Chemical Synthesis of RNA Sequences with 2′-*O*-[(Triisopropylsilyl)oxy]methyl-protected Ribonucleoside Phosphoramidites

This unit describes the chemical synthesis of oligoribonucleotides from 2′-*O*-[(triisopropylsilyl)oxy]methyl-protected phosphoramidites (TOM-phosphoramidites), including TOM-phosphoramidite preparation (see Basic Protocol 1), assembly on DNA synthesizers (see Basic Protocol 2), and subsequent deprotection (see Basic Protocol 3).

The preparation of the TOM-protected phosphoramidite building blocks is carried out by introducing a 2-cyanoethyl-*N*,*N*-diisopropylaminophosphinyl group to the 3′-*O* position of *N*-acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[(triisopropylsilyl)oxy]methyl ribonucleosides. The preparation of these TOM-protected ribonucleosides is described in *UNIT 2.9*.

The assembly of RNA sequences with TOM-phosphoramidites can be carried out under essentially the same conditions as the assembly of DNA sequences. With very minor exceptions, the same chemistry is employed and no additional equipment is required. Due to the minimal steric hindrance of the TOM protecting group, RNA syntheses with TOM-phosphoramidites are more efficient than syntheses with the traditional 2′- *O*-(*tert*-butyldimethylsilyl)phosphoramidites (TBDMS-phosphoramidites, *UNIT 3.6*). Specifically, better coupling yields are obtained with much shorter coupling times (99% at 2.5 min). This property, together with a reliable deprotection behavior, allows the efficient preparation of relatively long RNA sequences, even on a large scale and within a short time.

The assembly of oligoribonucleotides is carried out by stepwise addition of phosphoramidite building blocks to an immobilized nucleoside until the desired sequence has been obtained. Each addition of a new building block requires four reactions (detritylation, coupling, capping, oxidation) that make up one cycle. After the assembly, the detachment of the sequence from the solid support and the removal of the nucleobase- and phosphodiester-protecting groups are carried out under basic nucleophilic conditions with methylamine/water/ethanol, followed by removal of the remaining 2′-*O*-TOM protecting groups with tetra-*n*-butylammonium fluoride. After workup by size-exclusion chromatography, crude sequences are obtained, which ultimatively can be purified by high-performance liquid chromatography (HPLC; *UNIT 10.5*) or polyacrylamide gel electrophoresis (PAGE; *UNIT 10.4*).

CAUTION: All reactions must be performed in a well-ventilated fume hood to avoid exposure to 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite.

NOTE: Always use the highest quality of solvents and reagents. All reagents and solvents used for deprotection must be sterile and free of RNases (e.g., Fluka reagents for molecular biology).

PREPARATION OF 2′-*O*-TOM-PROTECTED PHOSPHORAMIDITES

BASIC PROTOCOL 1

The conversion of *N*-acetyl-5′-*O*-DMTr-2′-*O*-TOM-protected ribonucleosides into the corresponding phosphoramidite building blocks is shown in Figure 3.8.1. The structure of the TOM protecting group prevents its migration from the 2′-*O* to the 3′-*O* position, even under basic reaction conditions. Without extraction, the products are directly purified by chromatography on silica gel. In order to obtain pure phosphoramidite building blocks, the starting materials and the reaction solvent should be as dry as possible. For a

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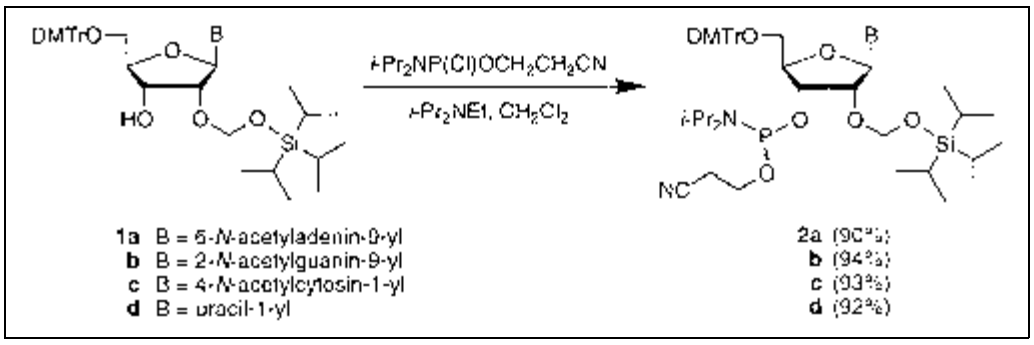

Figure 3.8.1 Preparation of the four 2′-*O*-TOM-protected ribonucleoside phosphoramidites **S.2a**-**S.2d**. DMTr, 4,4′-dimethoxytrityl; *i*-Pr_2NEt, *N*-ethyl-*N*,*N*-diisopropylamine; *i*-$Pr_2NP(Cl)OCH_2CH_2CN$, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite.

straightforward isolation, the reaction should be complete and the minimal amount of phosphitylation agent should be employed.

NOTE: The products are very acid sensitive; any contact with acids must be completely avoided.

Materials

Nitrogen or argon gas source
N-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[(triisopropylsilyl)oxy]methyl ribonucleosides (*UNIT 2.9*):
  N^6-Ac-5′-*O*-DMTr-2′-*O*-TOM-adenosine (**S.1a**)
  N^2-Ac-5′-*O*-DMTr-2′-*O*-TOM-guanosine (**S.1b**)
  N^4-Ac-5′-*O*-DMTr-2′-*O*-TOM-cytidine (**S.1c**)
  5′-*O*-DMTr-2′-*O*-TOM-uridine (**S.1d**)
Dichloromethane
N-Ethyl-*N*,*N*-diisopropylamine
2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (Aldrich)
Ethyl acetate
Hexane
Methanol
Anisaldehyde reagent (see recipe)
Silica gel (230 to 400 mesh, Fluka or Merck)
Sand

250-mL one-neck flask equipped with a stir bar
Rubber septum
Balloon
50-µL syringe
TLC plates (Merck silica gel 60, 4 × 10–cm)
254-nm UV lamp
5-cm-diameter chromatography column
Rotary evaporator with a vacuum pump or water aspirator

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

Prepare phosphoramidites

1. In a 250-mL one-neck flask equipped with a stir bar, a balloon filled with nitrogen or argon gas, and a rubber septum, dissolve 20 mmol of each dry *N*-Ac-5′-*O*-DMTr-2′-*O*-TOM-ribonucleoside (**S.1a-d**) in 70 mL dry dichloromethane.
2. While stirring, add 8.1 mL (50 mmol) *N*-ethyl-*N*,*N*-diisopropylamine and then slowly add 5.4 mL (25 mmol) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite.
3. Stir the clear reaction mixture overnight at room temperature.
4. Take ~50 µL of the clear reaction mixture with a syringe and dilute it with 0.5 mL dichloromethane in a small tube. Analyze by TLC (*APPENDIX 3D*) using 1:4 (v/v) hexane/ethyl acetate for A, C, and U, and 19:1 (v/v) dichloromethane/methanol for G. Co-spot the starting material for comparison. Visualize by exposure to a 254-nm UV lamp and stain with anisaldehyde reagent.

   The product typically migrates slightly faster than the starting material. In this reaction, two epimeric products are formed; consequently, the two pyrimidines show two distinct product spots.

   Addition of triethylamine to solvents is not necessary at this step.

5. *Optional:* If the reaction is not nearly complete (>5% starting material remaining), add an additional portion (~0.2 eq) of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, stir 2 hr at room temperature, and repeat TLC analysis.

   Try to limit the amount of phosphitylating agent as much as possible. If too much phosphitylating agent is used, it might be difficult to remove hydrolysis products (H-phosphonates) from the ribonucleoside phosphoramidites.

Isolate phosphoramidites

6. Prepare a slurry of 400 g silica gel (230 to 400 mesh) in 4:1 (v/v) hexane/ethyl acetate containing 3% triethylamine for A, C, and U, and in 1:1 (v/v) hexane/ethyl acetate containing 3% triethylamine for G.

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

7. Pour into a 5-cm-diameter chromatography column and add a layer of ~3 cm sand on top of the column.
8. Concentrate the reaction mixture (step 3) to a volume of ~40 mL in a rotary evaporator with a vacuum pump.
9. Load the reaction mixture carefully on top of the column and start the elution slowly using the same solvent mixture used to prepare the slurry. Continue until the dichloromethane (clearly visible as a transparent plug) has been eluted.
10. Continue eluting with gradually more polar solvent and collect 100-mL fractions. For A, C, and U, use 1 L each of hexane/ethyl acetate at 7:3, 6:4, 5:5, and so on. For G, use 1 L each of hexane/ethyl acetate at 1:1 and 1:3, then 100% ethyl acetate, and then add stepwise 1% methanol to the ethyl acetate.

    Always add 3% triethylamine to solvents.

11. Monitor fractions by TLC and pool fractions that contain pure 2′-*O*-TOM-substituted phosphoramidites.
12. Repeat column chromatography with the impure fractions on an appropriate smaller scale and combine fractions that contain pure products.

13. Evaporate to dryness in a rotary evaporator and dry overnight at high vacuum (<0.05 mbar).

Typically, ~90% pure 2′-O-TOM-protected phosphoramidites are obtained as a colorless, solid foam.

14. Check the purity of the material by ^{1}H- and ^{31}P-NMR.

Only pure products should be employed for RNA synthesis (see Basic Protocol 2). Small amounts of an H-phosphonate species (hydrolysis product of the phosphitylation agent) with a characteristic signal around δ = 10 ppm (^{31}P-NMR) can be tolerated. In pure and dry form, the products can be stored at −20°C for years without decomposition.

*N^6-Ac-5′-O-DMTr-2′-O-TOM-adenosine 3′-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (**S.2a**): 17.9 g (90%). TLC (hexane/ethyl acetate 3:7) 0.75; λ_{max} (acetonitrile): 270 (21,800), 235 (25,400); δ_H ($CDCl_3$) 0.88-0.90 (21 H, m, i-Pr_3Si), 1.09 and 1.18 and 1.19 (12 H, 3d, J = 6.8 Hz, Me), 2.38 (1 H, t, J = 6.4 Hz, CH_2), 2.62 (3 H, s, CH_3CO), 2.65 (1 H, dt, J = 3.3, 6.6 Hz, CH_2), 3.35 (1 H, ddd, J = 4.6, 7.5 Hz, 11.7, CH_2), 3.51-3.75 (5 H, m, CH_2, CH, H-5′), 3.77 and 3.78 (6 H, 2s, 2 OCH_3), 3.85-3.97 (1 H, m, CH_2), 4.36 and 4.41 (2q, J = 4.0 Hz, H-4′), 4.68 (m, H-3′), 4.92 and 4.95 and 4.99 (2 H, 3d, J = 5.0 Hz, OCH_2O), 5.17 and 5.20 (2t, J = 5.5 Hz, H-2′), 6.17 and 6.20 (2d, J = 5.8 Hz, H-1′), 6.76-6.80 (4 H, m, DMTr), 7.18-7.42 (9 H, m, DMTr), 8.12 and 8.15 (2s, H-2), 8.53 (br. s, HN), 8.54 and 8.56 (2s, H-8); δ_C ($CDCl_3$) 11.8 (d), 17.5 (q), 17.6 (q), 17.7 (q), 20.1 and 20.3 (2t, J_{CP} 7 Hz), 24.52 (q), 24.56 (q), 24.58 (q), 24.62 (q), 25.6 (q), 43.2 and 43.4 (2d, J_{CP} 13 Hz), 55.1 (q), 55.2 (q), 58.0 and 58.9 (2t, J_{CP} 18 Hz), 62.8 (t), 63.2 (t), 71.2 and 71.8, (2d, J_{CP} 16 Hz), 77.0 and 77.6 (2d, J_{CP} 4 Hz), 84.1 (d), 84.2 (d), 86.5 (s), 86.6 (s), 87.4 (d), 87.5 (d), 89.2 (t), 89.5 (t), 113.0 (d), 113.1 (d), 117.3 (s), 117.6 (s), 122.2 (s), 126.8 (d), 126.9 (d), 127.8 (d), 128.1 (d), 128.2 (d), 128.3 (d), 130.0 (d), 130.1 (d), 130.2 (d), 135.6 (s), 135.7 (s), 135.8 (s), 142.1 (d), 142.2 (d), 144.4 (s), 144.5 (s), 149.0 (s), 149.1 (s), 151.0 (s), 151.1 (s), 152.2 (d), 158.5 (s), 158.6 (s), 170.3 (s); δ_P ($CDCl_3$) 150.8, 151.5; m/z 998 (30, MH^+), 821 (63), 303 (100). Anal. calcd. for $C_{52}H_{72}N_7O_9SiP$: C, 62.57; H, 7.27; N, 9.82. Found: C, 62.62; H, 7.25; N, 9.72.*

*N^2-Ac-5′-O-DMTr-2′-O-TOM-guanosine 3′-(2-cyanoethyl-N,N-diisopropylphosphoramidite] (**S.2b**): 19.0 g (94%). TLC (CH_2Cl_2/methanol 19:1) 0.60; λ_{max} (acetonitrile): 276 (13,500), 250sh (19,700), 237 (24,600); δ_H ($CDCl_3$) 0.91-0.94 (21 H, m, Pr^i_3Si), 1.02-1.19 (12 H, 5d, J = 7 Hz, Me), 1.73 and 1.82 (2s, $COCH_3$), 2.25 and 2.75 (2m, 2 CH_2), 3.22 (0.5 H, dd, J = 3.7, 10.7 Hz, H-5′), 3.28 (0.5 H, dd, J = 5.3, 10.6 Hz, H-5′), 3.47-3.62 (3.5 H, m, CH, H-5′, CH_2), 3.68 (0.5H, m, CH_2), 3.75 and 3.76 and 3.77 and 3.78 (4s, 4 OCH_3), 3.92 and 4.04 (1.5 H, 2m, CH_2), 4.23 (0.5 H, q, J = 2.5 Hz, H-4′), 4.32 (0.5 H, br. dt, J ≈ 5, 2 Hz, H-4′), 4.52 (0.5 H, ddd, J = 2.0, 4.8, 12.1 Hz, H-3′), 4.62 (0.5 H, ddd, J = 4.8, 5.8, 10.6 Hz, H-3′), 4.91 (1 H, s, OCH_2O), 4.90 and 5.00 (1 H, 2d, J = 5.2 Hz, OCH_2O), 5.02 (0.5H, dd, J = 4.8, 7.4 Hz, H-2′), 5.05 (0.5 H, t, J = 5.8 Hz, H-2′), 5.87 (0.5 H, d, J = 5.7 Hz, H-1′), 5.97 (0.5 H, d, J = 7.4 Hz, H-1′), 6.76-6.82 (4 H, m, DMTr), 7.14-7.52 (9 H, m, DMTr), 7.74 and 7.80 (1 H, 2s, H-8), 8.29 and 8.57 (1 H, 2br. s, HN), 11.89 (1 H, br. s, HN); δ_C ($CDCl_3$) 11.8 (d), 17.6 (q), 17.7 (q), 17.8 (q), 20.1 (t, J_{CP} 3 Hz), 20.2 (t), 23.5 (q), 23.6 (q), 24.5 (q), 24.6 (q), 24.7 (q), 43.1 and 43.3 (2d, J_{CP} 13 Hz), 55.2 (q), 55.3 (q), 56.9 (t, J_{CP} 19 Hz), 58.8 (t, J_{CP} 13 Hz), 63.5 (t), 63.9 (t), 70.7 (d, J_{CP} 17 Hz), 71.7 (d, J_{CP} 14 Hz), 76.9 (d), 78.3 (d), 84.2 (d), 84.3 (d, J_{CP} 4 Hz), 86.2 (d), 86.3 (s), 86.7 (s), 88.9 (d), 89.4 (t), 89.5 (t), 113.1 (d), 113.2 (d), 117.5 (s), 118.1 (s), 122.0 (s), 122.7 (s), 127.0 (d), 127.1 (d), 127.9 (d), 128.0 (d), 128.1 (d), 130.0 (d), 130.1 (d), 130.2 (d), 135.6 (s), 135.8 (s), 136.0 (s), 136.3 (s), 137.7 (d), 139.1 (d), 144.6 (s), 145.0 (s), 146.8 (s), 147.1 (s), 148.0 (s), 148.5 (s), 155.6 (s), 158.6 (s), 158.7 (s), 171.5 (s), 171.6 (s); δ_P ($CDCl_3$) 149.9, 150.5; m/z 1014 (62, MH^+), 303 (100). Anal. calcd. for $C_{52}H_{72}N_7O_{10}SiP$: C, 61.58; H, 7.15; N, 9.67. Found C, 61.22; H, 7.19; N, 9.55.*

*N^4-Ac-5′-O-DMTr-2′-O-TOM-cytidine 3′-(2-cyanoethyl-N,N-diisopropylphosphoramidite] (**S.2c**): 18.1 g (93%). TLC (hexane/ethyl acetate 3:7) 0.75; λ_{max} (acetonitrile) 305 (6,700), 279 (6,200), 237 (29,000); δ_H ($CDCl_3$) 0.87-1.11 (21 H, m, i-Pr_3Si), 1.13 and 1.16 (2d, J = 6.8, 4 Hz, Me), 2.22 and 2.33 (2s, CH_3CO), 2.38 and 2.59 (2 H, 2t, J = 6.5 Hz, CH_2), 3.42-3.68 (5 H, m, CH_2, CH, H-5′), 3.80 and 3.81 and 3.814 and 3.82 (6 H, 4s, OCH_3), 3.91 (1 H, m, CH_2), 4.28-4.41 (2 H, m, H-2′,4′), 4.50 (ddd, J = 4.8, 8.0, 9.9 Hz, H-3′),*

5.15-5.22 (2 H, m, OCH_2O), 6.15 (0.5 H, d, J = 1.8 Hz, H-1′), 6.16 (0.5 H, d, J = 2.4 Hz, H-1′), 6.82-6.88 (4 H, m, DMTr), 6.96 and 7.03 (2d, J = 7.4 Hz, H-5), 7.24-7.47 (9 H, m, DMTr), 8.36 and 8.45 (2d, J = 7.4 Hz, H-6), 9.52 and 9.60 (2br. s, HN); δ_C ($CDCl_3$) 12.0 (d), 17.8 (q), 17.9 (q), 20.2 and 20.3 (2t, J_{CP} 7 Hz), 24.48 (q), 24.54 (q), 24.58 (q), 24.61 (q), 24.64 (q), 24.7 (q), 24.9 (q), 43.2 and 43.3 (2d, J_{CP} 7 Hz), 55.2 (q), 55.3 (q), 58.2 and 58.7 (2t, J_{CP} 20 Hz), 60.9 (t), 61.6 (t), 69.4 and 69.7, (2d, J_{CP} 14 Hz), 78.7 (d, J_{CP} 3 Hz), 78.9 (d), 82.3 (d, J_{CP} 3 Hz), 82.5 (d, J_{CP} 5 Hz), 87.0 (s), 87.2 (s), 89.6 (d), 89.7 (d), 89.8 (t), 96.5 (d), 113.2 (d), 113.3 (d), 117.4 (s), 117.7 (s), 127.2 (d), 128.0 (d), 128.2 (d), 128.4 (d), 130.1 (d), 130.2 (d), 130.3 (d), 130.4 (d), 135.2 (s), 135.3 (s), 135.4 (s), 135.5 (s), 144.1 (s), 144.3 (s), 145.0 (d), 145.1 (d), 154.9 (s), 158.7 (s), 162.6 (s), 162.7 (s), 170.5 (s); δ_P ($CDCl_3$) 150.6, 150.9; m/z 974 (21, MH^+), 303 (100). Anal. calcd. for $C_{51}H_{72}N_5O_{10}SiP$: C, 62.88; H, 7.45; N, 7.19. Found: C, 62.74; H, 7.56; N, 7.00.

*5′-O-DMTr-2′-O-TOM-uridine 3′-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (**S.2d**): 17.1 g (92%). TLC (hexane/ethyl acetate 1:1) 0.65; λ_{max} (acetonitrile) 264 (12,100), 236 (23,700); δ_H ($CDCl_3$) 1.02-1.05 (21 H, m, i-Pr_3Si), 1.06 and 1.17 (12 H, m, Me), 2.39 and 2.63 (2t, J = 6.7 Hz, CH_2), 3.39 (m, H-5′), 3.53-3.70 (4 H, m, CH, H′-5′, CH_2), 3.79 and 3.80 (6 H, 2 OCH_3), 3.81-3.96 (m, CH_2), 4.19 and 4.27 (2br. dt, J ≈ 5, 2 Hz, H-4′), 4.40-4.55 (m, H-2′,3′), 4.96-5.06 (2 H, m, OCH_2O), 5.32 and 5.36 (2d, J = 8.1 Hz, H-5), 6.12 (0.5 H, d, J = 5.1 Hz, H-1′), 6.14 (0.5 H, d, J = 4.9 Hz, H-1′), 6.82-6.85 (4 H, m, DMTr), 7.05-7.47 (9 H, m, DMTr), 7.80 and 7.86 (2d, J = 8.1 Hz, H-6), 8.33 (br. s, HN); δ_C ($CDCl_3$) 11.9 (d) 12.0 (d), 17.7 (q), 17.8 (q), 17.9 (q), 20.2 and 20.4 (2t, J_{CP} 7 Hz), 24.52 (q), 24.55 (q), 24.58 (q), 24.62 (q), 43.2 and 43.4 (2d, J_{CP} 12 Hz), 55.2 (q), 55.3 (q), 57.8 and 58.9 (2t, J_{CP} 18 Hz), 62.1 and 62.5 (2t), 70.4 and 70.9, (2d, J_{CP} 15 Hz), 77.4 and 78.1 (2d, J_{CP} 4 Hz), 83.3 (d, J_{CP} 4 Hz), 83.5 (d), 87.1 (s), 87.2 (s), 87.4 (d), 89.0 (d), 89.3 (t), 89.4 (t), 102.3 (d), 102.4 (d), 113.1 (d), 113.2 (d), 113.3 (d), 117.3 (s), 117.7 (s), 127.2 (d), 127.9 (d), 128.0 (d), 128.2 (d), 128.3 (d), 130.2 (d), 130.3 (d), 130.4 (d), 135.1 (s), 135.3 (s), 135.4 (s), 135.5 (s), 140.3 (d), 140.4 (d), 144.2 (s), 144.3 (s), 150.1 (s), 158.7 (s), 162.8 (s), 162.9 (s); δ_P ($CDCl_3$) 150.9, 151.3; m/z 933 (57, MH^+), 303 (100). Anal. calcd. for $C_{49}H_{69}N_4O_{10}SiP$: C, 63.07; H, 7.45; N, 6.00. Found: C, 62.84; H, 7.49; N, 5.98.*

ASSEMBLY OF 2′-*O*-TOM-PROTECTED PHOSPHORAMIDITES ON DNA SYNTHESIZERS

BASIC PROTOCOL 2

This protocol describes the setup and steps required for automated assembly of RNA sequences from TOM-phosphoramidites on 1-µmol and 10-µmol scales. The protocol was developed for GeneAssemblers (Amersham Pharmacia Biotech), but can easily be adapted to other automated DNA synthesizers. The authors recommend coupling times of 2.5 to 3.5 min and 6 to 9 min for 1.0-µmol and 10-µmol syntheses, respectively. The TOM-phosphoramidites allow preparation of RNA sequences consisting of up to 100 nt; however, the authors recommend first preparing shorter sequences (e.g., 20 nt) to gain experience in the handling of the intermediate and product sequences. The reactions carried out in each cycle are shown in Figure 3.8.2.

Any commercially available solid support containing the first immobilized ribonucleoside (or some modified ribonucleoside) can be employed. In the authors' hands, controlled-pore glass (CPG) supports give the best results, but polystyrene-based materials may be used with similar results. CPG supports with pore sizes of 500 and 1000 Å should be used for the synthesis of ≤47-mers and >47-mers, respectively.

Materials

2′-*O*-TOM-phosphoramidites (0.1 M in acetonitrile; Glen Research; see Basic Protocol 1)
Activator: 0.25 M 5-ethylthio-1*H*-tetrazole (SET, Glen Research) in acetonitrile
Argon source
Dry acetonitrile (<30 ppm water)
4A molecular sieves (optional)

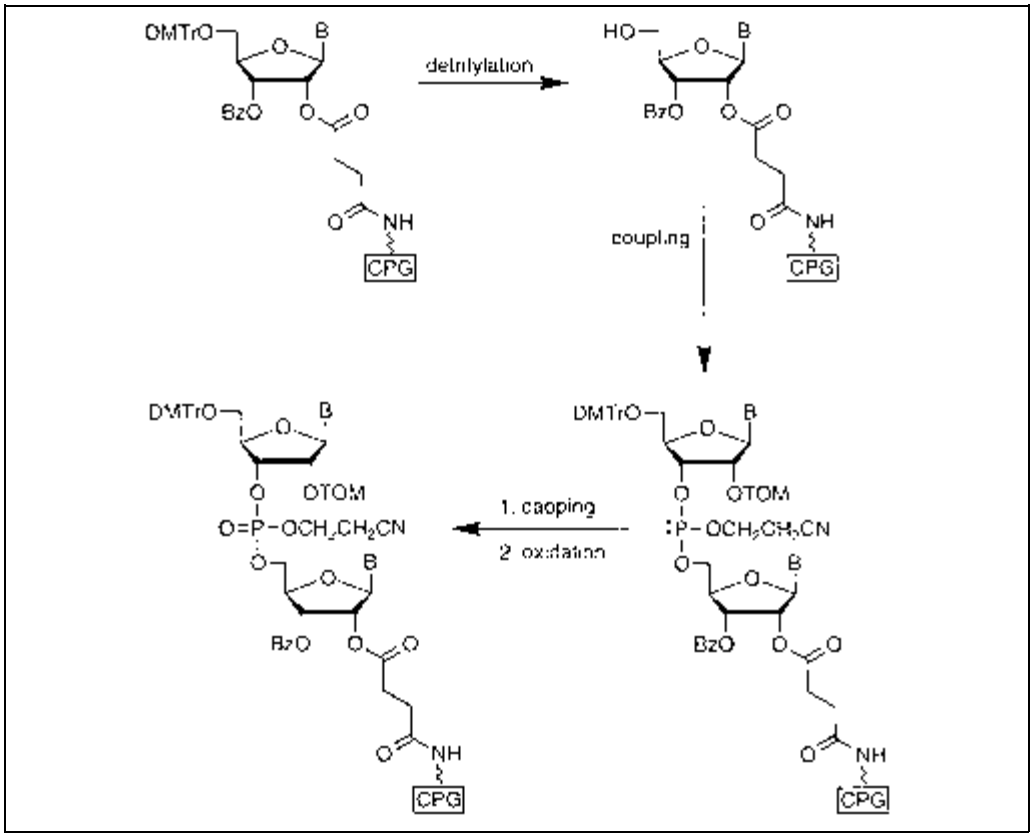

Figure 3.8.2 One coupling cycle—including detritylation, coupling, capping, and oxidation—required for the attachment of one nucleotide to the growing oligonucleotide chain. Additionally, after the last coupling cycle, the terminal dimethoxytrityl group is removed from the immobilized oligonucleotide. B, any *N*-protected nucleobase; Bz, benzoyl; CPG, controlled-pore glass; DMTr, 4,4′-dimethoxytrityl; TOM, [(triisopropylsilyl)oxy]methyl.

Detritylation solution (see recipe)
Oxidation solution (see recipe)
Capping solutions A and B (see recipes)
1,2-Dichloroethane (reagent grade)
Solid support, e.g., long-chain alkylamine controlled-pore glass (CPG) supports (500 or 1000 Å) for RNA synthesis derivatized with 5′-*O*-4,4′-dimethoxytrityl-ated ribonucleosides (Glen Research)

Vials and bottles for attachment of the phosphoramidites and reagents to the synthesizer
Automated DNA synthesizer (e.g., GeneAssembler; Amersham Pharmacia Biotech)
Synthesis column for 1-µmol or 10-µmol synthesis

Additional reagents and equipment for automated DNA synthesis (*APPENDIX 3C*)

1. Calculate the amount of phosphoramidites and activator (SET) required to assemble the desired sequence by multiplying the following by the number of nucleotides in the sequence, and adding an extra 50 mg phosphoramidites/50 mg SET to give enough material to purge the lines of the synthesizer.

   1-µmol scale: 12 mg phosphoramidite, 12 mg SET/coupling
   10-µmol scale: 40 mg phosphoramidite, 20 mg SET/coupling.

2. Place the calculated amounts of phosphoramidites and SET into the appropriate vials in an automated DNA synthesizer and flush with argon.

3. Use a syringe to add the amount of dry acetonitrile required to obtain ~0.1 M phosphoramidite (i.e., 1 mL/100 mg solid phosphoramidite) and ~0.25 M SET (1 mL/32 mg solid SET). Allow solids to dissolve.

   When working with the GeneAssembler, beads of 4A molecular sieves may also be added.

4. Create methods for the assembly of the desired sequence.

   In principle, methods employed for synthesis of DNA sequences can be used here. The authors recommend the steps listed in Table 3.8.1.

5. Connect the following reagents to the synthesizer according to the manufacturer's instructions:

   detritylation solution
   oxidation solution
   capping solutions A and B
   1,2-dichloroethane
   dry acetonitrile
   phosphoramidite solution
   SET solution.

6. Calculate the required amount of solid support (e.g., CPG support) according to its individual loading (as reported by the manufacturer) and the synthesis scale. Use a CPG support with a pore size of 500 Å for the synthesis of ≤47-mers, and a support with a pore size of 1000 Å for >47-mers. Load an empty synthesis column with the RNA support and connect it to the synthesizer.

   For example, when the loading is 40 µmol/g, use 25 mg and 250 mg for 1-µmol and 10-µmol syntheses, respectively.

7. Purge the lines of the synthesizer with all solutions and solvents.

8. Carry out the assembly according to manufacturer's instructions. Choose the option "DMTr off" in the setup menu.

   After the tenth coupling cycle, coupling yields of >99% can be expected by detritylation assay. If lower yields are obtained, see Troubleshooting.

9. Remove the synthesis column from the synthesizer and dry it by vacuum or under a stream of argon.

10. Deprotect the assembled, still fully protected oligoribonucleotide (see Basic Protocol 3).

DEPROTECTION OF RNA SEQUENCES ASSEMBLED FROM 2′-*O*-TOM-PROTECTED PHOSPHORAMIDITES

BASIC PROTOCOL 3

This protocol describes the two-step deprotection of RNA sequences assembled according to Basic Protocol 2. The two steps are presented schematically in Figure 3.8.3. In the first step, 10 M methylamine in 50% ethanol is used to detach the sequence from the solid support, eliminate the cyanoethyl groups, and remove the acetyl protecting groups from the nucleobases. In the second step, 1 M tetra-*n*-butylammonium fluoride in tetrahydrofuran is used to remove the 2′-*O*-TOM protecting groups. This reaction is quenched by addition of aqueous Tris·Cl buffer, pH 7.4. A final desalting procedure with commercially available size-exclusion cartridges yields the crude RNA sequence.

Table 3.8.1 Methods for Oligoribonucleotide Assembly on 1.0-µmol and 10-µmol Scales with TOM-Phosphoramidites[a]

1.0-µmol scale			10-µmol			Description
Time (min)	Step	Value[b]	Time (min)	Step	Value[b]	
Detritylation						
0.00	DETRIT		0.00	10DETRIT		Detritylation
1.50	VALVE POS	2.1	3.00	VALVE POS	2.1	Dichloroethane wash
2.50	VALVE POS	2.3	4.00	VALVE POS	2.3	Acetonitrile wash
2.70	INTEGRATE	0	5.00	INTEGRATE	0	Trityl assay
4.00	ML/MIN	0.00	8.00	ML/MIN	0.00	
Coupling						
4.00	LOOP TIMES	2	8.00	LOOP TIMES	4	
4.00	VALVE POS	1.8	8.00	VALVE POS	1.8	Activator to column
4.05	ML/MIN	0.90	8.05	ML/MIN	0.75	
4.15	ML/MIN	0.00	8.15	ML/MIN	0.00	
4.15	VALVE POS	1.X	8.15	VALVE POS	1.X	Amidite to column
4.20	ML/MIN	0.60	8.20	ML/MIN	1.00	
4.30	ML/MIN	0.00	8.30	ML/MIN	0.00	
4.30	VALVE POS	1.8	8.30	VALVE POS	1.8	Activator to column
4.35	ML/MIN	0.90	8.35	ML/MIN	0.75	
4.45	ML/MIN	0.00	8.45	ML/MIN	0.00	
4.45	END OF LOOP		8.45	END OF LOOP		
4.45	VALVE POS	1.1	8.45	VALVE POS	1.1	
4.50	ML/MIN	1.00	8.50	ML/MIN	1.00	
4.60	ML/MIN	0.00	9.00	ML/MIN	0.00	
4.60	LOOP TIMES	7	9.00	LOOP TIMES	7	
4.60	STEP VALVE	3	9.00	STEP VALVE	3	
4.60	END OF LOOP		9.00	END OF LOOP		
4.65	ML/MIN	2.50	9.05	ML/MIN	2.50	Coupling reaction
7.15	ML/MIN	0.00	16.05	ML/MIN	0.00	
7.15	STEP VALVE	3	16.05	STEP VALVE	3	
7.20	ML/MIN	2.50	16.10	ML/MIN	2.50	Acetonitrile wash
7.50	ML/MIN	0.00	17.10	ML/MIN	0.00	
Capping						
7.55	VALVE POS	2.5	17.15	VALVE POS	2.5	Capping A
7.60	ML/MIN	1.00	17.20	ML/MIN	1.00	
7.60	LOOP TIMES	4	17.20	LOOP TIMES	12	
7.60	VALVE POS	2.5	17.20	VALVE POS	2.5	Capping A
7.70	VALVE POS	2.6	17.30	VALVE POS	2.6	Capping B
7.80	END OF LOOP		17.40	END OF LOOP		
7.80	ML/MIN	0.00	17.40	ML/MIN	0.00	
7.85	VALVE POS	2.3	17.45	VALVE POS	2.3	Acetonitrile wash
7.85	ML/MIN	2.50	17.45	ML/MIN	2.50	
Oxidation						
8.15	VALVE POS	2.4	18.45	VALVE POS	2.4	Oxidation
8.75	VALVE POS	2.3	20.45	VALVE POS	2.3	Acetonitrile wash
10.25	ML/MIN	0.00	23.45	ML/MIN	0.00	
10.25	LOOP TIMES	7	23.45	LOOP TIMES	7	
10.25	STEP VALVE	3	23.45	STEP VALVE	3	
10.25	END OF LOOP		23.45	END OF LOOP		

[a]Synthesis performed on a GeneAssembler (Amersham Pharmacia Biotech). For 1.0-µmol scale: synthesis: 120 µl phosphoramidite solution (12 eq) and 360 µl activator solution (90 eq); detritylation: 1.5 min; coupling: 2.5 min; capping: 0.8 min; oxidation: 0.3 min. For 10-µmol scale: synthesis: 400 µl phosphoramidite solution (4 eq) and 600 µl activator solution (15 eq); detritylation: 3.0 min; coupling: 7.0 min; capping: 2.4 min; oxidation: 1.0 min.
[b]Description: VALVE POS 1.X: phosphoramidite (A, C, G, or U); 1.8: activator; 2.1: dichloroethane; 2.2: detritylation; 2.3: acetonitrile; 2.4: oxidation; 2.5: capping A; 2.6: capping B.

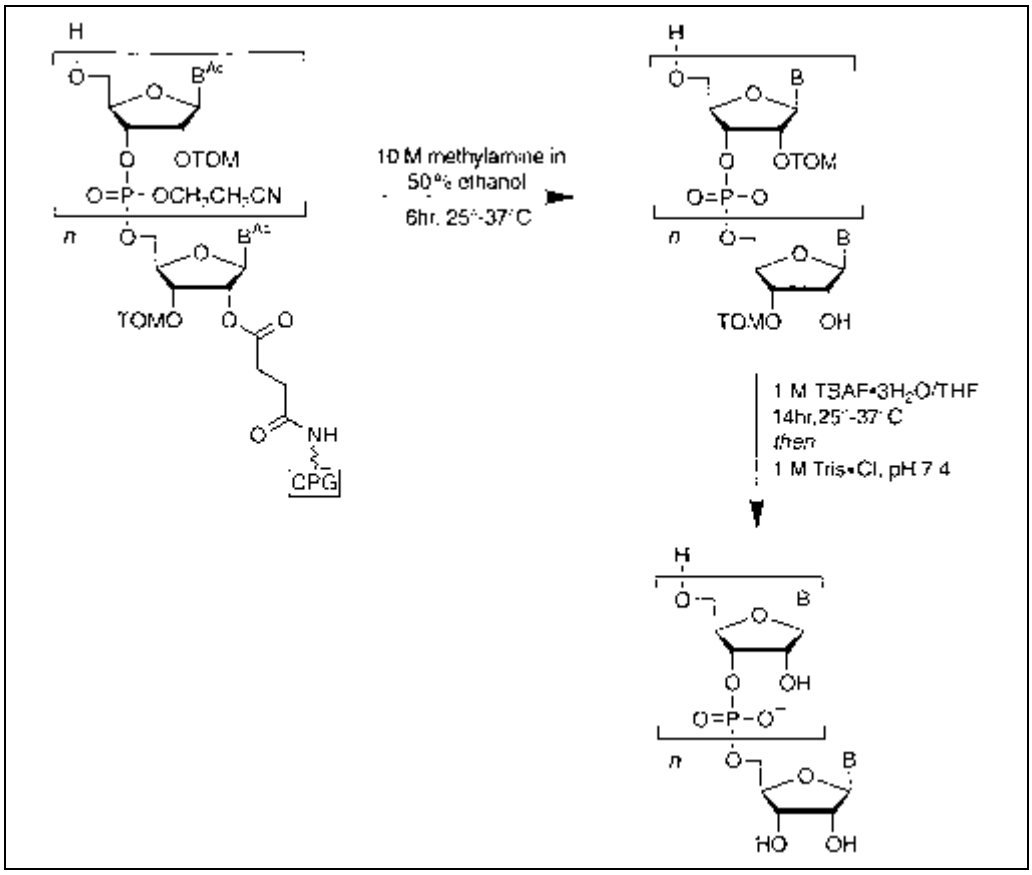

Figure 3.8.3 Two-step deprotection of oligoribonucleotide sequences assembled from TOM-phosphoramidites. B, any nucleobase; B^{Ac}, any *N*-acetylated nucleobase; CPG, controlled-pore glass; TBAF, tetra-*n*-butylammonium fluoride; TOM, [(triisopropylsilyl)oxy]methyl.

Materials

TOM-protected oligoribonucleotide attached to solid support (see Basic Protocol 2), in synthesis cartridge
12 M aqueous methylamine
8 M methylamine in ethanol (Fluka)
50% and 100% (v/v) ethanol
1 M tetra-*n*-butylammonium fluoride trihydrate (TBAF·$3H_2O$; Fluka) in tetrahydrofuran (THF)
N-Methylpyrrolidone (NMP) or dimethylformamide (DMF; optional)
1 M Tris·Cl, pH 7.4, RNase free, sterile (Fluka, for molecular biology)
3 M sodium acetate (*APPENDIX 2A*)

1.5-, 2-, and 5-mL twist-top vials
35°C incubator or heating block
Speedvac evaporator
NAP-10 columns (Amersham Pharmacia Biotech)

Additional reagents and equipment for oligoribonucleotide purification by HPLC (*UNIT 10.5*) or polyacrylamide gel electrophoresis (PAGE; *UNIT 10.4*)

Remove acetyl and cyanoethyl groups and solid support

1. Remove solid support from the synthesis cartridge and transfer it into 1.5-mL twist-top vials.

   For syntheses performed on a 1-µmol scale, a single 1.5-mL vial should suffice. For a 10-µmol scale, divide the solid support into four portions and add each to a separate 1.5-mL vial.

2. Add 0.5 mL of 12 M aqueous methylamine and 0.5 mL of 8 M methylamine in ethanol to each 1.5-mL vial.

3. Incubate 6 hr at 35°C with periodic shaking.

   Sequences with <60 nt can be incubated 6 hr at room temperature.

4. Centrifuge 3 min at 10,000 × *g*, 25°C, and transfer supernatant from each 1.5-mL vial into a 2-mL twist-top vial.

5. Wash solid support two times with 0.3 mL of 50% ethanol and place the washing solutions in the same 2-mL vial.

6. Evaporate to dryness in a Speedvac evaporator.

Remove TOM groups

7. To each 2-mL vial, add 1 mL of 1 M TBAF/THF and dissolve the crude intermediate sequence by vortexing or ultrasonicating. If intermediates are not dissolved after 10 min, add a few drops of NMP or DMF.

   The intermediates usually dissolve almost immediately. Short adenosine-rich RNA sequences or RNA hybrid sequences containing a majority of non-RNA nucleosides (e.g., DNA/2′-OMe-RNA-nucleosides) must be deprotected with 1 M TBAF in 1:1 THF/NMP.

8. Incubate the solution overnight at 35°C with periodic shaking.

   Sequences with <60 nt can be incubated overnight at room temperature.

9. Quench the reaction by adding 1 mL of 1 M Tris·Cl solution, pH 7.4.

   IMPORTANT NOTE*: Deprotected oligoribonucleotides are highly sensitive to nuclease degradation. Therefore, gloves should always be worn when manipulating deprotected RNA. Sterile vials and pipets, nuclease-free reagents, and UV-treated sterile water should always be used to limit potential exposure to nucleases. Work quickly and store the oligoribonucleotides at –20°C.*

10. Evaporate to a volume of 1 mL in a Speedvac evaporator.

    IMPORTANT NOTE*: This evaporation takes 20 to 40 min (depending on the setup). Do not overevaporate to dryness. Check the remaining volume frequently.*

Desalt oligoribonucleotide

11. Wash a NAP-10 column with 10 mL sterile water.

12. Place the oligoribonucleotide solution on top of the column and allow the solution to sink in slowly by gravity.

13. Place 1.5 mL sterile water on top of the column and collect the eluate (1.5 mL) in a 5-mL vial.

    This step is required to remove excess TBAF. Alternatively, desalting can be carried out by ion-exchange chromatography according to the protocol in UNIT 3.6.

    If the oligoribonucleotide will be used in its crude form, it should first be precipitated (steps 14 to 16). If it will be purified before use (step 17), precipitation is not required.

Precipitate (optional)

14. Evaporate eluate to dryness in a Speedvac evaporator and dissolve the residue in 0.5 mL sterile water.

15. Add 50 µl of 3 M sodium acetate solution followed by 3.5 mL of 100% ethanol. Store overnight at −20°C.

16. Centrifuge 3 min at 10,000 × *g*, 25°C, and carefully remove the supernatant.

    The precipitate consists of crude oligoribonucleotide in its sodium form.

Purify (optional)

17. Purify the oligoribonucleotide by HPLC (*UNIT 10.5*) or polyacrylamide gel electrophoresis (*UNIT 10.4*).

REAGENTS AND SOLUTIONS

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

Anisaldehyde reagent

In a clean container, mix 10 mL anisaldehyde with 180 mL 100% ethanol. Add slowly, while stirring, 10 mL concentrated sulfuric acid, followed by 2 mL acetic acid. Store reagent up to 3 months in the dark at 25°C; avoid contamination with acetone.

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.

Capping solution A

100 mL acetic anhydride
800 mL tetrahydrofuran (THF)
100 mL 2,6-lutidine
Store in a well-sealed bottle in a dark and cool (25°C) environment for up to 2 months

Capping solution B

160 mL *N*-methylimidazole
840 mL tetrahydrofurant (THF)
Store in a well-sealed bottle in a dark and cool (25°C) environment for up to 2 months

Detritylation solution

40 mL dichloroacetic acid
960 mL dry 1,2-dichloroethane
Store in a well-sealed bottle in a dark and cool (25°C) environment for up to 2 months

Oxidation solution

12.7 g iodine
700 mL tetrahydrofuran (THF)
100 mL pyridine
200 mL water

Dissolve iodine in THF, then add pyridine followed by water. Store in a well-sealed bottle in a dark and cool (25°C) environment for up to 2 months.

COMMENTARY

Background Information

During the last decade, many research groups have contributed to the development of a reliable chemical synthesis of nucleic acids. The synthesis of oligodeoxyribonucleotides by the phosphoramidite method (*UNITS 3.3, 3.5 & 3.6*) is probably the most evolved chemical process known thus far, and has almost reached perfection in terms of efficiency and automation.

This extremely powerful methodology can, in principle, also be applied to the synthesis of the structurally very similar RNA oligonucleotides. Compared to DNA, however, each nucleotide within an RNA strand contains an additional 2′-OH group, which is responsible for the instability of RNA under basic conditions (pH >12 at 25°C). This hydroxyl group must be protected during oligoribonucleotide assembly. Since the RNA products are base labile, the removal of these supplementary protecting groups is carried out separately, after removal of the 2-cyanoethyl groups and nucleobase-protecting groups and cleavage of the oligoribonucleotide from the support under basic nucleophilic conditions. As a consequence, the removal of these additional 2′-*O*-protecting groups must be completely orthogonal to all other acid-promoted and base-promoted deprotection conditions.

The large number of reported 2′-*O* ribonucleoside-protecting groups can essentially be divided into acid-labile (Griffin and Reese, 1964; Reese et al., 1991; Scaringe et al., 1998), photo-labile (Ohtsuka et al., 1974; Schwarz et al., 1992; Pitsch, 1997; Pitsch et al., 1999a), and fluoride-labile groups (Ogilvie et al., 1974; Usman et al., 1985; Pitsch et al., 1999a, b). Among them, the fluoride-labile *tert*-butyldimethylsilyl (TBDMS) group (Ogilvie et al., 1974) has the widest application. This is well reviewed in Beaucage and Caruthers (1996) and in *UNIT 3.6*. However, several factors—including the relatively sluggish coupling observed with RNA phosphoramidites as compared to the corresponding DNA phosphoramidites—has limited the length of routinely synthesized RNA sequences (*UNIT 3.6*). A very attractive alternative that leads to better coupling yields under shorter coupling times was the photolabile 2-nitrobenzyloxymethyl (NBOM) group (Schwartz et al., 1992; *UNIT 3.7*).

In a formal sense, the 2′-*O*-TOM protecting group represents a combination of the TBDMS and the NBOM protecting groups. It also displays some unique properties that render it a very valuable 2′-*O*-protecting group for the chemical synthesis of oligoribonucleotides. It is completely stable under all reaction conditions required for assembly and deprotection of RNA sequences. Its excellent stability towards both strongly acidic conditions (employed during detritylation) and strongly basic conditions (employed for deprotection of the nucleobase) is a consequence of the sterically very hindered triisopropylsilyl group. In contrast, the TOM group is very labile towards TBAF and is completely removed even in the presence of up to 20% water (Pitsch et al., 2001). Complete removal of the TBDMS group, in contrast, occurs only in the absence of water and requires drying of the TBAF solution with molecular sieves (Hogrefe et al., 1993).

Many interesting RNA sequences, such as tRNAs, ribozymes, and aptamers, consist of 60 to 80 nt. In order to obtain a reasonable overall product yield of ~50%, an individual coupling yield of ~99.3% must be achieved for each cycle in the assembly. Lower coupling yields result not only in small product yields, but also in complex crude products that are often very difficult to purify to homogeneity. The average coupling yields obtained with 2′-*O*-TOM phosphoramidites are generally >99% and, therefore, even relatively long RNA sequences (>70 nt) can be prepared routinely. Additionally, the short coupling times (<5 min) minimize side reactions during the coupling reaction.

In addition to high coupling yields, a reliable deprotection scheme is crucial for the efficient synthesis of longer sequences. The combination of 2′-*O*-TOM and *N*-acetyl protecting groups allows, for both deprotection processes, reaction times that correspond to >100 individual half-lives (with respect to the removal of one protecting group) without destruction of the oligonucleotide product. Therefore, it is possible to carry out the deprotection reactions even for extremely prolonged periods of time, assuring complete deprotection without risking concomitant destruction of the product (Pitsch et al., 1999a, 2001).

The chemical synthesis of oligonucleotides allows the more or less unrestricted incorporation of nucleobase, sugar, and backbone modifications, the preparation of hybrid sequences, and labeling with specific reporter groups. In this context, a great number of useful modified building blocks have been developed and are commercially available. By adapting the presented RNA chemistry to the established DNA

and RNA chemistry, the 2′-*O*-TOM-protected building blocks can be combined with all of these modifications and even with TBDMS-protected RNA phosphoramidites.

Critical Parameters

Phosphoramidite chemistry is extremely water-sensitive. The phosphoramidites and the activator should be completely dry. Only acetonitrile that contains <30 ppm water should be used as solvent for the coupling reaction or to dissolve the phosphoramidites and the activator. If working with the GeneAssembler from Amersham Pharmacia Biotech, molecular sieves may be added to the corresponding vials and bottles.

The synthesizer should be in good working condition. All lines should be washed after completion of each synthesis round. To prevent particles from reaching the valves, dust-free bottles, high-quality solvents and reagents, and appropriate filters should be used. Reagents should be replaced periodically with fresh supplies and should be stored in well-sealed bottles in a dark, cool environment.

Only sterile, RNase-free solvents, reagents, containers, vials, pipets, and other equipment should be used. Any contact between the oligoribonucleotides and body fluids (e.g., sweat, saliva) should be avoided. In all aqueous solutions and buffers, RNase-producing microorganisms may live and grow. Keep such solutions as sterile as possible and replace them often.

Good coupling yields should be obtained using the presented coupling conditions. Coupling times should not be significantly increased. Specifically, do not use the coupling conditions used for the assembly of 2′-*O*-TBDMS phosphoramidites in combination with SET as activator.

The deprotection under the presented conditions is straightforward. A longer deprotection time can be used, but the temperature should not exceed 35°C. Methylamine solutions should be stored at 4°C to prevent loss of concentration. The bottles should be opened only for a very brief time and should be replaced periodically. The TBAF solution should not be dried with molecular sieves, since this may lead to its decomposition. Ensure that the sequence is dissolved during TBAF treatment. If it is not, add a few drops of *N*-methylpyrrolidone (NMP), dimethylformamide (DMF), or dimethylsulfoxide (DMSO). Always quench the second deprotection reaction with Tris·Cl buffer according to the presented protocol.

The crude product should not be isolated by direct precipitation from the TBAF/THF solution. It should always be desalted by chromatography on Sephadex columns, NAP columns (see Basic Protocol 3), or ion-exchange cartridges (*UNIT 3.6*).

When the analysis of the crude products by HPLC or PAGE shows not one main peak (band), but a variety of peaks (or bands) or broad features, the product may exist in a variety of secondary structures. For complete denaturation, HPLC analysis can be performed at a higher temperature (with NucleoPac columns at neutral pH values, up to 90°C). Alternatively, 6 M urea can be added to HPLC and PAGE buffers.

Troubleshooting

Low coupling yield. Several conditions can lead to coupling yields that are <99% after the tenth coupling. (1) The acetonitrile may contain water. Substitute it with acetonitrile of better quality; add molecular sieves. (2) The synthesizer may be malfunctioning. Determine whether it is working properly by performing a DNA synthesis; also check the flow rate. (3) The phosphoramidites may contain water. Dry them under high vacuum (<0.05 mbar) overnight in a desiccator containing KOH pellets.

Multiple product peaks. Several conditions can also lead to the observation of several main product peaks or spots when the crude product is analyzed. (1) The sequence may exist in several secondary structures. Perform analysis at higher temperature and/or in the presence of 6 M urea. Isolate materials corresponding to the different features, heat them briefly at 95°C (at neutral pH), and reanalyze them separately. If the same pattern is observed, the sequence is not folding uniformly. (2) Deprotection may be incomplete. Use new deprotection solutions and reagents. Check (e.g., by carefully smelling) whether vials are sealed tightly so that methylamine can not evaporate. Check whether the intermediate product obtained after the first deprotection step completely dissolves in TBAF/THF. If it does not, add some polar solvent (NMP, DMF, DMSO). (3) The sequence was degraded by RNases. All materials and liquids that come into contact with the sequence must be free of contamination by RNases or microorganisms. Replace the Tris·Cl buffer. Use a different source of sterile water. Autoclave all vials, pipet, and other materials.

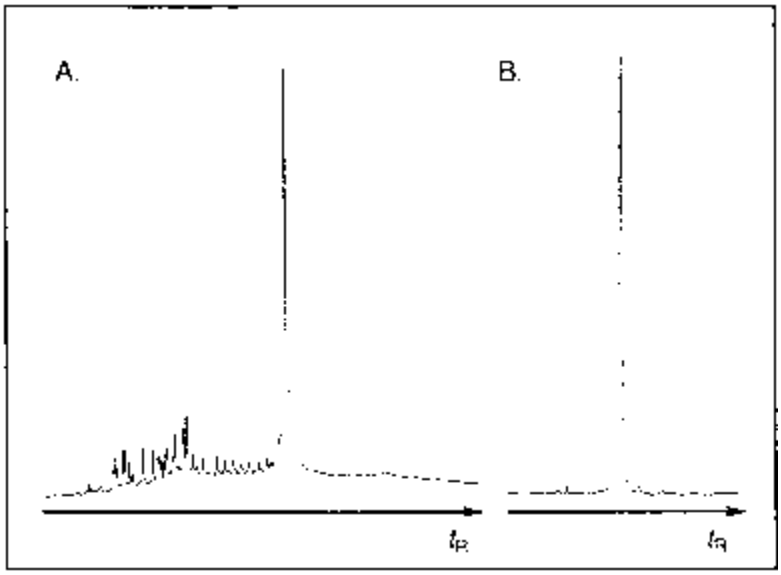

Figure 3.8.4 Capillary gel electrophoresis analysis of the 34-mer RNA sequence r(GGCGACCCUGAUGAGGCCGAAAGGCCGAAACCGU) prepared at the 10-µmol scale according to Basic Protocols 2 and 3; (**A)** crude product; (**B**) product after HPLC purification. Chromatography performed with BioFocus 3000 (Bio-Rad), coated BioCap XL-column 75 µm × 40 cm, "run-buffer" containing 6 M urea, elution with 15 kV at 40°C, detection at 260 nm.

Anticipated Results

With TOM-phosphoramidites, employing the methods presented here and observing the Critical Parameters, it should be possible to routinely obtain individual coupling yields >99% and complete deprotection. These properties allow routine preparation of oligoribonucleotides containing up to ~80 nt. The purification efficiency with such long oligomers depends strongly on the individual structure, and therefore on the sequence. With up to ~50 nt, HPLC purification with NucleoPac columns leads to very uniform products.

Figure 3.8.4 shows the results from capillary gel electrophoresis analysis of a crude 34-mer and the HPLC-purified 34-mer (prepared on a 10-µmol scale). Oligoribonucleotides >50-mers can be purified by PAGE; the purity of such oligoribonucleotide products usually exceeds 90%. In Figure 3.8.5, the gel analysis of

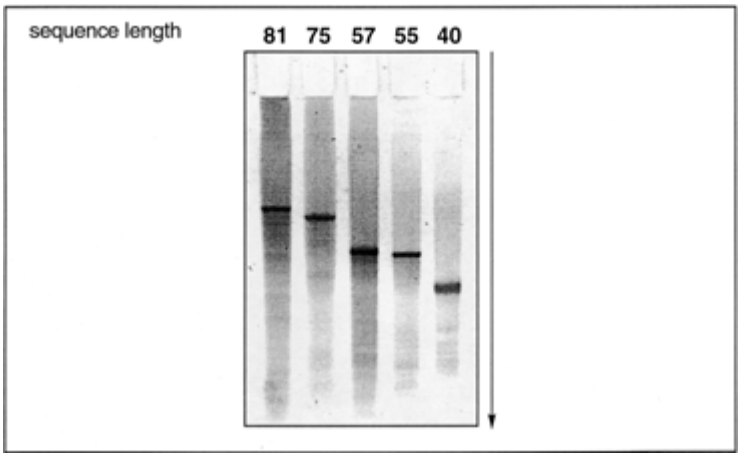

Figure 3.8.5 Polyacrylamide gel electrophoresis (PAGE) of five crude RNA sequences (sequence length indicated) prepared at the 1-µmol scale according to Basic Protocols 2 and 3. PAGE-conditions: 15% (w/v) acrylamide (29:1 acrylamide/bisacrylamide), 0.75 × 100 mm, 90 mM Tris-borate, pH 8.3, 7 M urea, 2 mM EDTA, elution at 200 V, stained with ethidium bromide.

five crude products with sequence lengths ranging from 40 to 81 nt is presented (all products were synthesized on a 1.5-µmol scale). Depending on the purification method, an overall yield of 10% to 40% purified RNA (based on solid support) can be expected.

Time Considerations

Preparation of TOM-phosphoramidites takes ~1 day each. Depending on the scale and the sequence length, the typical time for the assembly of an RNA sequence is 5 to 12 hr. It takes ~1.5 days to perform the deprotections and the final desalting according to Basic Protocol 3, although at least five deprotections can be carried out in parallel during this time. Purification of an RNA sequence typically requires 1 day (1-µmol synthesis scale).

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