Engineering Disulfide Cross-Links in RNA Via Air Oxidation

This unit describes methods for the synthesis of thiol-modified ribonucleosides, their incorporation into synthetic RNA, and the formation of intramolecular disulfide bonds in RNA by air oxidation. The disulfide bonds can be formed in quantitative yields between thiols positioned in close proximity in either RNA secondary or tertiary structure. Disulfide cross-links are useful tools to probe solution structures of RNA, monitor dynamic motions, and stabilize folded RNAs.

Several steps are involved in the successful formation of a disulfide-cross-linked RNA. First, a location for incorporation of the disulfide bond is selected (Glick, 1998). For best results, this should be based on the highest-resolution structural data available for the particular RNA. In most cases, it is desirable to avoid interfering with interactions that stabilize structure, such as hydrogen bonding. Once the site for the disulfide bond has been chosen, the necessary thiol-modified nucleosides must be chemically synthesized. In most cases, the authors use alkylthiol linkers on the 2′ hydroxyl and the N^3 position of pyrimidine residues, although linkers can be positioned at nearly any position of a nucleoside. The thiol-modified nucleoside phosphoramidites are synthesized with the thiol functionality protected as a *tert*-butyl disulfide, a protecting group that is stable under all conditions of solid-phase synthesis and subsequent manipulations.

Incorporation of the thiol-modified nucleosides is accomplished by solid-phase chemical synthesis of the RNA. Following removal of both the exocyclic amine-protecting groups and the *tert*-butyldimethyl silyl groups used to protect the 2′ hydroxyls, the full-length oligoribonucleotide is purified by denaturing polyacrylamide gel electrophoresis (PAGE). Reduction of the mixed disulfide with dithiothreitol (DTT) liberates the free thiols. These are then oxidized by vigorous stirring in air to form an intramolecular disulfide bond in quantitative yield.

The Basic Protocol describes the procedures for formation of intramolecular disulfide bonds by air oxidation of thiol-mediated RNA. Optimized protocols for solid-phase synthesis and deprotection of tRNAs (tRNA is used here as a model RNA) containing thiol-modified residues are presented in Support Protocol 1. Support Protocol 2 describes a method for purification of tRNAs containing thiol-modified residues. Support Protocol 3 describes a fluorescence assay to quantify the concentration of free thiol remaining during cross-link formation. Support Protocols 4, 7, and 8 describe the synthesis of three different thiol-containing nucleoside phosphoramidites, while Support Protocol 6 describes the synthesis of an intermediate compound needed for Support Protocols 7 and 8. Support Protocol 5 details the preparation of a thiol-modified controlled-pore glass (CPG) support used in the solid-phase synthesis of the modified RNA.

NOTE: All procedures should be conducted using sterile techniques. When handling the RNA, suitable precautions should be taken to avoid RNase contamination. Gloves must be worn during handling of all equipment. Treatment of solutions with DEPC to inactivate RNases is not necessary, but could be done if preferred.

CAUTION: Acrylamide is a neurotoxin, minimize inhalation and skin exposure. The reagents and solvents used in both solid-phase RNA synthesis and the synthesis of the thiol-modified phosphoramidites should be handled according to the manufacturer's safety data sheet for each reagent.

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Current Protocols in Nucleic Acid Chemistry (2000) 5.4.1-5.4.34
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BASIC PROTOCOL

FORMATION OF INTRAMOLECULAR DISULFIDE CROSS-LINKS IN RNA

The RNA used in this protocol has been synthesized with the thiol groups protected as *tert*-butyl disulfides. Liberation of the free thiols is achieved by reduction of the mixed disulfides with DTT or other reducing agents, such as glutathione. Following removal of the DTT by continuous flow dialysis, the intramolecular disulfide bond is formed in the dilute RNA solution by air oxidation in a mildly basic solution (e.g., pH 8.0).

Materials

tert-Butyl-disulfide-modified RNA (see Support Protocol 1)
100 mM sodium phosphate buffer, pH 8.3 (see recipe)
Dithiothreitol (DTT)
Sodium phosphate buffer/NaCl, pH 7.0 (see recipe)
5000-molecular-weight-cutoff (MWCO) cellulose ester membrane
200 mM $MgCl_2$ (see recipe)
0.1 N NaOH
TE buffer (*APPENDIX 2A*)
1.5 M sodium acetate (NaOAc), pH 5.5
Absolute ethanol

10-well (500 µL) microdialyzer (e.g., Spectrum)
0.2-µm bottle-top filter
Argon (Ar) tank
Peristaltic pump
70°C water bath
pH meter with microelectrode

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (e.g., *CPMB UNIT 7.6* and *APPENDIX 3B* of this manual) *or* for derivatization and fluorescence spectroscopy for quantitation of thiols (see Support Protocol 3)

Remove tert-butyl disulfide protecting groups to liberate free thiols

1. In a 0.5-mL microcentrifuge tube, dissolve the *tert*-butyl-disulfide-modified RNA (0.5 OD_{260} U) to a concentration of 50 µM in 16 µL of 100 mM sodium phosphate buffer, pH 8.3.

2. Add 0.054 mg DTT (200 eq per disulfide), close the tube, and incubate the mixture at 25°C for 12 hr.

   The authors have found that between 12 and 14 hr of incubation effects complete reduction of the tert-butyl disulfide protecting groups. This can be conveniently conducted overnight.

3. Open the tube and dilute the reaction mixture with 100 µL sodium phosphate buffer/NaCl, pH 7.0.

4. Assemble a 10-well continuous-flow microdialyzer with a 5000-MWCO cellulose ester membrane following the manufacturer's instructions.

   Other types of dialysis assemblies should work as well.

5. Filter 3 L sodium phosphate buffer/NaCl through a 0.2-µm bottle-top filter and sparge the filtered buffer with Ar for ≥30 min before use.

6. Fill the dialysis chamber with sodium phosphate buffer/NaCl, removing all air bubbles from under the wells by forcing them out one of the flow ports as the chamber fills.

7. Transfer the reduced RNA to a well of the microdialyzer and rinse the reaction tube with phosphate buffer/NaCl, adding the rinsings to the dialyzer well. Dialyze the RNA against the same buffer at a flow rate of 5.0 mL/min for 10 hr at room temperature.

   Control experiments show that this method completely removes the DTT.

8. Transfer the dialyzed RNA sample from the dialyzer well to a 1.5-mL microcentrifuge tube. Rinse the well of the dialyzer with 100 to 200 µL of sodium phosphate buffer/NaCl and add to the RNA sample.

Form intramolecular disulfide bonds via air oxidation

9. Dilute the reduced, dialyzed tRNA sample to a final RNA concentration of 1 to 4 µM with sodium phosphate buffer/NaCl to prevent intermolecular disulfide bonds from forming.

10. Add 200 mM $MgCl_2$ stock solution to 5 mM final concentration. Fold the RNA by heating the RNA sample to 70°C for 2 min in a water bath and then allowing the sample to cool to room temperature (15 to 30 min).

    This procedure has been optimized for yeast $tRNA^{Phe}$ samples; details of the folding protocol (i.e., time and temperature for refolding, with Mg^{2+} concentration) will be different for other RNAs.

11. Calibrate a microelectrode at pH 7.0. Immerse the electrode in the RNA sample. Adjust the pH of the sample to 8.0 with 0.1 N NaOH (typically 3 to 5 µL for a 400-µL sample).

12. Stir the RNA solution at room temperature exposed to air, loosely covering the tubes with a paper towel to minimize dust contamination.

13. Monitor disulfide bond formation by removing 5-µL aliquots of the reaction mixture and assessing them either by denaturing PAGE (e.g., *CPMB UNIT 7.6*) or by fluorescence spectroscopy after derivatization with 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (see Support Protocol 3). Disulfide bond formation is usually complete in ≤12 hr.

    Progress of cross-linking can be monitored throughout; however frequent monitoring is time-consuming and will decrease final yields.

    Cross-linked RNAs have slower gel mobility than the non-cross-linked RNAs (Sigurdsson and Eckstein, 1996).

    The fluorescence assay using 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (Parvari et al., 1983) is more sensitive than with Ellman's reagent.

14. Conduct native gel electrophoresis (e.g., *CPMB UNIT 2.7*) to verify formation of intramolecular cross-links.

15. Dialyze the cross-linked RNA against 1 L TE buffer using continuous-flow microdialysis as described in steps 4 to 8.

    Do not precipitate directly from the phosphate buffer, as recovered yields will be poor.

    Disulfide cross-link formation is usually quantitative, as judged by ethidium bromide staining of a sample analyzed by PAGE, and purification to remove non-cross-linked RNA is not necessary.

Purify RNA from eluate

16. Add ⅕ vol of 1.5 M NaOAc, pH 5.5, and 3 vol absolute ethanol. Mix thoroughly and place at –20°C overnight.
17. Centrifuge 1 hr at 16,000 × *g*, 4°C.
18. Decant the solution and dry the pellet of RNA under vacuum.
19. Combine the RNA into one tube by dissolving the pellets in water and transferring to one tube. Rinse the tubes at least 3 times with water to ensure transfer of all RNA. Evaporate the RNA solutions dryness under vacuum and store at –20°C until use.
20. Store the precipitated RNA at −20°C (stable at least 1 year).

SUPPORT PROTOCOL 1

SYNTHESIS OF RNA CONTAINING *tert*-BUTYLDISULFIDE-MODIFIED NUCLEOSIDES BY SOLID-PHASE METHODS

Incorporation of modified nucleosides into an RNA can be accomplished with the necessary phosphoramidites and solid-phase synthesis technology. The following procedure has been optimized to use standard, commercially available ribonucleoside phosphoramidites and reagents combined with thiol-modified nucleoside phosphoramidites to allow total chemical synthesis of RNAs at least 76 nucleotides long. Careful handling to minimize exposure of the phosphoramidites and reagents to moisture throughout the procedure enables milligram quantities of 76-mer RNAs containing modified nucleosides to be obtained from a single 1-µmol-scale synthesis.

Materials

Methylene chloride (CH_2Cl_2) in CaH_2
Acetonitrile (CH_3CN) in CaH_2
Thiol-modified nucleoside phosphoramidites (see Support Protocols 4, 7, and 8)
Nucleoside phosphoramidites
Argon (Ar)
Anhydrous ethanol
Absolute ethanol, room temperature and –20°C
Brine (saturated aqueous NaCl)
Nitrogen (N_2) stream
Ammonia gas (NH_3)
1.0 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF)
1.5 M sodium acetate (NaOAc), pH 5.5 (see recipe)
Ethyl acetate (EtOAc)

Rotoevaporator
Automated nucleic acid synthesizer
Trap-Pak molecular sieve bags (Perseptive Biosystems)
Rubber septa
Cannula
CPG column loaded with appropriate nucleoside (purchased, or see Support Protocol 5)
Desiccator
1- and 2-dram glass vials (oven dried >8 hr at 180°C)
Teflon tape
Dry bath, 55°C
Speedvac evaporator or equivalent
Spatula, RNase-free

5-in. (12.5-cm) glass pipets
Rotary shaker

Prepare thiol-modified phosphoramidites for automated synthesis

1. At least 24 hr before beginning the RNA synthesis, distill 1 L CH_2Cl_2 and 100 mL CH_3CN from CaH_2.

2. Dry the necessary thiol-modified phosphoramidites by coevaporation with distilled CH_3CN (~1 mL/50 mg) three times, then under high vacuum overnight.

   The quantity of phosphoramidite depends on both the RNA sequence (if the same or different nucleosides are used) and the number of sequences being synthesized at one time. One coupling typically takes 5 mg dissolved in 5 mL on a Perkin-Elmer Expedite 8909 synthesizer.

3. Prepare the automated nucleic acid synthesizer for RNA synthesis following the manufacturer's suggested protocols.

   Replacing all reagent solutions on the synthesizer with newly opened bottles of reagents immediately prior to use significantly improves both the quality and the yield of the synthesis. Although commercial preparations of 3% TCA can be used, it is recommended that solutions of 3% TCA be made with freshly distilled CH_2Cl_2 immediately prior to synthesis. The use of Trap-Pak molecular sieve bags to remove residual water in the solvent in CH_3CN solutions (one Trap-Pak per liter) is also recommended.

   The protocols described here have been conducted with both $A^{N\text{-}Bz}$, $C^{N\text{-}Bz}$, $G^{N\text{-}iBu}$, and U 5′-O-dimethoxytrityl, 2′-O-tert-butyldimethylsilyl, 3′-O-diisopropyl-β-cyanoethyl phosphoramidites as well as $A^{N\text{-}Pac}$ and $G^{N\text{-}Pac}$ 5′-O-dimethoxytrityl, 2′-O-tert-butyldimethylsilyl, and 3′-O-diisopropyl-β-cyanoethyl phosphoramidites. Other exocyclic amine-protecting groups should also be suitable for these protocols.

4. Backfill the flask(s) containing the thiol-modified phosphoramidite(s) with Ar and quickly seal with a rubber septum.

5. Working under Ar, dilute the thiol-modified phosphoramidite(s) with distilled CH_3CN to 50 mg/mL. Using a cannula, place the solution into an oven-dried bottle of appropriate size for the synthesizer and place on the synthesizer.

   This amidite concentration is recommended for an Expedite 8909 synthesizer (Perseptive Biosystems). Other synthesizers will also work; amidites should be diluted according to the manufacturer's protocols.

6. Prime the delivery lines with all ancillary reagents twice, and then prime the delivery lines with the amidite solutions twice, to ensure that the flow lines from each bottle of reagent and from the amidite bottle are filled.

7. Place a CPG column loaded with the appropriate nucleoside onto the synthesizer.

   See Glick (1991) for details on the loading of thiol-modified supports.

8. Begin the RNA synthesis following the manufacturer's protocols.

   Addition of CH_2Cl_2 wash steps both before and after TCA treatment improves coupling efficiencies (see Goodwin et al., 1996).

   RNA synthesis is very sensitive to water. Eliminate water from all reagents and handling procedures as much as possible. The use of freshly distilled CH_3CN and CH_2Cl_2 and newly opened dry reagents improves coupling efficiencies and allows the synthesis of RNAs exceeding 75 residues. It is also recommended that phosphoramidites be handled under an Ar atmosphere in a glove bag, especially in humid conditions. While solutions of A, C, and U phosphoramidites are stable for at least 5 days, the coupling efficiency of G phosphoramidites is increased with fresh dilution on a daily basis. A typical RNA coupling cycle

takes 15 min (Gait et al., 1991; Goodwin et al., 1994). The synthesis of one 76-mer tRNA sequence takes ~19.5 hr.

Deprotect synthetic RNA

9. Cool a 2-dram oven-dried vial under N_2.

10. Add 2 to 3 mL anhydrous ethanol to the vial and cool in a brine/ice water bath under N_2 for 2 to 5 min.

11. Remove the N_2 line and insert a needle attached to a lecture bottle of NH_3 and a vent. Saturate the ethanol by constant bubbling with NH_3 for at least 30 min.

    This procedure should be conducted in a fume hood.

12. Open the synthesis column and pour the solid support into a 1-dram oven-dried vial.

13. Quickly pour ~1 mL of the anhydrous ethanolic ammonia solution into the vial. Seal threads of the vial with Teflon tape and cap tightly. Seal the outside of the cap with Teflon tape and parafilm to prevent evaporation.

    The inner Teflon tape can be put on the vial before pouring the solution, but be careful not to pour the ethanolic ammonia onto the tape or the seal will not be adequate. Usually bubbling occurs as the solution is poured onto the resin.

14. Place the sealed vial in a 55°C dry bath for 18 hr.

15. Remove the vial from the dry bath and allow to cool to room temperature before opening. Carefully open the vial and pass a gentle stream of N_2 over the solution for 5 to 15 min to remove the ammonia.

    When the cap is removed, there should be a small release of pressure; this indicates that the ethanolic ammonia solution was thoroughly saturated during deprotection.

    A significant portion of the ethanol may evaporate during this procedure. If the solution becomes viscous (typically, with less than ~300 μL ethanol remaining), add 500 μL of anhydrous ethanol to the vial before proceeding to step 16.

16. Transfer the ethanol solution from the vial to an autoclaved 1.5-mL microcentrifuge tube and evaporate under vacuum to ≤400 μL total volume. Transfer any remaining solution to the microcentrifuge tube and then rinse the solid support with 500 μL anhydrous ethanol. Add the rinsing to the microcentrifuge tube and evaporate the solution under vacuum to ≤400 μL.

17. Thoroughly rinse the solid support with anhydrous ethanol five more times using 100 to 200 μL ethanol each time, adding the rinsings to the microcentrifuge tube. Evaporate the solution to dryness under vacuum. Coevaporate with 500 μL anhydrous ethanol. Repeat the coevaporation once more using 200 μL ethanol.

    The residue resulting from step 17 may be stored for a few days at −20°C before proceeding with the remainder of this protocol.

Desilylate synthetic RNA

18. Cool a 1-dram oven-dried vial in a desiccator.

19. Gently loosen the residue obtained after step 17 from the microcentrifuge tube with the tip of an RNase-free spatula. Carefully transfer the residue to the dried vial.

20. Add ~400 μL of 1.0 M TBAF in THF to the microcentrifuge tube with a glass pipet. Vortex, centrifuge, and transfer the solution to the vial with a glass pipet. Repeat this rinsing procedure twice more using ~300 μL TBAF each time.

TBAF solutions should be of the highest purity and lowest water content possible. For optimal results, bottles of TBAF should only be used once. TBAF is light sensitive.

21. Seal the vial with Teflon tape, parafilm, and cover in foil. Place the vial on a rotary shaker and shake at low speed for 24 hr.

22. Open the vial and quench the reaction with 1 mL of 1.5 M NaOAc, pH 5.5. Transfer the solution in two approximately equal aliquots into two 1.5-mL microcentrifuge tubes. Rinse the vial 5 times with 150 µL water and add the rinsings to the tubes.

23. Evaporate the solutions to ~600 µL total volume in each tube. Add 600 to 700 µL EtOAc to each tube and homogenize as thoroughly as possible. Centrifuge to separate the layers. Remove the upper layer (EtOAc) with a pipet and repeat the extraction a second time. Evaporate the solution under vacuum for ~10 min to remove residual EtOAc.

24. Transfer about half of the solution in each tube to another 1.5-mL microcentrifuge tube, resulting in four tubes containing ~300 µL solution each.

25. Add 1 mL absolute ethanol to each tube, mix thoroughly, and place at −20°C overnight.

26. Centrifuge all tubes for at least 1 hr at 16,000 × *g*, 4°C. Decant the supernatant from the pellet and then wash the pellet with 200 µL of –20°C absolute ethanol. Dry the pellet under vacuum.

27. Quantify the crude RNA by dissolving the pellets in water and measuring the absorbance of a 1-µL aliquot of the solution at 260 nm. Estimate the molar extinction coefficient (ε_{260}) using nearest-neighbor calculations.

    The ε_{260} calculation is done as described by Breslauer et al. (1986); see UNIT 7.3. *This assumes that the modified residues behave like their unmodified counterparts.*

    Both crude and purified RNA samples should be stored either pelleted from ethanol precipitation or dried down from aqueous solutions at −20°C.

28. Purify the crude RNA to single-nucleotide resolution (see Support Protocol 2).

PURIFICATION OF SYNTHETIC RNA CONTAINING *tert*-BUTYLDISULFIDE PROTECTED THIOL-MODIFIED NUCLEOSIDES

SUPPORT PROTOCOL 2

Single-nucleotide resolution of the products resulting from solid-phase synthesis, the full-length oligomer as well as failed sequences, can be achieved using denaturing PAGE (also see *APPENDIX 3B*). The procedures that are outlined have been optimized for the purification of a 76-mer tRNA sequence. These protocols can be modified for sequences of other lengths by adjusting the percentage of acrylamide in the gel matrix until both the full-length RNA and the sequence resulting from incomplete synthesis one residue from the end are resolved.

Materials

Crude synthetic tRNA (see Support Protocol 1)
80% formamide containing 0.05% xylene cyanol (XC) tracking dye
8% denaturing polyacrylamide solution (see recipe)
1× TBE electrophoresis buffer (*APPENDIX 2A*)
1× and 4× TAE electrophoresis buffer (*APPENDIX 2A*)
1.5 M sodium acetate (NaOAc), pH 5.5
Absolute ethanol

Power supply
Silica-gel plate

UVG-11 Mineralight lamp (254 nm, 115 V) or equivalent
Razor blade, RNase free
Hoefer Six-Pac electroeluter or equivalent
Inner elution tubes
Porous polyethylene plugs
Blotter-paper discs

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (e.g., see *CPMB UNIT 7.6* and *APPENDIX 3B* of this manual)

Separate RNA species by PAGE

1. Dissolve 20 to 40 OD_{260} U of crude synthetic tRNA in 60 µL water, mix thoroughly, and allow to stand for ~1 hr to thoroughly dissolve the RNA.

   There will probably also be insoluble material present.

2. Add 60 µL of 80% formamide/0.05% XC and centrifuge briefly to separate the insoluble matter.

3. Prepare an 8% denaturing polyacrylamide gel 31.0 cm × 38.5 cm × 0.8 mm, with 14.6-mm-wide wells (e.g., see *CPMB UNIT 7.6* and *APPENDIX 3B*).

   Electrophoresis conditions are outlined for the purification of a 76-nt tRNA sequence. For sequences of different lengths, adjust the percentage acrylamide and electrophoresis time accordingly.

4. Load 20 µL of the RNA sample into each of six wells, being careful not to load any insoluble material onto the gel.

5. Electrophorese the gel in 1× TBE at 55 W until the XC tracking dye has migrated ~11 inches (~4 to 4.5 hr).

   Under these conditions 76-mer RNA migrates with the XC—be careful not to run the RNA off the gel.

Electroelute full-length synthetic RNA from gel slice

6. Place the gel over a silica-gel plate in a dark location. Briefly shine 254-nm light on the gel to locate the RNA. With a clean RNase-free razor blade, excise the full-length RNA from the gel.

   RNA is photoreactive; minimize exposure of RNA to UV light.

7. Pack the RNA slices into three or four inner elution tubes containing 300 µL of 1× TAE using blotter-paper discs and porous polyethylene plugs according to manufacturer's directions. Cut off the bottom tip of the inner elution tube and insert into a 1.5-mL microcentrifuge tube containing 200 µL of 4× TAE. Electroelute in a Hoefer Six-Pac eluter for 90 min at +50 V.

   For efficient electroelution, remove all air bubbles from the inner elution tubes. Also, the elutions work better if the inner elution tubes are packed tightly with gel slices, but it is important not to crush the slices. If the amperage does not decrease below 0.2 mA during the course of electroelution, the gel slices can be recovered from the inner elution tube and soaked in 400 µL of 4× TAE at room temperature overnight. The eluted RNA should then be ethanol precipitated as described in the next step and combined with the RNA eluted during electroelution.

8. Remove the inner elution tube carefully, rinse the electrode with 20 µL water, and add to the eluted RNA. Remove the electrode and pipet all buffer remaining above the porous plug into the eluted RNA.

   The total volume should be ~350 µL.

Purify RNA from eluate

9. Add 70 µL of 1.5 M NaOAc, pH 5.5, and 1 mL absolute ethanol. Mix thoroughly and place at −20°C overnight.

10. Centrifuge 1 hr at 16,000 × *g*, 4°C.

11. Decant the solution and dry the pellet of RNA under vacuum.

12. Combine the RNA into one tube by dissolving the pellets in water and transferring to one tube. Rinse the tubes at least 3 times with water to ensure transfer of all RNA. Evaporate the RNA solutions dryness under vacuum and store at −20°C until use (stable at least 1 year).

QUANTIFICATION OF THIOLS IN RNA USING 7-DIETHYLAMINO-3-(4′-MALEIMIDYLPHENYL)-4-METHYLCOUMARIN

SUPPORT PROTOCOL 3

Reaction of free thiols with 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin produces a fluorescent adduct that can be quantified spectroscopically (Parvari et al., 1983). This provides a convenient method to determine when the cross-linking reaction is complete. The method can be used to accurately determine thiol concentrations as low as 0.5 µM.

Materials

RNA solution containing free thiols (see Basic Protocol)
5× CPM buffer (see recipe)
0.4 mM CPM in isopropyl alcohol (see recipe)
1% Triton X-100
Sodium phosphate buffer/NaCl, pH 7.0 (see recipe)
Fluorimeter and cuvette

1. To a 5-µL aliquot of an RNA solution containing free thiols, add 1 µL of 5× CPM buffer and 5 µL of 0.4 mM CPM in isopropyl alcohol. Incubate the sample 10 min at room temperature.

2. Dilute the reaction with 489 µL of 1% Triton X-100.

3. Prepare a blank consisting of:

   5 µL sodium phosphate buffer/NaCl, pH 7.0
   1 µL 5× CPM buffer
   5 µL 0.4 mM CPM in isopropyl alcohol
   489 µL Triton X-100.

4. Transfer blank solution to a fluorimeter cuvette and measure the fluorescence intensity at 480 nm (λ_{ex} = 390 nm).

5. In the same way, measure the fluorescence intensity of the RNA solution from step 2, and correcting for background fluorescence.

   A calibration curve can be constructed using DTT as a standard (0.5 to 5 mM is a useful range).

SUPPORT PROTOCOL 4

SYNTHESIS OF 5′-*O*-(4,4′-DIMETHOXYTRITYL)-2′-*O*-(*tert*-BUTYLDIMETHYLSILYL)-N^3-(ETHYL)URIDINE-3′-*O*-(*N*,*N*-DIISOPROPYL-β-CYANOETHYLPHOSPHORAMIDITE) *tert*-BUTYL DISULFIDE (S.5)

The preparation of a thiol-containing nucleoside phosphoramidite modified at the N^3 position of uridine is described. The synthesis of 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-N^3-(ethyl)uridine-3′-*O*-(*N*,*N*-diisopropyl-β-cyanoethylphosphoramidite) *tert*-butyl disulfide (**S.5**) proceeds in four sets of steps from uridine (Fig. 5.4.1). The 3′-*O*-*tert*-butyldimethylsilyl-protected isomer (**S.4**) obtained as a side product of the third set of steps is used to make a thiol-modified controlled-pore glass (CPG) support (Fig. 5.4.2; see Support Protocol 5).

Materials

Uridine
Distilled acetonitrile (CH_3CN)
Distilled triethylamine (Et_3N)
N,N-Dimethylformamide (DMF)
Chlorotrimethylsilane (TMSCl)
N_2
Petroleum ether
Diethyl ether (Et_2O)
Sodium hydride (NaH)
1-Tosyl-2-benzoylmercaptoethanol (see Glick et al., 1991)
48% (w/v) aqueous HF
Methylene chloride (CH_2Cl_2)
Brine (saturated aqueous NaCl)
Sodium sulfate (Na_2SO_4)

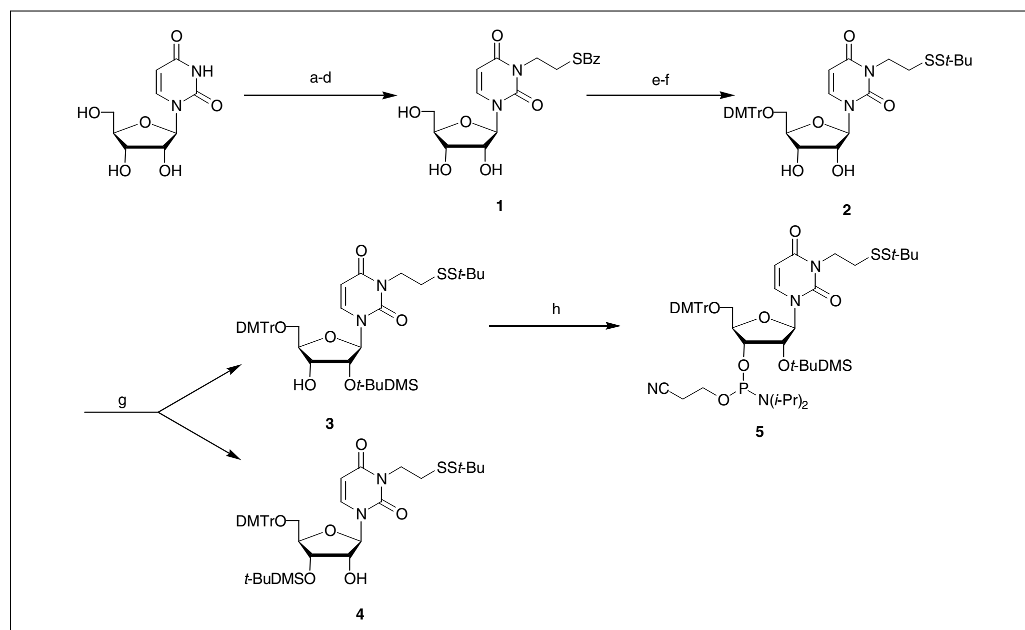

Figure 5.4.1 Synthesis of 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-N^3-(ethyl)uridine-3′-*O*-(*N*,*N*-diisopropyl-β-cyanoethylphosphoramidite) *tert*-butyl disulfide. (**a**) TMSCl, Et_3N, DMF; (**b**) NaH, DMF; (**c**) *p*-$TsOCH_2CH_2SBz$, DMF; (**d**) HF (aq); (**e**) DMTrCl, pyridine; (**f**) 1-(*tert*-butylthio)-1,2-hydrazinedicarboxmorpholide, LiOH, CH_3OH; (**g**) TBDMSCl, imidazole, DMF; (**h**) chloro-*N*,*N*-diisopropylamine-β-cyanoethyl phosphine, 2,4,6-collidine, *N*-methylimidazole, THF. Abbreviations: Bz, benzoyl; DMF, dimethylformamide; DMTr, 4,4′-dimethoxytrityl; TBDMS, *tert*-butyldimethylsilyl; THF, tetrahydrofuran; Ts, tosyl.

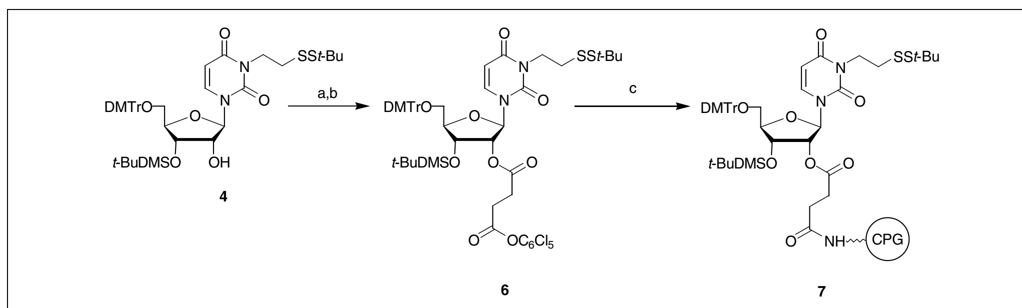

Figure 5.4.2 Synthesis of 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-2′-*O*-succinyl-CPG N^3-(ethyl)uridine *tert*-butyl disulfide. (**a**) succinic anhydride, DMAP, pyridine; (**b**) pentachlorophenol, DCC, DMAP, CH_2Cl_2; (**c**) CPG (1000 Å), Et_3N, DMF. Abbreviations: DCC, dicyclohexyl-carbodiimide; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide.

Methanol (CH_3OH)
Pyridine
4,4′-Dimethoxytrityl chloride (DMTrCl)
1-(*tert*-Butylthio)-1,2-hydrazine carboxmorpholide (see Wünsch et al., 1982)
$LiOH{\cdot}H_2O$
Ethyl acetate (EtOAc)
Imidazole
tert-Butyldimethylsilyl chloride (TBDMSCl)
Tetrahydrofuran (THF)
2,4,6-Collidine
N-Methylimidazole
Chloro-*N*,*N*-diisopropylamine-β-cyanoethyl phosphine

Additional reagents and equipment for flash chromatography (*APPENDIX 3E*)

Prepare N^3-(2-thiobenzoylethyl)uridine (S.1)

1. Dry 24.42 g uridine (100 mmol) by coevaporation with distilled CH_3CN.
2. Dissolve the dried uridine in 83.6 mL freshly distilled Et_3N (600 mmol, 6 eq) and 250 mL DMF.
3. Cool the solution to 4°C.
4. Slowly add 42 mL chlorotrimethylsilane (330 mmol, 3.3 eq) and stir the reaction under N_2 for 2 hr.
5. Remove salts that precipitate during the course of the reaction by filtration under N_2.
6. Triturate the residual salts in the filtrate with 1:1 (v/v) petroleum ether/diethyl ether.
7. Dissolve the residue in 400 mL DMF and cool to 4°C.
8. Add 4.40 g NaH (110 mmol, 1.1 eq) to the reaction mixture with stirring under N_2.
9. After hydrogen evolution subsides, add 37 g 1-tosyl-2-benzoylmercaptoethanol (110 mmol, 1.1 eq) and stir the reaction overnight at 45°C.

   This step is based on the procedure detailed by Glick (1991).

10. Cool the solution to room temperature and remove the silyl groups by the addition of 5 mL of 48% aqueous HF.

11. After 1 hr, dilute the reaction mixture with CH_2Cl_2 and wash successively with water and brine.

12. Dry the organic layer over Na_2SO_4 and concentrate under vacuum.

13. Purify the oily residue by flash chromatography (*APPENDIX 3E*) using 19:1 (v/v) CH_2Cl_2/CH_3OH to obtain **S.1** as a white foam (26 g, 63% yield).

Prepare 5′-O-(4,4′-dimethoxytrityl)-N^3-(ethyl)uridine tert-butyl disulfide (S.2)

14. Coevaporate 15.0 g compound **S.1** (37 mmol) once from 100 mL of 9:1 (v/v) CH_3CN/pyridine, then dissolve in 185 mL pyridine.

15. Add 15.0 g 4,4′-dimethoxytrityl chloride (44 mmol, 1.2 eq) in 2.5-g portions over a 6-hr period at 4°C with stirring.

16. Allow the reaction to warm to room temperature overnight while stirring under N_2.

17. Add 5 mL CH_3OH and stir the mixture an additional 10 min.

18. Remove the solvents under vacuum, and coevaporate the residue with 100 mL CH_3CN to yield a light yellow-orange foam.

19. Dissolve the crude tritylated product (14 g, 20 mmol) in 100 mL CH_3OH and add 8.31 g 1-(*tert*-butylthio)-1,2-hydrazinedicarboxmorpholide (Wünsch et al., 1982) (24 mmol, 1.2 eq) and 2.52 g $LiOH{\cdot}H_2O$ (60 mmol, 3.0 eq).

20. Stir the reaction under N_2 for 12 hr and then concentrate the mixture under vacuum.

21. Dissolve the residue in EtOAc and wash with brine.

22. Dry the organic layer over Na_2SO_4 and concentrate under vacuum.

23. Purify the residue by flash chromatography (*APPENDIX 3E*) using a step gradient of 3:2 to 2:3 (v/v) petroleum ether/EtOAc to obtain **S.2** as a white foam (9.7 g, 70% yield).

Prepare 5′-O-(4,4′-dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-N^3-(ethyl)uridine tert-butyl disulfide (S.3) and 5′-O-(4,4′-dimethoxytrityl)-3′-O-(tert-butyldimethyl-silyl)-N^3-(ethyl)uridine tert-butyl disulfide (S.4)

24. Dissolve 9.5 g compound **S.2** (13.6 mmol) in 50 mL DMF.

25. Add 2.31 g imidazole (34 mmol, 2.5 eq) and 2.58 g *tert*-butyldimethylsilyl chloride (17.1 mmol, 1.25 eq).

26. Stir the mixture overnight under N_2.

27. Dilute the reaction with EtOAc, wash the mixture with brine, dry over Na_2SO_4, and evaporate under vacuum.

28. Purify the residue by flash chromatography (*APPENDIX 3E*) using 9:1 (v/v) petroleum ether/EtOAc to obtain **S.3** and **S.4** as white foams (**S.3**: 3.9 g, 40% yield; **S.4**: 2.1 g, 25% yield).

Prepare 5′-O-(4,4′-dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-N^3-(ethyl)uridine-3′-O-(N,N-diisopropyl-β-cyanoethylphosphoramidite) tert-butyl disulfide (S.5)

29. Dissolve 0.81 g compound **S.3** (1.0 mmol) in 3.0 mL THF.

30. Add 1.0 mL 2,4,6-collidine (7.5 mmol, 7.5 eq) and 40 µL *N*-methylimidazole (0.5 mmol, 0.5 eq).

31. Add 0.56 mL chloro-*N*,*N*-diisopropylamine-β-cyanoethyl phosphine (2.5 mmol, 2.5 eq) dropwise while stirring under N_2.

32. After 2 hr, dilute the reaction with EtOAc and wash the mixture with $NaHCO_3$ and brine.

33. Dry the organic layer over Na_2SO_4, and concentrate under vacuum.

34. Purify the residue by flash chromatography (*APPENDIX 3E*) using 80:15:5 (v/v/v) petroleum ether/EtOAc/Et_3N to obtain **S.5** as a brittle white foam (0.82 g, 82% yield).

SYNTHESIS OF 5′-*O*-(4,4′-DIMETHOXYTRITYL)-3′-*O*-(*tert*-BUTYLDIMETHYLSILYL)-2′-*O*-SUCCINYL-CPG *N*^{3}-(ETHYL)URIDINE *tert*-BUTYL DISULFIDE CONTROLLED-PORE GLASS SUPPORT

SUPPORT PROTOCOL 5

5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-2′-*O*-succinyl-CPG *N*^{3}-(ethyl)-uridine *tert*-butyl disulfide (Fig. 5.4.2), a thiol-modified controlled-pore glass (CPG) support, is prepared using a side product (**S.4**) obtained in Support Protocol 4. This nucleoside support can be used to introduce a thiol modification at the 3′ terminus of an RNA species. The nucleoside loading concentration is usually 32 µmol/g (Schaller et al., 1963).

Materials

Compound **S.4** (see Support Protocol 4)
Pyridine
Succinic anhydride
4-Dimethylaminopyridine (DMAP)
Methylene chloride (CH_2Cl_2)
Brine (saturated aqueous NaCl)
Sodium sulfate (Na_2SO_4)
Pentachlorophenol
Dicyclohexylcarbodiimide (DCC)
Petroleum ether
Ethyl acetate (EtOAc)
Long-chain alkyl amino controlled-pore glass (CPG), 1000-Å pore size, 100 µmol amino groups/g, 120/200 mesh
N,*N*-Dimethylformamide (DMF)
Triethylamine (Et_3N)
Methanol (CH_3OH)
Diethyl ether (Et_2O)
Acetic anhydride

Additional reagents and equipment for flash chromatography (*APPENDIX 3E*)

Prepare 5′-O-(4,4′-dimethoxytrityl)-3′-O-(tert-butyldimethylsilyl)-2′-O-pentachlorophenylsuccinate N^3*-(ethyl)uridine tert-butyl disulfide (S.6)*

1. Dissolve 1.77 g compound **S.4** (2.2 mmol) in 12.0 mL pyridine under N_2.

2. Add 0.69 g succinic anhydride (6.6 mmol, 3.0 eq) and 0.133 g DMAP (1.1 mmol, 0.5 eq) and incubate 12 hr.

3. Concentrate the reaction mixture under vacuum.

4. Dissolve the residue in CH_2Cl_2 and wash with brine.

5. Dry the organic layer over Na_2SO_4 and concentrate under vacuum.

6. Dissolve the crude succinate in 25 mL CH_2Cl_2 and add 0.88 g pentachlorophenol (3.3 mmol, 1.5 eq), 67 mg DMAP (0.55 mmol, 0.25 eq), and 0.91 g DCC (4.4 mmol, 2.0 eq).
7. After 8 hr, add petroleum ether to precipitate dicyclohexylurea.
8. Gravity filter the reaction mixture and concentrate under vacuum.
9. Purify the residue by flash chromatography (*APPENDIX 3E*) using 4:1 (v/v) petroleum ether/EtOAc to obtain **S.6** as a white foam (2.3 g, 90% yield).

Prepare 5′-O-(4,4′-dimethoxytrityl)-3′-O-(tert-butyldimethylsilyl)-2′-O-succinyl-CPG N^3-(ethyl)uridine tert-butyl disulfide (S.7)

10. Suspend long-chain alkyl amino CPG in 4.0 mL DMF with 0.58 g compound **S.6** (0.5 mmol, 5 eq) and 0.14 mL Et_3N (1.0 mmol, 10 eq).
11. Gently swirl the mixture in the dark for 2 days.
12. Vacuum filter the support and rinse successively with 15 mL DMF, 50 mL CH_3OH, and 50 mL Et_2O.
13. Remove the residual solvents under vacuum.
14. Acetylate the unreacted amino groups by swirling the support for 1 hr with 0.70 mL acetic anhydride (7.0 mmol, 100 eq) and 10 mg DMAP (70 µmol, 1 eq) in 4 mL pyridine.
15. Rinse the support successively with 30 mL pyridine, 90 mL CH_3OH, and 90 mL Et_2O.
16. Remove the residual solvents under vacuum.

SUPPORT PROTOCOL 6

SYNTHESIS OF 3′,5′-*O*-(TETRAISOPROPYLDISILOXANE-1,3-DIYL)-2′-*O*-ALLYL-O^4-(2-NITROPHENYL) URIDINE (S.10) INTERMEDIATE

Preparation of thiol-modified nucleoside phosphoramidite modified at the 2′-hydroxyl position of pyrimidines proceeds from preparation of 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-allyl-O^4-(2-nitrophenyl)uridine. Preparation of this compound is described. First, uridine is converted to 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-O^4-(2-nitrophenyl) uridine (**S.9**) in two sets of steps as described by Sproat and Lamond (1991). This compound is then allylated using the procedure described by Sproat et al. (1991).

Materials

Uridine
Pyridine
1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane
Methylene chloride (CH_2Cl_2)
Methanol (CH_3OH)
Saturated aqueous sodium bicarbonate ($NaHCO_3$)
Sodium sulfate (Na_2SO_4)
Triethylamine (Et_3N)
Chlorotrimethylsilane
Brine (saturated aqueous NaCl)
2-Mesitylenesulfonyl chloride
4-Dimethylaminopyridine (DMAP)
2-Nitrophenol
1,4-Diazabicyclo[2.2.2]octane (DABCO)

p-Toluenesulfonic acid monohydrate
p-Dioxane
Ethyl acetate (EtOAc)
Petroleum ether
Tetrahydrofuran (THF)
Triphenylphosphine
Tris(dibenzylideneacetone) dipalladium(0)
Allyl ethyl carbonate

Additional reagents and equipment for flash chromatography (*APPENDIX 3E*)

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-uridine (S.8)

1. Dissolve 499 mg uridine (2.04 mmol) in 5.1 mL pyridine.
2. Cool to 0°C under N_2.
3. Dissolve 732 µL 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (2.29 mmol, 1.1 eq) in 0.5 mL CH_2Cl_2 and add dropwise to uridine solution.
4. Stir the reaction, allowing it to warm to room temperature, for 4 hr.
5. Dilute the reaction by adding 0.4 mL CH_3OH, and evaporate under vacuum.
6. Dissolve the residue in 15 mL CH_2Cl_2 and wash three times with saturated aqueous $NaHCO_3$.
7. Dry the organic layer over Na_2SO_4 and evaporate under vacuum.
8. Dissolve the residue in toluene and evaporate under vacuum to obtain **S.8** as a white foam (1.0 g, 100%).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-O^4-(2-nitrophenyl) uridine (S.9)

9. Dissolve 3.4 g compound **S.8** (6.9 mmol) in 35 mL CH_2Cl_2 and cool to 0°C.

   It may be necessary to repeat the synthesis of ***S.8*** *to obtain the necessary quantity for the synthesis of* ***S.9****.*

10. Add 9 mL Et_3N (64.6 mmol, 9.4 eq) followed by 6.4 mL chlorotrimethylsilane (50.4 mmol, 7.3 eq) and stir the reaction 4 hr under N_2.
11. Pour the reaction onto 100 mL saturated aqueous $NaHCO_3$ and stir for 10 min.
12. Wash the organic layer with brine, dry over Na_2SO_4, and concentrate under vacuum to a peach foam (3.8 g, 100%).
13. Dissolve the residue in 36 mL CH_2Cl_2 and 4.8 mL Et_3N (34.6 mmol, 5 eq).
14. Add 2.25 g 2-mesitylenesulfonyl chloride (10.3 mmol, 1.5 eq) and 0.5 g DMAP (3.5 mmol, 0.5 eq). Stir for 15 min.
15. Add 2.0 g 2-nitrophenol (14.4 mmol, 2.1 eq) and 0.42 g DABCO (3.7 mmol, 0.55 eq) and continue stirring for 3 hr.
16. Dilute the reaction with 40 mL CH_2Cl_2 and wash with saturated aqueous $NaHCO_3$.
17. Backwash the aqueous layer twice with CH_2Cl_2.
18. Dry the combined organic layers over Na_2SO_4 and concentrate under vacuum.
19. Dissolve the residue in 21 mL CH_2Cl_2 and add a solution of 2.70 g *p*-toluenesulfonic acid monohydrate in 21 mL *p*-dioxane.

20. Stir the reaction for 2 min and then quench by adding 2.5 mL Et_3N.

21. Pour the reaction onto saturated aqueous $NaHCO_3$ and backwash the aqueous layer with CH_2Cl_2 twice.

22. Dry the combined organic layers over Na_2SO_4 and concentrate under vacuum.

23. Purify the residue by flash chromatography (APPENDIX 3E) using 55% EtOAc in petroleum ether to obtain **S.9** (4.5 g, 6.9 mmol, 100%).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-O-allyl-O⁴-(2-nitrophenyl) uridine (S.10)

24. Dissolve the 4.5 g compound **S.9** in 37 mL THF (37 mL).

25. Add 371 mg triphenylphosphine (1.4 mmol, 0.2 eq) and 168 mg tris(dibenzylideneacetone) dipalladium(0) (0.2 mmol, 0.026 eq).

26. Add 1.9 mL allyl ethyl carbonate (14.0 mmol, 2 eq) dropwise.

27. Heat the reaction to reflux for 2 hr.

28. Cool the reaction to room temperature and concentrate under vacuum.

29. Purify the residue by flash chromatography (APPENDIX 3E) using 18% EtOAc in petroleum ether to obtain **S.10** (3.3 g, 72%).

SUPPORT PROTOCOL 7

SYNTHESIS OF 5′-*O*-(4,4′-DIMETHOXYTRITYL)-*N*⁴-(BENZOYL)-2′-*O*-(ETHYL)CYTIDINE-3′*O*-(*N,N*-DIISOPROPYL-β-CYANOETHYLPHOSPHORAMIDITE) *tert*-BUTYL DISULFIDE (S.20)

The intermediate prepared in Support Protocol 6 is converted to 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-*N*⁴-(benzoyl)-2′-*O*-allyl cytidine (**S.11**) using the method of Sproat and Lamond (1991) replacing isobutyryl chloride with benzoyl chloride. The synthesis of the fully protected phosphoramidite can be accomplished in nine sets of steps (Fig. 5.4.3).

Materials

Compound **S.10** (see Support Protocol 6)
Tetrahydrofuran (THF)
Ammonia (NH_3)
N_2
Methanol (CH_3OH)
Petroleum ether
Ethyl acetate (EtOAc)
Pyridine
N,N-Dimethylformamide (DMF)
Benzoic anhydride
Ammonium hydroxide (NH_4OH)
N-Methylmorpholine-*N*-oxide
Acetone
Osmium tetroxide (OsO_4)
Saturated aqueous sodium bisulfite
Diethyl ether (Et_2O)
Saturated aqueous sodium bicarbonate ($NaHCO_3$)
Brine (saturated aqueous NaCl)
Sodium sulfate (Na_2SO_4)

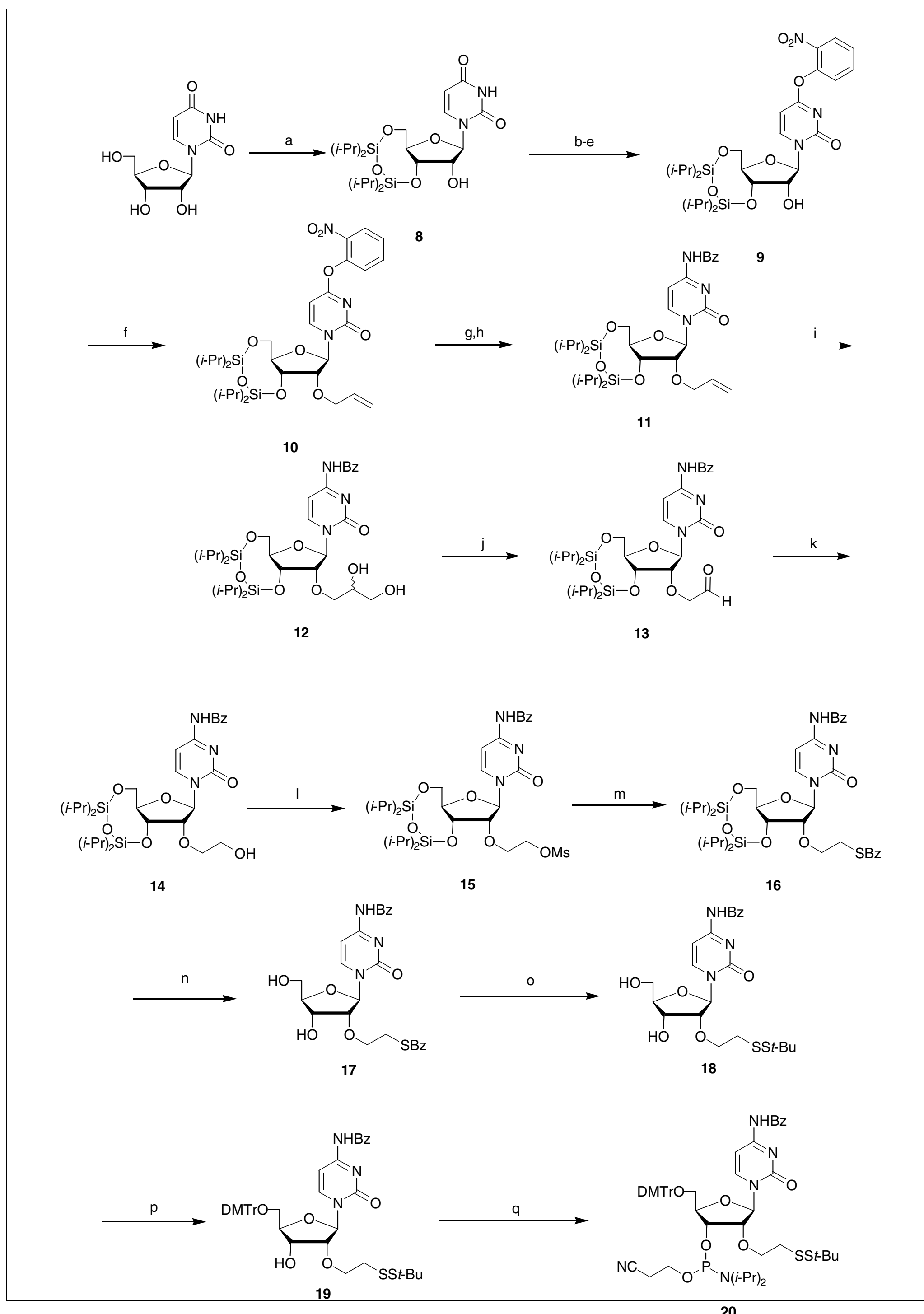

Figure 5.4.3 Synthesis of 5′-*O*-(4,4′-dimethoxytrityl)-N^4-(benzoyl)-2′-*O*-(ethyl)cytidine *tert*-butyl disulfide 3′-*O*-(*N,N*-diisopropyl-β-cyanoethylphosphoramidite). (**a**) pyridine, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, CH_2Cl_2; (**b**) chlorotrimethylsilane, Et_3N, CH_2Cl_2; (**c**) 2-mesitylenesulfonyl chloride, DMAP, Et_3N, CH_2Cl_2; (**d**) 2-nitrophenol, DABCO; (**e**) *p*-toluenesulfonic acid monohydrate, *p*-dioxane, CH_2Cl_2; (**f**) triphenylphosphine, tris(dibenzylideneacetone) dipalladium(0), allyl ethyl carbonate, THF; (**g**) NH_3, THF; (**h**) pyridine, benzoic anhydride, DMF; (**i**) OsO_4, *N*-methylmorpholine-*N*-oxide, acetone, H_2O; (**j**) $NaIO_4$, *p*-dioxane, H_2O; (**k**) $NaBH_4$, CH_3OH; (**l**) methanesulfonyl chloride, pyridine; (**m**) thiobenzoic acid, Et_3N, DMF; (**n**) HF (aq), CH_3CN; (**o**) 1-(*tert*-butylthio)-1,2-hydrazinedicarboxmorpholide, LiOH, CH_3OH, THF; (**p**) DMTrCl, Et_3N, DMF, DMAP; (**q**) chloro-*N,N*-diisopropylamine-β-cyanoethyl phosphine, *N,N*-diisopropylethylamine, CH_2Cl_2. Abbreviations: Bz, benzoyl; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; DMTr, 4,4′-dimethoxytrityl; Ms, methanesulfonyl; THF, tetrahydrofuran.

Methylene chloride (CH_2Cl_2)
p-Dioxane
Sodium periodate ($NaIO_4$)
Sodium borohydrate ($NaBH_4$)
Methanesulfonyl chloride
Triethylamine (Et_3N)
Thiobenzoic acid
Acetonitrile (CH_3CN)
48% (w/v) aqueous HF
1-(*tert*-Butylthio)-1,2-hydrazinedicarboxmorpholide (Wünsch et al., 1982)
$LiOH{\cdot}H_2O$
1 N aqueous sodium citrate
4-Dimethylaminopyridine (DMAP)
4,4′-Dimethoxytrityl chloride (DMTrCl)
N,N-Diisopropylethylamine
Chloro-*N,N*-diisopropylamine-β-cyanoethyl phosphine

Pressure tube (−78°C)
CO_2/isopropyl alcohol (*i*-PrOH) bath

Additional reagents and equipment for flash chromatography (APPENDIX 3E)

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)- N⁴-(benzoyl)-2′-O-allyl cytidine (S.11)

1. Dissolve 1.94 g compound **S.10** (2.99 mmol) in 8.5 mL THF in a pressure tube.
2. Cool the tube to −78°C using a CO_2/*i*-PrOH bath.
3. Add 5 g NH_3 (299 mmol, 100 eq).
4. Seal the reaction vessel and stir while allowing it to warm to room temperature for 62 hr.
5. Cool the reaction mixture to −78°C using CO_2/*i*-PrOH bath and open the pressure tube.
6. Allow the mixture to warm under N_2 to room temperature.
7. Concentrate under vacuum.
8. Purify the residue by flash chromatography (APPENDIX 3E) using a step gradient of 0 to 10% CH_3OH in 7:3 (v/v) petroleum ether/EtOAc.
9. Dissolve the purified residue in 14 mL pyridine and 6.5 mL DMF.
10. Add 0.72 g benzoic anhydride (3.20 mmol, 1.5 eq) and stir the reaction under N_2 for 12 hr at room temperature.
11. Cool the reaction to 0°C and add 0.4 mL water.
12. Stir the reaction 5 min at 0°C, then add 0.3 mL NH_4OH.
13. Continue stirring the reaction 10 min at 0°C, then concentrate the mixture under vacuum.
14. Purify the residue by flash chromatography (APPENDIX 3E) using 3:2 (v/v) petroleum ether/EtOAc to obtain **S.11** (1.42 g, 75% yield) as a white foam.

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)- 2′-O-(2,3-dihydroxypropyl)-cytidine (S.12)

15. Dissolve 1.34 g compound **S.11** (2.30 mmol) and 0.27 g *N*-methylmorpholine-*N*-oxide (2.34 mmol, 1.1 eq) in 21 mL of 6:1 (v/v) acetone/water.
16. Add 5 mg OsO_4 (21 µmol, 0.01 eq) and stir the reaction mixture in the dark for 2.5 hr.
17. Add 1 mL saturated aqueous sodium bisulfite to precipitate osmium salts.
18. Decant the solution and dissolve the light-brown residue in Et_2O.
19. Wash the solution with saturated aqueous $NaHCO_3$ and brine.
20. Dry the combined organic layers over Na_2SO_4 and concentrate under vacuum.
21. Purify the yellow residue by flash chromatography (*APPENDIX 3E*) using a step gradient of 19:1 to 37:3 CH_2Cl_2/CH_3OH to obtain **S.12** (1.27 g, 90% yield).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-N^4-(benzoyl)-2′-O-(ethanal)-cytidine (S.13)

22. Dissolve the 1.27 g compound **S.12** (1.91 mmol) in 20 mL of 3:1 (v/v) *p*-dioxane/water.
23. Add 0.49 g $NaIO_4$ (2.29 mmol, 1.2 eq) and stir the reaction mixture in the dark for 4.5 hr.
24. Dilute the reaction with Et_2O and wash with water.
25. Dry the organic layer over Na_2SO_4 and concentrate under vacuum.
26. Purify the residue by flash chromatography (*APPENDIX 3E*) using 19:1 (v/v) CH_2Cl_2/CH_3OH to obtain **S.13** as a white foam (2.94 g, 97% yield).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-N^4-(benzoyl)-2′-O-(2-hydroxyethyl)cytidine (S.14)

27. Dissolve 1.17 g compound **S.13** (1.85 mmol) in 19 mL CH_3OH.
28. Add 21 mg $NaBH_4$ (0.56 mmol, 0.3 eq) and stir the mixture in the dark under N_2 for 90 min.
29. Dilute the solution with Et_2O and wash with saturated aqueous $NaHCO_3$ and brine.
30. Wash the aqueous layer once with Et_2O.
31. Dry the combined organic layers over Na_2SO_4 and concentrate under vacuum.
32. Purify the residue by flash chromatography (*APPENDIX 3E*) using 19:1 (v/v) CH_2Cl_2/CH_3OH to obtain **S.14** as a white foam (1.09 g, 93% yield).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-N^4-(benzoyl)-2′-O-(ethyl-2-methylsulfonate)cytidine (S.15)

33. Dissolve the 1.09 g compound **S.14** (1.72 mmol) in 17 mL CH_2Cl_2 and 1.4 mL pyridine (17.2 mmol, 10 eq).
34. Cool under N_2 to 0°C.
35. Add 0.19 mL methanesulfonyl chloride (2.41 mmol, 1.2 eq) dropwise.
36. Stir the mixture under N_2 while gradually warming to room temperature overnight.

37. Dilute the reaction with Et_2O and wash with saturated aqueous $NaHCO_3$ and brine.

38. Dry the organic layer over Na_2SO_4 and concentrate under vacuum.

39. Purify the residue by flash chromatography (APPENDIX 3E) using 24:1 (v/v) CH_2Cl_2/CH_3OH to obtain **S.15** as a white foam (1.13 g, 92% yield).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-N^4-(benzoyl)-2′-O-(thiobenzoylethyl) cytidine (S.16)

40. Dissolve 1.04 g compound **S.15** (1.46 mmol) in 5.8 mL DMF.

41. Add 2.0 mL Et_3N (14.6 mmol, 10 eq) and 0.34 mL thiobenzoic acid (2.91 mmol, 2.0 eq).

42. Stir the mixture under N_2 in the dark overnight.

43. Dilute the solution with Et_2O and wash with saturated aqueous $NaHCO_3$ and brine.

44. Dry the organic layer over Na_2SO_4 and concentrate under vacuum to a dark orange-brown solid.

45. Purify the residue by flash chromatography (APPENDIX 3E) using a step gradient of 0 to 10% CH_3CN in 2:1 (v/v) petroleum ether/EtOAc to obtain **S.16** as a white foam (0.84 g, 76% yield).

Prepare N^4-(benzoyl)-2′-O-(thiobenzoylethyl)cytidine (S.17)

46. Dissolve 0.37 g compound **S.16** (0.49 mmol) in 4.3 mL CH_3CN.

47. Add 0.5 mL of 48% aqueous HF and stir the reaction mixture for 7 hr.

48. Dilute the solution with Et_2O and wash with H_2O.

49. Dry the organic layer over Na_2SO_4 and concentrate under vacuum.

50. Purify the pink residue by flash chromatography (APPENDIX 3E) using 19:1 (v/v) CH_2Cl_2/CH_3OH to obtain **S.17** as a white foam (0.25 g, 100% yield).

Prepare N^4-(benzoyl)-2′-O-(ethyl)cytidine tert-butyl disulfide (S.18)

51. Dissolve the 0.25 g compound **S.17** (0.49 mmol) in 3.8 mL of 1:1 (v/v) CH_3OH/THF.

52. Add 0.20 g 1-(*tert*-butylthio)-1,2-hydrazinedicarboxmorpholide (Wünsch et al., 1982) (0.58 mmol, 1.2 eq) and 41 mg $LiOH{\cdot}H_2O$ (0.97 mmol, 2.0 eq).

53. Stir the reaction under N_2 at 0°C for 45 min.

54. Dilute the reaction with EtOAc, and then wash with 1 N aqueous sodium citrate and the saturated aqueous $NaHCO_3$.

55. Dry the organic layer over Na_2SO_4 and concentrate under vacuum.

56. Purify the residue by flash chromatography (APPENDIX 3E) using 24:1 (v/v) CH_2Cl_2/CH_3OH to obtain **S.18** as a pink foam (0.18 g, 74% yield).

Prepare 5′-O-(4,4′-dimethoxytrityl)-N^4-(benzoyl)-2′-O-(ethyl)cytidine tert-butyl disulfide (S.19)

57. Dissolve 0.10 g compound **S.18** (0.21 mmol) and 13 mg DMAP (0.10 mmol, 0.5 eq) in 0.8 mL DMF and 26 µL pyridine (0.31 mmol, 1.5 eq).

58. Add 85 mg DMTrCl (0.25 mmol, 1.2 eq) and stir the reaction under N_2 for 6 hr.

59. Dilute the reaction with CH_2Cl_2 and wash with saturated aqueous $NaHCO_3$ and brine.

60. Dry the organic layer over Na_2SO_4 and concentrate under vacuum.

61. Purify the residue by flash chromatography (*APPENDIX 3E*) using 1:1 (v/v) petroleum ether/acetone to obtain **S.19** as a tan foam (0.13 g, 77% yield).

Prepare 5′-O-(4,4′-dimethoxytrityl)-N⁴-(benzoyl)-2′-O-(ethyl)cytidine 3′-O-(N,N-diisopropyl-β-cyanoethylphosphoramidite) tert-butyl disulfide (S.20)

62. Dissolve 55 mg compound **S.19** (0.07 mmol) in 0.3 mL CH_2Cl_2 containing 60 µL *N,N*-diisopropylethylamine (0.35 mmol, 5 eq).

63. Cool under N_2 to 0°C.

64. Add 23 µL chloro-*N,N*-diisopropylamine-β-cyanoethyl phosphine (0.10 mmol, 1.5 eq) dropwise and stir the reaction under N_2 while allowing it to warm to room temperature.

65. After 2 hr, quench the excess chloridate with 0.3 mL CH_3OH and concentrate the mixture under vacuum.

66. Purify the residue by flash chromatography (*APPENDIX 3E*) using 2:1 (v/v) petroleum ether/acetone to obtain **S.20** as a white foam (58 mg, 84% yield).

SUPPORT PROTOCOL 8

SYNTHESIS OF 5′-*O*-(4,4′-DIMETHOXYTRITYL)-2′-*O*-(ETHYL)URIDINE-3′-*O*-(*N,N*-DIISOPROPYL-β-CYANOETHYLPHOSPHORAMIDITE) *tert*-BUTYL DISULFIDE

The intermediate **S.10** prepared in Support Protocol 6 can be converted to 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-allyl uridine (**S.21**) using nitrobenzaldoxime and 1,1,3,3-tetramethylguanidine as described by Sproat and Lamond (1991). The synthesis of the fully protected phosphoramidites can be accomplished in nine sets of steps (Fig. 5.4.4).

Materials

2-Nitrobenzaldoxime
1,1,3,3-Tetramethylguanidine
Acetonitrile (CH_3CN)
Compound **S.10** (see Support Protocol 6)
Ethyl acetate (EtOAc)
Sodium sulfate (Na_2SO_4)
Methanol (CH_3OH)
Methylene chloride (CH_2Cl_2)
Acetone
N-Methylmorpholine-*N*-oxide
Osmium tetroxide (OsO_4)
Saturated aqueous sodium bisulfite
Celite
1,4-Dioxane
Sodium periodate ($NaIO_4$)
Diethyl ether (Et_2O)
Sodium bicarbonate ($NaHCO_3$)
Sodium borohydride ($NaBH_4$)
Brine (saturated aqueous NaCl)
Pyridine

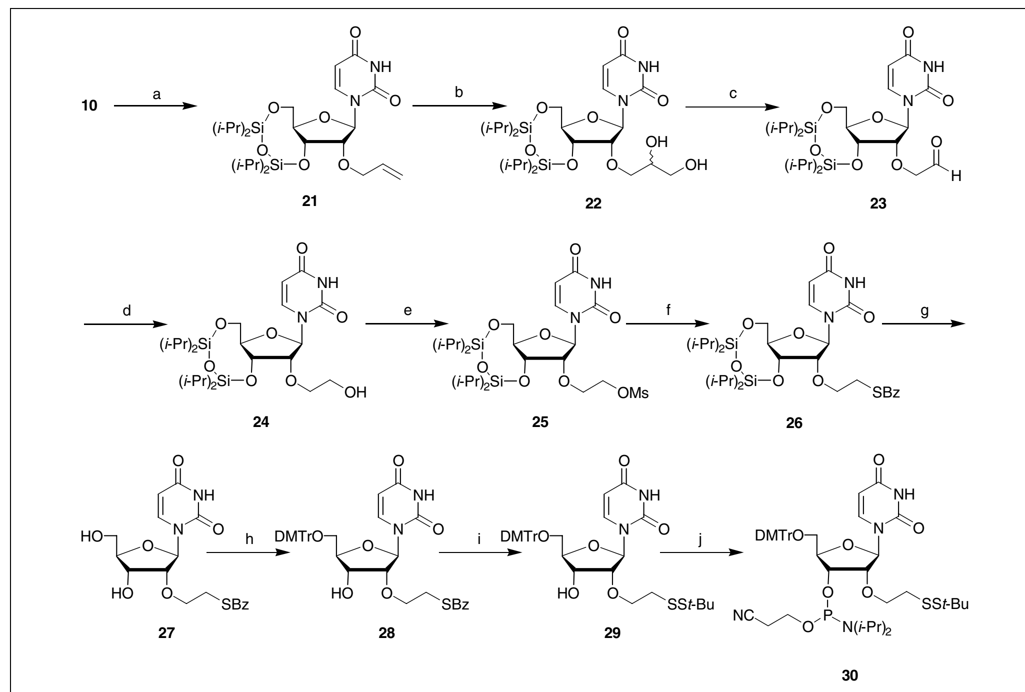

Figure 5.4.4 Synthesis of 3′-*O*-(*N,N*-diisopropyl-β-cyanoethyl-phosphoramidite)-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(ethyl)uridine *tert*-butyl disulfide. (**a**) 2-Nitrobenzaldoxime, 1,1,3,3-tetramethylguanidine, CH_3CN; (**b**) OsO_4, *N*-methylmorpholine-*N*-oxide, acetone, H_2O; (**c**) $NaIO_4$, *p*-dioxane, H_2O; (**d**) $NaBH_4$, CH_3OH; (**e**) methanesulfonyl chloride, pyridine; (**f**) thiobenzoic acid, Et_3N, DMF; (**g**) HF (aq), CH_3CN; (**h**) DMTrCl, Et_3N, DMF, DMAP; (**i**) 1-(*tert*-butylthio)-1,2-hydrazine-dicarboxmorpholide, LiOH, CH_3OH, THF; (**j**) chloro-*N,N*-diisopropylamine-β-cyanoethyl phosphine, *N,N*-diisopropylethylamine, CH_2Cl_2. Abbreviations: Bz, benzoyl; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; DMTr, 4,4′-dimethoxytrityl; Ms, methanesulfonyl.

Methanesulfonyl chloride
N,N-Dimethylformamide (DMF)
Triethylamine (Et_3N)
Thiobenzoic acid
48% (w/v) aqueous HF
4,4′-Dimethoxytrityl chloride (DMTrCl)
4-Dimethylaminopyridine (DMAP)
Tetrahydrofuran (THF)
$LiOH{\cdot}H_2O$
1-(*tert*-Butylthio)-1,2-hydrazinedicarboxmorpholide
1 N aqueous sodium citrate
N,N-Diisopropylethylamine
Chloro-*N,N*-diisopropylamine-β-cyanoethyl phosphine
Petroleum ether

Additional reagents and equipment for flash chromatography (*APPENDIX 3E*)

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-O-allyluridine (S.21)

1. Dissolve 2.1 g 2-nitrobenzaldoxime (12.3 mmol, 4 eq) and 1.4 mL 1,1,3,3-tetramethylguanidine (11.1 mmol, 3.6 eq) in 28 mL CH_3CN.

2. Stir this solution under N_2.

3. Add the solution to 2.0 g compound **S.10** (3.1 mmol).

4. Stir the reaction for 30 min at room temperature and then concentrate under vacuum.

5. Dissolve the residue in EtOAc and wash with water.

6. Backwash the water layer 3 times with EtOAc.

7. Combine the organic layers and dry over Na_2SO_4.

8. Concentrate under vacuum.

9. Purify the residue by flash chromatography (*APPENDIX 3E*) using a step gradient of 0 to 5% CH_3OH in CH_2Cl_2 to obtain **S.21** (1.61 g, 99%).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-O-(2,3-dihydroxypropyl)-uridine (S.22)

10. Dissolve 1.61 g compound **S.21** (3.06 mmol) in 30 mL of 6:1 (v/v) acetone/water.

11. Add 394 mg *N*-methylmorpholine-*N*-oxide (3.4 mmol, 1.1 eq) and 7.7 mg OsO_4 (0.03 mmol, 0.01 eq) and stir in the dark for 3 hr.

12. Precipitate the osmium salts with 2 mL saturated aqueous sodium bisulfite and pour the mixture through a funnel containing Celite.

13. Evaporate the solvent under vacuum to obtain **S.22** as a white foam (1.77 g, 100% yield).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-O-(ethanal)uridine (S.23)

14. Dissolve 437 mg compound **S.22** (0.8 mmol) in 7.8 mL of 3:1 (v/v) 1,4-dioxane/H_2O.

15. Add 209 mg $NaIO_4$ (1.0 mmol, 1.25 eq) and stir the reaction in the dark for 6 hr.

16. Dilute the solution with Et_2O and filter.

17. Wash the filtrate with saturated aqueous $NaHCO_3$, dry over Na_2SO_4, and concentrate under vacuum to obtain **S.23** as a pale-yellow foam (400 mg, 97% yield).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-O-(2-hydroxyethyl)uridine (S.24)

18. Dissolve 470 mg compound **S.23** (0.9 mmol) in 7.4 mL CH_3OH.

    *It may be necessary to repeat the synthesis of **S.22** to obtain the necessary quantity for the synthesis of **S.23.***

19. Add 16 mg $NaBH_4$ (0.4 mmol, 0.48 eq) and stir the mixture overnight under N_2.

20. Dilute the mixture with Et_2O, then wash once with saturated aqueous $NaHCO_3$ and twice with brine.

21. Dry the combined organic layers over Na_2SO_4, filter, and evaporate under vacuum to obtain **S.24** as a white foam (464 mg, 98% yield).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-O-(ethyl-2-methylsulfonate)uridine (S.25)

22. Dissolve 138 mg compound **S.24** (0.3 mmol) in a mixture of 2.6 mL CH_2Cl_2 and 0.21 mL pyridine.

23. Cool to 0°C.

24. Add 28 µL methanesulfonyl chloride (0.4 mmol, 14 eq) dropwise while stirring under N_2.

25. Allow the reaction to warm to room temperature over 18 hr.

26. Dilute the mixture with CH_2Cl_2 and wash once with saturated aqueous $NaHCO_3$.

27. Dry the organic layer over Na_2SO_4, filter, and evaporate under vacuum.

28. Purify the residue by flash chromatography (APPENDIX 3E) using 17:3 (v/v) CH_2Cl_2/acetone to obtain **S.25** as a white foam (133 mg, 84% yield).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2′-O-(thiobenzoylethyl)uridine (S.26)

29. Dissolve 552 mg compound **S.25** (0.9 mmol) in a mixture of 3.6 mL DMF and 1.3 mL Et_3N (9.1 mmol, 10.0 eq).

    *It may be necessary to repeat the synthesis of **S.25** to obtain the necessary quantity for the synthesis of **S.26**.*

30. Add 0.21 mL thiobenzoic acid (1.8 mmol, 2 eq) and stir overnight under N_2.

31. Dilute the mixture with Et_2O, then wash once with saturated aqueous $NaHCO_3$ and twice with brine.

32. Dry the combined organic extracts over Na_2SO_4, filter, and concentrate under vacuum.

34. Purify the residue by flash chromatography (APPENDIX 3E) using a step gradient of 47:3 to 4:1 (v/v) CH_2Cl_2/acetone to obtain **S.26** as a white foam (472 mg, 80% yield).

Prepare 2′-O-(thiobenzoylethyl)uridine (S.27)

35. Dissolve the 472 mg compound **S.26** (0.7 mmol) in 5.8 mL CH_3CN.

36. Add 1.3 mL of 48% aqueous HF and stir for 4 hr.

37. Dilute the mixture with EtOAc and wash once with H_2O.

38. Dry the organic layer over Na_2SO_4 and filter.

39. Evaporate under vacuum to obtain **S.27** as a white foam (327 mg, 100% yield).

Prepare 5′-O-(4,4′-dimethoxytrityl)-2′-O-(thiobenzoylethyl)uridine (S.28)

40. Dissolve 277 mg compound **S.27** (0.7 mmol) in 2.7 mL DMF and 82 μL pyridine (1.0 mmol, 1.5 eq).

41. Add 280 mg DMTrCl (0.8 mmol, 1.2 eq) and 54 mg DMAP (0.4 mmol, 0.65 eq).

42. Stir the reaction overnight under N_2.

43. Dilute the reaction with CH_2Cl_2 and wash the solution once with saturated aqueous $NaHCO_3$ and twice with brine.

44. Dry the combined organic extracts over Na_2SO_4, filter, and concentrate under vacuum.

45. Purify the residue by flash chromatography (APPENDIX 3E) using 48:1 (v/v) CH_2Cl_2/CH_3OH) to obtain **S.28** as a yellow foam (302 mg, 63% yield).

Prepare 5′-O-(4,4′-dimethoxytrityl)-2′-O-(ethyl)uridine tert-butyl disulfide (S.29)

46. Dissolve 293 mg compound **S.28** (0.4 mmol) in 3.3 mL of 1:1 (v/v) THF/CH_3OH.

47. Add 26 mg $LiOH \cdot H_2O$ (0.6 mmol, 1.5 eq).

48. After stirring for 2 min, add 164 mg 1-(*tert*-butylthio)-1,2-hydrazinedicarboxmorpholide (0.5 mmol, 1.2 eq) (Wünsch et al., 1982).

49. Stir the mixture for an additional 10 min under N_2.

50. Dilute the solution with CH_2Cl_2.

51. Wash the solution with 1 N aqueous sodium citrate, saturated aqueous $NaHCO_3$, and brine.

52. Dry the organic layer over Na_2SO_4.

53. Filter the solution and evaporate under vacuum.

54. Purify the residue by flash chromatography (*APPENDIX 3E*) using 49:1 (v/v) CH_2Cl_2/CH_3OH to obtain **S.29** as a white foam (285 mg, 100% yield).

Prepare 3′-O-(N,N-diisopropyl-β-cyanoethyl-phosphoramidite)-5′-O-(4,4′-dimethoxytrityl)-2′-O-(ethyl)uridine tert-butyl disulfide (S.30)

55. Dissolve 49 mg compound **S.29** (0.07 mmol) in 0.28 mL CH_2Cl_2 containing 62 µL *N*,*N*-diisopropylethylamine (0.4 mmol, 5 eq) and cool to 0°C.

56. Add 24 µL chloro-*N*,*N*-diisopropylamine-β-cyanoethyl phosphine (0.1 mmol, 1.5 eq) dropwise.

57. Stir the mixture for 2 hr under N_2.

58. Dilute the solution with CH_3OH.

59. Concentrate the solution under vacuum.

60. Purify the residue by flash chromatography (*APPENDIX 3E*) using 19:6 (v/v) petroleum ether/acetone to obtain **S.30** as a white foam (49 mg, 78% yield).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX.***

CPM buffer, 5×

7.6 g Tris·Cl (250 mM)
38 g NaCl (2.6 mM)
46.5 mg Na_2EDTA (0.5 mM)
0.38 mL Triton X-100 (0.15%, v/v)
H_2O to 200 mL
Adjust pH to 7.5 with 1 N NaOH or HCl
Add H_2O to 250 mL
Store up to 1 month at 25°C

CPM in isopropanol, 0.4 mM

Dissolve 1.6 mg of 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (Molecular Probes) in 10 mL HPLC-grade isopropanol. Store up to 1 week at 25°C.

Denaturing polyacrylamide gel solution, 8%

0.32 g bisacrylamide
9.28 g acrylamide
57.7 g urea (8 M final)
24 mL 5× TBE buffer (*APPENDIX 2A*)
H_2O to 120 mL
Prepare fresh daily

$MgCl_2$, 200 mM

Dissolve 10.2 g magnesium chloride hexahydrate in 250 mL water. Store at room temperature (stable for several months).

Sodium acetate, 1.5 M, pH 5.5

Dissolve 12.3 g anhydrous sodium acetate (NaOAc) in 100 mL water. Adjust pH to 5.5 with glacial acetic acid. Store up to 6 months at 25°C.

Sodium phosphate buffer, 100 mM, pH 8.3

17.8 g Na_2HPO_4
H_2O to 900 mL
Adjust pH to 8.3 with 1 N H_3PO_4 or NaOH
Add H_2O to 1 L
Store up to 6 months at 25°C

Sodium phosphate buffer/NaCl, pH 7.0

100 mM sodium phosphate buffer, pH 7.0 (*APPENDIX 2A*)
5 mM NaCl (*APPENDIX 2A*)
Check pH and adjust to 7.0 with 1 N H_3PO_4 or NaOH, if necessary
Store up to 6 months at 25°C

COMMENTARY

Background Information

Generation of cysteine mutants in proteins capable of forming disulfide bonds is a useful technique to stabilize structure as well as to probe structure, folding, and dynamics. Disulfide cross-links have also been incorporated into DNA to examine ground-state structure, trap non-ground-state structures, and examine protein-DNA and DNA-DNA interactions (Glick, 1998). Disulfide cross-links have more recently been incorporated into RNA sequences to stabilize secondary structure (Goodwin and Glick, 1994; Allerson and Verdine, 1995), to examine dynamic motion between helices in the *Tetrahymena* ribozyme (Cohen and Cech, 1997), and to probe solution conformation of a tRNA, the hammerhead ribozyme, and the hairpin ribozyme (Sigurdsson et al., 1995; Goodwin et al., 1996; Earnshaw et al., 1997; Maglott and Glick, 1998).

The methods that have been used to incorporate disulfide bonds into RNA have relied on either air oxidation or thiol-disulfide exchange. Although air oxidation is slower than thiol-disulfide exchange, using the methodology presented here it is possible to achieve quantitative conversion of the purified thiol-modified RNA to disulfide-cross-linked RNA. The ability to form intramolecular disulfide cross-links in quantitative yields makes it possible to produce milligram quantities of these constructs, which can then be used in a wide variety of biophysical and biochemical experiments. In addition, attachment of the alkylthiol tethers at positions other than the 5′ or 3′ hydroxyls allows for radiolabeling of constructs modified at the termini of the RNA.

Disulfide cross-links can be incorporated into RNA secondary and tertiary structures at many different locations. The specific thiol-modified nucleosides that are needed to create a particular cross-link depend on the location of the cross-links within the RNA. It is possible to form cross-links between two of the same thiol-modified nucleosides or between two different thiol-modified nucleosides. The preparation of nucleosides with thiol modifications at the N^3 position of uridine as well as at the 2′ hydroxyl of both cytidine and uridine is described in this unit. Preparation of many other thiol-modified nucleosides has been reported, including those with alkylthiols on both G and A (Glick, 1998). Use of particular bases depends on the specific application (see Glick, 1998). The thiol group in each of these nucleosides is protected as a *tert*-butyl disulfide, which is stable to all conditions of solid-phase synthesis. These protocols involve relatively simple synthetic organic chemistry techniques and standard glassware and equipment. In addition to the thiol-modified nucleosides described here, synthesis of both adenosine and guanosine derivatized with 2′-*O*-alkyl linkers has been achieved (Manoharan et al., 1993; Douglas et al., 1994; Gundlach et al., 1997).

Alkylthiol-modified nucleosides have also been described with linkers at, among others, the C5 position of pyrimidines (Sun et al., 1996), the C8 position of purines (Gundlach et al., 1997), and all exocyclic amine groups on both purines and pyrimidines (Allerson et al., 1997).

Compound Characterization

N[3]-(2-Thiobenzoylethyl)uridine (**S.1**). TLC (9:1 CH_2Cl_2/CH_3OH) $R_f = 0.41$. ^{1}H NMR (300 MHz, CD_3CN) δ 3.30 (2 H, t, *J* = 5 Hz, C*H*$_2$SC(O)Ar), 3.65-3.81 (2H, 2 dd, *J* = 2.5, 10 Hz, 5′, 5″), 3.96 (1H, m, 4′), 4.16-4.19 (4H, m, 2′, 3′, NC*H*$_2$), 5.66 (1H, d, *J* = 8.1 Hz, 6), 5.80 (1H, d, *J* = 3.8 Hz, 1′), 7.43-7.62 (3H, m, Ar), 7.81 (1H, d, *J* = 8.1 Hz, 5), 7.88 (2H, d, *J* = 7.3 Hz, Ar). ^{13}C NMR (75 MHz, CD_3CN) δ 27.4 (*C*H$_2$SC(O)Ar), 40.9 (N*C*H$_2$), 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 (S*C*(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}. MS (FAB, 3-NBA/trifluoroacetic acid) *m/z* 409 (M$^+$ + 1).

5′-*O*-(4,4′-Dimethoxytrityl)-*N*[3]-(ethyl)uridine *tert*-butyl disulfide (**S.2**). ^{1}H NMR (360 MHz, CD_3CN) δ 1.31 (9H, s, SS(C*H*$_3$)$_3$), 2.90 (2H, t, *J* = 6.0 Hz, C*H*$_2$SS), 3.36 (2H, m, 5′,5″), 3.75 (6H, s, 2 OC*H*$_3$), 4.02 (1H, m, 4′), 4.11 (2H, m, NC*H*$_2$), 4.18 (1H, m, 2′), 4.32 (1H, m, 3′), 5.38 (1H, d, *J* = 8.1 Hz, 6), 5.78 (1H, d, *J* = 3.2 Hz, 1′), 6.85-7.44 (13H, m, Ar), 7.73 (1H, d, *J* = 8.1 Hz, 5). ^{13}C NMR (90 MHz, CD_3CN) δ 30.1 (SSC(*C*H$_3$)$_3$), 37.2 (*C*H$_2$SS), 41.0 (N*C*H$_2$), 48.5 (SS*C*(CH$_3$)$_3$), 56.0 (O*C*H$_3$), 63.4 (5′), 70.5 (3′), 75.5 (2′), 83.8 (4′), 87.5 (O*C*(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}. MS (FAB, 3-NBA) *m/z* 695 (M$^+$ + 1).

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-*N*[3]-(ethyl)uridine *tert*-butyl disulfide (**S.3**) TLC (4:1 petroleum ether/EtOAc) $R_f = 0.30$. ^{1}H NMR (300 MHz, CD_3CN) δ 0.12 (6H, s, Si(C*H*$_3$)$_2$), 0.90 (9H, s, SiC(C*H*$_3$)$_3$), 1.30 (9H, s, SSC(C*H*$_3$)$_3$), 2.88 (2H, t, *J* = 8.0 Hz, C*H*$_2$HS), 3.38 (2H, 2 dd, *J* = 2.4, 11.0 Hz, 5′,5″), 3.74 (6H, s, 2 OC*H*$_3$), 4.05 (3H, m, 4′, NC*H*$_2$), 4.24 (1H, m, 2′), 4.31 (1H, m, 3′), 5.35 (1H, d, *J* = 8.2 Hz, 6), 5.83 (1H, d, *J* = 4.0 Hz, 1′), 6.84-7.43 (13H, m, Ar), 7.74 (1H, d, *J* = 8.1 Hz, 5). ^{13}C NMR (75 MHz, CD_3CN) δ –4.4 (Si(*C*H$_3$)$_2$), 18.8 (Si*C*(CH$_3$)$_3$), 26.3 (SiC(*C*H$_3$)$_3$), 30.2 (SSC(*C*H$_3$)$_3$), 37.5 (*C*H$_2$SS), 41.0 (N*C*H$_2$), 48.4 (SS*C*(CH$_3$)$_3$), 55.9 (O*C*H$_3$), 63.7 (5′), 71.2 (3′), 76.9 (2′), 84.1 (4′), 87.7 (O*C*(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}. MS (FAB, 3-NBA) *m/z* 809 (M$^+$ + 1).

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-*N*[3]-(ethyl)uridine *tert*-butyl disulfide (**S.4**) TLC (4:1 petroleum ether/EtOAc) $R_f = 0.14$. ^{1}H NMR (300 MHz, CD_3CN) δ 0.04, 0.05 (6H, 2 s, Si(C*H*$_3$)$_2$), 0.81 (9H, s, SiC(C*H*$_3$)$_3$), 1.30 (9H, s, SSC(C*H*$_3$)$_3$), 2.88 (1H, dd, *J* = 3.8, 11.1 Hz, 5′), 2.89 (2H, t, *J* = 7.6 Hz, C*H*$_2$SS), 3.46 (1H, dd, *J* = 2.4, 11.0 Hz, 5″), 3.74 (6H, s, 2 OC*H*$_3$), 4.00 (1H, m, 4′), 4.10 (3H, m, 2′, NC*H*$_2$), 4.33 (1H, m, 3′), 5.38 (1H, d, *J* = 8.1 Hz, 6), 5.80 (1H, d, *J* = 3.0 Hz, 1′), 6.84-7.42 (13H, m, Ar), 7.74 (1H, d, *J* = 8.1 Hz, 5). ^{13}C NMR (75 MHz, CD^3CN) δ –4.4, –4.1 (Si(*C*H$_3$)$_2$), 18.7 (Si*C*(CH$_3$)$_3$), 26.3 (SiC(*C*H$_3$)$_3$), 30.3 (SSC(*C*H$_3$)$_3$), 37.5 (*C*H$_2$SS), 41.0 (N*C*H$_2$), 48.3 (SS*C*(CH$_3$)$_3$, 56.0 (O*C*H$_3$), 63.3 (5′), 72.0 (3′), 75.4 (2′), 84.1 (4′), 87.7 (O*C*(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}. MS (FAB, 3-NBA) *m/z* 809 (M$^+$ + 1).

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-*N*[3]-(ethyl)uridine-3′-*O*-(*N*,*N*-diisopropyl-β-cyanoethylphosphoramidite) *tert*-butyl disulfide (**S.5**). TLC (80:15:5 petroleum ether/EtOAc/Et_3N) $R_f = 0.28$. ^{1}H NMR (300 MHz, CD_3CN) δ (two diastereomers) 0.09, 0.12 (6H, 2 s, Si(C*H*$_3$)$_2$), 0.83, 0.85 (9H, 2 s, SiC(C*H*$_3$)$_3$), 1.01, 1.14 (12H, d, *J* = 9 Hz, 2 NCH(C*H*$_3$)$_2$), 1.31 (9H, s, SSC(C*H*$_3$)$_3$), 2.45 (2H, m, OCH$_2$C*H*$_2$CN), 2.88 (2H, m, C*H*$_2$SS), 3.40 (2H, m, 5′, 5″), 3.50-3.90 (4H, m, OC*H*$_2$CH$_2$CN, 2 NC*H*(CH$_3$)$_2$), 3.75 (6H, s, 2 OC*H*$_3$), 4.10 (2H, m, NC*H*$_2$CH$_2$SS), 4.20-4.42 (3H, m, 2′, 3′, 4′), 5.37, 5.39 (1H, d, *J* = 8.1 Hz, 6), 5.84, 5.89 (1H, d, *J* = 6.6 Hz, 1′), 6.83-7.46 (13H, m, Ar), 7.72, 7.77 (1H, d, *J* = 8.1 Hz, 5). ^{13}C NMR (75 MHz, CD_3CN) δ (two diastereomers) –4.2 (Si(*C*H$_3$)$_2$), 18.9 (Si*C*(CH$_3$)$_3$), 21.1 (OCH$_2$*C*H$_2$CN), 25.0, 25.1, 25.2, 25.3 (NCH(*C*H$_3$)$_2$), 26.4 (SiC(*C*H$_3$)$_3$), 30.4 (SC(*C*H$_3$)$_3$), 37.8 (*C*H$_2$SS), 41.2 (N*C*H$_2$

CH_2SS), 44.1, 44.3, 44.5, 44.6 (N*C*H($CH_3)_2$), 48.5 (SS*C*($CH_3)_3$), 56.2 (OCH_3), 59.4, 60.0 (PO*C*H_2CH_2CN), 64.0, 64.2 (5′), 73.5, 73.7 (3′), 76.1, 76.5 (2′), 83.9, 84.0 (4′), 88.1 (O*C*($Ph)_3$), 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). ^{31}P NMR (202 MHz, CD_3CN) 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}. MS (FAB, 3-NBA) *m/z* 1009.5 (M^+ + 1). Anal. Calcd for $C_{51}H_{73}N_4O_9PS_2Si$: 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*-butyldimethylsilyl)-2′-*O*-pentachlorophenylsuccinate *N*3-(ethyl)uridine *tert*-butyl disulfide (**S.6**). TLC (4:1 petroleum ether/EtOAc) R_f = 0.68. ^{1}H NMR (360 MHz, CD_3CN) δ –0.07, 0.01 (6H, s, Si($CH_3)_2$), 0.79 (9H, s, SiC($CH_3)_3$), 1.29 (9H, s, SSC($CH_3)_3$), 2.79 (4H, m, succinate CH_2), 3.03 (2H, m, CH_2SS), 3.29-3.47 (2H, m, 5′, 5″), 3.76 (6H, s, 2 OCH_3), 3.96-4.12 (3H, m, 4′, NCH_2), 4.38 (1H, m, 3′), 5.43 (2H, m, 6, 2′), 5.97 (1H, d, J = 4.8 Hz, 1′), 6.21-7.45 (13H, m, Ar), 7.65 (1H, d, J = 8.1 Hz, 5). ^{13}C NMR (90 MHz, CD_3CN) δ –4.4, –4.1 (Si($CH_3)_2$), 18.6 (Si*C*($CH_3)_3$), 26.1 (SiC($CH_3)_3$), 29.4, 29.5 (succinate CH_2), 30.2 (SSC($CH_3)_3$), 37.0 (CH_2SS), 41.1 (NCH_2CH_2SS), 48.5 (SS*C*($CH_3)_3$), 56.0 (OCH_3), 63.6 (5′), 71.4 (3′), 76.1 (2′), 85.3 (4′), 88.0 (O*C*($Ph)_3$), 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 CO_2). IR (film; NaCl) ν 2956, 2931, 1786, 1751, 1713, 1672, 1608, 1509, 1455, 1390, 1363, 1253, 1229, 1177, 1154, 1107, 1036, 837 cm^{-1}. MS (FAB, 3-NBA) *m/z* 1173 (M^+ + 1). Anal. Calcd for $C_{52}H_{59}N_2O_{11}S_2SiCl_5$: C, 53.96; H, 5.10; N, 2.42. Found C, 54.03; H, 5.15; N, 2.40.

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-uridine (**S.8**). TLC (1:1 petroleum ether/EtOAc) R_f 0.28; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.03-1.11 (28H, m, isopropyl), 3.56-4.40 (5H, m, 2′,3′,4′,5′,5″), 5.70 (1H, dd, 5), 5.74 (1H, s, 1′), 7.71 (1H, d, 6), 8.83 (1H, s, NH); ^{13}C NMR (90 MHz, $CDCl_3$) δ 12.63-13.55 (isopropyl CHs), 16.98-17.65 (isopropyl CH_3s), 60.29 (5′), 68.85 (3′), 75.32 (2′), 82.05 (4′), 91.15 (1′), 102.18 (5), 140.10 (6), 150.48 (2), 163.84 (4); IR (KBr pellet) ν 3434, 3200, 3063, 2946, 2868, 1703, 1465, 1388, 1270, 1211, 1123, 1091, 1060, 1038, 993, 907, 885, 861, 809, 774, 705, 553 cm^{-1}; MS (CI, CH_4) *m/z* 487 (MH^+).

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-O^4-(2-nitrophenyl) uridine (**S.9**). TLC (1:1 petroleum ether/EtOAc) R_f 0.27; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00-1.11 (28H, m, isopropyl), 3.99-4.37 (5H, m, 2′,3′,4′,5′,5″), 5.74 (1H, s, 1′), 6.21 (1H, d, 5), 7.32 (1H, d, Ar), 7.41 (1H, ddd, Ar), 7.67 (1H, ddd, Ar), 8.13 (1H, dd, Ar), 8.23 (1H, d, 6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.89-13.70 (isopropyl CHs), 17.14-17.71 (isopropyl CH_3s), 60.71 (5′), 69.28 (3′), 75.29 (2′), 82.34 (4′), 92.46 (1′), 94.78 (5), 125.76, 126.14, 126.81, 134.88, 141.90, 145.08 (Ar), 145.19 (6), 154.68 (2), 171.10 (4).

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-allyl-O^4-(2-nitrophenyl) uridine (**S.10**). TLC (1:1 petroleum ether/EtOAc) R_f 0.68. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00-1.12 (28H, m, isopropyl), 3.93-4.44 (7H, m, 2′,3,4′,5′,5″, allyl CH_2), 5.16 (1H, ddd, =CH_{2a}), 5.39 (1H, m, =CH_{2b}), 5.76 (1H, s, 1′), 5.92 (1H, m, CH=), 6.19 (1H, d, 5), 7.33 (1H, dd, Ar), 7.41 (1H, ddd, Ar), 7.67 (1H, ddd, Ar), 8.13 (1H, dd, Ar), 8.38 (1H, d, 6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.92-13.79 (isopropyl CHs), 17.16-17.79 (isopropyl CH_3s), 59.85 (5′), 68.04 (3′), 71.51 (OCH_2), 81.02 (2′), 82.16 (4′), 90.46 (1′), 94.50 (5), 117.29 (=CH_2), 125.80, 126.13, 126.76, 134.73 (Ar), 134.82 (CH=), 142.00 (Ar), 144.77 (6), 145.26 (Ar) 154.72 (2), 171.06 (4); IR (KBr pellet) ν 3105, 2947, 2869, 1683, 1633, 1603,1549, 1531, 1454, 1365, 1351, 1288, 1220, 1166, 1126, 1073, 1041, 1014, 991, 886, 858, 780, 698 cm^{-1}; MS (CI, CH_4) *m/z* 648 (M^+).

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-N^4-(benzoyl)-2′-*O*-allyl cytidine (**S.11**). TLC (2:3 EtOAc/petroleum ether) R_f = 0.32. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96-1.11 (28H, m, 4 ($CH_3)_2$HSi, 4 ($CH_3)_2$*H*Si), 3.95-4.02 (2H, m, 2′-H, 5′-H_a), 4.16 (1H, dd, J = 4.1, 9.6 Hz, 3′-H), 4.24-4.32 (2H, m, 4′-*H*, 5′-H_b), 4.39-4.52 (2H, m, 7-$H_{a,b}$), 5.19 (1H, d, J = 10.5 Hz, 9-H_{trans}), 5.42 (1H, d, J = 16.0 Hz, 9-H_{cis}), 5.84 (1H, s, 1′-*H*), 5.87-6.00 (1H, m, 8-*H*), 7.47-7.62 (4H, m, 5-*H*, Ar*H*), 7.90-7.92 (2H, m, Ar*H*), 8.39 (1H, d, J = 7.5 Hz, 6-*H*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.6, 13.0, 13.2, 13.5 (($CH_3)_2$)*C*HSi), 16.9, 17.0, 17.1, 17.4, 17.5 (($CH_3)_2$)CHSi), 59.5 (5′), 67.8 (3′), 71.3 (7), 80.8 (2′), 81.9 (4′), 90.1 (1′), 96.1 (5), 117.2 (9), 127.5, 128.9 (Ar.), 133.0 (8, Ar.), 134.4 (6), 144.4 (2), 162.2 (4). IR (film; NaCl) ν 2946, 2868, 1699, 1667, 1620, 1553, 1488, 1264, 1126, 1040, 886 cm^{-1}. FAB MS (3-NBA) *m/z* 630 (M^+ + 1).

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(2,3-dihydroxypropyl)cytidine (**S.12**). TLC (19:1 CH_2Cl_2/CH_3OH) R_f = 0.23; ^1H NMR (360 MHz, $CDCl_3$) δ (two diastereomers) 0.98-1.11 (28H, m, 4 $(CH_3)_2)CHSi$, 4 $(CH_3)_2)CHSi$), 3.66 (1H, dd, *J* = 5.2, 10.7 Hz, $CH(OH)CH_{2a}OH$), 3.72-3.76 (2H, m, $CH_2CH(OH)CH_2$, $CH(OH)CH_{2b}OH$), 3.88-4.06 (4H, m, 2′, 5′, $OCH_2CH(OH)$), 4.15-4.28 (2H, m, 3′, 4′), 4.30 (1H, d, *J* = 13.5 Hz, 5″), 5.83 (1H, s, 1′), 7.49-7.63 (4H, m, 5, Ar), 7.90-7.92 (2H, m, Ar), 8.31-8.34 (1H, m, 6), 8.94 (1H, br s, N*H*). ^{13}C NMR (90 MHz, $CDCl_3$) δ (two diastereomers) 12.5, 12.6, 12.9, 13.0 ($(CH_3)_2CHSi$), 16.8, 16.9, 17.0, 17.3, 17.4, 17.4, 17.6 ($((CH_2CHSi)$), 59.2 (5′), 63.6, 63.9 ($CH(OH)CH_2OH$), 67.8 (3′), 70.4, 70.5 ($OCH_2CH(OH)$), 73.4, 74.3 (CH3)2CH(OH)CH_2), 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}. MS (FAB, 3-NBA) *m/z* 664 (M^+ + 1).

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-N^4-(benzoyl)-2′-*O*-(ethanal)cytidine (**S.13**). TLC (37:3 CH_2Cl_2/CH_3OH) R_f = 0.50; ^1H NMR (360 MHz, CDCl3) δ 0.98-1.11 (28H, m, 4 $(CH_3)_2CHSi$, 4 $(CH_3)_2CHSi$), 3.98-4.07 (2H, m, 2′, 5′), 4.18-4.31 (3H, m, 3′, 4′ ,5″), 4.47-4.59 (2H, m, OCH_2CHO), 5.86 (1H, s, 1′), 7.49-7.63 (4H, m, 5, Ar), 7.89-7.91 (2H, m, Ar), 8.37 (1H, d, *J* = 7.5 Hz, 6), 8.81 (1H, br s, N*H*), 9.81 (1H, s, CH_2CHO). ^{13}C NMR (90 MHz, $CDCl_3$) δ 12.3, 12.9, 13.0, 13.4 ($(CH_3)_2CHSi$), 16.7, 16.9, 17.3, 17.4 ($(CH_3)_2CHSi$), 59.2 (5′), 68.1 (3′), 76.2 (OCH_2CHO), 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 (CH_2CHO). IR (film; NaCl) ν 2946, 2868, 1700, 1667, 1619, 1484, 1253, 1130, 1064, 1040, 886, 703 cm^{-1}. MS (FAB, 3-NBA) *m/z* 632 (M^+ + 1).

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-N^4-(benzoyl)-2′-*O*-(2-hydroxyethyl)cytidine (**S.14**). TLC (37:3 CH_2Cl_2/CH_3OH) R_f = 0.56. ^1HNMR (360 MHz, $CDCl_3$) δ 0.99-1.10 (28H, m, 4 $(CH_3)_2CHSi$, 4 $(CH_3)_2CHSi$), 3.17 (1H, br s, CH_2CH_2OH), 3.72-3.77 (2H, m, CH_2CH_2OH), 3.93-4.03 (4H, m, 2′, 5′, OCH_2CH_2OH), 4.15-4.25 (2H, m, 3′, 4′), 4.30 (1H, d, *J* = 13.6 Hz, 5″), 5.83 (1H, s, 1′), 7.49-7.63 (4H, m, 5, Ar), 7.89-7.92 (2H, m, Ar), 8.35 (1H, d, *J* = 7.5 Hz, 6), 8.92 (1H, br s, N*H*). ^{13}C NMR (90 MHz, $CDCl_3$) δ 12.6, 12.9, 13.0, 13.4 ($(CH_3)_2CHSi$), 16.8, 16.9, 17.0, 17.3, 17.4, 17.4 ($(CH_3)_2CHSi$), 59.3 (5′), 61.7 (CH_2CH_2OH), 68.0 (3′), 73.1 (OCH_2CH_2OH), 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}. MS (FAB, 3-NBA) *m/z* 634 (M^+ + 1).

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-N^4-(benzoyl)-2′-*O*-(ethyl-2-methylsul- fonate)cytidine (**S.15**). TLC (19:1 CH_2Cl_2/CH_3OH) R_f = 0.35. ^1H NMR (360 MHz, $CDCl_3$) δ 0.97-1.11 (28H, m, 4 $(CH_3)_2CHSi$, 4 $(CH_3)_2CHSi$), 3.14 (3H, s, CH_3SO_2), 3.97-4.01 (2H, m, 2′, 5′), 4.17-4.21 (4H, m, 3′, 4′, $OCH_2HH_2OSO_2$), 4.30 (1H, d, *J* = 13.7 Hz, 5″), 4.46-4.49 (2H, m, $CH_2CH_2OSO_2$), 5.82 (1H, s, 1′), 7.50-7.62 (4H, m, 5, Ar), 7.89-7.92 (2H, m, Ar), 8.38 (1H, d, *J* = 7.5 Hz, 6). ^{13}C NMR (90 MHz, $CDCl_3$) δ 12.4, 12.9, 13.1, 13.4 ($(CH_3)_2CHSi$), 16.8, 16.9, 17.1, 17.3, 17.4, 17.4 ($(CH_3)_2CHSi$), 37.80 (CH_3SO_2), 59.3 (5′), 67.9 (3′), 69.0 ($CH_2CH_2OSO_2$), 69.5 (OCH_2 CH_2OSO_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} MS (FAB, 3-NBA) *m/z* 712 (M^+ + 1).

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-N^4-(benzoyl)-2′-*O*-(thiobenzoylethyl) cytidine (**S.16**). TLC (6:3:1 petroleum ether/EtOAc/CH_3CN) R_f = 0.57. ^1H NMR (360 MHz, $CDCl_3$) δ 0.98-1.11 (28H, m, 4 $(CH_3)_2CHSi$, 4 $(CH_3)_2CHSi$), 3.35-3.43 (2H, m, CH_2CH_2SBz), 3.97-4.02 (2H, m, 2′, 5′), 4.09-4.13 (2H, m, OCH_2CH_2SBz), 4.18 (1H, dd, *J* = 3.9, 9.6 Hz, 3′), 4.25 (1H, dd, *J* = 2.0, 9.6 Hz, 4′), 4.30 (1H, d, *J* = 13.5 Hz, 5″), 5.84 (1H, s, 1′), 7.40-7.62 (7H, m, 5, Ar), 7.91-7.99 (4H, m, Ar), 8.37 (1H, d, *J* = 7.4 Hz, 6), 8.97 (1H, br s, N*H*). ^{13}C NMR (90 MHz, $CDCl_3$) δ 12.5, 12.8, 13.1, 13.4 ($(CH_3)_2CHSi$), 16.8, 16.9, 17.1, 17.3, 17.4, 17.5 ($(CH_3)_2CHSi$), 29.1 (CH_2CH_2SBz), 59.4 (5′), 67.9 (3′), 69.9 (OCH_2CH_2SBz), 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 (M^+ + 1).

N^4-(Benzoyl)-2′-*O*-(thiobenzoylethyl)cytidine (**S.17**). TLC (19:1 CH_2Cl_2/CH_3OH) R_f = 0.21. ^1H NMR (360 MHz, $CDCl_3$) δ 3.25-3.42 (2H, m, CH_2SBz), 3.91-3.96 (2H, m, 2′, 5′), 4.05-4.19 (3H, m, 5″, OCH_2CH_2SBz), 4.20-4.25

(1H, m, 4′), 4.35-4.37 (1H, m, 3′), 5.87 (1H, s, 1′), 7.33-7.54 (7H, m, 5, Ar), 7.82-7.94 (4H, m, Ar), 8.57 (1H, d, *J* = 7.5 Hz, 6), 9.22 (1H, br s, N*H*). ^{13}C NMR (90 MHz, $CDCl_3$) δ 28.8 (CH_2SBz), 59.8 (5′), 67.4 (3′), 69.4 (OCH_2CH_2SBz), 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}. MS (FAB, 3-NBA) *m/z* 512 (M^+ + 1).

N^4-(Benzoyl)-2′-*O*-(ethyl)cytidine *tert*-butyl disulfide (**S.18**). TLC (24:1 CH_2Cl_2/CH_3OH) R_f = 0.29; ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.32 (9H, s, $C(CH_3)_3$), 2.93 (2H, t, *J* = 5.8 Hz, CH_2SS), 3.93-3.99 (2H, m, 2′, 5′), 4.09-4.18 (3H, m, 5″, OCH_2CH_2SS), 4.24-4.30 (1H, m, 4′), 4.33-4.41 (1H, m, 3′), 5.84 (1H, d, *J* = 1.7 Hz, 1′), 7.48-7.62 (4H, m, 5, Ar), 7.87-7.89 (2H, m, Ar), 8.48 (1H, d, *J* = 7.5 Hz, 6), 9.01 (1H, br s, N*H*). ^{13}C NMR (90 MHz, $CDCl_3$) δ 29.8 ($C(CH_3)_3$), 40.3 (CH_2SS), 48.0 ($C(CH_3)_3$), 60.3 (5′), 67.8 (3′), 69.0 (OCH_2CH_2SS), 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 (M^+ + 1).

5′-*O*-(4, 4′-Dimethoxytrityl)-N^4)-benzoyl)-2′-*O*-(ethyl)cytidine *tert*-butyl disulfide (**S.19**). TLC (1:1 acetone/petroleum ether) R_f = 0.23; ^{1}H NMR (360 MHz, CD_3CN) δ 1.30 (9H, s, $C(CH_3)_3$), 2.95 (2H, t, *J* = 6.3 Hz, CH_2SS), 3.39-3.44 (2H, m, OCH_2CH_2SS), 3.76 (6H, s, 2 OCH_3), 3.90-4.03 (3H, m, 2′, 5′, 5″), 4.12-4.19 (1H, m, 4′), 4.39-4.47 (1H, m, 3′), 5.84 (1H, d, *J* = 1.7 Hz, 1′), 6.87-6.89 (4H, m, Ar), 7.15-7.61 (13H, m, 5, Ar), 7.92-7.95 (2H, m, Ar), 8.46 (1H, d, *J* = 7.6 Hz, 6). ^{13}C NMR (90 MHz, CD_3CN) δ 30.2 ($C(CH_3)_3$), 41.0 (CH_2SS), 48.5 ($C(CH_3)_3$), 56.0 (OCH_3), 62.1 (5′), 68.9 (3′), 70.1 (OCH_2CH_2SS), 83.3 (2′), 83.6 (4′), 87.7 ($OC(Ph)_3$), 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}. MS (FAB, 3-NBA) *m/z* 798 (M^+ + 1).

5′-*O*-(4, 4′-Dimethoxytrityl)-N^4)-benzoyl)-2′-*O*-(ethyl)cytidine *tert*-butyl disulfide 3′-*O*-(*N,N*-diisopropyl β cyanoethylphosphoramidite) (**S.20**). TLC (2:1 petroleum ether/acetone) R_f = 0.40; ^{1}H NMR (300 MHz, CD_3CN) δ (two diastereomers) 1.05-1.20 (12H, m, 2 $NCH(CH_3)_2$), 1.30 (9H, s, $C(CH_3)_3$), 2.50-2.67 (2H, m, OCH_2CH_2CN), 2.94-3.01 (2H, m, CH_2SS), 3.41-3.71 (6H, m, OCH_2CH_2SS, OCH_2CH_2CN, 2 $NCH(CH_3)_2$), 3.79 (6H, s, 2 OCH_3), 3.80-4.22 (4H, m, 2′, 4′, 5′, 5″), 4.43-4.63 (1H, m, 3), 5.88 (1H, s, 1′), 6.87-6.92 (4H, m, Ar), 7.03-7.66 (13H, m, 5, Ar), 7.91-7.97 (2H, m, Ar), 8.42-8.55 (1H, 2 d, *J* = 7.6 Hz, 6). ^{13}C NMR (75 MHz, CD_3CN) δ (two diastereomers) 21.2, 21.3 (OCH_2CH_2CN), 24.9, 25.0, 25.2, 25.3 ($NCH(CH_3)_2$), 30.3 ($C(CH_3)_3$), 41.4 (CH_2SS), 44.1, 44.3 ($NCH(CH_3)_2$), 48.5 ($C(CH_3)_3$), 56.0 (OCH_3), 59.3, 59.6 (OCH_2CH_2CN), 61.8, 62.2 (5′), 70.2, 70.4 (3′), 70.8 (OCH_2CH_2SS), 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). ^{31}P NMR (202 MHz; CD_3CN) δ 147.57, 146.51. IR (film; NaCl) ν 2967, 2934, 1708, 1686, 1509, 1510, 1462, 1251, 1180, 1035, 979 cm^{-1}. MS (FAB, 3-NBA) *m/z* 998 (M^+ + 1). Anal. Calcd for $C_{52}H_{64}N_5O_9PS_2$: C, 62.56; H, 6.48; N, 7.02. Found: C, 62.41; H, 6.41; N, 6.92.

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-allyluridine (**S.21**). TLC (1:1 petroleum ether/EtOAc) R_f 0.59; ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.01-1.11 (28H, m, isopropyl), 3.88 (1H, m, 4′), 3.98 (1H, dd, OCH_{2a}), 4.18 (2H, m, 2′,3′), 4.26 (1H, dd, OCH_{2b}), 4.39 (2H, m, 5′,5″), 5.21 (1H, ddd, $=CH_{2a}$), 5.40 (1H, ddd, $=CH_{2b}$), 5.68 (1H, dd, 5), 5.76 (1H, s, 1′), 5.93 (1H, m, CH=), 7.93 (1H, d, 6), 9.14 (1H, s, NH); ^{13}C NMR (90 MHz, $CDCl_3$) δ 12.68-13.63 (isopropyl CHs), 17.00-17.70 (isopropyl CH_3s), 59.62 (5′), 68.25 (3′), 71.46 (OCH_2), 81.27 (2′), 82.00 (4′), 89.29 (1′), 101.69 (5), 117.59 ($=CH_2$), 134.51 (CH=), 139.77 (6), 150.06 (2), 163.61 (4); MS (DCI, CH_4) *m/z* 527 (M^+).

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(2,3-dihydroxypropyl)uridine (**S.22**). TLC (51:4 CH_2Cl_2/CH_3OH) R_f = 0.22; ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.02-1.10 (28H, m, 4 $SiCH(CH_3)_2$), 3.65-3.75 (3H, m, $CH_2CH(OH)CH_2OH$, $CH_2CH(OH)CH_2OH$), 3.86-4.04 (4H, m, 2′, 5″, $CH_2CH(OH)CH_2OH$), 4.12-4.17 (2H, m, 3′, 4′), 4.25 (1H, d, *J* = 13.6 Hz, 5′), 5.71 (1H, dd, *J* = 1.4, 8.1 Hz, 5), 5.74 (1H, d, *J* = 1.6 Hz, 1′), 7.88 (1H, dd, *J* = 1.9, 8.1 Hz, 6). ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.07-13.90 ($SiCH(CH_3)_2$), 17.18-17.82 ($SiCH(CH_3)_2$), 59.66 (5′), 68.58 (3′), 70.94 ($CH_2CH(OH)CH_2OH$), 74.20 ($CH_2CH(OH)CH_2OH$), 82.22 (4′), 83.99 (CH_2CH

(OH)CH_2OH), 89.43 (1′), 89.67 (2′), 102.33 (5), 139.24 (6), 150.82 (2), 163.33 (4). IR (Nujol) ν 3424, 3160, 3092, 2951, 2946, 2935, 2925, 2918, 2909, 2896, 2891, 2873, 2867, 2863, 2856, 2850, 2726, 1693, 1685, 1462, 1378, 1272, 1163, 1125, 1062, 1035, 1010, 991, 884, 703 cm^{-1}.

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(ethanal)uridine (**S.23**) TLC (51:5 CH_2Cl_2/CH_3OH) R_f = 0.48; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.03-1.12 (28 H, m, 4 $SiCH(CH_3)_2$), 3.71 (2 H, s, OCH_2CHO), 3.95-4.01 (2 H, m, 2′, 5″), 4.23-4.30 (2 H, m, 3′,4′), 4.45-4.47 (1 H, m, 5′), 5.70 (1 H, dd, *J* = 2.2, 8.1 Hz, 5), 5.78 (1 H, s, 1′), 7.90 (1 H, d, *J* = 8.1 Hz, 6), 8.93 (1 H, s, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.87-13.82 ($SiCH(CH_3)_2$), 17.11-17.78 ($SiCH(CH_3)_2$), 59.64 (5′), 68.95 (3′), 76.62 (OCH_2CHO), 82.00 (4′), 83.85 (2′), 89.32 (1′), 102.03 (5), 139.41 (6), 150.18 (2), 163.21 (4), 200.35 (OCH_2CHO). IR (KBr) ν 3482, 2947, 2868, 1700, 1685, 1464, 1386, 1272, 1256, 1166, 1131, 1096, 1063, 1056, 1038, 1012, 991, 886, 704 cm^{-1}.

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(2-hydroxyethyl)uridine (**S.24**). TLC (6:3:1 petroleum ether/EtOAc/CH_3CN) R_f = 0.34; ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.01-1.10 (28H, m, 4 $SiCH(CH_3)_2$), 3.74-3.76 (2H, m, CH_2CH_2OH), 3.92-4.00 (4H, m, 2′, 5′, CH_2CH_2OH), 4.17 (2H, m, 3′, 4′), 4.26 (1H, d, *J* = 13.7 Hz, 5′), 5.70 (1H, d, *J* = 8.1 Hz, 5), 5.75 (1H, s, 1′), 7.90 (1H, d, *J* = 8.1 Hz, 6), 9.96 (1H, s, NH). ^{13}C NMR (90 MHz, $CDCl_3$) δ 12.73-13.62 ($SiCH(CH_3)_2$), 16.97-17.65 ($SiCH(CH_3)_2$), 59.46 (5′), 61.78 ($OCH_2 CH_2OH$), 68.37 (3′), 73.20 (OCH_2CH_2OH), 81.99 (4′), 83.04 (2′), 89.62 (1′), 101.99 (5), 139.38 (6), 150.58 (2), 163.80 (4). IR (KBr) ν 2946, 2869, 1701, 1464, 1387, 1271, 1165, 1129, 1094, 1062, 1039, 992, 885, 705 cm^{-1}. MS (CI, CH_4) *m/z* 531 (M^+).

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(ethyl-2-methylsulfonate)uridine (**S.25**) TLC (17:3 CH_2Cl_2/acetone) R_f = 0.48; ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.03-1.12 (28H, m, 4 $SiCH(CH_3)_2$), 3.12 (3H, s, SCH_3), 3.89 (1H, d, *J* = 4.1 Hz, 2′), 3.97 (1H, dd, *J* = 2.2, 13.6 Hz, 5′), 4.10-4.15 (3H, m, 4′, OCH_2CH_2S), 4.20-4.23 (1H, m, 3′), 4.26 (1H, d, *J* = 13.6 Hz, 5″), 4.44-4.46 (2H, m, OCH_2CH_2S), 5.70 (1H, dd, *J* = 2.1, 8.2 Hz, 5), 5.73 (1H, s, 1′), 7.91 (1H, d, *J* = 8.2 Hz, 6), 8.95 (1H, s, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.85-13.74 ($SiCH(CH_3)_2$), 17.07-17.73 ($SiCH(CH_3)_2$), 38.00 (SCH_3), 59.59 (5′), 68.63 (3′), 69.29 (OCH_2CH_2OMs), 69.44 (OCH_2CH_2OMs), 81.93 (4′), 83.27 (2′), 88.96 (1′), 101.97 (5), 139.38 (6), 150.33 (2), 163.60 (4). IR (KBr) ν 3213, 3065, 2947, 2869, 1699, 1465, 1355, 1272, 1179, 1129, 1095, 1065, 1040, 993, 885, 705 cm^{-1}.

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(thiobenzoylethyl)uridine (**S.26**). TLC (4:1 CH_2Cl_2/acetone) R_f = 0.68; ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.00-1.10 (28H, m, 4 $SiCH(CH_3)_2$), 3.36 (2H, m, OCH_2CH_2S), 3.91 (1H, d, *J* = 3.3 Hz, 2′), 3.99 (1H, dd, *J* = 1.5, 13.6 Hz, 5′), 4.02-4.06 (2H, m, OCH_2CH_2S), 4.18 (2H, m, 3′, 4′), 4.25 (1H, d, *J* = 13.6 Hz, 5″), 5.68 (1H, dd, *J* = 1.7, 8.2 Hz, 5), 5.73 (1H, s, 1′), 7.44 (2H, m, Ar), 7.56 (1H, m, Ar), 7.90 (1H, d, *J* = 8.2 Hz, 6), 7.96 (1H, m, Ar), 9.47 (1H, s, NH). ^{13}C NMR (90 MHz, $CDCl_3$) δ 12.71-13.62 ($SiCH(CH_3)_2$), 17.00-17.70 ($SiCH(CH_3)_2$), 29.16 (OCH_2CH_2S), 59.57 (5′), 68.45 (3′), 70.12 (OCH_2CH_2S), 81.88 (4′), 82.60 (2′), 89.34 (1′), 101.71 (5), 127.45, 128.74, 133.53, 137.18 (Ar), 139.73 (6), 150.14 (2), 163.92 (4), 191.67 (CO). IR (KBr) ν 3205, 3058, 2945, 2868, 1699, 1463, 1387, 1271, 1207, 1164, 1126, 1093, 1064, 1040, 914, 885, 705, 690 cm^{-1}.

2′-*O*-(Thiobenzoylethyl)uridine (**S.27**). TLC (9:1 CH_2Cl_2/CH_3OH) R_f = 0.14; ^{1}H NMR (360 MHz, CD_3OD) δ 3.27-3.38 (2H, m, OCH_2CH_2S), 3.75 (1H, dd, *J* = 3.0, 12.4 Hz, 5′), 3.82-3.96 (2H, m, 2′, 5″), 3.99-4.02 (1H, m, 4′), 4.05-4.09 (2H, m, OCH_2CH_2S), 4.24 (1H, m, 3′), 5.67 (1H, d, *J* = 8.1 Hz, 5), 5.95 (1H, d, *J* = 3.7 Hz, 1′), 7.49 (2H, m, Ar*H*), 7.62 (1H, m, Ar*H*), 7.93 (2H, m, Ar*H*), 8.06 (1H, d, *J* = 8.1 Hz, 6). ^{13}C NMR (90 MHz, CD_3OD) δ 29.70 (OXH_2CH_2S), 61.77 (5′), 70.01 (3′), 70.65 (OCH_2CH_2S), 83.80 (4′), 86.26 (2′), 89.31 (1′), 102.74 (5), 128.27, 130.04, 134.94, 138.30 (Ar), 142.58 (6), 152.35 (2), 166.36 (4), 193.18 (CO). IR (KBr) ν 3473, 3422, 3168, 3107, 3051, 2954, 2916, 2874, 2860, 2572, 2540, 1693, 1672, 1647, 1615, 1446, 1388, 1267, 1209, 1143, 1111, 1098, 915, 770, 687 cm^{-1}. MS (CI, CH_4) *m/z* 409 (M^+ + 1).

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(thiobenzoylethyl)uridine (**S.28**). TLC (19:1 CH_2Cl_2/CH_3OH) R_f = 0.59. ^{1}H NMR (360 MHz, CD_3CN) δ 3.30-3.41 (5H, m, 2′, 5′, 5″, OCH_2CH_2S), 3.75 (6H, s, 2 OCH_3), 3.87-3.99 (3H, m, 4′, OCH_2CH_2S) 4.01-4.04 (1H, m, 3′), 5.24 (1H, d, *J* = 8.2 Hz, 5), 5.83 (1H, d, *J* = 1.7 Hz, 1′), 6.87 (4H, m, Ar*H*), 7.21-7.33 (9H, m, Ar*H*), 7.41-7.52 (5H, m, Ar*H*), 7.61-7.66 (1H, m, Ar*H*), 7.74 (1H, d, *J* = 8.2 Hz, 6), 7.93-7.96 (4H, m, Ar*H*). ^{13}C NMR (90 MHz, CD_3CN) δ

17.03 (OCH_2CH_2S), 56.02 (OCH_3), 63.11 (5′), 69.86 (3′), 70.28 (OCH_2CH_2S), 83.21 (2′), 84.04 (4′), 87.65 ($OC(Ph)_3$), 88.68 (1′), 102.55 (5), 114.24 (Ar), 128.06, 129.02, 129.14, 129,98, 131.17, 131.64, 134.81, 136.49, 136.73, 137.89 (Ar), 141.17 (6), 145.87 (Ar), 151.41 (2), 159.86 (Ar), 161.07 (4), 192.35 (SC(O)Ph). MS (CI, CH_4) *m/z* 710 (M^+).

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(ethyl)-uridine *tert*-butyl disulfide (**S.29**) TLC (49:1 CH_2Cl_2/CH_3OH) R_f = 0.12; ^1H NMR (360 MHz, acetone-d_6) δ 1.32 (9H, s, $C(CH_3)_3$) 3.00 (2H, t, J = 6.5 Hz, OCH_2CH_2S), 3.48 (2H, m, 5′, 5″), 3.80 (6 H, s, OCH_3), 3.94-4.07 (4H, m, 2′, 3′, OCH_2CH_2S), 4.15-4.17 (1H, m 4′), 5.29 (1H, d, J = 8.1 Hz, 5), 5.94 (1H, d, J = 2.5 Hz, 1′), 6.90-6.93 (4H, m, Ar*H*), 7.32-7.37 (7H, m, Ar*H*), 7.47-7.50 (2H, m, Ar*H*), 7.92 (1H, d, J = 8.1 Hz, 6). ^{13}C NMR (90 MHz, acetone-d_6) δ 29.28-30.56 (acetone, $C(CH_3)_3$), 40.79 (OCH_2CH_2S), 48.32 ($C(CH_3)_3$), 55.61 (OCH_3), 63.15 (5′), 69.94 (3′), 70.16 (OCH_2CH_2S), 83.30 (2′), 84.03 (4′), 87.58 (Ar), 88.75 (1′), 102.41 (5), 114.09, 127.83, 128.82, 129.13, 131.14, 136.37, 136.67 (Ar), 140.88 (6), 145.93 (Ar), 151.29 (2), 159.80 (Ar), 163.58 (4). MS (FAB, 3-NBA) *m/z* 695 (M^+ + 1).

3′-*O*-(*N*,*N*-Diisopropyl-β-cyanoethylphosphoramidite)-5′-*O*-(4,4′-dimethoxytrityl)-2-*O*-(ethyl)uridine *tert*-butyl disulfide (**S.30**). TLC (2:1 petroleum ether/acetone) R_f = 0.34; ^1H NMR (360 MHz, CD_3CN) δ (two diastereomers) 1.04-1.06 (4H, m, 2 $NCH(CH_3)_2$), 1.14-1.18 (12H, m, 2 $NCH(CH_3)_2$), 1.30, 1.31 (9H, 2 s, $SSC(CH_3)_3$), 2.51-2.69 (2H, m, OCH_2CH_2CN), 2.89-2.94 (2H, m, OCH_2CH_2S), 3.37-3.44 (2H, m, OCH_2CH_2CN), 3.58-3.66 (2H, m, 5′, 5″), 3.76, 3.77 (6H, 2 s, OCH_3), 3.80-3.95 (2H, m, OCH_2CH_2S), 4.06-4.12 (2H, m, 2′,4′), 4.40-4.53 (1H, m, 3′), 5.20, 5.21 (1H, 2 d, J = 8.1 Hz, 5), 5.85 (1H, m, 1′), 6.86-6.90 (4H, m, Ar*H*), 7.25-7.36 (7H, m, Ar*H*), 7.42-7.47 (2H, m Ar*H*), 7.73,7.82 (1H, 2 d, J = 8.1 Hz, 6). ^{13}C NMR (75 MHz, acetone-d_6) δ (two diastereomers) 20.96 ($NCH(CH_3)_2$), 25.02-25.32 ($NCH(CH_3)_2$), 29.14-30.68 (acetone, $SSC(CH_3)_3$), 41.17 (OCH_2CH_2S), 44.07, 44.24 (CH_2CN), 48.27 ($SSC(CH_3)_3$), 55.68 (OCH_3), 59.25-59.92 ($POCH_2$), 62.72, 63.00 (5′), 70.27, 70.41 (OCH_2CH_2S), 71.30, 71.48 (3′), 82.11, 82.81 (2′), 83.14-83.41 (4′), 87.78 (Ar), 89.04, 89.29 (1′), 102.53, 102.59 (5), 114.10 (Ar), 118.81, 118.96 (CN), 127.80, 128.72, 129.19, 131.15, 131.53, 136.27, 136.35, 136.45 (Ar), 140.65, 140.72 (6), 145.68, 145.75 (Ar), 151.21 (2), 159.80 (Ar), 163.37 (4). ^{31}P NMR (202 MHz, CD_3CN) δ 147.44, 146.90. IR (KBr) ν 3199, 3059, 2966, 2932, 2871, 2837, 2248, 1692, 1608, 1615, 1510, 1458, 1364, 1253, 1179, 1119, 1085, 1070, 1035, 980, 828, 810 cm^{-1}. MS (FAB, 3-NBA) *m/z* 895 (M^+ +1). Anal. Calcd for $C_{45}H_{59}N_4O_9PS_2$: C, 60.38. H, 6.64. N, 6.26. Found: C, 60.59. H, 6.59. N, 5.95.

Critical Parameters

There are three critical parameters for the formation of intramolecular disulfide bonds in RNA. First, prior to oxidation the reducing agent used to remove the thiol protecting groups, DTT, must be entirely removed from sample. Second, the concentration of the RNA must be very low (1 to 4 μM) to avoid intermolecular cross-link formation. Third, for efficient cross-link formation the pH of the solution must be maintained between 8.0 and 8.4. At pH values <8.0 the concentration of thiolate ion will be reduced and the rate of cross-link formation will be reduced. However, prolonged exposure of the RNA to pH values >8.4 may lead to degradation.

Troubleshooting

See Table 5.4.1 for discussion of common problems encountered with these procedures and methods to identify, solve, and avoid them.

Anticipated Results

The authors have achieved 100% conversion of *tert*-butyl disulfide–protected tRNAs to intramolecularly disulfide-cross-linked tRNAs at three different sites of disulfide incorporation. Similar results should be obtained for thiols positioned at proximal locations either in a folded tertiary structure or within secondary structure.

The solid-phase synthesis of tRNAs containing the thiol-modified nucleosides described here results in yields of 0.5 mg tRNA after purification to single-nucleotide resolution. Each thiol-modified nucleoside is incorporated into the RNA oligomer as efficiently as commercially available phosphoramidites.

Time Considerations

Conversion of the *tert*-butyl disulfide–protected RNA to disulfide-cross-linked RNA takes 36 to 48 hr; both the reduction of the *tert*-butyl disulfide protecting groups and the air oxidation can be done as overnight reactions. To ensure complete reduction of the *tert*-butyl disulfide protecting groups the

Table 5.4.1 Troubleshooting Guide for Disulfide Cross-Linking

Problem	Possible causes	Solution
RNA does not cross-link or does not cross-link quantitatively	Protecting groups were not completely reduced	Add more DTT and/or incubate longer. After DTT removal, thiols on the RNA can be quantified using the fluorescent assay.
	DTT was not completely removed before oxidation commenced	Dialyze at a slower rate; use more buffer; monitor the dialysate with the fluorescence assay to determine when all DTT has been removed
	pH of the reaction was <8.0	Adjust pH to 8.0
	RNA was not properly folded before oxidation commenced	Fold the RNA
	RNA was not pure	Repurify the starting thiol-modified RNA
	Thiols are not positioned close enough to react	Select alternate positions for modification
	Resulting cross-linked RNA would be a strained, high-energy structure	Select alternate positions for modification
Modified nucleosides incorporate poorly during RNA synthesis	Moisture was present in phosphoramidite	Dry amidites by evaporation from freshly distilled CH_3CN several times
		Handle amidites under Ar
		Repurify the phosphoramidite
		Dilute the amidite with CH_3CN immediately before coupling instead of at the beginning of the synthesis

RNA should be incubated with DTT for ≥12 hr, while >14 hr appears unnecessary. The slow rate of dialysis (5 mL/min), using 3 L of buffer over 10 hr, ensures that the DTT is completely removed and that the buffer exchange into the lower-salt buffer is complete. Cross-link formation between thiols positioned at locations that are proximal in the folded RNA generally occurs in <12 hr. It has taken up to 48 hr for completion of some cross-linking reactions; in such cases the pH is monitored periodically and adjusted as necessary, and water is also added to compensate for evaporation.

Synthesis and purification of thiol-modified tRNA takes between 1 and 2 weeks depending on the amount of RNA to be purified before proceeding with the cross-linking reaction. This includes 1 day to synthesize the necessary phosphoramidites before commencing the solid-phase synthesis.

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