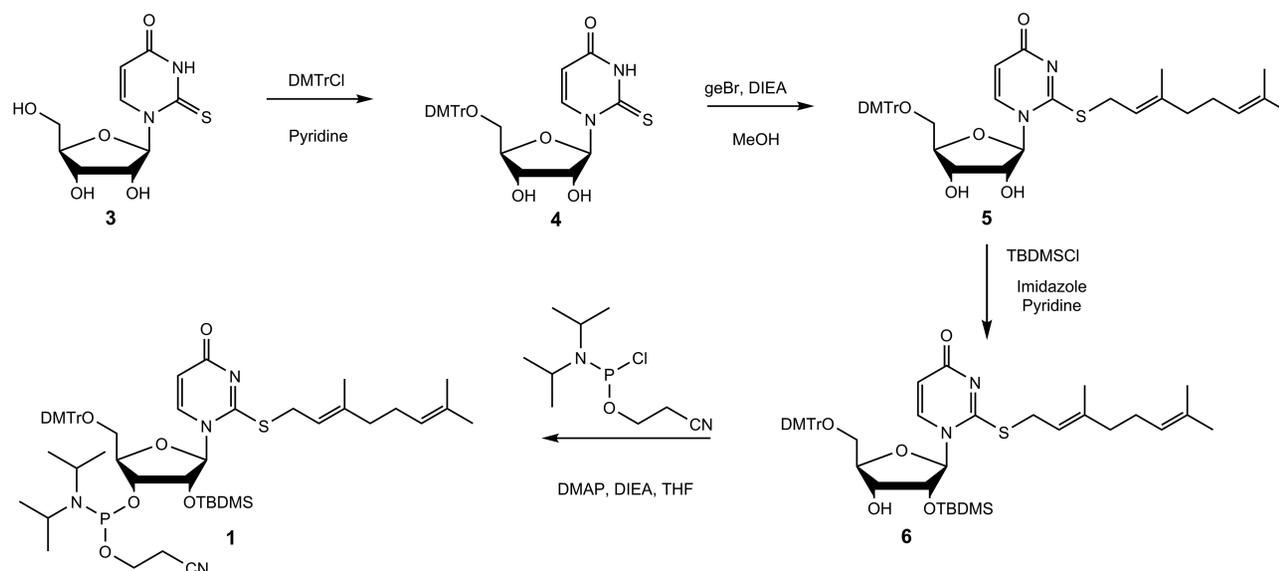
for automated solid phase synthesis. A 10-fold molar excess of each amidite was used, and coupling reactions were conducted for 10 min using BMT in ACN as an activator. Oxidation was carried out with an I₂/THF-H₂O-pyridine solution. After chain assembly was complete, the DMTr group was removed, and CPG-bound RNA was treated with TEA in ACN to remove the 2-cyanoethyl groups from the triester phosphate residues. Upon treatment with anhydrous 8 M ethanolic ammonia (8 h, rt) the oligoribonucleotide was released from the CPG-support, and the remaining base-labile groups were removed. Finally, 2'-OH groups were deprotected with a mixture of Et₃N·3HF in NMP. After purification (Figure 2A), 19 ODs of chromatographically pure geS2U–RNA were obtained (ca. 55%). The molecular mass (MW 5483) of geS was confirmed by MALDI-TOF mass spectrometry (*m/z* 5481) (Supplementary Figure S1).

Synthesis of geS via post-synthetic geranylation of CPG-linked S2U-RNA (method II). An alternative route to geS RNA involved the geranylation of 2-thiouridine in an already assembled oligomer still linked to the solid support (Scheme 1, method II). *S*-geranylation was previously reported at the nucleoside level (11,14). The applied reaction conditions (geranyl bromide, DIEA, methanol, 15 min., rt) seemed to be safe for canonical nucleosides, and we performed a similar reaction at the oligomer level. The S2U unit was incorporated into an RNA chain (*E. coli* ASL^{Lys}_{UUU}) using an appropriately protected S2U amidite (**2**) (2, Scheme 1). To avoid the loss of sulfur, a 0.25 M *tert*-butyl hydroperoxide solution in toluene was used in the oxidation steps (24). After some workup (see Materials and Methods), the support-bound RNA was dried in *vacuo* and *S*-alkylated with a mixture of geranyl bromide (>200 equivalents), TEA and EtOH (3 h, rt) (25). Further workup and chromatographic purification furnished 13 ODs of geS RNA (ca. 40% yield). IE-HPLC analysis of the crude product (Figure 2B) indicated almost quantitative conversion of S2U–RNA (Rt = 30.25 min.) to geS2U–RNA (Rt = 31.09 min.; see co-injection profile in Supplementary Figure S10). The structure of geS2U–RNA and the selectivity of geranylation were confirmed by MALDI-TOF mass spectrometry (*m/z* 5481) as well as enzymatic hydrolysis followed by analysis of nucleoside composition (Supplementary Figure S2A). RP-HPLC analysis revealed the presence of four canonical nucleosides and the reference geS2U (at Rt = 54 min) (Supplementary Figure S2B).

Synthesis of geS via post-synthetic geranylation of fully deprotected S2U-RNA (method III). In this approach, the synthesis of geS2U–RNA was based on the post-synthetic geranylation of fully deprotected S2U–RNA (Scheme 1, method III). The starting RNA, which contained 2-thiouridine (17-mer *E. coli* ASL, S), was obtained as described above. After final desalting, the product was lyophilized, treated with an emulsion of geranyl bromide and TEA in an ethanol/water solution, and then vigorously shaken for 3 h at room temperature. After a pass through a NAP-25 column, the crude geS2U–RNA product (geS) was purified by IE-HPLC according to the procedure described for methods I and II (Figure 2C). The identity of geS RNA (obtained in ca. 67% yield) was confirmed by MALDI-TOF



Scheme 1. A general scheme for geS2U-RNA synthesis. (I) direct incorporation of the geS2U unit using phosphoramidite **1**; (II) post-synthetic chemical geranylation of CPG-linked S2U-RNA with subsequent deprotection/cleavage from the solid support; (III) post-synthetic chemical geranylation of fully deprotected S2U-RNA; (IV) enzymatic geranylation of fully deprotected S2U-RNA with the recombinant SelU enzyme.



Scheme 2. Synthetic pathway of *S*-geranyl-2-thiouridine phosphoramidite **1**.

MS (m/z 5483, MW 5483, Supplementary Figure S3) and by comparison with a genuine sample obtained via chemical synthesis (method I). The RP-HPLC chromatogram of a mixture of S2U-RNA (Supplementary Figure S11A) with its geranylated product (geS2U-RNA, Supplementary Figure S11B) shown in Figure 2D clearly confirms that the

presence of a geranyl residue increases the hydrophobicity of geS over that of S.

Analysis of SelU catalytic activity

Preparation of recombinant SelU. SelU, a tRNA 2-selenouridine synthase from *E. coli*, was previously described as an enzyme that catalyzes two different reactions,

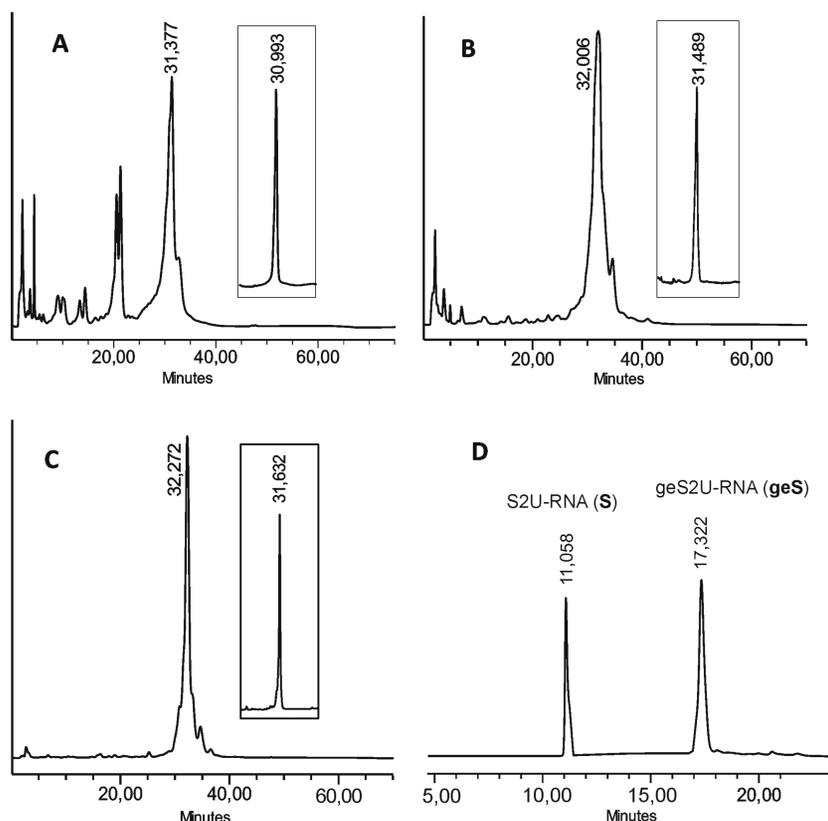


Figure 2. IE-HPLC analysis of the crude geS2U-RNA (geS) synthesized (A) *via* direct incorporation of geS2U unit with phosphoramidite **1** (method I, anion-exchange IE-HPLC analysis on a Source 15Q 4.6/100PE®) as described in the Materials and Methods); (B) *via* the post-synthetic geranylation of support-linked S2U-RNA (method II, IE-HPLC as described above); (C) *via* post-synthetic geranylation of fully deprotected S2U-RNA (S) (method III, anion-exchange IE-HPLC analysis on a Source 15Q 4.6/100PE®) according to the methodology described in the Materials and Methods). The insets indicate IE-HPLC analysis of purified oligoribonucleotides; (D) RP-HPLC co-injection of pure 5'-GUUGACUS2UUUAAUCAAC-3' (Rt = 11.0 min.) and 5'-GUUGACUgeS2UUUAAUCAAC-3' (Rt = 17.3 min.) using a C18 column (Ascentis®, 4.6 × 250 mm, 5 μm). The mobile phase composition was as follows: 100% A (0.1 M ammonium acetate) for 5 min.; linear gradient of 100% buffer A to 100% buffer B (40% acetonitrile in 0.1 M ammonium acetate) for 20 min.; maintain at 100% B for 5 min.

selenation (26) and geranylation of bacterial 5-(c)mm-2-thiouridine-containing tRNAs (11). We isolated the *selU* gene (1095 bp) from total bacterial RNA and cloned it into a pET28c expression vector between the sequence complementary to the T7 promoter and the region encoding a His6 affinity tag. The sequence of the insert was confirmed via sequencing. The recombinant protein SelU-His6 (MW of ca. 42 kDa) was overexpressed in BL21Star(DE3) and accounted for ~1% of the total bacterial protein pool (Supplementary Figure S12). The protein was purified up to 90% purity using HisPur Cobalt Resin according to the procedure described in the Materials and Methods. An improved preparation procedure allowed ~1.5 mg of protein to be obtained from 1 l of bacterial culture.

Enzymatic geranylation of S2U-RNA (method IV). Approximately 40 μg (1 OD) of 17-mer oligo-RNA (ASL^{Lys3}) with the sequence 5'-UCAGACUS2UUUAAUCUGA-3' (Supplementary Figure S4A, *m/z* 5342, MW 5349) and containing a single S2U substitution at the site corresponding to the wobble position of the intact tRNA was incubated with the SelU-His6 enzyme (10 μg) in the presence of a 5-fold molar excess of the ammonium salt of geranyl pyrophosphate. A set of reactions was performed in Tris-HCl

buffer (pH 8.0) with increasing amount of MgSO₄. After 24 h of incubation at 25°C, reaction mixtures were analyzed by RP-HPLC (Figure 3). The geS2U-RNA product (geS^h) appeared as a single peak at Rt = 39.1 min. (the S2U-RNA substrate peak was at Rt = 29.4 min). Samples collected during RP-HPLC were analyzed by MALDI-TOF mass spectrometry (Supplementary Figure S4), and the structure of geS^h was confirmed (*m/z* 5477, MW 5483).

Enzymatic geranylations with thermally denatured SelU or geranylations performed in Mg²⁺-free buffer were unsuccessful (Figure 3A and B, respectively). Reactions carried out in buffers containing 10 or 100 mM Mg²⁺ yielded the desired geS^h product in 9 or 11 % yield (Figure 3C and D), respectively. The geS^h oligomer was hydrolyzed with nuclease nP1 and calf intestine alkaline phosphatase. Nucleoside composition analysis by RP-HPLC then confirmed the presence of the geS2U nucleoside at Rt = 49.4 min (Supplementary Figure S13). The identity of the geS2U nucleoside was further confirmed via co-injection with an original geS2U sample and via comparison of UV spectra (Figure S14).

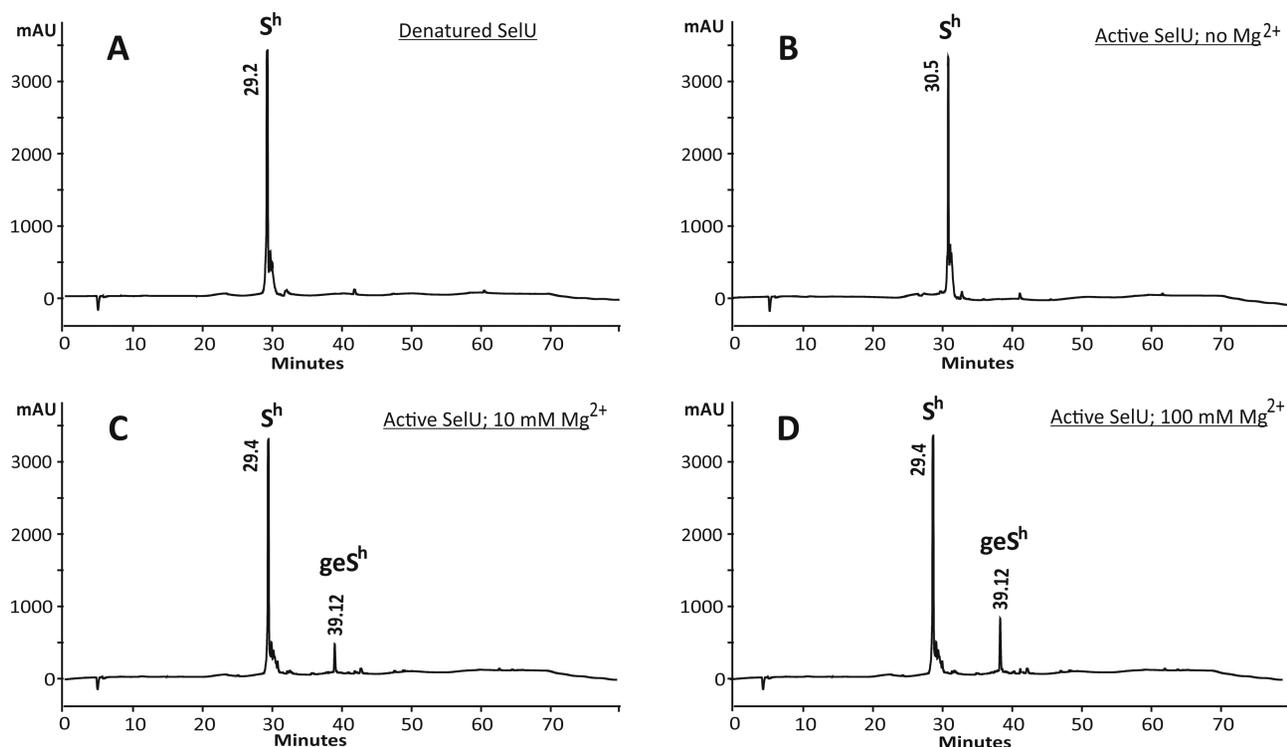


Figure 3. RP-HPLC analysis of the S2U-RNA (5'-UCAGACUS2UUUAAUCUGA-3', ASL of human tRNA^{Lys3}, S^h) geranylation with (A) denatured SelU enzyme in the reaction mixture; (B) active SelU enzyme but without Mg²⁺; (C) 10 mM Mg²⁺ and active SelU enzyme; (D) 100 mM Mg²⁺ and active SelU enzyme. Rt of S^h = 29.4 min., Rt of geS^h = 39.1 min.

Thermodynamic stability of RNA/RNA and RNA/DNA duplexes containing geS2U-RNA strands

To assess the thermodynamic stability of geS2U-RNA/RNA and geS2U-RNA/DNA duplexes, UV-monitored melting experiments were performed for a series of 12 duplexes (Table 1). The U, S and geS RNA strands (*E. coli* ASL with U, S2U or geS2U in the modification site, respectively) were annealed with complementary RNA or DNA strands containing either A/dA or G/dG opposite the modification site. Melting profiles are shown in Figure 4, and calculated T_m values as well as ΔG° values for ds \rightarrow ss dissociation (in kcal/mol) are given in Table 1. The full set of thermodynamic data (ΔH° , ΔS° and ΔG°) is provided in Supplementary Table S1. These results demonstrate that the geranyl group in geS/A greatly decreases the stability of the duplex compared to the U/A and S/A duplexes (containing U-A and S2U-A Watson-Crick base pairs): the free Gibbs' energy (ΔG°) increased by approximately 5 kcal/mol, while T_m is reduced by approximately 9°C and 13°C compared to the U/A and S/A duplexes, respectively. The stability of the U/G and S/G duplexes (with U-G and S2U-G wobble base pairs) was not changed significantly by geranylation. The highest T_m value was observed for the U/G duplex, while T_m values for S/G and geS/G were slightly decreased (by 1.5 and 3.5°C, respectively). ΔG° for the S/G and geS/G duplexes were increased by 1.0 and 1.7 kcal/mol, respectively. Surprisingly, the geS/G duplex was slightly more stable than the geS/A duplex, with $\Delta T_m = 3.2^\circ\text{C}$, and $\Delta\Delta G^\circ = -1.5$ kcal/mol. This difference disappeared (see Supplementary Figure S15)

upon addition of silver ions (27), which mediate formation of an additional bond in the geS/A duplex between the N3 in geS2U and the N1 in A (Figure 6). In this case, the T_m of the geS/A duplex increased by 3.0°C, reaching the T_m value of the geS/G duplex (Table 1).

Circular dichroism analysis of U, S and geS single stranded RNAs and their duplexes

The structures of U, S and geS single-stranded RNAs as well as their duplexes with A and G matrices were analyzed by CD spectroscopy. The measured spectra (Supplementary Figure S16) indicate that both single stranded oligomers (Supplementary Figure S16A) and their RNA/RNA duplexes (Supplementary Figure S16B and C) adopted typical A-type helical structures with a positive Cotton effect maxima at $\lambda \approx 265$ nm, a negative band at $\lambda \approx 210$ nm and a crossover point at 250 nm for ss RNA and ~ 240 nm for dsRNA. The S-geranyl group in the geS2U-RNA did not disturb the overall A-type helical structure of the RNA. As expected, we observed a shift of the crossover point from 240 to 260 nm in the case of RNA/DNA duplexes (Supplementary Figure S16D and E), suggesting that the structure of these duplexes is distorted from the A-RNA type. However, these duplexes remain more similar to the RNA rather than DNA homoduplexes.

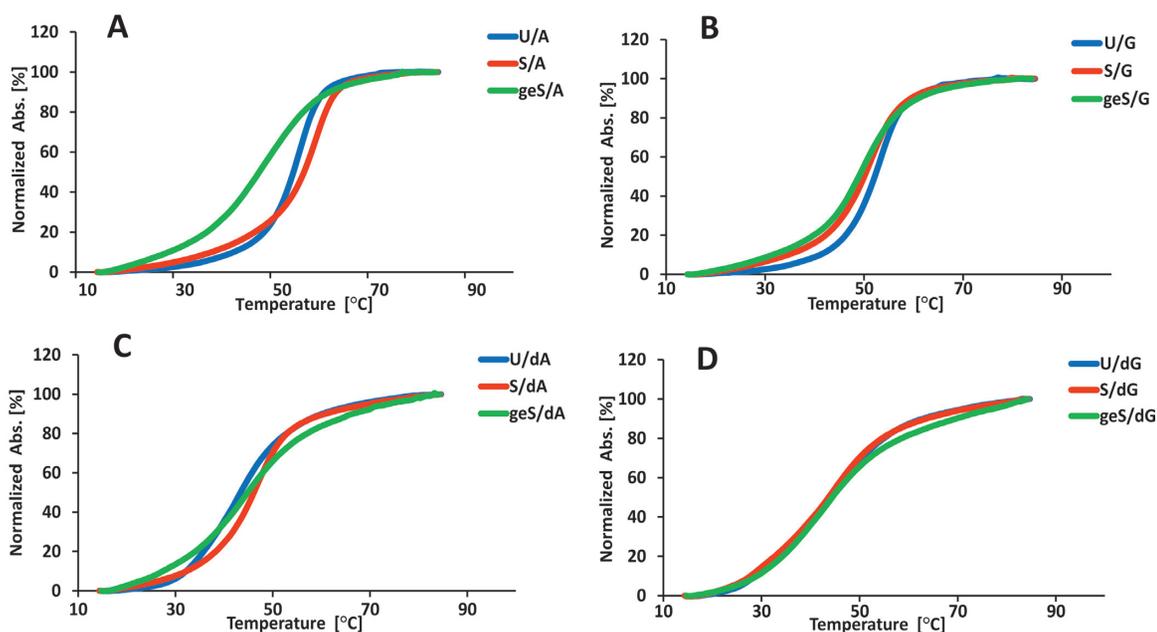


Figure 4. UV melting profiles for four sets of duplexes, (A) U/A, S/A and geS/A; (B) U/G, S/G and geS/G; (C) U/dA, S/dA and geS/dA; (D) U/dG, S/dG and geS/dG.

Table 2. Analysis of the lipophilic properties of *S*-geranyl-2-thiouridine-containing RNA (geS) in comparison to S2U-RNA (S) and unmodified RNA (U) oligonucleotides

RNA oligonucleotide	Log P
U	-1.74 ± 0.16
S	-1.64 ± 0.13
geS	-1.47 ± 0.09

Determination of the lipophilic properties of *S*-geranyl-RNA oligonucleotides

The influence of a single geS2U modification on the hydrophobic properties of a 17-mer RNA oligonucleotide was assessed by measuring the solubility of the oligonucleotide in a water/1-octanol biphasic system and comparing this measured solubility with the lipophilicities of reference U and S RNA strands. The log *P*-values determined for U-, S2U- and geS2U-containing oligo RNAs are given in Table 2. As expected, a single geS2U modification increased the lipophilicity of the entire RNA oligonucleotide by $\Delta\text{Log } P = 0.27$.

DISCUSSION

To determine the properties of RNA chains bearing *S*-geranylated units we synthesized two 17-nt long *S*-geranylated RNAs, i.e. ASL RNAs with sequences homologous to *E. coli* tRNA^{Lys} (geS) or to human tRNA^{Lys3} (geS^h) and containing the geS2U modification in the site corresponding to the wobble position. Three of the methods used to prepare geS2U-RNA oligomers (methods I, II and III) were purely chemical, while the fourth method involved enzyme-catalyzed geranylation of S2U-RNA (Scheme 1).

Thioalkyl groups are known as good leaving groups, so we optimized our synthetic protocols to retain geS2U in the assembled RNA chain. The geS2U unit was found to be stable under the conditions routinely used for automated solid phase RNA synthesis. The desilylating conditions used also left the *S*-geranyl group intact. We paid special attention to the alkaline cleavage of the oligonucleotide from the solid support and the removal of base-labile protecting groups (from nucleobases and from phosphate moieties). Routine treatment with aqueous ammonia/EtOH (3:1, v/v, 3 h incubation, rt) was too harsh; fortunately, anhydrous conditions (8 M NH₃/EtOH, 8 h, rt) did not cause any apparent degradation of geS2U. Furthermore, the geS oligonucleotide was obtained with good yield (approximately 55% following isolation by anion-exchange HPLC). In the second and third methods (Scheme 1), we performed selective geranylation of the sulfur atom in support-linked or released/deprotected RNA oligomers containing 2-thiouridine. In method II, detritylated CPG-linked S2U-RNA was alkylated with a >200-fold molar excess (25) of geranyl bromide following the removal of 2-cyanoethyl groups. Subsequent work-up followed by IE-HPLC purification (Figure 2B) produced the geS product with a yield (~40%) similar to method I. In method III, we successfully (~67% yield) performed the site-selective geranylation of fully deprotected S2U-RNA using conditions similar to those used in previous approach. The structure of geS and the site-selectivity of the post-synthetic geranylation were confirmed by MALDI-TOF mass spectrometry as well as by RNA enzymatic digestion and nucleoside composition analysis as described in the RESULTS section. It should be emphasized that, contrary to reports in the literature (15), the *S*-geranylated oligomers eluted later than the parent oligomers bearing the S2U unit in our RP-HPLC analyses. This behavior should be expected for compounds decorated with a highly lipophilic

geranyl substituent. The reported unexpected higher mobility (shorter retention time in RP-HPLC) of the geranylated product compared to its native precursor (T instead of geS2T) was explained by the Authors as 'a special structural alignment in the oligonucleotide' (15). Our observations are in agreement with recently obtained log *P* data determined at the level of R5geS2U nucleosides (R = H, mnm or cmnm), which indicate a higher lipophilicity of these compounds compared to their parent R5S2Us (16). In the present studies, we measured the partition coefficients of the U, S and geS oligonucleotides in a water/1-octanol biphasic system. As expected, we found that a single geranyl modification increases the hydrophobicity of the geS2U–RNA strand in comparison to the reference U ($\Delta\text{Log } P = 0.27$) and S ($\Delta\text{Log } P = 0.10$) RNA strands (Table 2). Although it has been suggested that the presence of a hydrophobic geranyl residue in the tRNAs affects their intracellular localization and transport through the cell membranes (11,15), recent studies have shown a lack of geranylated tRNA enrichment in outer or inner cellular membranes (9).

Several years ago, enzymatic *S*-geranylation of 2-thiouridine in transfer RNAs was observed (11). Interestingly, this activity was eventually assigned to a previously characterized enzyme, which is involved in selenophosphate-dependent selenation of the same 2-thiouridine–tRNA substrate (26). In *E. coli*, this enzyme was named tRNA selenouridine synthase (SelU, also named MnmH, SufY or YbbB). In earlier studies, we chemically transformed S2U to Se2U via *S*-geranyl-2-thiouridine and suggested a possible linear mechanism for the SelU-catalyzed reaction, in which geS2U exists as an intermediate in the cellular transformation of S2U to Se2U (Figure 5) (14). Such a possibility has been recently considered in advanced studies on tRNA selenouridine synthase (9).

The SelU enzyme exhibits intrinsic low geranylation activity (~10%) (10). However, some MnmH proteins with mutations in the rhodanese domain exhibit improved tRNA geranylation efficiencies (9). We measured the geranylation potential of a recombinant SelU–His6 enzyme on a human ASL^{Lys3} S2U–RNA model (S^h). The geranylation reaction, carried out in the presence of an excess of SelU–His6 and geranyl pyrophosphate, was not successful unless magnesium ions were added to the reaction mixture. Despite our efforts at improvement, the yield of enzymatically geranylated S2U–RNA was rather low (9% with 10 mM Mg²⁺ and 11% with 100 mM Mg²⁺) (Figure 3). We assume that our S2U–RNA used for enzymatic geranylation only weakly mimics natural tRNAs, as it is limited to the anticodon-stem-loop domain. Moreover, this model does not have a 5-(c)mnm side chain at 2-thiouridine, an important structural element for the geranylation activity of tRNA selenouridine synthase *in vivo* (9). The enzymatic activity of SelU may be lower because the enzyme contains a bound tRNA population in its native form, and a part of the bound tRNA population is *S*-geranylated. Therefore, the exchange of naturally bound tRNA (to which the enzyme exhibits high affinity) with a poor S2U–RNA substrate is disfavored unless magnesium ions are added; magnesium ions likely facilitate the formation of better accepted and structured ASL molecule (28).

The more fundamental question relates to the function of

the geS2U wobble units in recognizing the 3'-ending purines in synonymous mRNA codons (for Lys, Glu and Gln). We were able to demonstrate via UV-melting measurements of various RNA/RNA and RNA/DNA duplexes that the geranyl modification decreases the thermodynamic stability of the geS/A duplexes in comparison with the S/A and U/A duplexes, which contain S2U–A and U–A Watson–Crick base pairs. In contrast, the stability of the S/G and U/G duplexes containing S2U–G and U–G wobble base pairs does not differ significantly upon S2U geranylation. Moreover, the geS/G duplex is more stable than the geS/A duplex. The higher thermal stability of a model 11-bp DNA duplex containing a geS2T–dG base pair compared to a duplex containing a geS2T–dA base pair was demonstrated previously (15). All these data indicate that geranylation changes the reading preferences from A to G. This phenomenon has been attributed to changes in the hydrogen bonding properties of the *S*-geranylated 2-thiouridine via conversion of the N3H donor into an N3 acceptor site (Figure 6). A similar acceptor pattern could be observed in 4-pyrimidinone riboside (H2U), the product of S2U oxidative desulfuration (8). We have already shown that duplexes made of H2U–RNA with RNA or DNA strands are more stable when H2U is base-paired with G rather than A (6–8,29). This H2U-like 2-fold bond system is also present in geS2U and in prestructured (c)mnm5S2U nucleosides (30, Sochacka *et al.* submitted). These nucleosides all hybridize preferably to guanosine. Therefore, this new arrangement between the modified wobble nucleosides and guanosine offers precise recognition of synonymous 3'-G-ending codons.

The stability of the geS/A duplex is low because only one hydrogen bond is present between the O4 acceptor and HN6 donor centers. In addition, repulsive interactions are present between the N3 of geS2U and N1 of A. Our earlier modeling studies showed that the H2U–A base pair in RNA duplexes is bound by this single hydrogen bond (8). Adding silver ions, which mediate the formation of bonds between acceptor centers with lone ion pairs (31), resulted in the formation of an additional bond in the geS2U–A base pair (Figure 6) and an increase in Tm (3.0°C, Table 1). This result confirms the presence of two acceptor sites at the geS2U–A base pair.

Our circular dichroism analysis demonstrated that the *S*-geranylated unit in the RNA chain does not alter the structure of the single stranded geS chain and does not affect the overall conformation of the RNA/RNA duplexes, which preferentially adopt typical A-type helical structures. As expected, the structures of RNA/DNA duplexes are distorted from that of the A–RNA type; however, the structures of the duplexes remain more similar to that of the RNA homoduplex rather than the DNA homoduplex.

In conclusion, we describe four different methods of preparing *S*-geranylated RNA based on chemical and enzymatic site-specific geranylation of the sulfur atom in 2-thiouridine. The recombinant SelU–His6 protein accepts a simplified tRNA model consisting of the S2U–ASL domain but only in the presence of magnesium ions at millimolar concentrations. We demonstrate that the *S*-geranyl-2-thiouridine-containing model exhibits enhanced hydrophobicity compared to its parent S2U–RNA and non-modified

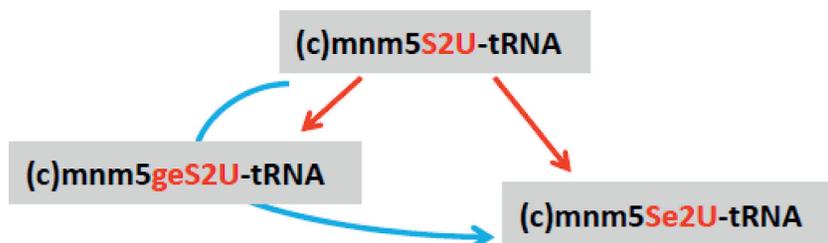


Figure 5. Proposed linear (blue arrow) and bidirectional (red arrows) pathways of SelU activity on the S2U-tRNA substrate.

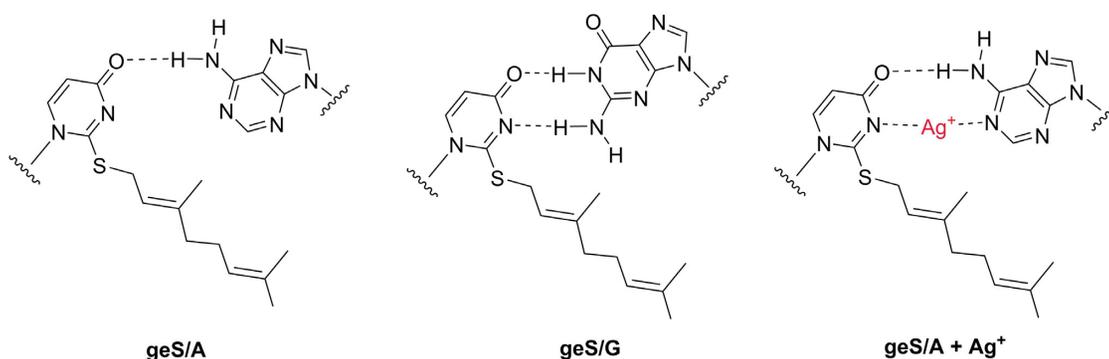


Figure 6. Base pairing modes of geS/A, geS/G and geS/A with Ag⁺.

RNA. Moreover, the *S*-geranyl-2-thiouridine-RNA displays improved affinity for RNA matrices with a G opposite the modification site. The geS/A duplex is thermodynamically less stable. These results further support the already proven biological property of *S*-geranylated tRNAs, which preferentially recognize 3'-G-ending codons over 3'-A-ending codons. We therefore conclude that nature introduced sulfur into wobble uridines to promote the reading of 3'-A-ending codons, while geranylation of S2U changes the reading preferences to 3'-G-ending codons. This original observation offers an explanation for a possible mechanism of gene expression regulation carried out by differentially modified wobble uridines (6).

SUPPLEMENTARY DATA

[Supplementary Data](#) are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Dr Piotr Guga for his critical reading of the manuscript, and Dr Milena Sobczak for her skillful technical assistance. This paper is dedicated to the late Prof. Mathias Sprinzl (1941–2016).

FUNDING

National Science Centre in Poland [Project Number UMO-2014/13/B/ST5/03979, 2015–2018, to B.N. and E.S., 2015–2018]; Lodz University of Technology and the Centre of Molecular and Macromolecular Studies of the Polish Academy of Sciences. Funding for open access charge: National Science Centre in Poland [UMO-2014/13/B/ST5/03979].

Conflict of interest statement. None declared.

REFERENCES

- Cantara, W.A., Crain, P.F., Rozenski, J., McCloskey, J.A., Harris, K.A., Zhang, X., Vendeix, F.A., Fabris, D. and Agris, P.F. (2011) The RNA Modification Database, RNAMDB: 2011 update. *Nucleic Acids Res.*, **39**, D195–D201.
- Machnicka, M.A., Milanowska, K., Osman Oglou, O., Purta, E., Kurkowska, M., Olchowik, A., Januszewski, W., Kalinowski, S., Dunin-Horkawicz, S., Rother, K.M. *et al.* (2013) MODOMICS: A database of RNA modification pathways—2013 update. *Nucleic Acids Res.*, **41**, D262–D267.
- Agris, P.F., Vendeix, F.A. and Graham, W.D. (2007) tRNA's wobble decoding of the genome: 40 years of modification. *J. Mol. Biol.*, **366**, 1–13.
- Motorin, Y. and Helm, M. (2010) tRNA stabilization by modified nucleotides. *Biochemistry*, **49**, 4934–4944.
- Jackman, J.E. and Alfonzo, J.D. (2013) Transfer RNA modifications: nature's combinatorial chemistry playground. *Wiley Interdiscip. Rev. RNA*, **4**, 35–48.
- Duchler, M., Leszczynska, G., Sochacka, E. and Nawrot, B. (2016) Nucleoside modifications in the regulation of gene expression: Focus on tRNA. *Cell Mol. Life Sci.*, **73**, 3075–3095.
- Nawrot, B., Sochacka, E. and Duchler, M. (2011) tRNA structural and functional changes induced by oxidative stress. *Cell Mol. Life Sci.*, **68**, 4023–4032.
- Sochacka, E., Szczepanowski, R.H., Cypryk, M., Sobczak, M., Janicka, M., Kraszewska, K., Bartos, P., Chwialkowska, A. and Nawrot, B. (2015) 2-Thiouracil deprived of thiocarbonyl function preferentially base pairs with guanine rather than adenine in RNA and DNA duplexes. *Nucleic Acids Res.*, **43**, 2499–2512.
- Jager, G., Chen, P. and Bjork, G.R. (2016) Transfer RNA bound to MnmH protein is enriched with geranylated tRNA—a possible intermediate in its selenation? *PLoS One*, **11**, e0153488.
- Chen, P., Crain, P.F., Nasvall, S.J., Pomerantz, S.C. and Bjork, G.R. (2005) A 'gain of function' mutation in a protein mediates production of novel modified nucleosides. *EMBO J.*, **24**, 1842–1851.
- Dumelin, C.E., Chen, Y., Leconte, A.M., Chen, Y.G. and Liu, D.R. (2012) Discovery and biological characterization of geranylated RNA in bacteria. *Nat. Chem. Biol.*, **11**, 913–919.

12. Palsuledesai, C.C. and Distefano, M.D. (2015) Protein prenylation: enzymes, therapeutics, and biotechnology applications. *ACS Chem. Biol.*, **10**, 51–62.
13. Veres, Z. and Stadtman, T.C. (1994) A purified selenophosphate-dependent enzyme from *Salmonella typhimurium* catalyzes the replacement of sulfur in 2-thiouridine residues in tRNAs with selenium. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 8092–8096.
14. Bartos, P., Maciaszek, A., Rosinska, A., Sochacka, E. and Nawrot, B. (2014) Transformation of a wobble 2-thiouridine to 2-selenouridine via S-geranyl-2-thiouridine as a possible cellular pathway. *Bioorg. Chem.*, **56**, 49–53.
15. Wang, R., Ranganathan, S.V., Basanta-Sanchez, M., Shen, F., Chen, A. and Sheng, J. (2015) Synthesis and base pairing studies of geranylated 2-thiothymidine, a natural variant of thymidine. *Chem. Commun.*, **51**, 16369–16372.
16. Leszczynska, G., Sadowska, K., Bartos, P., Nawrot, B. and Sochacka, E. (2016) S-geranylated 2-thiouridines of bacterial tRNAs: Chemical synthesis and physicochemical properties. *Eur. J. Org. Chem.*, 3482–3485.
17. Nawrot, B. and Sochacka, E. (2009) Preparation of short interfering RNA containing the modified nucleosides 2-thiouridine, pseudouridine or dihydrouridine. *Curr. Protoc. Nucleic Acid Chem.*, doi:10.1002/0471142700.nc1602s37.
18. Gehrke, C.W., Kuo, K.C., McCune, R.A. and Gerhardt, K.O. (1982) Quantitative enzymatic hydrolysis of tRNAs: Reversed-phase high-performance liquid chromatography of tRNA nucleosides. *J. Chromatogr. B Biomed. Sci. Appl.*, **230**, 297–308.
19. Gehrke, C.W. and Kuo, K.C. (1989) Ribonucleoside analysis by reversed-phase high-performance liquid chromatography. *J. Chromatogr.*, **471**, 3–36.
20. Damha, M.J. and Ogilvie, K.K. (1993) Oligoribonucleotide synthesis. The silyl-phosphoramidite method. *Methods Mol. Biol.* **20**, 81–114.
21. Agris, P.F., Malkiewicz, A., Kraszewski, A., Everett, K., Nawrot, B., Sochacka, E., Jankowska, J. and Guenther, R. (1995) Site-selected introduction of modified purine and pyrimidine ribonucleosides into RNA by automated phosphoramidite chemistry. *Biochimie*, **77**, 125–134.
22. Scaringe, S.A., Francklyn, C. and Usman, N. (1990) Chemical synthesis of biologically active oligoribonucleotides using beta-cyanoethyl protected ribonucleoside phosphoramidites. *Nucleic Acids Res.*, **18**, 5433–5441.
23. Kumar, R.K. and Davis, D.R. (1997) Synthesis and studies on the effect of 2-thiouridine and 4-thiouridine on sugar conformation and RNA duplex stability. *Nucleic Acids Res.*, **25**, 1272–1280.
24. Leszczynska, G., Pieta, J., Leonczak, P., Tomaszewska, A. and Malkiewicz, A. (2012) Site-specific incorporation of 5-methylaminomethyl-2-thiouridine and 2-thiouridine(s) into RNA sequences. *Tetrahedron Lett.*, **53**, 1214–1217.
25. Kierzek, E. and Kierzek, R. (2003) The synthesis of oligoribonucleotides containing N6-alkyladenosines and 2-methylthio-N6-alkyladenosines via post-synthetic modification of precursor oligomers. *Nucleic Acids Res.*, **31**, 4461–4471.
26. Wolfe, M.D., Ahmed, F., Lacourciere, G.M., Lauhon, C.T., Stadtman, T.C. and Larson, T.J. (2004) Functional diversity of the rhodanese homology domain: The *Escherichia coli* ybbB gene encodes a selenophosphate-dependent tRNA 2-selenouridine synthase. *J. Biol. Chem.*, **279**, 1801–1809.
27. Mei, H., Röhl, I. and Seela, F. (2013) Ag⁺-mediated DNA base pairing: extraordinarily stable pyrrolo-dC-pyrrolo-dC pairs binding two silver ions. *J. Org. Chem.*, **78**, 9457–9463.
28. Nobles, K.N., Yarian, C.S., Liu, G., Guenther, R.H. and Agris, P.F. (2002) Highly conserved modified nucleosides influence Mg²⁺-dependent tRNA folding. *Nucleic Acids Res.*, **30**, 4751–4760.
29. Sochacka, E., Kraszewska, K., Sochacki, M., Sobczak, M., Janicka, M. and Nawrot, B. (2011) The 2-thiouridine unit in the RNA strand is desulphured predominantly to 4-pyrimidinone nucleoside under in vitro oxidative stress conditions. *Chem. Commun.*, **47**, 4914–4916.
30. Rozov, A., Demeshkina, N., Khusainow, I., Westhof, E., Yusupov, M. and Yusupova, G. (2016) Novel base-pairing interactions at the tRNA wobble position crucial for accurate reading of the genetic code. *Nat. Commun.*, **7**, 10457.
31. Scharf, P. and Muller, J. (2013) Nucleic acids with metal-mediated base pairs and their applications. *ChemPlusChem.*, **78**, 20–34.