Preparation of 2′-*O*-[(Triisopropylsilyl)oxy]methyl-protected Ribonucleosides

This unit describes, in detail, the preparation of 2′-*O*-[(triisopropylsilyl)oxy]methyl (TOM)–protected phosphoramidite building blocks derived from the ribonucleosides adenosine, cytidine, guanosine, and uridine (see Basic Protocol). These building blocks are suitable for the chemical synthesis of RNA oligonucleotides under DNA-coupling conditions. Additionally, protocols are presented for the preparation of suitably protected purines, N^6-acetyl-5′-*O*-(4,4′-dimethoxytrityl)adenosine and N^2-acetyl-5′-*O*-(4,4′-dimethoxytrityl)guanosine (see Support Protocols 1 and 2, respectively), and for the protecting group reagent [(triisopropylsilyl)oxy]methyl chloride (TOM·Cl; see Support Protocol 3). The two corresponding pyrimidine derivatives are commercially available (e.g., ChemGenes). The preparation of phosphoramidites from these building blocks and the synthesis of oligoribonucleotides is described in *UNIT 3.8*.

CAUTION: All reactions must be performed in a well-ventilated fume hood to avoid exposure to dibutyltin dichloride and TOM·Cl. These procedures should be performed only by personnel trained and experienced in organic synthesis. Standard precautions to prevent excessive exposure to toxic chemicals and solvents should be followed. All reactions should first be performed on a small scale.

INTRODUCTION OF THE [(TRIISOPROPYLSILYL)OXY]METHYL GROUP INTO *N*-ACETYLATED, 5′-*O*-DIMETHOXYTRITYLATED RIBONUCLEOSIDES

BASIC PROTOCOL

The introduction of the TOM group into the four *N*-acetylated, 5′-*O*-dimethoxytritylated ribonucleosides is presented in Figure 2.9.1. Alkylation of the 2′,3′-diols of ribonucleosides is achieved by first forming their cyclic 2′,3′-di-*O*-dibutylstannyl derivatives under basic conditions with *n*-dibutyltin dichloride and *N*-ethyl-*N*,*N*-diisopropylamine in 1,2-dichloroethane for 1 hr at 25°C. These activated intermediates are subsequently treated with 1.1 to 1.3 equivalents of TOM·Cl for 20 min at 80°C. After the aqueous workup, the 2′-*O*-alkylated and 3′-*O*-alkylated nucleosides (**S.3a-d** and **S.2a-d**, respectively) are isolated in pure form by chromatography on silica gel (unreacted starting materials can also be recovered). Under these optimized reaction conditions, side reactions (alkylation of the base moieties) are observed only to a very small extent (<5%). Fair to good yields of the desired 2′-*O*-alkylated products **S.3a-d** are obtained (40% to 60%), along with various amounts of the 3′-*O*-alkylated isomers **S.2a-d** (5% to 30%). In all cases, the predominant first-eluting isomer is the desired 2′-*O*-alkylated compound.

In the following protocol, 5′-*O*-dimethoxytritylated, *N*-acetylated ribonucleoside derivatives are used as starting materials. However, employing the identical reaction conditions, the TOM group can also be introduced with similar results to 5′-*O*-dimethoxytritylated ribonucleosides that are protected with other *N*-acyl protecting groups (e.g., benzoyl, isobutyryl).

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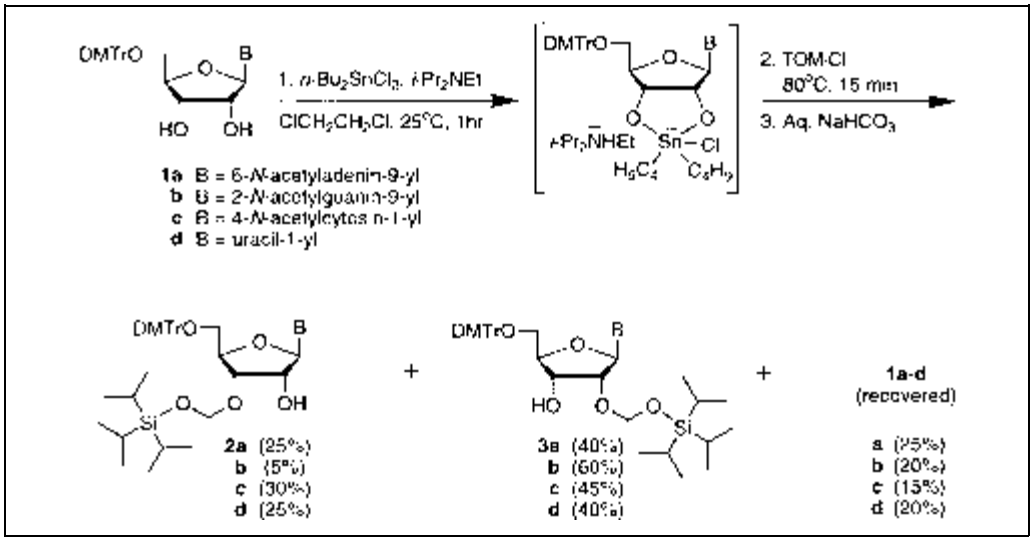

Figure 2.9.1 Preparation of the *N*-acetylated-5′-*O*-DMTr-2′-O-TOM-protected ribonucleosides **S.3a-d**. *n*-Bu_2SnCl_2, *n*-dibutyltin chloride; DMTr, 4,4′-dimethoxytrityl; *i*-Pr_2NEt, *N*-ethyl-*N*,*N*-diisopropylamine.

Materials

Nitrogen source
5′-*O*-Dimethoxytritylated, *N*-acetylated ribonucleosides:
  N^6-Ac-5′-*O*-DMTr-adenosine (**S.1a**; see Support Protocol 1)
  N^2-Ac-5′-*O*-DMTr-guanosine (**S.1b**; see Support Protocol 2)
  N^4-Ac-5′-*O*-DMTr-cytidine (**S.1c**; ChemGenes)
  5′-*O*-DMTr-uridine (**S.1d**; ChemGenes)
1,2-Dichloroethane (reagent grade)
N-Ethyl-*N*,*N*-diisopropylamine
n-Dibutyltin dichloride
[(Triisopropylsilyl)oxy]methyl chloride (TOM·Cl; **S.6**; see Support Protocol 3)
Ethyl acetate (for chromatography, technical grade)
Saturated aqueous sodium bicarbonate ($NaHCO_3$)
9:1 (v/v) ethyl acetate/hexane (for A, C, and U)
19:1 (v/v) dichloromethane/methanol (for G)
Anisaldehyde reagent (see recipe)
Dichloromethane (CH_2Cl_2) with and without 5% triethylamine (with TEA for G only)
Magnesium sulfate
Celite
Silica gel (230 to 400 mesh)
6:4, 5:5, 4:6, and so on (v/v) hexane/ethyl acetate containing 2% triethylamine (for A, C, and U)
Sand
Methanol (for G)
95:5:0.1 (v/v/v) dichloromethane/methanol/triethylamine

500-mL two-neck flask equipped with a reflux condenser and stir bar
Balloon
Rubber septum
80°C water bath
TLC plates (Merck silica gel 60, 4 × 10–cm)
254-nm UV lamp
1-L separatory funnel

1-L Erlenmeyer flask
6-cm glass filter
Rotary evaporator equipped with a vacuum pump or water aspirator
5-cm-diameter chromatography column

Additional reagents and equipment for thin-layer chromatography (TLC; *APPENDIX 3D*) and column chromatography (*APPENDIX 3E*)

Prepare TOM-protected ribonucleosides

1. In a 500-mL two-neck flask equipped with a reflux condenser, a stir bar, a balloon filled with N_2, and a rubber septum, dissolve 50 mmol of 5′-*O*-dimethoxytritylated, *N*-acetylated ribonucleoside in 200 mL of 1,2-dichloroethane.

2. While stirring, add 29.2 mL (180 mmol) *N*-ethyl-*N*,*N*-diisopropylamine and then slowly add 18.8 g (50 mmol) solid *n*-dibutyltin dichloride. Stir 1 hr at room temperature.

   It is extremely important that the dibutyltin dichloride be added after the N-ethyl-N,N-diisopropylamine.

   CAUTION*: Dibutyltin dichloride is very toxic and quite volatile. It must be handled in a well-ventilated fume hood.*

3. Place the flask in an 80°C water bath and stir 5 to 10 min.

4. Add 14 mL (65 mmol) TOM·Cl (**S.6**) with a syringe and continue stirring and heating for an additional 15 min at 80°C. Remove the flask from the water bath.

5. Using a syringe, transfer ~50 µL of the clear reaction mixture to a small tube containing 50 µL ethyl acetate/50 µl saturated aqueous $NaHCO_3$ and mix thoroughly.

6. Analyze the clear supernatant by TLC (*APPENDIX 3D*). Co-spot the starting material for comparison. Elute with 9:1 (v/v) ethyl acetate/hexane for adenosine, cytidine, and uridine derivatives, and with 19:1 (v/v) dichloromethane/methanol for the guanosine derivative.

7. Visualize by exposing the plate to a 254-nm UV lamp and staining it with anisaldehyde reagent.

   *Typically, the R_f value of the desired 2′-O-alkylated compound **S.3a-d** is ~0.55 and the R_f value of the 3′-O-alkylated byproduct **S.2a-d** is ~0.45. The R_f value of the starting material **S.1a-d** is ~0.1, and the R_f value of the nucleobase-alkylated byproduct is ~0.9.*

8. *Optional:* If the turnover is less than ~70% to 80% (~20% remaining starting material), return the reaction mixture to the 80°C water bath and add an additional 0.2 eq TOM·Cl. Stir an additional 15 min at 80°C and analyze again by TLC.

   No more TOM·Cl should be added if the amount of nucleobase-alkylated byproducts (R_f ~0.9) exceeds 5% to 10%.

9. Dilute the reaction mixture (step 4) with 250 mL dichloromethane and add it under stirring to 400 mL saturated aqueous $NaHCO_3$. Stir the biphasic mixture for 10 to 15 min.

   During this time a white solid precipitates.

10. Pour the mixture into a 1-L separatory funnel and allow the phases to separate. Pour the lower, yellow-colored organic phase (which contains some colorless, solid material) into a 1-L Erlenmeyer flask.

11. Extract the aqueous phase once more with 250 mL dichloromethane. Combine this organic phase with first organic phase in the 1-L Erlenmeyer flask.
12. Add 5 to 10 g solid magnesium sulfate and vacuum filter the organic phase by means of a 6-cm glass filter covered with 1.5 cm Celite.
13. Wash the Celite with dichloromethane and evaporate the filtrate to dryness in a rotary evaporator.

Isolate 2′-O-TOM-protected ribonucleosides

14. Prepare a slurry of 400 g silica gel in 6:4 (v/v) hexane/ethyl acetate containing 2% triethylamine for A, C, and U (**S.3a,c,d**) or in dichloromethane containing 5% triethylamine for G (**S.3b**).

    For detailed procedures for column chromatography, see APPENDIX 3E.

15. Pour slurry into a 5-cm-diameter column and carefully layer ~3 cm sand on top.
16. Dissolve the crude product in a minimal amount of dichloromethane and place it carefully on top of the column.
17. Elute first with 1 L of the solvent mixture used to pack the column, and then with gradually more polar solvent, collecting 200-mL fractions.
    a. For A, C, and U, use 1 L each of hexane/ethyl acetate at serial dilutions of 5:5, 4:6, and so on.
    b. For G, use 1-L portions of dichloromethane with increased methanol at 0.5% increments (1000:5, 1000:10, and so on).

    Always include the amount of triethylamine indicated in step 14.

18. Check fractions periodically by TLC. As soon as the two alkylation products are completely eluted, wash column with 3 L of 95:5:0.1 (v/v/v) dichloromethane/methanol/triethylamine in order to isolate unreacted starting material.
19. Pool fractions that contain pure 2′-*O*-TOM-substituted ribonucleosides (the first-eluting main product, R_f typically 0.55).
20. Pool fractions that contain impure product separately and repeat chromatography with these fractions on an appropriate smaller scale.
21. Combine fractions that contain pure product, evaporate to dryness in a rotary evaporator, and dry overnight at high vacuum (<0.05 mbar).

    Typically, ~15 to 20 g (40% to 60%) pure 2′-O-TOM-protected ribonucleoside derivatives are obtained in the form of a solid foam.

22. Check purity of the material by ^{1}H-NMR.

    Only pure products (purity >95%) should be used for phosphoramidite and oligoribonucleotide synthesis (UNIT 3.8).

    *N^6-Ac-5′-O-DMTr-2′-O-TOM-adenosine (**S.3a**): 15.9 g (40%). TLC (hexane/ethyl acetate 1:9) 0.60; $[a]_D^{25}$ +11.5 (c = 1.36, $CDCl_3$); λ_{max} (methanol) 272 (20,500), 234 (24,700); δ_H ($CDCl_3$) 0.99-1.08 (21 H, m, i-Pr_3Si); 2.61 (s, CH_3CO), 3.07 (br. d, J ≈ 3 Hz, HO-3′), 3.40 (dd, J = 3.9, 10.6 Hz, H-5′), 3.53 (dd, J = 3.5, 10.6 Hz, H′-5′), 3.78 (s, 2 OCH_3), 4.30 (q, J = 3.7 Hz, H-4′), 4.57 (br. q, J ≈ 3 Hz, H-3′), 4.96 (t, J = 5.3 Hz, H-2′), 4.98 and 5.14 (2d, J = 5.0 Hz, OCH_2O), 6.21 (d, J = 5.3 Hz, H-1′), 6.78-6.81 (4 H, m, DMTr), 7.23-7.44 (9 H, m, DMTr), 8.16 (s, H-2), 8.60 (s, H-8), 8.65 (br. s, HN); δ_C ($CDCl_3$) 11.8 (d), 17.8 (q), 25.9 (q), 55.3 (q), 63.4 (t), 71.0 (d), 82.2 (d), 84.6 (d), 86.8 (s), 87.4 (d), 91.0 (t), 113.4 (d), 122.5 (s), 127.2 (d), 128.1 (d), 128.4 (d), 130.3 (d), 135.9, (s), 142.1 (d), 144.8 (s), 149.4 (s), 151.4 (s), 152.7 (d), 158.9 (s), 170.7 (s); m/z 798 (29, MH^+), 303 (100). Anal. calcd. for $C_{43}H_{55}N_5O_8Si$: C, 64.72; H, 6.95; N, 8.78. Found: C, 64.64; H, 7.06; N, 8.74.*

*N^2-Ac-5′-O-DMTr-2′-O-TOM-guanosine (**S.3b**): 24.4 g (60%). TLC (CH_2Cl_2/methanol 19:1) 0.55; $[a]_D^{25}$ +7.7 (c = 0.85, $CDCl_3$); λ_{max} (methanol) 282sh (13,500), 275 (14,300), 260sh (17,500), 251 (19,200), 235 (26,500); δ_H ($CDCl_3$) 0.94-1.12 (21 H, m, i-Pr_3Si), 1.46 (s, CH_3CO), 3.03 (br. d, J ≈ 3 Hz, HO-3′), 3.13 (dd, J = 2.8, 10.6 Hz, H-5′), 3.53 (dd, J = 2.0, 10.6 Hz, H′-5′), 3.76 and 3.78 (2s, 2 OCH_3), 4.24 (q, J = 2.0 Hz, H-4′), 4.57 (ddd, J = 1.5, 5.0 Hz, 3.0, H-3′), 4.95 and 5.14 (2d, J = 4.7 Hz, OCH_2O), 5.09 (dd, J = 5.3, 7.5 Hz, H-2′), 5.90 (d, J = 7.5 Hz, H-1′), 6.78-6.83 (4 H, m, DMTr), 7.18-7.57 (9 H, m, DMTr), 7.75 (br. s, NH), 7.81 (s, H-8), 11.82 (br. s, NH); δ_C ($CDCl_3$) 11.8 (d), 17.8 (q), 23.7 (q), 55.4 (q), 63.9 (t), 70.8 (d), 81.5 (d), 84.5 (d), 86.5 (d), 86.9 (s), 91.1 (t), 113.5 (d), 122.4 (s), 127.3 (d), 128.2 (d), 128.3 (d), 130.3 (d), 135.9 (s), 136.3 (s), 139.1 (d), 145.3 (s), 147.4 (s), 148.7 (s), 156.3 (s), 159.0 (s), 171.8 (s). m/z 814 (17, MH^+), 303 (100). Anal. calcd. for $C_{43}H_{55}N_5O_9Si$: C, 63.45; H, 6.81; N, 8.60. Found: C, 63.34; H, 6.84; N, 8.35.*

*N^4-Ac-5′-O-DMTr-2′-O-TOM-cytidine (**S.3c**): 17.4 g (45%). TLC (hexane/ethyl acetate 1:9) 0.55; $[a]_D^{25}$ +46.8 (c = 0.94, $CDCl_3$); λ_{max} (methanol) 300 (6,800), 283 (6,800), 236 (27,200); δ_H ($CDCl_3$) 1.04-1.15 (21 H, m, i-Pr_3Si), 2.21 (s, CH_3CO), 3.34 (d, J = 8.4 Hz, HO-3′), 3.53 (dd, J = 2.5, 10.9 Hz, H-5′), 3.62 (dd, J = 1.8, 10.9 Hz, H'-5′), 3.81 and 3.82 (2s, 2 OCH_3), 4.09 (br. dt, J ≈ 9, 2 Hz, H-4′), 4.23 (d, J = 5.0 Hz, H-2′), 4.37 (m, H-3′), 5.15 and 5.28 (2d, J = 4.6 Hz, OCH_2O), 5.97 (s, H-1′), 6.84-6.88 (4 H, m, DMTr), 7.04 (d, J = 7.1 Hz, H-5), 7.24-7.44 (9 H, m, DMTr), 8.48 (d, J = 7.4 Hz, H-6), 8.62 (br. s, HN); δ_C ($CDCl_3$) 11.9 (d), 17.8 (q), 24.9 (q), 55.3 (q), 61.4 (t), 67.9 (d), 83.50 (d), 83.55 (d), 87.2 (s), 90.2 (d), 90.9 (t), 96.8 (d), 113.5 (d), 127.4 (d), 128.3 (d), 128.4 (d), 128.5 (d), 130.4 (d), 135.6 (s), 135.8 (s), 144.6 (s), 145.1 (d), 155.3 (s), 159.0 (s), 163.1 (s), 170.6 (s); m/z 774 (23, MH^+), 303 (100). Anal. calcd. for $C_{42}H_{55}N_3O_9Si$: C, 65.18; H, 7.16; N, 5.43. Found: C, 64.89; H, 7.18; N, 5.42.*

*5′-O-DMTr-2′-O-TOM-uridine (**S.3d**): 14.6 g (40%). TLC (hexane/ethyl acetate 1:4) 0.65; $[a]_D^{25}$ +42.9 (c = 1.00, $CDCl_3$); λ_{max} (methanol) 267 (13,100), 226 (28,600); δ_H ($CDCl_3$) 1.05-1.15 (21 H, m, i-Pr_3Si), 3.17 (br. d, J ≈ 3 Hz, HO-3′), 3.52 (br. d, J ≈ 11 Hz, H-5′), 3.55 (br. d, J ≈ 11 Hz, H′-5′), 3.80 (s, 2 OCH_3); 4.11 (m, H-4′), 4.26 (dd, J = 3.5, 5.0 Hz, H-2′), 4.55 (br. t, J ≈ 3 Hz, H-3′), 5.03 and 5.23 (2d, J = 5.0 Hz, OCH_2O), 5.29 (d, J = 8.1 Hz, H-5), 6.03 (d, J = 3.1 Hz, H-1′), 6.83-6.87 (4 H, m, DMTr); 7.24-7.40 (9 H, m, DMTr), 7.94 (d, J = 8.1 Hz, H-6), 8.38 (br. s, HN); δ_C ($CDCl_3$) 11.9 (d), 17.8 (q), 55.3 (q), 62.3 (t), 69.5 (d), 83.0 (d), 83.8 (d), 87.3 (s), 88.0 (d), 90.8 (t), 102.4 (d), 113.5 (d), 127.4 (d), 128.2 (d), 128.4 (d), 130.4 (d), 135.4 (s), 135.6 (s), 140.4 (d), 144.6 (s), 150.4 (s), 159.0 (s), 159.1 (s), 163.3 (s); m/z 732 (21, M^+), 303 (100). Anal. calcd. for $C_{40}H_{52}N_2O_9Si$: C, 65.55; H, 7.15; N, 3.82. Found: C, 65.29; H, 7.08; N, 4.02.*

PREPARATION OF N^6-ACETYL-5′-*O*-(4,4′-DIMETHOXYTRITYL)ADENOSINE

SUPPORT PROTOCOL 1

In this protocol, the preparation of N^6-acetyl-5′-*O*-(4,4′-dimethoxytrityl)adenosine (**S.1a**) is presented. In this two-step process (Figure 2.9.2), N^6-acetyladenosine (**S.4a**) is synthesized by first silylating the hydroxyl groups with trimethylsilyl chloride and then acetylating the amino group with acetyl chloride. Extractive work-up and cleavage of the trimethylsilyl ethers is carried out with acetic acid in methanol. The introduction of the 5′-*O*-(4,4′-dimethoxytrityl) group into the intermediates is carried out under standard conditions with 4,4′-dimethoxytrityl chloride/pyridine, giving the title compound (**S.1a**) in good yields. The intermediate (**S.4a**) is isolated by crystallization and the dimethoxytritylated compound (**S.1a**) is isolated by chromatography on silica gel.

Materials

Adenosine
Pyridine (reagent grade)
Nitrogen source
Trimethylsilyl chloride (TMS·Cl)
Acetonitrile (reagent grade), dry
Acetyl chloride

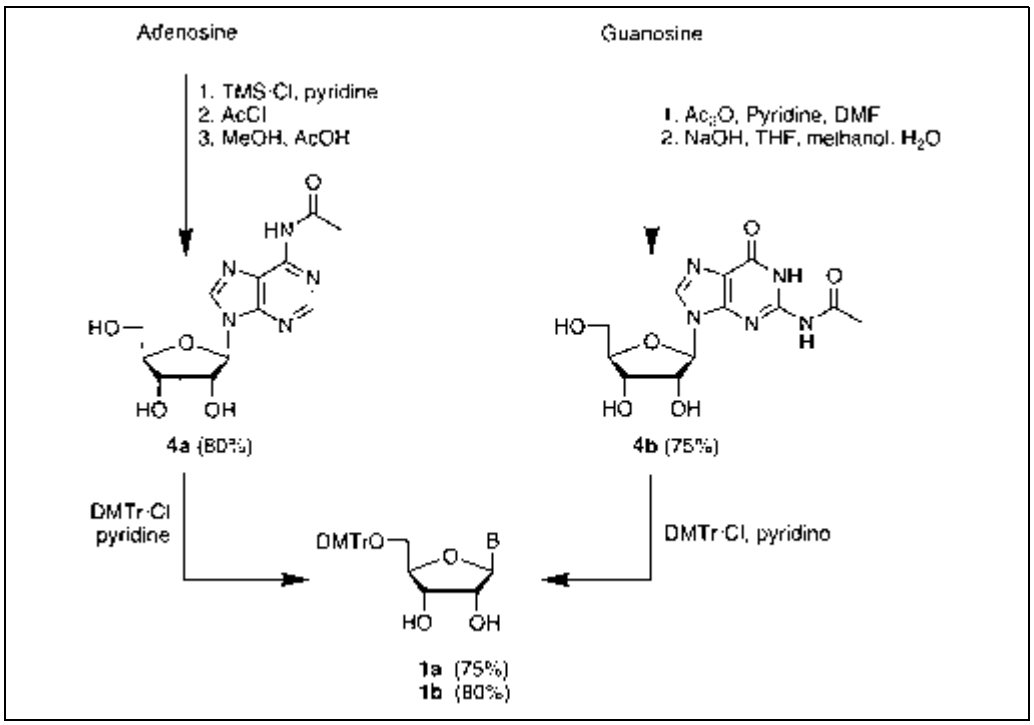

Figure 2.9.2 Preparation of the *N*-acetylated-5′-*O*-dimethoxytritylated purine ribonucleosides **S.1a** and **S.1b**. Ac, acetyl; DMTr, 4,4′-dimethoxytrityl.

Dichloromethane, precooled
Magnesium sulfate
Toluene
Acetic acid
Dimethoxytrityl chloride (DMTr·Cl)
Triethylamine
Silica gel (230 to 400 mesh)
Sand
Saturated sodium bicarbonate solution in water

250- and 500-mL two-neck flasks
Pressure-equilizing dropping funnel
Balloon
Rubber septum
Stir bar
1-L separatory funnels
Rotary evaporator with vacuum pump
1-L Erlenmeyer flasks
3-cm glass filter
3-cm-diameter chromatography column

Additional reagents and equipment for thin-layer chromatography (TLC, *APPENDIX 3D*) and column chromatography (*APPENDIX 3E*)

Acetylate adenosine

1. In an ice bath, prepare a suspension of 10 g adenosine (0.037 mol) in 75 mL pyridine in a 500-mL two-neck flask equipped with a pressure-equilizing dropping funnel, nitrogen-filled balloon, rubber septum, and stir bar.
2. Add 47.3 mL trimethylsilyl chloride (0.37 mol) dropwise to the precooled (4°C) suspension.

Preparation of 2′-*O*-[(Triisopropylsilyl)-oxy]methyl-protected Ribonucleosides

3. Remove the cooling bath and stir suspension 14 hr at room temperature.
4. Dilute with 110 mL dry acetonitrile and cool to 4°C using an ice bath.
5. Slowly add 4 mL (0.056 mol) acetyl chloride by syringe and stir 1 hr at 4°C.
6. Pour reaction mixture into a 1-L Erlenmeyer flask. While stirring, add 220 mL precooled (4°C) dichloromethane and 150 mL precooled (4°C) water. Pour into a 1-L separatory funnel.
7. Pour the lower organic layer into another 1-L separatory funnel. Add 200 mL ice-water to the funnel, shake, and pour the lower organic layer into a 1-L Erlenmeyer flask.
8. Add magnesium sulfate until a clear solution is obtained and vacuum filter by means of a 3-cm glass filter.
9. Evaporate the filtrate to dryness using a rotary evaporator.
10. Add 200 mL toluene and evaporate to dryness using a rotary evaporator. Repeat.

    Evaporation with toluene removes the pyridine.

11. Dissolve residue in 75 mL methanol and add 22.5 mL acetic acid. Keep the mixture at −20°C for 2 days to crystallize product.
12. Collect crystals by vacuum filtration on a 3-cm glass filter, wash them with a small amount of methanol, and dry them at high vacuum.

    *N^6-Acetyladenosine (**S.4a**): 9.1 g (80%) as off-white powder. TLC (CH_2Cl_2/methanol 7:3) 0.50; m.p. 175°C; $[a]_D^{25}$ -53.7 (c = 0.98, H_2O/methanol 1:1); λ_{max} (H_2O) 273 (16,700), 209 (21,700); δ_H (CD_3OD/D_2O 1:1) 2.36 (s, CH_3CO), 3.80 (dd, J = 3.3, 12.6 Hz, H-5′), 3.92 (dd, J = 2.8, 12.6 Hz, H′-5′), 4.23 (br. q, J ≈ 3 Hz, H-4′), 4.39 (dd, J = 3.4, 5.5 Hz, H-3′), 4.77 (t, J = 5.6 Hz, H-2′), 6.10 (d, J = 5.9 Hz, H-1′), 8.58 and 8.65 (2s, H-2 and H-8); δ_C (CD_3OD/D_2O 1:1) 24.9 (q), 63.2 (t), 72.4 (d), 75.7 (d), 87.8 (d), 90.8 (d), 125.1 (s), 145.3 (d), 150.9 (s), 153.0 (s), 153.5 (d), 173.4 (s); m/z 310 (100, MH^+), 178 (55), 107 (40). Anal. calcd. for $C_{12}H_{15}N_5O_5 \cdot (0.25H_2O)$: C, 45.93; H, 4.98; N, 22.31. Found: C, 46.00; H, 4.87; N, 22.23.*

Tritylate N^6-acetyladenosine

13. In a 250-mL two-neck flask equipped with a rubber septum, nitrogen-filled balloon, and stir bar, dissolve 7.7 g (0.025 mol) N^6-acetyladenosine (**S.4a**) in 80 mL pyridine.
14. Add 10.2 g (0.03 mol) dimethoxytrityl chloride in four 2.55-g portions at 20-min intervals. Keep the solution 1 hr at room temperature.
15. Pour reaction mixture into a 1-L Erlenmeyer flask. While stirring, add 250 mL dichloromethane and 150 mL water. Pour the mixture into a 1-L separatory funnel. Pour the lower organic layer into a 1-L Erlenmeyer flask.
16. Wash the aqueous phase with 100 mL dichloromethane. Allow phases to separate and then pour the organic phase into the same 1-L Erlenmeyer flask.
17. Add magnesium sulfate until a clear solution is obtained, filter by means of a 3-cm glass filter, and evaporate the filtrate to dryness in a rotary evaporator.
18. Add 100 mL toluene and evaporate again to dryness in a rotary evaporator. Repeat.

    Evaporation with toluene removes the pyridine.

19. Prepare a slurry of 40 g silica gel in 98:2 (v/v) dichloromethane/triethylamine and pour into a 3-cm-diameter chromatography column. Add ~2-cm layer of sand on top of the column.
20. Dissolve the crude product in a minimal amount of dichloromethane and place it carefully on top of the column.
21. Start eluting with 250 mL of 98:2 (v/v) dichloromethane/triethylamine and continue eluting with gradually more polar solvent by adding stepwise 1% methanol (250 mL for each step). Collect 100-mL fractions.

    Always add 2% triethylamine to solvents.
22. Check fractions periodically by TLC (*APPENDIX 3D*). Pool the fractions that contain the main product [$R_f = 0.55$ in 9:1 (v/v) dichloromethane/methanol] and evaporate to dryness using a rotary evaporator.
23. Dissolve residue in 150 mL dichloromethane, add 100 mL saturated sodium bicarbonate solution, and stir 5 min.
24. Transfer mixture to a 1-L separatory funnel. Pour the lower organic layer into a 1-L Erlenmeyer flask.
25. Wash the aqueous phase with 100 mL dichloromethane. Allow phases to separate and pour the organic phase into the same 1-L Erlenmeyer flask. Add magnesium sulfate until a clear solution is obtained and vacuum filter by means of a 3-cm glass filter.
26. Evaporate to dryness using a rotary evaporator and dry overnight at high vacuum (<0.05 mbar).

    *N^6-Ac-5′-O-DMTr-adenosine (**S.1a**): 11.4 g (75%) as yellow, solid foam. TLC (CH_2Cl_2/methanol 9:1) 0.50; $[a]_D^{25}$ –7.4 (c = 0.87, $CDCl_3$); λ_{max} (methanol) 272 (18,700), 234 (22,700); δ_H ($CDCl_3$) 2.58 (s), 3.31 (dd, J = 3.4, 10.6 Hz, H-5′), 3.44 (dd, J = 3.1, 10.6 Hz, H′-5′), 3.53 (br. s, OH), 3.75 (s, $2OCH_3$), 4.43 (br. q, J ≈ 2.5 Hz, H-4'), 4.49 (dd, J = 2.5, 5.3 Hz, H-3′), 4.88 (t, J = 5.3 Hz, H-2′), 5.74 (br. s, OH), 6.05 (d, J = 5.6 Hz, H-1′), 6.72-6.75 (4 H, m, DMTr), 7.16-7.29 (9 H, m, DMTr), 8.23 (s, H-2), 8.60 (s, H-8), 8.94 (br. s, NH); δ_C ($CDCl_3$) 25.7 (q), 55.3 (q), 63.7 (t), 72.7 (d), 76.0 (d), 86.1 (d), 86.8 (s), 90.7 (d), 113.4 (d), 122.2 (s), 127.2 (d), 128.1 (d), 128.2 (d), 130.1 (d), 130.2 (d), 135.7 (s), 141.7 (d), 144.6 (s), 149.6 (s), 150.8 (s), 152.2 (d), 158.9 (s), 170.7 (s); m/z 612 (17, MH^+), 303 (100). Anal. calcd. for $C_{33}H_{33}N_5O_7$: C, 64.80; H, 5.44; N, 11.45. Found: C, 64.56; H, 5.55; N, 11.18.*

SUPPORT PROTOCOL 2

PREPARATION OF N^2-ACETYL-5′-*O*-(4,4′-DIMETHOXYTRITYL)GUANOSINE

As with the protected adenosine (see Support Protocol 1), the preparation N^2-acetyl-5′-*O*-(4,4′-dimethoxytrityl)guanosine (**S.1b**) is a two-step process. N^2-Acetylguanosine (**S.4b**) is prepared by peracetylation of guanosine with acetic anhydride in dimethylformamide/pyridine, followed by cleavage of the *O*-bound acetyl groups with NaOH in tetrahydrofuran/methanol/water. Tritylation is performed as in Support Protocol 1, although dimethylformamide is used as a co-solvent in the reaction with the guanosine derivative. Isolation of the intermediate (**S.4b**) is performed by chromatography and crystallization, and isolation of the product (**S.1b**) is performed by chromatography.

Additional Materials *(also see Support Protocol 1)*

Guanosine
Dimethylformamide
Acetic anhydride
1 M hydrochloric acid
Methanol

Tetrahydrofuran
10 M NaOH

250-mL two-neck flasks
Reflux condenser
135°C bath
Distillation apparatus (15 × 2.9–cm Vigreux column equipped with a condenser and water aspirator)
500-mL flask
4A molecular sieves

Acetylate guanosine

1. In a 250-mL two-neck flask equipped with a reflux condenser, stir bar, and nitrogen-filled balloon, prepare a mixture of:

   5.7 g (0.02 mol) guanosine
   25 mL dimethylformamide
   25 mL pyridine
   25 mL acetic anhydride.

2. Heat 3 hr at reflux in a 135°C bath.
3. Remove all liquids by distillation on a 15 × 2.9–cm Vigreux column equipped with a condenser and water aspirator, at 100°C and 30 mbar, and dissolve the residue in 100 mL dichloromethane.
4. Extract with 100 mL of 1 M hydrochloric acid.
5. Extract the organic phase with 100 mL saturated sodium bicarbonate solution.
6. Dry organic phase with magnesium sulfate until a clear solution is obtained and vacuum filter by means of a 3-cm glass filter.
7. Add 15 g silica gel to the filtrate and evaporate to dryness using a rotary evaporator.
8. Place the powder on top of a chromatography column containing 20 g silica gel in dichloromethane. Elute with 500 mL dichloromethane, and then with 250 mL each (in order) 98:2, 96:4, 94:6, 92:8, and 90:10 (v/v) dichloromethane/methanol. Collect 100-mL fractions.
9. Check fractions periodically by TLC (*APPENDIX 3D*). Pool the fractions containing the intermediate N^2-acetyl-2′,3′, 5′-tri-*O*-acetylguanosine (R_f = 0.5 in 95:5 [v/v] dichloromethane/methanol) in a 500-mL flask and evaporate them to dryness using a rotary evaporator.
10. Dissolve the residue in 200 mL of 10:8:7 (v/v/v) tetrahydrofuran/methanol/water and add 2 mL of 10 M NaOH. Stir solution 20 min at room temperature.
11. Add 2.5 mL acetic acid and concentrate the solution to 40 mL using a rotary evaporator. Keep the solution 1 day at 4°C to crystallize the product.
12. Collect crystals by vacuum filtration by means of a 3-cm glass filter, wash with a small amount of water, and dry at high vacuum (<0.05 mbar).

*N^2-Acetylguanosine (**S.4b**): 5.05 g (77%) as an off-white powder. TLC (CH_2Cl_2/methanol 7:3) 0.30; m.p. 226°C; $[a]_D^{25}$ -19.0 (c = 0.94, H_2O); λ_{max} (H_2O) 280sh (11,200), 259 (16,300); δ_H (D_2O) 2.28 (s, CH_3CO), 3.83 (dd, J = 4.0, 12.6 Hz, H-5′), 3.92 (dd, J = 1.0, 12.6 Hz, H′-5′), 4.21 (m, H-4′), 4.45 (t, J = 4.7 Hz, H-3′), 4.72 (t, J = 4.6 Hz, H-2′), 5.97 (d, J = 4.4 Hz, H-1′), 8.18 (s, H-8). δ_C (D_2O) 26.2 (q), 64.0 (t), 72.9 (d), 76.8 (d), 87.8 (d), 90.9 (d), 122.9 (s), 142.5 (d), 150.6 (s), 152.1 (s), 160.2 (s), 178.3 (s); m/z 326 (100, MH^+). Anal. calcd. for $C_{12}H_{15}N_5O_6 \cdot (0.33H_2O)$: C, 43.51; H, 4.77; N, 21.14. Found: C, 43.65; H, 5.14; N, 21.16.*

Tritylate N^2-acetylguanosine

13. In a 250-mL two-neck flask equipped with a rubber septum, nitrogen-filled balloon, and stir bar, dissolve 8.3 g (0.026 mol) N^2-acetylguanosine (**S.4b**) in 75 mL pyridine and 25 mL dimethylformamide.

14. Add 5 g of 4A molecular sieves and stir mixture 1 hr at room temperature.

15. Perform tritylation reaction and purification of product as described for adenosine (see Support Protocol 1, steps 14 to 26).

*N^2-Ac-5′-O-DMTr-guanosine (**S.1b**): 13.4 g (81%) as a yellow powder. TLC (CH_2Cl_2/methanol 9:1) 0.35; m.p. 174°C; $[a]_D^{25}$ + 15.5 (c = 0.93, methanol); λ_{max} (methanol) 282 sh (13,700) 274 (14,600), 260 sh (18,300), 251 sh (19,600), 235 (25,800); δ_H ($CDCl_3$/CD_3OD 3:1) 2.02 (s, CH_3CO), 3.20 (dd, J = 4.3, 10.5 Hz, H-5′), 3.26 (dd, J = 2.8, 10.5 Hz, H-5′), 3.62 (s, 2 OCH_3), 3.69 (br. s, H-4′), 4.22 (t, J = 4.9 Hz, H-3′), 4.45 (t, J = 4.9 Hz, H-2′), 5.76 (d, J = 4.8 Hz, H-1′), 6.64 (6 H, m, DMTr), 7.03-7.26 (9 H, m, DMTr), 7.74 (s, H-8); δ_C ($CDCl_3$/CD_3OD 3:1) 23.4 (q), 55.1 (q), 63.6 (t), 71.0 (d), 74.8 (d), 84.2 (d), 86.6 (d), 88.6 (s), 113.2 (d), 120.8 (s), 127.0 (d), 127.9 (d), 128.2 (d), 130.1 (d), 135.8 (s), 137.8 (d), 144.6 (s), 147.9 (s), 148.8 (s), 156.2 (s), 158.7 (s), 173.3 (s); m/z 628 (74, MH^+), 303 (100). Anal. calcd. for $C_{32}H_{35}N_4O_8$: C, 63.15; H, 5.30; N, 11.16; Found: C, 63.08; H, 5.35; N, 10.91.*

SUPPORT PROTOCOL 3

PREPARATION OF [(TRIISOPROPYLSILYL)OXY]METHYL CHLORIDE

This protocol describes the two-step synthesis of the reagent TOM·Cl, required for introduction of the 2′-*O*-TOM group into 5′-*O*-dimethoxytritylated, nucleobase-protected ribonucleosides. The two reactions are illustrated in Figure 2.9.3. In the first reaction, the adduct formed from formaldehyde and ethanethiol in situ according to a procedure by Gundersen et al. (1989) is converted with triisopropylsilyl chloride (TIPS·Cl)/imidazole into the corresponding silylated *S*,*O*-acetal (**S.5**). Upon treatment of this intermediate with sulfuryl chloride, TOM·Cl (**S.6**) is formed. The reaction can easily be performed on a larger scale than that presented; however, the authors recommend that it be carried out first on the scale described here. Both the intermediate (*S*,*O*-acetal) and TOM·Cl are isolated by distillation. After distillation, TOM·Cl can be stored for at least 1 year in pure form at −20°C.

Materials

Paraformaldehyde
Ethanethiol
Nitrogen or argon gas
10 M aqueous NaOH

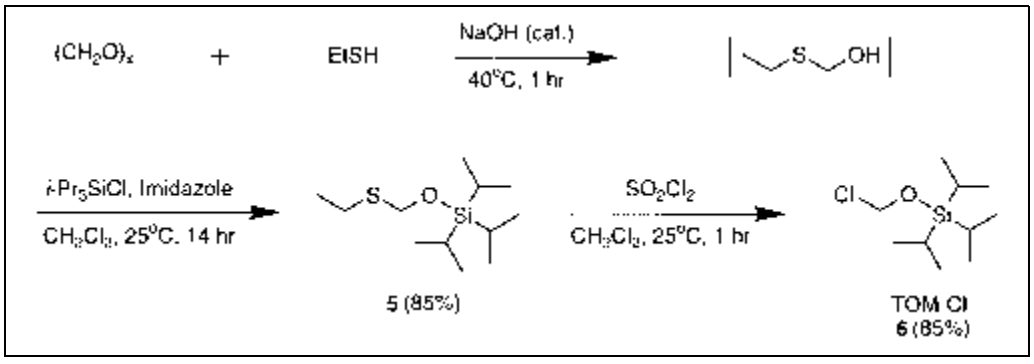

Figure 2.9.3 Preparation of [(triisopropylsilyl)oxy]methyl chloride (TOM·Cl; **S.6**). Et, ethyl; *i*-Pr, isopropyl.

Dichloromethane (CH_2Cl_2; reagent grade)
Imidazole
Triisopropylsilyl chloride (TIPS·Cl)
Hexane (technical grade)
10% (w/v) NaH_2PO_4
Anhydrous magnesium sulfate ($MgSO_4$)
Sulfuryl chloride (SO_2Cl_2)

250- and 1000-mL one-neck round-bottom flasks
Balloon
Oil bath, 40°C
1-L separatory funnel
3-cm glass filter
Rotary evaporator with a vacuum pump
10-cm 14.5-mm Vigreux distillation column
Vacuum distillation equipment (head, thermometer, and so on) with high-vacuum pump
250-mL two-neck flask
Rubber septum
Syringes

Prepare [(triisopropylsilyl)oxy]methylthioethyl ether

1. In a well-ventilated fume hood, mix 3.06 g (0.102 mol) paraformaldehyde with 7.5 mL (0.102 mol) ethanethiol in a 250-mL one-neck round-bottom flask equipped with a magnetic stir bar and a balloon filled with nitrogen or argon gas.
2. Place the suspension in an ice bath and stir until the temperature reaches 4°C (~5 min).
3. While the solution stirs, remove the gas-filled balloon, add one drop 10 M aqueous NaOH and then quickly replace the balloon. Let the reaction mixture warm up slowly by removing it from the ice bath.

   At a temperature between 15° and 23°C, a sudden exothermic reaction occurs, forming a colorless solution. The violent reaction results from depolymerization of the paraformaldehyde. If it does not occur spontaneously, heat the reaction mixture gently until the depolymerization reaction starts. Do not heat above 40°C.
4. Place the flask in a preheated oil bath and stir 1 hr at 40°C.
5. Cool the reaction mixture to room temperature and dilute with 100 mL dichloromethane.
6. Add 13.9 g (0.204 mol) solid imidazole, quickly followed by 20.5 mL (0.097 mol) triisopropylsilyl chloride. Replace the balloon.

   The imidazole does not need to be completely dissolved before addition of the triisopropylsilyl chloride. It is very important that the triisopropylsilyl chloride be added quickly.
7. Stir the resulting colorless suspension overnight at room temperature.
8. Pour the reaction mixture into a 1-L flask containing a stirred mixture of 250 mL hexane and 150 mL of 10% NaH_2PO_4.
9. Separate the two phases in a 1-L separatory funnel.
10. Re-extract the aqueous phase with 200 mL hexane and combine the organic extracts.

11. Dry the extract over anhydrous magnesium sulfate, vacuum filter by means of a 3-cm glass filter, and evaporate the solvents in a rotary evaporator with a vacuum pump.

12. Distill the residue in vacuo by means of a 10-cm, 14.5-mm Vigreux column using a vacuum that is <0.1 mbar and a heating bath temperature that does not exceed 100°C. Collect the main fraction, which typically distills at 70°C (0.05 mbar).

13. Check the purity of the product by ^{1}H-NMR.

    If the purity of the product is <95%, repeat the distillation carefully. Typically, the product is obtained in yields between 80% and 90%.

    *{[(Triisopropylsilyl)oxy]methyl}thioethyl ether (**S.5**): 20.6 g (85%) as a colorless liquid, b.p. 72°C (0.05 torr); δ_H ($CDCl_3$) 1.08-1.18 (21 H, m, i-Pr_3Si), 1.31 (t, J = 7.4 Hz, CH_3), 2.71 (q, J = 7.4 Hz, CH_2), 4.88 (s, CH_2); δ_C ($CDCl_3$) 12.0 (CH), 15.0 (CH_3), 17.9 (CH_3), 24.7 (CH_2), 66.1 (CH_2).*

Prepare [(triisopropylsilyl)oxy]methyl chloride

14. In a 250-mL two-neck round-bottom flask equipped with a rubber septum and a magnetic stir bar, and connected to a continuous nitrogen flow, dissolve 17.6 g (0.07 mol) {[(triisopropylsilyl)oxy]methyl}thioethyl ether (**S.5**) in 50 mL dry dichloromethane. Place the colorless solution in an ice bath and stir for 10 min.

    CAUTION*: This reaction must be carried out in a well-ventilated hood, since one byproduct of this reaction (ethanesulfenyl chloride) is toxic and an irritant.*

15. Over a 15-min period, add 5.75 mL (0.07 mol) sulfuryl chloride by syringe, remove the ice bath, and continue stirring.

    The solution turns gradually more yellow. During the reaction, sulfur dioxide and ethanesulfenyl chloride are produced.

16. Stir 1 hr at room temperature.

17. Connect the flask to a dry rotary evaporator and start to evaporate slowly and carefully.

    The yellow ethanesulfenyl chloride distills together with the dichloromethane and the residue turns colorless again.

18. Remove the solvent completely.

    The reaction mixture contains the partially dissolved gaseous compounds sulfur dioxide and ethanesulfenyl chloride; apply initially only a gentle vacuum.

19. Distill the residue in vacuo by means of a 10-cm, 14.5-mm Vigreux column. Use a vacuum that is <0.05 mbar and a heating bath temperature that does not exceed 80°C. Collect the main fraction, which typically distills at 40°C (0.01 mbar).

20. Check the purity of the product by ^{1}H-NMR.

    If the purity of the product is <85%, repeat the distillation carefully. Typically, the product is obtained in yields between 90% and 95%.

    *[(Triisopropylsilyl)oxy]methyl chloride (**S.6**): 14.8 g (95 %) as a colorless liquid, b.p. 40°C (0.01 torr); δ_H ($CDCl_3$) 1.08-1.10 (21 H, m, i-Pr_3Si), 5.66 (s, CH_2); δ_C ($CDCl_3$) 11.8 (CH), 17.7 (CH_3), 76.6 (CH_2).*

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. Use dry solvents (reagent grade) for all reactions. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Anisaldehyde reagent

In a clean container, mix 10 mL anisaldehyde with 180 mL ethanol. Add slowly, while stirring, 10 mL concentrated sulfuric acid, followed by 2 mL acetic acid. Store in the dark for up to 3 months at 25°C.

Contamination with acetone must be avoided. For staining, TLC plates are dipped into this mixture and heated with a heat gun until dark spots, corresponding to the reaction products, appear on a pink to red background.

COMMENTARY

Background Information

The chemical synthesis of DNA sequences based on 5′-*O*-dimethoxytritylated phosphoramidite building blocks is straightforward and very reliable. This extremely powerful methodology can, in principle, also be applied to the synthesis of RNA oligonucleotides, which are structurally very similar. However, compared to DNA, each RNA nucleoside contains an additional 2′-OH group that must be protected during chemical assembly of the nucleotide chain. Since RNA products are base labile, the removal of these supplementary protecting groups is carried out separately, after removing all other protecting groups under basic, nucleophilic conditions (Beaucage and Caruthers, 1996).

Among the large number of 2′-*O*-protecting groups investigated thus far, the fluoride-labile *tert*-butyldimethylsilyl (TBDMS) group (Ogilvie et al., 1974; *UNIT 3.6*) has the widest application. However, several factors have limited the length of routinely synthesized RNA sequences using this protecting group. In particular, relatively low coupling yields of ≤98% are obtained with these RNA phosphoramidites despite rather long coupling times of ~15 min, compared to >99% with DNA phosphoramidites using coupling times of ~2 min. A very attractive alternative was the photolabile 2-nitrobenzyloxymethyl (NBOM) group (Schwartz et al., 1992; *UNIT 3.7*), which leads to coupling yields of ~99% under DNA coupling conditions. The superior coupling behavior of these building blocks is presumably the result of the minimal steric demand of the NBOM group, achieved by connecting the photocleavable 2-nitrobenzyl protecting group via a sterically nondemanding formaldehyde acetal linker to the 2′-*O* position of the ribonucleosides (Schwartz et al., 1992; Pitsch, 1997). The 2′-*O*-TOM protecting group represents an advantageous combination of the TBDMS and NBOM protecting groups. First, it is completely stable towards all reaction conditions applied during the assembly and the first (basic) deprotection step. Second, it does not interfere with the coupling reaction and leads to very good coupling yields under DNA coupling conditions (99% with a coupling time of 2.5 min). Third, its final cleavage occurs quantitatively without concomitant destruction of the RNA product sequences (*UNIT 3.8*).

The introduction of the TOM group into partially protected ribonucleosides is carried out according to a method originally developed for the introduction of the related NBOM group (Pitsch, 1997, Wu and Pitsch, 1998; Pitsch et al., 1999). The separation of the two regioisomeric TOM-substituted ribonucleosides, formed during the dibutyltin-mediated alkylation reaction, is straightforward and leads to uniform products. In contrast to the TBDMS protecting group, the TOM group does not migrate from the 2′-*O* to the 3′-*O* position, even under strongly basic conditions. These factors allow the routine preparation of very pure phosphoramidites, which result in the exclusive formation of 3′→5′ phosphodiester moieties. Furthermore, due to the stability of the 2′-*O*-TOM group, a variety of nucleobase and sugar manipulations can be carried out after its introduction (Wu and Pitsch, 1998; Stutz et al., 2000).

Although the Basic Protocol in this unit uses *N*-acetylated-5′-*O*-dimethoxytritylated starting nucleosides, the authors have also introduced the TOM group into a large number of different nucleosides, including nucleosides with different acyl-type nucleobase-protecting groups, unnatural backbones, or noncanonical nucleobases. To date, the reaction has failed only with 5′-*O*-dimethoxytritylated inosine, which consequently was prepared from 2′-*O*-TOM-substituted adenosine by deamination.

Alkylation of dialkyl-stannylated nucleosides with TOM·Cl occurred under a variety of reaction conditions. Performing the reaction at 80°C and using 1,2-dichloroethane as solvent favors the formation of the 2′-*O*-alkylated products, whereas formation of the 3′-*O*-alkylated products can predominate at lower temperatures and with other solvents (tetrahydrofuran, acetonitrile, benzene). Slightly higher yields (5% to 10%) of 2′-*O*-TOM-protected nucleosides can sometimes be obtained by forming the stannylated nucleosides with *tert*-Bu_2SnCl_2 instead of *n*-Bu_2SnCl_2. However, since *tert*-Bu_2SnCl_2 is very expensive, the authors use it only for the alkylation of precious nucleosides.

Critical Parameters

In general, there are two important considerations for a successful preparation of 2′-*O*-TOM-protected ribonucleosides. The reaction always results in the formation of a mixture of 2′-*O*- and 3′-*O*-alkylated products. Fortunately, the chromatographic behavior (on silica gel) of these two regioisomers differs substantially ($\Delta R_f \geq 0.1$). In all experiments to date, the more quickly migrating isomer has always been the desired 2′-*O*-alkylated product, which by careful chromatography could be obtained in pure form. The unambiguous identification of the products is conveniently carried out by ^{1}H-NMR spectroscopy. Irradiation experiments provide the identification of the signals from the sugar-bound protons and the remaining hydroxyl protons. By irradiation (or D_2O-exchange) of the latter, their connection to either C3′ or C2′ can be established, and therefore the alkylation sites can be identified (Pitsch, 1997).

Under the authors' preferred reaction conditions, employing only 1.1 to 1.3 eq of TOM·Cl, a significant portion of starting material can be recovered. It is recommended that a larger excess of TOM·Cl not be used, since this generally results not in better product yields, but only in the formation of nucleobase-alkylated byproducts, which are quite difficult to remove from the desired product. As a rule, the addition of extra equivalents of TOM·Cl should be stopped when more than ~5% of nucleobase-alkylated byproducts (eluting substantially faster then the 2′-*O*-alkylated product) are detected by TLC analysis. The unreacted starting materials can be isolated by elution with 95:5 (v/v) dichloromethane/methanol. If required, the 3′-*O*-alkylated regioisomers can be transformed into the starting materials by short treatment (e.g., 30 min) with 0.25 M Bu_4NF/THF or 0.25 M, tetraethylammonium fluoride/acetonitrile.

Anticipated Results

Introduction of the TOM group into nucleosides depends on the structure of the starting material and on the reaction conditions. In the authors' experience, yields may range from 25% and 60%. The successful separation of the two diastereoisomers formed can require careful optimization of the chromatography conditions. However, the unique properties of TOM-protected phosphoramidites for RNA synthesis justify the efforts required for their introduction.

Time Considerations

Each of the intermediates and products described in this unit can be prepared and isolated within 1 or 2 days.

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