A Base-Labile Protecting Group (Fluorenylmethoxycarbonyl) for the 5′-Hydroxy Function of Nucleosides

Once the exocyclic amino functions of a nucleoside have been protected (*UNIT 2.1*), further functionalization aimed towards assembly into oligonucleotides requires consideration of the specific regioselective protection of the hydroxy groups present in the monomeric building block. Some years ago it was found that, in the case of 2′-deoxynucleosides, the two hydroxy groups (3′- and 5′-) could be satisfactorily differentiated by the application of the triarylmethyl (trityl) family of acid-labile protecting groups for temporary protection of the primary 5′-hydroxy function (*UNIT 2.3*). In the case of the ribonucleoside series, however, the differentiation of the three hydroxy groups (2′-, 3′-, and 5′-) has to be achieved at the extremely high level of efficiency required for the iterative steps involved in oligonucleotide assemblies carried out on synthesizer machines. This increased complexity has led to a number of alternative synthetic pathways to oligoribonucleotides, some of which are now commercialized; other pathways remain to be explored more profoundly.

Most of the present oligoribonucleotide synthesis strategies adhere to the popular 5′-*O*-trityl chemistry and aim at finding solutions for 2′-*O*-protection (*UNIT 2.2*), such as the *t*-butyldimethylsilyl (TBDMS) group, which can be removed by treatment with fluoride ion. In contrast, the authors chose to take advantage of the well-established ketal-type group for 2′-*O*-protection, which allows final deprotection under particularly mild and isomerization-free acidolytic aqueous conditions at the end of the synthesis (*UNIT 2.2*), and investigated for 5′-protection the base-labile 9-fluorenylmethoxycarbonyl (FMOC) group as a valuable orthogonal complement. The following Basic Protocol presents a reliable procedure for the regioselective introduction of the FMOC group at the 5′-hydroxy position of nucleosides, starting with deoxythymidine or with 4-methoxytetrahydropyran-4-yl (MTHP)–protected ribonucleosides. The Basic Protocol includes steps for the isolation and characterization of all four 5′-*O*-FMOC-2′-*O*-MTHP-protected ribonucleosides and of 5′-*O*-FMOC-2′-deoxythymidine, which serves as a reference for the deoxyribonucleoside series. No modification is required for 5′-*O*-FMOC-2′-dA^{Bz,} -dC^{Bz}, or -dG$^{i\text{-}Bu}$ (yields are ~65% for dG$^{i\text{-}Bu}$ and 70% to 80% for dA^{Bz} and dC^{Bz}; Balgobin and Chattopadhyaya, 1987; Ma and Sonveaux, 1987). Additionally, four Support Protocols describe the preparation of the starting *N*-protected-2′-*O*-MTHP-ribonucleosides, which are not commercially available and are considered key intermediates for the preparation of the 5′-*O*-FMOC-protected ribonucleoside building blocks.

Characterization of the various products requires experience in thin-layer chromatography, short column (flash) chromatography, and proton NMR.

ACYLATION OF THE 5′-HYDROXY GROUP OF 2′-*O*-(4-METHOXYTETRAHYDROPYRAN-4-YL)-RIBONUCLEOSIDES WITH 9-FLUORENYLMETHOXYCARBONYL CHLORIDE

BASIC PROTOCOL

The procedure describes the acylation of the 5′-hydroxy function of *N*-base- and 2′-*O*-protected ribonucleosides with 9-fluorenylmethoxycarbonyl (FMOC) chloride in pyridine at moderately low temperature. The FMOC workup procedure and the chromatographic isolation and ^{1}H-NMR spectroscopic characterization of the resulting 5′-*O*-(9-fluorenylmethyl)carbonates **S.2a** to **S.2e** (Fig. 2.4.1) are presented in this protocol. The starting intermediates are the four 2′-*O*-MTHP-protected ribonucleotides rU$_{MTHP}$, rC$^{Bz}_{MTHP}$, rA$^{Bz}_{MTHP}$, and rG$^{i\text{-}Bu}_{MTHP}$ (**S.1a** to **S.1d**; Fig. 2.4.1 and Fig. 2.4.2) and

Contributed by Michael J. Gait and Christian Lehmann
Current Protocols in Nucleic Acid Chemistry (2000) 2.4.1-2.4.22

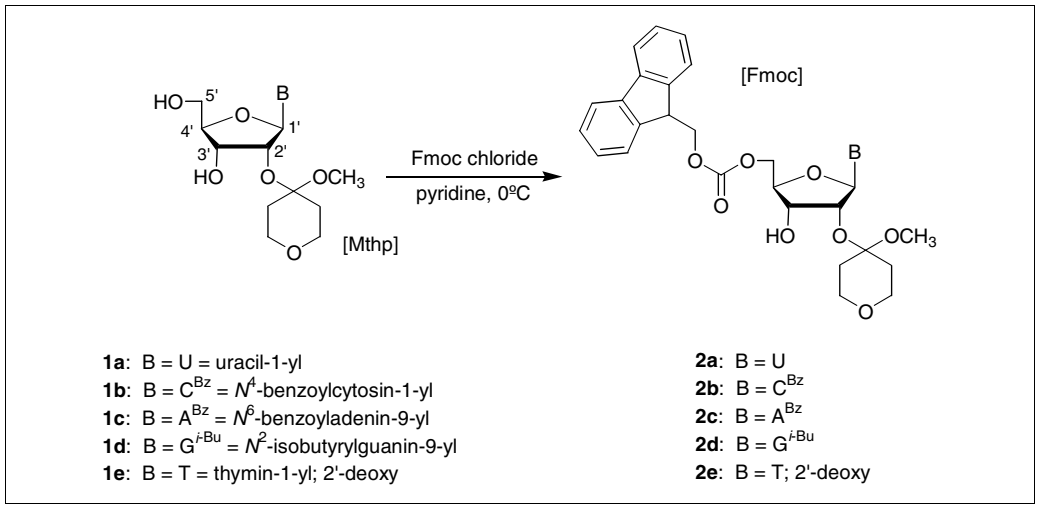

Figure 2.4.1 General procedure for the preparation of 5′-*O*-fluorenylmethoxycarbonyl-2′-*O*-(4-methoxytetrahydropyran-4-yl)-ribonucleosides (**S.2a-d**) and of 5′-*O*-fluorenylmethoxycarbonyl-2′-deoxythymidine (**S.2e**).

deoxythymidine (dT; **S.1e**; Fig. 2.4.1). Support Protocols 1 to 4 provide a summary for the preparation of the starting key intermediates **S.1a** to **S.1d**.

Materials

2′-Deoxythymidine or 2′-*O*-MTHP-ribonucleoside: 2′-*O*-(4-methoxytetrahydropyran-4-yl)-rU, -rC^{Bz}, -rA^{Bz}, or -rG$^{i\text{-}Bu}$ (see Support Protocols 1 to 4)

Dry pyridine, freshly distilled from calcium hydride (5 g/L) after refluxing for 2 hr under an inert atmosphere

Nitrogen gas

9-Fluorenylmethoxycarbonyl (FMOC) chloride (Cambridge Research Biochemicals), recrystallized from ether/pentane in a large desiccator (vapor diffusion method)

Diethyl ether (analytical grade)

Chloroform stabilized with 1% ethanol (commercially available analytical grade)

Methanol (analytical grade)

Anisaldehyde reagent (see recipe)

Ethane-1,2-diol (analytical grade)

Saturated aqueous sodium bicarbonate solution

Sodium sulfate (anhydrous)

Toluene (analytical grade)

Ethanol (analytical grade)

Pentane (analytical grade)

Silica-coated thin-layer chromatography (TLC) plate with fluorescent indicator Kieselgel 60F$_{254}$ (Merck 5554 glass plates or 5744 aluminium foils)

UV light source

D4 glass-filter crucible

Short column (diameter 5 cm; length ~10 cm) containing 50 g Kieselgel 60H without calcium sulfate (Merck 7736 or Fluka 60770, particle size 5-40 μm; or Merck 11677, particle size 15 μm), preconditioned with chloroform

Glass-fiber tissue

CAUTION: Wear gloves and perform all operations involving TLC solvents and reagents in a well-ventilated fume hood.

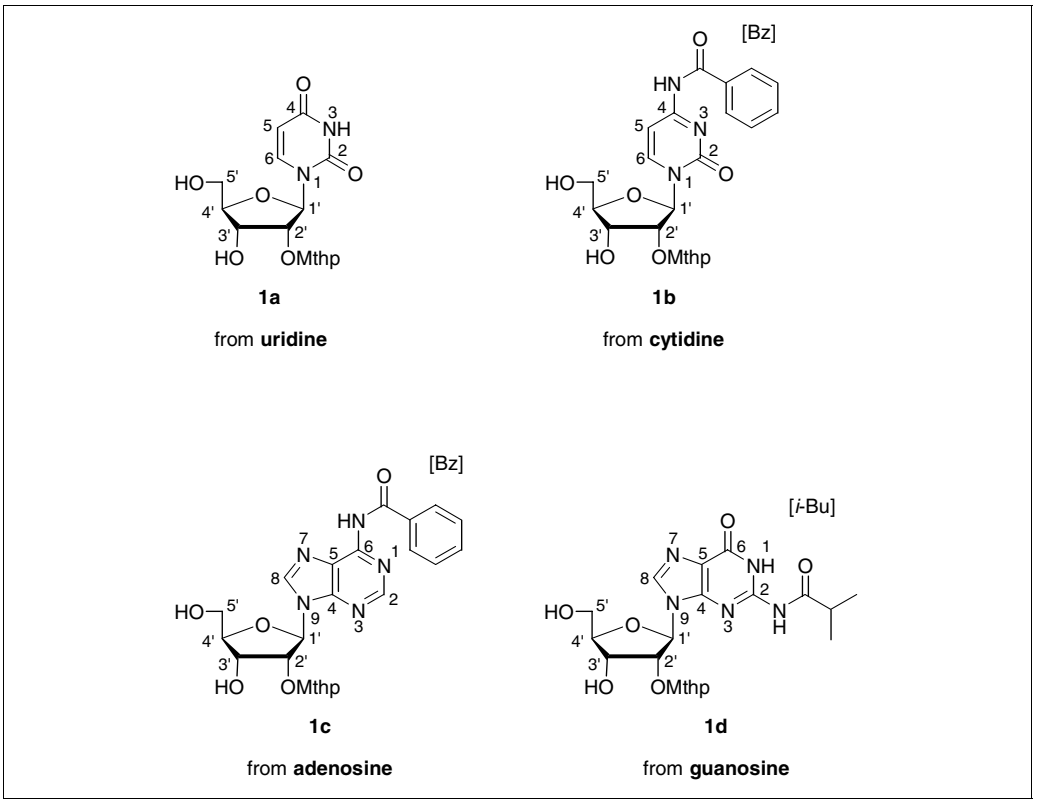

Figure 2.4.2 2′-*O*-(4-Methoxytetrahydropyran-4-yl)-ribonucleosides used as starting intermediates for FMOC protection. Preparation of these intermediates is shown in Figures 2.4.3 to 2.4.6.

Introduce FMOC group

1. Coevaporate 3.0 mmol of 2′-*O*-MTHP-ribonucleoside or deoxythymidine derivative (**S.1a** to **S.1e**) twice from 25 mL dry pyridine under reduced pressure using a rotary evaporator, and then apply a dry nitrogen atmosphere.

2. Dissolve the substrate in a third 25-mL portion of dry pyridine and cool to 0°C.

3. Stir the solution and add 3.6 mmol crystalline FMOC chloride. Stir under nitrogen for 30 min at 0°C.

   Normally, all the FMOC chloride will have dissolved by this time.

Check reaction by TLC

4. Resolve products on a silica-coated TLC plate with fluorescent indicator. Develop with diethyl ether followed by 9:1 (v/v) chloroform/methanol.

   Predevelopment in diethyl ether is used to disperse the pyridine.

5. Visualize under UV light (254 nm).

6. Stain by spraying with anisaldehyde reagent and heating on a hot plate at 80°C.

   Coloring plates containing nucleoside materials (summarized in Jork et al., 1990) is necessary for compounds that do not absorb strongly in the 254-nm region (i.e., unprotected rU_{MTHP} and dT).

   *TLC shows complete conversion into products with higher R_f values than the starting nucleosides. The major component is the 5′-O-FMOC derivative (see respective R_f values for each nucleoside following step 18; when starting with **S.1b**, the 5′-derivative R_f = 0.54). A very minor component at slightly higher R_f is identified as the 3′-O-FMOC derivative*

*(R_f = 0.68 when starting with **S.1b**). Some 3′,5′-di-O-FMOC derivative can be found at a very much higher R_f (0.80 when starting with **S.1b**).*

If complete conversion is not observed, the reaction in step 3 can be continued. It should not exceed 2 hr, however, as this leads to increased production of the 3′,5′-di-O-FMOC derivative.

Isolate product by chromatography

7. Terminate the reaction by adding 200 µL of ethane-1,2-diol and concentrate the mixture to an oil using a rotary evaporator with a vacuum pump.

8. Dissolve the oil in 150 mL chloroform and wash with 50 mL saturated aqueous sodium bicarbonate solution. Remove the aqueous phase and wash twice with 50 mL chloroform.

9. Dry the combined chloroform extracts over ~20 g anhydrous sodium sulfate.

10. Filter the dried extract under vacuum with a D4 glass filter crucible, and concentrate under reduced pressure (step 7).

11. Coevaporate the residual oil from ~50 mL each toluene (twice), ethanol (once), and chloroform (once).

    This removes traces of pyridine that are still contained in the dried, concentrated extract (step 10).

12. Apply as concentrated as possible (i.e., in ~5 mL) to a short 50-g Kieselgel 60H column preconditioned with chloroform. Apply a circular piece of glass-fiber tissue (not sea sand) to the top of the column.

    Short-column chromatography is performed according to Hunt and Rigby (1967) and is similar to the flash chromatography described by Still et al. (1978).

13. Elute minor side products first with chloroform (first 1 to 2 column volumes; 20-mL fraction size), then elute the major product-containing fractions with a gradient of 1% to 5% (v/v) ethanol/chloroform. Do not use >1 atm pressure for elution (Hunt and Rigby, 1967; Still et al., 1978).

14. Combine the product-containing fractions and concentrate to a foam.

15. Dissolve foam in 5 mL chloroform, precipitate by dropwise addition to 500 mL rapidly stirring pentane, and decant off most of the supernatant (~450 mL).

16. Collect solids by centrifuging 10 min at 3000 rpm *g*, 15°C, and decant the pentane.

17. Wash twice by resuspending in 50 mL fresh pentane and repeating the centrifugation. Dry the resulting white powder (**S.2a** to **S.2e**) in vacuo using a high vacuum from an oil pump.

Characterize the product

18. Characterize the final product by TLC and ^{1}H-NMR.

    *Chemical characterization data is provided below for **S.2a** to **S.2e**. ^{1}H-NMR spectra (250 MHz) were measured on a Bruker WM 250 instrument in DMSO-d_6. Chemical shifts (δ) are given in ppm downfield from tetramethylsilane (TMS, 0 ppm). Coupling constants (J) are given in Hz. D_2O exchange was performed on all samples.*

    *5′-O-FMOC-2′-O-MTHP-uridine (**S.2a**): yield 64%; R_f (silica, chloroform/methanol 9:1): 0.43; ^{1}H-NMR (250 MHz, DMSO-d_6): 1.6-1.9 (m×m, 4H; -CH_2-(3,5) of MTHP), 2.98 (s, 3H; H_3CO- of MTHP), 3.3-3.7 (m×m, 4H; -CH_2-(2,6) of MTHP), 3.9-4.6 (m×m, 8H; -CH-(4′, 3′, 2′), -CH_2-(5′), fluorenyl-H9 and -CH_2-), 5.4 (dbr, 1H, exchangeable with D_2O;*

3′-OH), 5.63 (d, J = 8.0 , 1H; uridyl-H5), 5.99 (d, J = 6.3, 1H; -CH-(1′)), 7.29-7.95 (m×m, 9H; uridyl-H6, fluorenyl-aromatic H), 11.4 (sbr, 1H, exchangeable; uridyl-N3-H).

*5′-O-FMOC-2′-O-MTHP-4-N-benzoyl-cytidine (**S.2b**): yield 61%; R_f (silica, chloroform/methanol 9:1): 0.54; ^{1}H-NMR (250 MHz, DMSO-d_6): 1.5-1.9 (m×m, 4H; -CH_2-(3,5) of MTHP), 2.95 (s, 3H; H_3CO- of MTHP), 3.2-3.7 (m×m, 4H; -CH_2-(2,6) of MTHP), 4.00-4.35 (m×m, 5H; -CH-(4′, 3′, 2′), -CH_2-(5′)), 4.47 (t, J = 6.0, 1H; fluorenyl-H9), 4.54 (d, J = 6.0, 2H; fluorenyl-CH_2-), 5.4 (d, J = 5, 1H, exchangeable with D_2O; 3′-OH), 6.09 (d, J = 6.4, 1H; -CH-(1′)), 7.3-8.3 (m×m, 15H; cytidyl-H5 and -H6, fluorenyl- and benzoyl-aromatic H), 11.3 (sbr, 1H, exchangeable; cytidyl-N4-H).*

*5′-O-FMOC-2′-O-MTHP-6-N-benzoyl-adenosine (**S.2c**): yield 65%; R_f (silica, chloroform/methanol 9:1): 0.56; ^{1}H-NMR (250 MHz ,DMSO-d_6): 1.4-1.9 (m×m, 4H; -CH_2-(3,5) of MTHP), 2.62 (s, 3H; H_3CO- of MTHP), 3.2-3.7 (m×m, 4H; -CH_2-(2,6) of MTHP), 4.18-4.48 (m×m, 4H; -CH-(4′, 3′), -CH_2-(5′)), 4.30 (t, J = 6.0, 1H; fluorenyl-H9), 4.51 (d, J = 6.0, 2H; fluorenyl-CH_2-), 5.08 (d×d, $J_{2'-1'}$ = 6.8, $J_{2'-3'}$ = 5.0; 1H; -CH-(2′)), 5.6 (d, J = 4.5, 1H, exchangeable with D_2O; 3′-OH), 6.21 (d, J = 6.8, 1H; -CH-(1′)), 7.2-8.4 (m×m, 13H; fluorenyl- and benzoyl-aromatic H), 8.73 and 8.74 (2s, 2H; adenyl-H2 and H8), 11.3 (sbr, 1H, exchangeable; adenyl-N6-H).*

*5′-O-FMOC-2′-O-MTHP-2-N-isobutyryl-guanosine (**S.2d**): yield 52%; R_f (silica, chloroform/methanol 9:1): 0.37; ^{1}H-NMR (250 MHz, DMSO-d_6): 1.12 (2d, J = 7.0, 6H; $(H_3C)_2CH$-)), 1.4-1.9 (m×m, 4H; -CH_2-(3,5) of MTHP), 2.67 (s, 3H; H_3CO- of MTHP), 2.75 (septet, J = 7.0, 1H; $(H_3C)_2CH$-)), 3.2-3.7 (m×m, 4H; -CH_2-(2,6) of MTHP), 4.12-4.37 (m×m, 4H; -CH-(4′, 3′), -CH_2-(5′)), 4.34 (m(t), 1H; fluorenyl-H9), 4.53 (d, J = 6.0, 2H; fluorenyl-CH_2-), 4.81 (d×d, $J_{2'-1'}$ = 7.5, $J_{2'-3'}$ = 4.7, 1H; -CH-(2′)), 5.4 (d, J = 4, 1H, exchangeable with D_2O; 3′-OH), 5.99 (d, J = 7.5, 1H; -CH-(1′)), 7.2-8.0 (m×m, 8H; fluorenyl-aromatic H), 8.25 (s, 1H; guanyl-H8), 11.6 and 12.1 (2sbr, 2H, exchangeable; guanyl-N1-H and N2-H).*

*5′-O-FMOC-2′-deoxythymidine (**S.2e**): obtained in analytically pure form by subsequent crystallization from ethyl acetate (76%; mp. 183°-185°C (decomp.); calc.: C 64.65, H 5.21, N 6.03%; found: C 64.54, H 5.09, N 5.82%); R_f (silica, chloroform/methanol 9:1): 0.44; ^{1}H-NMR (250 MHz, DMSO-d_6): 1.69 (s, 3H; thymidyl-5-CH_3), 2.10 (m, 2H; -CH_2-(2′)), 3.90 (m, 1H; fluorenyl-H9), 4.10-4.40 (m×m, 4H; -CH-(4′, 3′), -CH_2-(5′)), 4.58 (m(d), 2H; fluorenyl-CH_2-), 5.44 (d, J = 4.4, 1H, exchangeable with D_2O; 3′-OH), 6.17 (t, J = 6.3, 1H; -CH-(1′)), 7.29-7.91 (m×m, 9H; thymidyl-H6, fluorenyl-aromatic H), 11.3 (sbr, 1H, exchangeable; thymidyl-N3-H).*

PREPARATION OF 2′-*O*-(4-METHOXYTETRAHYDROPYRAN-4-YL)-URIDINE (S.1a) FROM URIDINE

SUPPORT PROTOCOL 1

Starting from uridine (**S.3**; Fig. 2.4.3), the desired key intermediate **S.1a** is obtained by a series of well-described reactions that handle the three ribonucleoside hydroxy groups differentially without protection of the base (Fromageot et al., 1967; Reese et al., 1970; van Boom and Wreesmann, 1984). Chromatography is necessary only in the last step of the sequence, which makes the procedure particularly useful for the preparation of large amounts of material. In the first step, reaction of uridine (**S.3**) with trimethyl orthoacetate gives the 2′,3′-methoxyethylidene derivative **S.4**, which is then acylated at the 5′-OH group to yield **S.5**. This derivative is cleaved by partial acidic hydrolysis to give a mixture of 2′,5′-di-*O*- and 3′,5′-di-*O*-acetates, of which the latter (**S.6**) can be fractionally crystallized in good yield. Acid-catalyzed ketalization with 5,6-dihydro-4-methoxy-2*H*-pyran followed by ammonolysis subsequently gives 2′-*O*-MTHP-uridine (**S.1a**).

Additional Materials *(also see Basic Protocol)*

Uridine (Sigma or Fluka), dried before use for 2 hr at 50°C over phosphorus pentoxide in vacuo

Toluene-*p*-sulfonic acid monohydrate (analytical grade)

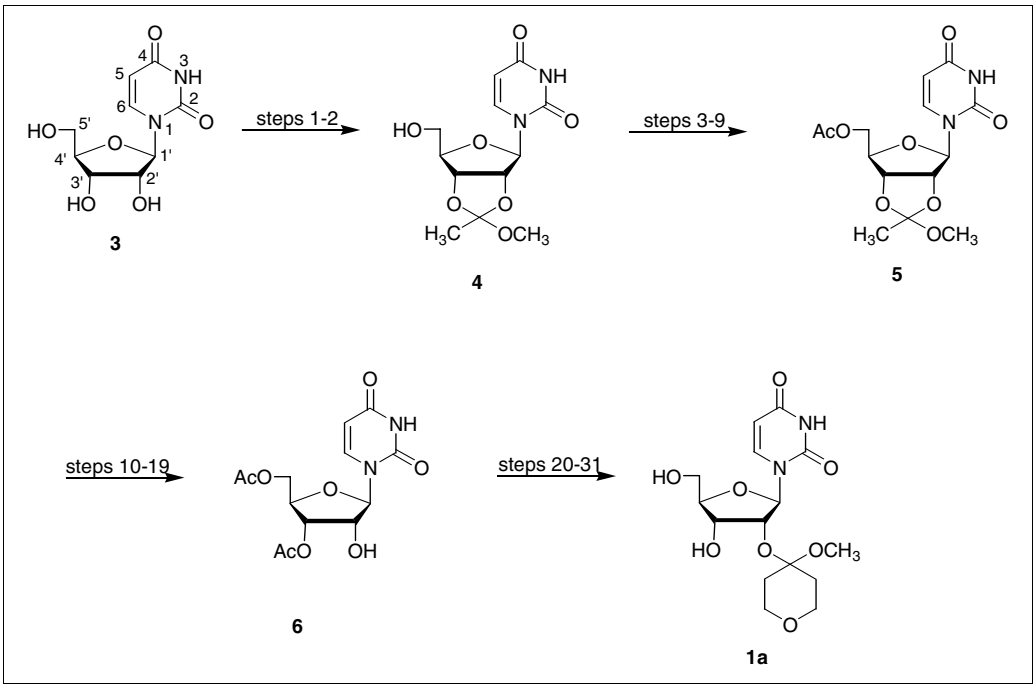

Figure 2.4.3 Scheme showing the preparation of 2′-*O*-MTHP-uridine (**S.1a**) from uridine (see Support Protocol 1).

Dioxane (analytical grade), dried by keeping over activated basic aluminum oxide, then distilled before use from sodium/benzophenone after refluxing until the blue color indicates complete dryness

Trimethyl orthoacetate (purum, 97%), dried by refluxing over calcium hydride (5 g/L) and distilled under inert atmosphere

Pyridine (analytical grade), dried by refluxing over calcium hydride (5 g/L) and then distilled under inert atmosphere

Acetic anhydride (analytical grade)

Methylene chloride (analytical grade)

Saturated (~1 M) aqueous sodium hydrogen carbonate

Magnesium sulfate

Formic acid (analytical grade)

5,6-Dihydro-4-methoxy-2*H*-pyran (analytical grade; Sigma or Fluka pract., ~90%; b.p.$_{20}$ 59° to 61°C)

Half-saturated methanolic ammonia solution (see recipe)

500-mL separatory funnel

Prepare 2′,3′-methoxyethylidene derivative (S.4)

1. Prepare a mixture of

   24.2 g dry uridine (**S.3**; 100 mmol)
   100 mg toluene-*p*-sulfonic acid monohydrate
   150 mL dry dioxane
   17 mL dry trimethyl orthoacetate
   and stir for 6 hr at room temperature.

2. Check the reaction by TLC (see Basic Protocol, steps 4 to 6).

   *TLC should show complete conversion of the starting material (R_f = 0.04) to the less polar cyclic orthoester **S.4** (R_f = 0.38).*

Prepare 5′-acetylated derivative (S.5)

3. Combine 150 mL dry pyridine and 50 mL acetic anhydride. Add this acylating solution dropwise over 15 min at room temperature to the stirred reaction mixture (step 1), and leave overnight.

4. Check the reaction by TLC.

   *TLC should show that the acetylation has gone to completion to yield a product (**S.5**) with higher mobility (R_f = 0.63).*

5. Add 50 mL methanol dropwise to the reaction mixture at 0°C to destroy excess acetic anhydride.

6. Concentrate the mixture to a colorless oil under reduced pressure using a rotary evaporator.

7. Dissolve in 250 mL methylene chloride and wash with 100 mL of 1 M aqueous sodium hydrogen carbonate.

8. Extract the aqueous layer five times with 100 mL methylene chloride for each extraction.

9. Dry the combined organic layers over 25 g magnesium sulfate and concentrate to a thick oil.

Prepare 3′,5′-di-O-acetyluridine (S.6)

10. Coevaporate the resulting oil twice from 100 mL ethanol to remove traces of pyridine.

11. Dissolve **S.5** in 150 mL of 9:1 (v/v) formic acid/water and incubate 15 min at room temperature.

12. Check the reaction by TLC.

    TLC should show that the cleavage of the methoxyethylidene function is complete, yielding 2′,5′-di-O- and 3′,5′-di-O-acetyluridines ($R_f \approx 0.4$).

13. Evaporate the aqueous formic acid under reduced pressure with a rotary evaporator, and coevaporate the residue twice with 100 mL toluene and twice with 100 mL ethanol.

14. Dissolve in 250 mL chloroform and transfer to a 500-mL separatory funnel containing 150 mL saturated aqueous sodium hydrogen carbonate solution. Shake carefully and separate out the chloroform layer.

15. Extract the aqueous layer twelve times with 50-mL portions of chloroform.

16. Dry the combined chloroform extracts over ~80 g anhydrous sodium sulfate, filter (see Basic Protocol, step 10), and evaporate to a glass.

17. Dissolve the crude product in 500 to 1000 mL boiling ethanol and store the solution for 2 days at room temperature.

18. Filter off the crystals and dry at high vacuum.

19. Determine the isomeric purity by ^{1}H-NMR spectroscopy. Recrystallize the product if the 2′,5′-isomer is present.

    *3′,5′-Di-O-acetyluridine (**S.6**): yield 23.9 g (73% from **S.3**; m.p. = 152°-155°C); R_f (silica, chloroform/methanol 9:1): 0.42; ^{1}H-NMR (250 MHz, DMSO-d_6/D_2O, 1:1, containing three drops of acetic acid per mL): 7.57 (d, J = 7.5, 1H; uridyl-H6), 5.89 (d, J = 7.5, 1H; uridyl-H5), 5.88 (d, J = 5.5, 1H; -CH-(1′)); the 2′,5′-isomer shows a slightly different*

^{1}H-NMR spectrum (same conditions): 7.57 (d, J = 7.5, 1H; uridyl-H6), 5.89 (d, J = 7.5, 1H; uridyl-H5), 5.86 (d, J = 6.0, 1H; -CH-(1′)).

Prepare 2′-O-MTHP-uridine (S.1a)

20. Combine 138 mg toluene-*p*-sulfonic acid monohydrate and 250 mL dry dioxane and stir until clear.

21. Add 32.8 g of 3′,5′-di-*O*-acetyluridine (**S.6**; 100 mmol) and 57 g of 5,6-dihydro-4-methoxy-2*H*-pyran (57 g, 5.0 eq) to the mixture and stir for 20 hr at room temperature.

22. Check the reaction by TLC.

    TLC should indicate complete reaction of the starting material.

23. Cool the clear solution in an ice water bath.

    The solution should be below 0°C to absorb exothermicity during neutralization.

24. Neutralize the reaction mixture by adding 800 µl of half-saturated methanolic ammonia solution.

25. Concentrate under reduced pressure and redissolve in 500 mL methanolic ammonia. Let stand overnight and check the reaction by TLC.

    TLC should show complete deacetylation.

26. Evaporate off methanolic ammonia and apply the residue to a column of silica gel (300 g; 12 × 7 cm) packed in methylene chloride.

    Short-column chromatography is performed according to Hunt and Rigby (1967) and is similar to the flash chromatography described by Still et al. (1978). Do not use air pressure >1 atm.

27. Elute the column with the following methylene chloride/methanol solutions: 500 mL of 98:2 (v/v); 500 mL of 96:4 (v/v); 1000 mL of 9:1 (v/v); and 1000 mL of 8:2 (v/v). Combine the product-containing fractions and evaporate to a colorless glass.

28. Dissolve the residue in 25 mL chloroform, precipitate by dropwise addition to 2500 mL rapidly stirring pentane, and decant off most of the supernatant (>2000 mL).

29. Collect solids by centrifuging 10 min at 3000 rpm, 15°C, and decant the pentane.

30. Wash twice by resuspending in 250 mL fresh pentane and repeating the centrifugation. Dry the resulting white powder (**S.1a**) in vacuo.

31. Characterize the final product by TLC and ^{1}H-NMR.

    *2′-O-MTHP-uridine (**S.1a**): yield 26.1 g (73% from **S.6**); R_f (silica, chloroform/methanol 9:1): 0.24; ^{1}H-NMR (250 MHz, DMSO-d_6): 1.50-1.90 (m×m, 4H; -CH_2-(3,5) of MTHP), 2.95 (s, 3H; H_3CO- of MTHP), 3.35-3-50 (m, 2H, -CH_2-(5′)), 3.5-3.8 (m×m, 4H; -CH_2-(2,6) of MTHP), 3.90 (m, 1H; -CH-(4′)), 3.96 (m, 1H, -CH-(3′)), 4.25-4.36 (m, 1H; -CH-(2′)), 5.10-5.25 (d×tbr, 2H, exchangeable with D_2O; 3′/5′-OH), 5.73 (d, J = 8.1, 1H; uridyl-H5), 6.00 (d, J = 7.8, 1H; -CH-(1′)), 7.93 (d, 1H; uridyl-H6), 11.4 (sbr, 1H, exchangeable; uridyl-N3-H).*

PREPARATION OF 2′-*O*-(4-METHOXYTETRAHYDROPYRAN-4-YL)-4-*N*-BENZOYLCYTIDINE (S.1b) FROM CYTIDINE

In the first step of this procedure, cytidine (**S.7**; Fig. 2.4.4) is simultaneously protected on its 3′- and 5′-hydroxy groups by the bifunctional protecting reagent 1,3-dichloro-1,1,3,3-disiloxane (Markiewicz and Wiewerowski, 1985). The derivative **S.8** is formed exclusively, due to the higher reactivity of the sterically more accessible 5′-hydroxy group and the subsequently favorable cyclization to the 3′-hydroxy group. Selective benzoylation on N4 of the base is then achieved by treatment with the active ester 1-hydroxybenzotriazolyl benzoate to give **S.9**. The latter compound is ketalized at the 2′-hydroxy group upon reaction with 5,6-dihydro-4-methoxy-2*H*-pyran. Then a solution of *n*-tetrabutylammonium fluoride in acetonitrile removes the disiloxane protecting group to give 2′-*O*-MTHP-4-*N*-benzoylcytidine (**S.1b**) in good yield (cf. Reese et al., 1970; van Boom and Wreesmann, 1984). Chromatography is required only for the last step of the sequence.

Additional Materials *(also see Basic Protocol and Support Protocol 1)*

Cytidine (**S.7**; Sigma or Fluka), dried before use for 2 hr at 50°C over phosphorus pentoxide in vacuo

N,*N*-Dimethylformamide, dried by stirring overnight at room temperature with calcium hydride (5 g/L) and subsequent distillation under reduced pressure (b.p. 70° to 80°C, 20 to 30 mmHg)

Dry pyridine (see Support Protocol 1 for drying procedure)

1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (see Support Protocol 5)

2 M triethylammonium bicarbonate buffer (see recipe)

Acetone (analytical grade)

1-Hydroxybenzotriazole (Fluka), dried before use for 72 hr at 50°C over phosphorus pentoxide in vacuo

Triethylamine, dried by refluxing 2 hr over calcium hydride (5 g/L) followed by distillation

Benzoyl chloride (Fluka, puriss.)

Acetonitrile (analytical grade)

1 M *n*-tetrabutylammonium fluoride (Aldrich/Fluka) in acetonitrile

CAUTION: 1-Hydroxybenzotriazole may explode at higher temperatures.

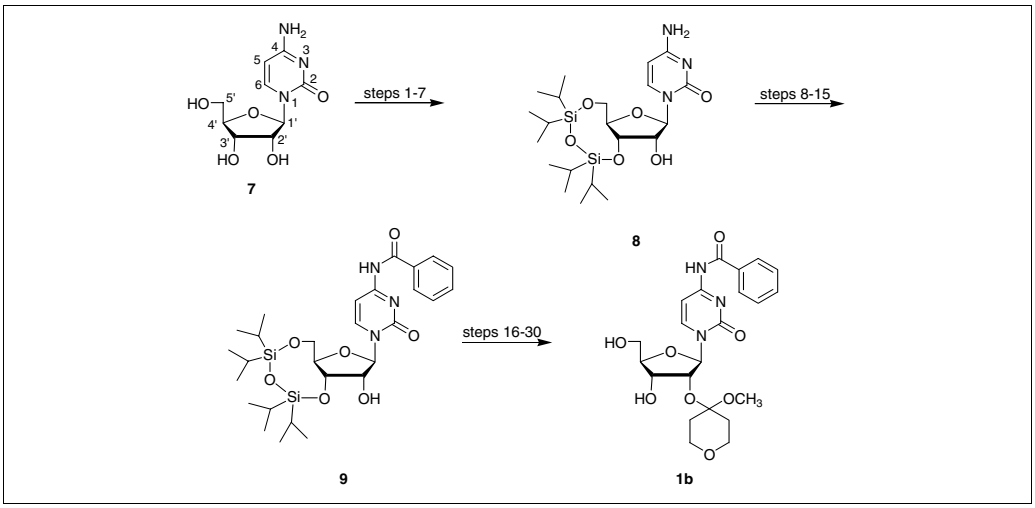

Figure 2.4.4 Scheme showing the preparation of 2′-*O*-MTHP-4-*N*-benzoylcytidine (**S.1b**) from cytidine (see Support Protocol 2).

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

1. Prepare and stir a suspension of the following:

   12.12 g dry cytidine (**S.7**; 50 mmol)
   200 mL dry *N*,*N*-dimethylformamide
   40 mL dry pyridine (40 mL).

2. Add 18 mL of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (62.5 mmol) to 50 mL dry *N*,*N*-dimethylformamide and add dropwise to the stirred cytidine solution over a period of 15 min. Stir the reaction mixture for 1 hr at room temperature.

3. Check the reaction by TLC (see Basic Protocol, steps 4 to 6).

   *TLC should indicate complete reaction of the starting material (R_f < 0.05) to **S.8** (R_f = 0.28).*

4. Neutralize with 75 mL of 2 M triethylammonium bicarbonate buffer and concentrate under reduced pressure to a small volume (100 mL) using a rotary evaporator.

5. Dissolve in 1000 mL methylene chloride and wash with 500 mL of 1 M aqueous sodium hydrogen carbonate followed by 500 mL water.

6. Dry the organic layer over 50 g magnesium sulfate and concentrate to a colorless oil.

7. Crystallize from 500 mL acetone to produce pure 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)cytidine.

   *3′,5′-O-(Tetraisopropyldisiloxane-1,3-diyl)cytidine (**S.8**): yield 19.5 g (85%), m.p. 226-228 (decomp.), R_f = 0.28. ^{1}H-NMR ($CDCl_3$/CD_3OD): 8.00 (d, J = 7.5, 1H; cytidyl-H6), 7.58 (d, J = 7.5, 1H; cytidyl-H5), 5.64 (d, J = 5.5, 1H; -CH-(1′)); ^{13}C-NMR ($CDCl_3$/CD_3OD): 166.2 (C4), 156.3 (C2), 140.6 (C6), 94.7 (C5), 91.5 (C1′), 81.6 (C4′), 75.1 (C3′), 68.2 (C2′), 60.0 (C5′), 17.4, 17.0 13.5, 13.0, 12.5 (TIPS).*

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoyl-cytidine (S.9)

8. Prepare and stir a solution of the following:

   10.0 g dry 1-hydroxybenzotriazole (75 mmol)
   300 mL dry dioxane
   21 mL dry triethylamine (150 mmol).

9. Combine 8.70 mL benzoyl chloride (75 mmol) and 50 mL dry dioxane, and add dropwise to the above mixture (step 8) over 15 min. Stir for 1 hr at room temperature.

10. Filter off the precipitated triethylammonium chloride salts under inert atmosphere using a glass filter crucible, and add the filtrate to a solution containing 24.25 g of 2′-*O*-(tetraisopropyldisiloxane-1,3-diyl)cytidine (**S.8**; 50 mmol) in 150 mL dry *N*,*N*-dimethylformamide.

11. Evaporate off a volume of ~100 mL under reduced pressure and stir the residue at room temperature for 3 days. Check the reaction by TLC.

    TLC should indicate complete reaction of the starting material.

12. Add 5 mL water and concentrate the reaction mixture under reduced pressure to a small volume (100 mL).

13. Dissolve in 1000 mL methylene chloride and wash as in step 5.

14. Dry the organic layer over 50 g magnesium sulfate and concentrate to a light brown oil.

15. Crystallize from a minimal amount of refluxing acetonitrile to yield analytically pure 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-4-*N*-benzoylcytidine (**S.9**).

*3′,5′-O-(Tetraisopropyldisiloxane-1,3-diyl)-4-N-benzoylcytidine (**S.9**): yield 22.6 g (77% in first crop). R_f = 0.40 (silica, chloroform/methanol 9:1). Elemental analysis: calc.; C 57.04, H 7.30, N 7.26%; found: C 56.36, H 7.08, N 7.26%.*

Prepare 2′-O-MTHP-4-N-benzoylcytidine (S.1b)

16. Dissolve 4.75 g toluene-*p*-sulfonic acid monohydrate (25 mmol) in 125 mL dry dioxane and evaporate under reduced pressure to a colorless oil to remove traces of water.

17. Under an inert atmosphere, add 29.45 g of 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-4-*N*-benzoylcytidine (**S.9**; 50 mmol) followed by 250 mL dry dioxane.

18. To the resulting clear solution, add 29 mL of 5,6-dihydro-4-methoxy-2*H*-pyran (28.5 g; 250 mmol). Stir 20 hr at room temperature and check the reaction by TLC.

TLC should show complete reaction of the starting material.

19. Neutralize the reaction with 3.5 mL half-saturated methanolic ammonia solution (check with moist pH indicator paper) and concentrate immediately under reduced pressure to a small volume (100 mL).

20. Dissolve in 1000 mL methylene chloride and wash as in step 5.

21. Dry the organic layer over 50 g magnesium sulfate and concentrate to a glass.

22. Add 250 mL of 1 M *n*-tetrabutylammonium fluoride in acetonitrile, stir for 1 hr at room temperature, and check the reaction by TLC.

TLC should show complete removal of the silyl protecting group.

n-Tetrabutylammonium fluoride in acetonitrile must be prepared by the experimenter. Commercially available solutions in tetrahydrofuran can also be used.

23. Concentrate under reduced pressure to a small volume (100 mL). Dissolve in methylene chloride, wash, and dry as in steps 20 and 21.

24. Dissolve with a minimal amount of dichloromethane and apply to a column of silica gel (300 g; 12 × 7 cm) packed in methylene chloride.

Short-column chromatography is performed according to Hunt and Rigby (1967) and is similar to the flash chromatography described by Still et al. (1978). Do not use air pressure >1 atm.

25. Elute the column with the following methylene chloride/methanol solutions: 500 mL of 98:2 (v/v); 500 mL of 96:4 (v/v); and 1000 mL of 9:1 (v/v).

26. Collect the main product-containing fractions and evaporate to a glass.

27. Dissolve the residue in 25 mL chloroform, precipitate by dropwise addition to 2500 mL rapidly stirring pentane, and decant off most of the supernatant (>2000 mL).

28. Collect the solids by centrifuging 10 min at 3000 rpm *g*, 15°C, and decant the pentane.

29. Wash twice by resuspending in 250 mL fresh pentane and repeating the centrifugation. Dry the resulting white powder (**S.1b**) in vacuo.

30. Characterize the final product by TLC and ^{1}H-NMR.

*2′-O-MTHP-4-N-benzoylcytidine (**S.1b**): yield 16.1 g (70% from **S.9**); R_f (silica, chloroform/methanol 9:1): 0.43; ^{1}H-NMR (250 MHz, DMSO-d_6): 1.50-1.90 (m×m, 4H; -CH_2-*

(3,5) of MTHP), 2.93 (s, 3H; H_3CO- of MTHP), 3.40-3.60 (m, 2H, -CH_2-(5′)), 3.5-3.8 (m×m, 4H; -CH_2-(2,6) of MTHP), 3.96 (m, 1H; -CH-(4′)), 4.02 (m, 1H, -CH-(3′)), 4.39 (m(d×d), 1H; -CH-(2′)), 5.19 (d, J = 6.5, 1H, exchangeable with D_2O; 3′-OH), 5.25 (t, J = 2.7, 1H, exchangeable with D_2O; 5′-OH), 6.14 (d, J = 6.8, 1H; -CH-(1′)), 7.36 (d, J = 7.7, 1H; cytidyl-H5), 7.4-8.1 (m×m, 5H; benzoyl-aromatic H), 8.43 (d, 1H; cytidyl-H6), 11.3 (sbr, 1H, exchangeable with D_2O; cytidyl-N4-H).

SUPPORT PROTOCOL 3

PREPARATION OF 2′-*O*-(4-METHOXYTETRAHYDROPYRAN-4-YL)-6-*N*-BENZOYLADENOSINE (S.1c) FROM ADENOSINE

In this protocol, adenosine (**S.10**, Fig. 2.4.5) is base protected prior to 3′,5′-disilylation by benzoylation of its crystalline 2′,3′,5′-tri-*O*-acetyl-derivative (**S.11**), yielding 6-*N*-benzoyladenosine (**S.12**) after deacetylation in situ. The chosen route (cf. Schaller et al., 1963; Reese et al., 1970; Büchi and Khorana, 1972; Jones, 1984; van Boom and Wreesmann, 1984; Markiewicz and Wiewerowski, 1985) is then comparable to the cytidine case (see Support Protocol 2): 3′,5′-disilylation to yield **S.13**, followed by acid-catalyzed ketalization of the 2′-OH by 5,6-dihydro-4-methoxy-2*H*-pyran, and cleavage of the silyl protecting group by fluoride ion to yield 2′-*O*-MTHP-6-*N*-benzoyladenosine (**S.1c**) in good chromatographically isolated yield.

Additional Materials *(also see Basic Protocol and Support Protocols 1 and 2)*

Adenosine (**S.10**; Sigma or Fluka), dried before use for 2 hr at 50°C over phosphorus pentoxide in vacuo
Dry pyridine (see Support Protocol 1 for drying procedure)
Acetic anhydride, fractionally distilled with 10% toluene to remove acetic acid
25% (w/v) sodium methoxide in methanol (pract., Aldrich, Fluka)
Acetic acid (analytical grade)
Diethyl ether (analytical grade)
Imidazole (analytical grade)
0.1 M hydrochloric acid

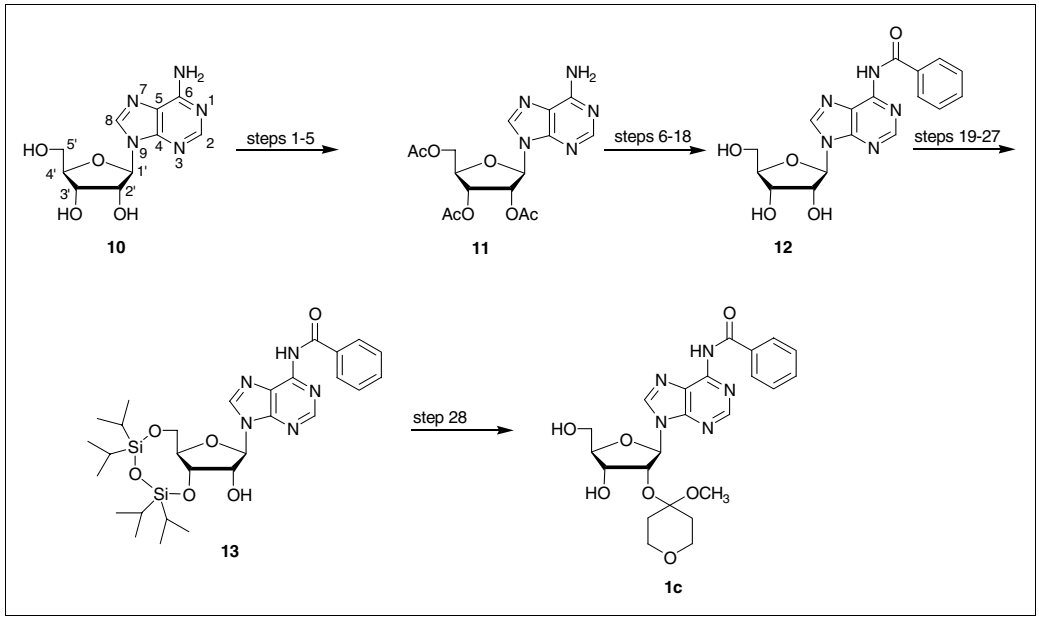

Figure 2.4.5 Scheme showing the preparation of 2′-*O*-MTHP-6-*N*-benzoyladenosine (**S.1c**) from adenosine (see Support Protocol 3). The final step from **S.13** to **S.1c** is performed as in steps 16 to 30 of Support Protocol 2 (see Fig. 2.4.4).

Prepare 2′,3′,5′-tri-O-acetyladenosine (S.11)

1. Prepare and stir a suspension of 10.0 g adenosine (**S.10**; 37.4 mmol) in 50 mL dry pyridine.
2. Add 23 mL acetic anhydride (0.24 mol) and stir overnight at room temperature.
3. Cool in an ice bath and quench with 40 mL methanol.
4. Remove from the ice bath and stir 1 hr at room temperature.
5. Concentrate to dryness, and recrystallize the white solid residue twice from 100 mL hot ethanol to yield 12.0 g (82%) tri-*O*-acetyladenosine (**S.11**). Check the product by TLC (see Basic Protocol, steps 4 to 6).

   The product is sufficiently pure (R_f = 0.31) for use in the subsequent steps.

Prepare 6-N-benzoyladenosine (S.12)

6. Dissolve crystalline **S.11** in 50 mL pyridine, cool in an ice bath, and add 5.3 mL benzoyl chloride (45.8 mmol).
7. Let the reaction mixture warm to room temperature and stir for 2 hr.
8. Quench with 6 mL water and stir for another 30 min.
9. Concentrate to a syrup using a rotary evaporator.
10. Partition the residue between 100 mL chloroform and 100 mL saturated aqueous sodium hydrogen carbonate solution. Reextract the aqueous phase with two 50-mL portions of chloroform.
11. Dry the combined organic extracts over ~20 g anhydrous sodium sulfate and evaporate to a glass (R_f = 0.71).
12. Redissolve in 50 mL of 1:1 (v/v) methanol/pyridine, cool on ice, and then add 15 mL (~3 eq) of 25% sodium methoxide in methanol.
13. Let the reaction mixture warm to room temperature and monitor by TLC.

    Deacetylation to the more polar product (R_f = 0.24) occurs in <15 min.

14. Neutralize with 4.0 mL acetic acid and evaporate to a small volume (~$\frac{1}{3}$) under reduced pressure.
15. Pour the concentrated product mixture into 100 mL saturated aqueous sodium hydrogen carbonate and extract with 200 mL chloroform.
16. Collect the precipitate that settles between the chloroform and aqueous layers by centrifuging 10 min at 3000 rpm, 15°C.
17. Resuspend in ~100 mL water and centrifuge again. Repeat with ~100 mL each ethanol and then diethyl ether.
18. Dry the white solid first in air and then under high vacuum to yield 7.15 g (63%) **S.12** as a white solid. Check the product by TLC.

    The product is sufficiently pure (R_f = 0.24) for use in the subsequent steps.

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-6-N-benzoyladenosine (S.13)

19. Dissolve 2.40 g of 6-*N*-benzoyladenosine (**S.12**; 6.46 mmol) in 32 mL dry pyridine.
20. Add 2.16 g imidazole followed by 2.50 mL of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (7.9 mmol; 1.2 eq).

21. Stir the reaction overnight. Check the reaction by TLC.

    TLC should show complete reaction of the starting material.

22. Add 10 mL methanol, stir for another 15 min, and concentrate under reduced pressure.

23. Take up the residue in 50 mL chloroform and wash sequentially with 25 mL saturated sodium hydrogen carbonate solution, 50 mL of 0.1 M hydrochloric acid, and 30 mL water.

24. Evaporate the organic layer.

25. Remove traces of pyridine by coevaporating with ~50 mL (once each) toluene, ethanol, and chloroform.

26. Purify the crude product by short-column chromatography (see Basic Protocol, step 12). Elute with chloroform until the product appears, then with chloroform containing 2% (v/v) ethanol (~700 mL total in 20-mL fractions). Do not use >1 atm pressure for elution (Hunt and Rigby, 1967; Still et al., 1978).

27. Combine the product-containing fractions, evaporate the solvent, and dry under high vacuum to yield 3.21 g (81%) chromatographically pure **S.13** as a colorless foam. Check by TLC (R_f = 0.58).

Prepare 2′-O-MTHP-6-N-benzoyladenosine (S.1c)

28. Introduce the MTHP protecting group and perform desilylation (see Support Protocol 2, steps 16 to 30), replacing **S.9** (29.45 g; 50 mmol) with the corresponding adenosine derivative **S.13** (30.84 g; 50 mmol) to yield **S.1c** as a dry white powder.

    *2′-O-MTHP-6-N-benzoyladenosine (**S.1c**): yield 18.9 g (78% from **S.13**); R_f (silica, chloroform/methanol 9:1): 0.30; ^{1}H-NMR (250 MHz, DMSO-d_6): 1.40-1.90 (m×m, 4H; -CH_2-(3,5) of MTHP), 2.55 (s, 3H; H_3CO- of MTHP), 3.20-3.80 (m×m, 2H, -CH_2-(5′)), 3.3-3.8 (m×m, 4H; -CH_2-(2,6) of MTHP), 4.05 (m, 1H; -CH-(4′)), 4.18 (m, 1H, -CH-(3′)), 5.00 (m(d×d), 1H; -CH-(2′)), 5.27 (t, J = 5.5, 1H, exchangeable with D_2O; 5′-OH), 5.33 (d, J = 4.5, 1H, exchangeable with D_2O; 3′-OH), 6.19 (d, J = 7.4, 1H; -CH-(1′)), 7.5-8.1 (m×m, 5H; benzoyl-aromatic H), 8.77 (2s, 2H; adenyl-H2/8), 11.3 (sbr, 1H, exchangeable with D_2O; adenyl-N6-H).*

SUPPORT PROTOCOL 4

PREPARATION OF 2′-*O*-(4-METHOXYTETRAHYDROPYRAN-4-YL)-2-*N*-ISOBUTYRYLGUANOSINE (S.1d) FROM GUANOSINE

Guanosine (**S.14**; Fig. 2.4.6) is base protected on N2 (**S.16**) by reaction with isobutyryl chloride after transiently blocking the 2′-, 3′-, and 5′-hydroxy groups by persilylation (**S.15**). The chosen route is then comparable to the cytidine and adenosine cases: 3′,5′-disilylation to **S.17**, followed by acid-catalyzed ketalization of the 2′-OH by 5,6-dihydro-4-methoxy-2*H*-pyran and cleavage of the silyl protecting group by fluoride ion to yield 2′-*O*-MTHP-2-*N*-isobutyrylguanosine (**S.1d**) in satisfactory isolated yield. Generally, the higher basicity and polarity of guanosine derivatives renders them more difficult to handle, and the yields are frequently compromised by loss of material in aqueous extraction media or on polar silica gel phases. Nevertheless, the proposed sequence is a viable route that is based on well-described literature procedures (Reese et al., 1970; Ti et al., 1982; Jones, 1984; van Boom and Wreesmann, 1984; Markiewicz and Wiewerowski, 1985; McLaughlin et al., 1985).

Additional Materials *(also see Basic Protocol and Support Protocols 1 and 2)*

Guanosine (**S.14**; Sigma or Fluka), dried before use for 2 hr at 50°C over phosphorus pentoxide in vacuo

Dry pyridine (see Support Protocol 1 for drying procedure)

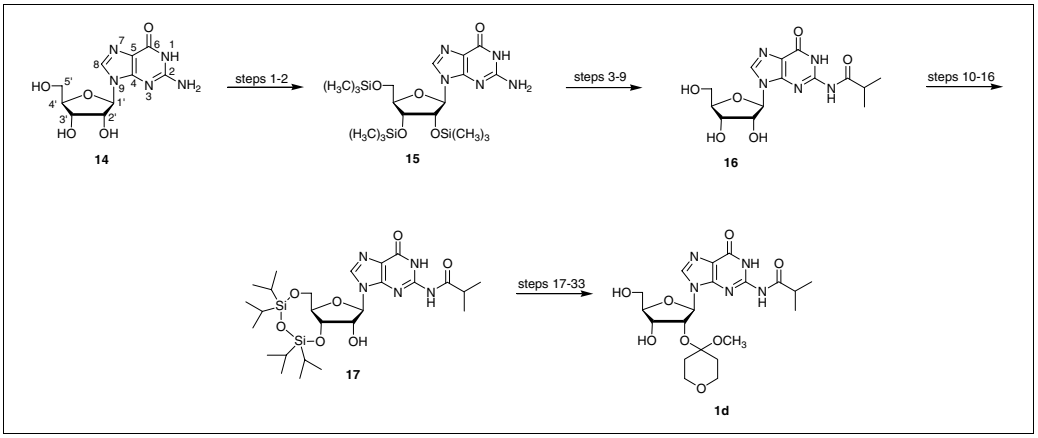

Figure 2.4.6 Scheme showing the preparation of 2′-*O*-MTHP-2-*N*-isobutyrylguanosine (**S.1d**) from guanosine (see Support Protocol 4).

Trimethylsilyl chloride (analytical grade)
Isobutyryl chloride (analytical grade)
Concentrated aqueous ammonia (~25%)
Methylene chloride (analytical grade)
Phosphorus pentoxide

Prepare 2-N-isobutyrylguanosine (S.16)

1. Suspend 14.15 g dry guanosine (**S.14**; 50 mmol) in 200 mL dry pyridine.
2. Add 47.5 mL trimethylsilyl chloride (375 mmol) and stir for 3 hr at ambient temperature to produce **S.15**.
3. Cool to 0°C and add 15.6 mL isobutyryl chloride (150 mmol), dropwise, over a period of 30 min.
4. Allow to warm to room temperature and stir overnight.
5. Cool again to 0°C and quench by adding 50 mL water. Stir for another 10 min at room temperature.
6. Add 100 mL concentrated aqueous ammonia to the clear solution and stir for an additional 30 min.
7. Pour the mixture into 800 mL water and extract with 150 mL methylene chloride.
8. Evaporate the aqueous phase to dryness and crystallize three times from 50 to 100 mL boiling water.
9. Dry the resulting solids for 2 days under high vacuum over phosphorus pentoxide to obtain 7.25 g (41%) 2-*N*-isobutyrylguanosine (**S.16**) as a grayish-white, chromatographically homogenous powder. Check by TLC (see Basic Protocol, steps 4 to 6; $R_f = 0.074$).

Prepare 3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)-2-N-isobutyrylguanosine (S.17)

10. Dissolve 7.05 g of 2-*N*-isobutyrylguanosine (**S.16**; 20.0 mmol) in 125 mL dry pyridine.
11. Add 6.6 mL of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (24 mmol) and stir overnight. Check the reaction by TLC.

TLC shows complete reaction of the starting material.

12. Evaporate the suspension and partition between 250 mL chloroform and 250 mL saturated aqueous sodium hydrogen carbonate solution.

13. Reextract the aqueous layer twice with 125 mL chloroform, dry the organic layer over ~30 g sodium sulfate, and evaporate to a glass using a rotary evaporator.

14. Remove traces of pyridine by azeotropic rotoevaporation from 100 mL toluene (twice), 100 mL ethanol (once), and 100 mL chloroform (once).

15. Purify the crude product by short-column chromatography using 200 g silica gel (see Basic Protocol, step 12). Elute with chloroform until the product appears, then with chloroform containing 2% (v/v) ethanol (~1500 mL total in 30-mL fractions). Do not use >1 atm pressure for elution (Hunt and Rigby, 1967; Still et al., 1978).

16. Combine the product-containing fractions, evaporate the solvent, and dry under high vacuum to give 7.25 g (61%) chromatographically pure **S.17** as a colorless foam. Check by TLC ($R_f = 0.55$).

Prepare 2′-O-MTHP-2-N-isobutyrylguanosine (S.1d)

17. Dissolve 0.95 g of toluene-*p*-sulfonic acid monohydrate (5 mmol) in 25 mL dry dioxane and evaporate under reduced pressure to a colorless oil to remove traces of water.

18. Under an inert atmosphere, add 5.95 g of 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2-*N*-isobutyrylguanosine (**S.17**; 10 mmol) followed by 50 mL dry dioxane.

19. To the resulting clear solution, add 5.8 mL of 5,6-dihydro-4-methoxy-2*H*-pyran (5.7 g, 50 mmol). Stir 20 hr at room temperature and check the reaction by TLC.

    TLC should show complete reaction of the starting material.

20. Neutralize the reaction with 0.8 mL half-saturated methanolic ammonia solution and concentrate immediately under reduced pressure to a small volume (20 mL).

21. Dissolve the residue in 200 mL chloroform and wash with 100 mL of 1 M aqueous sodium hydrogen carbonate solution.

22. Dry the organic layer over 50 g magnesium sulfate and concentrate to a glass.

23. Add 50 mL of 1 M *n*-tetrabutylammonium fluoride in acetonitrile, stir for 1 hr at room temperature, and check the reaction by TLC.

    TLC should show complete removal of the silyl group.

24. Concentrate under reduced pressure to a small volume (20 mL). Dissolve in chloroform and wash as in step 21.

25. Reextract the aqueous phase twice with 100 mL chloroform.

26. Dry the organic layer over 10 g anhydrous sodium sulfate and concentrate to a glass.

27. Dissolve the residue with minimal amount of chloroform and apply to a column of silica gel (100 g; 8 × 5 cm) packed in chloroform.

28. Elute the column with chloroform until the product appears, and then collect 20-mL fractions with the following chloroform/ethanol solutions: 300 mL of 98:2 (v/v); 300 mL of 96:4 (v/v), 600 mL of 9:1 (v/v).

29. Collect the main product-containing fractions and evaporate to a glass.

30. Dissolve the residue in 25 mL chloroform, precipitate by dropwise addition to 500 mL rapidly stirring pentane, and decant off most of the supernatant (>400 mL).

31. Collect the solids by centrifuging 10 min at 3000 rpm, 15°C, and decant the pentane.

32. Wash the solids twice by resuspending in 100 mL fresh pentane and repeating the centrifugation. Dry the resulting white powder (**S.1d**) in vacuo.

33. Characterize the final product by TLC and ^{1}H-NMR.

*2′-O-MTHP-2-N-isobutyrylguanosine (**S.1d**): yield 2.42 g (52% from **S.17**); R_f (silica, chloroform/methanol 9:1): 0.29; ^{1}H-NMR (250 MHz, DMSO-d_6): 1.12 (2d, J = 6.8, 6H; $(H_3C)_2CH$-)), 1.40-1.90 (m×m, 4H; -CH_2-(3,5) of MTHP), 2.55 (s, 3H; H_3CO- of MTHP), 2.75 (septet, J = 6.8, 1H; $(H_3C)_2CH$-)), 3.19-3.33 (m×m, 2H, -CH_2-(5′)), 3.4-3.7 (m×m, 4H; -CH_2-(2,6) of MTHP), 3.98 (m(t), 1H; -CH-(4′)), 4.10 (m(t), 1H, -CH-(3′)), 4.74 (m(d×d), 1H; -CH-(2′)), 5.77 (m(t), 1H, exchangeable with D_2O; 5′-OH), 5.19 (m(d), 1H, exchangeable with D_2O; 3′-OH), 6.98 (d, J = 7.9, 1H; -CH-(1′)), 7.5-8.1 (m×m, 5H; benzoyl-aromatic H), 8.31 (s, 1H; guanyl-H8), 11.7/12.1 (2sbr, 2H, exchangeable with D_2O; guanyl-N1-H and N2-H).*

PREPARATION OF 1,3-DICHLORO-1,1,3,3-TETRAISOPROPYLDISILOXANE

SUPPORT PROTOCOL 5

Although this reagent is commercially available (e.g., Fluka), this protocol provides a convenient procedure for its preparation.

Materials

Magnesium curls
Dry diethyl ether, distilled from phosphorus pentoxide (30 g/L)
Isopropyl bromide, distilled from calcium hydride (5 g/L)
Trichlorosilane, freshly distilled
0.1 N hydrochloric acid
Magnesium sulfate
Methylene chloride (analytical grade), dried by passage through activated basic alumina
Chlorine gas, dried over concentrated sulfuric acid
NaCl plates for infrared (IR) spectroscopy

Additional reagents and equipment for IR spectroscopy

Prepare 1,1,3,3-tetraisopropyldisiloxane

1. Combine 64 g magnesium curls and 200 mL dry diethyl ether.

2. Add a solution of 270 mL isopropyl bromide in 400 mL dry diethyl ether dropwise to the solution.

3. Stir the reaction mixture mechanically and heat under reflux for 3.5 hr.

4. Add a solution of 100 mL trichlorosilane and 400 mL dry diethyl ether dropwise to the stirred solution (step 3) and heat under reflux overnight.

5. Quench by adding 800 mL of 0.1 N hydrochloric acid in a dropwise fashion.

6. Stir the mixture and heat under reflux for another 3.5 hr.

7. Separate out the organic layer and extract the aqueous layer three times with 300 mL diethyl ether.

8. Dry the combined organic layers over ~50 g magnesium sulfate and concentrate to a colorless oil under reduced pressure.

9. Distill the residue to produce pure 1,1,3,3-tetraisopropyldisiloxane (100 g; b.p. 80° to 90°C, 10 mmHg) as a colorless oil.

Prepare 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane

10. Dissolve the above product (100 g of 1,1,3,3-tetraisopropyldisiloxane) in 500 mL methylene chloride and pass a stream of dry chlorine gas through the solution.

    Protect the apparatus from atmospheric moisture using a paraffin oil bubbler. Calcium chloride drying tubes are entirely inefficient and will lead to reaction failure.

11. When the temperature rises to ~27° to 30°C, cool the reaction mixture to 17° to 20°C by immersing it in an ice water bath while continuing the stream of chlorine gas.

12. After 2 hr, and then after every hour, withdraw a small sample of the reaction mixture and analyze by IR spectroscopy using NaCl plates.

13. Stop the chlorination when the IR spectrum indicates the disappearance of the absorption band at 2100 cm^{-1} (Si-H).

14. Evaporate off the volatile compounds and distill the residue under diminished pressure (b.p. 85° to 90°C, 2 mmHg) to give 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (70 g) as a colorless oil. Store dry at 0°C for up to several months or even years.

REAGENTS AND SOLUTIONS

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

Anisaldehyde reagent

1 mL 4-methoxybenzaldehyde
2 mL concentrated sulfuric acid
10 mL glacial acetic acid
87 mL methanol (analytical grade)
Store up to 6 months at room temperature

All analytical-grade reagents are preferred if available.

Methanolic ammonia solution, half-saturated (~8 M)

Pass dry ammonia gas (passed over solid potassium hydroxide pellets) through 500 mL analytical-grade methanol at –20°C until saturation. Dilute with 500 mL analytical-grade methanol. Store up to 4 weeks at –18°C.

Triethylammonium bicarbonate buffer, 2 M

Combine 825 mL analytical-grade triethylamine and 2175 mL water. Saturate with carbon dioxide gas at 0°C until the pH of the clear solution reaches 7.5. Store up to 4 weeks at 4°C.

COMMENTARY

Background Information

Within the field of solid-phase peptide synthesis, the introduction of a base-labile protecting group for the nucleophilic (amino-) center has been very successful, exceeding by far the status of alternative and becoming a standard technique in many laboratories (Atherton and Sheppard, 1989, and references cited therein). The base-sensitive amino-protecting 9-fluorenylmethoxycarbonyl (FMOC) group, first introduced by Carpino and Han in 1970 (for a review see Carpino, 1987), is strictly orthogo-

nal to the acid-labile protecting groups used for the side chains. Orthogonality is fulfilled when permanent side-chain protecting groups remain completely stable during peptide assembly while being repeatedly exposed to the conditions applied for removal of the temporary terminal protecting group. For solid-phase oligonucleotide assemblies, the use of 5′-*O*-FMOC chemistry may provide an opportunity for such orthogonality, and also avoids the problem of depurination occasionally observed during repeated acidolytic steps required for 5′-*O*-detritylation.

Compared to solid-phase peptide synthesis, functional protection is less demanding in oligonucleotide synthesis due to the smaller number of building blocks. In the deoxyribonucleotide series, very satisfactory protecting groups have resulted from the pioneering efforts in the chemical synthesis of genes by the group of Khorana (e.g., Schaller et al., 1963; Büchi and Khorana, 1972). For example, permanent protection of the exocyclic amino functions as *N*-acyl and *N*-aroyl derivatives, respectively, is compatible with nonnucleophilic conditions, but the protecting groups may be cleaved at the very end of the synthesis by hydrolysis in aqueous ammonium hydroxide at elevated temperature.

On the other hand, an adequate treatment of the protecting group strategy in RNA synthesis requires the differential handling of the 2′-, 3′-, and 5′-hydroxy groups. If phosphoramidite chemistry is chosen for chain extension, the central problem is the selection of a suitable combination of the permanent 2′-hydroxyl-protecting group together with a temporary 5′-hydroxyl-protecting group. The former must remain intact until the very end of the synthesis, including base deprotection, whereafter it must be removed very cleanly and under conditions that leave the phosphate-ribose backbone in complete constitutional and regiochemical integrity. This was convincingly shown both for the acetal (tetrahydropyranyl or THP) as well as the presently applied 4-methoxytetrahydropyranyl (MTHP) ketal-type of 2′-hydroxyl-protecting groups some years ago by the research group of Reese (Norman et al., 1984). It was demonstrated by HPLC analysis that diuridine and diadenosine phosphates are stable in 0.01 N hydrochloric acid for time periods over ten times longer than that required for complete 2′-deprotection. Moreover, the authors (Christodoulou et al., 1986) and others (Reese and Skone, 1985) showed that if a conventional acid-labile group such as di-*O*-(*p*-anisyl)phenylmethyl (dimethoxytrityl or DMTr) or 9-phenylxanthen-9-yl (pixyl or Px) is used to protect the 5′ position, concomitant cleavage of the 2′-*O*-THP or 2′-*O*-MTHP takes place to an unacceptable extent. For orthogonal protection of the 5′-hydroxy group, the authors therefore chose the base-labile FMOC protecting group (Fig. 2.4.1 and Fig. 2.4.7).

FMOC was first proposed for 5′-*O*-protection in the deoxyribonucleotide series for solid-phase synthesis of an octathymidylic acid fragment (Gioeli and Chattopadhyaya, 1982), and was later used in the same laboratory for the regioselective construction of a 2′-*O*-pixyl-protected dinucleotide (Pathak and Chattopadhyaya, 1985). Later, the results reported for oligothymidylic acids were extended to deoxyribonucleotide sequences containing 4-*N*-benzoyl-2′-deoxycytidine and 6-*N*-benzoyl-2′-deoxyadenosine, whereby the above procedure for the regioselective introduction of the FMOC group was again confirmed (Ma and Sonveaux, 1987; Balgobin and Chattopadhyaya, 1987). An approach for a solution-phase RNA synthesis combining an acid-labile acetal group (1-ethoxyethyl) for 2′-protection with 5′-*O*-FMOC was proposed by Fukuda et al. (1988). However, the drawback of using chiral protecting groups on chiral nucleoside building blocks has been commented on with crystallographic data (Lehmann et al., 1991).

Further efforts to incorporate the FMOC strategy within the framework of solid-phase

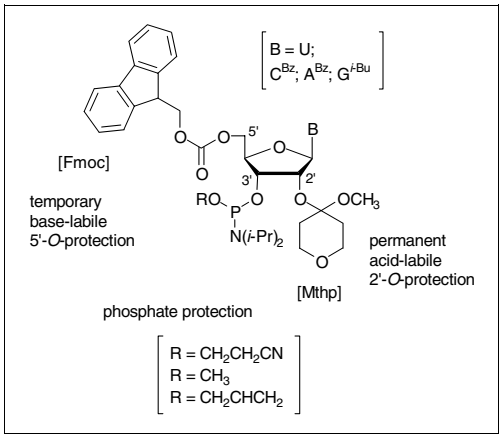

Figure 2.4.7 Orthogonal protection scheme for solid-phase RNA synthesis applying a temporary base-labile (FMOC) 5′-*O*-protecting group and a permanent acid-labile (MTHP) 2′-*O*-protecting group within the phosphoramidite strategy. Compatibility with current variants of phoshate internucleotide protection as well as anchorage to the solid support are discussed in the text.

oligonucleotide chemistry were aimed at avoiding unwanted side reactions during deprotection. Primarily, it was reported (Gao et al., 1985) that, under strongly basic deprotection conditions (5% 1,8-diazabicyclo-[5.4.0]undec-7-ene [DBU] in acetonitrile), methyl group transfer to the N3 position of thymine (and presumably uracil) occurs via an intramolecular S_N2-reaction from the phosphotriester linkage if the common methyl phosphoramidite chemistry is applied. It was not possible to use *H*-phosphonates, since the latter are decomposed immediately under the action of base (Lehmann et al., 1989). The authors therefore first applied cyanoethyl phosphoramidites, presently in use for DNA synthesis, conscious of the fact that probably some or all of the phosphate protection is lost during FMOC cleavage. Nevertheless, the results were remarkably encouraging, and it was possible to assemble oligomers of up to 20 residues containing all four ribonucleosides in good yield and in regiochemically homogenous form.

The fact that phosphoramidite building blocks are of a significantly basic nature and are prone to hydrolysis to the corresponding *H*-phosphonates on normal silica gel surfaces demands deactivation of such surfaces by the addition of base to the eluent. If the kind of base (which should be nonnucleophilic and of a low pK_a) is chosen inadequately or the 5′-*O*-FMOC-nucleoside-3′-*O*-phosphoramidite is exposed to higher concentrations of base at elevated temperatures, some loss of the protecting group may occur during isolation, which may lead to a danger of double couplings. The authors therefore recommend (Lehmann et al., 1989) repeated coevaporation of the derived phosphoramidite fractions from toluene under high vacuum followed by immediate precipitation of the product. An evaluation of the less basic *N*-morpholino phosphoramidite derivatives (McBride and Caruthers, 1983) may be advantageous in the present context.

From the more recent literature, two improvements to the FMOC strategy emerge as particularly noteworthy. First, it was possible to apply a safer protection of the phosphate moiety by use of the allyl group as described for a variety of solid-phase protocols by Hayakawa et al. (1990). Moreover it was found (Bergmann et al., 1995) that the final deprotection conditions normally used for removal of the base amino protecting groups (concentrated ammonia, several hours at 55°C) simultaneously take off the allyl groups on the internucleotide phosphate linkage. A second improvement consists of the development of a different linker unit to the solid support, which is of the acetal type and hence is acid labile (2′,3′-*O*-methylidene-4-phenoxyacetyl; Palom et al., 1993). This allows cleavage of the FMOC group under nucleophilic conditions (10% piperidine in *N*,*N*-dimethylformamide) that are significantly less basic and are therefore compatible with an oligonucleotide assembly via methyl phosphoramidites. In addition, two recently introduced alternatives to the FMOC group (BSMOC and MSPOC; Carpino and Mansour, 1999), which are cleaved with lower concentrations of weak nucleophilic bases such as piperidine or morpholine, may be particularly well suited for this oligonucleotide assembly scheme. The linker arm to the support previously used by the authors (succinyl-sarcosyl; Brown et al., 1989) is stable under strongly basic conditions (up to 5% DBU in CH_3CN), but is cleaved by exposure to nucleophiles.

Finally, the authors would like to encourage the exploitation of new chemistries potentially compatible with the FMOC strategy. For instance, it might be possible to combine the photolabile *o*-nitrobenzyloxymethyl group (Schwartz et al., 1992; Pitsch, 1997) with the base-labile FMOC group to further increase the scope of this remarkably versatile tool in the chemical synthesis of biooligomers.

Critical Parameters and Troubleshooting

The synthesis outlined in the Basic Protocol is fairly short and straightforward. Other than careful attention to basic organic synthesis techniques, little troubleshooting advice needs to be offered. Critical to achieving the expected yields is strict adherence to the given reaction and isolation conditions.

The syntheses of the starting materials are considerably more involved, although the expected yields are quite good. Success is dependent upon careful operational planning inherent to multistep syntheses.

Anticipated Results

Following the synthesis strategy outlined in the Basic Protocol, yields of between 52% and 65% can be achieved for ribonucleosides protected at the 5′ hydroxyl by FMOC, and 76% can be achieved for 5′-*O*-FMOC-2′-deoxythymidine. Similarly, good to excellent yields are reported in the literature for deoxyguanosine, deoxyadenosine, and deoxycytidine derivatives (Balgobin and Chattopadhyaya, 1987; Ma and Sonveaux, 1987). The four Support

Protocols for the preparation of the starting materials (2′-*O*-MTHP-protected ribonucleosides) provide yields of ~70%, except for the synthesis of 2′-*O*-MTHP-2-*N*-isobutyrylguanosine, which provide a yield of ~52%.

Time Considerations

The procedure described in the Basic Protocol may be carried out within 1 to 2 days starting from 2′-*O*-MTHP-protected ribonucleosides. The preparation of the MTHP-protected starting intermediates can take 2 to 3 weeks per nucleoside derivative. The authors suggest starting with uridine (the simplest case) and allowing time for crystallization of the 3′,5′-diacetyl intermediate.

Literature Cited

Atherton, E. and Sheppard, R.C. 1989. Solid-Phase Peptide Synthesis: A Practical Approach. IRL Press, Oxford.

Balgobin, N. and Chattopadhyaya, J.B. 1987. Solid phase synthesis of DNA under a non-depurinating condition with a base labile 5′-protecting group (Fmoc) using phosphite-amidite approach. *Nucleosides Nucleotides* 6:461-463.

Bergmann, F., Kueng, E., Iaiza, P., and Bannwarth, W. 1995. Allyl as internucleotide protecting group in DNA synthesis to be cleaved off by ammonia. *Tetrahedron* 51:6971-6976.

Brown, T., Pritchard, C.E., Turner, G., and Salisbury, S.A. 1989. A new base-stable linker for solid-phase oligonucleotide synthesis. *J. Chem. Soc., Chem. Commun.* 891-893.

Büchi, H. and Khorana, H.G. 1972. Total synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast. Chemical synthesis of an icosadeoxyribonucleotide corresponding to the nucleotide sequence 31 to 50. *J. Mol. Biol.* 72:251-288.

Carpino, L.A. 1987. The 9-fluorenylmethoxycarbonyl family of base-sensitive amino-protecting groups. *Acc. Chem. Res.* 20:401-407.

Carpino, L.A. and Han, G.Y. 1970. the 9-fluorenylmethoxycarbonyl function, a new base-sensitive amino-protecting group. *J. Am. Chem. Soc.* 92:5748-5749.

Carpino, L.A. and Mansour, E.M.E. 1999. The 2-methylsulfonyl-3-phenyl-1-prop-2-enyloxycarbonyl (MSPOC) amino-protecting group. *J. Org. Chem.* 64:8399-8401.

Christodoulou, C., Agrawal, S., and Gait, M.J. 1986. Incompatibility of acid-labile 2′ and 5′ protecting groups for solid-phase synthesis of oligoribonucleotides. *Tetrahedron Lett.* 27:1521-1522.

Fromageot, W.P.M., Griffin, B.E., Reese, C.B., and Sulston, J.E. 1967. Monoacylation of ribonucleosides and derivatives via orthoester exchange. *Tetrahedron* 23:2315-2331.

Fukuda, T., Hamana, T., and Marumoto, R. 1988. Synthesis of RNA oligomers using 9-fluorenylmethoxycarbonyl (Fmoc) group for 5′-hydroxyl protection. *Nucl. Acids Res.* 16:13-16.

Gao, X., Gaffney, B.L., Senior, M., Riddle, R.R., and Jones, R.A. 1985. Methylation of thymine residues during oligonucleotide synthesis. *Nucl. Acids Res.* 13:573-584.

Gioeli, C. and Chattopadhyaya, J.B. 1982. The fluoren-9-ylmethoxycarbonyl group for the protection of hydroxy-groups; its application in the synthesis of an octathymidylic acid fragment. *J. Chem. Soc. Chem. Commun.* 1982:672-674.

Hayakawa, Y., Uchiyama, M., Kato, H., and Noyori, R. 1990. Allylic protection of internucleotide linkage. *Tetrahedron Lett.* 26:6505-6508.

Hunt, B.J. and Ribgy, W. 1967. Short column chromatography. *Chem. Ind.* 1967:1868-1869.

Jones, R.A. 1984. Preparation of protected deoxyribonucleotides. *In* Oligonucleotide Synthesis: A Practical Approach (M.J. Gait, ed.) pp. 23-34. IRL Press, Oxford.

Jork, H., Funk, W., Fischer, W., and Wimmer, H. 1990. Thin-Layer Chromatography, Vol. Ia: Reagents and Detection Methods, pp. 195-198. VCH-Verlagsgesellschaft, Weinheim.

Lehmann, C., Xu, Y.-Z., Christodoulou, C., Tan, Z.-K., and Gait, M.J. 1989. Solid-phase synthesis of oligoribonucleotides using 9-fluorenylmethoxy-carbonyl (Fmoc) for 5′-hydroxyl protection. *Nucl. Acids Res.* 17:2379-2390.

Lehmann, C., Xu, Y.-Z., Christodoulou, C., Gait, M.J., Van Meervelt, L., Moore, M., and Kennard, O. 1991. 3′/5′-Regioselectivity of introduction of the 9-fluorenylmethoxy-carbonyl group to 2′-O-tetrahydropyran-2-yl and 2′-O-(4-methoxytetrahydropyran-4-yl)-nucleosides: Useful intermediates for solid-phase RNA synthesis. *Nucleosides Nucleotides* 10:1599-1614.

Ma, Y. and Sonveaux, E. 1987. The 9-fluorenylmethyloxycarbonyl (Fmoc) group as a 5′-*O* base labile protecting group in solid supported oligonucleotide synthesis. *Nucleosides Nucleotides* 6:491-493.

Markiewicz, W.T. and Wiewerowski, M. 1985. Simultaneous protection of 3′- and 5′-hydroxyl groups of nucleosides. *In* Nucleic Acid Chemistry, Section III: Nucleosides (L.B. Townsend and R.S. Tipson, eds.) pp. 229-231. John Wiley & Sons, New York.

McBride, L.J. and Caruthers, M.H. 1983. An investigation of several deoxynucleoside phosphoramidites useful for synthesizing deoxyoligonucleotides. *Tetrahedron Lett.* 24:245-248.

McLaughlin, L.W., Piel, N., and Hellmann, T. 1985. Preparation of protected ribonucleotides suitable for chemical oligoribonucleotide synthesis. *Synthesis* 1985:322-323.

Norman, D.G., Reese, C.B., and Serafinowska, H.T. 1984. The protection of 2′-hydroxy functions in oligoribonucleotide synthesis. *Tetrahedron Lett.* 25:3015-3018.

Palom, Y., Alazzouzi, E-M., Gordillo, F., Grandas, A., and Pedroso, E. 1993. An acid-labile linker for solid-phase oligoribonucleotide synthesis using Fmoc group for 5′-hydroxyl protection. *Tetrahedron Lett.* 34:2195-2198.

Pathak, T. and Chattopadhyaya, J. 1985. The 2′-hydroxyl function assisted cleavage of the internucleotide phosphotriester bond of a ribonucleotide under acidic conditions. *Acta Chem. Scand.* B39:799-806.

Pitsch, S. 1997. An efficient synthesis of enantiomeric ribonucleic acid from D-glucose. *Helv. Chim. Acta* 80:2286-2314.

Reese, C.B. and Skone, P.A. 1985. Action of acid on oligoribonucleotide phosphotriester intermediates. Effect of released vicinal hydroxy functions. *Nucl. Acids Res.* 13:5215-5231.

Reese, C.B., Saffhill, R., and Sulston, J. 1970. 4-Methoxytetrahydropyran-4-yl: A symmetrical alternative to the tetrahydropyranyl group. *Tetrahedron* 26:1023-1030.

Schaller, H., Weiman, G., Lerch, B., and Khorana, H.G. 1963. Protected derivatives of deoxyribonucleotides and new syntheses of deoxyribonucleotide-3′ phosphates. *J. Am. Chem. Soc.* 85:3821-3827.

Schwartz, M.E., Breaker, R.R., Asteriadis, G.T., deBear, J.S., and Gough, G.R. 1992. Rapid synthesis of oligoribonucleotides using 2′-*O*-(*o*-nitrobenzyloxymethyl)-protected monomers. *Bioorg. Med. Chem. Lett.* 2:1019-1024.

Still, W.C., Kahn, M., and Mitra, A. 1978. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 43:2923-2925.

Ti, G.S., Gaffney, B.L., and Jones, R.A. 1982. Transient protection: Efficient one-flask synthesis of protected deoxynucleosides. *J. Am. Chem. Soc.* 104:1316-1319.

van Boom, J.H. and Wreesmann, C.T.J. 1984. Chemical synthesis of small oligoribonucleotides in solution. *In* Oligonucleotide Synthesis: A Practical Approach (M.J. Gait, ed.) pp. 153-183. IRL Press, Oxford.

Key-References

Blackburn, M. and Gait, M.J. (eds.) 1996. Nucleic Acids in Chemistry and Biology. Oxford University Press, New York.

In particular, chapter 3 on chemical synthesis is very recommendable as an illustrative overview to the present topics.

Gait, M.J. (ed.) 1984. Oligonucleotide Synthesis: A Practical Approach, IRL Press, Oxford.

Basic principles of oligonucleotide synthesis are illustrated by practical advice through further step-by-step protocols; some of the chapters may be regarded as primers for units in the present volume.

Lehmann et al., 1989. See above.

The procedure in the Basic Protocol is first described for the four 2′-O-MTHP-protected ribonucleosides. For more background information on particular complications arising when the chiral 2′-O-tetrahydropyranyl (THP) group is used, see Lehmann et al., 1991.

Contributed by Michael J. Gait
MRC Laboratory of Molecular Biology
Cambridge, United Kingdom

Christian Lehmann
Institute of Organic Chemistry
University of Lausanne
Lausanne, Switzerland