Synthesis and Characterization of the Native Anticodon Domain of *E. coli* tRNA^{Lys}: Simultaneous Incorporation of Modified Nucleosides mnm^{5}s^{2}U, t^{6}A, and Pseudouridine Using Phosphoramidite Chemistry

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The anticodon domain of *E. coli* tRNA^{Lys} contains the hypermodified nucleosides mnm^{5}s^{2}U and t^{6}A at positions 34 and 37, respectively, along with a more common ψ at position 39. The combination of these three nucleotides represents one of the most extensively modified RNA domains in nature. 2-Cyanoethyl diisopropylphosphoramidites of the hypermodified nucleosides mnm^{5}s^{2}U and t^{6}A were each synthesized with protecting groups suitable for automated RNA oligonucleotide synthesis. The 17 nucleotide anticodon stem-loop of *E. coli* tRNA^{Lys} was then assembled from these synthons using phosphoramidite coupling chemistry. Coupling efficiencies for the two hypermodified nucleosides and for pseudouridine phosphoramidite were all greater than 98%. A mild deprotection scheme was developed to accommodate the highly functionalized RNA. High coupling yields, mild deprotection, and efficient HPLC purification allowed us to obtain 1.8 mg of purified RNA from a 1 μmol scale RNA synthesis. Our efficient synthetic protocol will allow for biophysical investigation of this rather unique tRNA species wherein nucleoside modification has been shown to play a role in codon-anticodon recognition, tRNA aminoacyl synthetase recognition, and programmed ribosomal frameshifting. The human analogue, tRNA^{Lys,3}, is the specific tRNA primer for HIV-1 reverse transcriptase and has a similar modification pattern.

The anticodon domain of *E. coli* tRNA^{Lys} contains hypermodified nucleosides at the wobble position (mnm^{5}s^2-U34) and at the conserved purine immediately adjacent to the anticodon (t^{6}A37) in addition to a pseudouridine (ψ39) at the base of the stem.[1] These modifications are critically important for correct function of this tRNA in protein synthesis as undermodified tRNAs have poor or altered mRNA binding,[2,3] and are not properly recognized by the cognate lysyl-tRNA aminoacyl synthetase.[4,5] Anticodon modification also seems to be functionally important in programmed frameshifting required for expression of certain bacterial genes,[6,7] and modification is required for proper function of human tRNA^{Lys,3} as the primer for HIV reverse transcriptase.[8] In an attempt to explain the novel biochemical properties of this tRNA, a model of the tRNA anticodon domain has been proposed where the two hypermodified nucleosides interact directly.[9] However, the stem-loop shown in Figure 1 is constrained in a manner distinctly different from the model pentamer, and extensive biochemical and genetic studies indicate the functional tRNA anticodon domain includes the entire loop and at least the first two base pairs of the stem.[10,11]

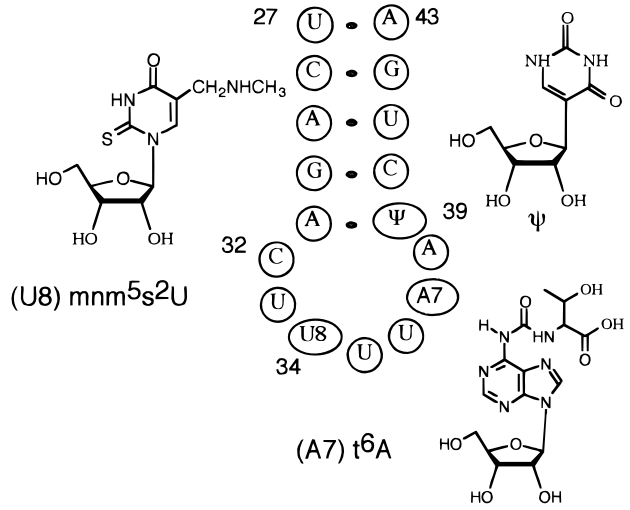

Figure 1. Secondary structure of the 17 nucleotide anticodon domain of *E.coli* tRNA^{Lys} and structures for the hypermodified nucleosides.

The mnm^{5}s^{2}U and t^{6}A nucleosides have previously been incorporated into an RNA pentamer using the

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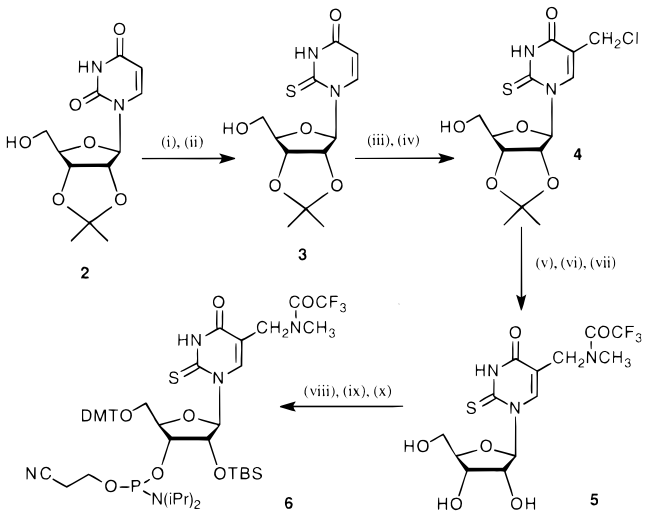
phosphotriester method in solution.[9] The nucleoside phosphoramidite of the mnm^{5}U nucleoside lacking the 2-thio group has also been reported,[12] but no details were provided about the RNA yields or physical characterization of the RNA product. The amine protecting strategy we employed was the same as reported previously for the mnm^{5}U and mnm^{5}s^{2}U nucleosides.[12−14] The phosphoramidite method has been used recently to synthesize a tRNA$_f^{Met}$ anticodon stem-loop containing t^{6}A at position 37.[15] The protecting strategy employed was similar to that which we report with the exception that a base-labile 2-(4-nitrophenyl)ethyl group was used to protect the side chain carboxylate. The general synthetic strategy we describe will allow for biochemical study and high-resolution structural determination of tRNA^{Lys} as well as other tRNA isoacceptors where either mnm^{5}s^{2}U or t^{6}A is found. Preliminary NMR data indicate the modified anticodon domain is structurally distinct from both unmodified tRNA^{Lys} and the modified pentamer.[9,16]

For compatibility with the standard RNA phosphoramidite chemistry, the two functional group challenges of mnm^{5}s^{2}U are the sulfur at position 2 and the secondary amine side chain. Sulfur was introduced into the nucleobase of 2′,3′-isopropylidineuridine via H_2S treatment of the 5′,2-anhydro nucleoside formed under Mitsunobo conditions to give **3**.[17] The 5-methylaminomethyl side chain was elaborated in a series of high-yielding steps and then protected as the base labile trifluoroacetate.[17,18] The 2′,3′-acetonide was removed with mild acid to generate the N-protected nucleoside **5** (Scheme 1).[18] Synthesis of the protected phosphoramidite **6** was then accomplished using typical RNA phosphoramidite procedures.[19]

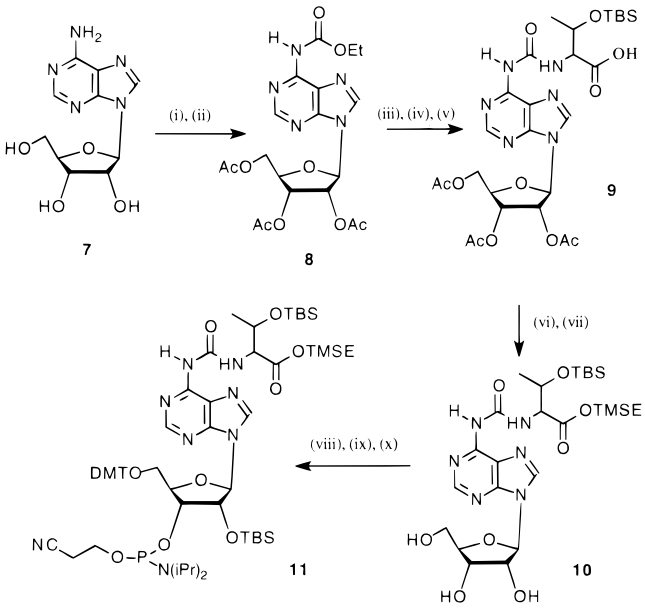
The hypermodified nucleoside t^{6}A presents several challenges for incorporation into oligonucleotides via phosphoramidite chemistry. The secondary hydroxyl and the carboxylate require protection, and the carbamoyl linkage is relatively base-sensitive, requiring some care if one chooses to use the commercial A, C, and G RNA phosphoramidites having base labile amine protecting groups. The t^{6}A nucleoside was synthesized according to literature procedures.[20] Compound **9** was obtained by TBS-OTf treatment[21] of the sugar-protected t^{6}A nucleoside, then selective hydrolysis of the labile TBS-ester. The carboxylate was protected as the stable TMSE alkyl ester[22] followed by selective removal of the acetates[20] to give **10** (Scheme 2). Elaboration of **10** to phosphoramidite **11** proceeded in high yield by the same general procedure as for **6** to give an overall yield of 2.2% from adenosine.

Scheme 1[a]



[a] Key: (i) Ph_3P, DEAD, 1,4-dioxane, 95%; (ii) H_2S, pyridine, 90%; (iii) $(CHO)_n$, 0.5 M NH_3, 90%; (iv) TMS-Cl, 1,4-dioxane; (v) CH_3NH_2, methanol, 60%; (vi) TMS-Cl, pyridine; TFAA, 70%; (vii) 20% acetic acid, 95%; (viii) DMT-Cl, pyridine, 90%; (ix) TBS-Cl, imidazole, pyridine, 70%; (x) $[(CH_3)_2CH]_2NP(Cl)OCH_2CH_2CN$, DMAP, DIEA, THF, 90%.

Scheme 2[a]



[a] Key: (i) $(CH_3CO)_2O$, pyridine, 95%; (ii) ethylchloroformate, pyridine, 75%; (iii) L-threonine, pyridine, 90%; (iv) TBS-OTf, Et_3N, CH_2Cl_2, 95%; (v) 2 M NH_3/methanol, 95%; (vi) DCC, TMSE-OH, pyridine, 91%; (vii) 2 M NH_3/methanol, 90%; (viii) DMT-Cl, pyridine, 90%; (ix) TBS-Cl, imidazole, pyridine, 75%; (x) $[(CH_3)_2CH]_2NP(Cl)OCH_2CH_2CN$, DMAP, DIEA, THF, 90%.

The fully modified anticodon domain in Figure 1 was synthesized using commercial PAC-protected phosphoramidites (Glen Research) and standard coupling chemistry[19] with the exception of tBuOOH oxidation.[23−25] Trityl assays indicated >98% coupling for all phosphoramidites; this was verified by HPLC and MALDI/MS of the crude, deprotected RNA. To avoid reaction of the secondary

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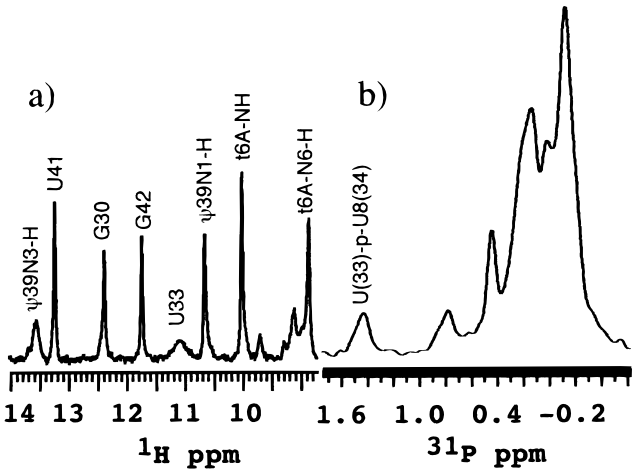

Figure 2. (a) ^{1}H NMR imino spectrum of the native anticodon stem-loop of *E. coli* tRNA^{Lys}; (b) ^{31}P NMR spectrum showing the downfield resonance characteristic of a "U-turn" between U33 and mnm^{5}s^{2}U34. Sample conditions were 1.0 mM RNA in 0.25 mL of 10 mM sodium phosphate, pH 6.8 containing 50 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$, and 5% deuterium oxide.

amine of mnm^{5}s^{2}U with acrylonitrile generated during base-deprotection[26] we used a two-step deprotection procedure wherein the column bound RNA is treated first with *tert*-butylamine and then with NH_3/ethanol.[27] After ammonia treatment the RNA solution was dried, then treated with $Et_3N{\cdot}HF$ to remove the TBS ethers and the TMS-ethyl ester.[28] The fully deprotected RNA was then purified by HPLC.[29] MALDI-MS was used to verify that the purified oligonucleotides had the correct molecular weight. The presence of the major and modified nucleotides was verified by digesting the RNA to mononucleosides and then analyzing the digest by LC/MS.[30,31]

The ^{1}H NMR spectrum in Figure 2a shows the downfield, imino spectral region where N−H bound protons in slow exchange with water resonate.[32] There are a number of distinctive features evident for the tRNA^{Lys} hairpin. The three strong peaks at 13.23, 12.38, and 11.75 are from the central base pairs of the stem. The most downfield resonance is for the N3−H proton of ψ39, which forms a Watson−Crick base pair with A31.[16] The resonance at 10.64 is the ψ39 N1−H proton, while the peaks at 9.94, and 8.85 are the amide and H6 protons of t^{6}A. Resonances are seen in both the ^{1}H and ^{31}P NMR spectra that indicate a stable structure reminiscent of the yeast tRNA^{Phe} crystal structure.[33,34] In the ^{1}H spectrum a weak peak is seen at 11.10 ppm for the U33 N3−H proton hydrogen bonded to the phosphate between U35 and U36, a key feature of a "U-turn" structure.[33,35] The chemical shift of this peak is rather unique and is downfield of a merely slow exchanging imino, consistent with our interpretation of the imino-phosphate interaction. The published spectra of tRNA^{Phe} have an almost identical resonance and we have unambiguously assigned the imino spectrum of a tRNA^{Leu} anticodon domain which has a strong peak for the U33−NH to phosphate interaction. These analogous studies, the chemical shift arguments, and the exchange behavior argue in favor of our assignment for tRNA^{Lys}, but the broad peak has prevented us from obtaining direct NOE confirmation. Additional support for our assignment and interpretation comes from the ^{31}P NMR spectrum (Figure 2b) which shows a downfield shifted phosphorus assigned to the U33-p-mnm^{5}s^{2}U34 phosphate.[36−38] Earlier work from our laboratory showed that in the unmodified tRNA^{Lys} anticodon, no U-turn is present.[16] These results clearly show that the hypermodified nucleosides in tRNA^{Lys} have a dramatic effect on the structure of this tRNA. In addition to the imino protons, we have used standard NMR experiments[32] to completely assign the base and sugar protons, including stereospecific assignments for some of the H5′, H5″ protons. A number of NOE cross-peaks supporting the U-turn structure[39] are seen in the 2D NOESY spectrum (Supporting Information). The efficient chemical synthesis methodology we have presented will enable us to pursue the high-resolution NMR structure of the anticodon domain and to investigate the structural effects of the individual mnm^{5}s^{2}U and t^{6}A hypermodified nucleosides.

Experimental Section

General Methods. Melting points are uncorrected. ^{1}H and ^{31}P NMR spectra were collected in deuterated solvents on either 200 or 500 MHz spectrometers. ^{1}H chemical shifts are reported relative to tetramethylsilane at 0.0 ppm and referenced to the residual proton signal of the deuterated solvent. ^{31}P chemical shifts are relative to external phosphoric acid at 0.0 ppm. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60F-254 plates. Column chromatography was carried out with Merck silica gel 60 (230−400 mesh). Chemical reagents were purchased from Aldrich except where noted and used without further purification. Spectral grade acetonitrile (Baxter) was used for HPLC. Instrumentation and sample preparation for MALDI MS and ESI LC/MS are described below. Elemental analysis was done by Galbraith Laboratories (Knoxville, TN).

5′-*O*-(4,4′-Dimethoxytrityl)-5-(*N*-trifluoroacetyl)methylaminomethyl-2-thiouridine (5a). 2′,3′-*O*-Isopropylidene-2-thiouridine **(3)** was synthesized from 2′,3′-*O*-isopropylideneuridine (**2**) (Aldrich) by the method of Malkiewicz et al.[17] 5-Methylaminomethyl-2-thiouridine was synthesized as described by Ikeda et al.[18] and the secondary amino side chain protected as the trifluoroacetate to give the N-protected nucleoside **5**.[17] Intermediate **5** (1.0 g, 2.5 mmol) was dissolved in 25 mL of dry pyridine and 4,4′-dimethoxytrityl chloride (1.05 g, 3.12 mmol, 1.25 equiv) added to the stirred solution. The reaction was continued for 12 h and then diluted with 75 mL of CH_2Cl_2 and washed with 2 × 20 mL of 5% $NaHCO_3$ and 20 mL of saturated NaCl. The organic layer was dried over sodium sulfate and solvent removed under reduced pressure.

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The residual pyridine was removed by coevaporation with dry toluene, and the resulting foam carried forward.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-5-(*N*-trifluoroacetyl)methylaminomethyl-2-thiouridine. The 5′-DMT nucleoside (1.0 g, 1.42 mmol, 1.0 equiv) was dissolved in 12 mL of dry pyridine under Ar atmosphere. To this were added imidazole (0.22 g, 3.55 mmol, 2.5 equiv) and *tert*-butyldimethylsilyl chloride (0.238 g, 1.78 mmol, 1.25 equiv). The reaction mixture was stirred for 24 h, at which time the starting material was converted to a nearly equimolar mixture of 2′ and 3′ TBS products. The reaction was diluted with 75 mL of CH_2Cl_2 and extracted with 5% sodium bicarbonate. The organic layer was dried over sodium sulfate and solvent removed under reduced pressure. The 2′ TBS isomer was isolated by flash chromatography on silica gel using CH_2Cl_2: ethyl acetate (19:1) to yield 0.5 g, 44% of a white foam. The 2′ and 3′ isomers have R_f' s of 0.4 and 0.3 respectively on silica gel TLC. A 2D COSY NMR experiment was used to confirm the identities of each isomer from the correlations from H2′ to H2′OH for the 3′ TBS isomer and from the H3′ to H3′OH for the 2′ TBS isomer. The 3′ TBS compound could be isomerized to an equimolar mixture of 2′ and 3′ isomers by stirring in methanol with a trace of triethylamine. This isomerization and chromatography was repeated twice in order to obtain additional quantities of the pure 2′ TBS isomer for an overall yield of 70%: mp 96−98 °C; ^{1}H NMR (DMSO-d_6) δ 0.1 (s, 3H), 0.2 (s, 3H), 0.85 (s, 9H), 2.5 (d, 3H), 3 (d, 2H), 3.2 (s, 3H), 3.3 (s, 3H) 5.0 to 4.3 (m, 5H), 6.6 (d, 1H), 6.90 (d, 4H), 7.30 (m), 7.8 (s, 1H), 12.8 (s, 1H); HRMS (FAB) MH+ calcd for $C_{40}H_{48}N_3O_8Si_1S_1F_3$ 816.2962, obsd 816.2932. Anal. Calcd for $C_{40}H_{48}N_3O_8Si_1S_1F_3$: C, 58.95; H, 5.81; N, 5.15. Found: C, 58.78; H, 6.22; N, 4.91.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-5-(*N*-trifluoroacetyl)methylaminomethyl-2-thiouridine-3′-(cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (6). The 5′-DMT, 2′-TBS nucleoside (0.60 g, 0.74 mmol, 1.0 equiv) was dissolved in 12 mL of dry THF under Ar atmosphere. To this were added DMAP (0.018 g, 0.14 mmol, 0.2 equiv) and diisopropylethylamine (0.316 mL, 1.62 mmol, 2.2 equiv). The solution was stirred while adding 2-cyanoethyl *N*,*N*-diisopropylphosphonamidic chloride (0.328 mL, 1.48 mmol, 2.0 equiv). After 30 min, a white precipitate formed, and the stirring was continued for 3 h. The reaction was followed by taking a 50 μL aliquot of the reaction mixture, removing the solvent, oxidizing for 3 min with tBuOOH/toluene, followed by evaporation and then silica gel TLC. The amidite product is found at the origin while the unreacted starting material is unaffected by the oxidation procedure. This protocol is very helpful since the starting material and products migrate with identical Rf in the solvent systems we investigated. The reaction was quenched by adding 100 mL of ethyl acetate, and then extracting with saturated $NaHCO_3$ followed by saturated NaCl. The organic layer was separated, dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel using CH_2Cl_2/acetonitrile (18:1) to yield 0.47 g (64%) of compound **6** as a white foam. The material exists as 1:1 ratio of stereoisomers about phosphorus and two chemical shifts are observed for some of the NMR resonances. The secondary shifts are indicated in parentheses: mp 88−90 °C; ^{1}H NMR (acetone-d_6): δ 0.1(0.2) (s, 3H), 0.3(0.4) (s, 3H), 0.85-(0.9) (s, 9H), 4.7 to 2.5 (m, 18H), 7.6 to 6.6(m, 14H), 7.9(8.0) (s, 1H); ^{31}P NMR (acetone-d_6) 155.79, 155.57; HRMS (FAB) MH+ calcd for $C_{49}H_{66}N_5O_9Si_1S_1F_3P_1$ 1016.4040, obsd 1016.3991. Anal. Calcd for $C_{49}H_{66}N_5O_9Si_1S_1F_3,P_1$: C, 57.97; H, 6.35; N, 6.89. Found: C, 57.59, H; 6.49; N, 5.98.

(2,3,5-Tri-*O*-acetyl-*N*-[(9-β-D-Ribofuranosyl-9*H*-purin-6-yl)carbamoyl])-L-threonine. The modified nucleoside t^{6}A was synthesized as the 2′, 3′, 5′-triacetate according to literature procedures.[20] ^{1}H NMR spectra were consistent with that previously reported and mass spectrometry was used to confirm the identity of the starting material.

(2,3,5-Tri-*O*-acetyl-*N*-[(9-β-D-Ribofuranosyl-9*H*-purin-6-yl)carbamoyl])-*O*-*tert*-butyldimethylsilyl-L-threonine (9). *tert*-Butyldimethylsilyl triflate (7.6 mL, 29.6 mm, 4 equiv) was added to a solution of t^{6}A triacetate (4 g, 7.4 mmol, 1 equiv) and triethylamine (5.6 mL, 37.2 mmol, 5 equiv) in 100 mL of dry dichloromethane.[21] The reaction mixture was stirred for 4 h, at which time TLC indicated that both the secondary hydroxyl and the carboxyl group were converted to their TBS derivatives. The reaction mixture was diluted with 50 mL of dichloromethane and washed with cold, 5% sodium bicarbonate. The organic layer was dried over anhydrous sodium sulfate and solvent removed under reduced pressure. The residue was triturated with dry ether, separating the soluble product from insoluble triethylammonium triflate. The ether solution was then dried over anhydrous sodium sulfate. The ether was removed under reduced pressure and the dry product treated with 25 mL of 2 M ammonia in methanol for 1 min to remove the labile TBS-ester. The ammonia solution was concentrated under reduced pressure to give 4.3 g of the dry product **9** (95% yield). This crude material was carried to the next step without purification.

(2,3,5-Tri-*O*-acetyl-*N*-[(9-β-D-Ribofuranosyl-9*H*-purin-6-yl)carbamoyl])-*O*-*tert*-butyldimethylsilyl-L-threonine Trimethylsilylethyl Ester (9a). Compound **9** (2.5 g, 3.82 mmol, 1 equiv) was dissolved in 20 mL of anhydrous pyridine. DCC (987 mg, 4.8 mmol, 1.25 equiv) was added, and the mixture was stirred at room temperature for 5 min. To this was added 2-trimethylsilylethanol (700uL, 4.8 mmol, 1.25 equiv) and the reaction mixture stirred for 18 h, at which time TLC indicated the reaction was complete.[22] The reaction mixture was diluted with 100 mL ethyl acetate and washed with saturated sodium bicarbonate. The organic layer was dried over sodium sulfate and solvent removed under reduced pressure. The crude product was purified by flash chromatography on silica gel using EtOAc/CH_2Cl_2 (1:1) to yield 2.6 g (91%) of the protected nucleoside as a white foam: mp 96−98 °C; ^{1}H NMR (Me_2SO-d_6) δ 0.0 (s, 9H), 0.2 (s, 3H), 0.3 (s, 3H), 0.8 (s, 9H), 1.0 (d, 4H), 1.2 (d, 3H), 1.95 (s, 3H), 2.0 (s, 3H), 2.05 (s, 3H), 5.0 to 4.3 (m, 5H), 5.65 (t, 1H), 6.05 (t, 1H), 6.3 (d, 1H), 8.4 (s, 1H), 8.65 (s, 1H), 9.85 (d, 1H), and 10.05 (s, 1H); HRMS (FAB) MH+ calcd for $C_{32}H_{52}N_6O_{11}Si_2$ 753.3311, obsd 753.3285.

***N*-[(9-β-D-Ribofuranosyl-9*H*-purin-6-yl)carbamoyl)]-*O*-*tert*-butyldimethylsilyl-L-threonine Trimethylsilylethyl Ester (10).** The triacetyl protected nucleoside (4.6 g, 6.1 mm, 1 equiv) was dissolved in 100 mL of 2 M ammonia in methanol and the solution stirred for 6 h at room temperature and then evaporated to dryness under reduced pressure. Crude yield: 3.6 g (95%). The reaction was quite clean as judged by thin-layer chromatography, and for preparative purposes this material was carried forward without purification. Analytical samples were prepared by flash chromatography on silica gel using EtOAc/methanol (98:2): mp 102−104 °C; ^{1}H NMR (Me_2SO-d6): δ 0.0 (s, 9H), 0.2 (s, 3H), 0.3 (s, 3H), 0.8 (s, 9H), 1.0 (d, 4H), 1.2 (d, 3H), 5.0 to 4.3 (m, 5H), 5.65 (t, 1H), 6.05 (t, 1H), 6.3 (d, 1H), 8.4 (s, 1H), 8.65 (s, 1H), 9.85 (d, 1H), and 10.05 (s, 1H); MS (FAB) MH$^+$ 627.2, calcd for $C_{26}H_{46}N_6O_8Si_2$ 627.9.

5′-*O*-(4,4′-Dimethoxytrityl)-*N*-[(9-β-D-ribofuranosyl-9*H*-purin-6-yl)carbamoyl)]-*O*-*tert*-butyldimethylsilyl-L-threonine Trimethylsilylethyl Ester (10a). Protected nucleoside **10** (1 g, 1.6 mmol, 1 equiv) was dissolved in 20 mL of anhydrous pyridine under Ar atmosphere. 4,4′-Dimethoxytrityl chloride (750 mg, 2.2 mmol, 1.4 equiv) was added, and the mixture was stirred at room temperature for 5 h. The reaction mixture was diluted with 100 mL of CH_2Cl_2 and washed with saturated sodium bicarbonate. The organic layer was dried over sodium sulfate and solvent removed under reduced pressure. The crude product was purified by flash chromatography on silica gel using EtOAc/MeOH (20:1) to yield 1.4 g (95%) of the 5′-DMT nucleoside as white foam: ^{1}H NMR (Me_2SO-d_6) δ 0.0 (s, 9H), 0.2 (s, 3H), 0.3 (s, 3H), 0.8 (s, 9H), 1.0 (d, 4H), 1.2 (d, 3H), 3.2 (s, 3H), 3.3 (s, 3H) 5.0 to 4.3 (m, 5H), 5.65 (t, 1H), 6.05 (t, 1H), 6.3 (d, 1H), 6.90 (d, 4H), 7.30 (m, 9H), 8.4 (s, 1H), 8.65 (s, 1H), 9.85 (d, 1H), and 10.05 (s, 1H); MS (FAB) MH+ 928.4, calcd for $C_{47}H_{64}N_6O_{10}Si_2$ 929.0.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-*N*-[(9-β-D-ribofuranosyl-9*H*-purin-6-yl)carbamoyl)]-*O*-*tert*-butyldimethylsilyl-L-threonine Trimethylsilylethyl Ester (10b). The 5′-DMT nucleoside **10a** (1.4 g, 1.5 mm, 1

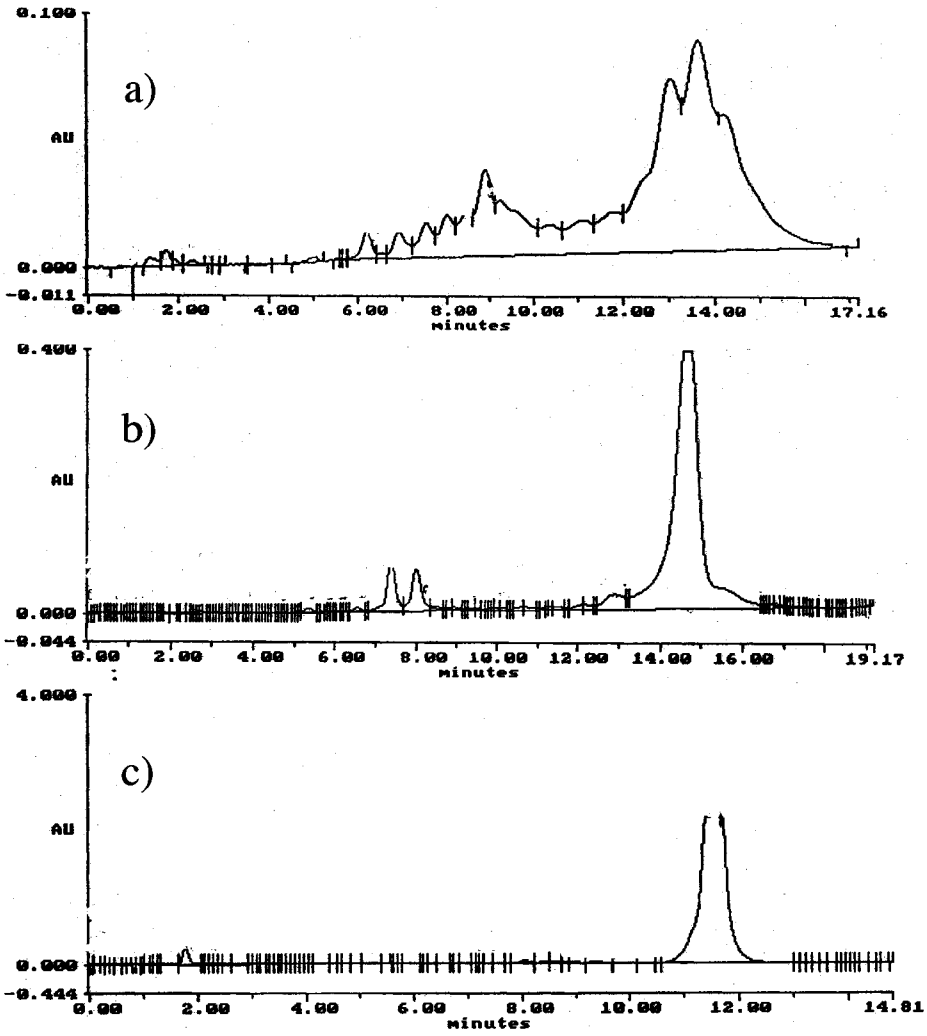

Figure 3. Ion- exchange HPLC of the native *E. coli* tRNA^{Lys} stem-loop. HPLC was done at 60 °C using a 1 × 15 cm Pharmacia Resource Q column. The mobile phase was 0.02 M ammonium acetate and the RNA eluted with a gradient of 20−600 mM $LiClO_4$. (a) The crude material obtained using the standard, mild deprotection conditions commonly used for PAC protected RNA. There are multiple "full-length" species corresponding to material where the t^6A side chain has been lost, and where the methylamino side chain is adducted with acrylonitrile; (b) an analytical (10 μg) injection of crude material after deprotection with the optimized protocol; (c) an analytical trace of the pooled, lyophilized, and dialyzed RNA used for NMR.

equiv.) was dissolved in 25 mL of anhydrous pyridine under Ar atmosphere. To this were added imidazole (260 mg, 4.2 mm, 2.8 equiv) and *tert*-butyldimethylsilyl chloride (281 mg, 2.1 mm, 1.4 equiv). The reaction mixture was stirred for 24 h, at which time the starting material was converted to a nearly equimolar mixture of 2′ and 3′ TBS products. The reaction mixture was diluted with 100 mL of CH_2Cl_2 and washed with saturated sodium bicarbonate. The organic layer was dried over sodium sulfate and solvent removed under reduced pressure. The crude product was purified by flash chromatography on silica gel using EtOAc/CH_2Cl_2 (6:1) to yield 703 mg (45%) of the 5′-DMT, 2′-TBS nucleoside as a white foam. The 2′ and 3′ isomers have a R_f's of 0.4 and 0.2 respectively on silica gel TLC with EtOAc/CH_2Cl_2 (6:1) as the eluting solvent. The identities were confirmed by 2D COSY NMR as described for the mnm^5s^2U nucleoside. The 3′ TBS compound can be isomerized to an equimolar mixture of 2′ and 3′ isomers by stirring in methanol with a trace of triethylamine. The isomerization and chromatography was repeated twice to obtain additional, pure 2′-TBS material: mp 94−96 °C; ^1H NMR (Me_2SO-d_6) δ −0.2 (s, 3H), −0.1 (s, 3H), 0.0 (s, 9H), 0.2 (s, 3H), 0.3 (s, 3H), 0.75 (s, 9H), 0.85 (s, 9H), 1.0 (d, 4H), 1.2 (d, 3H), 3.2 (s, 3H), 3.3 (s, 3H), 5.0 to 4.3 (m, 5H), 5.65 (t, 1H), 6.05 (t, 1H), 6.3 (d, 1H), 6.90 (d, 4H), 7.30 (m, 9H), 8.4 (s, 1H), 8.65 (s, 1H), 9.85 (d, 1H), and 10.05 (s, 1H); HRMS (FAB) MH+ calcd for $C_{53}H_{79}N_6O_{10}Si_3$ 1043.5166, obsd 1043.5175.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-*N*-[(9-β-D-ribofuranosyl-9*H*-purin-6-yl)carbamoyl)]-*O*-*tert*-butyldimethylsilyl-L-threoninetrimethylsilylethyl Ester 3′-(Cyanoethyl *N*,*N*-diisopropylphosphoramidite) (11). 5′-DMT, 2′-TBS-t^6A (550 mg, 0.53 mmol, 1 equiv) was dissolved in 10 mL of anhydrous THF under Ar atmosphere. To this were added DMAP (13 mg, 0.11 mmol, 0.2 equiv) and diisopropylethylamine (186 μL, 1.06 mmol, 2 equiv). The reaction mixture was stirred for 5 min before adding 2-cyanoethyl *N*,*N*-diisopropylphosphoramidic chloride (237 μL, 1.06 mmol, 2 equiv). After 10 min a white precipitate was formed, and the stirring continued for 3 h. The reaction was followed by taking a 50 μL aliquot of the reaction mixture, removing the solvent, oxidizing for 3 min with tBuOOH/toluene, followed by evaporation and then silica gel TLC. The oxidized amidite product is found at the origin while the unreacted starting material is unaffected by the oxidation procedure. This protocol is helpful since the starting material and the products migrate with identical RF in the solvent systems that we investigated. The reaction mixture was diluted with 50 mL of dichloromethane and washed with 5% sodium bicarbonate. The organic layer was dried over sodium sulfate and solvent removed under reduced pressure. The crude product was purified by flash chromatography on silica gel using EtOAc: CH_2Cl_2 (6:1) to yield 526 mg (80%) of compound **11** as a white foam. The material exists as a 1:1 ratio of stereoisomers about

phosphorus and two chemical shifts are observed for some of the resonances. The secondary shifts are indicated in the parenthesis: ^{1}H NMR (acetone-d_6) δ −0.2 (s, 3H), −0.1 (s, 3H), 0.0 (s, 9H), 0.2 (s, 3H), 0.3 (s, 3H), 0.75 (s, 9H), 0.85 (s, 9H), 1.0 (d, 4H), 1.2 (d,3H), 3.2 (s, 3H), 3.3 (s, 3H), 4.3 to 3.8 (m, 14H), 5.0 to 4.3 (m, 5H), 5.6 (5.65) (t, 1H), 5.8 (5.85) (t, 1H), 6.7 (d, 1H), 6.90 (d, 4H), 7.30 (m, 9H), 8.8 (8.85) (s, 1H), 8.9 (8.95) (s, 1H), and 10.55 (d, 1H); ^{31}P NMR (acetone-d_6) 154.74, 153.15. HRMS (FAB) MH+ calcd for $C_{62}H_{97}N_8O_{11}Si_3P_1$ 1243.6244, obsd 1243.6143. The purity was verified by ^{1}H NMR as shown in Figure S8 (Supporting Information).

Oligonucleotide Synthesis. The oligoribonucleotides were synthesized on an Applied Biosystems 394 oligoribonucleotide synthesizer on a 1 μmole scale using 0.05 M acetonitrile solutions of PAC amidites (PAC A, isopropyl PAC G, and acetyl C) from Glen Research. The concentrations of t^6A and mnm^5s^2U amidites were 0.12 M each. The phosphoramidites were coupled for 25 min. The normal I_2/H_2O oxidation solution was used up until the mnm^5s^2U nucleoside and then 2 × 5 min oxidation cycles with 10% tBuOOH in ACN was used from that point forward.

RNA Deprotection and Purification. The CPG-bound RNA sequences were transferred from the column to a screw cap glass vial, to which was added 1 mL of 10% *tert*-butylamine in dry pyridine.[27] The solution was stirred at rt for 1 h. The solvent was removed in vacuo, and the residue was treated with 3 mL of NH_4OH/ethanol (3:1 v/v) at rt for 1 h and at 55 °C for 4 h. The supernatant was decanted, the support material washed with an additional 1.0 mL of NH_4OH/ethanol, and the combined washings were lyophilized on a Speed-Vac concentrator. The dried material was dissolved in 1 mL of neat $Et_3N\cdot 3HF$ (Aldrich) and stirred at room temperature for 9−12 h.[28,40] The reaction was quenched by adding 0.1 mL of water, and the RNA was precipitated by adding 10 mL of *n*-butanol and allowing the solution to stand at −20 °C for 6 h. The precipitated RNA was recovered by centrifugation and dried under reduced pressure in a Speed-Vac concentrator. The residue was then dissolved in 1 mL of 1 M tetrabutylammonium fluoride in THF and stirred at rt for 12−18 h.[19] The solution was passed through a NAP-25 (Pharmacia) size exclusion column to remove the tetrabutylammonium counterions. The RNA-containing eluate (as monitored by UV absorption at 260 nm) was lyophilized and then purified by anion exchange HPLC (Figure 3).[29]

Fractions containing full length material as judged by HPLC were collected, lyophilized, and then dialyzed against 2 × 1 L of 0.1 M NaCl, 0.1 mM EDTA, and then against 2 × 1 L of deionized water. The dialyzed material was lyophilized to yield 1.8 mg of RNA, a yield of 32% based on a theoretical column loading of 1 micromole.

Sample Preparation for MALDI. All matrix compounds used in this work were purchased from Sigma Chemical Co. and were used without further purification. Matrix and co-matrix solutions were prepared as follows. 0.04 g of 6-aza-2-thiothymine (ATT matrix) was added to 500 μL of 50% ACN and mixed on a Vortex mixer for 30 s. Ammonium citrate co-matrix solution was prepared by dissolving 0.023 g of ammonium citrate in 1 mL of water. Oligonucleotide concentrations for most of the samples were ~10 pmol/μL. For each spectrum, 0.5 μL of the sample solution was spotted onto a gold plated sample well and allowed to air-dry. A 0.5 μL aliquot of co-matrix solution was added to the dry sample and allowed to air-dry. A 0.5 μL aliquot of matrix solution was then added to the sample/co-matrix mixture in the sample well. After the sample had again dried, the MALDI probe was inserted into the mass spectrometer.

MALDI Mass Spectrometry. MALDI spectra were obtained on a reflectron MALDI/TOF mass spectrometer (Model: Perseptive Voyager-DE STR, PE Applied Biosystems Co., Foster City, CA) equipped with a delayed extraction ion source, and a pulsed linear detector. A nitrogen laser at 337 nm (3 ns pulse width) was used to desorb the ions in the source region. The time-of-flight data were either externally calibrated or mass converted using ion peaks of known masses. Background pressure within the instrument was less than 1 × 10^{-7} Torr as measured by a Bayard-Alpert ion gauge located below the source.

Combined Liquid Chromatography/Mass Spectrometry (LC/MS) Analysis of the Nucleoside Content of Oligonucleotide 1. The fully modified oligonucleotide **1** was digested to nucleosides using nuclease P1, phosphodiesterase I, and alkaline phosphatase.[30] The resulting nucleoside mixture was analyzed by LC/MS using a HP 1090 liquid chromatograph interfaced to a Fisons Quattro II triple quadrupole mass spectrometer (Manchester, U.K.) using electrospray ionization. As shown in Figure S2, the HPLC elution monitored at 260 nm indicates that the RNA oligonucleotide contains the 4 major nucleosides as well as ψ (3.78 min), mnm^5s^2U (13.89 min), and t^6A (19.89 min). The seven nucleosides in this sample elute with the correct retention times and their identities were verified by mass spectrometry as they eluted.[31]

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Supporting Information Available: MALDI-MS spectrum, LC/MS nucleoside analysis, and 2D NOESYs for oligonucleotide **1**. ^{1}H NMR spectra for **5a**, **5b**, **10a**, and **11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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