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Synthesis of the tRNA^{Lys,3} Anticodon Stem-Loop Domain Containing the Hypermodified ms^{2}t^{6}A Nucleoside

Ashok C. Bajji† and Darrell R. Davis*

Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah 84112

davis@adenosine.pharm.utah.edu

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The synthesis of a protected form of the hypermodified nucleoside, *N*-[(9-*β*-D-ribofuranosyl-2-methylthiopurin-6-yl)carbamoyl]threonine, (ms^{2}t^{6}A) is reported. The hypermodified nucleoside was subsequently elaborated to the protected nucleoside phosphophoramidite using a protecting group strategy compatible with standard RNA oligonucleotide chemistry. The phosphoramidite reagent was then used to synthesize the 17-nucleotide RNA hairpin having the sequence of the anticodon stem-loop (ASL) domain of human tRNA^{Lys,3}, the primer for HIV-1 reverse transcriptase. Introduction of the modification at position 37 of the tRNA ASL modestly decreases the thermodynamic stability of the RNA hairpin as has been seen previously for the prokaryotic t^{6}A nucleoside lacking the 2-methylthio substituent. 2D NOESY NMR spectra of the ms^{2}t^{6}A containing tRNA ASL indicate that the threonyl side chain adopts a conformation similar to that seen in the solution structure of the analogous t^{6}A containing *E. coli* tRNA^{Lys}, despite the presence of the bulky methylthio group. This synthetic approach allows for site-specific incorporation of the hypermodified nucleoside and should facilitate future studies directed at understanding the roles of nucleoside modification in modulating the stability and specificity of biologically important RNA−RNA interactions. Our synthesis of the ms^{2}t^{6}A containing RNAs demonstrates that this methodology is suitable for obtaining quantities of RNA required for structural studies of the HIV primer tRNA.

Introduction

tRNA^{Lys,3} is the specific RNA primer for HIV-1 reverse transcription.[1] The transcription initiation complex involves two specific RNA−RNA interactions between the human tRNA and the 5′ region of the HIV-1 genomic RNA. The first is a Watson−Crick RNA duplex involving the 3′ 18 nucleotides of the tRNA and the complementary primer-binding site (PBS) of the HIV genome, while the second is a less well-understood interaction between a conserved "A-rich loop" in the HIV genome and the anticodon domain of tRNA^{Lys,3}.[2,3] Genetic studies have shown that the ability to form an anticodon/A-loop interaction is necessary for stable tRNA primer maintenance, and with the appropriate complementarity to the anticodon several different tRNAs can be made to serve as primers.[4] The importance of the tRNA anticodon/A-loop interaction appears to be that this is a recognition event that helps validate that the reverse transcription complex is "correct", allowing DNA synthesis to then proceed from this "initiation" complex to the "elongation" complex.[5]

Interestingly, the integrity of the anticodon/A-loop interaction is strongly dependent on one or more hypermodified nucleosides present in the anticodon stem-loop (ASL) domain of tRNA^{Lys,3}. Chemical probing has established the presence of a high-affinity interaction in vitro, and the extensive investigations by Ehresmann and coworkers of the anticodon/A-loop interaction have shown that while an unmodified tRNA transcript interacts with the 18 nucleotide PBS, no interaction is detected between the unmodified anticodon nucleotides and the A-loop.[6] Despite the relatively complex nucleoside structures, chemical synthesis is the method of choice for site-specifically introducing hypermodified nucleosides into RNA oligonucleotides due to the lack of an appropriate biosynthetic system. The ability to synthesize the tRNA ASL domain containing single modified nucleosides, or any combination of the natural modified nucleosides, is a powerful tool for studying the structural effects of these nucleosides on the tRNA anticodon.[7,8]

The t^{6}A family of nucleosides consists of related modified adenosines at position 37 found adjacent to the anticodon of tRNAs with U36 as the last codon nucleotide

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* To whom correspondence should be addressed. Phone: (801) 581-7006. Fax: (801) 581-7087.

† Current address: Myriad Genetics, Salt Lake City, UT.

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(Figure 1).[9] The adenosine base is derivatized by either threonine or glycine connected to N6 via a carbamoyl linkage. The nucleosides can then be further modified by N6 methylation,[10] and by methylthiolation at position 2 of the base,[11] but remarkably little is known about their structural and functional role.[12] The highly functionalized threonine group been proposed to be a specific coordination site for metal ions,[13] but there is some dispute about this effect.[14] Recent measurements of the thermodynamic effect of t^{6}A modification have shown that t^{6}A has a modest destabilizing effect on the ASL T$_m$,[15,16] but no dramatic structural or stability effects were seen upon titration with magnesium.[17] In the context of the tRNA^{Lys} ASL, we have shown that t^{6}A37 helps to promote a canonical U-turn structure,[16] which could be important in lowering the entropic cost of the tRNA binding either a cognate codon during translation[18] or the HIV A-loop. In a series of classic studies by Grosjean and co-workers, the kinetic and thermodynamic effects of a wide variety of tRNA modifications were investigated using temperature jump methods.[19] They found that t^{6}A37 has a stabilizing effect on tRNA−tRNA complexes in the range of 1−4-fold[20] and that the 2-methylthio is likely to have a somewhat stronger effect, but still in the neighborhood of 4-fold.[21] Unfortunately, since they were limited to natural tRNAs for these studies, they were restricted in some cases to using partially complementary tRNAs, and the combinations available resulted in an inability to separate the methylthio and threonyl effects within the same sequence context.

A recent X-ray crystal structure of tRNA^{Lys,3} at 3.3 Å resolution established the overall fold of the tRNA; however, the resolution was insufficient to accurately position the threonyl side chain of ms^{2}t^{6}A relative to the base.[22] The deposited coordinates (PDB ID:1FIR) suggest the side chain adopts a position different from that seen in the high-resolution X-ray structure of t^{6}A nucleoside[23] and also different from what we had determined for the *E. coli* tRNA^{Lys} ASL containing t^{6}A37 lacking the methylthio group.[16] In the current study, we have now synthesized the protected ms^{2}t^{6}A nucleoside and subsequently elaborated this nucleoside to the phosphoramidite. This phosphoramidite reagent was used in a modified RNA synthesis protocol with very mild deprotection conditions to synthesize the tRNA^{Lys,3} ASL RNA oligonucleotide. NMR spectra of the ms^{2}t^{6}A37 containing 17 nucleotide tRNA^{Lys,3} ASL indicate a nucleoside conformation similar to that of the free t^{6}A nucleoside and similar to that determined for t^{6}A within native *E. coli* tRNA^{Lys}.

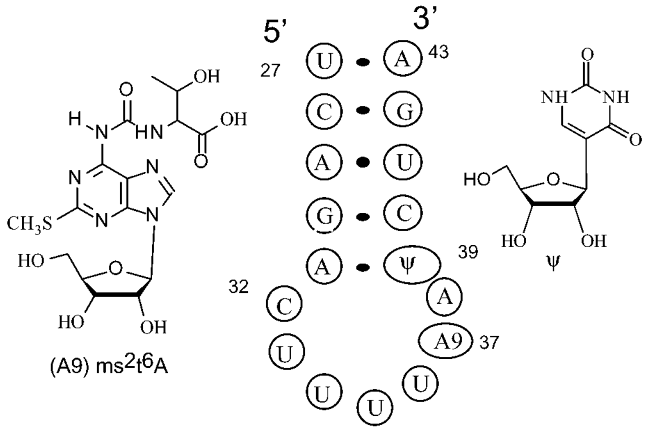

FIGURE 1. Secondary structure of the 17-nucleotide anticodon stem-loop domain of human tRNA^{Lys,3}. The structures of the modified nucleosides at tRNA positions 37 and 39 are shown. Native tRNA^{Lys,3} also has a hypermodified mcm^{5}s^{2}U nucleoside at position 34.

Results and Discussion

The present study describes the efficient synthesis of N-[[9-(*β*-D-ribofuranosyl)-2-methylthiopurine-6-yl]carbamoyl]-L-threonine nucleoside phosphoramidite (Scheme 1) and its incorporation into the anticodon stem loop domain of tRNA^{Lys,3} (Figure 1). To the best of our knowledge, the synthesis of the ms^{2}t^{6}A nucleoside has not been previously reported. However, numerous studies have been done on the simpler t^{6}A nucleoside, which lacks the methylthio group at the 2-position. A standard literature protocol for synthesizing the t^6 nucleoside that we have used previously involves displacing the ethoxy group of ethyl [9-(2,3,5-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine-6-yl]carbamate with L-threonine.[8,24] A recent report from Herdewijn and co-workers[25] describes an alternate approach to the nucleoside where they reinvestigate an early route to the nucleoside that involved reacting adenosine triacetate with the protected isocyanate of L-threonine.[26] Recognizing that an advantage of the isocyanate strategy for making protected phosphoramidites was its convergence, Herdewijn and co-workers addressed the poor coupling with threonine isocyanate by inverting the reactant functionalities. They generated the protected adenosine N6-isocyanate in situ and then

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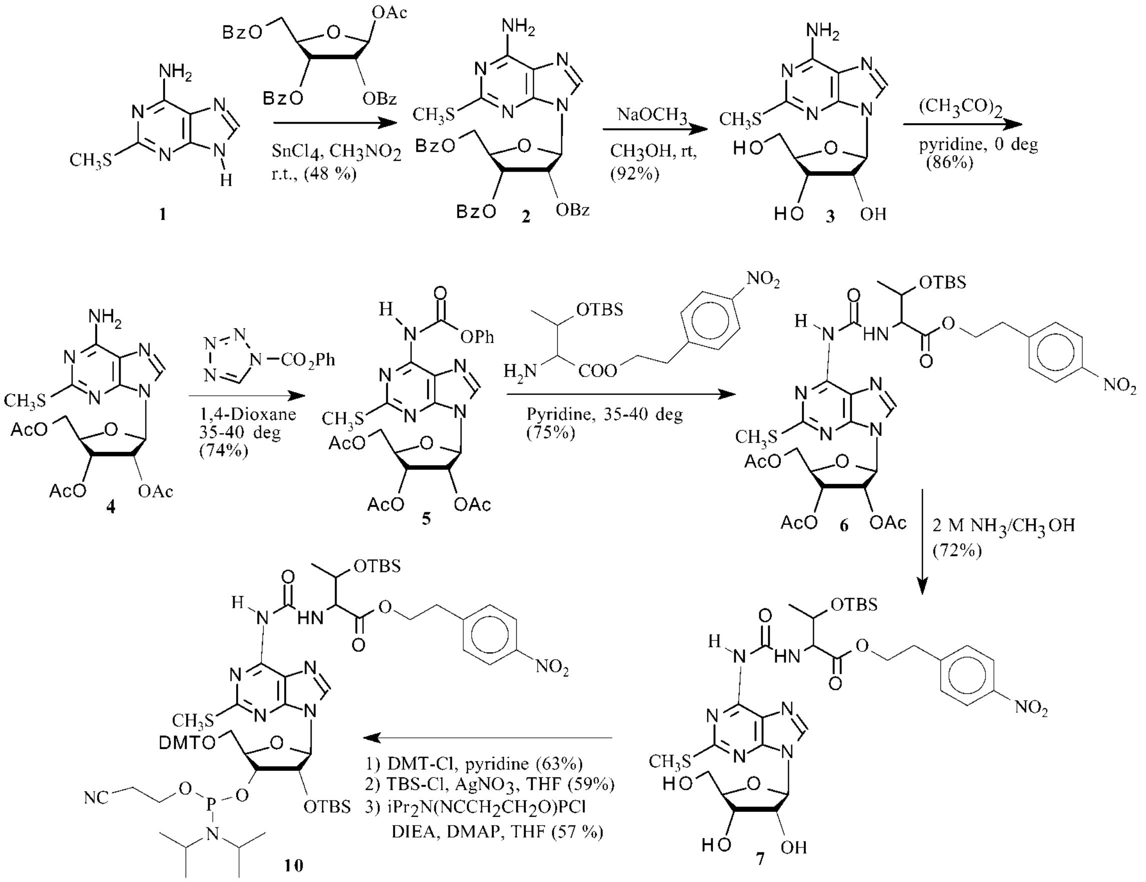
SCHEME 1. ms^{2}t^{6}A Phosphoramidite Synthesis



subsequently coupled with the L-threonine derivative to synthesize protected t^{6}A with a combined 19% yield for the activation and coupling steps. Although the coupling yield was still modest, by using threonine with the protecting groups desired for the final product, the overall yield to the protected phosphoramidite was approximately 5% starting from adenosine. We noticed that this approach was somewhat reminiscent of work by Adamiak and co-workers where they made a heptamer oligonucleotide corresponding to the t^{6}A containing anticodon loop of an eukaryotic initiator tRNA using the phosphotriester approach.[27,28] The strategy we envisioned was to use the high-yielding coupling step of Adamiak, but in the context of Herdewijn's relatively labile threonine nitrophenylethyl carboxylic ester. Since simply generating the starting 2-methylthioadenosine required several steps, some improvement over the published methods was viewed as critical for making the milligram quantities of RNA required for structural studies.

ms^{2}t^{6}A Nucleoside Phosphoramidite Synthesis. 2-Methylthioadenine base **1** was synthesized in four steps starting from thiourea and malononitrile following literature procedures in 21% overall yield.[29,30] Compound **1** could also be made from commercially available 4,6-diamino-2-mercaptopyridine in three steps. The nucleoside base was then coupled with protected ribose under Lewis acid conditions without prior base silylation to yield the 2′,3′,5′-tri-*O*-benzoyl 2-methylthioadenosine nucleoside in 48% yield.[31] Unfortunately, the nucleoside tri-*O*-benzoate was found to be unsuitable since we could not get complete removal of the benzoyl groups after threonine coupling without simultaneous t^{6}A side-chain hydrolysis. Therefore, the benzoyl groups were removed from compound **2** with sodium methoxide in methanol and then the sugar hydroxyls reprotected as the acetates. The logical alternative would be to directly produce the triacetate by coupling the nucleobase with tetraacetyl ribose, but the yield for this reaction was poor in our hands. The [9-(2,3,5-tri-*O*-acetyl-*β*-D-ribofuranosyl)-2-methylthiopurin-6-yl]phenylcarbamate **5** was prepared in 74% yield by reacting **4** and phenoxycarbonyl tetrazole in 1,4-dioxane at 35−40 °C for 16 h.[28] The carbamate **5** was then combined with the 2-(4-nitrophenyl)ethyl-*O*-*tert*-butyldimethylsilyl-L-threonine ester[25] in pyridine at 35−40 °C to give fully protected ms^{2}t^{6}A **6** in 75% yield. The acetyl groups of **6** were removed by treating with 2 M ammonia in methanol at room temperature for 3 h to afford 72% yield of the threonine protected nucleoside **7**. By reacting the protected amino acid with the carbamate, we achieved an overall coupling yield of 55% for protected ms^{2}t^{6}A compared to 19% reported previously for t^{6}A.[25]

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The acetate deprotection is a critical step since the carbamoyl linkage is base sensitive, and extended reaction times result in significant degradation of the nucleoside. The 5′ hydroxy group of **7** was then protected using DMT-Cl in pyridine to give the 5′ dimethoxytrityl ether nucleoside in 63% yield. Treatment of the 5′ DMT derivative with TBS-Cl in THF in the presence of $AgNO_3$ and pyridine gave the 2′-TBS derivative in 59% yield and a small amount of the 3′-TBS isomer.[32] Reaction of the 2′-TBS-protected nucleoside with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in THF for 4 h in the presence of diisopropylethylamine with DMAP catalyst afforded the target phosphoramidite **8** in 57% yield.[33]

RNA Synthesis, Deprotection, and Purification. The modified tRNA^{Lys,3} anticodon domain in Figure 1 was synthesized using commercial PAC-protected phosphoramidites (Glen Research) and standard coupling chemistry with *t*-BuOOH oxidation.[34,35] The methylthio should be stable to normal I_2/water oxidation, but we have noticed little decrease in coupling yields when using *t*-BuOOH oxidation and therefore used 10% tBuOOH/acetonitrile for all oxidation steps to establish conditions that would also be compatible with 2-thiouridine nucleosides. Trityl assays indicated >97% coupling for all the phosphoramidites; this was verified by HPLC and MALDI/MS of the fully deprotected RNA. The deprotection was carried out by first treating the column-bound RNA with 10% DBU in THF for 1.5 h at 40 °C to remove the nitrophenylethyl group. After DBU/THF treatment, the supernatant was removed, and the support-bound RNA was reacted with a solution of 33% ethanolic methylamine for 2 h. This procedure is similar to that of Herdewijn and co-workers, and we also found that a two-step base treatment is necessary to deprotect the RNA while maintaining the sensitive side chain.[25] The RNA solution was then dried in vacuo and treated with $Et_3N\cdot 3HF$ to remove the TBS ethers. The fully deprotected RNA was then purified by HPLC,[36] and MALDI-MS was used to verify the identity of the purified oligonucleotide as previously described.[8]

Effect of ms^{2}t^{6}A Modification on Stem-Loop Stability. Previous studies from our laboratory and others have shown that t^{6}A37 slightly destablizes the tRNA^{Lys} ASL compared to A37.[15,16] Thermal melting studies of the RNA oligomer in Figure 1 showed that ms^{2}t^{6}A37 causes a somewhat greater decrease in the T_m; 5 °C compared to the 2 °C decrease seen for t^{6}A. The imino NMR spectrum shown in Figure 2 indicates that the effect of ms^{2}t^{6}A on the base of the anticodon stem is similar to that seen for t^{6}A. The N3-H imino resonance for ψ39 is completely melted out indicating that the A31-ψ39 base pair is destabilized, and the ψ N1-H is also broad compared to the spectrum of the tRNA ASL with A37 acquired under similar conditions.[37] This loss of hydrogen bonding probably occurs from stacking destabilization, although a detailed chemical shift analysis of the 2D NMR spectra will be necessary to fully characterize these structural changes.

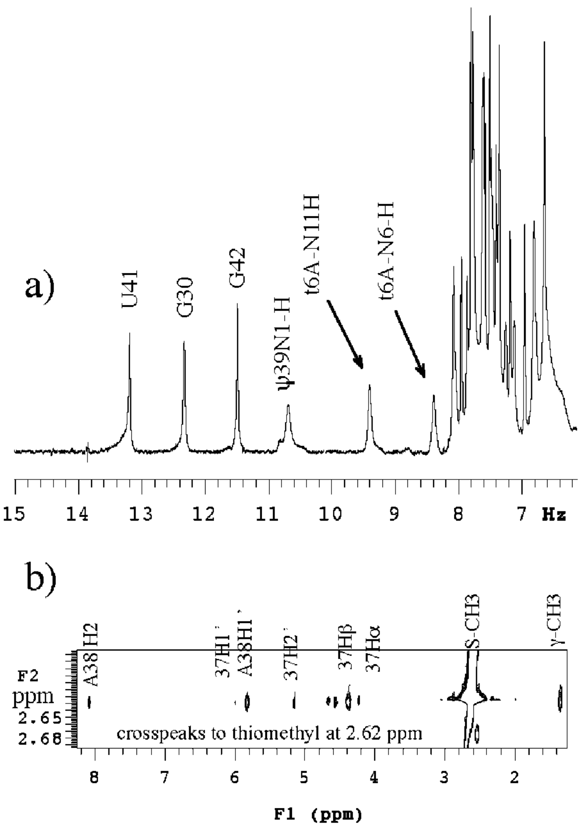

FIGURE 2. NMR spectra of the 17-mer RNA shown in Figure 1. The RNA concentration was 1 mM in 25 mM sodium phosphate buffer, pH 7.0 containing 100 mM NaCl and 10 mM $MgCl_2$: (a) downfield spectral region showing the hydrogen-bonded imino protons and side-chain amide resonances; (b) expanded region of the 2D NOESY spectrum showing the cross-peaks from the methylthio group diagonal peak at 2.62 ppm. These NOE connectivities indicate that the threonyl side chain is oriented trans relative to the imidazole ring of adenosine as shown in Figure 4.

CD spectra (Figure 3) were also collected in order gain insight into the structural environment of the hypermodified nucleoside. The CD spectrum has an overall appearance that is consistent with a largely A-form RNA structure, characterized by the dominant maximum at 260 nm. The additional, long wavelength band at 300 nm is due to the methylthio group. The temperature dependence of the CD spectrum indicates that although there is a significant base-stacking loss as the stem melts, the environment of the methylthio group changes little over this temperature range. While one should be cautious about drawing detailed structural conclusions from CD spectra, this behavior is consistent with our preliminary 2D NMR experiments. The 300 nm ellipticity is a potential reporter of stacking interactions involving ms^{2}t^{6}A as the signal could change significantly when the tRNA ASL forms a complex with a complementary RNA.

Conformation of the Threonyl Side Chain in ms^{2}t^{6}A Containing tRNA^{Lys,3} ASL. High-resolution structures of t^{6}A nucleoside and NMR studies of t^{6}A containing RNAs have shown that the threonyl side chain adopts an orientation trans (distal) to the adenosine imidazole ring that would be stabilized by a potential

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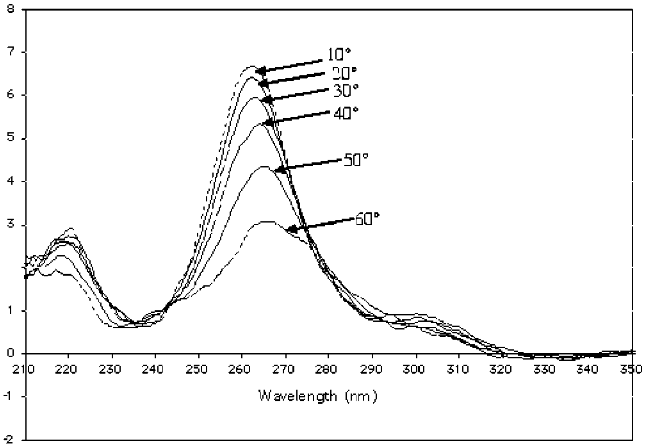

FIGURE 3. CD spectrum of a 5 μM solution of the modified $tRNA^{Lys}$ ASL. These spectra were collected from 10 to 60 °C in the NMR buffer absent magnesium. The spectrum shows a characteristic profile for a predominantly A-form RNA structure with an additional ellipticity maximum at 300 nm from the methylthio group.

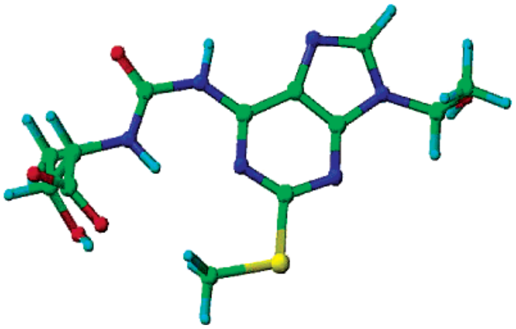

FIGURE 4. Geometry-optimized model of ms^2t^6A nucleoside.

hydrogen bond from NH-11 to N1.[16,23] Quantum chemical calculations on 2-methylthio t^6A have indicated that although the bulky methylthio could sterically interfere with the trans orientation as seen for 2-methylthio N^6-isopentenyladeonsine, in ms^2t^6A the methylthio orients away from N1 as shown in Figure 4. This allows for a hydrogen bond stabilized threonine conformation just as seen for t^6A.[38] In the recent X-ray structure of $tRNA^{Lys,3}$, the resolution was insufficient to accurately position the modified nucleoside side chains, but the carbamoyl threonine conformation was modeled into the structure with an extended conformation lacking an N11-H to N1 hydrogen bond.[22] The 2D NOESY NMR spectrum in Figure 2b for our synthetic $tRNA^{Lys,3}$ ASL shows numerous NOEs from the easily assigned methylthio at 2.62 ppm to protons of the ms^2t^6A37 nucleoside and also shows NOEs to assigned protons on adjacent nucleotides. From this NMR data, it seems clear that the threonine side chain is oriented trans as seen for t^6A in the *E. coli* $tRNA^{Lys}$ [16] and also consistent with the low energy conformation calculated by Tewari.[38]

Our structural studies are still preliminary, but the thermodynamic and NMR data presented here indicate that the structural effect of the threonyl group in ms^2t^6A containing tRNAs will be similar to the simpler t^6A nucleoside in a tRNA with the same anticodon sequence. Within the context of a canonical tRNA structure, this would then orient the large, hydrophobic methylthio group over the 5′ codon nucleotide of a codon−anticodon complex. This type of stacking interaction would be analogous to the 3′ end effect that has been quantitated by Turner and co-workers[39] and explain the methylthio stabilization of complementary tRNAs.[21] Methylthio-mediated 3′-stacking stabilization is also a possible explanation for the importance of nucleoside modification in stabilizing the HIV A-loop interaction with the $tRNA^{Lys,3}$ anticodon.

Experimental Section

General Methods. Melting points are recorded by the open capillary method and are uncorrected. Standard nucleoside phosphoramidite procedures were used to synthesize the protected phosphoramidite of pseudouridine.[33,40] ^1H and ^{31}P NMR of phosphoramidites and precursors were acquired in deuterated solvents on either 200 or 400 MHz spectrometers. The NMR spectra of the modified 17-mer tRNA ASL were collected at 500 MHz. ^1H chemical shifts are reported relative to tetramethylsilane at 0.0 ppm and referenced to the residual proton signal of the deuterated solvents. ^{31}P chemical shifts are relative to external phosphoric acid at 0.0 ppm. Analytical thin-layer chromatography (TLC) was carried out on silica gel 60F-254 plates. Flash column chromatography was carried out with silica gel 60 (230−400 mesh). Chemical reagents were purchased from commercial suppliers and used without further purification. Instrumentation and sample preparation for MALDI MS and ESI LC/MS have been described previously.[8]

2′,3′,5′-Tri-*O*-benzoyl-2-methylthioadenosine(2). To a suspension of 2-methylthioadenine **1**[29,30] (10.0 g, 55.18 mmol) and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (33.40 g, 66.21 mmol) in anhydrous nitromethane (160 mL) was added $SnCl_4$ (10 mL) dropwise at 0 °C. The temperature of the reaction mixture was slowly raised to room temperature and stirring continued for 16 h at room temperature. At the end of this period, the solvent was evaporated, and the brown residue was triturated with a saturated solution of $NaHCO_3$ and the solid collected by filtration. The crude product was purified by flash chromatography using a mixture of dichloromethane and methanol (95:5) to give product **2** (16.58 g, 48%) as a pale yellow solid: mp 108−110 °C; ^1H NMR (DMSO-d_6, 200 MHz) δ (ppm) 2.56 (s, 3H), 4.72−4.99 (m, 3H), 6.25 (t, $J = 6.0$ Hz, 1H), 6.45−6.60 (m, 4H), 7.39−7.62 (m, 9H), 7.93−8.01 (m, 6H), 8.10 (s, 1H).

2-Methylthioadenosine (3). To a solution of **2** (16.0 g, 25.73 mmol) in methanol (150 mL) was added freshly prepared sodium methoxide (0.7 g of sodium in 5.0 mL of anhydrous methanol). The mixture was stirred at 25 °C for 4 h, neutralized with Dowex 50 (H^+, 200-mesh), and filtered. The solvent was evaporated completely, and the crude product was then triturated with 5% ethyl acetate in petroleum ether and filtered. The product was washed with diethyl ether and dried to afford **3** (7.4 g, 92%): mp 222−224 °C (lit. mp[41] 225−228.5 °C). This product was sufficiently pure to use for the next step and therefore carried forward without further purification: ^1H NMR (DMSO-d_6, 200 MHz) δ (ppm) 2.45 (s, 3H), 3.20−3.74 (m, 4H), 3.85 (b, 1H), 4.29 (bs, 1H), 5.30 (bs, 1H), 5.82 (d, $J = 5.8$ Hz, 1H), 7.59 (bs, 2H), 8.21 (s, 1H); FABMS (MH^+) calcd for $C_{11}H_{15}N_5O_4S$ 312.4, obsd 313.22.

2′,3′,5′-Tri-*O*-acetyl-2-methylthioadenosine (4). A mixture of 2-methylthioadenosine **3** (6.0 g, 19.15 mmol), anhydrous

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pyridine (50 mL), and acetic anhydride (30 mL) was stirred at 0 °C for 4 h. The excess reagent and pyridine were removed under reduced pressure and coevaporated with toluene (2 × 50 mL). The crude product was crystallized from a mixture of ethyl acetate and petroleum ether to afford **4** (7.2 g, 86%) as a colorless solid: mp 159−161 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ (ppm) 1.94 (s, 3H), 2.05 (s, 3H), 2.09 (s, 3H), 2.47 (s, 3H), 4.14−4.20 (m, 1H), 4.30−4.42 (m, 2H). 5.67 (t, J = 5.9 Hz, 1H), 6.04 (t, 5.9 Hz, 1H), 6.14 (d, J = 4.2 Hz, 1H), 7.45 (s, 2H), 8.17 (s, 1H); ^{13}C NMR (DMSO-d_6) δ (ppm) 13.69, 20.27, 20.33, 62.55, 69.69, 71.87, 78.72, 80.34, 117.00, 139.35, 149.55, 155.48, 164.93, 169.36, 169.46, 170.01; FABMS (MH$^+$) calcd for $C_{17}H_{21}N_5SO_7$ 439.44, obsd 440.0.

[9-(2′,3′,5′-Tri-*O*-acetyl-*β*-D-ribofuranosyl)-2-methylthiopurin-6-yl]phenylcarbamate (5). 2-Methylthioadenosine triacetate **4** (1.4 g, 3.18 mmol) was dissolved in 5 mL of 1,4-dioxane and phenoxycarbonyl tetrazole (1.82 g, 9.56 mmol)[28] added to the stirred solution. The solvent was removed in vacuo and then the suspension/evaporation step repeated twice before addition of 5 mL of 1,4-dioxane a final time. The thick slurry was heated at 35−40 °C for 16 h. At the end of this period, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography using a mixture of dichloromethane and ethyl acetate (8:2) to afford **5** (1.32 g, 74%). The ^{1}H NMR spectrum shows that the product is relatively pure; however, minor peaks are seen at 6.8 and 9.3 ppm indicating a persistent impurity that resisted multiple attempts to obtain absolutely clean material. Regardless, a sharp melting point was obtained: mp 120−122 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ (ppm) 1.94 (s, 3H), 1.97 (s, 3H), 2.06 (s, 3H), 2.75 (s, 3H), 4.10−4.29 (m, 1H), 4.30−4.45 (m, 2H), 5.68 (t, 1H, J = 5.4 Hz), 6.05 (t, 1H, J = 6.0), 6.24 (d, 1H, J = 4.3 Hz), 7.10−7.39 (m, 3H), 7.40−7.52 (m, 2H), 8.50 (s, 1H), 11.20 (s, 1H); MS (FAB) MH$^+$ calcd for $C_{24}H_{25}N_5O_9S$ 559.5, obsd 559.9.

***N*-[[9-(2′,3′,5′-Tri-*O*-acetyl-*β*-D-ribofuranosyl)-2-methylthiopurin-6-yl]carbamoyl]-*O*-*tert*-butyldimethylsilyl-L-threonine 2-(4-Nitrophenyl)ethyl Ester (6).** To a solution of **5** (3.07 g, 5.48 mmol) in pyridine (20 mL) was added *O*-(*tert*-butyldimethylsilyl)-L-threonine 2-(4-nitrophenyl)ethyl ester (4.19 g, 10.97 mmol). The protected amino acid was synthesized as described previously.[25] The reaction mixture was stirred at 35−40 °C for 12 h. At the end of this period, the solvent was evaporated to dryness, and the residue was coevaporated with toluene (2 × 15 mL). The pale yellow residue was purified by flash chromatography using dichloromethane and ethyl acetate (6:1) to afford **6** (3.5 g, 75%) as a pale yellow solid: mp 67−69 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm) −0.05 (s, 3H), 0.04 (s, 3H), 0.82 (s, 9H), 1.19 (d, 3H, J = 6.2 Hz), 1.96 (s, 3H), 2.05 (s, 3H), 2.59 (s, 3H), 3.25 (t, 2H, J = 6.4 Hz), 4.18−4.42 (m, 7H), 5.63 (t, 1H, J = 5.4 Hz), 6.08−6.13 (m, 1H), 6.25 (d, 1H, J = 5.5 Hz), 7.56 (d, 2H, J = 7.5 Hz), 8.09 (d, 2H, J = 7.5 Hz), 8.51 (s, 1H), 9.82 (d, 1H, J = 6.5 Hz), 10.02 (s, 1H); HRMS (FAB) MH$^+$ calcd for $C_{36}H_{50}N_7O_{13}$-SiS 848.2878, obsd 848.2910.

***N*-[[9-(*β*-D-Ribofuranosyl)-2-methylthiopurin-6-yl]carbamoyl]-*O*-*tert*-butyldimethylsilyl-L-threonine 2-(4-Nitrophenyl)ethyl Ester (7).** Compound **6** (3.5 g, 4.12 mmol) was dissolved in 2 M ammonia in methanol (25 mL) and stirred at room temperature for 2 h. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography using a mixture of dichloromethane and methanol (9:1) to afford **7** (2.15 g, 72%) as an off-white solid: mp 78−79 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm) −0.06 (s, 3H), 0.03 (s, 3H), 0.82 (s, 9H), 1.19 (d, 3H, J = 6.2 Hz), 2.56 (s, 3H), 3.10 (t, 2H, J = 6.4 Hz), 3.45−3.48 (m, 1H), 3.60−3.72 (m,1H), 3.86−3.89 (m, 1H), 4.18−4.44 (m, 5H), 4.55−4.62 (m, 1H), 5.00 (t, 1H, J = 4.7 Hz), 5.21 (d, 1H, J = 4.3 Hz), 5.48 (d, 1H, J = 5.2 Hz), 5.92 (d, 1H, J = 5.2 Hz), 7.59 (d, 2H, J = 7.6 Hz), 8.15 (d, 2H, J = 7.6 Hz), 8.61 (s, 1H), 9.30 (d, 1H, J = 6.6 Hz), 10.01 (s, 1H); HRMS (FAB) MH$^+$ calcd for $C_{30}H_{44}N_7O_{10}SiS$ 722.2639, obsd 722.2667.

***N*-[[9-(5′-*O*-(4,4′-Dimethoxytrityl)-*β*-D-ribofuranosyl)-2-methylthiopurin-6-yl]carbamoyl]-*O*-*tert*-butyldimethylsilyl-L-threonine 2-(4-Nitrophenyl)ethyl Ester (8).** A mixture of **7** (1.34 g, 1.85 mmol) and 4,4′-dimethoxytrityl chloride (0.81 g, 2.41 mmol) in anhydrous pyridine (10 mL) was stirred at 25 °C under nitrogen atmosphere for 16 h. At the end of this period, the solvent was removed under reduced pressure, and the residue was coevaporated with toluene (2 × 20 mL). The product was purified by flash chromatography using a mixture of dichloromethane and methanol (9:1) to give the 5′-dimethoxytrityl compound (1.2 g, 63%) as a yellow solid: mp 104−106 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm) −0.6 (s, 3H), 0.03 (s, 3H), 0.08 (s, 9H), 1.21 (d, 3H, J = 6.0 Hz), 2.42 (s, 3H), 3.08 (t, 3H, J = 6.1 Hz), 3.12−3.28 (m, 2H), 3.71 (s, 6H), 4.02−4.10 (m, 1H), 4.21−4.48 (m, 5H), 4.74 (t, 1H, J = 4.7 Hz), 5.23 (d, 1H, J = 5.0 Hz), 5.58 (d, 1H, J = 5.2 Hz), 5.96 (d, 1H, J = 5.1 Hz), 6.72−6.82 (m, 4H), 7.15−7.35 (m, 9H), 7.50 (d, 2H, J = 7.6 Hz), 8.01 (d, 1H, J = 7.6 Hz), 8.42 (s, 1H) 9. 21 (d, 1H, J = 6.8 Hz), 9.99 (s, 1H); MS (FAB) MH$^+$ calcd for $C_{51}H_{61}N_7O_{12}SiS$ 1024.2, obsd 1024.1.

***N*-[[9-(2′-*O*-*tert*-Butyldimethylsilyl-5′-*O*-(4,4′-dimethoxytrityl)-*β*-D-ribofuranosyl)-2-methylthiopurin-6-yl]carbamoyl]-*O*-*tert*-butyldimethylsilyl-L-threonine 2-(4-Nitrophenylethyl) Ester (9).** To a solution of the 5′-dimethoxytrityl compound **8** (0.4 g, 0.39 mmol) in dry THF (5 mL) was added $AgNO_3$ (0.079 g, 0.47 mmol), and the reaction mixture was stirred at room temperature for 30 min. To this reaction mixture were added TBS-Cl (0.076 g, 0.50 mmol) and pyridine (50 μL), and stirring was continued at room temperature for 12 h. At the end of this period, the mixture was filtered through Celite into a saturated solution of $NaHCO_3$ (50 mL) and extracted with dichloromethane (2 × 30 mL). The organic layer was washed with water (50 mL), dried (Na_2SO_4), and filtered, and the solvent was removed under reduced pressure to give predominantly the 2′-TBS isomer and less than 10% of the 3′-TBS isomer. The crude mixture was purified by flash chromatography over silica gel using a mixture of dichloromethane and ethyl acetate (6:1) to afford the 2′-TBS isomer (0.26 g, 59%) as a pale yellow solid: mp 96−97 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm) −0.08 (s, 3H), −0.06 (s, 3H), 0.0 (s, 3H), 0.02 (s, 3H), 0.75 (s, 3H), 0.08 (s, 9H), 1.18 (d, 3H, J = 6.5 Hz), 2.37 (s, 3H), 3.02 (t, 2H, J = 6.3 Hz), 3.18−3.25 (m, 1H), 3.68 (s, 3H), 3.71 (s, 3H), 4.08−4.12 (m, 1H), 4.25−4. 40 (m, 4H), 4.42−4.48 (m, 1H), 4.88 (t, 1H, J = 5.1 Hz), 5.18 (d, 1H, J = 5.5 Hz), 6.0 (d, 1H, J = 5.6 Hz), 6.28−6.70 (m, 4H), 7.15−7.28 (m, 7H), 7.36 (d, 2H, J = 7.3 Hz), 7.49 (d, 2H, J = 7.4 Hz), 7.99 (d, 2H, J = 7.4 Hz), 8.42 (s, 1H), 9.22 (d, 1H, J = 6.7 Hz), 9.99 (s, 1H); MS (FAB) MH$^+$ calcd $C_{57}H_{75}N_7O_{12}Si_2S$ 1138.4, obsd 1138.1.

***N*-[[9-(2′-*O*-*tert*-Butyldimethylsilyl-3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-5′-*O*-(4,4′-dimethoxytrityl)-*β*-D-ribofuranosyl)-2-methylthiopurin-6-yl]carbamoyl]-*O*-*tert*-butyldimethylsilyl-L-threonine, 2-(4-Nitrophenylethyl) Ester (10).** The above 5′-DMT, 2′-TBS derivative (0.24 g, 0.21 mmol) and *N*,*N*-dimethylaminopyridine (5.1 mg, 0.042 mmol) were dissolved in dry THF under argon atmosphere. To the reaction mixture were added sequentially diisopropylethylamine (110 μL, 0.63 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (94 μL, 0.42 mmol) with stirring at 25 °C. After 30 min, a white solid was formed, and the stirring was continued for an additional 3 h. The reaction was followed by taking 50 μL of the aliquot of the reaction mixture, removing the solvent, oxidizing for 5 min with *t*-BuOOH/toluene, evaporating the toluene, and analysis by silica gel TLC. The oxidized amidite product is found at the origin, while unreacted starting material is not affected by the oxidation procedure. This protocol is helpful since the starting material and the products migrate with the identical R_f's in the solvent systems that we investigated. Upon completion, the reaction mixture was extracted with ethyl acetate (50 mL), washed with 5% sodium bicarbonate solution (25 mL), and finally with water (2 × 25 mL). The organic layer was dried (Na_2SO_4) and filtered, and the solvent evaporated under reduced pressure. The mixture was purified by flash chromatography using a mixture of dichloromethane and ethyl acetate (6:1) to yield the target nucleoside phosphoramidite as a mixture of diastereomers (0.16 g, 57%): mp 77−81 °C; ^{31}P (DMSO-d_6) δ (ppm) 149.14, 150.30. The ^1H and ^{31}P NMR spectra are included in the Supporting Information and indicate the product is homogeneous although the existence of two diastereomers significantly complicates the ^1H NMR spectrum.

RNA Oligonucleotide Synthesis. The oligonucleotide was synthesized using an Applied Biosystems 394 oligonucleotide synthesizer on a 1 μmol scale using 0.05 M acetonitrile solutions of PAC amidites (PAC A, isopropyl PAC G, and acetyl C) from Glen Research. The concentration of ms^{2}t^{6}A amidite was 0.12 M. The phosphoramidites were coupled for 25 min. The normal I_2/H_2O oxidation solution was replaced with 10% *t*-BuOOH in acetonitrile, and 2 × 5 min oxidation cycles were used for each coupling step.

RNA Deprotection and Purification. The CPG-bound RNA was transferred from the column to a screw-cap glass vial, to which was added 1 mL of 10% DBU in THF (v/v).[25] The solution was magnetically stirred at 45 °C for 2 h. The supernatant was decanted, the support was washed with an additional 1.0 mL of THF, and the support was dried on a Speed-Vac concentrator. The dried support was treated with a solution of 33% ethanolic methylamine (Fluka) for 2 h. The supernatant was decanted, the support was washed with an additional 1.0 mL of dimethylamine solution, and the combined washings were lyophilized on the Speed-Vac. The dried material was dissolved in 1 mL of neat $Et_3N\cdot 3HF$ and stirred at room temperature for 9−12 h.[42] 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. After centrifugation, the RNA pellet was dissolved in 0.5 mL of water and purified by anion-exchange HPLC using a Pharmacia Resource Q anion-exchange column (1 × 15 cm) and eluting with a linear gradient of lithium perchlorate at 50 °C.[36] The fractions containing full length material as judged by HPLC were collected, lyophilized, and then dialyzed against 3 × 1 L of deionized water. The dialyzed material was lyophilized on a Speed-Vac and then directly dissolved in the appropriate buffer for further characterization.

CD and UV Spectroscopy. Temperature-dependent CD and UV spectroscopy were conducted on RNA sample concentrations of 5 μM in either sodium cacodylate or sodium phosphate buffers, pH 7.4. The buffers contained 100 mM NaCl, 0.1 mM EDTA, and either 0, 5, or 10 mM $MgCl_2$. For the temperature-dependent UV studies, the temperature was raised from 10−90 °C in 1° steps with a 1 min equilibration period between measurements. CD spectra were acquired in a 1 cm water-jacketed CD cell. The spectra were acquired from 10 to 60 °C in 10° increments. The sample was allowed to equilibrate for 10 min between measurements.

Molecular Modeling. A molecular model of ms^{2}t^{6}A was built using Spartan 5.1.1 (Wavefunction, Inc.). The nucleoside was constructed with a truncated sugar according to the specification of Kollman and co-workers in order to ultimately derive RESP charges for the AMBER force field.[43] A preliminary low energy conformation was obtained using the AM1 force field in Spartan and then the geometry was optimized by ab initio Hartree−Fock calculations with the 6-31G+ basis set using Gaussian 98.[44]

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Supporting Information Available: RNA MALDI-MS, HPLC trace, and NMR spectra for compounds **2**, **4**−**10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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