# Palladium-Mediated C5 Substitution of Pyrimidine Nucleosides

One of the most efficient ways to link a reporter group to oligonucleotides is through the incorporation of a modified nucleoside during automated oligonucleotide synthesis. Most techniques, which make use of synthetic oligonucleotides, function by hybridization to a complementary sequence. In order to avoid interference with hybridization, reporter groups should ideally be attached so that they do not interfere with hybridization or destabilize dsDNA. Two different types of tethers are described here—a rigid amidopropynyl linker and a flexible aminoethylthioether linker. The rigid amidopropynyl tether, linked through C5 of deoxyuridine, is sufficiently long and positioned such that a reporter group attached at the distal end lies outside the major groove of a DNA duplex.

Basic Protocol 1 describes a detailed procedure for the synthesis of one example of deoxyuridine modified by an amidopropynyl-linked reporter group, 5-(3-nicotinamidopropyn-1-yl)-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine (Fig. 1.1.1). The procedure is general and may be applied to other amidopropynyl-linked functional groups. The nicotinoyl group was used only as an illustration of the strategy for incorporating a functional group on the amimopropynyl tether. For use in oligonucleotide synthesis, the C5-modified deoxyuridine is converted to a 3′-phosphoramite as described in *UNIT 3.3*.

Basic Protocol 2 outlines the synthesis of 5-(3-acetamido-1-thiapropyl)-2′-deoxyuridine (Fig. 1.1.2). In contrast to the amidopropynyl tether, the more conformationally flexible thioether tether was designed to allow positioning of a molecular tool (e.g., chemical cleavage reagent or cross-linking reagent) on a complementary nucleic acid by hybridization of the modified oligonucleotide. The thiapropyl linker is capable of bridging the

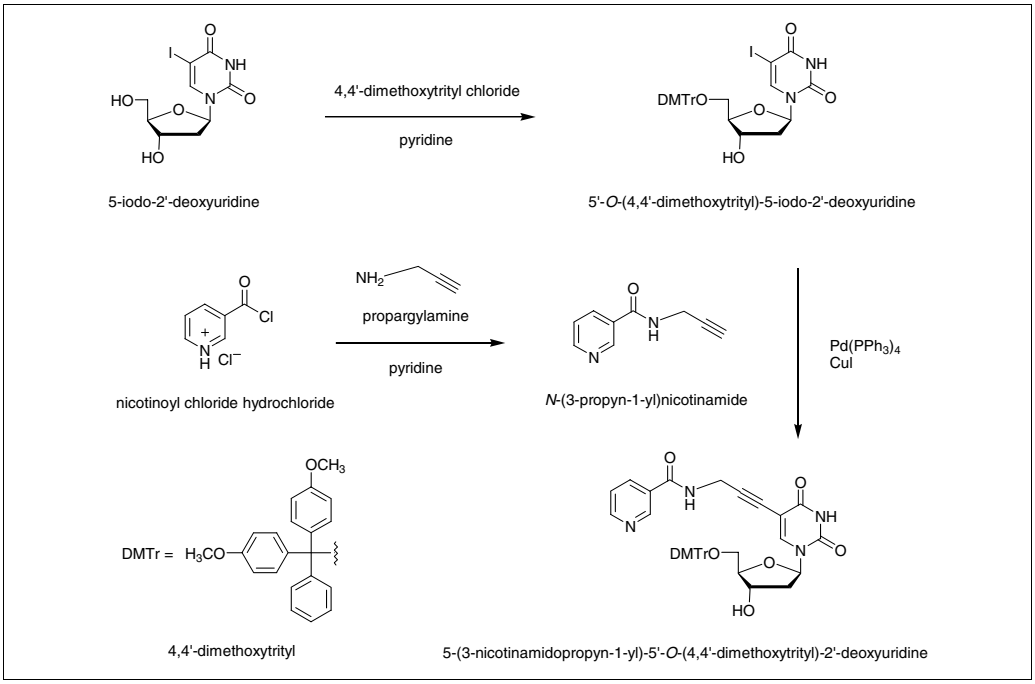


**Figure 1.1.1** Synthetic scheme for the preparation of 5-(3-nicotinamidopropyn-1-yl)-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine from 5-iodo-2′-deoxyuridine. The structure of 4,4′-dimethoxytrityl (DMTr) is shown in the lower left.

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span between two helices. For use in oligodeoxyribonucleotide synthesis, the N-acylated 5-(3-amino-1-thiapropyl)-2′-deoxyuridine is transformed to the 5′-dimethoxytrityl (DMTr) derivative as illustrated in Basic Protocol 1 for 5-iodo-2′-deoxyuridine, and is then converted to the 3′-phosphoramite as described in *UNIT 3.3*. Support Protocols 1 and 2 describe the preparation of two reagents needed for Basic Protocol 2—*N*,*N*′-bis(trifluoroacetyl)cystamine and *N*-acetoxysuccinimide, respectively.

***BASIC PROTOCOL 1***

## SYNTHESIS OF 5-(3-NICOTINAMIDOPROPYN-1-YL)-5′-*O*-(4,4′-DIMETHOXYTRITYL)-2′-DEOXYURIDINE

The sequence of reactions outlined here (see Fig. 1.1.1) illustrate conditions that are useful for the synthesis of a wide variety of reporter groups linked through C5 of deoxyuridine. The protocol includes three steps: synthesis of the N-acylated 3-aminopropyne (3-nicotinamidopropyne, in this example), reaction of 5-iodo-2′-deoxyuridine with 4,4′-dimethoxytrityl chloride, and palladium-catalyzed coupling of 3-nicotinamidopropyne with 5′-*O*-(4,4′-dimethoxytrityl)-5-iodo-2′-deoxyuridine. For the introduction of a different reporter group, 3-aminopropyne can be N-acylated by RCOCl (an acid chloride) or RC(O)OC(O)R (an anhydride) to obtain $RC(O)NHCH_2C{\equiv}CH$, in which R is the desired reporter group.

*CAUTION:* All reactions should be run in a suitable fume hood to avoid inhalation of toxic vapors.

### *Materials*

Nicotinoyl chloride hydrochloride
Pyridine, anhydrous
Nitrogen ($N_2$) stream
Triethylamine, freshly distilled (dried and purified by distillation at atmospheric pressure over calcium hydride; boiling point = 89° to 90°C)
Propargylamine, reagent grade (typically 99% pure)
Dichloromethane, reagent grade
10% (w/v) hydrochloric acid in water

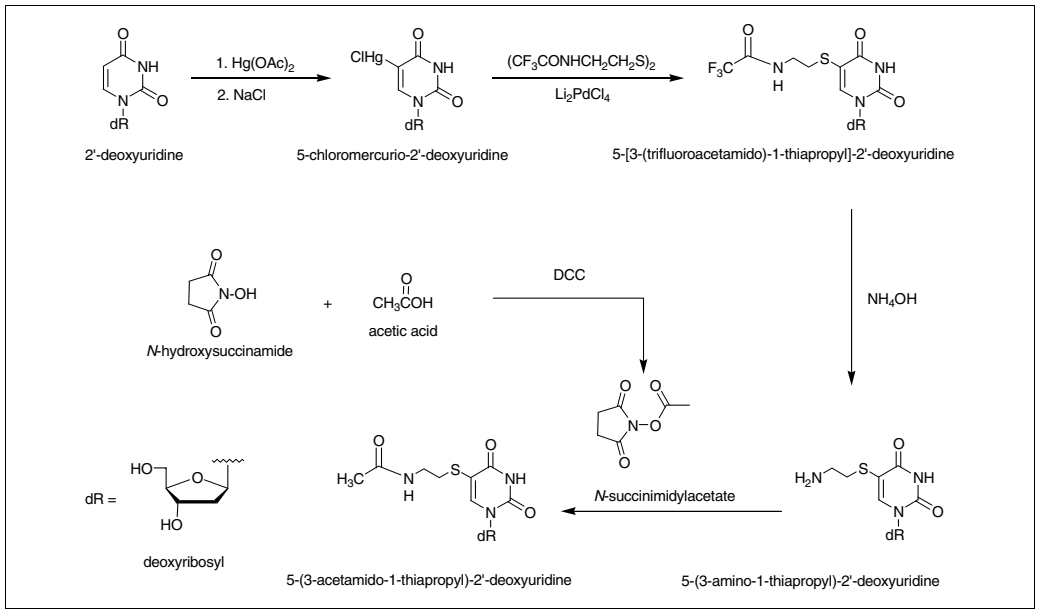


**Figure 1.1.2** Synthetic scheme for the preparation of 5-(3-acetamido-1-thiapropyl)-2′-deoxyuridine from 2′-deoxyuridine. The structure of deoxyribosyl (dR) is shown in the lower left. DCC, dicyclohexylcarbodiimide.

Sodium sulfate, anhydrous
Silica gel (230 to 400 mesh)
Methanol, reagent grade
5-Iodo-2′-deoxyuridine
4,4′-Dimethoxytrityl chloride
Diethyl ether, anhydrous
*N,N*-Dimethylformamide, anhydrous
Argon gas (optional)
Tetrakis(triphenylphosphine)palladium, $[(C_6H_5)_3P]_4Pd$
Copper(I) iodide
5% (w/v) $Na_2EDTA$ in water
Ethyl acetate, reagent grade

25- and 50-mL round-bottom flasks
Inert atmosphere/vacuum manifold (see Fig. 1.1.3)
500-µL and 1-mL syringes with stainless steel needles
125- and 250-mL Ehrlenmeyer flask
100-mL separatory funnel
Filter funnel and Whatman no. 1 filter paper
Chromatotron and radial chromatography plate coated with silica gel (2-mm thickness; Harrison Research)
Rotary evaporator with vacuum pump and water aspirator
Glass column (2-cm i.d. × ≥20-cm length) with stopcock

Additional reagents and equipment for thin-layer chromatography (TLC; *APPENDIX 4D*)

***Synthesize N-(3-propyn-1-yl)nicotinamide***

1. In a dry 25-mL round-bottom flask containing a ½-in. magnetic stir bar, add 1.068 g nicotinoyl chloride hydrochloride (6 mmol) to 10 mL of anhydrous pyridine under a nitrogen stream.

   *It is important that the flask be dry because nicotinoyl chloride reacts with water to give nicotinic acid. Glassware can be effectively dried by heating in a drying oven at 120°C for 2 hr. A small magnetic stir bar is generally dried at the same time as the flask and added to the flask prior to addition of the reagents.*

   *A general setup for running small-scale reactions under a dry nitrogen atmosphere is shown in Figure 1.1.3. The apparatus is configured for air-sensitive palladium-catalyzed reactions. For most other reactions, it is not necessary to bubble nitrogen through the reaction mixture at inlet (b).*

2. Add 500 µL triethylamine (700 mg, 7 mmol) with a 1-mL syringe and stainless steel needle, and stir the mixture on a magnetic stirrer at room temperature until the triethylamine is completely in solution.

   CAUTION: *Wear reagent-impermeable protective gloves. Triethylamine and propargylamine are corrosive.*

3. Add 250 µL propargylamine (365 mg, 6.6 mmol) dropwise to the reaction mixture with a 500-µL syringe and stainless steel needle, and continue stirring under nitrogen at room temperature for 4 hr.

4. Transfer contents of the flask to a 125-mL Ehrlenmeyer flask containing 40 mL water. Stir the mixture briefly, transfer to a 100-mL separatory funnel, and extract three times with 40 mL reagent-grade dichloromethane.

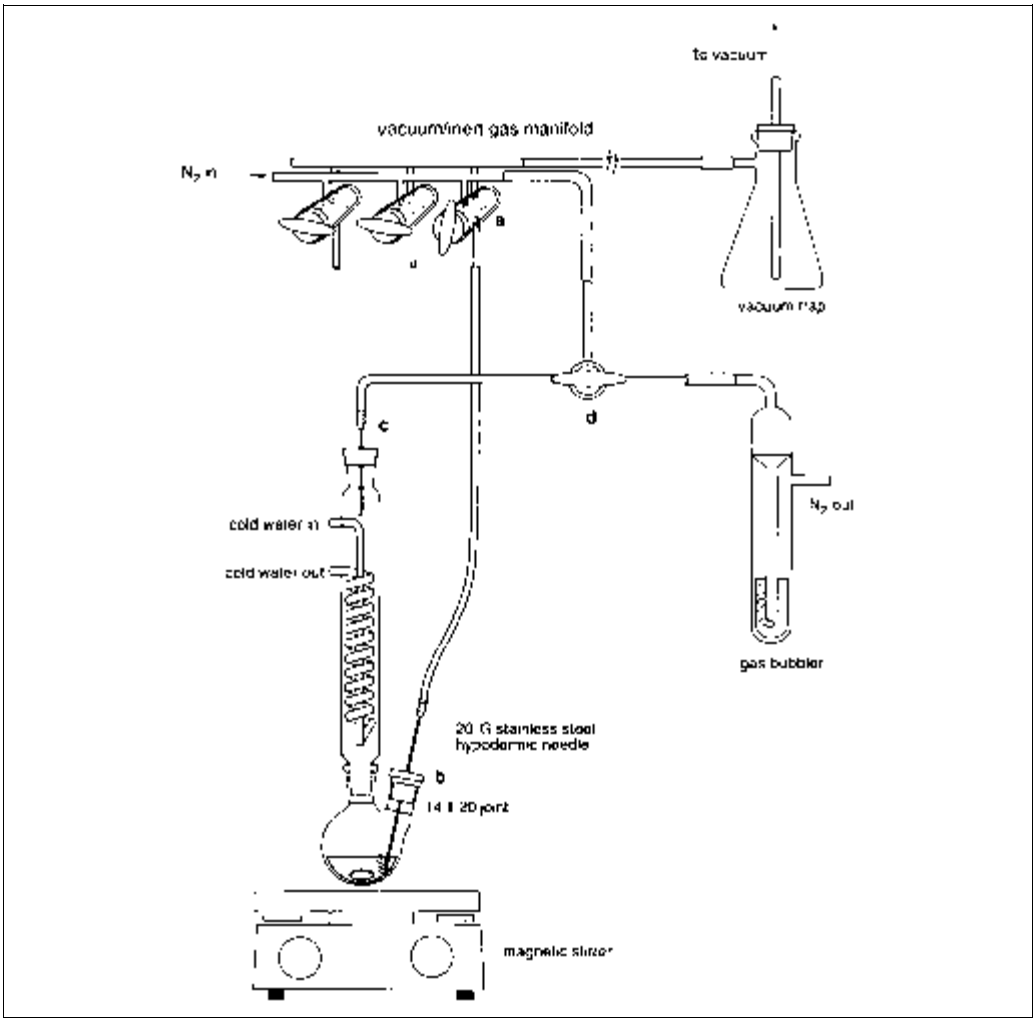


**Figure 1.1.3** Inert atmosphere/vacuum manifold setup for running reactions in a dry, oxygen-free atmosphere. As shown, the inert gas can be introduced into the reaction flask through stopcock (**a**) and a hypodermic needle inserted at (**b**; 14/20 standard taper joint). The slow stream of inert gas then passes through stopcock (**d**) and out through the gas bubbler. Care must be taken to avoid completely closing the system while the insert gas is being introduced under pressure through the manifold. The setup requires a source of dry nitrogen. For very oxygen-sensitive reactions, the solution is purged by bubbling the inert gas (nitrogen or argon) directly through the solution by lowering the stainless steal hypodermic needle into the solution. The needle is then pulled up above the level of the solution and the flask and condenser evacuated through stopcock (**a**) with stopcock (**d**) positioned to allow only the inert gas to pass through to the gas bubbler.

5. Wash the combined organic extracts twice with 20 mL of 10% hydrochloric acid and then once with 10 mL water.

6. Transfer the combined dichloromethane solution to a 250-mL Erlenmeyer flask and add 0.5 g anhydrous sodium sulfate. Swirl the solution for a few minutes and then allow to stand for 30 min.

7. Remove the drying agent by gravity filtration through a filter funnel fitted with Whatman no. 1 filter paper.

8. Wash the solid with 10 mL dichloromethane and remove solvent under reduced pressure using a rotary evaporator and water aspirator at room temperature to obtain the crude product.

*With a water aspirator and a water bath temperature of 25°C, the dichloromethane can generally be completely removed within 30 min. Longer periods of time may be required for complete removal of other higher-boiling-temperature solvents, such as methanol (step 9).*

9. Purify the crude product by radial chromatography according to manufacturer's instructions using a chromatotron plate with 2-mm silica gel thickness. Elute with 96:4 (v/v) dichloromethane/methanol and collect the effluent in ~10-mL fractions.

   *The silica gel plates may be purchased from the manufacturer or prepared according to the manufacturer's instructions.*

   *Alternatively, the crude product can be purified by column chromatography on silica gel (230 to 400 mesh; 12 × 2 cm) and eluted with the same solvent to give ~10-mL fractions.*

10. Analyze fractions by thin-layer chromatrography (TLC) on silica gel. Develop TLC plates with 96:4 (v/v) dichloromethane/methanol.

11. Combine all fractions that contain the desired product ($R_f$ = 0.26). Evaporate the solvent under reduced pressure (see step 8) to obtain *N*-(3-propyn-1-yl)nicotinamide (784 mg, 78%) as a white solid.

    *The compound is stable at room temperature and can be stored in a capped amber glass vial that has been purged with nitrogen. The authors do not generally store the product for >1 month, but it may be stable for a longer period of time. Unless otherwise specified, all intermediates synthesized as part of this protocol are stored under these conditions.*

12. Analyze the product by mass spectrometry (MS) and by proton and carbon nuclear magnetic resonance (NMR) spectroscopy.

    *N-(3-Propyn-1-yl)nicotinamide has the following spectroscopic characteristics:*

    *MS-EI 160 ($M^+$), 106, 78*

    *MS-CI 161 $(M + H)^+$*

    *$^1$H NMR 250 MHz ($CHCl_3$-$d_1$) δ 9.0 (d, J = 1.6 Hz, H-2 aromatic, 1H), 8.74 (m, H-6, 1H), 8.16 (m, H-5, 1H), 7.41 (m, H-4, 1H), 6.79 (br s, H-N, 1H), 4.28 (dd, $J_1$ = 5.2 Hz, $J_2$ = 2.5 Hz, N-$CH_2$-, 2H), 2.32 (t, J = 2.5 Hz, CCH, 1H)*

    *$^{13}$C NMR 62.9 MHz ($CHCl_3$-$d_1$) δ 29.90, 72.32, 79.0, 123.62, 135.26, 147.94, 152.61*

    *Analysis calculated for $C_9H_8N_2O$: C, 67.5; H, 5.0; N, 17.5; observed: C, 67.24; H, 4.82; N, 17.65. All values are given as percentages.*

    *The same procedure may be used for the synthesis of other amide derivatives of propargylamine from carboxylic acid chlorides or anhydrides. The $R_f$ and the spectral characteristics will differ depending on the nature of the acyl group.*

### *Synthesize 5′-O-(4,4′-dimethoxytrityl)-5-iodo-2′-deoxyuridine*

13. In a 50-mL round-bottom flask containing a ¾-in. egg-shaped magnetic stir bar, dissolve 354 mg of 5-iodo-2′-deoxyuridine (1 mmol) in 10 mL anhydrous pyridine.

    CAUTION: *The reaction must be performed in a well-vented fume hood.*

    *This reaction is sensitive to water, and anhydrous solvent(s) must be used under inert atmosphere. Anhydrous pyridine obtained in Sure/Seal bottles (e.g., Aldrich) is suitable for use in this reaction without further drying. Otherwise, the pyridine should be dried over solid KOH and distilled over Linde type 5Å molecular sieves and solid KOH. Pyridine has a fairly high boiling point (115°C).*

14. Evaporate approximately half the solvent using a rotary evaporator connected to a vacuum pump.

*It is advisable to use a vacuum pump rather than a water aspirator in order to rapidly evaporate the pyridine. This procedure removes water from the reaction mixture that may have been associated with the nucleoside by way of a pyridine-water azeotrope. With a good vacuum (<1 mmHg) it is possible to concentrate the reaction mixture in 10 to 30 min.*

15. Add 406 mg 4,4′-dimethoxytrityl chloride (1.2 mmol) to the resulting solution and stir the mixture overnight at room temperature under a dry nitrogen atmosphere (Figure 1.1.3).

    *In this case it is not necessary to bubble nitrogen through the reaction mixture. The apparatus shown in Figure 1.1.3 provides a means to keep the reaction mixture dry.*

    *4,4′-Dimethoxytrityl chloride may deteriorate if stored for long periods of time. The reagent should be light orange in color; do not use if it appears red. Although the reaction with 5-iodo-2′-deoxyuridine is allowed to run overnight, with pure reagents the reaction is typically compete within 1 to 2 hr.*

16. Add 15 mL ice-cold water to the reaction mixture and then extract twice with 20 mL dichloromethane.

17. Combine the two organic extracts, wash with 10 mL water, and dry over anhydrous sodium sulfate (step 6).

18. Remove the drying agent, wash, and remove solvent as described (steps 7 and 8).

19. Dissolve the residue in ~0.5 mL dichloromethane, introduce the resulting solution to a 10 × 2–cm column of 230- to 400-mesh silica gel, and elute with 98:2 (v/v) dichloromethane/methanol.

20. Collect the effluent in ~10-mL fractions and analyze by TLC on silica gel. Develop the plates with 98:2 (v/v) dichloromethane/methanol.

21. Combine fractions that contain the desired product ($R_f$ = 0.36) and evaporate the solvent under reduced pressure (step 8).

22. Add 2 mL anhydrous diethyl ether to the residue and evaporate under vacuum (step 8). Repeat this step until a white foam (646 mg, 98.5% yield) is obtained.

    *It is important to continue the evaporation until a foam is obtained. Otherwise, the product will contain unacceptable amounts of solvents that may interfere with the subsequent reaction. This can generally be accomplished by using a rotary evaporator connected to a water aspirator, with the flask partially immersed in a room temperature water bath. The product may be stored under dry nitrogen in an amber bottle in the dark for a few weeks.*

23. Analyze the product by mass spectrometry and by proton and carbon NMR spectroscopy.

    *5′-O-(4,4′-Dimethoxytrityl)-5-iodo-2′-deoxyuridine has the following spectroscopic characteristics:*

    *$^1$H NMR 250 MHz ($CHCl_3$-$d_1$) δ 8.38 (s, $N_3$-H, 1H), 8.13 (s, H-6, 1H), 7.46 to 7.23 (m, DMTr aromatic protons, 9H), 6.85 (d, J = 8.8 Hz, DMTr aromatic protons, 4H), 6.30 (dd, $J_1$ = 7.6 Hz, $J_2$ = 5.5 Hz, H-1′, 1H), 4.54 (m, H-3′, 1H), 4.08 (m, H-4′, 1H), 3.80 (s, $OCH_3$, 6H), 3.40 (m, H-5′, 2H), 2.33 and 2.44 (two sets of multiplets, H-2′, 2H), 1.98 (br s, 3′-OH, 1H)*

    *$^{13}$C NMR 62.9 MHz ($CHCl_3$-$d_1$) δ 159.83, 158.66, 149.7, 144.26, 135.39, 135.28, 130.08, 128.11, 128.01, 127.10, 123.80, 113.37, 87.06, 86.45, 85.55, 72.46, 68.51, 63.41, 55.27, 41.46*

    *MS-PD m/z calculated for $C_{30}H_{29}IN_2O_7$: 656; observed: 656 ($M^+$).*

*The same procedure may be applied to other C5-substituted deoxyuridine derivatives such as 5-(3-acetamido-1-thiapropyl)-2′-deoxyuridine or 5-(3-trifluoroacetamido-1-thiapropyl)-2′-deoxyuridine (see Basic Protocol 2). The $R_f$ and spectral characteristics will differ depending on the nature of the C5 substituent.*

### *Synthesize 5-(3-nicotinamidopropyn-1-yl)-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine*

24. In a two-neck 25-mL round-bottom flask containing a ½-in. magnetic stir bar (see Fig. 1.1.3), dissolve 328 mg of 5′-*O*-(4,4′-dimethoxytrityl)-5-iodo-2′-deoxyuridine (0.5 mmol; step 22) in 4 mL anhydrous *N,N*-dimethylformamide.

    *Anhydrous N,N-dimethylformamide obtained in Sure/Seal bottles (Aldrich) may be used without further drying. Alternatively, N,N-dimethylformamide may be dried by distillation at reduced pressure (boiling point = 76°C at 39 mmHg) over Linde type 4Å molecular sieves.*

25. Remove any dissolved oxygen in the solution by alternating three times between a vacuum and inert gas (dry nitrogen or argon) in the reaction apparatus.

    *As shown in Figure 1.1.3, the inert gas can be introduced directly to the solution through a tube attached at stopcock (a) and a hypodermic needle inserted at (b). When pulling a vacuum through stopcock (a), the needle must be lifted above the solution in the flask.*

    *The subsequent coupling reaction proceeds by way of a zero-valent palladium complex. Although the dry reagents may be weighed out in the atmosphere, the reaction is very sensitive to oxygen once the reagents are in solution. A complex mixture of products may result if all oxygen is not removed from the system at this point.*

26. Add the following reagents in the order indicated and then stir the reaction mixture at room temperature under nitrogen for 8 hr.

    300 µL freshly distilled triethylamine (218 mg, 2.15 mmol)

    240 mg *N*-(3-propyn-1-yl)nicotinamide (1.5 mmol; step 10)

    58 mg tetrakis(triphenylphosphine)palladium (0.05 mmol)

    19 mg copper(I) iodide (0.1 mmol).

    *Experience with many different alkyne-coupling reactions has shown that it is not always possible to predict which palladium reagent will work most effectively. In some cases (e.g., with 3,3-dimethoxypropyne), bis(triphenylphosphine)palladium dichloride is more effective.*

27. Add 10 mL of 5% $Na_2EDTA$ to the reaction mixture and extract twice with 30 mL dichloromethane.

28. Combine extracts and wash with 10 mL water. Dry the organic solution over anhydrous sodium sulfate (step 6).

29. Remove sodium sulfate by filtration (step 7), wash, and evaporate the solvent (step 8) to obtain the crude product as a foamy solid.

30. Purify the crude product by radial chromatography as in step 9, but elute with 50:50:5 (v/v/v) ethyl acetate/dichloromethane/methanol.

    *Alternatively, the crude product can be purified by column chromatography on silica gel (230 to 400 mesh; 10 × 2 cm) and eluted with 99:1 (v/v) dichloromethane/methanol.*

31. Analyze fractions by TLC on silica gel. Develop TLC plates with 99:1 (v/v) dichloromethane/methanol.

32. Combine all fractions that contain the desired product ($R_f$ = 0.39). Evaporate the solvent under reduced pressure (step 8).

33. Add 2 mL diethyl ether to the residue and evaporate under vacuum (step 8). Repeat this step until a white foam (215 mg, 62.5% yield) is obtained.

    *The product may be stored a short period of time (a few weeks) under nitrogen in an amber bottle. Normally, it is converted immediately to the phosphoramidite for incorporation into an oligonucleotide.*

34. Analyze the product by mass spectrometry and by proton and carbon NMR spectroscopy.

    *5′-O-(4,4′-Dimethoxytrityl)-5-(3-nicotinamidopropyn-1-yl)-2′-deoxyuridine has the following spectroscopic characteristics:*

    *$^1$H NMR 500 MHz ($CHCl_3$-$d_1$) δ 12.84 (s, $N_3$-H, 1H) 8.90 (d, J = 1.5, H-2 pyridine ring, 1H), 8.68 (dd, $J_1$ = 4.5 Hz, $J_2$ = 2 Hz, H-4 pyridine moiety, 1H), 8.22 (s, H-6 pyrimidine moiety, 1H), 7.90 (m, H-6 pyridine moiety, 1H), 7.65 (s, NH-CO-,1H), 7.47 to 7.17 (several sets, m, DMTr aromatic protons overlapped with H-5 pyridine ring, 10H), 6.80 (d, J = 7 Hz, DMTr aromatic protons, 4H), 6.14 (t, J = 10 Hz, H-1′, 1H), 4.59 (m, H-3′, 1H), 4.18 (m, -$CH_2$N- and H-4′, 3H), 3.85 (s, $OCH_3$, 6H), 3.35 (m, H-5′, 1H), 3.45 (m, H-5″, 1H), 2.56 (m, H-2′, 1H), 2.32 (m, H-2″, 1H)*

    *$^{13}$C NMR 62.9 MHz ($CHCl_3$-$d_1$) δ 162.42, 158.30, 151.84, 149.61, 149.18, 148.47, 148.26, 142.75, 135.58, 135.40, 135.19, 134.80, 129.79, 129.50, 127.72, 126.66, 123.33, 123.23, 122.94, 122.54, 113.05, 86.70, 65.61, 70.83, 63.42, 54.95, 41.69, 30.32*

    *MS-CI m/z calculated for $C_{39}H_{36}N_4O_8$: 688; observed: 689 $(M + H)^+$*

    *MS-PD m/z observed: 688.8 $(M)^+$, 303 $(DMTr)^+$*

    *Analysis calculated for $C_{39}H_{36}N_4O_8$: C, 68.01; H, 5.21; N, 8.13; observed: C, 67.72; H, 5.26; N, 8.31. All values are given as percentage.*

    *Other alkynes will give 5-substituted 2′-deoxyuridine products with different $R_f$ values and spectral characteristics.*

***BASIC PROTOCOL 2***

## SYNTHESIS OF 5-(3-ACETAMIDO-1-THIAPROPYL)-2′-DEOXYURIDINE

This protocol describes synthesis of 5-(3-acetamido-1-thiapropyl)-2′-deoxyuridine. The sequence of reactions outlined here (Fig. 1.1.2) illustrates conditions useful for the synthesis of different functional groups linked through an aminothiapropyl tether to C5 of deoxyuridine. The protocol includes four steps: preparation of 5-chloromercurio-2′-deoxyuridine, palladium-mediated coupling with *N*,*N*′-bis(trifluoroacetyl)cystamine, removal of the trifluoroacetyl protecting group by ammonia, and coupling of an active ester to the pendant amino group. The latter reaction is illustrated with the active ester *N*-acetoxysuccinimide. Other active esters may be used to link modifying groups to the aminothiapropyl tether. For example, a modified nucleoside that has a bipyridine ligand linked to the amino group of 5-(3-amino-1-thiapropyl)-2′-deoxyuridine was synthesized via an active ester of 4-carboxy-4′-methylbipyridine. This modified nucleotide was used to prepare oligonucleotides that function as metal-mediated sequence-specific nucleases (Bergstrom and Chen, 1996).

*CAUTION:* Mercuric acetate and organomercury compounds are highly toxic. Wear gloves and properly dispose of all waste materials generated by this procedure.

**Palladium-Mediated C5 Substitution of Pyrimidine Nucleosides**

### *Materials*

2′-Deoxyuridine
Mercury(II) acetate

30% (w/v) and 0.1 M sodium chloride (reagent grade) in water
Ethanol, anhydrous
Diethyl ether, anhydrous
*N*,*N*′-Bis(trifluoroacetyl)cystamine (see Support Protocol 1)
0.1 M $Li_2PdCl_4$ solution (see recipe)
Hydrogen sulfide ($H_2S$)
Methanol, reagent grade
Chloroform, reagent grade
Silica gel (230 to 400 mesh)
Concentrated ammonium hydroxide
Dry ice/acetone (for freezing)
*N*-Acetoxysuccinimide (see Support Protocol 2)
Triethylamine, freshly distilled (dried and purified by distillation at atmospheric pressure over calcium hydride; boiling point = 89° to 90°C)
Tetrahydrofuran, anhydrous
Ethyl acetate, reagent grade

100- and 200-mL round-bottom flasks
Temperature-controlled magnetic stirrer
Mortar and pestle
Whatman no. 1 filter paper
100-mm-diameter porcelain Buchner funnel
Rotary evaporator with water aspirator
Glass chromatography column (2-cm i.d. × ≥20-cm length) with stopcock
Lyophilizer

***Synthesize 5-chloromercurio-2′-deoxyuridine***

1. Dissolve 7.094 g of 2′-deoxyuridine (31.09 mmol) in 30 mL water in a 200-mL round-bottom flask and add a 1-in. magnetic stir bar.

2. Dissolve 10.449 g mercury(II) acetate (32.8 mmol) in 45 mL water and add to the deoxyuridine.

3. Add 25 mL water and stir on a magnetic stirrer at 50°C for 2 hr.

   *During the course of the reaction, a dense white suspension of acetoxymercuriodeoxyuridine is formed. This is converted to the chloromercurio derivative in step 4.*

4. While stirring, cool the reaction to 40°C and add 15 mL of 30% sodium chloride (4.5 g, 0.077 moles).

5. Cool the reaction mixture to room temperature and filter the solution using a 100-mm-diameter porcelain Buckner funnel and Whatman no. 1 filter paper.

6. Wash the fine white precipitate sequentially with 60 mL of 0.1 M NaCl, 40 mL water, 20 mL ethanol, and 30 mL anhydrous diethyl ether.

7. Dry the white precipitate in a vacuum oven under vacuum at 80°C to obtain 5-chloromercurio-2′-deoxyuridine (13.95 g; 97% yield; melting point = 210.5° to 211°C).

   *5-Chloromercurio-2′-deoxyuridine is neither water nor air sensitive. It has been successfully stored without decomposition in amber bottles at room temperature for >5 years.*

8. Analyze the product by IR, UV, and $^1H$ NMR spectroscopy.

   *5-Chloromercurio-2′-deoxyuridine has the following spectroscopic characteristics:*

*$^1$H NMR (1.0 M KCN/$D_2O$) δ 7.70 (s, 1H), 6.35 (t, 1H, J = 6.5 Hz), 4.47 (m, 1H), 3.99 (m, 1H), 3.83 (m, 2H), 2.36 (2H, dd, J = 6 Hz)*

*IR (KBr) 3365, 1714, 1642, 1440, 1275, 1089, 1040 $cm^{-1}$*

*UV (pH 1.0) $\lambda_{max}$ 266 (ε = 10,440), $\lambda_{min}$ 239 (ε = 4,180); (pH 9.0) $\lambda_{max}$ 266 (ε = 10,120), $\lambda_{min}$ 242 (ε = 5,440); (pH 12.3) $\lambda_{max}$ 267 (ε = 8,870), $\lambda_{min}$ 253 (ε = 7,070)*

*Analysis calculated for $C_9H_{11}N_2O_5HgCl$: C 23.34; H 2.39; N 6.05; observed: C 23.54; H 2.32; N 5.89.*

***Synthesize 5-[3-(trifluoroacetamido)-1-thiapropyl]-2′-deoxyuridine***

9. Grind 5-chloromercurio-2′-deoxyuridine to a fine powder using a mortar and pestle.

   CAUTION: *In the process of grinding, the powder tends to accumulate static electricity and may be difficult to contain in the mortar and pestle. The mortar should be placed on a surface that can be easily cleaned. It is preferable to carry out this step in a hood to avoid breathing the powder. Grind ≥10% more material than required for the subsequent step to make up for loss that occurs during this process.*

10. Place 0.926 g finely ground 5-chloromercurio-2′-deoxyuridine (2.0 mmol) and 1.720 g *N*,*N*′-bis(trifluoroacetyl)cystamine (5 mmol) in a 100-mL round-bottom flask.

11. Add 40 mL of 0.1 M $Li_2PdCl_4$ solution to the flask and stir on a magnetic stir plate for 16 hr at ambient temperature.

    *The mixture turns orange to yellow shortly after the reagents are combined, and usually yields a clear orange-yellow solution within a few hours.*

12. Rapidly bubble hydrogen sulfide through the solution for 30 sec. Filter the mixture through a filter funnel by gravity filtration through Whatman no. 1 filter paper, and wash the solid with 20 mL reagent-grade methanol.

    CAUTION: *Hydrogen sulfide gas is highly toxic. All operations should be conducted in a well-ventilated fume hood. Hydrogen sulfide may be obtained in ½-lb (227-g) lecture bottles.*

13. Using a rotary evaporator with a water aspirator, evaporate the solvent from the filtrate under reduced pressure to give an oil.

    *Because the methanol solution still contains hydrogen sulfide gas, this evaporation should be done using a rotary evaporator located inside a fume hood. Water aspirator pressure is normally sufficient to remove the methanol within 30 min at room temperature.*

14. Purify the crude product on a 12 × 2–cm, 230- to 400-mesh silica gel column, eluting with a linear chloroform/methanol gradient ranging from 10% to 18% methanol.

15. Combine fractions that contain material with $R_f$ = 0.30 ($CH_3OH$-$CHCl_3$ 1:9 v/v) or $R_f$ = 0.71 ($CH_3OH$-$CHCl_3$ 1:3 v/v), and evaporate the solvent on a rotary evaporator using a water aspirator to obtain 5-[3-(trifluoroacetamido)-1-thiapropyl]-2′-deoxyuridine (0.41 g; 51% yield).

    *5-[3-(Trifluoroacetamido)-1-thiapropyl]-2′-deoxyuridine is neither water nor air sensitive. It can be stored without decomposition in amber bottles at room temperature for many years.*

16. Analyze the product by MS and by IR, $^1$H, and $^{13}$C NMR spectroscopy.

    *5-[3-(trifluoroacetamido)-1-thiapropyl]-2′-deoxyuridine has the following spectroscopic characteristics:*

    *MS-FAB m/z calculated for $C_{13}H_{16}F_3N_3O_6S$: 399.071; observed: 400.079 $(M + H)^+$*

*$^1$H NMR 300 MHz ($CH_3OH$-$d_4$) δ 8.32 (s, 1H, H-6), 6.27 (t, 1H, J = 6.6 Hz, H-1′), 4.43 (m, 1H, H-3′), 3.95 (m, 1H, H-4′), 3.79 (m, 2H, H-5′), 3.47 (t, 2H, J = 6.0 Hz, H-3″), 2.87 (m, 2H, H-2′), 2.32 (t, 2H, J = 6.0 Hz, H-2″)*

*$^{13}$C NMR 125 MHz ($CH_3OH$-$d_4$) 164.5 (C4), 159.0 (q, J = 286.6 Hz, C5″), 106.8 (C5), 89.0 (C1′), 86.8 (C4′), 72.0 (C3′), 62.7 (C5′), 41.4 (C2′), 39.7 (C3″), 33.5 (C2″)*

*IR (KBr): 3550-2900 (br, O-H), 3423, 3443 (N-H), 1723, 1692, 1651 (C=O), 1660, 1553, 1461 (C=C), 1179, 1271 $cm^{-1}$*

*Analysis calculated for $C_{13}H_{16}F_3N_3O_6S$: C, 39.08; H, 4.01; N, 10.52; S, 8.03; observed: C, 39.35; H, 3.63; N, 10.38; S, 8.22.*

***Remove trifluoroacetyl protecting group***

17. In a 100-mL round-bottom flask containing a 1-in. magnetic stir bar, dissolve 615 mg of 5-[3-(trifluoroacetamido)-1-thiapropyl]-2′-deoxyuridine (1.5 mmol) in 10 mL methanol.

18. Add 30 mL concentrated ammonium hydroxide, cap the reaction container, and stir at room temperature for 16 hr.

19. Remove excess ammonia and methanol under reduced pressure using a rotary evaporator with a water aspirator. Freeze the remainder of the solution with a mixture of dry ice and acetone.

    *The methanol and ammonia can generally be removed on a rotary evaporator in 30 min or less at room temperature.*

20. Remove water by lyophilization and dissolve the remaining solid in 3 mL anhydrous ethanol.

***Synthesize 5-(3-acetamido-1-thiapropyl)-2′-deoxyuridine***

21. Prepare a solution of 315 mg *N*-acetoxysuccinimide (2 mmol) and 350 µL freshly distilled triethylamine (2.5 mmol) in 2 mL anhydrous tetrahydrofuran. Add to ethanolic solution and stir at room temperature for 4 hr.

    *Other active esters may be used in place of N-acetoxysuccinimide to place a different functional group on the tether. The resulting chromatographic characteristics and spectroscopic properties will change accordingly.*

22. Remove the solvent under reduced pressure using a rotary evaporator and a water aspirator to obtain the crude product.

23. Purify the crude product by column chromatography using a 12 × 2–cm silica gel column, and eluting with a gradient of 100% ethyl acetate to 80:20 (v/v) ethyl acetate/methanol.

24. Collect effluent in ~10-mL fractions and analyze by TLC on silica gel, developing the plates with 85:15 (v/v) ethyl acetate/methanol.

25. Combine all fractions that contain the desired product ($R_f$ = 0.32). Evaporate the solvent under reduced pressure using a rotary evaporator and a water aspirator to obtain 5-[3-acetamido-1-thiapropyl]-2′-deoxyuridine (462 mg; 87% yield) as a white solid.

    *The product may be stored indefinitely under nitrogen in an amber bottle.*

26. Analyze the product by MS and by UV, $^1$H, and $^{13}$C NMR spectroscopy.

    *5-(3-Acetamido-1-thiapropyl)-2′-deoxyuridine has the following spectroscopic characteristics:*

*MS-FAB m/z calculated for $C_{13}H_{19}N_3O_6S$: 345.1073; observed: 346.1072 $(M + H)^+$*

*$^1H$ NMR 300 MHz ($CH_3OH$-$d_4$) δ 8.33 (s, H-6, 1H), 6.25 (t, J = 7 Hz, H-1′), 4.41 (m, H-3′, 1H), 3.93 (m, H-4′, 1H), 3.78 (m, H-5′, 2H), 3.31 (m, $SCH_2$-, 2H), 2.78 (t, J = 6 Hz, -$CH_2N$-, 2H), 2.29 (m, H-2′, 2H), 1.95 (s, acetyl group's $CH_3$, 3H)*

*$^{13}C$ NMR 125 MHz (DMSO-$d_6$) 169.3, 161.65, 150.0, 142.55, 106.8, 87.54, 84.58, 70.23, 61.1, 39.94, 37.88, 32.1, 22.6*

*UV (methanol) $\lambda_{max}$: 282.4, 202.0 nm*

*Analysis calculated for $C_{13}H_{19}N_3O_6S$: C, 45.2; H, 5.5; N, 12.2; S, 9.3; observed: C, 44.88; H, 5.37; N, 12.27; S, 9.32.*

***SUPPORT PROTOCOL 1***

## SYNTHESIS OF *N,N′*-BIS(TRIFLUOROACETYL)CYSTAMINE

The trifluoroacetyl group has found wide application as a base-sensitive protecting group. It can be removed by ammonia under the same conditions used to deprotect oligodeoxyribonucleotides following synthesis by the phosphoramidite method. Amines are most commonly converted to trifluoracetyl derivatives by treatment with trifluoroacetic anhydride and a tertiary amine.

*CAUTION:* Both triethylamine and trifluoroacetic anhydride are corrosive. Wear gloves and work only in a suitable hood.

### *Materials*

Chloroform, reagent grade
Cystamine dihydrochloride
Triethylamine, freshly distilled (dried and purified by distillation at atmospheric pressure over calcium hydride; boiling point = 89° to 90°C)
Trifluoroacetic anhydride
10% (w/v) $NaHCO_3$
2 N HCl
Sodium sulfate, anhydrous
Methanol, reagent grade
Ethyl acetate, reagent grade
Hexane, reagent grade

1-liter round-bottom flask
Drying tube containing Drierite
5-mL syringe
1-liter separatory funnel
Rotary evaporator with water aspirator
Vacuum oven at 35°C
Buchner funnel and Whatman no. 1 filter paper

### *Synthesize N,N′-bis(trifluoroacetyl)cystamine*

1. Filter reagent-grade chloroform through a short column of basic alumina and add 500 mL to a 1-liter round-bottom flask containing a magnetic stir bar and capped by a drying tube containing Drierite.

   *Reagent-grade chloroform typically contains ethanol to inhibit decomposition, which produces HCl. The basic alumina removes the ethanol.*

2. Add 9.0 g cystamine dihydrochloride (40 mmol) and 20.2 g triethylamine (27.8 mL, 0.2 mol) to the filtered chloroform.

3. Cool the flask in a cold water bath (0° to 5°C) while slowly adding 1.5 mL trifluoroacetic anhydride (18.5 g, 88 mmol) with a 5-mL syringe.

   *Trifluoroacetic anhydride, which hydrolyzes to trifluoroacetic acid when exposed to water, should be stored in a hood and protected from moisture.*

4. Stir the reaction mixture at room temperature overnight.

5. Transfer the reaction mixture to a 1-liter separatory funnel and wash sequentially with 250 mL water, 10% $NaHCO_3$, 2 N HCl, and water again.

6. Dry the chloroform solution over anhydrous sodium sulfate for 1 hr (see Basic Protocol 1, step 6).

7. Add 30 mL reagent-grade methanol, filter to remove the solid sodium sulfate (see Basic Protocol 1, step 7), and evaporate the solvent on a rotary evaporator using a water aspirator to yield a light yellow paste.

8. Add 100 mL of 2 N HCl to give a slurry of light yellow crystals, and stir for 20 min at room temperature.

9. Filter the slurry and wash the solid product with 100 mL of 2 N HCl and then with 100 mL water.

10. Dry the solid product in a vacuum oven at 35°C overnight.

    *The product is of suitable purity for use in palladium coupling reactions. To obtain analytically pure product, recrystallize as described below. The product may be stored indefinitely in a closed bottle at room temperature.*

***Recrystallize product***

11. Dissolve product in a minimum of hot methanol.

12. Slowly add ethyl acetate to the warm methanol solution and allow to cool.

    *On cooling to room temperature, a colorless crystalline product separates (melting point = 111°C).*

13. Collect crystals by vacuum filtration through a Buchner funnel using Whatman no. 1 filter paper.

14. Collect a second crop by adding hexane to the warmed methanol/ethyl acetate solution, and cooling and collecting crystals again.

    *The total yield of crystalline product is 10.8 g (84%). The product may be stored indefinitely in a closed bottle at room temperature.*

***Analyze product***

15. Analyze the product by $^1H$ and $^{13}C$ NMR spectroscopy.

    *$^1H$ NMR (250 MHz, acetone-$d_6$) δ 2.70 (t, $CH_2$ 4H, J = 10.4 Hz), 2.68 (q, $CH_2$ 4H)*

    *$^{13}C$ NMR (62.9 MHz, acetone-$d_6$) δ 37.2 ($CH_2$, J = 10.4 Hz), 39.6 ($CH_2$), 117.0 ($CF_3$, J = 287 Hz), 157.8 (C=O).*

## SYNTHESIS OF *N*-ACETOXYSUCCINIMIDE

*N*-Hydroxysuccinamide esters are generally prepared from carboxylic acids by reaction with dicyclohexylcarbodiimide and *N*-hydroxysuccinimide. The procedure outlined below can be applied to the synthesis of other *N*-hydroxysuccinimide esters. Active esters of some compound classes (e.g., amino acids) are commercially available.

***Materials***

*N*-Hydroxysuccinamide
Tetrahydrofuran, anhydrous
Nitrogen ($N_2$) gas
Glacial acetic acid
Dicyclohexylcarbodiimide (DCC)
Silica gel (optional; 230 to 400 mesh)
Ethyl acetate, reagent grade
Methanol, reagent grade

Vacuum manifold apparatus (Fig. 1.1.3) modified with a 10-mL conical flask and a 500-µL syringe
Filter funnel and Whatman no. 1 filter paper
Glass chromatography column (optional; 2-cm i.d. × 10-cm length) with stopcock

1. In a 10-mL conical flask containing a small magnetic stir bar, prepare a solution of 345.3 mg *N*-hydroxysuccinamide (3 mmol) in 1 mL anhydrous tetrahydrofuran under an inert atmosphere (e.g., nitrogen).

   *The setup shown in Figure 1.1.3 may be used with the inert gas inlet at (b) replaced by a 500-µL syringe containing glacial acetic acid (step 2). The apparatus is maintained under a positive pressure of nitrogen by the inlet line at (c).*

2. Add 174 µL glacial acetic acid (3 mmol).

3. Dissolve 620 mg dicyclohexylcarbodiimide (3 mmol) in 1 mL tetrahydrofuran and add to the reaction mixture. Stir the resulting solution at room temperature overnight (12 hr).

4. Remove the white precipitate (dicyclohexylurea) by filtration through Whatman no. 1 filter paper in a filter funnel.

5. *Optional:* Purify filtrate on a 10 × 2–cm silica gel column, eluting with 9:1 (v/v) ethyl acetate/methanol. Collect appropriate fractions ($R_f = 0.45$) and evaporate on a rotary evaporator using a water aspirator to give a white powder melting at 120°C.

   *Although N-acetoxysuccinimide can be used without purification, it is generally preferable to purify if the product is to be stored. The product may be stored indefinitely under dry nitrogen in an amber bottle.*

6. Analyze by MS and by proton NMR spectroscopy.

   The purified product has the following spectroscopic characteristics:

   *$R_f$ = 0.45 ($CH_2Cl_2$, silica)*

   *$^1$H NMR (500 MHz, $CHCl_3$-$d_1$) δ 2.84 (s, $CH_2$ 4H), 2.34 (s, $CH_3$ 3H)*

   *MS-CI calculated for $C_6H_7NO_4$: 157; observed m/z: 158 $(M + H)^+$.*

## 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*****.**

### *0.1 M $Li_2PdCl_4$ solution*

1.77 g $PdCl_2$ (0.01 mol)
0.84 g LiCl (0.02 mol)
70 mL anhydrous methanol
Stir at room temperature for 24 hr

Adjust volume to 100 mL with methanol

*The solution is generally not stored for more than a few weeks in a stoppered flask at room temperature.*

## COMMENTARY

### Background Information

The C5 position of pyrimidine nucleosides is nearly ideal as a site for tethering molecular reporter groups and other molecular devices to oligodeoxyribonucleotides, as groups of different sizes may be attached without adversely affecting DNA duplex formation. In recent years, a variety of specialized probe moieties such as biotin (Langer et al., 1981; Shimkus et al., 1985; Cook et al., 1988), fluorophores (Prober et al., 1987; Tesler et al., 1989; Hagmar et al., 1995), paramagnetic probes (Spaltenstein et al., 1988, 1989; Kirchner et al., 1990), pendant catalytic moieties (Dreyer and Dervan, 1985; Bashkin et al., 1994; Kwiatkowski et al., 1994; Bergstrom and Chen, 1996; Shah et al., 1996), and cross-linkers (Gibson and Benkovic, 1987; Tabone et al., 1994; Chaudhuri and Kool, 1995; Meyer and Hanna, 1996) have been coupled to deoxyuridine and then incorporated into nucleic acids. The use of C5 linkers to functionalize nucleic acids has been reviewed (Goodchild, 1990). Although many kinds of linkers have been used to attach reporter groups to C5, alkynyl groups appear to be preferable because they enhance duplex stability (Sagi et al., 1993; Ahmadian et al., 1998).

Synthesis of 5-carboxamidopropynyl-2′-deoxyuridine derivatives was initiated by 4,4′-dimethoxytrityl protection of the readily available 5-iodo-2′-deoxyuridine following a standard procedure (Jones, 1984). Tritylation of 5′-hydroxyl of 5-iodo-2′-deoxyuridine prior to the coupling reaction eliminated the need for toluyl protection and deprotection of the nucleoside hydroxyl groups (Robins and Barr, 1981, 1983). Palladium-mediated coupling reactions were carried out in dimethylformamide following a procedure similar to that reported by Hobbs (1989).

The steps outlined in the preparation of 5-(3-nicotinamidopropyn-1-yl)-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine provide a blueprint and strategy for the incorporation of many different kinds of reporter groups. As long as the desired reporter group has a reactive acyl functional group (carboxylic acid anhydride, chloride, or active ester), it is likely that it can be coupled to propargylamine and subsequently linked to deoxyuridine by the organopalladium coupling reaction.

The thioether-linked deoxyuridine derivative 5-[3-(trifluoroacetamido)-1-thiapropyl]-2′-deoxyuridine was synthesized by the procedure reported by Bergstrom et al. (Bergstrom et al., 1991; Ahmadian et al., 1998) via a palladium-mediated reaction of the disulfide *N*,*N*′-bis(trifluoroacetyl)cystamine with 5-chloromercurio-2′-deoxyuridine. Trifluoroacetyl functions as a base-sensitive protecting group that can be easily removed with aqueous ammonia. Because the trifluoroacetamido group is a poor ligand for palladium, it does not interfere with the palladium-mediated coupling reaction. Groups that are substantially more electron rich (such as acetamido) interfere with the coupling reaction. For this reason, the coupling reaction must be carried out prior to attaching electron-rich ligands to the cystamine amino group.

5-[3-(Trifluoroacetamido)-1-thiapropyl]-2′-deoxyuridine may be incorporated into oligonucleotides as its 5′-*O*-dimethoxytrityl-3′-phosphoramidite derivative (procedure not given). The trifluoroacetyl protecting group is subsequently cleaved during the final ammonia deprotection of the oligonucleotide. This leaves the amino group available for conjugation to reporter groups or molecular tools at the oligonucleotide stage. Alternatively, 5-[3-(trifluoroacetamido)-1-thiapropyl]-2′-deoxyuridine may be deprotected and conjugated to the desired reporter groups or molecular tool prior to transformation to the 5′-*O*-dimethoxytrityl-3′-phosphoramidite derivative and incorporation into the oligonucleotide.

Synthesis of 5-[3-(trifluoroacetamido)-1-thiapropyl]-2′-deoxyuridine requires 5-chloromercurio-2′-deoxyuridine. This intermediate is obtained in high yield by direct electrophilic mercuration of 2′-deoxyuridine with mercuric acetate in aqueous solution (Bergstrom and Ruth, 1977). An important difference between the synthesis of the alkynyl-linked dU and the thioether-linked dU is that the former requires use of a protected dU and must be done with complete exclusion of oxygen, while the latter is not air sensitive and does not require protecting groups.

**Table 1.1.1** Estimated Times for Completion of Syntheses

| Protocol | Time (hr) | Synthesis |
|---|---|---|
| Basic Protocol 1 | 18 | N-(3-Propyn-1-yl)nicotinamide |
| | 20 | 5′-*O*-(4,4′-Dimethoxytrityl)-5-iodo-2′-deoxyuridine |
| | 15 | 5-(3-Nicotinamidopropyn-1-yl)-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine |
| Basic Protocol 2 | 16 | 5-Chloromercurio-2′-deoxyuridine |
| | 21 | 5-[3-(Trifluoroacetamido)-1-thiapropyl]-2′-deoxyuridine |
| | 32 | 5-(3-Acetamido-1-thiapropyl)-2′-deoxyuridine |
| Support Protocol 1 | 36 | *N*,*N*′-Bis(trifluoroacetyl)cystamine |
| Support Protocol 2 | 14 | *N*-Acetoxysuccinimide |

There are alternative types of linkers as well as alternative methods for preparing alkynyl and thioalkyl linkers. In addition to the alkynyl and thioether linkages described here, alkyl and alkenyl linkers may also be obtained through organopalladium coupling methodology (Bergstrom and Ogawa, 1978). Examples of reporter groups linked through alkyl and alkenyl linkers are included in Literature Cited. The advantage of the alkynyl linker is the ease of preparation of the C5-substituted deoxyuridine that contains the 5′ protecting group (dimethoxytrityl) needed for subsequent oligonucleotide synthesis via the phosphoramidite methodology. In addition to the nicotinamidopropyne coupling reaction, the preparation of a series of other carboxamidopropynyl derivatives has been described (Ahmadian et al., 1998). Since organopalladium reactions can tolerate a wide variety of functional groups (e.g., hydroxyl, amido, carboxamide, ester, cyano, nitro, keto), there may be relatively few limitations on the nature of the group that can be introduced at C5 as a component of carboxamidopropyne. Deoxyuridine C5 substitution is preferred over deoxycytidine N4 or deoxyadenosine N6 substitution because the latter two modifications destabilize double-stranded DNA.

## Critical Parameters

The most critical parameter in each reaction is the purity of the reactants and the reagents. Even fresh commercial reagents should be checked by TLC and $^1$H NMR for purity and identity. 4,4′-Dimethoxytrityl chloride is sensitive to moisture and will deteriorate over time. The best results are obtained with a freshly opened bottle of the reagent. Alternatively, the authors store and transfer 4,4′-dimethoxytrityl chloride in a Vacuum Atmospheres dry box under a dry nitrogen atmosphere (any dry box should be suitable). The condensation reaction between 5′-*O*-(4,4′-dimethoxytrityl)-5-iodo-2′-deoxyuridine and the alkyne requires air-sensitive tetrakis(triphenylphosphine)palladium. This reagent should also be stored and transferred under nitrogen or argon. Again, it is preferable to use freshly opened reagents, as they are supplied in sealed glass ampules that may be difficult to keep free of oxygen once opened.

When planning the construction of a C5-modified nucleoside for introduction into oligonucleotides, the intermediates must not contain functional groups that are likely to interfere either with phosphoramidite preparation (e.g., hydroxyl) or oligonucleotide synthesis.

## Anticipated Results

If the procedures are followed as described in this unit, the yields of isolated product should be comparable to that reported here. The palladium-catalyzed coupling reactions are especially sensitive to reagents and conditions. If an alkyne other than the one described in the protocol is used for the procedure, it may be necessary to try palladium catalysts other than tetrakis(triphenylphosophine)palladium. If the products are to be used to construct phosphoramidites for oligonucleotide synthesis, 50 mg of the C5-substituted 4,4′-dimethoxytrityl (DMTr) derivative is generally sufficient to obtain enough product to accomplish one coupling reaction on a 1-μmol synthesis scale.

## Time Considerations

The time required to complete each procedure is summarized in Table 1.1.1. The estimated times do not include the time necessary to purify solvents. In some cases, these may be purchased and used without further purification.

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