Synthesis of Oligoribonucleotides Using the 2-Nitrobenzyloxymethyl Group for 2′-Hydroxyl Protection

The methodology for oligoribonucleotide construction described in the Basic Protocol uses a DNA synthesizer to add protected ribonucleoside phosphoramidite monomers one after another in defined sequence to a 3′-terminal nucleoside that is attached to a solid support. The monomers used are the 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidites of N-protected 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-nitrobenzyloxymethyl) ribonucleosides. Their structures are shown in Figure 3.7.1. After a target oligoribonucleotide has been synthesized, it is treated with ammonia to release it from the support and to remove protecting groups from its nucleobases and phosphates. Finally, it is irradiated with long-wave UV light, which removes the 2-nitrobenzyloxymethyl groups from the 2′-hydroxyls, to give the unmodified RNA. The preparation of the 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidites is described in the Support Protocol.

SYNTHESIS AND DEPROTECTION OF N-PROTECTED 2′-*O*-(2-NITROBENZYLOXYMETHYL) OLIGORIBONUCLEOTIDES

BASIC PROTOCOL

This protocol describes the steps involved in constructing oligoribonucleotides using a DNA synthesizer and starting with phosphoramidites of the 2′-*O*-(2-nitrobenzyloxymethyl) derivatives of the four common ribonucleosides U, C, A, and G. Details of those parts of the operation that are carried out on the synthesizer will vary depending on the make and model used. However, the above monomers are compatible with all the usual reagents used in these instruments and, with the few exceptions noted below, the manufacturer's directions for setup and synthesis should be followed in each specific case. A generalized discussion of the use of DNA synthesizers can be found in *APPENDIX 3C*. In contrast, the deprotection methodology that constitutes the second section of the protocol is unique to oligoribonucleotides constructed from these particular monomers, and is set out in detail below.

Materials

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(2-nitrobenzyloxymethyl)-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidites (U, C, A, and G; see Support Protocol)
Phosphorus pentoxide
Anhydrous acetonitrile (Aldrich)
0.45 M tetrazole (Amersham Pharmacia Biotech) in anhyrdous acetonitrile
Pyridine, HPLC grade (Aldrich)
Concentrated ammonium hydroxide, 4°C
50% (v/v) aqueous pyridine
50% (v/v) aqueous 2-methyl-2-propanol, HPLC grade
0.2 M formic acid
0.2 M ammonium hydroxide

DNA synthesizer with ancillary reagents
Amidite vials fitted with septa
5-mL syringes and 21-G needles
Synthesis column containing support derivatized with *O*-acyl ribonucleosides (Glen Research; see Critical Parameters)
10.2-cm pressure tube (e.g., Ace Glass) fitted with Teflon screw-in cap and Teflon-encapsulated O ring

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Current Protocols in Nucleic Acid Chemistry (2000) 3.7.1-3.7.8

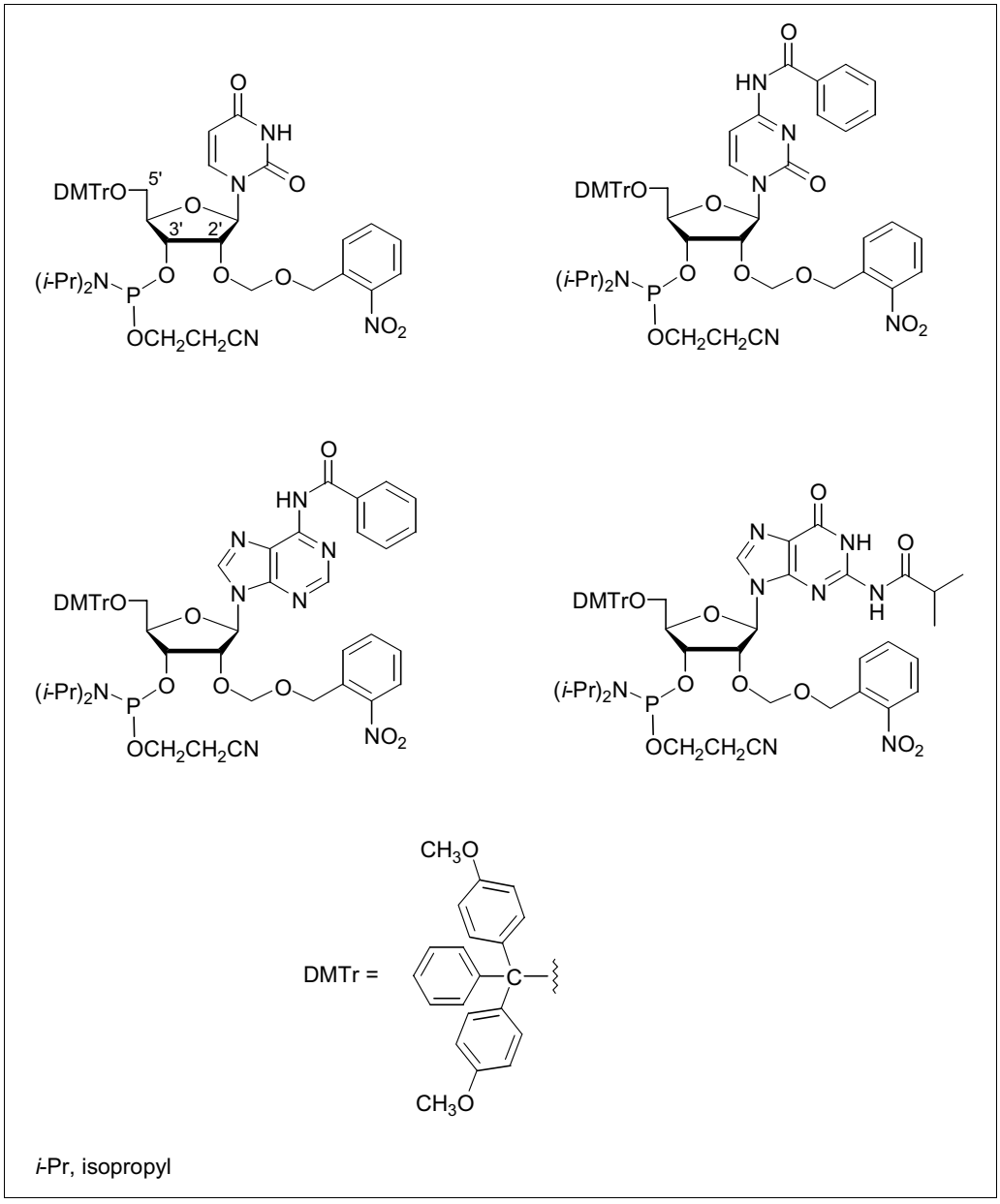

Figure 3.7.1 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(2-nitrobenzyloxymethyl)-3′-*O*-phosphoramidites of the four common ribonucleosides. Chemical structures are shown for (clockwise from upper-left corner): uridine phosphoramidite, cytidine phosphoramidite, guanosine phosphoramidite, and adenosine phosphoramidite. DMTr, dimethoxytrityl group (bottom).

Yellow lights
Covered water bath, 55°C
30-mL Buchner funnel (medium porosity)
500-mL filter flask
Vacuum aspirator
500-mL round-bottom flask
Rotary evaporator connected to water aspirator, with bath temperature set <40°C
250-mL Pyrex Erlenmeyer flasks
Long-wave (365-nm) UV lamp, containing two 15-W black-light tubes behind a UV-transmitting glass filter measuring ~10 × 30 cm

NOTE: The amidites are sensitive to UV light (see Critical Parameters).

Synthesize oligoribonucleotides

1. Determine volumes of amidite solutions needed to carry out synthesis of the target oligoribonucleotide, following the manufacturer's instructions for the DNA synthesizer.

2. Use the following concentrations to calculate the required weight of each amidite.

U	137 mg/mL
C	152 mg/mL
A	156 mg/mL
G	153 mg/mL.

These values correspond to a concentration of 0.15 M for each amidite.

3. Weigh out the calculated quantity of each 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-nitrobenzyloxymethyl)-3′-*O*-(2-cyanoethyl-*N,N*-diisopropyl) phosphoramidite into a separate amidite vial.

4. Dry amidites 12 to 16 hr in a vacuum desiccator over phosphorus pentoxide, then cap vials with rubber septa.

The phosphorus pentoxide should be allowed to absorb a little atmospheric moisture before being placed in the desiccator, so that the amidites are not contacted by loose dust from the compound's surface.

5. Dissolve each amidite in the required volume of anhydrous acetonitrile (as determined in step 1), using a fresh 5-mL syringe and 21-G needle to deliver the solvent through the septum of the vial.

A syringe and needle should be used to avoid moisture contamination.

6. Fit amidite vials onto the DNA synthesizer as part of the general setup procedure, following the manufacturer's specific directions.

7. Use 0.45 M tetrazole in anhydrous acetonitrile as the phosphoramidite activator solution.

8. Set the coupling time at 3 min.

9. Install a synthesis column containing support derivatized with *O*-acyl ribonucleosides and carry out the synthesis.

The amidites and growing oligoribonucleotide must be protected from light.

10. Perform the final detritylation on the machine, then remove the synthesis column and either begin the deprotection process immediately, or store the column up to 1 week at –20°C.

Deprotect oligoribonucleotides

11. Empty derivatized support from the synthesis column into a 10.2-cm pressure tube containing 2 mL pyridine. Rinse column with pyridine if necessary, but do not allow total amount of pyridine in the pressure tube to exceed 4 mL.

NOTE: *Steps 11 through 16 should be carried out under yellow lights (see Critical Parameters).*

12. Cool pressure tube in ice and fill with cold concentrated ammonium hydroxide to just below the level of the screw cap.

A volume (~16 mL) of ammonium hydroxide should be used that minimizes air space in the closed tube. Only full-strength ammonium hydroxide from a recently opened bottle should be used. The bottle should be sealed and stored at 4°C when not in use.

13. Screw in a Teflon cap fitted with a Teflon-encapsulated O ring, making sure seal is tight. Wrap tube in aluminum foil and heat 24 hr in a covered water bath at 55°C. Invert tube occasionally to mix contents.

14. Chill tube on ice, open carefully, and quickly filter contents through a 30-mL medium-porosity Buchner funnel into a 500-mL filter flask using a vacuum aspirator.

    CAUTION: *This should be performed in a fume hood because the mixture bubbles violently as ammonia is released.*

15. Transfer filtrate into a 500-mL round-bottom flask, using two 10-mL washes of 50% aqueous pyridine.

16. Evaporate solution to ~10 mL using a rotary evaporator connected to a water aspirator. Add 100 mL water and again evaporate to 10 mL.

    The evaporation should be carried out at the lowest bath temperature (<40°C) consistent with efficient removal of solvent. The solution should not be evaporated to dryness.

17. Add 100 mL of 50% aqueous 2-methyl-2-propanol and evaporate to ~5 mL. Repeat this process once more to remove traces of pyridine.

18. Transfer solution into a graduated beaker and dilute with 50% aqueous 2-methyl-2-propanol so that the final volume corresponds to a concentration of 1 A_{260} unit of oligonucleotide/mL. Stir solution.

    The volume can be roughly calculated using the formula $V = 10L \times S$, where V is the final volume in milliliters, L is the number of nucleotides in the oligomer, and S is the scale of the reaction in micromoles.

19. Adjust pH of the solution to 3.7 by careful dropwise addition of 0.2 M formic acid. Do not overshoot.

    Removal of the 2′-hydroxyl-protecting groups is pH dependent, and is most efficient at pH 3.7.

20. Place 50-mL aliquots of the solution in 250-mL Pyrex Erlenmeyer flasks.

    Pyrex is transparent to long-wave UV light.

21. In a 4°C cold room, set up flasks on the flat glass filter of a long-wave UV lamp. Irradiate solutions 4.5 hr with intermittent swirling.

    A brilliant sky-blue fluorescence develops as photolysis products derived from the 2-nitrobenzyl group accumulate in the solution.

    CAUTION: *UV light can cause eye damage. Wear protective glasses and shield the irradiation setup with a sheet of aluminum foil to prevent inadvertent exposure of others.*

22. Recombine solutions and adjust pH back up to 7 to 8 by careful addition of 0.2 M ammonium hydroxide with stirring.

    RNA is degraded by base at a pH above 8. Step 23 should be carried out immediately if too much ammonium hydroxide is added by mistake.

23. Evaporate solution to a volume of 1 to 2 mL under vacuum using the rotary evaporator.

    The deprotected oligoribonucleotide can now be purified by anion-exchange HPLC or by gel electrophoresis (UNITS 10.4 & 10.5).

SYNTHESIS AND PURIFICATION OF 3′-*O*-(2-CYANOETHYL-*N*,*N*-DIISOPROPYL) PHOSPHORAMIDITES OF 5′-*O*-(4,4′-DIMETHOXYTRITYL)-2′-*O*-(2-NITROBENZYLOXYMETHYL) RIBONUCLEOSIDES

This section describes a method for obtaining the phosphoramidite monomers of U, C, A, and G used in the Basic Protocol.

Materials

N-Protected 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-nitrobenzyloxymethyl) ribonucleosides (*UNIT 2.5*)
Pyridine, HPLC grade (Aldrich)
Anhydrous toluene (Aldrich)
Anhydrous tetrahydrofuran (Aldrich)
N,N-Diisopropylethylamine (Aldrich)
2-Cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (Aldrich)
99:1 (v/v) chloroform/methanol containing 3% (v/v) triethylamine
Ethyl acetate
1 M sodium carbonate (for U, A, G) *or* 1 M sodium bicarbonate (for C), ice cold
Merck 60 silica gel (230 to 400 mesh ASTM)
4:1 (v/v) ethyl acetate/hexane containing 0.1% (v/v) triethylamine (for A, C, and U)
0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% (v/v) methanol in dichloromethane containing 5% (v/v) triethylamine (for G)
Anhydrous diethyl ether
Dry argon

100-mL flask fitted with a septum
Rotary evaporator, with its air inlet connected to a Drierite gas-drying unit, connected interchangeably to water aspirator and vacuum pump
1-mL syringe and 21-C needle
3 × 60–cm flash chromatography column, with reservoir and flow controller
100-mL round-bottom flask

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

NOTE: Triethylamine, *N,N*-diisopropylethylamine, and pyridine should be kept dry over calcium hydride granules.

Synthesize phosphoramidites

1. Using a rotary evaporator connected to a water aspirator, dry 1 mmol of any N-protected 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-nitrobenzyloxymethyl) ribonucleoside in a 100-mL flask by two successive co-evaporations with each of the following solvents: 20 mL pyridine, 20 mL anhydrous toluene, and 20 mL anhydrous tetrahydrofuran.
2. Dissolve residue in 3 mL anhydrous tetrahydrofuran and add 0.7 mL (4 mmol) *N,N*-diisopropylethylamine. Add a stir-bar, seal flask with a septum, and begin stirring.
3. Using a 1-mL syringe and 21-G needle, add 0.44 mL (2 mmol) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite dropwise over a 2-min period through the septum. Continue stirring 2 hr at room temperature.

4. Examine reaction mixture by TLC (*APPENDIX 3D*) in 99:1 chloroform/methanol containing 3% triethylamine.

   The product should appear under short-wave UV light as two closely spaced bands of approximately equal intensity, corresponding to its two diastereomeric forms.

5. Dissolve reaction mixture in 150 mL ethyl acetate and extract three times with 75 mL ice-cold 1 M sodium carbonate (1 M sodium bicarbonate for phosphoramidite of C).

6. Evaporate ethyl acetate solution to an oil using the rotary evaporator with the water aspirator.

Purify phosphoramidites

7. Prepare a 3 × 60–cm flash chromatography column with a 100-mL bed volume of Merck 60 silica gel packed in starting solvent.

8a. *For phosphoramidites of U, C, and A:* Dissolve phosphoramidite oil in 2 to 3 mL starting solvent and apply to chromatography column. Use 600 mL of 4:1 ethyl acetate/hexane containing 0.1% triethylamine to elute. Collect 15-mL fractions and assay by TLC in 99:1 chloroform/methanol containing 3% triethylamine.

8b. *For phosphoramidites of G:* Dissolve phosphoramidite oil in 2 to 3 mL starting solvent and apply to chromatography column. Use a stepwise gradient of 50 mL each of 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% methanol in dichloromethane containing 5% triethylamine to elute. Collect 15-mL fractions and assay by TLC in 99:1 chloroform/methanol containing 3% triethylamine.

9. Combine fractions containing pure product in a 100-mL round-bottom flask and evaporate solution to an oil using the rotary evaporator with the water aspirator.

10. Dissolve residue in 1 to 2 mL anhydrous diethyl ether. Concentrate solution on the rotary evaporator first using the water aspirator, and finally under high vacuum (i.e., with a vacuum pump) until a stable foam is formed.

11. Fill flask with dry argon, seal it, and wrap in aluminum foil. Store amidite up to 1 year at −80°C in the dark.

COMMENTARY

Background Information

Ribonucleoside phosphoramidite monomers with their 2′-hydroxyls protected by 2-nitrobenzyloxymethyl groups (Schwartz et al., 1992) were introduced at a time when phosphoramidites with other modes of 2′-protection required very long coupling times on synthesizers, often >10 min per condensation. The nitrobenzyloxymethyl compounds were unique in needing coupling times not much longer than those of DNA amidites (i.e., ~2 min). This enhanced reactivity may be due to the presence of the methylenedioxy "arm" that forms part of the 2-nitrobenzyloxymethyl group and allows it the flexibility to remain out of the way of the reactive phosphoramidite center. However, problems of low reactivity with other types of amidites have been largely overcome by the introduction of newer, more powerful activating agents such as 5-ethylthio-H^1-tretrazole (Vinayak et al., 1994), and machine-assisted synthesis of RNA is now generally as rapid and simple as that of DNA. The protocol described in this unit, for example, has been used to prepare biologically functional RNA of up to 33 nucleotides in length.

Compound Characterization

The ^{31}P nuclear magnetic resonance (^{31}P-NMR) chemical shifts for the 2′-*O*-(2-nitrobenzyloxymethyl)-protected phosphoramidites of U, C, A, and G are given in Table 3.7.1. If these monomers have been properly prepared and purified, no extraneous peaks should be present in their NMR spectra. Oligoribonucleotides made from these monomers can be charac-

Table 3.7.1 ^{31}P-NMR Chemical Shifts of 2′-*O*-(2-Nitrobenzyloxymethyl)-Protected Ribonucleoside Phosphoramidites[a]

Ribonucleoside	Downfield (ppm)	Upfield (ppm)
A	151.359	151.095
C	152.552	151.127
G	151.408	151.141
U	151.501	150.471

[a]In acetonitrile-d_3, relative to 85% H_3PO_4 as external standard at 0 ppm.

terized, after deprotection and purification, using an RNA sequencing kit (nuclease method, e.g., USB) or by digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase followed by reversed-phase HPLC analysis of the digest.

Critical Parameters

The techniques for RNA synthesis described in this protocol have been thoroughly tested using polystyrene supports in conjunction with an easily prepared universal adapter that allows any sequence, DNA or RNA, to be assembled on any kind of support (Schwartz et al., 1995). Consequently, this system is strongly recommended. Use of controlled-pore glass has been less well studied. Supports of this type should be chosen with caution as they are attacked by hot ammonia solutions, and the effects of significant amounts of dissolved silica on the deprotection of longer oligoribonucleotides by the methods described here are unknown. In any case, synthesis columns that contain a support derivatized with base-labile *O*-acyl ribonucleosides (e.g., the 3′-RNA supports supplied by Glen Research) should be used in this protocol. Only these nucleosides will be completely deprotected under the conditions specified.

The ribonucleoside phosphoramidites are prepared using the method of Sinha et al. (1984). As in all such syntheses, it is essential to maintain anhydrous conditions. Make sure all glassware is dry and avoid exposing amidite reagents or their solutions to atmospheric moisture. Take further precautions when using these particular amidites because of their sensitivity to UV light. Enough of this radiation exists as a component of standard laboratory lighting to cause slow loss of 2-nitrobenzyloxymethyl groups. Loss of 2′-protecting groups in RNA leads to chain cleavage during the ammonia treatment that is part of the deprotection process. Take steps to minimize exposure not only of the amidites but also of the synthesized oligoribonucleotides to UV light. This means replacing overhead fluorescent bulbs in a designated work area with yellow bulbs, such as GE or Sylvania Golds. Wrap amidite vials, synthesis columns, and the vessels used in the deprotection process (up to the point where ammonia is removed from the mixtures) in aluminum foil.

Once the oligomers are deprotected, they become vulnerable to the action of ribonucleases, which are everywhere in the laboratory, including on the experimenter's skin. Always wear latex gloves when handling RNA, heat all glassware at 250°C for a few hours before use, and maintain a supply of freshly distilled water in covered containers at 4°C. Store pH electrodes in solutions containing thymol crystals.

Anticipated Results

Yields of ribonucleoside phosphoramidites should be 80% to 90%. In oligoribonucleotide syntheses, the amounts of isolated, deprotected oligoribonucleotides that are obtained decrease with increasing length. However, for products up to 20 nucleotides long, yields in the range of 10% to 30%, based on support-bound starting nucleoside, can be expected.

Time Considerations

The ribonucleoside phosphoramidites each require one day for preparation and purification. Oligoribonucleotide synthesis takes a day, and deprotection requires a further 2 to 3 days.

Literature Cited

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

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Key References

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

Provides an overview of the use of 2-nitrobenzyloxymethyl as a 2′-hydroxyl-protecting group in oligoribonucleotide synthesis.

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