

Design, Synthesis, and Analysis of Yeast tRNA^{Phe} Analogs Possessing Intra- and Interhelical Disulfide Cross-Links[†]

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Abstract: Disulfide cross-links have been site-specifically incorporated into unmodified yeast tRNA^{Phe} by total chemical synthesis. One cross-link is between positions 1 and 72 in the amino-acid acceptor stem, and it was prepared by replacing G1 and C72 with N³-(thioethyl)uridine. A second cross-link is in the central D-region of yeast tRNA^{Phe} between 11 and 25, and it was synthesized by replacing C11 and C25 with 2'-O-alkylthiol modified cytosine residues. Air oxidation to form the cross-link at both sites occurs in 12 h and is nearly quantitative. Analysis of the cross-linked products by native and denaturing PAGE along with Pb(II) cleavage experiments demonstrates that the cross-linked molecules are monomeric and suggests that the disulfide bridges do not significantly alter the structure of the modified tRNAs relative to the parent sequence. The finding that cross-link formation between thiol-derivatized residues correlates with the position of these groups in the crystal structure of native yeast tRNA^{Phe} and that the modifications apparently do not perturb native structure suggests that this methodology should be applicable to the study of RNA structure, dynamics, and folding.

Introduction

Although significant progress has been made in elucidating the folding pathways and structure of ribonucleic acids, our current understanding of these properties lags behind that for DNA.¹ Indeed, biophysical analysis of RNA can be quite difficult because oligoribonucleotides often self-associate and/or equilibrate between several different conformations, particularly at the concentrations required for many high-resolution biophysical measurements.² To help circumvent these problems, an array of chemical and biochemical approaches has been developed to explore RNA conformation and dynamics. For example, RNA structure and folding have been probed by footprinting with base- and phosphate-specific-modifying reagents, transition metal complexes, and a broad spectrum of nucleases.³ Complementary to these experiments has been the design, synthesis, and site-specific incorporation of residues possessing modifications to the base and/or sugar–phosphate backbone.⁴ These latter experiments have proved particularly useful for precisely assessing the role of various functional groups in both the structure and function of RNA.

In previous work we described a method to probe for the presence of helical termini on A-form RNA.^{4g} Specifically, incorporation of N³-thioethyluridine at both the 3' and 5' termini of duplex RNA, followed by air oxidation, produces intra-helical disulfide cross-links. RNAs possessing this cross-link are both

conformationally homogeneous and thermally more stable than their wild-type parent sequences. In this report we show that both inter- and intrahelical disulfide cross-links can be site-specifically placed into unmodified yeast tRNA^{Phe}, a relatively large oligoribonucleotide, by total chemical synthesis.⁵ The ability to form these cross-links correlates with predictions based on the crystal structure of native yeast tRNA^{Phe}, and the cross-links do not appear to significantly alter the structure of the thiol/disulfide-modified tRNAs relative to the parent sequence. Collectively, these results demonstrate that the mild and selective chemistry of disulfide formation is compatible with RNA of larger and more complex structure.

Results

Design. Yeast tRNA^{Phe} was selected for these studies because a high-resolution X-ray crystal structure of the native oligomer is available⁶ and solution measurements indicate that the conformation of fully unmodified yeast tRNA^{Phe} is similar to that of the native molecule.⁷ Sites for modification and design of the actual cross-links were guided by the following criteria.

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First, all of the alkylthiol modifications were designed so as not to interfere with noncovalent interactions that stabilize the folded tertiary structure, such as hydrogen bonding and Mg^{2+} binding. Second, all of the alkylthiol linkers are on the periphery of the folded tRNA molecule where they should be accessible to the various reagents required for oxidation, reduction, and alkylation of the sulfhydryl groups. Third, the thiols that comprise each cross-link are within 10 Å of each other so that cross-link formation should proceed relatively rapidly, and the (entropic) stability conferred by the cross-link will be maximized.

Two initial sites were chosen based on these criteria. The first site (**I**) is between G1 and C72 in the amino-acid acceptor stem (Figure 1A,B). This site is distal from any tertiary interactions in the folded structure, and an intrahelical cross-link bridging these two bases should not grossly perturb native geometry. A cross-link between these residues can be incorporated by replacing G1 and C72 with N^3 -(thioethyl)uridines (**U^S**).⁴⁸ Although replacing both G1 and C72 with **U^S** can result in a loss of Watson–Crick hydrogen bonding, due to end-fraying effects at the termini of duplexes, base-pairing is reduced and we have found that terminal cross-links are not energetically unfavorable.^{48,9} Furthermore, cross-links positioned at helical termini do not appear to perturb the conformation of adjacent base pairs.⁹

The second site for modification (**II**) is in the central D-region of yeast tRNA^{Phe} between C11 and C25 (Figure 1C,D). The X-ray structure of native yeast tRNA^{Phe} indicates that the 2'-hydroxyl groups on these residues converge to ~9 Å and do

not interact with other sites on the folded tRNA. Modeling studies suggest that replacing C11 and C25 with 2'-O-(thioethyl)cytosine (**C^S**) will afford a cross-link between these two hydroxyl groups that neither disrupts base-pairing interactions involving C11 and C25 nor introduces significant structural perturbations in the parent structure. In addition, we chose site **II** because it is not precisely known where along the folding pathway of yeast tRNA^{Phe} the D-region assembles, and by measuring the rate at which **II_{tbu}** and **II_{xl}** fold as a function of temperature, it should be possible to elucidate at what point this region of tertiary structure forms.^{1h,10}

Monomer Synthesis. Synthesis of the fully protected N^3 -(thioethyl)uridine phosphoramidite was conducted as previously described.⁴⁸ Briefly, the N^3 -position of 2',3',5'-tris-O-(trimethylsilyl)uridine was deprotonated with NaH and alkylated with O-tosyl-S-benzoyl mercaptoethanol.¹¹ Removal of the silyl groups with aqueous HF afforded **1** in 63% yield from uridine (Scheme 1). Preparation of the modified nucleoside for solid-phase synthesis was accomplished by protection of the 5'-hydroxyl group as a 4,4'-dimethoxytrityl ether, exchange of the thiobenzoyl to the *tert*-butyl mixed disulfide,¹² and silylation of the 2'-hydroxyl group. To incorporate **U^S** at the 5'-terminus of site **I**, the 3'-hydroxyl was activated as a β -cyanoethyl *N,N*-diisopropylphosphoramidite using conditions that suppress silyl group migration.¹³ For incorporation of **U^S** at the 3'-terminus of site **I**, intermediate **4** was coupled to controlled pore glass (CPG, 1000 Å) through a succinate linker. The loading concentration of the modified monomer, as ascertained by the trityl cation release assay, was typically 32 μ mol/g.¹⁴

Synthesis of fully protected **C^S** proceeded from 3',5'-O-(tetraisopropylidisiloxane-1,3-diyl)- N^4 -benzoyl-2'-O-allylcytidine, which was prepared as described previously by Sproat and co-workers.¹⁵ The allyl group was dihydroxylated using OsO₄,¹⁶ oxidatively cleaved with NaIO₄, and reduced with NaBH₄ to afford the saturated alcohol **10** in good yield (Scheme 2). Mesylation of the primary alcohol followed by displacement with thiobenzoic acid afforded the thiobenzoyl intermediate **12**, which upon removal of the silyl protecting group with aqueous HF was exchanged to the *tert*-butyl mixed disulfide. Protection of the 5'-hydroxyl and activation of the 3'-hydroxyl following standard procedures¹⁷ provided the desired **C^S** monomer for use in solid-phase synthesis.

RNA Synthesis and Cross-Link Formation. Solid-phase synthesis of **I_{tbu}** and **II_{tbu}** was conducted using standard β -cyanoethyl *N,N*-diisopropylphosphoramidite chemistry as previously described (the coupling efficiency of **U^S** and **C^S** is $\geq 98.5\%$).¹⁸ All tRNA syntheses were conducted on a 1 μ mol scale, and each synthesis afforded between 80 and 130 OD₂₆₀ units of crude material, from which ~10% of pure RNA could be isolated by denaturing polyacrylamide gel electrophoresis

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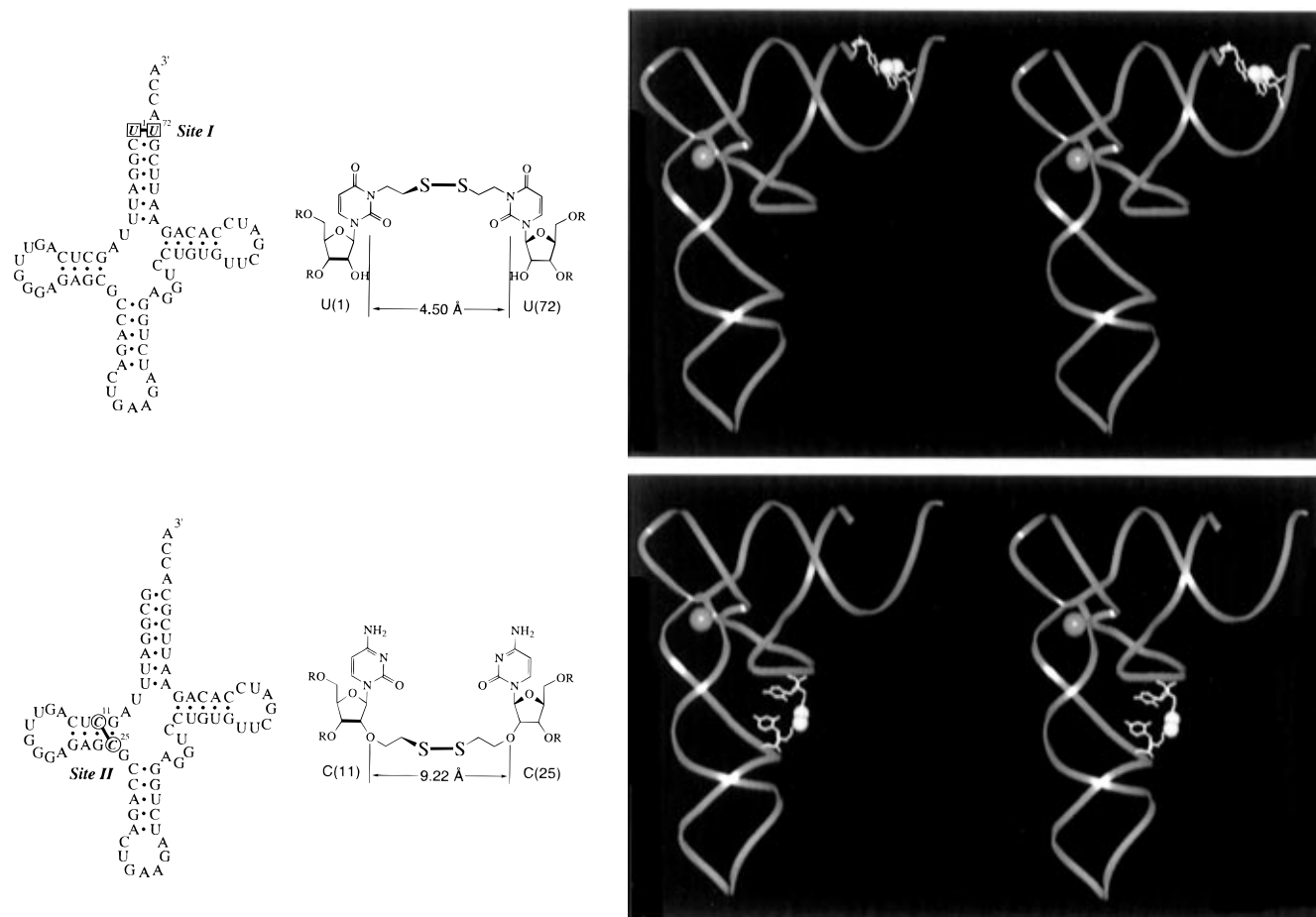


Figure 1. Location and chemical composition of cross-links. (A, top left) Primary sequence and secondary structure of unmodified yeast tRNA^{Phe} showing position and chemical structure of site I. Note that, for site I, a truncated version of the tRNA lacking the last four bases on the 3' terminus was synthesized. (B, top right) Model of yeast tRNA^{Phe} crystal structure showing the site I cross-link (stereoview). The purple sphere is a lead atom in its binding site, and the yellow spheres are the sulfur atoms that comprise the cross-link. The cross-link was constructed using the Biosym INSIGHT/DISCOVER software. Molecular mechanics and dynamics calculations show that the alkylthiol linker is fully staggered and the C-S-S-C dihedral angle is ~90°. (C, bottom left) Primary sequence and secondary structure of unmodified yeast tRNA^{Phe} showing position and chemical structure of the site II cross-link. (D, bottom right) Model of yeast tRNA^{Phe} showing the site II cross-link (stereoview). The coloring scheme is the same as in part B. The fully base-, sugar-, and phosphate-deprotected sequences still possessing the thiol protecting groups are designated **I_{tBu}** and **II_{tBu}** for sites I and II, respectively. The corresponding cross-linked molecules are designated **I_{XL}** and **II_{XL}**.

(PAGE). Prior to cross-link formation, the *tert*-butyl protecting groups on the thiols must be removed. However, we could not monitor the progress of thiol deprotection because the reduced and *tert*-butyl protected tRNAs cannot be resolved by PAGE, ion-exchange, or reversed-phase HPLC, and the initial protected tRNAs and reduced products have similar spectral properties. By contrast, reduced and *tert*-butyl protected alkylthiol-modified DNAs can be separated by HPLC.¹⁹ Therefore, the tDNA sequences corresponding to **I_{tBu}** and **II_{tBu}** were synthesized and removal of the thiol protecting groups was followed by reversed-phase HPLC to select conditions for the quantitative removal of the thiol protecting groups. On the basis of the results from these model studies, purified samples of **I_{tBu}** and **II_{tBu}** were reduced by treatment with dithiothreitol (DTT; 200 equiv/disulfide in 100 mM sodium phosphate, pH 8.3) for 12 h. Following dialysis to remove excess DTT, a near quantitative recovery of the reduced tRNAs was obtained, and these materials were used immediately to form the desired cross-links.

Disulfide bond formation was initiated by heat denaturing and slowly cooling the reduced oligomers in the presence of Mg²⁺ to ensure proper folding.²⁰ The pH was then adjusted to 8.0, and the tRNA solutions were stirred exposed to air to effect

cross-link formation. To follow the progress of disulfide bond formation, aliquots of each reaction mixture were treated with 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM), which produces a fluorescent adduct with thiols (~5 pmol of thiol can be detected).²¹ After 12 h, the fluorescence signal was at background and the cross-linked tRNAs were isolated by ethanol precipitation.²² Denaturing PAGE analysis of the crude reaction mixtures shows a single major band (~95%) with only minor amounts of higher molecular weight products, demonstrating that disulfide bond formation is near quantitative (Figure 2). Intramolecular cross-links do not form if the tRNAs are not folded (i.e., in the absence of Mg²⁺), which suggests that the thiol linkers are in close proximity in the folded structure, and that the alkylthiol modifications do not provide a kinetic or thermodynamic impediment to tertiary folding.

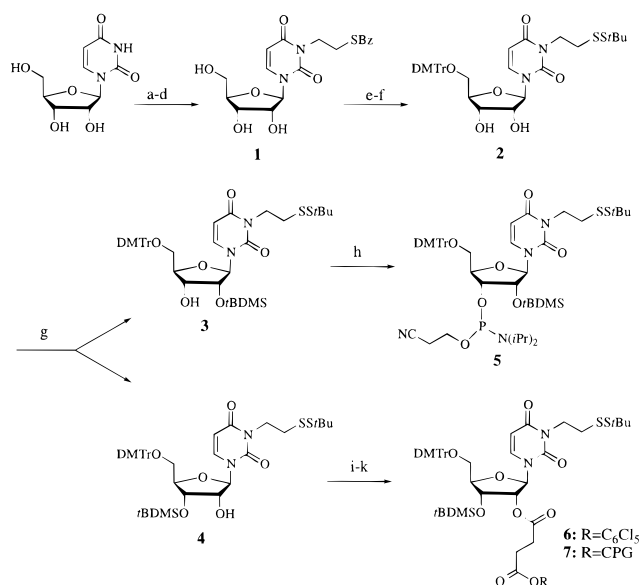
Both **I_{XL}** and **II_{XL}** migrate more slowly than either unmodified yeast tRNA^{Phe} or their corresponding *tert*-butyl disulfide

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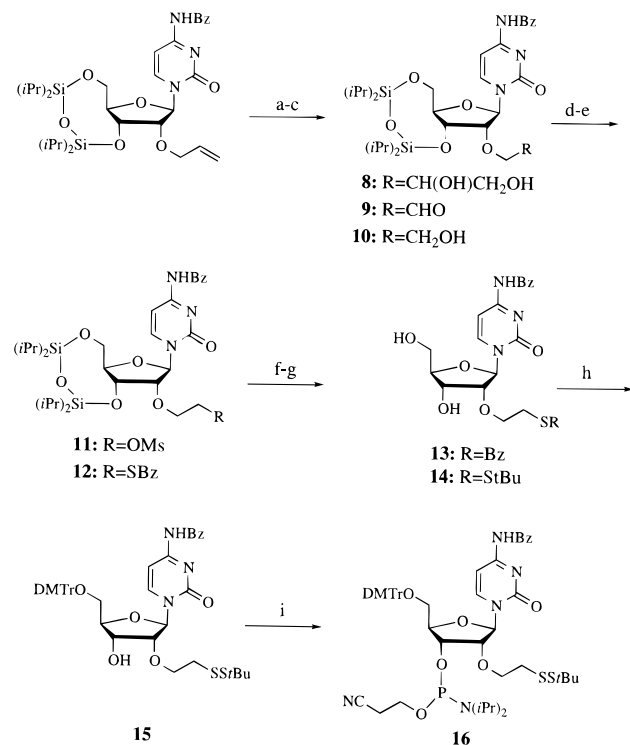
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Scheme 1^a

^a (a) TMSCl, Et₃N, DMF; (b) NaH, DMF; (c) *p*TsOCH₂CH₂SBz, DMF; (d) HF (aqueous) (63% from a); (e) DMTrCl, pyridine; (f) 1-(*tert*-butylthio)-1,2-hydrazinedicarboxymorpholine, LiOH, MeOH (70% from e); (g) TBDMSCl, imidazole, DMF (65%; **3:4**, 3:2); (h) 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite, 2,4,6-collidine, *N*-methylimidazole, THF (82%); (i) succinic anhydride, DMAP, pyridine; (j) pentachlorophenol, DCC, DMAP, CH₂Cl₂ (90% from i); (k) CPG (1000 Å), Et₃N, DMF.

Scheme 2^a

^a (a) OsO₄, *N*-methylmorpholine *N*-oxide, acetone, H₂O (90%); (b) NaIO₄, *p*-dioxane, H₂O (97%); (c) NaBH₄, MeOH (93%); (d) methanesulfonyl chloride, pyridine (92%); (e) thiobenzoic acid, Et₃N, DMF (76%); (f) HF (aqueous), CH₃CN (100%); (g) 1-(*tert*-butylthio)-1,2-hydrazinedicarboxymorpholine, LiOH, MeOH, THF (74%); (h) DMTrCl, Et₃N, DMF (77%); (i) 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite, *N,N*-diisopropylethylamine, CH₂Cl₂ (84%).

protected precursors by denaturing PAGE (Figure 3). The observation that **I_{XL}** migrates slower than **II_{XL}** on denaturing gels can be explained given that **I_{XL}** is a 72-base-long macro-

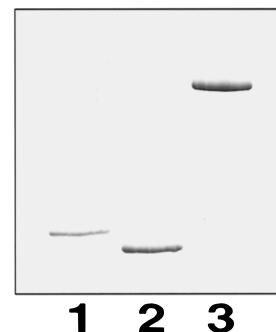


Figure 2. PAGE analysis of site I disulfide cross-link formation reaction (15% polyacrylamide denaturing gel visualized with Stains-All). Lane 1, purified native yeast tRNA^{Phe}; lane 2, purified **I_{tBu}**; lane 3, crude **I_{XL}** after 12 h. Similar results are obtained for formation of **II_{XL}** (data not shown).

cycle whereas **II_{XL}** contains a smaller, internal loop. By contrast, unmodified yeast tRNA^{Phe} and all four thiol-modified tRNAs run similarly under nondenaturing conditions, which indicates that **I_{XL}** and **II_{XL}** are monomeric. Furthermore, these results suggest that the modifications do not significantly alter the folded structure of the tRNAs relative to the parent sequence.

Further evidence that the modified tRNAs fold properly was obtained by Pb(II)-induced cleavage, which is a very sensitive assay of tRNA^{Phe} tertiary structure.²⁰ Specifically, a Pb(II) binding site between the D- and T-loops forms upon proper folding of tRNA^{Phe} into its canonical three-dimensional motif (see Figure 1B,D).²³ After binding of Pb(II) in this site, heating at pH 7 produces cleavage of the phosphodiester backbone, predominantly between U17 and G18. In the absence of Mg²⁺, none of the tRNAs fold properly and only minor nonspecific hydrolysis is observed (Figure 4). By contrast, Pb(II) cleavage of either unmodified yeast tRNA^{Phe}, **I_{XL}**, or **II_{XL}** annealed in the presence of Mg²⁺ affords a single major band corresponding to strand scission between U17 and G18 in the D-loop. The conclusion that the alkylthiol linkers do not significantly alter native structure is also supported by UV thermal denaturation experiments. Specifically, the *T_m* values for both **I_{tBu}** and **II_{tBu}** are within ~1 °C of the *T_m* value of unmodified yeast tRNA^{Phe} (Figure 5). Furthermore, the shapes of the melting transitions for both **I_{tBu}** and **II_{tBu}** are nearly superimposable with that for unmodified yeast tRNA^{Phe}, which suggests that the alkylthiol-modified tRNAs and the parent sequence denature along similar pathways.

Discussion

In 1966 Lipsett showed that iodine oxidation of the two native 4-thiouridine residues in tRNA^{Tyr} from *Escherichia coli* affords a unique intramolecular disulfide cross-link.²⁴ Although the potential of these disulfide cross-links as probes of both structure and function was noted in this early work, neither methods to synthesize RNA site-specifically labeled with thiol groups nor sensitive biochemical and structural assays to analyze the cross-linked products were available. Building on chemistry developed over the past few years to cross-link various DNA secondary structures with disulfide bonds,^{11,19,25} we showed that disulfide cross-links also can be placed within small RNA sequences without significantly perturbing native structure.^{4g} More recently, both Eckstein²⁶ and Verdine²⁷ have described the use of disulfide cross-links to probe the conformation of

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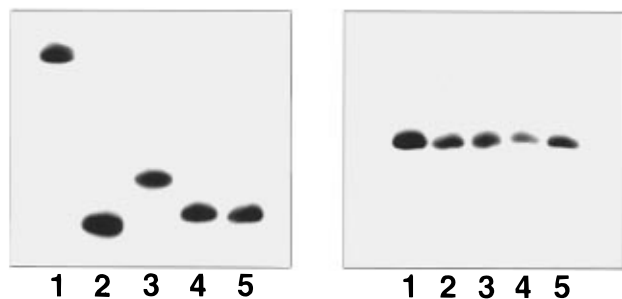


Figure 3. PAGE analysis of ³²P-end-labeled tRNAs. Left: 15% polyacrylamide denaturing gel; lane 1, **I_{XL}**; lane 2, **I_{tBu}**; lane 3, **II_{XL}**; lane 4, **II_{tBu}**; lane 5, unmodified yeast tRNA^{Phe}. Right: 15% polyacrylamide nondenaturing gel; lane 1, **I_{XL}**; lane 2, **I_{tBu}**; lane 3, **II_{XL}**; lane 4, **II_{tBu}**; lane 5, unmodified yeast tRNA^{Phe}.

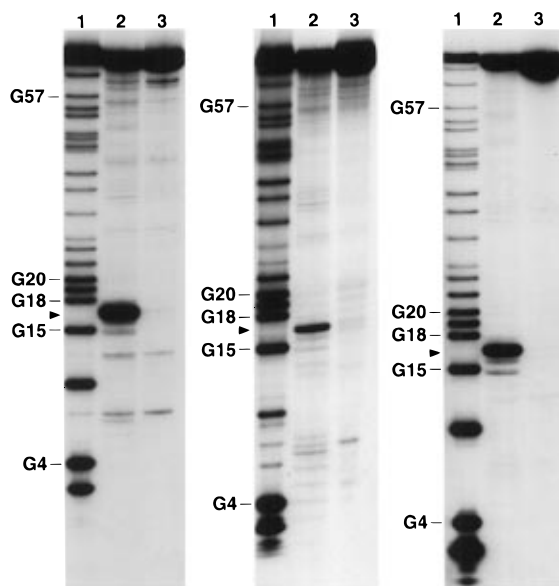


Figure 4. Lead cleavage analysis of ³²P-end-labeled tRNAs. The cleavage reactions with Pb(OAc)₂ were conducted at 40 °C for 10 min as previously described.²⁰ After cleavage reactions involving **I_{XL}** and **II_{XL}**, the disulfide cross-link was reduced with DTT and the thiol groups were blocked with *N*-ethylmaleimide to prevent reoxidation prior to or during electrophoresis.³² Lead cleavage of **I_{tBu}** and **II_{tBu}** affords similar results (data not shown). Left: **I_{XL}**; lane 1, RNase T₁ ladder; lane 2, Pb(II) plus 5 mM MgCl₂; lane 3, Pb(II) only. Middle: **II_{XL}**; lane 1, RNase T₁ ladder; lane 2, Pb(II) plus 5 mM MgCl₂; lane 3, Pb(II) only. Right: unmodified yeast tRNA^{Phe}; lane 1, RNase T₁ ladder; lane 2, Pb(II) plus 5 mM MgCl₂; lane 3, Pb(II) only. Note that the reaction products for the three tRNAs were analyzed on different gels, which gives rise to the small differences in band migration and band intensities.

the hammerhead ribozyme and to stabilize a small RNA hairpin, respectively.

Other cross-links, not based on disulfide chemistry, have been used to examine RNA structure and function. For example, ethylene glycol-based linkers bridging the 5'- and 3'-terminal hydroxyl groups on opposing strands of duplex RNA have been

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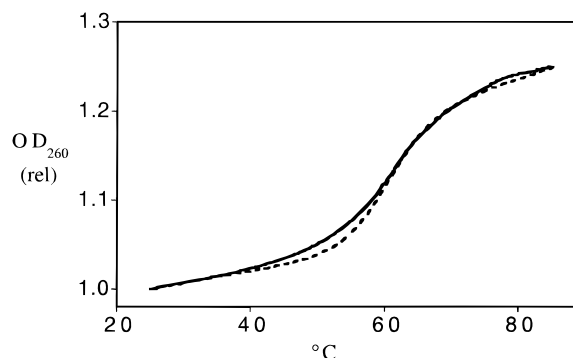


Figure 5. Representative normalized UV thermal denaturation curves for unmodified tRNA^{Phe} (—) and **II_{tBu}** (---). Spectra were measured in pH 7.2 buffer as described in the Experimental Section. The *T_m* values for unmodified yeast tRNA^{Phe} and **II_{tBu}** are 67.3 and 68.3 °C, respectively. Similar results are obtained with **I_{tBu}** (data not shown). The cross-linked tRNAs melt several degrees higher than their *tert*-butyl protected counterparts.

used to investigate both the hammerhead ribozyme and an analog of HIV-1 TAR RNA.²⁸ However, glycol and related cross-links are necessarily incorporated during solid-phase synthesis and can only be placed between sites immediately (and linearly) adjacent in sequence. RNA cross-links have also been introduced postsynthetically. For example, UV irradiation of unmodified yeast tRNA^{Phe} yields a pyrimidine–pyrimidine photoadduct between C48 and U59 in the T-region of the folded sequence.²⁹ However, formation of this and related cross-links³⁰ generally depends on the fortuitous predisposition of the two reacting groups and therefore is not a flexible strategy to examine RNA structure and folding.³¹ Moreover, only the disulfide cross-links are easily cleaved (by treatment with reducing agents), which is an important requirement for several types of footprinting experiments.³²

We have demonstrated that disulfide cross-links can be site-specifically incorporated into unmodified yeast tRNA^{Phe} and that cross-link formation between thiol-derivatized loci correlates with the position of these groups in the crystal structure of native yeast tRNA^{Phe}. Within the resolution of our assays, the thiol/disulfide modifications do not alter the structure or folding of the derivatized tRNAs. In principle, incorporation of disulfide cross-links as described in this work is not restricted to RNAs for which high-resolution structural data already exists. Since disulfide bond formation is mild and highly selective, such cross-links can be used to verify theoretical models of RNA geometry. By varying the length of the alkyl thiol linkers, information on the distance between structural elements can be investigated. In this regard, incorporating disulfide cross-links is complementary to other techniques used to study RNA folding and structure, including transient electric birefringence³³ and fluo-

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rescence resonance energy transfer.³⁴ Furthermore, the ability to ligate RNA fragments enzymatically opens the way to prepare very large molecules site-specifically labeled with thiol groups.³⁵ Finally, by analogy to experiments devised for proteins containing cysteine residues, the ability to place thiol groups in RNA should facilitate examination of both the conformational flexibility³⁶ and folding pathways^{1,10} of ribonucleic acids.

Experimental Section

General. Reagents were purchased from Aldrich Chemical Company. Controlled-pore glass (1000 Å) was obtained from CPG, Inc. 7-Diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin was obtained from Molecular Probes, Inc. RNase T₁ was obtained from United States Biochemical. CH₃CN, CH₂Cl₂, *N,N*-diisopropylethylamine, pyridine, and triethylamine (Et₃N) were each distilled from CaH₂ under N₂. CH₃OH was dried by distillation from Mg(OCH₃)₂. Tetrahydrofuran (THF) was distilled from sodium and benzophenone under N₂. *N,N*-Dimethylformamide (DMF) was dried by storing for 1 week over activated 4 Å molecular sieves and then decanting under N₂ onto fresh sieves prior to use. Silica gel (32–63 mesh) for flash chromatography was obtained from ICN Biochemicals. Glass-backed silica gel 60 plates for thin-layer chromatography (TLC) were precoated with a 0.25-mm-thick layer of Kieselgel 60F-254 and were obtained from E. Merck. All reactions were performed at room temperature unless otherwise noted. ¹H and ¹³C NMR spectra were measured on either a Bruker AM 360 MHz spectrometer or a Bruker AM 300 MHz spectrometer. ³¹P NMR spectra were measured on a Bruker AMX 500 spectrometer. All NMR spectra were measured at ambient probe temperature using the residual solvent proton peak as an internal reference, except for ³¹P NMR, which was referenced to trimethyl phosphate ($\delta = 0.00$). Infrared spectra (IR) were measured on a Nicolet Model 5D Fourier-transform spectrophotometer. Bands are reported in reciprocal centimeters (cm⁻¹) and were calibrated by comparison with the 1601 cm⁻¹ stretch in polystyrene. Mass spectra (MS) were measured on a VG Instruments Model 7070 spectrometer with 3-nitrobenzyl alcohol (3-NBA) as a matrix or by electron ionization (EI). Yields refer to chromatographically and spectroscopically homogeneous materials.

The unmodified and *tert*-butyl disulfide-modified tRNA^{Ph} sequences were synthesized on an Expedite 8909 DNA/RNA synthesizer using standard β -cyanoethyl diisopropylphosphoramidites purchased from PerSeptive Biosystems. Synthesis, deprotection, desilylation, and purification of tRNA sequences was performed as previously described.¹⁸ Dialysis of tRNA samples was conducted by buffer exchange (5 mL/min) using a Spectrum microdialyzer fitted with a cellulose ester membrane (5000 molecular weight cutoff).

N³-(2-Thiobenzoyl)uridine (1). Freshly distilled Et₃N (83.6 mL, 600 mmol, 6 equiv) was added to a solution of uridine (24.42 g, 100 mmol) in DMF (250 mL) and cooled to 4 °C. Chlorotrimethylsilane (42 mL, 330 mmol, 3.3 equiv) was added slowly, and the reaction mixture was stirred under N₂ for 2 h. Salts that had precipitated during the course of the reaction were removed by filtration under N₂, and the residual salts in the filtrate were triturated with petroleum ether: diethyl ether (Et₂O) (1:1). Sodium hydride (4.40 g, 110 mmol, 1.1 equiv) was added to the crude reaction mixture at 4 °C with stirring under N₂. After hydrogen evolution subsided, *O*-tosyl-5-benzoylmercaptoethanol¹¹ (37 g, 110 mmol, 1.1 equiv) was added and the reaction mixture was stirred at 45 °C overnight. The solution was cooled to room temperature, and silyl groups were removed by the addition of HF (5 mL, 48%). After 1 h the reaction mixture was diluted with

CH₂Cl₂ and washed with H₂O and brine. The organic layer was dried over Na₂SO₄ and concentrated *in vacuo*. The oily residue was purified by flash chromatography (CH₂Cl₂:CH₃OH, 19:1) to afford **1** as a white foam (26 g, 63% yield): TLC (CH₂Cl₂:CH₃OH, 9:1) *R*_f = 0.41; ¹H NMR (300 MHz, CD₃CN) δ 3.30 (2 H, t, *J* = 5 Hz, CH₂SC(O)Ar), 3.65–3.81 (2 H, 2 dd, *J* = 2.5, 10 Hz, 5', 5''), 3.96 (1 H, m, 4'), 4.16–4.19 (4 H, m, 2', 3', NCH₂), 5.66 (1 H, d, *J* = 8.1 Hz, 6), 5.80 (1 H, d, *J* = 3.8 Hz, 1'), 7.43–7.62 (3 H, m, Ar), 7.81 (1 H, d, *J* = 8.1 Hz, 5), 7.88 (2 H, d, *J* = 7.3 Hz, Ar); ¹³C NMR (75 MHz, CD₃CN) δ 27.4 (CH₂SC(O)Ar), 40.9 (NCH₂), 62.1 (5'), 70.8 (3'), 75.6 (2'), 85.9 (4'), 91.8 (1'), 102.1 (5), 128.0, 129.9, 134.7, 138.0 (Ar), 140.3 (6), 152.4 (2), 163.9 (4), 192.4 (SC(O)Ar); IR (KBr) ν 3548, 3448, 3086, 3060, 2944, 2901, 1706, 1673, 1655, 1462, 1388, 1286, 1211, 1124, 1079, 919, 813, 779, 695, 647, 569 cm⁻¹; FAB MS (3-NBA/trifluoroacetic acid) *m/z* 409 (*M*⁺ + 1).

5'-O-(4,4'-Dimethoxytrityl)-N³-ethyluridine *tert*-Butyl Disulfide (2). Compound **1** (15.0 g, 37 mmol) was coevaporated once from CH₃CN:pyridine (100 mL, 9:1) and dissolved in pyridine (185 mL), and 4,4'-dimethoxytrityl chloride (15.0 g, 44 mmol, 1.2 equiv) was added in portions (2.5 g each) over a 6 h period at 4 °C with stirring. The reaction was allowed to warm to room temperature overnight with stirring under N₂, after which time CH₃OH (5 mL) was added. The mixture was stirred for an additional 10 min, the solvents were removed *in vacuo*, and coevaporation of the residue with CH₃CN (100 mL) yielded a light yellow-orange foam (24.4 g, 93%). 1-(*tert*-Butylthio)-1,2-hydrazinedicarboxymorpholide¹² (8.31 g, 24 mmol, 1.2 equiv) and LiOH·H₂O (2.52 g, 60 mmol, 3.0 equiv) were added to the crude tritylated product (14 g, 20 mmol) in CH₃OH (100 mL). The reaction mixture was stirred under N₂ for 12 h, after which the reaction mixture was concentrated *in vacuo*, then dissolved in ethyl acetate (EtOAc), and washed with brine. The organic layer was dried over Na₂SO₄ and concentrated *in vacuo*, and the residue was purified by flash chromatography (EtOAc:petroleum ether, step gradient of 2:3 to 3:2) to afford **2** as a white foam (9.7 g, 70% yield): ¹H NMR (360 MHz, CD₃CN) δ 1.31 (9 H, s, SS(CH₃)₃), 2.90 (2 H, t, *J* = 6.0 Hz, CH₂SS), 3.36 (2 H, m, 5', 5''), 3.75 (6 H, s, 2 OCH₃), 4.02 (1 H, m, 4'), 4.11 (2 H, m, NCH₂), 4.18 (1 H, m, 2'), 4.32 (1 H, m, 3'), 5.38 (1 H, d, *J* = 8.1 Hz, 6), 5.78 (1 H, d, *J* = 3.2 Hz, 1'), 6.85–7.44 (13 H, m, Ar), 7.73 (1 H, d, *J* = 8.1 Hz, 5); ¹³C NMR (90 MHz, CD₃CN) δ 30.1 (SSC(CH₃)₃), 37.2 (CH₂SS), 41.0 (NCH₂), 48.5 (SSC(CH₃)₃), 56.0 (OCH₃), 63.4 (5'), 70.5 (3'), 75.5 (2'), 83.8 (4'), 87.5 (OC(Ph)₃), 91.4 (1'), 101.9 (5), 114.2 (Ar), 128.0, 129.0, 131.1, 136.5, 136.7 (Ar), 139.7 (6), 145.9 (Ar), 152.0 (2), 159.8 (Ar), 163.3 (4); IR (film; NaCl) ν 3452, 2959, 2940, 1708, 1665, 1608, 1509, 1457, 1252, 1177, 1103, 1035, 829, 810, 702 cm⁻¹; FAB MS (3-NBA) *m/z* 695 (*M*⁺ + 1).

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(*tert*-butyldimethylsilyl)-N³-ethyluridine *tert*-Butyl Disulfide (3) and 5'-O-(4,4'-Dimethoxytrityl)-3'-O-(*tert*-butyldimethylsilyl)-N³-ethyluridine *tert*-Butyl Disulfide (4). Compound **2** (9.5 g, 13.6 mmol) was dissolved in DMF (50 mL). Imidazole (2.31 g, 34 mmol, 2.5 equiv) and *tert*-butyldimethylsilyl chloride (2.58 g, 17.1 mmol, 1.25 equiv) were added, and the mixture was stirred overnight under N₂. The reaction mixture was diluted with EtOAc, and the mixture was washed with brine and dried over Na₂SO₄. The residue was purified by flash chromatography (petroleum ether:EtOAc, 9:1) to afford **3** and **4** as white foams (**3**, 3.9 g, 40% yield; **4**, 2.1 g, 25% yield): Compound **3**: TLC (petroleum ether:EtOAc, 4:1) *R*_f = 0.30; ¹H NMR (300 MHz, CD₃CN) δ 0.12 (6 H, s, Si(CH₃)₂), 0.90 (9 H, s, SiC(CH₃)₃), 1.30 (9 H, s, SSC(CH₃)₃), 2.88 (2 H, t, *J* = 8.0 Hz, CH₂SS), 3.38 (2 H, 2 dd, *J* = 2.4, 11.0 Hz, 5', 5''), 3.74 (6 H, s, 2 OCH₃), 4.05 (3 H, m, 4', NCH₂), 4.24 (1 H, m, 2'), 4.31 (1 H, m, 3'), 5.35 (1 H, d, *J* = 8.2 Hz, 6), 5.83 (1 H, d, *J* = 4.0 Hz, 1'), 6.84–7.43 (13 H, m, Ar), 7.74 (1 H, d, *J* = 8.1 Hz, 5); ¹³C NMR (75 MHz, CD₃CN) δ -4.4 (Si(CH₃)₂), 18.8 (SiC(CH₃)₃), 26.3 (SiC(CH₃)₃), 30.2 (SSC(CH₃)₃), 37.5 (CH₂SS), 41.0 (NCH₂), 48.4 (SSC(CH₃)₃), 55.9 (OCH₃), 63.7 (5'), 71.2 (3'), 76.9 (2'), 84.1 (4'), 87.7 (OC(Ph)₃), 90.6 (1'), 102.1 (5), 114.1 (Ar), 127.8, 128.8, 128.9, 130.9, 136.3, 136.5 (Ar), 139.3 (6), 145.6 (Ar), 151.7 (2), 159.7 (Ar), 162.8 (4); IR (film; NaCl) ν 3856, 3546, 2955, 2930, 2857, 2361, 2334, 1710, 1668, 1608, 1509, 1456, 1253, 1177, 1122, 1036, 836 cm⁻¹; FAB MS (3-NBA) *m/z* 809 (*M*⁺ + 1). Compound **4**: TLC (petroleum ether:EtOAc, 4:1) *R*_f = 0.14; ¹H NMR (300 MHz, CD₃CN) δ 0.04, 0.05 (6 H, 2 s, Si(CH₃)₂), 0.81 (9 H, s, SiC(CH₃)₃), 1.30 (9 H, s, SSC(CH₃)₃), 2.88 (1

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H, dd, $J = 3.8, 11.1$ Hz, 5'), 2.89 (2 H, t, $J = 7.6$ Hz, CH₂SS), 3.46 (1 H, dd, $J = 2.4, 11.0$ Hz, 5'), 3.74 (6 H, s, 2 OCH₃), 4.00 (1 H, m, 4'), 4.10 (3 H, m, 2', NCH₂), 4.33 (1 H, m, 3'), 5.38 (1 H, d, $J = 8.1$ Hz, 6), 5.80 (1 H, d, $J = 3.0$ Hz, 1'), 6.84–7.42 (13 H, m, Ar), 7.74 (1 H, d, $J = 8.1$ Hz, 5); ¹³C NMR (75 MHz, CD₃CN) δ -4.4, -4.1 (Si(CH₃)₂), 18.7 (Si(CH₃)₃), 26.3 (Si(CH₃)₃), 30.3 (SSC(CH₃)₃), 37.5 (CH₂SS), 41.0 (NCH₂), 48.3 (SSC(CH₃)₃), 56.0 (OCH₃), 63.3 (5'), 72.0 (3'), 75.4 (2'), 84.1 (4'), 87.7 (OC(Ph)₃), 92.0 (1'), 102.1 (5), 114.1 (Ar), 127.7, 128.7, 129.0, 131.0, 136.4 (Ar), 139.6 (6), 145.5 (Ar), 151.7 (2), 159.7 (Ar), 162.9 (4); IR (film; NaCl) ν 3869, 2955, 2929, 2858, 2364, 2334, 1710, 1670, 1509, 1456, 1252, 1176, 1116, 1035, 836 cm⁻¹; FAB MS (3-NBA) m/z 809 (M⁺ + 1).

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(tert-butylidimethylsilyl)-N³-ethyluridine 3'-O-(β -Cyanoethyl N,N-diisopropylphosphoramidite) tert-Butyl Disulfide (5). Compound 3 (0.81 g, 1.0 mmol) was dissolved in THF (3.0 mL), and 2,4,6-collidine (1.0 mL, 7.5 mmol, 7.5 equiv) and N-methylimidazole (40 μ L, 0.5 mmol, 0.5 equiv) were added. 2-Cyanoethyl N,N-diisopropylphosphoramidochloridite (0.56 mL, 2.5 mmol, 2.5 equiv) was then added dropwise with stirring under N₂. After 2 h, the reaction mixture was diluted with EtOAc and the mixture washed with NaHCO₃ and brine, dried over Na₂SO₄, and concentrated *in vacuo*. The residue was purified by flash chromatography (petroleum ether:EtOAc:Et₃N, 80:15:5) to afford 5 as a brittle white foam (0.82 g, 82% yield): TLC (petroleum ether:EtOAc:Et₃N, 80:15:5) $R_f = 0.28$; ¹H NMR (300 MHz, CD₃CN) δ (two diastereomers) 0.09, 0.12 (6 H, 2 s, Si(CH₃)₂), 0.83, 0.85 (9 H, 2 s, Si(CH₃)₃), 1.01, 1.14 (12 H, d, $J = 9$ Hz, 2 NCH(CH₃)₂), 1.31 (9 H, s, SSC(CH₃)₃), 2.45 (2 H, m, OCH₂CH₂CN), 2.88 (2 H, m, CH₂SS), 3.40 (2 H, m, 5', 5''), 3.50–3.90 (4 H, m, OCH₂CH₂CN, 2 NCH(CH₃)₂), 3.75 (6 H, s, 2 OCH₃), 4.10 (2 H, m, NCH₂CH₂SS), 4.20–4.42 (3 H, m, 2', 3', 4'), 5.37, 5.39 (1 H, d, $J = 8.1$ Hz, 6), 5.84, 5.89 (1 H, d, $J = 6.6$ Hz, 1'), 6.83–7.46 (13 H, m, Ar), 7.72, 7.77 (1 H, d, $J = 8.1$ Hz, 5); ¹³C NMR (75 MHz, CD₃CN) δ (two diastereomers) -4.2 (Si(CH₃)₂), 18.9 (Si(CH₃)₃), 21.1 (OCH₂CH₂CN), 25.0, 25.1, 25.2, 25.3 (NCH(CH₃)₂), 26.4 (Si(CH₃)₃), 30.4 (SC(CH₃)₃), 37.8 (CH₂SS), 41.2 (NCH₂CH₂SS), 44.1, 44.3, 44.5, 44.6 (NCH(CH₃)₂), 48.5 (SSC(CH₃)₃), 56.2 (OCH₃), 59.4, 60.0 (POCH₂CH₂CN), 64.0, 64.2 (5'), 73.5, 73.7 (3'), 76.1, 76.5 (2'), 83.9, 84.0 (4'), 88.1 (OC(Ph)₃), 90.3, 90.4 (1'), 102.4, 102.5 (5), 114.4 (Ar), 128.1, 129.0, 129.2, 129.3, 131.2, 136.4, 136.5, 136.6 (Ar), 139.3, 139.4 (6), 145.7, 145.8 (Ar), 152.1 (2), 160.0 (Ar), 162.9, 163.0 (4); ³¹P NMR (202 MHz, CD₃CN) δ (two diastereomers) 147.4, 146.8; IR (KBr) ν 2965, 2931, 2859, 2254, 1712, 1671, 1611, 1509, 1456, 1391, 1365, 1253, 1179, 1037, 979, 836 cm⁻¹; FAB MS (3-NBA) m/z 1009.5 (M⁺ + 1). Anal. Calcd for C₅₁H₇₃N₄O₄PS₂Si: C, 60.70; H, 7.28; N, 5.55. Found: C, 60.77; H, 7.24; N, 5.47.

5'-O-(4,4'-Dimethoxytrityl)-3'-O-(tert-butylidimethylsilyl)-N³-ethyluridine 2'-O-Pentachlorophenylsuccinate tert-Butyl Disulfide (6). Compound 4 (1.77 g, 2.2 mmol) was dissolved in pyridine (12.0 mL) under N₂ followed by the addition of succinic anhydride (0.69 g, 6.6 mmol, 3.0 equiv) and 4-(dimethylamino)pyridine (DMAP) (0.133 g, 1.1 mmol, 0.5 equiv). After 12 h the reaction mixture was concentrated *in vacuo* and the residue was dissolved in CH₂Cl₂, washed with brine, dried over Na₂SO₄, and concentrated *in vacuo*. The crude succinate was then dissolved in CH₂Cl₂ (25 mL), and pentachlorophenol (0.88 g, 3.3 mmol, 1.5 equiv), DMAP (67 mg, 0.55 mmol, 0.25 equiv), and dicyclohexylcarbodiimide (0.91 g, 4.4 mmol, 2.0 equiv) were added. After 8 h, petroleum ether was added to precipitate dicyclohexylurea, and the reaction mixture was filtered and concentrated *in vacuo*. Purification of the residue by flash chromatography (petroleum ether:EtOAc, 4:1) afforded 6 as a white foam (2.3 g, 90% yield): TLC (petroleum ether:EtOAc, 4:1) $R_f = 0.68$; ¹H NMR (360 MHz, CD₃CN) δ -0.07, 0.01 (6 H, s, Si(CH₃)₂), 0.79 (9 H, s, Si(CH₃)₃), 1.29 (9 H, s, SSC(CH₃)₃), 2.79 (4 H, m, succinate CH₂), 3.03 (2 H, m, CH₂-SS), 3.29–3.47 (2 H, m, 5', 5''), 3.76 (6 H, s, 2 OCH₃), 3.96–4.12 (3 H, m, 4', NCH₂), 4.38 (1 H, m, 3'), 5.43 (2 H, m, 6, 2'), 5.97 (1 H, d, $J = 4.8$ Hz, 1'), 6.21–7.45 (13 H, m, Ar), 7.65 (1 H, d, $J = 8.1$ Hz, 5); ¹³C NMR (90 MHz, CD₃CN) δ -4.4, -4.1 (Si(CH₃)₂), 18.6 (Si(CH₃)₃), 26.1 (Si(CH₃)₃), 29.4, 29.5 (succinate CH₂), 30.2 (SSC(CH₃)₃), 37.0 (CH₂SS), 41.1 (NCH₂CH₂SS), 48.5 (SSC(CH₃)₃), 56.0 (OCH₃), 63.6 (5'), 71.4 (3'), 76.1 (2'), 85.3 (4'), 88.0 (OC(Ph)₃), 88.7 (1'), 102.5 (5), 114.2 (Ar), 128.1, 129.0, 129.1, 131.2, 136.4 (Ar), 139.4 (6), 145.7 (Ar), 152.0 (2), 159.9 (Ar), 163.1 (4), 169.6, 171.8

(succinate CO₂); IR (film; NaCl) ν 2956, 2931, 1786, 1751, 1713, 1672, 1608, 1509, 1455, 1390, 1363, 1253, 1229, 1177, 1154, 1107, 1036, 837 cm⁻¹; FAB MS (3-NBA) m/z 1173 (M⁺ + 1). Anal. Calcd for C₅₂H₅₉N₂O₁₁S₂SiCl₅: C, 53.96; H, 5.10; N, 2.42. Found C, 54.03; H, 5.15; N, 2.40.

5'-O-(4,4'-Dimethoxytrityl)-3'-O-(tert-butylidimethylsilyl)-N³-ethyluridine 2'-O-(CPG succinyl) tert-Butyl Disulfide (7). Long-chain alkyl amino controlled-pore glass (1.0 g, 1000 Å pore size, 100 μ mol amino groups/g, 120/200 mesh) was suspended in DMF (4.0 mL) with 6 (0.58 g, 0.5 mmol, 5 equiv) and Et₃N (0.14 mL, 1.0 mmol, 10 equiv). The mixture was gently swirled in the dark for 2 days. The support was then vacuum filtered and rinsed successively with DMF (15 mL), CH₃OH (50 mL), and Et₂O (50 mL), and the residual solvents were removed *in vacuo*. The unreacted amino groups were acetylated by swirling the support for 1 h with acetic anhydride (0.70 mL, 7.0 mmol, 100 equiv) and DMAP (10 mg, 70 μ mol, 1 equiv) in pyridine (4 mL). The support was rinsed successively with pyridine (30 mL), CH₃OH (90 mL), and Et₂O (90 mL), and the residual solvents were removed *in vacuo*. The nucleoside loading concentration was 32 μ mol/g.¹⁴

3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)-N⁴-benzoyl-2'-O-(2,3-dihydroxypropyl)cytidine (8). 3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)-N⁴-benzoyl-2'-O-allylcytidine¹⁵ (1.34 g, 2.30 mmol) and N-methylmorpholine N-oxide (0.27 g, 2.34 mmol, 1.1 equiv) were dissolved in acetone:H₂O (21 mL, 6:1), and OsO₄ (5 mg, 21 μ mol, 0.01 equiv) was added. The reaction mixture was stirred in the dark for 2.5 h, after which an aqueous solution of saturated sodium bisulfite (1 mL) was added to precipitate osmium salts. The solution was decanted, and the light brown residue was diluted with Et₂O and washed with saturated NaHCO₃ and brine. The combined organic layers were dried over Na₂SO₄ and concentrated *in vacuo*. The yellow residue was purified by flash chromatography (CH₂Cl₂:CH₃OH, step gradient of 19:1 to 37:3) to afford 8 as a brown foam (1.27 g, 90% yield): TLC (CH₂Cl₂:CH₃OH, 19:1) $R_f = 0.23$; ¹H NMR (360 MHz, CDCl₃) δ (two diastereomers) 98–1.11 (28 H, m, 4 (CH₃)₂CHSi, 4 (CH₃)₂CHSi), 3.66 (1 H, dd, $J = 5.2, 10.7$ Hz, CH(OH)CH₂aOH), 3.72–3.76 (2 H, m, CH₂CH(OH)CH₂, CH(OH)CH₂bOH), 3.88–4.06 (4 H, m, 2', 5', OCH₂-CH(OH)), 4.15–4.28 (2 H, m, 3', 4'), 4.30 (1 H, d, $J = 13.5$ Hz, 5'), 5.83 (1 H, s, 1'), 7.49–7.63 (4 H, m, 5, Ar), 7.90–7.92 (2 H, m, Ar), 8.31–8.34 (1 H, m, 6), 8.94 (1 H, br s, NH); ¹³C NMR (90 MHz, CDCl₃) δ (two diastereomers) 12.5, 12.6, 12.9, 13.0 ((CH₃)₂CHSi), 16.8, 16.9, 17.0, 17.3, 17.4, 17.6 ((CH₃)₂CHSi), 59.2 (5'), 63.6, 63.9 (CH(OH)CH₂OH), 67.8 (3'), 70.4, 70.5 (OCH₂CH(OH)), 73.4, 74.3 (CH₂CH(OH)CH₂), 82.0 (2'), 83.1, 83.3 (4'), 89.9, 90.2 (1'), 96.3 (5), 127.6, 129.0, 132.8 (Ar), 133.3 (6), 144.3 (2), 162.5 (4); IR (film; NaCl) ν 3367, 2946, 2868, 1700, 1655, 1617, 1486, 1257, 1126, 1040, 886, 704 cm⁻¹; FAB MS (3-NBA) m/z 664 (M⁺ + 1).

3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)-N⁴-benzoyl-2'-O-(formyl-methyl)cytidine (9). Compound 8 (1.27 g, 1.91 mmol) was dissolved in *p*-dioxane:H₂O (20 mL, 3:1), and NaIO₄ (0.49 g, 2.29 mmol, 1.2 equiv) was added. The reaction mixture was stirred in the dark for 4.5 h and then diluted with Et₂O and washed with water. The organic layer was dried over Na₂SO₄ and concentrated *in vacuo*. Purification of the residue by flash chromatography (CH₂Cl₂:CH₃OH, 19:1) afforded 9 as a white foam (2.94 g, 97% yield): TLC (CH₂Cl₂:CH₃OH, 37:3) $R_f = 0.50$; ¹H NMR (360 MHz, CDCl₃) δ 0.98–1.11 (28 H, m, 4 (CH₃)₂CHSi, 4 (CH₃)₂CHSi), 3.98–4.07 (2 H, m, 2', 5'), 4.18–4.31 (3 H, m, 3', 4', 5''), 4.47–4.59 (2 H, m, OCH₂CHO), 5.86 (1 H, s, 1'), 7.49–7.63 (4 H, m, 5, Ar), 7.89–7.91 (2 H, m, Ar), 8.37 (1 H, d, $J = 7.5$ Hz, 6), 8.81 (1 H, br s, NH), 9.81 (1 H, s, CH₂CHO); ¹³C NMR (90 MHz, CDCl₃) δ 12.3, 12.9, 13.0, 13.4 ((CH₃)₂CHSi), 16.7, 16.9, 17.3, 17.4 ((CH₃)₂CHSi), 59.2 (5'), 68.1 (3'), 76.2 (OCH₂CHO), 81.8 (2'), 83.0 (4'), 89.7 (1'), 96.2 (5), 127.5, 129.0, 132.8 (Ar), 133.2 (6), 144.3 (2), 162.4 (4), 200.7 (CH₂CHO); IR (film; NaCl) ν 2946, 2868, 1700, 1667, 1619, 1484, 1253, 1130, 1064, 1040, 886, 703 cm⁻¹; FAB MS (3-NBA) m/z 632 (M⁺ + 1).

3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)-N⁴-benzoyl-2'-O-(2-hydroxyethyl)cytidine (10). Compound 9 (1.17 g, 1.85 mmol) was dissolved in CH₃OH (19 mL), and NaBH₄ (21 mg, 0.56 mmol, 0.3 equiv) was added. The mixture was stirred under N₂ in the dark for 90 min and then diluted with Et₂O and washed with saturated NaHCO₃ and brine. The aqueous layer was washed once with Et₂O, and the

combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash chromatography (CH_2Cl_2 : CH_3OH , 19:1) to afford **10** as a white foam (1.09 g, 93% yield): TLC (CH_2Cl_2 : CH_3OH , 37:3) R_f = 0.56; ^1H NMR (360 MHz, CDCl_3) δ 0.99–1.10 (28 H, m, 4 $(\text{CH}_3)_2\text{CHSi}$, 4 $(\text{CH}_3)_2\text{CHSi}$), 3.17 (1 H, br s, $\text{CH}_2\text{CH}_2\text{OH}$), 3.72–3.77 (2 H, m, $\text{CH}_2\text{CH}_2\text{OH}$), 3.93–4.03 (4 H, m, 2', 5', $\text{OCH}_2\text{CH}_2\text{OH}$), 4.15–4.25 (2 H, m, 3', 4'), 4.30 (1 H, d, J = 13.6 Hz, 5''), 5.83 (1 H, s, 1'), 7.49–7.63 (4 H, m, 5, Ar), 7.89–7.92 (2 H, m, Ar), 8.35 (1 H, d, J = 7.5 Hz, 6), 8.92 (1 H, br s, NH); ^{13}C NMR (90 MHz, CDCl_3) δ 12.6, 12.9, 13.0, 13.4 ($(\text{CH}_3)_2\text{CHSi}$), 16.8, 16.9, 17.0, 17.3, 17.4, 17.4 ($(\text{CH}_3)_2\text{CHSi}$), 59.3 (5'), 61.7 ($\text{CH}_2\text{CH}_2\text{OH}$), 68.0 (3'), 73.1 ($\text{OCH}_2\text{CH}_2\text{OH}$), 82.0 (2'), 82.3 (4'), 90.5 (1'), 96.2 (5), 127.5, 129.0, 132.9 (Ar), 133.2 (6), 144.3 (2), 162.4 (4); IR (film; NaCl) ν 2946, 2868, 1699, 1664, 1619, 1485, 1263, 1128, 1074, 1063, 1040, 886, 703 cm^{-1} ; FAB MS (3-NBA) m/z 634 ($\text{M}^+ + 1$).

3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)- N^4 -benzoyl-2'-O-(2-methylsulfonyl)ethylcytidine (11). Compound **10** (1.09 g, 1.72 mmol) was dissolved in CH_2Cl_2 (17 mL) and pyridine (1.4 mL, 17.18 mmol, 10 equiv) and cooled under N_2 to 0 $^\circ\text{C}$, and methanesulfonyl chloride (0.19 mL, 2.41 mmol, 1.2 equiv) was added dropwise. The mixture was stirred under N_2 while gradually warming to room temperature overnight. The reaction mixture was diluted with Et_2O and washed with saturated NaHCO_3 and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*, and purification of the residue by flash chromatography (CH_2Cl_2 : CH_3OH , 24:1) afforded **11** as a white foam (1.13 g, 92% yield): TLC (CH_2Cl_2 : CH_3OH , 19:1) R_f = 0.35; ^1H NMR (360 MHz, CDCl_3) δ 0.97–1.11 (28 H, m, 4 $(\text{CH}_3)_2\text{CHSi}$, 4 $(\text{CH}_3)_2\text{CHSi}$), 3.14 (3 H, s, CH_3SO_2), 3.97–4.01 (2 H, m, 2', 5'), 4.17–4.21 (4 H, m, 3', 4', $\text{OCH}_2\text{CH}_2\text{OSO}_2$), 4.30 (1 H, d, J = 13.7 Hz, 5''), 4.46–4.49 (2 H, m, $\text{CH}_2\text{CH}_2\text{OSO}_2$), 5.82 (1 H, s, 1'), 7.50–7.62 (4 H, m, 5, Ar), 7.89–7.92 (2 H, m, Ar), 8.38 (1 H, d, J = 7.5 Hz, 6); ^{13}C NMR (90 MHz, CDCl_3) δ 12.4, 12.9, 13.1, 13.4 ($(\text{CH}_3)_2\text{CHSi}$), 16.8, 16.9, 17.1, 17.3, 17.4, 17.4 ($(\text{CH}_3)_2\text{CHSi}$), 37.80 (CH_3SO_2), 59.3 (5'), 67.9 (3'), 69.0 ($\text{CH}_2\text{CH}_2\text{OSO}_2$), 69.5 ($\text{OCH}_2\text{CH}_2\text{OSO}_2$), 81.9 (2'), 82.4 (4'), 89.5 (1'), 96.2 (5), 127.6, 129.1, 132.8 (Ar), 133.3 (6), 144.5 (2), 162.4 (4); IR (film; NaCl) ν 2945, 2868, 1664, 1484, 1170, 1128, 1074, 1063, 1040, 885, 704 cm^{-1} ; FAB MS (3-NBA) m/z 712 ($\text{M}^+ + 1$).

3',5'-O-(Tetraisopropylidisiloxane-1,3-diyl)- N^4 -benzoyl-2'-O-(2-thiobenzoyl)ethylcytidine (12). Compound **11** (1.04 g, 1.46 mmol) was dissolved in DMF (5.8 mL) and Et_3N (2.0 mL, 14.6 mmol, 10 equiv), and thiobenzoic acid (0.34 mL, 2.91 mmol, 2.0 equiv) was added. The mixture was stirred under N_2 in the dark overnight, then diluted with Et_2O , and washed with saturated NaHCO_3 and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to give a dark orange-brown solid. The residue was purified by flash chromatography (step gradient of 0 to 10% CH_3CN in petroleum ether: EtOAc , 2:1) to afford **12** as a white foam (0.84 g, 76% yield): TLC (petroleum ether: EtOAc : CH_3CN , 6:3:1) R_f = 0.57; ^1H NMR (360 MHz, CDCl_3) δ 0.98–1.11 (28 H, m, 4 $(\text{CH}_3)_2\text{CHSi}$, 4 $(\text{CH}_3)_2\text{CHSi}$), 3.35–3.43 (2 H, m, $\text{CH}_2\text{CH}_2\text{SBz}$), 3.97–4.02 (2 H, m, 2', 5'), 4.09–4.13 (2 H, m, $\text{OCH}_2\text{CH}_2\text{SBz}$), 4.18 (1 H, dd, J = 3.9, 9.6 Hz, 3'), 4.25 (1 H, dd, J = 2.0, 9.6 Hz, 4'), 4.30 (1 H, d, J = 13.5 Hz, 5''), 5.84 (1 H, s, 1'), 7.40–7.62 (7 H, m, 5, Ar), 7.91–7.99 (4 H, m, Ar), 8.37 (1 H, d, J = 7.4 Hz, 6), 8.97 (1 H, br s, NH); ^{13}C NMR (90 MHz, CDCl_3) δ 12.5, 12.8, 13.1, 13.4 ($(\text{CH}_3)_2\text{CHSi}$), 16.8, 16.9, 17.1, 17.3, 17.4, 17.5 ($(\text{CH}_3)_2\text{CHSi}$), 29.1 ($\text{CH}_2\text{CH}_2\text{SBz}$), 59.4 (5'), 67.9 (3'), 69.9 ($\text{OCH}_2\text{CH}_2\text{SBz}$), 81.8 (2'), 81.9 (4'), 90.0 (1'), 96.0 (5), 127.2, 127.6, 128.5, 129.0, 133.0 (Ar), 133.2 (6), 137.1 (Ar), 144.6 (2), 162.3 (4), 191.5 (Ar); IR (film; NaCl) ν 2945, 2868, 1699, 1667, 1620, 1489, 1373, 1265, 1126, 1075, 1063, 1040, 691 cm^{-1} ; EI MS m/z 754 ($\text{M}^+ + 1$).

N^4 -Benzoyl-2'-O-(2-thiobenzoyl)ethylcytidine (13). Compound **12** (0.37 g, 0.49 mmol) was dissolved in CH_3CN (4.3 mL), and HF (0.5 mL, 48%) was added. The reaction mixture was stirred for 7 h, after which the solution was diluted with Et_2O and washed with H_2O . The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The pink residue was purified by flash chromatography (CH_2Cl_2 : CH_3OH , 19:1) to afford **13** as a white foam (0.25 g, 100% yield): TLC (CH_2Cl_2 : CH_3OH , 19:1) R_f = 0.21; ^1H NMR (360 MHz, CDCl_3) δ 3.25–3.42 (2 H, m, CH_2SBz), 3.91–3.96 (2 H, m, 2', 5'), 4.05–4.19 (3 H, m, 5'', $\text{OCH}_2\text{CH}_2\text{SBz}$), 4.20–4.25 (1 H, m, 4'), 4.35–4.37 (1 H, m,

3'), 5.87 (1 H, s, 1'), 7.33–7.54 (7 H, m, 5, Ar), 7.82–7.94 (4 H, m, Ar), 8.57 (1 H, d, J = 7.5 Hz, 6), 9.22 (1 H, br s, NH); ^{13}C NMR (90 MHz, CDCl_3) δ 28.8 (CH_2SBz), 59.8 (5'), 67.4 (3'), 69.4 ($\text{OCH}_2\text{CH}_2\text{SBz}$), 82.0 (2'), 84.6 (4'), 90.1 (1'), 96.7 (5), 126.8, 127.2, 127.6, 128.3, 128.6, 128.8, 132.9, 133.1 (Ar), 133.5 (6), 136.7 (Ar), 146.0 (2), 162.6 (4), 191.4 (Ar); IR (film; NaCl) ν 3345, 2928, 1699, 1658, 1617, 1558, 1485, 1379, 1258, 1111, 1067, 913, 705, 689 cm^{-1} ; FAB MS (3-NBA) m/z 512 ($\text{M}^+ + 1$).

N^4 -Benzoyl-2'-O-ethylcytidine *tert*-Butyl Disulfide (14). Compound **13** (0.25 g, 0.49 mmol) was dissolved in a CH_3OH :THF mixture (3.8 mL, 1:1) and 1-(*tert*-butylthio)-1,2-hydrazinedicarboxymorpholide¹² (0.20 g, 0.58 mmol, 1.2 equiv) and $\text{LiOH}\cdot\text{H}_2\text{O}$ (41 mg, 0.97 mmol, 2.0 equiv) were added. The reaction mixture was stirred under N_2 at 0 $^\circ\text{C}$ for 45 min, diluted with EtOAc , and washed with 1 N citric acid, saturated NaHCO_3 , and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*, and the residue was purified by flash chromatography (CH_2Cl_2 : CH_3OH , 24:1) to afford **14** as a pink foam (0.18 g, 74% yield): TLC (CH_2Cl_2 : CH_3OH , 24:1) R_f = 0.29; ^1H NMR (360 MHz, CDCl_3) δ 1.32 (9 H, s, $\text{C}(\text{CH}_3)_3$), 2.93 (2 H, t, J = 5.8 Hz, CH_2SS), 3.93–3.99 (2 H, m, 2', 5'), 4.09–4.18 (3 H, m, 5'', $\text{OCH}_2\text{CH}_2\text{SS}$), 4.24–4.30 (1 H, m, 4'), 4.33–4.41 (1 H, m, 3'), 5.84 (1 H, d, J = 1.7 Hz, 1'), 7.48–7.62 (4 H, m, 5, Ar), 7.87–7.89 (2 H, m, Ar), 8.48 (1 H, d, J = 7.5 Hz, 6), 9.01 (1 H, br s, NH); ^{13}C NMR (90 MHz, CDCl_3) δ 29.8 ($\text{C}(\text{CH}_3)_3$), 40.3 (CH_2SS), 48.0 ($\text{C}(\text{CH}_3)_3$), 60.3 (5'), 67.8 (3'), 69.0 ($\text{OCH}_2\text{CH}_2\text{SS}$), 81.7 (2'), 85.0 (4'), 90.9 (1'), 96.7 (5), 127.6, 129.0, 132.9 (Ar), 133.3 (6), 146.4 (2), 162.5 (4); IR (film; NaCl) ν 3374, 2960, 2922, 1699, 1648, 1617, 1558, 1487, 1379, 1260, 1110 cm^{-1} ; FAB MS (3-NBA) m/z 496 ($\text{M}^+ + 1$).

5'-O-(4,4'-Dimethoxytrityl)- N^4 -benzoyl-2'-O-ethylcytidine *tert*-Butyl Disulfide (15). Compound **14** (0.10 g, 0.21 mmol) and DMAP (13 mg, 0.10 mmol, 0.5 equiv) were dissolved in DMF (0.8 mL) and pyridine (26 μL , 0.31 mmol, 1.5 equiv), and 4,4'-dimethoxytrityl chloride (85 mg, 0.25 mmol, 1.2 equiv) was added. The reaction mixture was stirred under N_2 for 6 h, diluted with CH_2Cl_2 , and washed with saturated NaHCO_3 and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash chromatography (acetone:petroleum ether, 1:1) to afford **15** as a tan foam (0.13 g, 77% yield): TLC (acetone:petroleum ether, 1:1) R_f = 0.23; ^1H NMR (360 MHz, CD_3CN) δ 1.30 (9 H, s, $\text{C}(\text{CH}_3)_3$), 2.95 (2 H, t, J = 6.3 Hz, CH_2SS), 3.39–3.44 (2 H, m, $\text{OCH}_2\text{CH}_2\text{SS}$), 3.76 (6 H, s, 2 OCH_3), 3.90–4.03 (3 H, m, 2', 5', 5''), 4.12–4.19 (1 H, m, 4'), 4.39–4.47 (1 H, m, 3'), 5.84 (1 H, d, J = 1.7 Hz, 1'), 6.87–6.89 (4 H, m, Ar), 7.15–7.61 (13 H, m, 5, Ar), 7.92–7.95 (2 H, m, Ar), 8.46 (1 H, d, J = 7.6 Hz, 6); ^{13}C NMR (90 MHz, CD_3CN) δ 30.2 ($\text{C}(\text{CH}_3)_3$), 41.0 (CH_2SS), 48.5 ($\text{C}(\text{CH}_3)_3$), 56.0 (OCH_3), 62.1 (5'), 68.9 (3'), 70.1 ($\text{OCH}_2\text{CH}_2\text{SS}$), 83.3 (2'), 83.6 (4'), 87.7 ($\text{OC}(\text{Ph})_3$), 90.2 (1'), 97.2 (5), 114.3, 128.1, 129.1, 129.1, 129.2, 129.7, 131.0, 131.2, (Ar), 133.9 (6), 134.5, 136.6, 137.0, 145.5 (Ar), 145.9 (2), 155.7, 159.8 (Ar), 163.9 (4), 168.2 (Ar); IR (KBr) ν 3392, 2959, 2924, 1700, 1667, 1610, 1553, 1510, 1482, 1377, 1252, 1113, 1033, 704 cm^{-1} ; FAB MS (3-NBA) m/z 798 ($\text{M}^+ + 1$).

5'-O-(4,4'-Dimethoxytrityl)- N^4 -benzoyl-2'-O-ethylcytidine *tert*-Butyl Disulfide 3'-O-(β -Cyanoethyl N,N -diisopropylphosphoramidite) (16). Compound **15** (55 mg, 0.07 mmol) was dissolved in CH_2Cl_2 (0.3 mL) and N,N -diisopropylethylamine (60 μL , 0.35 mmol, 5 equiv) and cooled under N_2 to 0 $^\circ\text{C}$. 2-Cyanoethyl N,N -diisopropylphosphoramidochloridite (23 μL , 0.10 mmol, 1.5 equiv) was added dropwise, and the reaction mixture stirred under N_2 while being warmed to room temperature. After 2 h the excess chloridate was quenched with CH_3OH (0.3 mL), and the mixture was concentrated *in vacuo*. The residue was purified by flash chromatography (petroleum ether:acetone, 2:1) to afford **16** as a white foam (58 mg, 84% yield): TLC (petroleum ether:acetone, 2:1) R_f = 0.40; ^1H NMR (300 MHz, CD_3CN) δ (two diastereomers) 1.05–1.20 (12 H, m, 2 $\text{NCH}(\text{CH}_3)_2$), 1.30 (9 H, s, $\text{C}(\text{CH}_3)_3$), 2.50–2.67 (2 H, m, $\text{OCH}_2\text{CH}_2\text{CN}$), 2.94–3.01 (2 H, m, CH_2SS), 3.41–3.71 (6 H, m, $\text{OCH}_2\text{CH}_2\text{SS}$, $\text{OCH}_2\text{CH}_2\text{CN}$, 2 $\text{NCH}(\text{CH}_3)_2$), 3.79 (6 H, s, 2 OCH_3), 3.80–4.22 (4 H, m, 2', 4', 5', 5''), 4.43–4.63 (1 H, m, 3), 5.88 (1 H, s, 1'), 6.87–6.92 (4 H, m, Ar), 7.03–7.66 (13 H, m, 5, Ar), 7.91–7.97 (2 H, m, Ar), 8.42–8.55 (1 H, d, J = 7.6 Hz, 6); ^{13}C NMR (75 MHz, CD_3CN) δ (two diastereomers) 21.2, 21.3 ($\text{OCH}_2\text{CH}_2\text{CN}$), 24.9, 25.0, 25.2, 25.3 ($\text{NCH}(\text{CH}_3)_2$), 30.3 ($\text{C}(\text{CH}_3)_3$), 41.4 (CH_2SS), 44.1, 44.3 ($\text{NCH}(\text{CH}_3)_2$), 48.5 ($\text{C}(\text{CH}_3)_3$), 56.0 (OCH_3),

59.3, 59.6 OCH₂CH₂CN), 61.8, 62.2 (5'), 70.2, 70.4 (3'), 70.8 (OCH₂-CH₂SS), 82.2 (2'), 83.1 (4'), 87.8 (OC(Ph)₃), 90.8, 91.1 (1'), 97.3 (5), 114.2, 128.1, 129.0, 129.0, 129.3, 129.6, 131.2 (Ar), 133.8 (6), 134.5, 136.6, 136.7, 145.4 (Ar), 145.6 (2), 155.4, 159.8 (Ar), 163.6 (4); ³¹P NMR (202 MHz, CD₃CN) δ 147.57, 146.51; IR (film; NaCl) ν 2967, 2934, 1708, 1686, 1509, 1510, 1462, 1251, 1180, 1035, 979 cm⁻¹; FAB MS (3-NBA) m/z 998 (M⁺ + 1). Anal. Calcd for C₅₂H₆₄N₅O₉PS₂: C, 62.56; H, 6.48; N, 7.02. Found: C, 62.41; H, 6.41; N, 6.92.

Disulfide Cross-Link Formation. The requisite *tert*-butyl disulfide-modified tRNA (~3 OD₂₆₀ units; 130 μ g, 5 nmol) was dissolved in phosphate buffer (50 μ L, 100 mM Na₂HPO₄, pH 8.3), and DTT (54 μ g, 200 equiv/disulfide) was added. The mixture was sealed and incubated at 25 °C for 12 h. The reaction mixture was then diluted with phosphate buffer (200 μ L, 5 mM Na₂HPO₄, 5 mM NaCl, pH 7.0) and dialyzed against the same buffer (3 L) at room temperature for 10 h. The reduced and dialyzed tRNA was diluted in phosphate buffer (pH 7.0) to a final concentration of 10 μ M, heated to 70 °C for 1 min, and allowed to cool to ~40 °C. At this time MgCl₂ (0.1 M) was added to a final [Mg²⁺] of 5 mM. After the solution was equilibrated at room temperature for 30 min, the pH was adjusted to 8.0 with NaOH (0.1 M), and the reaction mixture was stirred at room temperature, exposed to air. At 6 h intervals, an aliquot (5 μ L) of the reaction mixture was mixed with 5X CPM buffer (1 μ L, 250 mM Tris-HCl, 2.6 M NaCl, 0.5 mM EDTA, 0.15% (v/v) Triton X-100, pH 7.5) and CPM (5 μ L of a 0.4 mM CPM stock solution in HPLC-grade *i*-PrOH).²¹ The mixture was incubated at room temperature for 10 min and then diluted with 1% Triton X-100 (489 μ L). The fluorescence intensity at 480 nm (λ_{ex} = 390 nm) was corrected for background fluorescence, and the amount of free thiol remaining was quantified on the basis of a calibration curve constructed for N³-(thioethyl)uridine.⁴⁸ After 12 h, no free thiol groups were present and the reaction mixture was either ethanol precipitated or dialyzed against TE buffer (3 L, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 10 h) and stored at -20 °C.

UV Thermal Denaturation. tRNA samples for UV thermal denaturation experiments were dialyzed against phosphate buffer (3 L, 5 mM Na₂HPO₄, 5 mM NaCl, pH 7.2, 10 h), and aliquots of tRNA were diluted with the same degassed buffer (final volume of 1 mL). tRNA solutions were heat-denatured at 90 °C for 1 min and then cooled to 10 °C at a cooling rate of 3 °C/min. For experiments with Mg²⁺ in the buffer, MgCl₂ (100 mM MgCl₂ in 5 mM Na₂HPO₄, 5 mM NaCl, pH 7.2) was added to a final [Mg²⁺] of 5 mM after the sample had cooled to ~40 °C. The tRNA samples were equilibrated at 10 °C for 30 min prior to thermal denaturation. The heating rate was 0.5 °C/min with absorbance monitored at 260 nm and a data interval of 0.1 °C. The melting temperature, *T*_m, was obtained by nonlinear regression analysis of the denaturation curve as described by Blatt et al.³⁷

³²P-Labeling of tRNA. tRNA samples (5 pmol, ~3 × 10⁻³ OD₂₆₀ unit) were dissolved in kinase buffer (10 μ L, 70 mM Tris-HCl, 10 mM MgCl₂, 0.1 mM EDTA, 5% glycerol, pH 7.5) containing T4 polynucleotide kinase (10 units) and [γ -³²P]ATP (10 μ Ci, 33 pmol, 6000 Ci/mmol). The mixture was incubated at 37 °C for 30 min. Formamide (10 μ L, 80% v/v) containing bromophenol blue (0.1% w/v) and xylene cyanole (0.1% w/v) tracking dyes was added to each sample, and the mixture was electrophoresed on a denaturing gel (15% polyacrylamide, 29:1 acrylamide:bisacrylamide, 8 M urea, 31.0 cm × 38.5 cm × 0.8 mm) at constant power (55 W) in TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.3) for 5 h. The labeled tRNA was excised from the gel and electroeluted into 4X TAE (300 μ L, 160 mM Tris, 80 mM acetic acid, 8 mM EDTA, pH 7.5) using a Hoefer SixPac microelectroeluter at +50 V for 1 h. The eluted tRNA was concentrated *in vacuo* to 50 μ L and precipitated with sodium acetate (NaOAc) (50 μ L, 3 M, pH 5.5) and ethanol (EtOH) (300 μ L, 100% v/v) to remove residual EDTA and urea. The specific activity (cpm/ μ L) of each sample was determined by Cerenkov counting.

Denaturing Analytical PAGE. An aliquot of an aqueous solution of each ³²P-end-labeled tRNA (7500 cpm) was diluted with H₂O (total

volume of 4 μ L) and formamide (4 μ L, 80% v/v) containing bromophenol blue (0.1% w/v) and xylene cyanole (0.1% w/v) tracking dyes. The samples were loaded onto a denaturing gel (15% polyacrylamide, 29:1 acrylamide:bisacrylamide, 8 M urea, 31.0 cm × 38.5 cm × 0.4 mm) and electrophoresed at constant power (55 W) in TBE until the xylene cyanole dye had migrated 27 cm. The gel was autoradiographed for 20 h at -78 °C on FUJI RX film using an intensifying screen.

Native Analytical PAGE. An aliquot of an aqueous solution of each ³²P-end-labeled tRNA (7500 cpm) was concentrated *in vacuo* and dissolved in 5X Tris-HEPES buffer (2 μ L, 170 mM Tris, 330 mM HEPES, 0.5 mM EDTA, 10 mM MgCl₂, pH 7.5) and H₂O (2.5 μ L). The samples were heated to 80 °C for 2 min and allowed to cool to 45 °C. MgCl₂ (0.5 μ L, 120 mM) was added, and the samples were equilibrated at room temperature for about 15 min. Sucrose loading buffer (5 μ L, 25% w/v) was then added, and each sample was placed on ice for 2 min prior to loading onto a native gel (15% polyacrylamide, 29:1 acrylamide:bisacrylamide; 14 cm × 16 cm × 0.8 mm) which had been equilibrated at 4 °C for 1 h after polymerization. The samples were electrophoresed at constant voltage (300 V) for 10 h in 1X Tris-HEPES buffer at 10 °C using a Hoefer SE600 electrophoresis apparatus attached to a recirculating water bath, until the xylene cyanole tracking dye (in a blank lane) had migrated 15 cm.

Lead(II) Autocleavage. An aliquot of an aqueous solution of each ³²P-end-labeled tRNA (7.5 × 10⁴ cpm) was concentrated *in vacuo* and dissolved in native precleavage buffer (4 μ L, 15 mM MOPS, 1.5 mM spermine, pH 7.0) or semidenaturing precleavage buffer (10 μ L, 15 mM MOPS, pH 7.0) along with unlabeled carrier tRNA (50 μ g), and heat-denatured at 70 °C for 1 min. The samples were slowly cooled to ~40 °C, and MgCl₂ (6 μ L, 25 mM in native precleavage buffer; final concentration of 15 mM) was added to the native reaction mixture. After equilibration at room temperature for 30 min, Pb(OAc)₂ (0.2 μ L, 10 mM, final concentration of 200 μ M) was added. The reaction mixtures were incubated at 40 °C for 10 min and quenched with NaOAc (80 μ L, 1.5 mM, pH 5.5), and the tRNAs were precipitated with glycogen (1 μ L, 20 μ g/ μ L) and EtOH (250 μ L, 100% v/v) at -80 °C for 15 min. Following centrifugation (16000 *g*, 4 °C, 30 min) the supernatants were removed and the pellets gently blotted dry. For lead-cleavage reactions of disulfide cross-linked tRNAs, the pellets were dissolved in an aqueous solution of DTT (20 μ L, 50 mM) and incubated at 37 °C for 1 h. The tRNAs were precipitated with NaOAc (80 μ L, 1.5 M, pH 5.5), glycogen (1 μ L), and EtOH (250 μ L, 100% v/v) at -80 °C for 15 min. The reduced sequences were dissolved in Tris-HCl (45 μ L, 50 mM, pH 8.0) at 37 °C and treated with aliquots of *N*-ethylmaleimide (5 μ L, 50 mM in H₂O) every 30 min for 1.5 h to block the thiols from reoxidation.³² The reduced, blocked tRNA was ethanol precipitated, and the pellets were rinsed once with EtOH (80% v/v), blotted dry, and dissolved in urea loading buffer (5 μ L, 9.8 M) for electrophoresis. To locate the cleavage sites, hydroxide and RNase T₁ cleavage ladders were run in parallel with the lead cleavage lanes. The sequencing and lead cleavage reactions were electrophoresed on a denaturing gel (15% polyacrylamide, 29:1 acrylamide:bisacrylamide, 8 M urea, 31.0 cm × 38.5 cm × 0.4 mm) at constant power (55 W) in TBE, and the product bands were visualized by autoradiography.

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