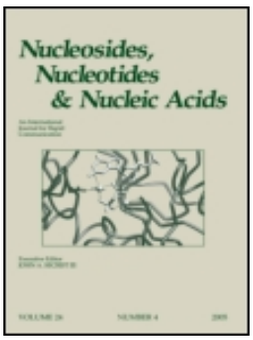

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CHEMICAL SYNTHESIS OF 4-THIOURIDINE-CONTAINING SUBSTRATE ANALOGUES OF tRNA:PSEUDOURIDINE-55 SYNTHASE: PHOTOCROSS-LINKING STUDIES

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Abstract: For site specific incorporation of 4-thiouridine into oligoribonucleotides a new phosphoramidite is proposed. It makes use of the *S*-pivaloyloxymethyl group for the protection of the thiol function. This group is easily introduced and removed without modification of the standard protocol for solid phase synthesis of RNA. Three 4-thiouridine-containing oligoribonucleotides (21-mers), corresponding to tRNA minisubstrates of yeast tRNA:pseudouridine-55 synthase (Pus4) were prepared. These 4-thiouridine containing substrates were characterized and used as photoaffinity probe of the enzyme:substrate complex. Irradiation resulted in the specific photocross-linking of these oligoribonucleotides with purified recombinant tRNA:pseudouridine-55 synthase.

Photoaffinity labelling which allows to map specific contacts between macromolecules, is a powerful tool for studying interactions between a protein and an RNA as well as between two RNAs.[1] Among natural modified nucleosides, 4-thiouridine (s^4U) is an outstanding photoaffinity probe.[2] Indeed, irradiation of this photoprobe with light of ≈360 nm specifically converts s^4U to highly reactive species that can form stable covalent bonds with molecules within the neighbourhood of the modified residue. The mechanisms of s^4U cross-linking reactions involve both cycloaddition and radical pathways.[3]

RNA-oligonucleotides (natural or synthetic) containing 4-thiouridine have been successfully used to cross-link pre-tRNA to RNA ligase,[4] for mapping selected regions between tRNA, rRNA or mRNA with ribosomal proteins,[5] RNA with "tat" protein and the reverse transcriptase of HIV[6] and pre-mRNA with snRNA .[7] For these experiments, s^4U-containing RNA were prepared either by *in vitro* transcription reaction using s^4UTP together with UTP, thus giving rise to a collection of substrates with randomly incorporated s^4U in place of U;[4,5,8] or by site-specific incorporation of s^4U using

enzymatic recombinant methods.[6a,7] Chemical site-specific incorporation either, via the phosphoramidite approach, or by post-synthetic modifications, has also been reported.[6c,9]

Concerning tRNA modification enzymes, the only cross-linking experiments reported so far were performed with tRNA-substrates bearing non-natural 5-fluorouridine (5-FU) and 5-nitrouridine (5-N02U) in tRNAs or tRNA fragments. In these experiments, uracil was randomly or completely replaced by 5-FU or 5-N02U by substituting 5-fluoro-UTP or 5-nitro-UTP for UTP in an *in vitro* transcription reaction. These artificial modified tRNA analogues were incubated with *E. coli* tRNA(uracil-5-) methyltransferase (RUMT) in the absence of AdoMet,[10] or with tRNA pseudouridine synthases (Pusp) from yeast,[11] or *E. coli*.[12] As a result, time-dependent binary covalent complexes were formed with the concomitant inactivation of the enzymes. Formation of these enzyme-tRNA complexes possibly correspond to steady-state intermediates of the catalytic reaction and therefore involves aminoacid residues of the active site (affinity mapping). In the case of *E. coli* RUMT, a cysteine residue linked to atom C4 of uracil was identified,[10a] while for *E. coli* tRNA:pseudouridine synthase (Pus-39) an aspartic residue, probably linked to C-1' of ribose, seems to play a catalytic role.[12]

Photochemical cross-linking of tRNA-like substrates bearing a photoactivable 4-thiouridine, is an alternative way to identify amino acids implicated in nucleotide modifications or located in close proximity of the activable probe. The method requires to define an efficient strategy to incorporate s^4U at selected position(s) of the RNA substrate. Herein, we describe the preparation of a new phosphoramidite derivative **5** (Figure1) allowing the facile introduction of a s^4U residue into oligoribonucleotides using a standard protocol for the chemical synthesis of RNA.[13] To test the validity of the method, we have incorporated 4-thiouridine at defined positions within of a mini-substrate consisting in a 21 nucleotides stem-loop sequence which can serve as a substrate for the yeast tRNA:pseudouridine-55 synthase (Pus4). In a preceding paper, we demonstrated that the uridine in the loop corresponding to U-55 in tRNA was quantitatively modified to pseudouridine when such a minisubstrate was incubated with a yeast extract.[14] Herein we show that irradiation of the s^4U-substituted minisubstrates incubated with purified recombinant yeast Pus4p allows to obtain photocross-linked products.

Preparation of 4-thiouridine phosphoramidite **5:** For the incorporation of thio-modified nucleosides into oligonucleotides by the phosphoramidite approach, protection of the base at the *S*-position is recommended because of the highly nucleophilic character of the thiol group. This function might interfere with the phosphitylation step in oligonucleotide synthesis. Moreover, it could be also sensitive to oxidation by iodine,

which is necessary for the conversion of P(III) into P(V). Recently, *S*-pivaloyloxymethylation was found to give satisfactory results when using phosphoramidite chemistry to prepare oligonucleotides substituted with 4-thio-2'-deoxyuridine.[15] However, other protecting groups and convertible base strategies have also been proposed. The incorporation of 4-thio-2'-deoxyuridine into DNA-oligonucleotides has been accomplished using a 2-cyanoethyl group for the critical protection of the thiol function.[16] In RNA synthesis with 4-thiouridine, the cyanoethyl group was employed in conjunction with the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) group for the 2'-OH protection.[9c] In another strategy, the introduction of the convertible 4-triazolylpyrimidine-2-one nucleoside into an RNA-oligonucleotide sequence allowed the introduction of 4-thiouridine by adding a treatment with thiolacetic acid to the deprotection steps.[9a] Compared to these methods application of *S*-pivaloyloxymethylation presents the advantage of allowing the preparation of modified oligonucleotides without introducing an additional deprotection step during the synthesis.

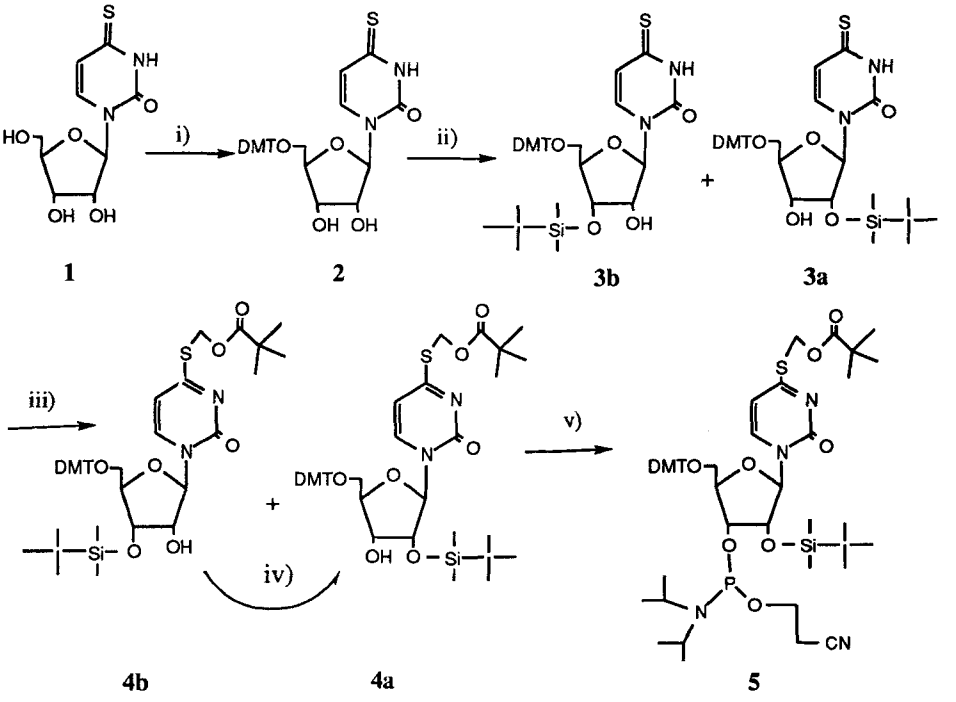

Figure 1: *Reagents and conditions* : i) Dimethoxytrityl chloride, Pyridine, ii) tert-Butyldimethylsilyl chloride, Imidazole, Dimethylformamide iii) Pivaloyloxymethyl chloride, K_2CO_3, Acetone iv), EtOH/Et_3N, v) *N*-Diisopropylethylamine, 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite, Dichloromethane. All reactions were performed at room temperature.

The synthesis of 4-thiouridine phosphoramidite **5** is delineated in Figure 1. The 5'-hydroxyl of 4-thiouridine was protected by dimethoxytritylation using dimethoxytrityl chloride to give compound **2** in 65% yield. Subsequent treatment of 5'-*O*-dimethoxytrityl-4-thiouridine **2** with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) gave a mixture of 2'- and 3'-*O*-TBDMS derivatives (**3a** and **3b** in 70% yield, equimolecular). No special effort was made to separate these compounds since, under the alkaline conditions required for the *S*-pivaloyloxymethylation step, migration of the silyl group from the 2'- to the 3'-*O*-position was expected to occur. Thus, pivaloyloxymethyl chloride was added to an acetone solution of compounds **3a** and **3b** in the presence of potassium carbonate to give a mixture of derivatives **4a** and **4b** which could be easily separated and characterized (56% yield for **4a**). Notice that the final yield of **4a** could be increased after isomerization of the 3'-*O*-TBDMS derivative **4b** into its 2'-*O*-TBDMS isomer **4a** under basic conditions. Finally, reaction of **4a** with 2-cyanoethyl *N*-diisopropyl-chlorophosphoramidite afforded the desired phosphoramidite **5** in 69% yield.

Synthesis, deprotection, purification and characterization of oligoribonucleotides containing site-specific 4-thiouridine: Phosphoramidite **5** served to incorporate 4-thiouridine at specific locations into synthetic RNA oligonucleotides, using standard automated synthesis. Oligoribonucleotides (21 mers) depicted in Figure 2, correspond to

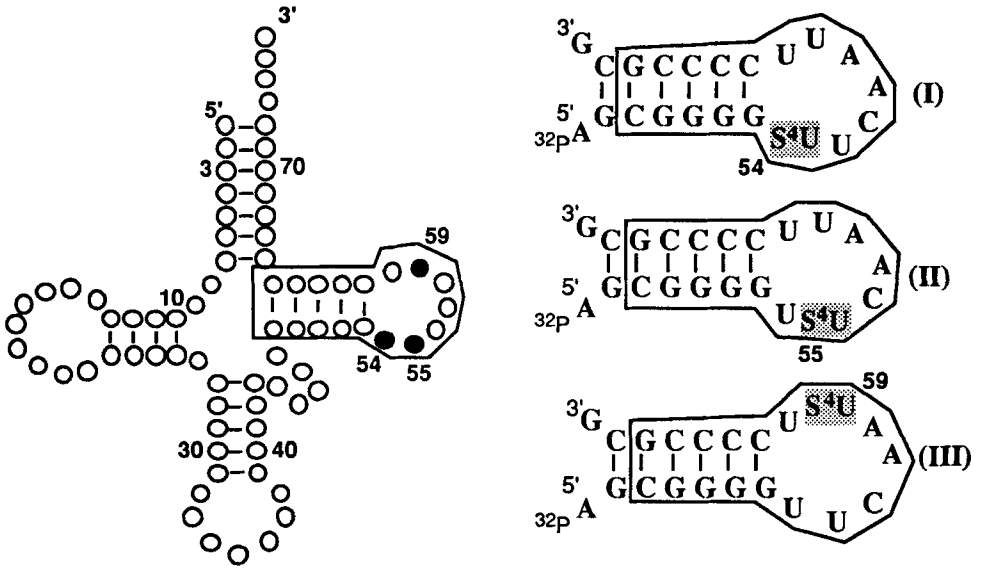

Figure 2: Schematic cloverleaf structure of a tRNA and synthetic oligoribonucleotides corresponding to the T-Ψ loop of tRNA containing a 4-thiouridine at the positions indicated.

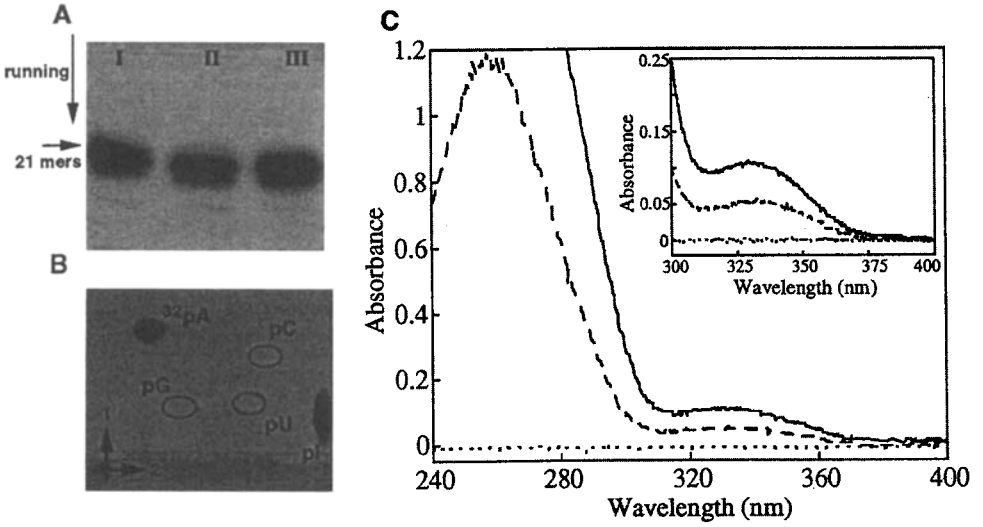

Figure 3: Characterization of the s^4U-containing oligoribonucleotides. Panel A: Autoradiography of the 5'-[^{32}P]-labelled oligoribonucleotides I, II and III after denaturing gel electrophoresis. Only the major radioactive band corresponding to the full-length oligoribonucleotide (upper band in each lane) was used in all subsequent experiments. Lower-bands correspond to the 20 mer oligoribonucleotides bearing a 5'-terminal guanosine. Panel B: Autoradiography of 2D-t.l.c. after P1 hydrolysis of the 5'-[^{32}P] labelled oligonucleotide II. 5'-[^{32}P]-AMP is detected by autoradiography, the other nucleoside 5'-monophosphates are visualized by UV shadowing. Panel C: UV spectrum of oligonucleotide II at two different concentrations in water, pH 6.0.

a stem composed of six G.C base-pairs prolonged by the seven nucleotides that are normally present in the T-Ψ loop of yeast $tRNA^{Asp}$, except that 4-thiouridine was incorporated in place of uridine at position 54, 55 or 59, respectively (the numbering system is that conventionally adopted[17] for full length tRNA). At the end of the synthetic cycle the 5'-*O*-dimethoxytrityl group was removed and the oligoribonucleotide cleaved from the support. The cyanoethyl phosphate protecting groups, as well as all of the base-protecting groups, were removed by ethanolic ammonium hydroxide treatment, whereas the last 2'-*O*-silyl protecting groups were removed in the presence of triethylamine tris-hydrofluoride (TEA,3HF).[18] Desalting was done by HPLC on a reverse-phase column and the full-length (21 mer) oligoribonucleotide was finally purified by two successive runs of electrophoresis on a 15% polyacrylamide gel in the presence of 8M urea. The second electrophoresis was performed with oligoribonucleotide labelled at its 5'-terminal nucleotide with [^{32}P]-orthophosphate (Figure 3, panel A). Only the major radioactive bands corresponding to full-length oligoribonucleotides (upper band in each lane) were used in all subsequent experiments. We verified, by 2D-t.l.c. of nuclease P1-hydrolysates, that the last nucleotide incorporated into each of the 4-thiouridine-

containing oligoribonucleotides was 5'-AMP (Figure 3, panel B). Finally, the presence of 4-thiouridine was confirmed by UV spectroscopy. Indeed, 4-thiouridine presents a characteristic absorption maximum near 330 nm that has been detected in each of the three synthetized oligoribonucleotides (Figure 3, panel C).

Specific cross-linking between synthetic tRNA variants and yeast tRNA:pseudouridine-55 synthase (Pus4): In a previous paper, we demonstrated that the minimal substrate that can be modified by the yeast tRNA:pseudouridine-55 synthase is a stem-loop composed of at least 3 base-pairs in the stem and 7 nucleotides in the loop.[14] We also established that the nucleotide sequence GUUC was required at positions 53-56 and that nucleosides 58 and 61 have to be A and C, respectively. These nucleotides correspond to the conserved residues G-53, U-54, U-55, C-56, A-58 and C-61, that are present in all yeast tRNAs.

To minimize natural stem-loop structure perturbation, only uridines at positions 54, 55 and 59 of the loop were substituted by 4-thiouridine in the photoactivable oligoribonucleotides (Figure 2). These s^4U-containing minisubstrates were irradiated in the presence of yeast tRNA:pseudouridine-55 synthase. Irradiation were performed at a wavelength of 360nm ±10nm, close to the absorption maximum of 4-thiouridine. The incubation time was : 0 min (control experiment), 5 min and 15 min. The enzyme was in two-fold excess relative to the oligoribonucleotide-substrate concentration. To facilitate the subsequent detection of the photocross-linked product, freshly prepared 5'-[^{32}P]-labelled oligonucleotides were used. After the UV-treatment, the samples were analyzed by electrophoresis on a 8% polyacrylamide gel under denaturing SDS-conditions (Figure 4). Each of the three oligoribonucleotides containing 4-thiouridine at position 54, 55 and 59, respectively, were photocross-linked to the enzyme. However, the oligoribonucleotides with s^4U-54 (I, panel A) and s^4U-55 (II, panel B) cross-linked much better (about 5% of the radiolabelled oligonucleotides in both cases) than that with s^4U-59 (III, panel C) (less than 0.5%). Longer irradiation times, up to 30 min, did not increase the yield of the cross-linking reactions. Such a low yield of cross-linking with the s^4U-59-containing minisubstrate III can be rationalized taking into account the peculiar flexibility of U-59 within the TΨ:stem-loop in solution. As shown by footprinting experiments,[14,19] the G-53:C-61 base-pair at the end of the stem tends to stack over the U-54 or T-54:A-58 reverse-Hoogsteen base-pair in trans, leading the two pyrimidines at positions 59 and 60 to preferentially bulge out the loop structure. Moreover, recent NMR studies, in conjunction with restrained molecular dynamics (MD) methods, demonstrated the peculiar flexibility of the two pyrimidines at positions 59 and 60 in an isolated 17-mers T-arm fragment of tRNA.[20] Therefore, it may well be that the

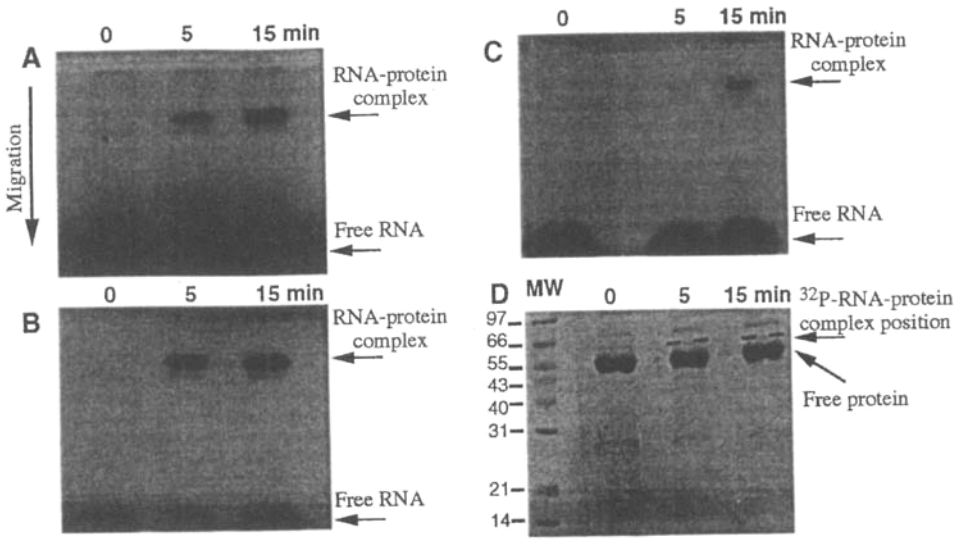

Figure 4: RNA-protein photocross-linking experiments. Panels A, B and C: Autoradiography of the RNA-protein complex (upper band) and the free [^{32}P]-oligoribonucleotide (bottom of the gel) after SDS-gel electrophoresis. Panel D: Same gel as in panel B, but stained by Coomassie blue prior to autoradiography. Dashed lines above each protein bands correspond to positions of the [^{32}P]-RNA-protein complex.

spatial orientation of this U-59 within the dynamic structure of the stem-loop, is such that it is less able to contact the enzyme and consequently to cross-link with an amino acid of the protein when U-59 is replaced by s^{4}U-59.

The molecular mass of the cross-linked complexes has been evaluated to be about 60 kDa, as compared with the molecular mass of 55 kDa for the free enzyme, visualized by Coomassie blue staining. Thus the radioactive bands in each panel of Figure 4, (Panels A, B, C) correspond to a 1:1 covalent complex between tRNA:pseudouridine-55 synthase and the 21-nucleotide-long RNA (molecular weight 6 kDa). No photocross-linked products were detectable when the tRNA:pseudouridine-55 synthase was replaced by *Bombyx mori* alanyl-tRNA synthetase or bovine serum albumin (data not shown), thus attesting to the specificity and selectivity of the above cross-linking experiments with the tRNA:modification enzyme. Likewise, when analyzed on a 15% polyacrylamide gel in the presence of 8 M urea, no detectable photocross-linked RNA-RNA could be detected in our assay conditions, even in the absence of enzyme in the reaction mixture. The reason is probably that the oligoribonucleotide concentration (about 10 μM) was too low for a non-specific RNA-RNA cross-linking reaction to occur.[4,5c]

For site-specific incorporation of 4-thiouridine into oligoribonucleotides we have developed a new phosphoramidite **5** in which the thiol group is protected by *S*-

pivaloyloxymethylation. The advantages of this new protection methodology are its easy application starting from 4-thiouridine, and its compatibility within the standard protocol of RNA synthesis. In the present study, we show that 4-thiouridine-containing oligoribonucleotides, which are valuable tools for RNA cross-linking studies with proteins, can also be used with tRNA:modification enzymes. In this particular case, identification of covalent bonds with amino acids within the neighbourhood of the photoactivable residue should help to better understand how enzymes, such as yeast tRNA:pseudouridine-55 synthase, bind to their RNA substrate. However, establishment of the detailed recognition scheme between the enzyme and its modified tRNA substrates, will depend on complementary informations to be provided by studies of the crystalline structure of both the enzyme and the enzyme-substrate complex.

EXPERIMENTAL SECTION

Reagents and Enzymes: γ-[^{32}P]-ATP (3000 Ci/mmol) was from Amersham (UK). Tris, dithiothreitol (DTT), *Penicillium citricum* nuclease P_1 and bovin serum albumin were from Sigma (France). T_4 polynucleotide kinase was from Boehringer-Mannheim (Germany). Isopropyl-ß-D-thiogalactoside (IPTG) was from MBI Fermentas (Vilnius, Lithuania). Purified alanyl-tRNA synthetase from *Bombyx mori* (MW 115kDa) was a gift from Dr J.D. Digman from Medical College of Ohio (USA).[21] C18 reverse phase chromatography columns were from Vydac (USA). Thin layer cellulose and silica gel plates were from Schleicher & Schuell (Germany). All other chemicals, including 4-thiouridine, were from Aldrich (France) and from Sigma (France). NMR chemical shifts (δ) are given in ppm relative to TMS (^{1}H) or external PO_4H_3 (^{31}P). Standard RNA phosphoramidites were from PerSeptive Biosystems (France).

5'-*O*-Dimethoxytrityl-4-thiouridine (2). A solution of 4-thiouridine **1** (2.0g, 7.72 mmol, 1 equiv.), dried by evaporation from dry pyridine (2 x 10 ml), in 30 ml of dry pyridine was treated overnight at room temperature under argon atmosphere with 4,4'-dimethoxytrityl chloride (2.8g, 8.3 mmol, 1.1 equiv.). After solvent removal the residue was dissolved in 100 ml of methylene chloride. The organic phase was washed with a dilute sodium bicarbonate aqueous solution followed by a saturated sodium chloride solution. The organic layer was separated and dried over anhydrous sodium sulfate. The solvent was removed in *vacuo* and the residue was purified by flash column chromatography over silica gel using heptane-ethyl acetate (5:5 then 2:8) as eluent to give 2.81g (65%) of compound **2** as a white foam. ^{1}H NMR ($CDCl_3$, 300 MHz): 8.60 (s, 1H, NH), 7.79 (d, J=7Hz, 1H, H-6), 7.38-6.82 (m, 13H, Ar.), 6.04 (d, J=7Hz, 1H, H-5), 5.94 (bs, 1H, H-1'), 4.49 (bs, 1H, H-3'), 4.44 (bs, 1H, H-2'), 4.23 (bs, 1H, H-

4'), 3.75 (s, 6H, OMe), 3.50(bs, 2H, H-5'). Analysis: $C_{30}H_{30}N_2O_7S$ Calc. C 64.04, H 5.37, N 4.98. Found C 64.38, H 5.42, N 4.77.

2'-*O*-tert-Butyldimethylsilyl-5'-*O*-dimethoxytrityl-4-thiouridine (3a) and 3'-*O*-*tert*-butyldimethylsilyl-5'-*O*-dimethoxytrityl-4-thiouridine (3b). 5'-*O*-Dimethoxytrityl-4-thiouridine **2** (1.25g, 2.2 mmol, 1 equiv.) dried by co-evaporation with pyridine (2 x 10 ml), was dissolved in 3 ml of dry dimethylformamide under an argon atmosphere. Imidazole (0.38g, 5.6 mmol, 2.5 equiv.) and tert-butyldimethylsilyl chloride (0.36g, 2.4 mmol, 1.1 equiv.) were added to the solution which was stirred for 24 hours at room temperature. Progress of the reaction was followed by t.l.c. (solvent heptane:ethyl acetate 5:5). The reaction mixture was diluted with 100 ml of ethyl acetate and the solution washed twice with 100 ml of water. The organic layer was dried over anhydrous sodium sulfate. The solvent was removed in *vacuo* and the residue filtered over a silica gel column using a gradient of heptane-ethyl acetate (9:1 to 1:9) as eluent, yielding 1.1g (70%) of a mixture of 2'- and 3'-*O*-(*tert*-butyldimethylsilyl)-5'-dimethoxytrityl-4-thiouridine (**3a** and **3b**).

4-*S*-Pivaloyloxymethyl-2'-*O*-*tert*-butyldimethylsilyl-5'-*O*-dimethoxytrityl-4-thiouridine (4a). To a stirred solution of a mixture of compounds **3a** and **3b** (1.1g, 1.4 mmol, 1 equiv.) in acetone (15 ml), was added K_2CO_3 (1.1g, 8 mmol, 5.7 equiv.). After 15 min, pivaloyloxymethyl chloride (0.5 ml, 3.5 mmol, 2.5 equiv.) was added dropwise and the reaction stirred for 24 h. The mixture was filtered on Celite, the filtrate concentrated under reduced pressure, diluted with 50 ml CH_2Cl_2. The organic phase was washed twice with 50 ml of a saturated aqueous sodium chloride solution and dried over sodium sulfate. The solvent was removed under reduced pressure and the residue was chromatographed over a silica gel column. Elution with an heptane-ethyl acetate gradient (9/1 to 3/7) gave (0.62g, 56%) of compound **4a** and (0.45g, 40%) of compound **4b** as foams. Compound **4a**: UV λ_{max} 265 nm, $\varepsilon = 14.10^3$; λ_{max} 305 nm, $\varepsilon = 12.10^3$. ^1H NMR ($CDCl_3$, 300 MHz): 8.08 (d, J=7Hz, 1H, H-6), 7.12-6.61 (m, 14H, arom., H-5), 5.57-5.46 (m, 3H, $SC\underline{H}_2O$, H-1'), 4.07-3.81 (m, 3H, H-3',H-2', H-4'), 3.54 (s, 6H, OMe), 3.31 (m, 2H, H-5'), 0.91 (s, 9H, t-Bu), 0.70 (s, 9H, t-Bu), 0.10 (s, 3H, Me), -0.10 (s, 3H, Me). Analysis: $C_{42}H_{54}N_2O_9SSi$, H_2O. Calc. C 62.35, H 6.98, N 3.46. Found C 62.71, H 6.89, N 3.62. Compound **4b**: ^1H NMR ($CDCl_3$, 300 MHz): 7.99 (d, J=7Hz, 1H, H-6), 7.07-6.52 (m, 14H, arom., H-5), 5.60-5.50 (m, 3H, $SC\underline{H}_2O$, H-1'), 4.12 (m, 1H, H-4'), 3.82 (m, 2H, H-3',H-2'), 3.54 (s, 6H, OMe), 3.42 (d, J=12Hz, 1H, H-5'), 3.04 (d, J=12Hz, 1H, H-5''), 0.92 (s, 9H, t-Bu), 0.57 (s, 9H, t-Bu), 0.19 (s, 3H, Me), -1.06 (s, 3H, Me). Analysis: $C_{42}H_{54}N_2O_9SSi$, $1/2H_2O$. Calc. C 63.05, H 6.93, N 3.51. Found C 62.80, H 6.63, N 3.86.

Isomerisation of 4-*S*-pivaloyloxymethyl-5'-O-dimethoxytrityl-3'-O-*tert*-butyldimethylsilyl-4-thiouridine (4b) into 4-*S*-pivaloyloxy-methyl-5'-*O*-

dimethoxytrityl-2'-*O*-*tert*-butyldimethylsilyl-4-thio-uridine (4a). A solution of compound **4b** (0.40g, 0.5 mmol) in 6 ml EtOH/0.25% Et_3N (v/v) was stirred for 2h at room temperature. Progress of the reaction was monitored by t.l.c. (solvent : ethyl acetate:heptane 5:5) until a stable **4a/4b** ratio was observed. After solvent evaporation compound **4a** (0.17g, yield 43%) was isolated by column chromatography as described previously.

4-*S*-Pivaloyloxymethyl-5'-*O*-dimethoxytrityl-2'-*O*-*tert*-butyldimethylsilyl-4-thiouridine-3'-(cyanoethyl)-*N*,*N*'-diisopropyl-phosphoramidite (5). To a solution of compound **4a** (200 mg, 0.25 mmol, 1 equiv.) and *N*-diisopropylethylamine (140 µl, 0.75 mmol, 3 equiv.) in 1 ml of anhydrous dichloromethane, was added 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (70 µl, 0.3 mmol, 1.2 equiv.). After 2 h, the reaction mixture was diluted by addition of 10 ml of dichloromethane, and the solution extracted with 5% aqueous sodium bicarbonate solution followed by a NaCl-saturated solution. The organic layer was separated and dried over anhydrous sodium sulfate and the solvent removed in *vacuo*. The residue was purified by flash chromatography on silica gel using heptane-ethyl acetate (8:2) as eluent to yield 170 mg of compound **5** as a white powder (yield : 69%). ^1H NMR ($CDCl_3$, 300 MHz): 8.15-8.05 (2d, J=7.5Hz, 1H, H-6), 7.15-6.58 (m, 14H, arom., H-5), 5.62-55.30 (m, 3H, $SC\underline{H}_2O$, H-1'), 4.12-4.10 (m, 3H, H-2', H-3', H-4'), 3.58 (s, 6H, OMe), 3.68-3.47 (m, 6H, H-5',$C\underline{H}N$, $C\underline{H}_2O$), 2.37 (t, 2H, CH_2CN), 0.95 (s, 9H, t-Bu), 0.69 (s, 9H, t-Bu), 1.02-0.80 (m, 12H, Me), 0.06 (s, 3H, Me), -0.06 (s, 3H, Me). ^{31}P NMR($CDCl_3$): 151.25, 149.56.

Oligoribonucleotide synthesis and purification: Oligoribonucleotide syntheses were performed on an Applied Biosystems 392 DNA/RNA Synthesizer on a 1 µmol scale using commercial phosphoramidites and the standard RNA synthesis protocol.[13] Average stepwise coupling yields and final 21 mer yields were in the 90.4-98.2% and 52.8-65.4% ranges, respectively. After removal of the terminal 5'-*O*-dimethoxytrityl group, oligonucleotides were cleaved from the support by treatment (1h) with a freshly prepared ethanol solution of 28% ammonia (3:1). The solution was maintained for 48h at room temperature to remove the alkali-labile protecting groups. Solvents were evaporated and the residue coevaporated with methanol in *vacuo*. Finally, the residue was taken up in bidistilled water, transferred to an Eppendorf tube and lyophilized.

To remove the 2'-*O*-silyl group, 8 A_{260} units of a suspension of crude oligoribonucleotide in 80 µl of triethylamine trihydrofluoride (10 µl/ODU-oligonucleotide) were kept at room temperature for 24 h under strong agitation. After addition of 16 µl of water (2 µl/ODU-oligonucleotide), the deprotected oligonucleotides were rapidly desalted using a C18 reverse-phase HPLC column (5µm, 300Å, 46x150mm). An acetonitrile gradient of 1-50% over 40 min (flow rate 0.8 ml/min) was

used for the run in a buffer solution containing 50 mM ammonium acetate, pH 5.0. Oligoribonucleotide fractions were lyophilized, resuspended in water and stored at -20°C. Purification of the full-length oligonucleotide was done by preparative 15% polyacrylamide gel electrophoresis in the presence of 8M urea. Oligoribonucleotides were recovered from the gel by overnight elution with a salt solution containing 0.5 M ammonium acetate buffer (pH 5.0), 10 mM magnesium acetate, 0.1 mM EDTA, 0.1% (w/v) sodium dodecylsulfate at room temperature. The eluted oligonucleotides were ethanol-precipitated at -20°C, washed with cold 70% ethanol, dried under vacuum and redissolved in water. They were stored at -20°C until used. A second run of purification on 15% polyacrylamide gel was performed after labelling the 5'-terminal nucleotide with [^{32}P] using T4-Polynucleotide kinase and [γ-^{32}P]-ATP. The radioactive band corresponding to the full-length (21 mers) oligoribonucleotide was recovered from the gel as described above and used for all subsequent experiments.

Characterization of oligonucleotides: i) An aliquot of each oligonucleotide was radiolabelled at its 5' end with T4-polynucleotide kinase and [γ-^{32}P]-ATP. The exact length of each oligonucleotide (21 mers) was checked by PAGE on a 15% gel in the presence of 8M urea. Their migrations were compared to oligodeoxynucleotide size-markers (Pharmacia, 8 to 32 nucleotides). ii) The presence of the 5' terminal adenosine was verified on the full length 5'-[^{32}P] labelled oligonucleotide by enzymatic hydrolysis with nuclease P1 according to a previously described procedure.[22] After migration on two-dimensional thin-layer chromatography (2D-t.l.c.) with appropriate solvents, 5'-P-mononucleotides were identified by comparison with reference's maps .[23] Only the last incorporated 5'-nucleotide was revealed by autoradiography, since it is the only [^{32}P] radiolabelled nucleotide. iii) The presence of 4-thiouridine in synthetic oligonucleotides was verified by UV spectroscopy. Each oligoribonucleotides containing 4-thiouridine exhibited UV spectrum with a maximum at 260 nm and a minor maximum at 330 nm, corresponding to the unsaturated thiocarbonyl function.

Yeast tRNA:pseudouridine-55 synthase preparation (Pus4): The enzyme which was used corresponds to recombinant yeast tRNA:pseudouridine-55 synthase (Pus4). pET28b-plasmid, bearing the *PUS4* gene downstream to the T7-promoter under the control of the lac-UV5 promoter, was used to transform *E. coli* cells BL21(DE3). Induction was performed by IPTG for 2h in MM9 medium. Cell growth, disruption, centrifugation and supernatant treatment were previously described.[24] The recombinant protein bears an N-terminal His_6-Tag and was purified to near homogeneity (90%) by step-elution on a Ni^{2+}-NTA-agarose column (Qiagen, Germany).

UV cross-linking: UV cross-linking of yeast tRNA:pseudourine-55 synthase to the TΨ stem-loop variants was carried out in 100 mM Tris-HCl, pH 8, 10 mM $MgSO_4$, 0.1

mM EDTA, 100 mM ammonium acetate and 1 mM DTT, in a total volume of 10 µl. TΨ:stem-loop variants containing 4-thiouridine (10 µM oligoribonucleotide and 500 cps 5'-[^{32}P]-oligoribonucleotide) were pre-renaturated to the correct folding by heating at 65°C for 3 min then slowly cooled to room temperature before addition of 20 µM recombinant protein. Reaction mixtures were irradiated using an HBO 200 watts superpressure mercury lamp. The wavelength was selected by mean of a Bausch and Lomb monochromator (360nm ±10nm). A Schott WG 345 filter was added at the exit slit of the monochromator to prevent any contamination by short wavelength light. To examine potential protein-RNA cross-links generated by irradiation, 10 µl of loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM 2-mercaptoethanol, 0.001% bromophenol blue) was added. The samples were then heated for 3 min at 70°C before electrophoresis at room temperature on a 8% SDS-gel. The gel was stained by Coomassie blue, dried and exposed overnight to autoradiography. Quantification of the photoproducts was performed by scanning on the PhosphoImager (Molecular Dynamics, USA) using ImageQuant software.

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