Oligoribonucleotides with 2′-*O*-(*tert*-Butyldimethylsilyl) Groups

This unit describes current methodologies to chemically synthesize oligoribonucleotides on solid support by means of automated DNA synthesizers. It also includes an updated collection of protocols describing the deprotection of base-labile phosphate, and nucleobase protecting groups, and fluoride-labile 2′-*O*-(*tert*-butyldimethylsilyl) protecting groups of crude synthetic oligoribonucleotides.

Small-scale synthesis (i.e., 0.2 µmol scale) of oligoribonucleotide provides ~400 to 600 µg of purified material, which is usually enough for a wide range of biochemical applications. Such synthesis can be readily achieved using the exocyclic amine-protected 5′-*O*-dimethoxytrityl-2′-*O*-*tert*-butyldimethylsilyl-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) -ribonucleoside phosphoramidites in combination with the acidic 5-ethylthio-1*H*-tetrazole (SET, pKa = 4.28) as the activator (Wincott et al., 1995; Vinayak et al., 1995; see Basic Protocol 1). Alternatively, the use of less acidic activators such as 4,5-dicyanoimidazole (DCI, pKa = 5.2; Vargeese et al., 1998) or 1*H*-tetrazole (TET, pKa = 4.8; Usman et al., 1987; Scaringe et al., 1990; Usman and Cedergren, 1992) also allows for efficient oligoribonucleotide synthesis at comparable scales. DCI is better suited for larger-scale (i.e., >500 µmol) syntheses due to lesser acidity (Vargeese et al., 1998) that may limit the activator-induced detritylation of incoming phosphoramidite during extended coupling time (Krotz et al., 1997).

Historically, crude oligoribonucleotides have been fully deprotected with a 3:1 mixture of concentrated ammonium hydroxide/ethanol followed by an *n*-tetrabutylammonium fluoride (TBAF) treatment (Usman et al., 1987; Stawinsky et al., 1988; see Basic Protocol 2). The use of aqueous methylamine followed by treatment with the triethylamine trihydrofluoride complex (see Alternate Protocol 1) constitutes a significant improvement in the deprotection process (Wincott et al., 1995; Vinayak et al., 1995), alleviating premature deprotection of the 2′-hydroxyl during the basic treatment, eliminating the well-known sensitivity of the TBAF to water (Hogrefe et al., 1993), and shortening the overall deprotection time considerably. Crude oligoribonucleotides can also be efficiently deprotected in a "one-pot" reaction using anhydrous methylamine and neat triethylamine trihydrofluoride (see Alternate Protocol 2). This alternate deprotection protocol eliminates the time-consuming evaporation step, thereby reducing the overall deprotection time to 45 min, which allows for a high-throughput production mode (Bellon, 2000).

AUTOMATED OLIGORIBONUCLEOTIDE SYNTHESIS

BASIC PROTOCOL 1

This protocol describes automated chemical synthesis of oligoribonucleotides by means of the phosphoramidite method (*UNIT 3.5*) according to the synthetic scheme pictured in Figure 3.6.1. The procedure described below was developed for the ABI 394 DNA/RNA synthesizer at the 0.2 µmol scale although it can be modified to utilize any standard synthesizer.

Materials

Aminomethyl polystyrene (RNA primer solid support) derivatized with 5′-*O*-DMTr-2′-*O*-TBDMS-3′-*O*-succinyl ribonucleosides (Amersham Pharmacia Biotech)

Contributed by Laurent Bellon

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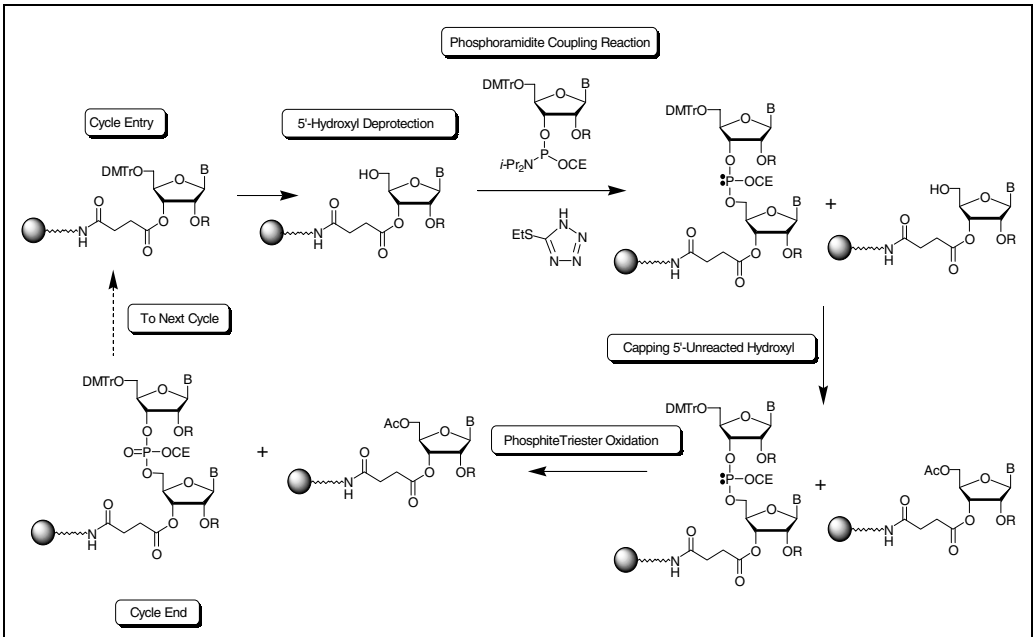

Figure 3.6.1 Synthesis of oligoribonucleotide with 2′-*O*-TBDMS groups on solid-support via the phosphoramidite approach.

RNA phosphoramidites (Amersham Pharmacia Biotech; diluted on the synthesizer to 0.1 M in acetonitrile, using automated protocols)
5′-*O*-DMTr-N^6-(phenoxyacetyl)-2′-*O*-TBDMS-adenosine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropyl) phosphoramidite
5′-*O*-DMTr-N^2-(isopropylphenoxyacetyl)-2′-*O*-TBDMS-guanosine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropyl) phosphoramidite
5′-*O*-DMTr-N^4-(acetyl)-2′-*O*-TBDMS-cytidine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropyl) phosphoramidite
5′-*O*-DMTr-2′-*O*-TBDMS-uridine-3′-*O*-(β-cyanoethyl-*N,N*-diisopropyl) phosphoramidite.
3% (v/v) TCA in methylene chloride (PE Biosystems)
Cap A: 10% (v/v) acetic anhydride/10% (v/v) 2,6-lutidine in THF (PE Biosystems)
Cap B: 16% (v/v) 1-methyl imidazole in THF (PE Biosystems)
Iodine solution: 16.9 mM I_2/49 mM pyridine/9% (v/v) water in THF (PE Biosystems)
Synthesis grade acetonitrile (Burdick & Jackson)
Activator (prepare in acetonitrile): 0.25 M 5-ethylthio-1*H*-tetrazole (SET), made from solid (American International Chemical) *or* 0.5 M 4,5-dicyanoimidazole solution (DCI), made from solid (Proligo) *or* 0.45 M 1*H*-tetrazole (TET; Glen Research).

Synthesis columns for 0.2-µmol-scale syntheses (PE Biosystems)
ABI 394 DNA/RNA synthesizer (PE Biosystems)

1. Load an empty synthesis column with ~8 mg of the RNA primer solid-support (i.e., ~25 µmol/g) corresponding to the first nucleotide at the 3′- end of the oligoribonucleotide.

Table 3.6.1 Equivalents to the Synthesis Scale and Wait Time Required for Optimal Solid-Phase Synthesis

Reagents[a]	Equivalents	Wait time (sec)
Phosphoramidites	15	465
Activator:		
SET	39	465
DCI	80	465
TET	70	465
Acetic anhydride (Cap A)	655	5
1-Methylimidazole (Cap B)	1245	5
TCA	700	10
Iodine solution	21	15

[a]Descriptions of these reagents have been shortened; see Basic Protocol 1 materials list for complete information.

2. Perform synthesis on an ABI 394 synthesizer according to the cycle outlined in Tables 3.6.1 and 3.6.2.

   See materials list above as well as Tables 3.6.1 and 3.6.2 for synthesis materials.

3. At the end of the synthesis, perform a manual detritylation cycle on the synthesizer (optional) to remove the dimethoxytrityl group at the 5′-end of the oligonucleotide.

4. Remove the synthesis column from the synthesizer and dry it either under a stream of argon or in a vacuum dessicator for 10 to 15 min.

5. Deprotect the oligoribonucleotide (see Basic Protocol 2, Alternate Protocol 1, or Alternate Protocol 2).

OLIGORIBONUCLEOTIDE DEPROTECTION WITH NH_4OH/ETHANOL AND TBAF

BASIC PROTOCOL 2

This protocol describes a deprotection scheme using a 3:1 cocktail of concentrated ammonium hydroxide and ethanol to cleave the oligoribonucleotide from the solid support, perform the β-elimination of the cyanoethyl phosphodiester protecting group, and cleave the exocyclic *N*-acyl protecting groups. A subsequent treatment with *n*-tetrabutylammonium fluoride effects cleavage of the *tert*-butyldimethylsilyl group protecting the 2′-hydroxyl functionality. (Fig. 3.6.2).

Materials

Oligoribonucleotide attached to solid support (see Basic Protocol 1)
3:1 (v/v) 29% ammonium hydroxide (Mallinckrodt Baker)/100% ethanol (prepare immediately before use)
3:1:1 (v/v/v) ethanol/acetonitrile/H_2O
1.0 M *n*-tetrabutylammonium fluoride (TBAF) in THF (Aldrich)
50 mM and 2 M triethylammonium bicarbonate (TEAB), pH 7.8 (see recipe)

Heating blocks
4-mL glass screw-top vial with Teflon lined lid (Wheaton)
14-mL centrifuge tubes (Falcon)
Qiagen-tip 500 column (Qiagen)

1. Transfer the dried oligoribonucleotide on solid support from the synthesis column (see Basic Protocol 1) to a 4-mL glass screw top vial with Teflon-lined lid.

Table 3.6.2 Summary of a 0.2 µmol–Scale Cycle for Oligoribonucleotide Synthesis on ABI 394 DNA/RNA Synthesizer[a]

Step	Function	Time (sec)
Wash steps		
1	Acetonitrile to waste	3
2	Acetonitrile to column	10
3	Argon reverse flush	8
4	Argon block flush	4
Chain extension steps		
5	Activator to waste	1.7
6	Amidite + activator to column	1.2
7	Push to column	NA
8	Wait	150
9	Push to column	0.1
10	Wait	45
11	Repeat steps 9-10 (6 times)	
12	Argon flush to waste	0.1
Wash steps		
13	Acetonitrile to waste	4
14	Repeat steps 3 and 4	
Capping steps		
15	Cap A and B to column	4
16	Wait	5
Wash steps		
17	Repeat steps 13 and 14	
Oxidation steps		
18	Iodine to column	4
19	Wait	15
Wash steps		
20	Repeat steps 13 and 14	
21	Acetonitrile to column	10
22	Argon flush to waste	4
23	Acetonitrile to column	10
24	Repeat steps 3 and 4	
25	Repeat steps 21, 23 and 24	
Detritylation steps		
26	TCA/DCM to column	6
27	Wait	5
28	Argon trityl flush	5
29	Repeat steps 26-28	
Wash steps		
30	Acetonitrile to column	10
31	Argon trityl flush	5
32	Repeat steps 2, 3, and 4	
33	End	

[a]Delivery flow rate are ~3.1 mL/min for phosphoramidites and activators and ~3.6 mL/min for all other reagents.

2. Add 2 mL of a 3:1 ammonium hydroxide/ethanol solution to the vial, screw cap on tightly, and place in a heat block for 4 hr at 65°C.
3. Remove the vial from the heat block, place it in a block kept at room temperature, and place the block in a –20°C freezer until cooled (i.e., ~30 min).

   IMPORTANT NOTE: *To avoid loss of contents, it is important to cool the sample vial in step 4 before opening the screw cap.*

4. Decant the solution (containing the deprotected oligonucleotide) into a 14-mL Falcon tube. Add 1 mL of ethanol/acetonitrile/H_2O solution, vortex well, and allow the support to settle. Decant wash and add to deprotected oligonucleotide solution. Repeat wash twice.

   Due to the presence of the hydrophobic 2′-O-TBDMS groups on the RNA, this organic wash helps increase the recovery yield.

5. Evaporate the combined supernatant from step 4 in the 14-mL tube on a Speedvac evaporator (i.e., ~2.5 hr on medium heat).
6. Add 1 mL of 1.0 M TBAF to the 14-mL tube containing the dried RNA and allow to react at room temperature for 24 hr.
7. Quench the desilylation reaction by adding 9 mL of 50 mM TEAB, then refrigerate at 4°C until ready for desalting.

   Deprotected oligoribonucleotides are highly sensitive to nuclease degradation. Therefore, gloves should always be worn when manipulating deprotected synthetic RNA; sterile disposable containers, nuclease-free laboratory reagents, and Milli-Q water should always be used to limit potential exposure to nucleases.

8. Prewash the Qiagen-tip 500 cartridge with 10 mL of 50 mM TEAB.
9. Load the quenched reaction in TEAB onto the Qiagen-tip 500 anion-exchange cartridge.
10. Wash the loaded cartridge with 10 mL of 50 mM TEAB and discard the eluent. Elute the RNA with 10 mL of 2 M TEAB into a sterile tube, and dry to a white powder on a Speedvac evaporator.

OLIGORIBONUCLEOTIDE DEPROTECTION WITH AQUEOUS METHYLAMINE AND TRIETHYLAMINE TRIHYDROFLUORIDE

ALTERNATE PROTOCOL 1

This protocol describes a deprotection scheme using aqueous methylamine and triethylamine trihydrofluoride as alternate reagents to effect nucleobase, 2′-hydroxyl and phosphodiester deprotection (Fig. 3.6.2.). Also see *APPENDIX 3C*, Basic Protocol 3, for general discussion of RNA oligonucleotide of deprotection.

Additional Materials *(also see Basic Protocol 2)*

40% (w/v) aqueous methylamine (Aldrich)
Triethylamine trihydrofluoride/NMP/TEA solution (see recipe)
3 M aqueous sodium acetate (e.g., Fluka)
n-butanol
70% aqueous ethanol

1. Transfer the oligoribonucleotide attached to the solid-support from the synthesis column (see Basic Protocol 1) to a 4-mL glass screw top vial.
2. Add 1 mL of 40% aqueous methylamine to the vial, screw the cap on tightly, and place in a heat block at 65°C for 10 min.

3. Remove the vial from the heat block, place it in block kept at room temperature, and place the block in −20°C freezer until cooled (e.g., ~20 min).

4. Decant the solution into a 14-mL tube. Add 1 mL of 3:1:1 ethanol/acetonitrile/H_2O solution, vortex well, and allow the support to settle. Decant wash solution and add to deprotection solution. Repeat wash twice, for a total of three washes.

5. Evaporate the combined supernatants in the tube on a Speedvac evaporator (i.e., ~2.5 hr on medium heat).

6. Add 0.3 mL of triethylamine trihydrofluoride/NMP/TEA solution to the tube containing the dried RNA, cap the tube, and place on a heat block for 90 min at 65°C. After incubation, bring to room temperature.

7. Precipitate the oligoribonucleotide directly from the desilylation reaction by adding 25 µL of 3 M aqueous sodium acetate, followed by 1 mL of *n*-butanol.

   If the oligoribonucleotide has been synthesized trityl-off, proceed to quenching and desalting steps (see Basic Protocol 2, steps 7 to 10).

8. Cool the mixture to −20°C for 2 hr to overnight and centrifuge 30 min at 4000 × *g* (3750 rpm in a Beckman GS-GR rotor), 4°C.

9. Decant the solution and wash the pellet with 70% ethanol. Centrifuge 10 min at 4000 × *g* (3750 rpm in a Beckman GS-GR rotor), 4°C. Decant the supernatant and dry the oligoribonucleotide pellet by using a Speedvac evaporator.

   This precipitation procedure cannot be applied to the TBAF procedure (see Basic Protocol 2) because of the high organic content of the desilylation reaction.

   Alternately, if a trityl-on deprotected oligoribonucleotide is sought, quench the desilylation reaction by adding 5 mL of 1.5 M ammonium bicarbonate, pH 7.5 (see recipe in Reagents and Solutions).

ALTERNATE PROTOCOL 2

"ONE-POT" OLIGORIBONUCLEOTIDE DEPROTECTION WITH ANHYDROUS METHYLAMINE AND NEAT TRIETHYLAMINE TRIHYDROFLUORIDE

This protocol describes an expedited deprotection scheme for oligoribonucleotides using anhydrous ethanolic methylamine and triethylamine trihydrofluoride to effect nucleobase, 2′-hydroxyl, and phosphodiester deprotection (Fig. 3.6.2).

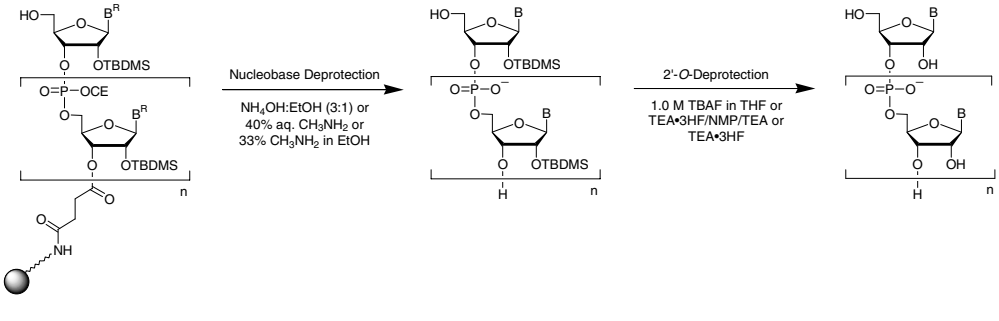

Figure 3.6.2 Deprotection of chemically synthesized oligoribonucleotides with 2′-*O*-TBDMS groups.

Additional Materials *(also see Basic Protocol 2)*

1:1 (v/v) mixture of 33% ethanolic methylamine and anhydrous DMSO
1.5 M ammonium bicarbonate, pH 7.5 (see recipe)
Acetonitrile
1:1:1 (v/v/v) acetonitrile/methanol/H_2O
RNase-free H_2O/DEPC-treated
C_{18} SepPak cartridges (Waters)

1. Transfer the dried solid support-bound oligoribonucleotide from the synthesis column to a 4-mL glass screw top vial.
2. Add 0.8 mL of a 1:1 (v/v) mixture of 33% methylamine/DMSO to the vial, screw the cap on tightly, and place on a heat block at 65°C for 15 min.

   DMSO is useful for solubilizing the partially deprotected oligoribonucleotide and helps prevent alkaline hydrolysis of the fully deprotected RNA.
3. Remove the vial from the heat block and place it in a block kept at room temperature.
4. Add 0.1 mL of neat triethylamine trihydrofluoride, vortex well, and place on a heating block for 15 min at 65°C. Cool the sample vial at room temperature and then at –20°C for 10 min.

   The solution usually gels after the addition of triethylamine trihydrofluoride.
5. Quench the reaction by adding 1 mL of 1.5 M ammonium bicarbonate, pH 7.5. Allow the support to settle and decant the supernatant.

   If sample is not cool enough, addition of ammonium bicarbonate solution may lead to significant effervescence.
6. Prewash the C_{18} Sep-pak cartridge successively with 10 mL of acetonitrile, 10 mL of 1:1:1 acetonitrile/methanol/H_2O solution, and 20 mL of RNase free H_2O.
7. Apply the quenched solution to the prewashed C_{18} Sep-pak cartridge and wash the loaded cartridge with 10 mL of RNase-free H_2O to remove salts.
8. Elute the product from the column by using 10 mL of 1:1:1 acetonitrile/methanol/H_2O and evaporate the eluate to dryness on a Speedvac.

   This desalting step will detritylate a trityl-on deprotected oligoribonucleotide. The precipitation procedure of Alternate Protocol 1 (steps 7 to 9) cannot be applied to the "one-pot" deprotection protocol because of the high organic content.

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****.*

Ammonium bicarbonate,1.5 M (pH 7.5)

Weigh 118.59 g of solid NH_4HCO_3 and bring up to 1 L with Milli-Q water. Store the buffer solution up to 2 months at 4°C.

Triethylamine trihydrofluoride/NMP/TEA solution, 1.5:0.75:1 (v/v/v)

Combine, in the following order, 0.75 mL 1-methyl-2-pyrrolidinone (NMP), 1.0 mL triethylamine (TEA), and 1.5 mL triethylamine trihydrofluoride. If the reagent is not used immediately after preparation, store it in a capped container on a warm (55° to 65°C) heat block.

The reagent will form an intractable gel if allowed to stand at room temperature.

Triethylammonium bicarbonate (TEAB), 50 mM and 2 M (pH 7.8)

Place a 4-L bottle containing 3 L of Super-Q water in an ethanol/ice bath. Bubble carbon dioxide through the water. After 15 min add 279 mL of neat triethylamine every 2 hr for 8 hr (total 4 additions). After 8 hr of CO_2 saturation, check the pH on an aliquot. Continue bubbling CO_2 until the pH reaches 7.8 then bring to 4 L with water. Store the buffer solution up to 6 months at 4°C. To prepare 50 mM TEAB, dilute 2M TEAB solution 40-fold with water.

COMMENTARY

Background Information

Synthesis of oligoribonucleotides

Similar to that of oligodeoxyribonucleotides, the chemical synthesis of oligoribonucleotides on solid support is routinely performed via the phosphoramidite method (Fig. 3.6.1; also see *UNITS 3.3 & 3.5*). However, the additional 2′-hydroxyl function of the ribofuranosyl sugar requires suitable protection during oligoribonucleotide synthesis. Among the various protecting groups available, trialkylsilyl ethers (Ogilvie et al., 1976, 1977; Wincott and Usman, 1994), and particularly the *tert*-butyldimethylsilyl ether (TBDMS; Usman et al., 1985), have been the most extensively studied, since fluoride-mediated silyl ether deprotection is quite orthogonal to the other acid- and base-labile protecting groups commonly used in oligonucleotide chemistry. Because of the steric bulk introduced by the TBDMS group, coupling 2′-*O*-TBDMS protected ribophosphoramidites to a growing oligonucleotide chain is notoriously more sluggish as compared to their 2′-deoxy analogs. The elongation cycle for automated oligoribonucleotide synthesis is similar to that of oligodeoxynucleotides and consists of the detritylation, coupling, capping, and oxidation steps. Each of these steps is critical for successful RNA synthesis. One important difference regarding the detritylation step is the possibility of using higher concentrations of haloacids due to the reduced sensitivity of ribofuranosylnucleotides to acid-mediated depurination. The oxidation and capping steps do not differ significantly from traditional oligodeoxyribonucleotide synthesis. Conversely, the coupling step has been the focus of much attention through the development of a number of phosphate protecting groups (Usman et al., 1987; Sinha et al., 1983; Schwarz et al., 1984; Hamamoto et al., 1986; Kayakawa et al., 1990; Wada and Sekine, 1994; Ravikumar and Cole, 1994) and different phosphoramidite dialkylamino functions (Lyttle et al., 1991; Sinha et al., 1984; Beaucage and Caruthers, 1981) aimed at providing faster coupling rates. Although some of these modifications have been commercialized, the phosphoramidites carrying the 2-cyanoethyl-*N*,*N*-diisopropyl combination (Sinha et al., 1983, 1984) are predominantly used. According to this chemistry, the coupling time for 2′-*O*-TBDMS ribophosphoramidites is considerably longer than that of the 2′-deoxy series for synthesis scales ranging from 0.2 to 2.5 µmol. Particularly important to commercially available ribophosphoramidites is the acidic activation step that allows rapid conversion of stable *N*,*N*-diisopropyl phophoramidite to azolide intermediates. These highly reactive transient species are then coupled to the free 5′-hydroxyl of any oligonucleotide bound to a support to form phosphite triester internucleotidic bonds which are further oxidized to the acid-stable pentavalent phosphotriester linkages. Dramatic improvements in oligoribonucleotide synthesis are achieved when activators more acidic than the standard 1-*H*-tetrazole (pKa = 4.8) are used. Such activators may include 5-(4-nitrophenyl)-1*H*-tetrazole (pKa = 3.7) (Sproat et al., 1989) or 5-ethylthio-1*H*-tetrazole (pKa = 4.28) (Wincott et al., 1995). These tetrazole derivatives are supposedly more efficient at protonating the trivalent phosphorus. Once protonated, this electrophilic phosphorus center reacts with a tetrazole molecule to displace an *N*,*N*-diisopropylamino group. 5-Ethylthio-1*H*-tetrazole (SET) became the preferred activator for oligoribonucleotides, and has been used in a number of reports describing successful RNA synthesis. However, the acidic properties of 5-ethylthio-1*H*-tetrazole can cripple larger-scale oligoribonucleotide syntheses because of concomitant acidic activator–mediated detritylation of nucleoside phosphoramidites, occurring as a result of an extended coupling reaction time required for satisfactory coupling efficiency (Krotz et al., 1997). This problem spearheaded the development of less acidic but more nucleophilic activators (Vargeese et al., 1998) like 4,5-dicyanoimidazole (pKa = 5.2) or benzimi-

dazolium triflate (pKa = 4.5) (Hayakawa et al., 1996). Although no mechanistic studies have been presented, these activators are reported to speed up coupling reaction rates due to the increased nucleophilicity of their conjugated base while exhibiting sufficient acidity to protonate the tricoordinated phosphorus center.

Automated oligoribonucleotide synthesis can be easily performed on long chain alkylamine controlled pore glass (CPG; Pon et al., 1988; *UNITS 3.1 & 3.2*). Non-swellable highly cross-linked polystyrene solid-supports have shown to be generally superior to CPG due to their increased mechanical and chemical resistance (McCollum and Andrus, 1991).

Deprotection of oligoribonucleotides

The deprotection of crude oligoribonucleotides traditionally requires a basic treatment, which allows for the transamination of the *N*-acyl exocyclic protecting groups, β-elimination of the 2-cyanoethyl phosphate protecting group, and cleavage of the succinic ester bond linking the oligoribonucleotide to the solid support (Fig. 3.6.2). Of these three concomitant reactions, base deprotection is by far the most rate-limiting step. Once these reactions are accomplished, a fluoride treatment removes the substituted silyl ether function protecting the 2′-hydroxyl of the ribofuranose ring.

Nucleobase deprotection

In the early days of oligoribonucleotide chemistry, the heterocyclic amino function of each nucleobase was almost exclusively protected with the benzoyl (rA and rC) and isobutyryl (rG) groups. Although these protecting groups on synthetic DNA are efficiently removed by treatment with concentrated ammonium hydroxide, the presence of the 2′-*O*-TBDMS group in synthesized RNA necessitated the use of ethanolic ammonia (Lyttle, 1993) or a solution of ammonium hydroxide in ethanol (3:1, v/v) for 12 to 16 hours at 55°C to minimize the cleavage of the silyl group (Stawinski, 1988; Wu et al., 1989). Premature deprotection of the 2′-hydroxyl under basic conditions results in extensive oligoribonucleotide chain cleavage from intramolecular nucleophilic attack of the 2′-hydroxyl group on the phophodiester function. To circumvent this unwanted side reaction and shorten the deprotection time, two strategies were investigated over the last decade.

One of these strategies involves the use of various combinations of hydrazine, ethanolamine, and alcohol (Polushin et al., 1991) or more nucleophilic alkylamines (Wincott et al., 1995; Reddy et al., 1995) for oligoribonucleotide deprotection. For example, 40% aqueous methylamine, in place of or in addition to 30% ammonium hydroxide, cleaves the nucleobase *N*-acyl protecting groups in a few minutes at 65°C or ~1 hr at room temperature. However, this procedure requires the use of N^4-acetyl cytidine phosphoramidite derivatives to avoid a well documented transamination side reaction (Reddy et al., 1994).

The second strategy relates to the development of base-labile amino-protecting groups that would further shorten exposure of the 2′-*O*-TBDMS group to basic conditions. These groups belong to the phenoxyacetyl (PAC) (Wu et al., 1988; Chaix et al., 1989) and amidine-type (McBride et al., 1986) protecting groups, mainly used for ribopurine phosphoramidites given that the N^4-acetyl protection of cytidine appears quite optimal. Amidine protecting groups include acetamidines (McBride et al., 1986) and dialkylformamidines (Vinayak et al., 1992), which can be cleaved in 2 to 3 hours at 55°C using concentrated ammonium hydroxide/ethanol (3:1, v/v). The main advantage of the dimethylformamidine protecting group when used in conjunction with 2′-deoxyribonucleoside phosphoramidites (Vu et al., 1990) is that it confers an increased resistance towards depurination during the detritylation step. However, because oligoribonucleotides are inherently less sensitive to depurination, *N*-phenoxyacetyl protection has been preferred for the commercial manufacturing of these biomolecules. The phenoxyacetyl and 4-*tert*-butyl- or (4-isopropylphenoxy)acetyl protecting groups (Sinha et al., 1993) are considerably more labile than amidines under basic conditions. Typically, these can be quantitatively cleaved from the exocyclic amino function of nucleobases after a 15 min to 1 hr incubation in concentrated ammonium hydroxide/ethanol (3:1) at 65°C, or 2 to 4 hr at room temperature.

The use of "fast deprotecting groups" of the PAC family does not preclude the concomitant use of more nucleophilic alkylamines. Indeed, combining these two strategies allows for expedited base-labile removal of protecting groups as exemplified in Alternate Protocol 1.

2′-O-tert-butyldimethylsilyl deprotection

The fluoride-sensitive *tert*-butyldimethylsilyl group allows for an efficient orthogonal deprotection of the 2′-hydroxyl of synthetic RNA. The use of fluoride-based reagents is the

preferred methodology for the removal of the 2′-*O*-TBDMS group, although Sekine and colleagues (Kawahara et al., 1996) recently reported the application of an acid-catalyzed desilylation scheme to oligoribonucleotides. After completion of nucleobase and phosphotriester deprotection, and subsequent evaporation of the basic solution, addition of *n*-tetrabutylammonium fluoride (TBAF) in THF to the partially deprotected RNA cleaves the 2′-*O*-TBDMS group at room temperature within 24 hr (Basic Protocol 2). However, limited solubility of the RNA in this apolar solvent hampers the efficient deprotection of longer oligoribonucleotides. To circumvent this problem, RNA dissolution in either dimethylsulfoxide (Gasparutto et al., 1992) or 50% ethanol (Scaringe, 1995) has been investigated. The notorious water sensitivity of TBAF (Hogrefe et al., 1993) and the desalting step required after quenching the desilylation reaction have led to the development of other fluoride-based reagents. Triethylamine trihydrofluoride (Alternate Protocol 1) has been shown to be a superior reagent either neat (Pirrung et al., 1994; Westman and Stromberg, 1994) or in combination with polar aprotic solvents such as dimethylformamide (Vinayak et al., 1995) or 1-methyl-2-pyrrolidinone (Wincott et al., 1995). This reagent promotes 2′-*O*-TBDMS deprotection within 30 to 90 min at 65°C or 4 to 8 hr at room temperature. Using triethylamine trihydrofluoride, the time-consuming desalting step may be replaced by a sodium acetate/1-butanol precipitation procedure (Wincott et al., 1995) that is not compatible with the high organic content present in TBAF deprotection mixtures. Further application of neat triethylamine trihydrofluoride in a "one-pot deprotection" procedure (Alternate Protocol 2; Bellon, 2000) that employs a mixture containing anhydrous ethanolic methylamine allows for expedititious RNA deprotection. This procedure requires a quenching step with ammonium bicarbonate if one desires to retain the trityl group on the oligonucleotide, and a subsequent desalting/purification on a reverse-phase cartridge because the presence of ethanol and dimethylsulfoxide prevent RNA precipitation from butanol.

HPLC analysis and purification of synthetic RNA is now well-documented (Wincott et al., 1995; Sproat et al., 1995; and Vinayak et al., 1995). In particular, the use of perchlorate-based buffer (Na or Li form) in conjunction with anion-exchange Nucleo Pac columns, allows for easy purification of full-length RNA product from truncated sequences.

Critical Parameters

Automated oligoribonucleotide synthesis according to the phosphoramidite method has not significantly evolved over the last decade. Therefore, all precautions mentioned in the major textbooks (Gait, 1984; Eckstein, 1991; Agrawal, 1993) still remain valid. It is particularly important to emphasize that phosphoramidite chemistry is highly water sensitive. Great care should therefore be taken to ensure that the phosphoramidites and activator are dissolved in strictly anhydrous acetonitrile. All ancillary reagents (i.e., acetonitrile, detrilylation, capping, and oxidation solutions) are commercially available, guaranteeing high performance reproducibility in the syntheses and relieving the chemist from time-consuming anhydrous distillations. Sterile, disposable pipet tips and plastic tubes should be used for storing and handling RNA. Troubleshooting an oligoribonucleotide synthesis is a relatively easy task when the trityl assay is used to spectrophotometrically monitor the synthesis (Gait, 1984; see also *APPENDIX 3C*, Basic Protocol 1, Support Protocol 1).

Deprotection of oligoribonucleotides according to the three protocols presented in this unit is quite straightforward. However, Alternate Protocol 2 or the "one-pot" deprotection protocol should not be used in conjunction with controlled-pore-glass (CPG) synthesized RNA because of the inherent incompatibility between triethylamine trihydrofluoride and the silyl components of CPG. Because all the basic solutions used are composed of gaseous amines dissolved in water or ethanol, freshly opened bottles will ensure that the effective concentration of the amine (i.e., 29% NH_4OH or 40% methylamine in water) is close to its stated nominal value. Typically, reagent bottles should be replaced every two weeks if opened on a regular basis. This may be especially true for the TBAF solution in THF (used in Basic Protocol 2) because a low water content is critical for efficient desilylation. The deprotection times at 65°C are suggested for 2 mL of basic solution, and need to be extended if larger amounts of reagents are used.

Finally, deprotected oligoribonucleotides are highly sensitive to nuclease degradation. Therefore, gloves should always be worn when manipulating deprotected synthetic RNA; sterile disposable containers and Milli-Q water should also always be used to limit potential exposure to nucleases. DEPC treated water should be used when desalting RNA on C18 cartridges.

Anticipated Results

Because of the iterative nature of solid-phase oligoribonucleotide synthesis, only modest chemical yields of oligoribonucleotide can be produced, especially for longer synthetic RNAs (i.e., >30 residues). When applying Basic Protocol 1 using 5-ethylthio-1*H*-tetrazole, a realistic averaged stepwise chemical yield (ASWY) of 97.5% can be routinely obtained for RNA synthesis. This ASWY is determined by the ratio $(\mu mol\ FLR/\mu mol\ scale)^{1/n} \times 100$ where µmol FLR is the amount of full length RNA in the crude mixture, µmol scale is the synthesis scale, and *n* is the number of synthesis cycles. This corresponds to an isolated yield of 41% for the all-RNA 36-mer pictured in Figure 3.6.3.A. Figure 3.6.3.B and C shows HPLC profiles of the same sequence synthesized according to Basic Protocol 1 using the alternate activators DCI and TET, respectively. Figure 3.6.3 indicates that 5-ethylthio-1*H*-tetrazole is the activator of choice for small scale RNA synthesis.

At small scale (i.e., <10 µmol), the quality of oligoribonucleotides generated from the presented deprotection protocols does not vary much. Therefore, one should consider the time requirement as an important parameter in selecting a particular protocol. However, given that oligoribonucleotide synthesis often incorporates one or more modified synthons that may be more or less sensitive to the basic or fluoride treatments used, a careful examination of chemical compatibility should be performed prior to selecting the optimal deprotection method.

Time Considerations

Typically, the duration of the 0.2 µmol elongation cycle of an oligoribonucleotide is ~12 min. It will thus take ~8 hr to synthesize an all-RNA 37-mer. It requires about 32 hr to perform oligoribonucleotide deprotection according to Basic Protocol 2, whereas processing time can be cut to <8 hrs when adopting Alternate Protocol 1. When using Alternate ("one-pot") Protocol 2, deprotection time can be shortened to a mere 45 min from start to finish.

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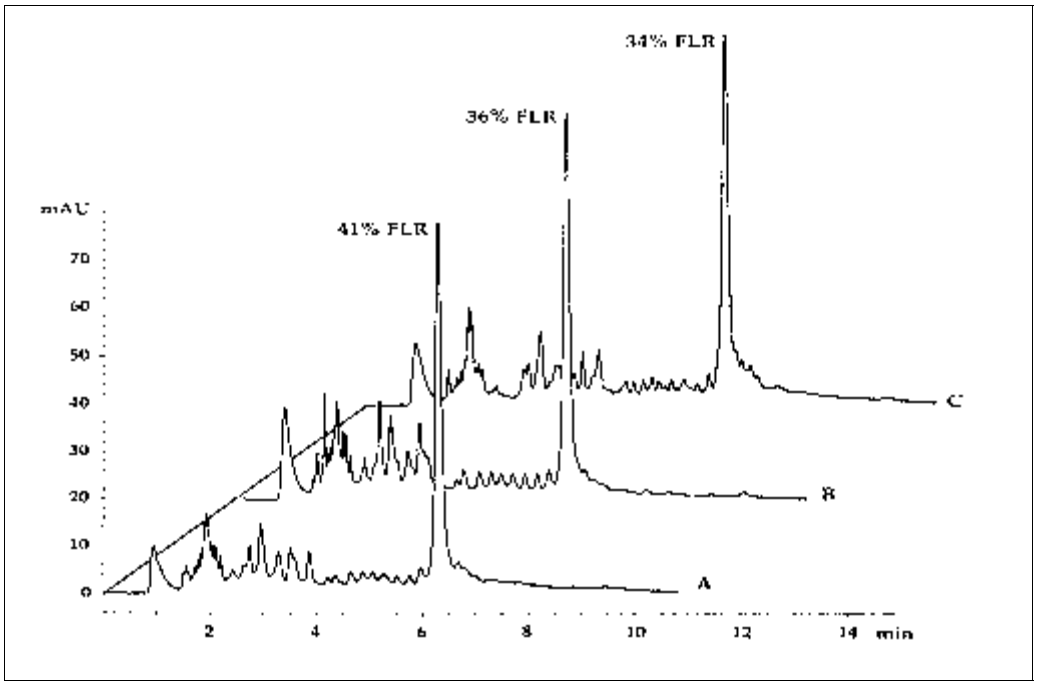

Figure 3.6.3 HPLC chromatograms of a 36-mer oligoribonucleotide (5′- GUU UUC CCU GAU GAG GCC GAA AGG CCG AAA UUC UCC -3′) synthesized at the 0.2 µmol scale according to the basic synthesis protocol and deprotected using the "one-pot" deprotection protocol (Alternate Protocol 2). (**A**) 0.25 M SET, (**B**) 0.5 M DCI, (**C**) 0.45M TET. Results obtained using Dionex NucleoPac PA-100 22 × 250–mm column at 50°C. Buffer A (1 mM Tris, 20 mM $NaClO_4$), buffer B (1 mM Tris, 300 mM $NaClO_4$). Gradient 40% B to 70% B in 12 min, flow rate = 1.5 mL/min (see *UNIT 10.5*).

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