Chemical synthesis of the 5-taurinomethyl(-2-thio)uridine modified anticodon arm of the human mitochondrial tRNA_{Leu(UUR)} and tRNA_{Lys}

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ABSTRACT
5-Taurinomethyluridine (τm5U) and 5-taurinomethyl-2-thiouridine (τm5s2U) are located at the wobble position of human mitochondrial (hmt) tRNA_{Leu(UUR)} and tRNA_{Lys}, respectively. Both hypermodified units restrict decoding of the third codon letter to A and G. Pathogenic mutations in the genes encoding hmt-tRNA_{Leu(UUR)} and hmt-tRNA_{Lys} are responsible for the loss of the discussed modifications and, as a consequence, for the occurrence of severe mitochondrial dysfunctions (MELAS, MERRF). Synthetic oligoribonucleotides bearing modified nucleosides are a versatile tool for studying mechanisms of genetic message translation and accompanying pathologies at nucleoside resolution. In this paper, we present site-specific chemical incorporation of τm5U and τm5s2U into 17-mers related to the sequence of the anticodon arms hmt-tRNA_{Leu(UUR)} and hmt-tRNA_{Lys}, respectively employing phosphoramidite chemistry on CPG support. Selected protecting groups for the sulfonic acid (4-(tert-butyldiphenylsiloxy)-2,2-dimethylbutyl) and the exoamine function (-C(O)CF3) are compatible with the blockage of the canonical monomeric units. The synthesis of τm5s2U-modified RNA fragment was performed under conditions eliminating the formation of side products of 2-thiocarbonyl group oxidation and/or oxidative desulphurization. The structure of the final oligomers was confirmed by mass spectroscopy and enzymatic cleavage data.

Keywords: human mitochondrial tRNA; modified ribonucleosides; 5-taurinomethyluridine; 5-taurinomethyl-2-thiouridine; phosphoramidite chemistry

INTRODUCTION
Mitochondria have a largely autonomic system of genetic message expression. The human mitochondrial genome encodes 13 proteins responsible for oxidative phosphorylation process (OXPHOS), two rRNAs, and 22 tRNAs (Florentz et al. 2003; Sissler et al. 2008). Knowledge about the detailed structure of mitochondrial tRNAs (mt-tRNAs) is still limited (Suzuki et al. 2011b). Research using specific chemical and enzymatic structure probes (Sissler et al. 2008) has shown that the secondary (2D) and tertiary (3D) arrangements of mt-tRNAs more or less deviate from the classical cloverleaf and L-shaped organization characteristic of their cytosolic counterparts (Helm et al. 2000; Jühling et al. 2012; Wende et al. 2014). mt-tRNAs are also significantly less prone to post-transcriptional modifications than cytosolic tRNAs (Watanabe 2010).

5-Taurinomethyluridine (τm5U, Fig. 1A) and 5-taurinomethyl-2-thiouridine (τm5s2U, 2, Fig. 1A) are present at the wobble position of human mitochondrial (hmt) tRNAs specific for Leu, Trp, and Lys, Glu, Gln, respectively (Kirino et al. 2004; Watanabe 2010). Like other xm5(s2)U-type wobble uridines, nucleosides 1, 2 decode A and G as the third codon letter (Kurata et al. 2008). The genes encoding hmt-tRNA_{Leu(UUR)} and hmt-tRNA_{Lys} are highly susceptible to point mutations. The most often occurring transitions A3243G, T3271C, and A8344G result in the absence of nucleosides 1, 2 (Suzuki et al. 2011a,b). Deficiency of the taurine-modified uridines 1, 2 is considered to be a key factor responsible for the severe mitochondrial diseases MELAS and MERRF (Suzuki et al. 2011a,b). In fact, the taurine residue linked to the atom C-5 by a methylene group has been found critical for G decoding (Kirino et al. 2004; Kurata et al. 2008), while the absence of both modifying functions (the C-5 substituent and the 2-thiocarbonyl group) results in a translational defect for the cognate codons UUA(G) and a disruption of protein biosynthesis (Suzuki et al. 2011a).

The abundance of mt-tRNAs is very poor as compared with their cytosolic homologues (Sissler et al. 2008) limiting

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An RNA fragment recombination technique and the 5′ to programmed ribosomes and as elements of a cell-free system (Kurata et al. 2008). The 5′ crystal 30S ribosomal subunit programmed with UUA(G) was introduced into the A-site of the geometry of the codon-anticodon mini-helix, the con-formation for highly reactive functional groups, in particular the sulfonic acid residue, is required. An attempt to employ phe-nyl protection for the sulfonic acid residue of tm5U and tm3′s′U phosphoramidites has been reported (Ogata and Wada 2006). However, their use in oligoribonucleotide solid-phase synthesis was limited to RNA dimers tm3′(s′)UpU (Ogata and Wada 2008).

This paper presents a milligram scale chemical synthesis of tm3′U and tm3′s′U-modified oligoribonucleotides (phosphoramidite chemistry on solid support) related to the sequence of the hmt-tRNAs anticodon arm domain specific for Leu and Lys, respectively (Fig. 1B). The 4-((tert-butyl-diphenylsilanyloxy)-2,2-dimethylbutyl group (neoO-dPS) was selected as the most useful protection for sulfonic acid residue of taurine derivatives.

RESULTS AND DISCUSSION

The incorporation of nucleosides 1 and 2 into the RNA sequences using phosphoramidite chemistry on CPG support requires such protection of the highly reactive aliphatic amine function as well as the sulfonic acid residue that would be compatible with the blockage of canonical monomeric units. The aliphatic amine function was protected with a base-labile trifluoroacetyl group, following the methodology developed previously for the incorporation of mm3′(s′)U into RNA oligomers (Malkiewicz and Sochacka 1983; Agris et al. 1995; Leszczyńska et al. 2011, 2012).

The most appropriate method for the protection of alkyl or aryl sulfonic acids, including taurine, is their transformation into sulfonate esters (Robert et al. 1997; Klán et al. 2002; Wrobel et al. 2002; Yan and Müller 2004; Avitabile et al. 2005; Seeberger et al. 2007; Hussain et al. 2008; Ali et al. 2009; Miller 2010). The masking of sulfonic acids as sulfonamides has also been proposed (Klamann and Hofbauer 1953; Richman and Atkins 1974; Andrianov et al. 2002; Hofbauer and Veronese 2004). The cleavage of most of the reported protecting groups employs, however, strongly acidic or basic conditions unsuitable for oligoribonucleotide synthesis. Substituted phenyl esters of taurine have been utilized for solid support synthesis of dimers with tm3′(s′)U at the 5′ end (Ogata and Wada 2006, 2008). However, this blockage has proven too labile to be used in the synthesis of longer, hypermodified RNA sequences (G Leszczyńska and A Malkiewicz, unpubl.). In search of alternative protection for the sulfonic acid function, model N-Boc taurine esters have been synthesized (Fig. 2) and their stability/cleavage has been examined under the typ-ical reaction conditions of RNA synthesis on solid support.

By analogy with the protection of the t6A carboxyl group (Boudou et al. 2000; Sundaram et al. 2000; Bajji and Davis 2002; Bajji et al. 2002; Esch et al. 2007; Bíbille et al. 2009), variously substituted N-Boc taurine 2-phenylethyl esters (Fig. 2, 3a–3g) removable via the β-elimination process (10% DBU/MeCN) have been prepared (Leszczyńska et al. 2013). Several attempts to prepare 2-(trimethylsilyl)ethyl ester (3j) which could be removed by treatment with fluoride anions have not been successful (data not shown). As an

FIGURE 1. (A) The structure of 5-taurinomethyluridine (tm5U, 1) and 5-taurinomethyl-2-thiouridine (tm3′s′U, 2). (B) The sequence and secondary structure of the human mitochondrial tRNALeu(UUR) and tRNALeu(UUG) anticodon stem and loop (hmt-ASLLeu(UUR) and hmt-ASLLeu(UUG)) bearing tm5U and tm3′s′U, respectively. The native sequence of hmt-ASLLeu(UUR) has pseudouridine (Ψ) at position 27, while in the sequence of hmt-ASLLeu(UUG) there are two pseudouridines (Ψ) at positions 27 and 28, and N′-[{3′β-D-ribofuranosyl-9H-purin-6-yl} carbamoyl]-L-threonine (t6A) at position 37.
alternative, we have synthesized and tested 4-((tert-butylidiphensilyl)oxy)-2,2-dimethylbutyl ester of N-Boc taurine (Fig. 2, 3b; Seeberger et al. 2007) and its analog, 4-((tert-butylidimethylsilyl)oxy)-2,2-dimethylbutyl ester (3i).

The esters 3a–3i were stable in 8 M ethanolic ammonia (24 h, room temperature [rt]), offering a simple way for the simultaneous deblocking of base-labile protecting groups, e.g., 2-cyanoethyl, -tac, and the cleavage of oligomers from CPG support without the risk of amide formation. Among 2-arylethyl esters 3a–3g only 2-(p-nitrophenyl)ethyl (3a), 2-(p-trifluoromethylphenyl)ethyl (3b), and 2-(2,4,5-trifluorophenylethyl)ethyl (3g) esters of N-Boc taurine were found to be easily deprotected under β-elimination conditions (10% DBU/MeCN, 40 min, 45°C). Both p-substituted phenylethyl esters 3a, 3b were, however, significantly unstable also in the presence of other tertiary amines, e.g., Et3N or iPr2NEt, which excluded their use in the synthesis of τm5U and τm3s2U phosphoramidites. Examination of the stability of N-Boc taurine 4-((tert-butylidiphensilyl)oxy)-2,2-dimethylbutyl ester (3h) as well as its analog, 4-((tert-butylidimethylsilyl)oxy)-2,2-dimethylbutyl ester (3i), revealed their effective cleavage with a standard desilylating reagent, 1 M TBAF/NMP (24 h, rt) as well as 1 M TEAF/NMP (24 h, rt) and triethylamine trihydrofluoride (TEA•3HF/NMP, 24 h, rt). In contrast to neoO-dPS sulfonate 3h, ester 3i was unstable under detritylation conditions (3% TCA/DCM).

The usefulness of neoO-dPS as a protecting group for sulfonic acid residue has been previously confirmed in the synthesis of various N-substituted taurines (Seeberger et al. 2007). The removal of neoO-dPS was performed with a small excess of TBAF in THF via cleavage of the Si-O bond, and then spontaneous cyclization of the desilylated intermediate (Seeberger et al. 2007).

In summary, evaluation of the stability of various N-Boc taurine esters (Fig. 2) allowed for the selection of two protecting groups for the sulfonic acid residue, the 2-(2,4,5-trifluorophenyl)ethyl group and the 4-((tert-butylidiphensilyl)oxy)-2,2-dimethylbutyl group. An attempt to introduce the 2-(2,4,5-trifluorophenyl)ethyl-protected τm5U and τm3s2U phosphoramidites into RNA sequences resulted, however, in very low yields of the target oligoribonucleotides (data not shown). The use of strong basic DBU solution required for the 2-(2,4,5-trifluorophenyl)ethyl deprotection caused a Michael-type addition of 2,4,5-trifluorostyrene to RNA and the loss of the 2-thiocarbonyl moiety in the case of τm3s2U-modified oligomer. The use of scavengers of 2,4,5-trifluorostyrene did not increase the yields of synthesis.

Finally, the 4-((tert-butylidiphensilyl)oxy)-2,2-dimethylbutyl group (neoO-dPS) was effectively used for the synthesis of τm5(s2)U phosphoramidites 16a/16b (Fig. 3) and then for their incorporation into target RNA sequences.

Initially, the synthesis of 16a/16b involved the introduction of 5′-O-DMTr and 2′-O-TBDMS protecting groups after the installation of a taurine ester side chain at position C-5.1. However, the steric hindrance around the 5′-hydroxyl group, caused by the presence of a large substituent at the atom C-5, decreased the yield of dimethoxytritylation to 5% (data not shown). As an alternative strategy, a fully protected taurine skeleton was installed after 5′-O-DMTr and 2′-O-TBDMS protection of the sugar moiety (Fig. 3). 5-Hydroxymethyl-2′,3′-O-isopropylidene(-2-thio)uridine (4a/4b) was used for the preparation of 5-azidomethyl(-2-thio)uridine derivatives 6a/6b (Seio et al. 1998). The treatment of 4a/4b with an excess of trimethylsilyl chloride in 1,4-dioxane at 60°C gave 5-chloromethyl-2′,3′-O-isopropylidene(-2-thio)uridine 5a/5b, which without purification was reacted with an excess of sodium azide in DMF at 60°C. In comparison with the synthesis of 6a, a similar method of preparation of the 2-thio derivative 6b was considerably less effective (40% vs. 70%).

The 2′,3′-O-isopropylidene group was removed from 6a/6b by treatment with 50% aqueous (aq.) trifluoroacetic acid (Myerscough et al. 1992) to afford 7a/7b in 90% yield. Subsequently, incorporation of the 5′-O-DMTr, and then 2′(3′)-O-TBDMS, protecting groups was performed according to the standard procedures (Damha and Ogilvie 1993). A mixture of 2′ and 3′ TBDMS isomers (9a/9b and 10a/10b, respectively) was separated by column chromatography on silica gel only in the amount required for spectral analysis. The separation of 2′ and 3′ TBDMS isomers at this stage was not convenient because the alkaline conditions of the subsequent reaction led to spontaneous isomerization giving an equimolar mixture of 2′ and 3′ regioisomers. Therefore, a mixture of azides 9a, 10a or their 2-thio analogous 9b, 10b was reduced to a suitable mixture of amines 11a, 12a or 11b, 12b, respectively, by treatment with triphenylphosphine in anhydrous pyridine followed by 25% aq. ammonia (Seio et al. 1998). The resulting mixtures of regioisomers 11a, 12a or 11b, 12b were effectively separated by flash chromatography. The storage of 3′ isomers 12a/12b in methanol for a longer period of time resulted in their partial isomerization, enabling improvement of the overall yield of 2′-TBDMS derivatives 11a/11b. Separated 5-aminomethyl(-2-thio)uridine 11a/11b was then used as a donor in a Michael-type addition to 4-((tert-butylidiphensilyl)oxy)-2,2-dimethylbutyl ethenesulfonate (13) (Baxter et al. 2000; Ogata and

FIGURE 2. Protecting groups selected for sulfonic acid residue of taurine; TBDPS, tert-butylidiphensilyl; TBDMS, tert-butylidimethylsilyl; TMS, trimethylsilyl.
Wada 2006; Seeberger et al. 2007). This strategy of synthesis of a fully protected taurine skeleton is more effective than procedures involving 5-chloromethyl- or 5-formyluridine as substrates (Leszczynska et al. 2013) and in the case of β-amino acids it should be considered as the method of choice. To exclude the isomerization of 2′-O-TBDMS 11a/11b to 3′-regiomers the reaction of ethenesulfonate 13 with 11a/11b requires an aprotic solvent. Consequently, an equimolar amount of amine 11a/11b and 4-(tert-butyldiphenylsiloxy)-2,2-dimethylbutyl ethenesulfonate (13) was mixed in DCM for 72 h at rt. The resulting material 14a/14b was purified by column chromatography in 70% yield. Following the previously reported procedure (Malkiewicz et al. 1983), the amine function of 14a/14b was protected with trifluoroacetyl to afford 15a/15b in 80% yield. Phosphitylation of 15a/15b was performed with 2-cyanoethyl N,N-diisopropylaminochlorophosphoramidite under standard conditions (Damha and Ogilvie 1993) giving τm5(s2)U phosphoramidites 16a/16b in ~85% yield.

Fully protected phosphoramidites 16a/16b were used for the synthesis of analogs of the anticodon arm domain of human mt-tRNA

Leu(UUR) and mt-tRNAlys modified with τm5U and τm5s2U, respectively (Fig. 1B). The synthesis of oligomers was conducted manually on a 5-μmol scale using commercial tac-protected phosphoramidites of the canonical units and 5-(3,5-bis(trifluoromethyl)phenyl)-1H-tetrazole as the activator. The couplings were conducted in 8 molar excess of A, U, C, and G amidites for 8 min, while the modified units were used in 12 molar excess and coupled twice, each time using 6 molar excess of an amidite and 12-min coupling time. Coupling yields were in the range of 90%–95%.

It is generally known that the 2-thiocarbonyl group of 2-thiouridine derivatives reacts with various oxidizing reagents used in the oligoribonucleotide synthesis giving products of 2-thio → 2-oxo transformation and/or oxidative desulfurization (s2 → H2) (Sochacka 2001; Okamoto et al. 2006). In model studies (Leszczynska et al. 2011, 2012), a 0.02 M iodine solution (8 equiv, 2 min) in THF-H2O-pyridine (Okamoto et al. 2006) was selected as the most promising oxidizing agent, leading to very small amounts of side products of 2-thiocarbonyl group degradation. In contrast to diluted iodine solution, 0.25 M tBuOOH (8 equiv, 2 min) in toluene or acetonitrile gave considerable amounts of side products.

“Trityl-off” CPG-bound RNA was treated with Et3N in CH3CN, and then with 8 M ethanolic ammonia. The two-step deprotection procedure made it possible to avoid the reaction of heterobase residues with acrylonitrile generated during the deprotection of phosphate residue (Capaldi et al. 2003). For the simultaneous removal of the TBDMS and neoO-dPS protecting groups in τm5U-modified RNA, Et3N·3HF was effectively employed. An alternative use of 1 M Bu4NF gave the desired product, but the yield of the oligomer was drastically reduced. In the case of τm5s2U-modified RNA, desilylation was performed with several reagents. We found that the only effective condition for the complete removal of neoO-dPS and TBDMS protections without observable degradation of the 2-thiocarbonyl function was the treatment with 1 M Et4NF in NMP (24 h, rt). The use of

FIGURE 3. Chemical synthesis of τm5U and τm5s2U phosphoramidites. TMSCl, trimethylsilyl chloride; TFA, trifluoroacetic acid; DMTcCl, 4,4′-dimethoxytrityl chloride; TBDMS, tert-butyldimethylsilyl chloride; TBDP, tert-butyldiphenylsilyl group.
Et₃N·3HF caused a significant loss of the 2-thiocarbonyl function in the τₚₙ₅ₛ₂U-modified oligomer. The excess of the desilylating reagent was deactivated by the addition of ethoxytrimethylsilane for Et₃N·3HF or phosphate buffer for Et₄NF. Crude products were purified by preparative IE-HPLC (Fig. 4A). The homogeneity and composition of synthetic oligoribonucleotides were verified by MALDI-TOF mass spectrometry (Supplemental Material) as well as RNA enzymatic digestion (Gehrke et al. 1982; Gehrke and Kuo 1989) to the expected mixture of nucleosides whose composition was tested by RP HPLC, and the data were compared with those recorded under identical conditions for modified nucleosides (Wada et al. 2002) as a reference (Fig. 4C,D).

CONCLUSIONS
The 4-(tert-butyldiphenylsilanyloxy)-2,2-dimethylbutyl (neoO-dPS) protecting group has been adopted for solid supported synthesis of oligoribonucleotides (phosphoramidite chemistry) bearing taurine-modified wobble uridines 1, 2. NeoO-dPS blockage is compatible with the protection of commercially available canonical monomeric units resistant to treatment with 8 M ethanolic ammonia and removable under mild, neutral conditions with fluoride anions. The usefulness of the discussed methodology was verified by the site-specific insertion of nucleosides 1, 2 into the anticodon arm sequence of hmt-tRNALeu, Lys. The presented work enables effective chemical synthesis of hypermodified RNA sequences which can be used for model studies on the mechanism of decoding processes in mitochondria and their pathologies on a molecular level.

MATERIALS AND METHODS
NMR spectra were recorded on a Bruker Avance DPX 250 spectrometer at 250.0 (¹H), 62.9 (¹³C), and 101.3 (³¹P) MHz or a Bruker Avance II Plus 700 spectrometer at 700.0 (¹H) and 176.0 (¹³C) MHz. Chemical shifts are reported in ppm relative to TMS (internal standard) for ¹H and ¹³C, and 85% phosphoric acid (external standard) for ³¹P. Chemical shifts are described as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and bs (broad singlet). Coupling constants (J) are reported in hertz. IR spectra were recorded on a Bruker FT-IR ALPHA spectrometer equipped with a platinum ATR QuickSnap module. High-resolution mass spectra were obtained from a Finnigan MAT 95 spectrometer (FAB ionization) and Maldi SYNAPT G2-S HDMS (ESI ionization). MALDI-TOF spectra were recorded on an Applied Biosystems Voyager-Elite mass spectrometer. Thin layer chromatography was done on Merck 60F254 coated plates, and Merck silica gel 60 (mesh 230–400) was used for column chromatography. HPLC was performed with a Waters chromatograph interfaced with a 996 spectral diode array detector.

5-Azidomethyl-2′,3′-O-isopropylidene(-2-thio)uridine (6a/6b)
Nucleoside 4a/4b (13.0 mmol, 1.0 equiv) was dissolved in 1,4-dioxane (128 mL), and then trimethylsilyl chloride (8 mL, 65 mmol, 5 equiv) was added. After being stirred for 4.5 h (4a)/7 h (4b) at 60°C, the mixture was cooled to rt, and anhydrous acetone (58 mL) was added. Stirring was continued for 1.5 h at rt. The mixture was then concentrated under reduced pressure and co-evaporated with anhydrous 1,4-dioxane. The resulting foam 5a/5b was dissolved in DMF (118 mL) and treated with NaOAc (5.1 g, 78.0 mmol, 6.0 equiv). The reaction mixture was stirred for 3 h at 60°C. NaCl precipitate was filtered off. The filtrate was concentrated under reduced pressure. The solid residue was dissolved in CH₂Cl₂/py (4:1, v/v); 58 mL and washed with H₂O (23 mL). The organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. Pyridine was removed by co-evaporation with anhydrous toluene. The resulting foam was purified by column chromatography.

Compound 6a was purified on a silica gel column with 5% MeOH in CHCl₃ as eluent to obtain a white foam in 69% yield. Spectroscopic data were in agreement with those presented previously (Seio et al. 1998).
Compound 6b was purified on a silica gel column with 2% MeOH in CHCl₃ as eluent to obtain a light yellow foam in 40% yield. TLC Rf = 0.52 (CHCl₃/MeOH, 9:1 v/v); ¹H NMR (700 MHz, CDCl₃): δ 1.37 (s, 3H), 1.62 (s, 3H), 3.91 (dd, 1H, J = 2.80 Hz, J = 11.90 Hz), 4.09 (dd, 1H, J = 2.10 Hz, J = 11.90 Hz), 4.19 (q, 2H, J = 11.20 Hz), 4.35–4.36 (m, 1H), 4.77 (dd, 1H, J = 2.10 Hz, J = 5.60 Hz), 6.49 (q, 1H, J = 3.50 Hz, 6.84 (d, 1H, J = 2.80 Hz), 8.15 (s, 1H), 10.40 (s, 1H); ¹³C NMR (176 MHz, CDCl₃): δ 17.36, 22.48, 28.23, 46.75, 54.66, 62.78, 80.19, 86.56, 87.60, 95.17, 115.04, 140.28, 160.45, 176.16; IR (ATR): 2106 cm⁻¹; HRMS (EI): calcd for C₁₃H₁₇N₅O₅NaS [M + Na]⁺ 378.0848, found 378.0847.

5-Azidomethyl(-2-thio)uridine (7a/7b)

Nucleoside 6a/6b (4.5 mmol, 1 equiv) was dissolved in 50%aq. trifluoroacetic acid (13 mL). After being stirred for 1.5 h at rt, anhydrous toluene (18 mL) was added, and the mixture was concentrated under reduced pressure. The solid residue was co-evaporated with anhydrous toluene and purified by column chromatography.

Compound 7a was purified on a silica gel column with 12% MeOH in CHCl₃ as eluent to obtain a white foam in 93% yield. TLC Rf = 0.18 (CHCl₃/MeOH 9:1, v/v); ¹H NMR (700 MHz, DMSO-d₆): δ 3.61 (dd, 1H, J = 3.50 Hz, J = 10.50 Hz), 3.76 (m, 1H), 3.90 (s, 1H), 3.91 (q, 1H, J = 3.50 Hz, J = 10.50 Hz), 3.65 (d, 1H, J = 14.00 Hz), 3.80 (s, 2H), 4.22–4.24 (m, 1H), 4.32 (d, 1H, J = 7.00 Hz), 4.45–4.47 (m, 1H), 4.58–4.59 (m, 1H), 4.81 (d, 1H, J = 4.90 Hz), 6.65 (d, 1H, J = 2.80 Hz), 6.92–7.51 (m, 13H), 8.01 (s, 1H), 11.46 (s, 1H); ¹³C NMR (176 MHz, DMSO-d₆): δ 26.49, 28.24, 46.20, 60.19, 86.56, 87.60, 95.17, 115.04, 140.28, 160.45, 175.16; IR (ATR): 2089 cm⁻¹; HRMS (FAB⁺): calcd for C₁₃H₁₇N₅O₅S [M – H]⁻ 640.1842, found 640.1842.

5-Azidomethyl-2-O-(tert-butyl dimethysilyl)-5′-O-(4,4′-dimethoxytrityl) (-2-thio)uridine (9a/9b) and 5-Azidomethyl-3′-O-(tert-butyl dimethysilyl)-5′-O-(4,4′-dimethoxytrityl) (-2-thio)uridine (10a/10b)

The 5′-DMT nucleoside 8a/8b (1.7 mmol, 1.0 equiv) was dissolved in anhydrous pyridine (17 mL), then imidazole (0.345 g, 5.0 mmol, 3 equiv) and tert-butyl dimethysil chloride (0.312 g, 2.1 mmol, 1.2 equiv) were added. After being stirred for 24 h at rt, the reaction was quenched with H₂O (20 mL). The resulting solution was extracted with CHCl₃ (3 × 35 mL). The combined organic layers were washed with water (35 mL), dried over MgSO₄, and the solvent was removed under reduced pressure. An equimolar mixture of 2′- and 3′-TBDMS isomers was purified by column chromatography and separated only in an amount sufficient for spectral analysis. Mixture of 2′- and 3′-TBDMS isomers (9a, 10a) was purified on a silica gel column with 3% aceton in DCM as eluent to obtain a white foam in 82% yield. Compound 9a: TLC Rf = 0.69 (DCM/acetonate 9:1, v/v); ¹H NMR (700 MHz, acetone-d₆): δ 0.17 (s, 3H), 0.18 (s, 3H), 0.94 (s, 3H), 3.41 (d, 1H, J = 14.00 Hz), 3.44 (dd, 1H, J = 2.8 Hz, J = 10.5 Hz), 3.46 (d, 1H, J = 3.50 Hz, J = 10.50 Hz), 3.64 (d, 1H, J = 13.30 Hz), 3.80 (s, 2H), 3.89 (d, 1H, J = 5.60 Hz), 4.17–4.19 (m, 1H), 4.38–4.40 (m, 1H), 4.53 (t, 1H, J = 4.90 Hz), 5.99 (d, 1H, J = 4.90 Hz), 6.91–7.50 (m, 13H), 7.91 (s, 1H), 10.33 (s, 1H); ¹³C NMR (176 MHz, acetone-d₆): δ 7.54, 17.47, 25.29, 46.75, 54.68, 63.22, 70.76, 76.11, 83.54, 86.78, 88.88, 109.19, 113.22, 127.02, 127.94, 128.17, 130.14, 134.50, 136.35, 139.05, 144.86, 150.24, 158.95, 158.97, 162.39; IR (ATR): 2100 cm⁻¹; HRMS (FAB⁺): calcd for C₁₃H₁₇N₅O₅S [M – H]⁻ 714.2959, found 714.2944. Compound 10a: TLC Rf = 0.48 (DCM/acetonate 9:1, v/v); ¹H NMR (700 MHz, (CD₃)₂CO): δ 0.05 (s, 3H), 0.12 (s, 3H), 0.87 (s, 9H), 3.37 (dd, 1H, J = 3.50 Hz, J = 10.50 Hz), 3.49–3.52 (m, 2H), 3.71 (d, 1H, J = 13.30 Hz), 3.77 (d, 1H, J = 8.00 Hz), 4.13–4.14 (m, 1H), 4.40–4.42 (m, 1H), 4.51 (t, 1H, J = 4.90 Hz), 5.96 (d, 1H, J = 4.90 Hz), 6.91–7.50 (m, 13H), 7.91 (s, 1H), 10.29 (s, 1H); ¹³C NMR (176 MHz, acetone-d₆): δ 4.05, 4.26, 18.81.
5-Aminomethyl-2′-O-(tert-butylidimethylsilyl)-5′-O-(4,4′-dimethoxytrityl)-(2-thio)uridine (11a/11b)

A mixture of 2′- and 3′-TBDMS isomers 9a, 10a/9b, 10b (1.36 mmol, 1.0 equiv) was dissolved in anhydrous pyridine (8.1 mL) and Ph3P (0.64 g, 2.5 mmol, 1.8 equiv) was added. After being stirred for 24 h at rt, 25% NH4OH (8.1 mL) was added. The solution was stirred for 1 h at rt and then extracted with CHCl3 (3 × 30 mL). The combined organic layers were dried over MgSO4 and the solvent was removed under reduced pressure. The solid residue was co-evaporated with anhydrous toluene and purified by column chromatography to obtain the pure compound 11a/11b.

Compound 11a was purified on a silica gel column with 4% MeOH in CHCl3 as eluent to obtain a white foam in 86% yield. Compound 11b was purified on a silica gel column with CHCl3 as eluent to obtain a white foam in 65% yield. TLC Rf = 0.45 (CHCl3/MeOH 9:1, v/v); 1H NMR (700 MHz, DMSO-d6): δ 0.05 (s, 3H), 0.07 (s, 3H), 0.83 (s, 9H), 1.03 (s, 9H), 1.30 (s, 9H), 1.64 (t, 2H, J = 7.00 Hz), 2.88–2.92 (m, 4H), 3.01 (d, 1H, J = 13.30 Hz), 3.33 (s, 1H, J = 14.00 Hz), 3.53–3.54 (m, 6H), 3.71–3.73 (m, 2H), 3.83 (t, 2H, J = 7.00 Hz), 3.91 (s, 2H), 4.25–4.26 (m, 1H), 4.45 (t, 1H, J = 4.90 Hz), 4.54 (t, 1H, J = 4.20 Hz), 6.29 (d, 1H, J = 3.50 Hz), 6.96–7.91 (m, 23H), 8.00 (s, 1H); 13C NMR (176 MHz, DMSO-d6): δ −5.24, −4.87, 18.00, 19.13, 23.99, 25.65, 26.90, 33.56, 40.94, 43.20, 45.62, 49.82, 54.73, 54.75, 60.55, 63.14, 70.75, 76.28, 77.37, 83.68, 87.10, 89.15, 112.93, 113.59, 113.61, 127.29, 128.19, 128.49, 128.85, 130.47, 130.49, 133.89, 135.65, 135.68, 135.79, 137.08, 145.12, 150.56, 159.19, 163.63; HRMS (ESI): calcd for C61H82N3O12Si2S [M + H]+ 1136.5187, found 1136.5187.

Compound 14b was purified on a silica gel column with 1% MeOH in CHCl3 as eluent to obtain a white foam in 70% yield. TLC Rf = 0.45 (CHCl3/MeOH 98:2, v/v); 1H NMR (700 MHz, CD3OD): δ 0.27 (s, 3H), 0.38 (s, 3H), 0.92 (s, 6H), 1.03 (s, 9H), 1.30 (s, 9H), 1.64 (t, 2H, J = 7.00 Hz), 2.88–2.92 (m, 4H), 3.01 (d, 1H, J = 13.30 Hz), 3.33 (s, 1H, J = 14.00 Hz), 3.53–3.54 (m, 6H), 3.71–3.73 (m, 2H), 3.83 (t, 2H, J = 7.00 Hz), 3.91 (s, 2H), 4.25–4.26 (m, 1H), 4.45 (t, 1H, J = 4.90 Hz), 4.54 (t, 1H, J = 4.20 Hz), 6.29 (d, 1H, J = 3.50 Hz), 6.96–7.91 (m, 23H), 8.00 (s, 1H); 13C NMR (176 MHz, CD3OD): δ −5.24, −4.87, 18.00, 19.13, 23.99, 25.65, 26.90, 33.56, 40.94, 43.20, 45.62, 49.82, 54.73, 54.75, 60.55, 63.14, 70.75, 76.28, 77.37, 83.68, 87.10, 89.15, 112.93, 113.59, 113.61, 127.29, 128.19, 128.49, 128.85, 130.47, 130.49, 133.89, 135.65, 135.68, 135.79, 137.08, 145.12, 150.56, 159.19, 163.63; HRMS (ESI): calcd for C61H82N3O12Si2S [M + H]+ 1136.5158, found 1136.5158.
Nucleoside 14a/14b (0.52 mmol, 1.0 equiv) was dissolved in anhydrous pyridine (11 mL), cooled in an ice bath, and trifluoroacetic anhydride (211 µL, 1.56 mmol, 3.0 equiv) was added dropwise. The mixture was stirred at rt for 2 h. The reaction was quenched with 5%aq. NaHCO₃ (30 mL). The resulting solution was extracted with CHCl₃ (3 x 40 mL). The combined organic layers were dried over MgSO₄, filtered, and concentrated under reduced pressure. Pyridine was removed by co-evaporation with anhydrous toluene, and the resulting foam was purified by column chromatography affording products 15a/15b as rotamers about the −N(C(O)CF₃ amide bond (two chemical shifts were observed for some ¹H and ¹³C NMR resonances; secondary shifts in ¹³C NMR spectra are given in parentheses).

Compound 15a was purified on a silica gel column with 2% MeOH in CHCl₃, as eluent to obtain a white foam in 81% yield. TLC Rf = 0.51 (CHCl₃/MeOH 98:2, v/v); ¹H NMR (700 MHz, CDCl₃): δ 0.30 (s, 2.4H), 0.36 (s, 0.6H), 0.37 (s, 2.4H), 0.48 (s, 0.6H), 1.01 (s, 6H), 1.06 (s, 2.7H), 1.10 (s, 1.8H), 1.34 (s, 1.8H), 1.35 (s, 2.7H), 1.64 (t, 2.5H, J = 6.7 Hz), 1.71 (t, 1.6H, J = 7.00 Hz), 3.22–3.28 (m, 1H), 3.31–3.35 (m, 1H), 3.56–3.57 (m, 6H), 3.66 (dd, 0.8H, J = 11.20 Hz, J = 4.20 Hz), 3.71 (dd, 0.2H, J = 11.20 Hz, J = 4.20 Hz), 3.80 (d, 1.0H, J = 14.00 Hz), 3.84–3.87 (m, 1H), 3.90 (t, 2H, J = 7.00 Hz), 3.93 (d, 1.0H, J = 14.00 Hz), 4.09–4.13 (m, 2H), 4.20–4.24 (m, 1H), 4.35–4.40 (m, 1H), 4.46–4.48 (m, 1H), 4.56–4.59 (m, 1H), 6.20 (d, 0.2H, J = 2.80 Hz), 6.22 (d, 0.8H, J = 4.20 Hz), 7.00–7.96 (m, 23H), 8.34 (s, 1H); ¹³C NMR (176 MHz, acetone-d₆): δ −3.21 (−3.18), −3.10 (−3.04).

Oligonucleotide synthesis

Oligoribonucleotides were synthesized manually on a 5-µmol scale using slightly modified Sproat’s procedure (Sproat 2005). Commercially available monomeric units A, C, U, and G were protected with DMT and TBDMS on the 5′- and 2′-hydroxy functions, respectively.
spectively, and the exocyclic amine functions of A, C, and G were masked with 4-tert-butylphenoxacyctyl (tac) (Proligo). Typical tA (tac)-succinyl-CPG (Proligo) support and 0.1 M acetonitrile solutions of monomeric units were used. A, U, C, and G amides were coupled in 8 molar excess for 8 min in the presence of Activator 42 (0.25 M solution of 5-(3,5-bis(trifluoromethyl)phenyl)-1H-tetrazole in CH3CN), while modified units were used in 12 molar excess and coupled twice, each time using 6 molar excess of amide and 12 min coupling time. Capping was performed with tac anhydride (Fast protection Cap A:Cap B 1:1 v/v) for 2 min. A 0.02 M iodine solution in THF-H2O-pyridine (90.5:0.45:9.05 v/v/v; 8 equiv) was used as an oxidizing agent for 2 min for each oxidation step.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available for this article.

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