Synthesis of Endcap Dimethoxytrityl Phosphoramidites for Endcapped Oligonucleotides

This unit describes the preparation of short endcapped DNA duplexes. Endcaps may be either aromatic (hydrophobic) or aliphatic (hydrophilic or hydrophobic) molecules that specifically cross-link the 5′ end of one strand with the 3′ end of the complementary strand in a DNA duplex. Endcaps may be viewed as a replacement of the loop region nucleotides of a DNA hairpin, with the added advantage of increased thermal stability. Specific cross-links at the terminus of a DNA duplex can be engineered through thiol-disulfide exchange with thiol-modified nucleosides incorporated at the end of the oligonucleotide strands (*UNIT 5.1*). The endcap approach, instead, requires the incorporation of the endcap into the sequence during oligonucleotide synthesis. Many different types of molecules may be used as endcaps, including oligo(ethylene glycol) linkers (*UNIT 5.3*), unsubstituted alkyl chains (Altmann et al., 1995), and aromatic molecules such as stilbene diethers (Lewis et al., 1999), carboxamides (Letsinger and Wu, 1995; Lewis et al., 2000), azobenzene (Yamana et al., 1996, 1998), and naphthalene diimides (Bevers et al., 1998, 2000).

Basic Protocol 1 describes the synthesis of a naphthalene diimide hydrophobic endcap that prefers to base stack with GC base pairs. The phosphoramidite derivative of the endcap can be readily synthesized in three short steps for incorporation into an oligonucleotide sequence. The preparation of a terthiophene hydrophobic endcap is described in Basic Protocol 2, again requiring just three synthetic steps to obtain the phosphoramidite derivative. The terthiophene endcap has higher lipophilicity than the naphthalene diimide endcap and provides higher stability when stacked over an AT base pair. Finally, Basic Protocol 3 outlines the preparation of a hydrophilic 2,2′-oxydiacetamide endcap, which provides lower enhancement in stability as compared to either of the hydrophobic endcaps, but a more rigid and well-defined structure than the oligo(ethylene glycol) endcaps.

Synthesis of endcapped oligonucleotides can be carried out using standard automated synthesis protocols with only minor modifications. The naphthalene diimide endcap is base sensitive and hence exposure to strong base such as ammonia must be avoided. Decomposition of the naphthalene diimide endcap can be avoided by using ultramild phosphoramidites (Glen Research) during oligonucleotide synthesis. The resulting oligonucleotide can be cleaved from the support as well as deprotected in a 0.05 M solution of potassium carbonate in methanol. The other requirement is to extend the time of the coupling cycle during the incorporation of the endcap phosphoramidite to 15 min during oligonucleotide synthesis.

The endcapped oligonucleotides can be purified by denaturing PAGE (*UNIT 10.4*) or by reversed-phase HPLC (*UNIT 10.5*). MALDI-TOF mass spectrometry (*UNIT 10.1*) can be effectively used to characterize the endcapped oligonucleotides.

SYNTHESIS OF *N*-[3-*O*-(2-CYANOETHYL-*N*,*N*-DIISOPROPYLPHOSHORAMIDITE)PROPYL]-*N*′-[3-(4,4′-DIMETHOXYTRITYLOXY)PROPYL]-NAPHTHALENE-1,4,5,8-TETRACARBOXYLIC DIIMIDE

BASIC PROTOCOL 1

The sequence of reactions outlined in this protocol can be seen in Figure 5.6.1. The alkyl substituents of the naphthalene diimide central core are derived from 3-aminopropanol. This can be easily substituted by another aminoalcohol to provide an endcap that is either

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Current Protocols in Nucleic Acid Chemistry (2003) 5.6.1-5.6.15

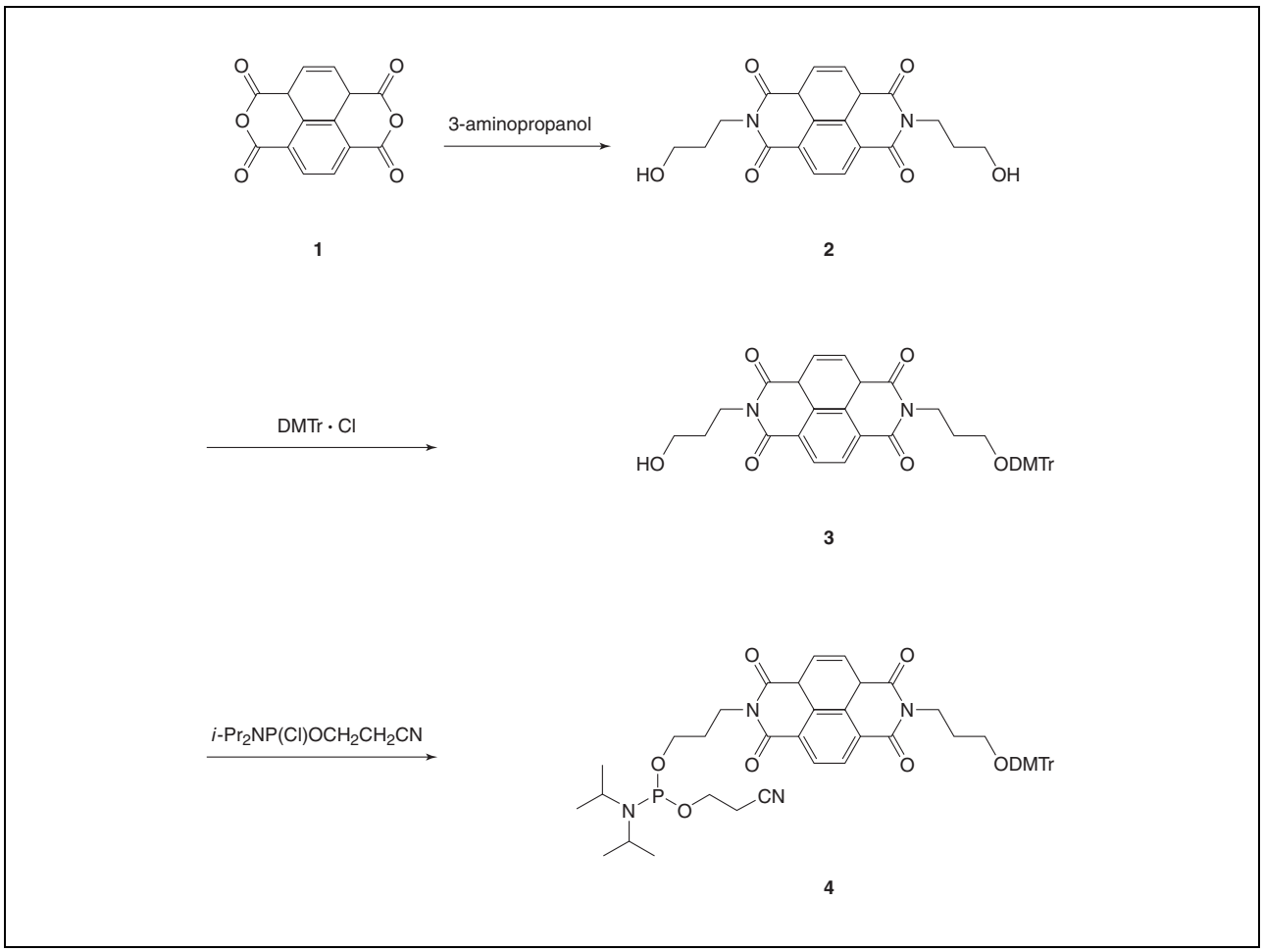

Figure 5.6.1 Synthetic scheme for the preparation of the phosphoramidite derivative of the naphthalene diimide endcap. Abbreviations: DMTr·Cl, 4,4′-dimethoxytrityl chloride; i-Pr$_2$NP(Cl)OCH$_2$CH$_2$CN, 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite.

shorter (2-aminoethanol) or longer (4-aminobutanol), but this will yield slightly less stable duplexes.

Materials

Napthalene-1,4,5,8-tetracarboxylic dianhydride (**S.1**; Figure 5.6.1)
3-Aminopropanol
2 M sodium carbonate
Chloroform, reagent grade
Activated charcoal
Methanol, reagent grade
Dichloromethane, reagent grade
Pyridine, anhydrous
4,4′-Dimethoxytrityl chloride (DMTr·Cl)
5% (w/v) sodium bicarbonate
Sodium sulfate, anhydrous
Silica gel (230 to 400 mesh, 60 Å, E Merck)
Triethylamine, reagent grade
Hexanes, reagent grade
Ethyl acetate, reagent grade
Dichloromethane, anhydrous
Diisopropylethylamine

2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite
Ethyl acetate, prewashed with 5% (w/v) sodium bicarbonate

25-, 50-, 100-, and 250-mL round-bottom flasks
Buchner funnel
Filtration flask
Whatman no. 1 filter paper
Vacuum oven
Water-cooled reflux condenser
Oil bath
Filter funnel, prewarmed (~50° to 60°C)
Rotary evaporator equipped with a water aspirator and vacuum pump
Nitrogen atmosphere (see inert atmosphere/vacuum manifold in *UNIT 1.1*, Fig. 1.1.3)
125-mL Erlenmeyer flasks
125-mL separatory funnels
Glass funnel
Glass wool
2 × 20–cm and 3 × 18–cm glass chromatography columns
25-mL pear-shaped flask
1-mL syringe and stainless steel needle

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

CAUTION: Exposure to pyridine and its vapors should be minimized. All reactions should be performed in a fume hood. The reactions for dimethoxytrityl protection and the phosphitylation of the dimethoxytrityl-protected endcap are sensitive to moisture and the glassware used for the reactions must be scrupulously dry.

NOTE: All listed reagents are available from Sigma-Aldrich.

Synthesize S.2

1. Suspend 2.14 g (8 mmol) naphthalene-1,4,5,8-tetracarboxylic dianhydride (**S.1**) in 150 mL water in a 250-mL round-bottom flask containing a stir bar.
2. Add 1.5 g (20 mmol) 3-aminopropanol drop-wise over 2 min while stirring the contents of the flask. Warm to 70°C and stir on a magnetic stirrer for 2 hr.
3. Cool the flask to room temperature and filter the solution using a Buchner funnel fitted with Whatman no. 1 filter paper attached to a filtration flask. Wash the residue on the filter paper with 50 mL of 2 M sodium carbonate and then with 200 mL of water. Allow the residue to dry on the filter paper.
4. Transfer the dry residue to a 100-mL round-bottom flask and dry overnight under vacuum (in a vacuum oven at <1 Torr and room temperature) to obtain a beige solid.
5. Dissolve the crude product in 25 mL chloroform and reflux the solution in the flask by attaching a water-cooled reflux condenser to the flask and heating the flask in an oil bath. When the solution begins refluxing (61°C), add 50 mg activated charcoal and reflux the solution for an additional 10 min.
6. Quickly filter the solution through a warm filter funnel (~50° to 60°C) fitted with Whatman no. 1 paper.
7. Remove the solvent under reduced pressure on a rotary evaporator equipped with a water aspirator to yield *N*,*N*′-bis(3-hydroxypropyl)-naphthalene-1,4,5,8-tetracarboxylic diimide (**S.2**) as an off-white powder.

8. Analyze the product by TLC (*APPENDIX 3D*) on silica gel, developing the plate with 10% (v/v) methanol in reagent-grade dichloromethane ($R_f = 0.4$).

Tritylate to give S.3

9. Coevaporate 382 mg (1 mmol) **S.2** three times with 20-mL portions of anhydrous pyridine in a 50-mL round-bottom flask using a rotary evaporator connected to a vacuum pump.
10. Dissolve the contents of the flask in 20 mL anhydrous pyridine and add 406 mg (1.2 mmol) DMTr·Cl while maintaining the flask under a dry nitrogen atmosphere. Stir the contents of the flask for 2 hr under nitrogen.
11. Remove the solvent on a rotary evaporator connected to a vacuum pump and dissolve the mixture of crude products in 50 mL reagent-grade dichloromethane.
12. Transfer the solution to a 125-mL separatory funnel and wash with 50 mL of 5% sodium bicarbonate solution and then with three 50-mL portions of water.
13. Transfer the dichloromethane solution to a 125-mL Erlenmeyer flask and add 1 g anhydrous sodium sulfate. Swirl the solution for a few minutes and allow to stand for 10 min.
14. Remove the drying agent by filtering the solution through a glass funnel with a glass wool plug.
15. Remove the solvent under reduced pressure using a rotary evaporator and water aspirator.
16. Purify the product (**S.3**) by flash chromatography (*APPENDIX 3E*) on a 3 × 18–cm column of silica gel neutralized by pretreatment with 1% (v/v) triethylamine in hexanes. Elute with hexanes and ethyl acetate, starting with 100% hexanes and increasing the amount of ethyl acetate to 70% in 10% increments every 100-mL fraction.

    Although chromatography of the DMTr derivative can be performed without using any triethylamine in the mobile phase, it is recommended that between 0.5% and 1% triethylamine be incorporated in the elution solvent. For more details on the purification of DMTr derivatives see UNIT 2.3.

17. Analyze fractions by TLC on silica gel, developing the plates with 50% (v/v) ethyl acetate in hexanes. Pool all fractions containing the product ($R_f = 0.3$) in a 250-mL round-bottom flask and remove the solvent under reduced pressure (see step 15).
18. Dry the purified *N*-(3-hydroxypropyl)-*N*′-[3-(4,4′-dimethoxytrityloxy)propyl]-naphthalene-1,4,5,8-tetracarboxylic diimide (**S.3**) overnight under vacuum (in a vacuum oven at <1 Torr and room temperature) to produce a yellow foam.

Synthesize phosphoramidite S.4

19. Dissolve 267 mg (0.379 mmol) **S.3** in 5 mL anhydrous dichloromethane in a 25-mL pear-shaped flask under a stream of dry nitrogen.
20. Add 234 µL (1.346 mmol) diisopropylethylamine. Then add 100 µL (0.448 mmol) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite drop-wise through a 1-mL syringe while gently swirling the flask.
21. Stir the reaction gently under nitrogen at room temperature for 1 hr.

22. Pour the contents of the flask into 20 mL ethyl acetate (previously washed with 5% sodium bicarbonate solution) in a 125-mL separatory funnel. Wash the ethyl acetate solution with three 20-mL portions of 5% sodium bicarbonate solution and then with three 10-mL portions of water.

23. Dry the ethyl acetate solution over anhydrous sodium sulfate (steps 13 and 14).

24. Remove the solvent under reduced pressure (step 15).

25. Purify the product (**S.4**) by flash chromatography on a 2 × 20–cm silica gel column pretreated with 1% (v/v) triethylamine in hexanes, eluting with 40% ethyl acetate and 1% triethylamine in hexanes. Collect the eluate in 10-mL fractions.

26. Analyze by TLC on silica gel, developing the plates with 40% ethyl acetate and 1% triethylamine in hexanes. Pool all fractions containing the product (R_f = 0.85) in a 250-mL round-bottom flask.

27. Remove the solvent under reduced pressure on a rotary evaporator connected to a water aspirator to produce *N*-[3-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-propyl]-*N*′-[3-(4,4′-dimethoxytrityloxy)propyl]-naphthalene-1,4,5,8-tetracarboxylic diimide (**S.4**) as an oily residue.

28. Dissolve the residue in 2 mL anhydrous dichloromethane and evaporate under vacuum on a rotary evaporator connected to a water aspirator. Repeat this step until a yellow foam is obtained and dry the foam overnight under vacuum using a vacuum pump at <1 Torr.

The phosphoramidite may be stored for short periods of time (<10 days) at 4°C under anhydrous conditions, but best results are obtained if it is incorporated into an oligonucleotide sequence within a few days of its preparation.

SYNTHESIS OF 5-[3-*O*-(2-CYANOETHYL-*N*,*N*-DIISOPROPYLPHOSPHORAMIDITE)PROPYL]-5″-[3-(4,4′-DIMETHOXYTRITYLOXY)PROPYL]-2,2′:5′,2″-TERTHIOPHENE

BASIC PROTOCOL 2

The scheme in Figure 5.6.2 outlines the synthesis of the phosphoramidite derivative of a terthiophene endcap.

Materials

2,2′:5′,2″-Terthiophene (**S.5**; Fig. 5.6.2)
Tetrahydrofuran (THF), anhydrous
Dry ice/acetone freezing bath
n-Butyllithium
Boron trifluoride diethyl etherate
Trimethylene oxide
Saturated sodium bicarbonate
Diethyl ether, reagent grade
Brine solution (saturated aqueous NaCl)
Sodium sulfate, anhydrous
Silica gel (230 to 400 mesh, 60 Å, E Merck)
Hexanes, reagent grade
Ethyl acetate, reagent grade
Pyridine, anhydrous
4,4′-Dimethoxytrityl chloride (DMTr·Cl)
Dichloromethane, anhydrous
5% (w/v) sodium bicarbonate

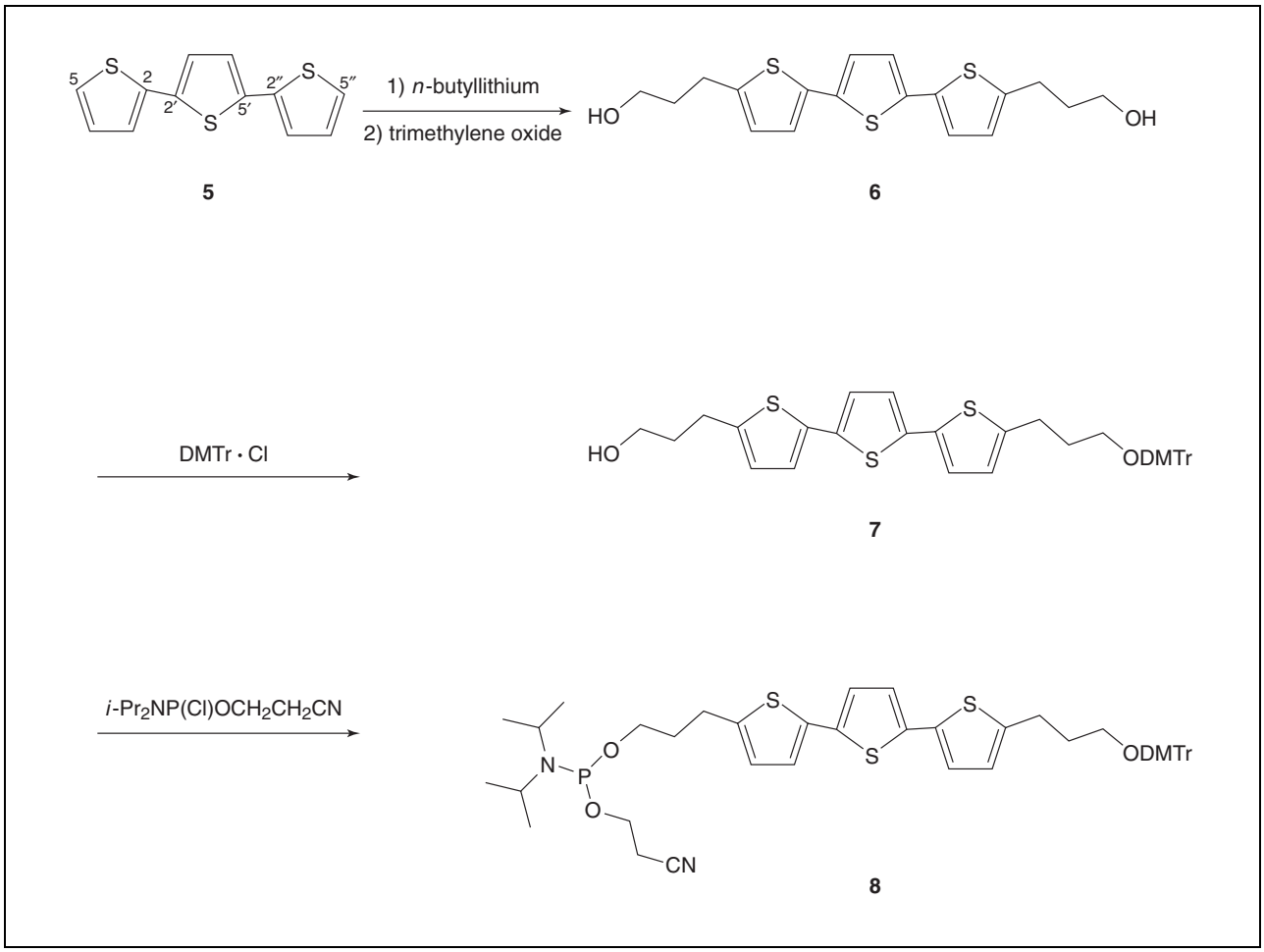

Figure 5.6.2 Synthetic scheme for the preparation of the phosphoramidite derivative of the terthiophene endcap. Abbreviations: DMTr·Cl, 4,4′-dimethoxytrityl chloride; *i*-$Pr_2NP(Cl)OCH_2CH_2CN$, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite.

Triethylamine
Diisopropylethylamine
2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite

25-mL round-bottom flasks
Nitrogen atmosphere (see inert atmosphere/vacuum manifold in *UNIT 1.1*, Fig 1.1.3)
1-mL syringe and stainless steel needle
125-mL separatory funnels
125-mL Erlenmeyer flasks
Glass funnel
Glass wool
Rotary evaporator with water aspirator and vacuum pump
2 × 20–cm and 4 × 20–cm glass chromatography columns

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

NOTE: All listed reagents are available from Sigma-Aldrich.

Synthesize S.6

1. Dissolve 333 mg (1.3 mmol) 2,2′:5′,2″-terthiophene (**S.5**) in 15 mL anhydrous THF in a 25-mL round-bottom flask containing a Teflon-coated 0.5-in. (1.3-cm) magnetic stir bar under a stream of nitrogen.
2. Cool the solution to −78°C by immersing the flask in a dry ice/acetone freezing bath.
3. Add 1.8 mL (1.3 mmol) *n*-butyllithium and warm the reaction to 0°C by transferring the flask to an ice bath.
4. Stir the reaction for 2 hr on a magnetic stirrer.
5. Add 384 µL (3 mmol) boron trifluoride diethyl etherate and then add 198 µL (3 mmol) trimethylene oxide with a 1-mL syringe.
6. Stir the reaction for 40 min on a magnetic stirrer.
7. Quench the reaction by addition of 10 mL saturated sodium bicarbonate solution and then allow the reaction to warm to room temperature.
8. Transfer the contents of the flask to a 125-mL separatory funnel and extract the aqueous layer with 30 mL diethyl ether.
9. Wash the ether layer with 25 mL brine solution.
10. Transfer the ether layer to a 125-mL Erlenmeyer flask and add 0.5 g anhydrous sodium sulfate.
11. Remove the drying reagent by filtering through a glass funnel fitted with a glass wool plug.
12. Remove the solvent under reduced pressure using a rotary evaporator and water aspirator.
13. Purify the product (**S.6**) by flash chromatography (*APPENDIX 3E*) on a 4 × 20–cm silica gel column, eluting first with hexanes, then with 35% (v/v) ethyl acetate in hexanes, and finally with 65% ethyl acetate in hexanes.
14. Analyze the fractions by TLC (*APPENDIX 3D*) on silica gel, developing the plates in 50% (v/v) ethyl acetate in hexanes. Combine all fractions containing the product (R_f = 0.13) and remove the solvent under reduced pressure (step 12).
15. Dry 5,5″-bis(3-hydroxypropyl)-2,2′:5′,2″-terthiophene (**S.6**) overnight under vacuum using a rotary evaporator with a water aspirator.

Tritylate to give S.7

16. Coevaporate 138 mg (0.38 mmol) **S.6** with 10 mL anhydrous pyridine in a 25-mL round-bottom flask using a rotary evaporator connected to a vacuum pump.
17. Dissolve the contents of the flask in 5 mL anhydrous pyridine and add 167 mg (0.49 mmol) DMTr·Cl while maintaining the flask under a dry nitrogen atmosphere. Stir the contents of the flask for 3 hr under nitrogen.
18. Remove the solvent on a rotary evaporator connected to a vacuum pump and dissolve the mixture of crude products in 25 mL anhydrous dichloromethane.
19. Transfer the solution to a 125-mL separatory funnel and wash with 25 mL of 5% sodium bicarbonate solution followed by three 20-mL portions of water.
20. Dry the solution with anhydrous sodium sulfate (steps 10 and 11).

21. Remove the solvent under reduced pressure (step 12).

22. Purify the product (**S.7**) by flash chromatography on a 2 × 20–cm silica gel column, neutralized by pretreatment with 1% (v/v) triethylamine in hexanes. Elute with 70% (v/v) ethyl acetate in hexanes.

    Although chromatography of the DMTr derivative can be performed without using any triethylamine in the mobile phase, it is recommended that between 0.5% and 1% triethylamine be incorporated in the elution solvent. For more details on the purification of DMTr derivatives see UNIT 2.3.

23. Analyze fractions by TLC using 50% (v/v) ethyl acetate in hexanes and combine all fractions containing the product (R_f = 0.31). Remove the solvent under reduced pressure (step 12).

24. Dissolve the residue in 2 mL anhydrous dichloromethane and evaporate under vacuum on a rotary evaporator connected to a water aspirator. Repeat this step until the product 5-(3-hydroxypropyl)-5″-[3-(4,4′-dimethoxytrityloxy)propyl]-2,2′:5′,2″-terthiophene (**S.7**) solidifies into a foam.

Synthesize phosphoramidite S.8

25. Dissolve 83 mg (0.125 mmol) **S.7** in 5 mL anhydrous dichloromethane in a 25-mL round-bottom flask under a stream of nitrogen.

26. Add 78 µL (0.446 mmol) diisopropylethylamine to the flask and then add 50 µL (0.225 mmol) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite drop-wise through a 1-mL syringe while gently swirling the flask.

27. Stir the reaction gently under nitrogen for 1 hr.

28. Pour the contents of the flask into 20 mL ethyl acetate in a 125-mL separatory funnel. Wash the ethyl acetate solution with three 20-mL portions of 5% sodium bicarbonate solution followed by three 10-mL portions of water.

29. Dry the ethyl acetate solution over anhydrous sodium sulfate (see steps 10 and 11).

30. Remove the solvent under reduced pressure (step 12).

31. Purify the product **S.8** by flash chromatography on a 2 × 20–cm silica gel column, eluting with 75% (v/v) ethyl acetate in hexanes.

32. Analyze by TLC using 25% ethyl acetate and 1% triethylamine in hexanes and combine all fractions containing the product (R_f = 0.5). Remove the solvent under reduced pressure (step 12).

33. Dissolve the residue in 2 mL anhydrous dichloromethane and evaporate under vacuum on a rotary evaporator connected to a water aspirator. Repeat this step until the product 5-[3-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)propyl]-5″-[3-(4,4′-dimethoxytrityloxy)propyl]-2,2′:5′,2″-terthiophene (**S.8**) solidifies into a foam.

    The phosphoramidite may be stored for short periods of time (<10 days) at 4°C under anhydrous conditions, but best results are obtained if it is incorporated into an oligonucleotide sequence within a few days of its preparation.

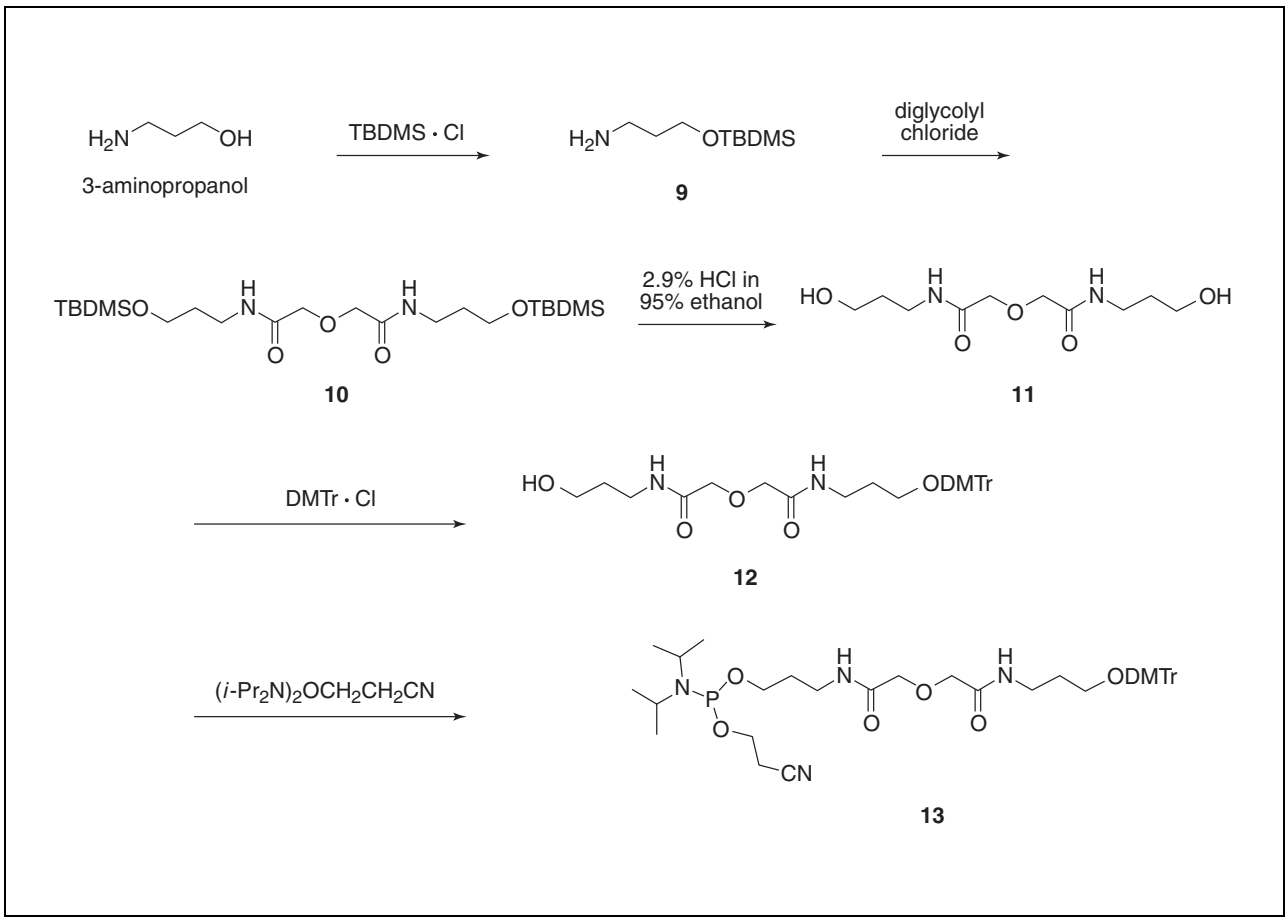

Figure 5.6.3 Synthetic scheme for the preparation of the phosphoramidite derivative of the 2,2′-oxydiacetamide endcap. Abbreviations: DMTr·Cl, 4,4′-dimethoxytrityl chloride; $(i\text{-Pr}_2\text{N})_2\text{OCH}_2\text{CH}_2\text{CN}$, 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite; TBDMS·Cl, *tert*-butyldimethylsilyl chloride.

SYNTHESIS OF *N*-[3-*O*-(2-CYANOETHYL-*N*,*N*-DIISOPROPYLPHOSPHORAMIDITE)PROPYL]-*N*′-[3-(4,4′-DIMETHOXYTRITYLOXY)PROPYL]-2,2′-OXYDIACETAMIDE

BASIC PROTOCOL 3

The sequence of reactions in this protocol describes the synthesis of the phosphoramidite derivative of an aliphatic hydrophilic endcap. The synthetic scheme is shown in Figure 5.6.3. All intermediate compounds up to the trityl-protected endcap are stable for storage purposes and may be prepared in larger quantities. It is advisable to prepare the final phosphoramidite derivative in the required quantity only when needed for oligonucleotide synthesis.

Materials

3-Aminopropanol
Dichloromethane, anhydrous
Triethylamine (TEA), anhydrous (preferably freshly distilled)
tert-Butyldimethylsilyl chloride (TBDMS·Cl)
4,4-Dimethylaminopyridine
Brine solution (saturated NaCl)
Sodium sulfate, anhydrous
Diglycolyl chloride
5% (v/v) acetic acid
5% (w/v) sodium bicarbonate

Concentrated HCl
95% (v/v) ethanol
Methanol, reagent grade
Hexanes, reagent grade
Pyridine, anhydrous (preferably freshly distilled)
4,4′-Dimethoxytrityl chloride (DMTr·Cl)
Silica gel (230 to 400 mesh, 60 Å, E Merck)
Dichloromethane, reagent grade
1% (w/v) sodium hydroxide (optional)
Acetonitrile, anhydrous
2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite
1*H*-Tetrazole

25-, 50-, 100- and 250-mL round-bottom flasks
Nitrogen atmosphere (see inert atmosphere/vacuum manifold in *UNIT 1.1*, Fig. 1.1.3)
1-mL syringe and stainless steel needles
125- and 250-mL separatory funnels
125- and 250-mL Erlenmeyer flasks
Glass funnels
Glass wool
Rotary evaporator with a water aspirator and vacuum pump
2 × 20–cm and 4 × 25–cm glass chromatography columns

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

NOTE: All listed reagents are available from Sigma-Aldrich.

Silylate 3-aminopropanol to give S.9

1. Dissolve 7 g (1 eq, 0.09 mol) 3-aminopropanol in 90 mL anhydrous dichloromethane in a dry 250-mL round-bottom flask containing a magnetic stir bar, under a stream of nitrogen.
2. Cool the flask in an ice bath and add 14.37 ml (1.1 eq, 0.1 mol) anhydrous triethylamine, 15.93 g (1.1 eq, 0.1 mol) TBDMS·Cl, and 0.5 g 4,4-dimethylaminopyridine to the flask. Stir the mixture overnight under nitrogen.

   Addition of TBDMS·Cl results in a vigorous reaction that generates heat and results in the formation of a white precipitate. It is important to cool the reaction flask in an ice bath. The contents of the reaction flask will slowly return to room temperature when the ice in the bath has melted, and the reaction will proceed at room temperature for the duration.
3. Add 50 mL water to the reaction mixture and stir vigorously for 10 min to dissolve the white precipitate.
4. Transfer the contents of the flask to a 250-mL separatory funnel and discard the aqueous layer. Wash the organic fraction with three 30-mL fractions of water followed by three 30-mL fractions of brine solution.
5. Transfer the dichloromethane solution to a 250-mL Erlenmeyer flask and add 1 g anhydrous sodium sulfate. Swirl the solution for a few minutes and then allow to stand for 10 min.
6. Remove the drying agent by filtering the solution through a glass funnel with a glass wool plug.
7. Remove the solvent under reduced pressure using a rotary evaporator and water aspirator at room temperature to obtain the product 3-*tert*-butyldimethylsilyloxypropylamine (**S.9**) as a colorless oil.

Synthesize diacetamide S.10

8. Dissolve 8.17 g (2 eq, 0.04 mol) **S.9** in 30 mL anhydrous dichloromethane in a 100-mL round-bottom flask containing a dry stir bar. Cool the flask in an ice bath.

9. Add 7.6 mL (3 eq, 0.05 mol) anhydrous triethylamine to the flask and then add 2.26 mL (1 eq, 0.018 mol) diglycolyl chloride drop-wise while stirring the reaction mixture on a magnetic stirrer.

   The reaction mixture will turn brown on addition of the diglycolyl chloride.

10. After addition of diglycolyl chloride is complete, remove the flask from the ice bath and stir the contents overnight at room temperature.

11. Add 40 mL water to the reaction mixture and stir vigorously for 10 min.

12. Transfer to a 125-mL separatory funnel and discard the aqueous layer. Wash the organic layer with two 30-mL portions of 5% acetic acid solution, then with two 30-mL portions of 5% sodium bicarbonate solution, and finally with two 60-mL portions of brine solution.

13. Transfer the dichloromethane solution to a 125-mL Erlenmeyer flask containing 0.5 g anhydrous sodium sulfate, swirl the solution for a few minutes, and allow the solution to stand for 10 min.

14. Remove the drying agent by filtering the solution through a glass funnel with a glass wool plug.

15. Remove the dichloromethane under reduced pressure using a rotary evaporator and water aspirator at room temperature to obtain the product *N*,*N*′-bis(3-*tert*-butyldimethylsilyloxypropyl)-2,2′-oxydiacetamide (**S.10**) as a brown oil. Dry the oil overnight in a 25-mL round-bottom flask under high vacuum (in a vacuum pump at <1 Torr).

Desilylate to give S.11

16. Prepare a 2.9% (w/w) solution of concentrated HCl in 95% ethanol.

17. Dissolve 7.63 g (0.016 mol) **S.10** in 15 mL of 95% ethanol in a 50-mL round-bottom flask containing a stir bar.

18. Add 15 mL of the HCl solution to the flask and stir the contents of the flask for 20 min on a magnetic stirrer.

19. Remove the solvent under reduced pressure using a rotary evaporator and water aspirator at room temperature to produce a brown oil.

20. Dissolve the brown oil in 15 mL methanol and transfer to a 125-mL separatory funnel. Wash the methanol layer with three 20-mL portions of hexanes.

21. Remove the solvent under reduced pressure using a rotary evaporator and water aspirator at room temperature to obtain the product *N*,*N*′-bis(3-hydroxypropyl)-2,2′-oxydiacetamide (**S.11**) as a brown oil.

Tritylate to give S.12

22. Coevaporate 7.63 g (0.016 mol) **S.11** three times with 20-mL portions of anhydrous pyridine in a 100-mL round-bottom flask using a rotary evaporator connected to a vacuum pump. Dissolve the contents of the flask in 30 mL anhydrous pyridine.

23. Maintain the solution under a nitrogen atmosphere and add 5.707 g (0.016 mol) DMTr·Cl. Stir the contents of the flask overnight under a nitrogen atmosphere.

24. Remove the solvent under reduced pressure using a rotary evaporator connected to a vacuum pump and purify the compound **S.12** by flash chromatography (*APPENDIX 3E*) on a 4 × 25–cm silica gel column, eluting with 0% to 1% (v/v) methanol in dichloromethane containing 1% (v/v) triethylamine. Collect the effluent in 10-mL fractions.

25. Analyze by TLC (*APPENDIX 3E*) on silica gel, developing with 1% (v/v) methanol and 1% (v/v) triethylamine in dichloromethane. Combine the fractions that contain the desired product (R_f = 0.27) and evaporate the solvent under reduced pressure on a rotary evaporator connected to a water aspirator to obtain the product as a foam.

26. *Optional:* If the product does not foam, dissolve the oily residue in 50 mL dichloromethane and transfer to a 125-mL separatory funnel. Wash the dichloromethane solution with 25 mL of 1% sodium hydroxide. Dry the dichloromethane solution over anhydrous sodium sulfate and remove the solvent under reduced pressure (steps 13 to 15).

27. Dry the product *N*-(3-hydroxypropyl)-*N*′-[3-(4,4′-dimethoxytrityloxy)propyl]-2,2′-oxydiacetamide (**S.12**) overnight in vacuo (in a vacuum pump at <1 Torr) to obtain a light brown foam.

Synthesize phosphoramidite S.13

28. Coevaporate 123.5 mg (0.224 mmol) **S.12** with anhydrous acetonitrile and then with anhydrous dichloromethane in a 25-mL round-bottom flask on a rotary evaporator connected to a water aspirator. Dry the material overnight under vacuum (in a vacuum pump at <1 Torr).

29. Dissolve the contents of the flask in 2 mL anhydrous dichloromethane and then add 0.078 mL (0.234 mmol) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (using a 1-mL syringe) and 15.9 mg (0.224 mmol) 1*H*-tetrazole over 15 min while maintaining the flask under nitrogen. Stir the contents of the flask under nitrogen for 2 hr.

30. Analyze the reaction by TLC on silica gel, developing the plate with 5:4:1 (v/v/v) hexanes/dichloromethane/triethylamine.

31. Purify the product *N*-[3-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-propyl]-*N*′-[3-(4,4′ -dimethoxytrityloxy)propyl]-2,2′-oxydiacetamide (**S.13**) on a 2 × 20–cm column of silica gel, eluting with 5:4:1 hexanes/dichloromethane/triethylamine.

32. Combine the fractions containing the product (R_f = 0.2) and remove the solvent under reduced pressure on a rotary evaporator connected to a water aspirator. Dry the product overnight under vacuum by connecting the flask to a vacuum pump (<1 Torr).

The phosphoramidite may be stored for short periods of (<10 days) at 4°C under anhydrous conditions, but best results are obtained if it is incorporated into an oligonucleotide sequence within a few days of its preparation.

COMMENTARY

Background Information

Oligonucleotides bearing specific cross-links generally display greater stability than their uncross-linked counterparts. This can be most easily observed in their melting behavior, with cross-linked oligonucleotides having higher melting temperature (T_m) values. The authors' goal was to develop both hydrophobic and hydrophilic endcaps that not only stabilize very short oligonucleotide duplexes (e.g., 4 bp) but also offer a variety of environments that may be appropriate for different applications.

Hydrophobic endcaps such as stilbene diether and stilbene dicarboxamide (Letsinger

and Wu, 1995; Lewis et al., 1995) have been used to both stabilize DNA hairpins as well as study photo-induced charge separation and charge recombination (Lewis et al., 2000) and charge transfer (Lewis et al., 1997, 2001). Endcapped oligonucleotides are capable of specific protein binding as demonstrated by a stilbene dicarboxamide–endcapped oligonucleotide that mimics the REV responsive element and binds with equal affinity to the REV protein of HIV-1 (Nelson et al., 1996). Stilbene diether–endcapped duplexes have been shown to crystallize in a pinwheel arrangement with four oligonucleotide duplexes per asymmetric subunit (Lewis et al., 1999, 2000). Naphthalene and perylene diimide endcaps have been used to stabilize both duplex and triplex oligonucleotides (Bevers et al., 1998, 2000). An azobenzene endcap has also been used to stabilize short duplexes (Yamana et al., 1996, 1998). Applications of aliphatic hydrophilic endcaps are discussed in *UNIT 5.3.*

Table 5.6.1 shows the melting temperature values for hairpin sequences with 4-bp stems and 4-nt loops. Replacement of the nucleotide loop by an endcap gives the results shown in Table 5.6.2. Both aromatic hydrophobic endcaps provide an overall increase in T_m values over the aliphatic endcaps. The more lipophilic terthiophene endcap provides the highest stability when it is stacked over an AT base pair, whereas the naphthalene diimide endcap shows the greatest enhancement in melting temperature when stacked over a GC base pair. In both cases, the endcapped duplexes show significantly higher melting temperatures than their hairpin counterparts.

The hydrophilic 2,2′-oxydiacetamide linker displays lower T_m values than either of the aromatic endcaps or the hexa(ethylene glycol) linker. However, the 2,2′-oxydiacetamide linker may offer some advantage when it is important to have a very hydrophilic moiety of minimal size with greater rigidity than the hexa(ethylene glycol) linker.

The length spanned by these endcaps can be tuned by changing the chain length of the hydroxyalkyl portion of the endcaps. However, the authors have found that the length provided by the aminopropyl unit is optimal for obtaining the highest T_m values.

Compound Characterization

*N,N′-Bis(3-hydroxypropyl)-naphthalene-1,4,5,8-tetracarboxylic diimide (**S.2**).* ^{1}H NMR (250 MHz, $CDCl_3$, δ): 8.8 (s, 4H, ar), 4.38 (t, J = 6.3 Hz, 3H, CH_2), 3.65 (t, J = 5.7 Hz, 3H, CH_2), 2.02 (m, 3H, CH_2). ^{13}C NMR (250 MHz, DMSO, δ): 162.0 130.7 129.4 125.6 59.9 38.0 30.7. MS: (+ve ESI) (M+H) 383, (–ve ESI) (M) 382. HRMS (FAB): calc. 383.1243; actual 383.1249.

Table 5.6.1 Melting Temperatures for 4-bp Stem Hairpin Oligonucleotides

Sequence[a]	T_m
ATGCTTTTGCAT	58.4°C
ATGCAAAAGCAT	62.0°C
GCTATTTTTAGC	38.5°C
GCTAAAAATAGC	50.9°C

[a]The underlined nucleotides form the loop of the hairpin oligonucleotide.

Table 5.6.2 Melting Temperatures for Endcapped Oligonucleotides

Sequence[a]	Type of endcap	T_m
ATGC-X-GCAT	Hexa(ethylene glycol)	61.4°C
ATGC-X-GCAT	Naphthalene diimide	74.6°C
GCTA-X-TAGC	Naphthalene diimide	61.6°C
ATGC-X-GCAT	Terthiophene	62.3°C
GCTA-X-TAGC	Terthiophene	65.8°C
ATGC-X-GCAT	2,2′-Oxydiacetamide	51°C
GCTA-X-TAGC	2,2′-Oxydiacetamide	41.7°C

[a]X refers to the location of the endcap.

*N-(3-Hydroxypropyl)-N′-[3-(4,4′-dimethoxytrityloxy)propyl]-naphthalene-1,4,5,8-tetracarboxylic diimide (**S.3**).* ^{1}H NMR (250 MHz, CD_2Cl_2, δ): 8.69 (s, 4H. naphthalene), 7.43 – 7.18 (m, 9H, DMTr), 6.8 – 7.9 (m, 4H, DMTr), 4.2 (m, 4H, CH_2), 3.74 (s, 6H, OCH_3), 3.68 (m, 2H, CH_2), 3.09 (t, J = 6.1 Hz, 2H, CH_2), 2.1 (m, 4H, CH_2). HRMS (FAB): calc. 684.2471; actual 684.2464.

*N-[3-O-(2-Cyanoethyl-N,N-diisopropylphosphoramidite)propyl]-N′-[3-(4,4′-dimethoxytrityloxypropyl]-naphthalene-1,4,5,8-tetracarboxylic diimide (**S.4**).* ^{31}P NMR: 147.8 (referenced to external 85% H_3PO_4).

*5,5″-Bis(3-hydroxypropyl)-2,2′:5′,2″-terthiophene (**S.6**).* ^{1}H NMR (250 MHz, $CDCl_3$, δ): 6.90 (d, 4H), 6.70 (d, 2H), 3.75 (t, 4 H), 2.90 (t, 4H), 1.95 (m, 4H).

*5-(3-Hydroxypropyl)-5″-[3-(4,4′-dimethoxytrityloxy)propyl]-2,2′:5′,2″-terthiophene (**S.7**).* ^{1}H NMR (250 MHz, $CDCl_3$, δ): 6.6-7.5 (m, 19H), 3.8 (s, 6H), 3.7 (t, 2 H), 3.2 (t, 2H), 2.9 (m, 4H), 1.95 (m, 4H).

*5-[3-O-(2-Cyanoethyl-N,N-diisopropylphosphoramidite)propyl]-5″-[3-(4,4′-dimethoxytrityloxy)propyl]-2,2′:5′,2″-terthiophene (**S.8**).* ^{1}H NMR: 6.6-7.2 (m, 19H), 2.6-3.9 (1s, 1m, 12H), 3.2 (t, 2H), 2.9 (m, 4H), 2.7 (t, 2H), 2.5 (m, 2H), 2.0 (m, 4H), 1.2 (d, 12H), 1.0 (t, 4H). ^{31}P NMR: 170.

*3-tert-Butyldimethylsilyloxypropylamine (**S.9**).* ^{1}H NMR (250 MHz, $CDCl_3$, δ): –0.010 (s, 6H), 0.831 (s, 9H), 1.595 (p, 2H), 2.737 (t, 2H), 3.637 (t, 2H). ^{13}C NMR (300 MHz, $CDCl_3$, δ): -5.4173, 18.2349, 25.8746, 36.2497, 39.3576, 61.1958. HRMS (ESI, +): calc. 190.1627; actual 190.1630.

*N,N′-Bis(3-tert-butyldimethylsilyloxypropyl)-2,2′-oxydiacetamide (**S.10**).* ^{1}H NMR (250 MHz, $CDCl_3$, δ): 2.019 (s, 10H), 2.854 (s, 18H), 3.709 (p, 4H), 5.380 (q, 4H), 5.686 (t, 4H), 5.976 (s, 4H). ^{13}C NMR (300 MHz, $CDCl_3$, δ): –5.476, 18.244, 25.848, 31.676, 37.352, 61.907, 71.270. HRMS (ESI, +): calc. 476.3102; actual 476.3091.

*N,N′-Bis(3-hydroxypropyl)-2,2′-oxydiacetamide (**S.11**).* ^{1}H NMR (250 MHz, CD_3OD, δ): 744 (q, 4H), 3.37 (t, 4H), 3.60 (t, 4H), 4.039 (s, 4H). ^{13}C NMR (250 MHz, CD_3OD, δ): 33.098, 37.290, 60.547, 71.409, 171.729. HRMS (ESI, +): calc. 249.1450; actual 249.1446.

*N-(3-Hydroxypropyl)-N′-[3-(4,4′-dimethoxytrityloxy)propyl]-2,2′-oxydiacetamide (**S.12**).* ^{1}H NMR (250 MHz, CD_2Cl_2, δ): 1.602 (p, 2H), 1.805 (p, 2H), 3.164 (t, 2H), 3.283-3.429 (m, 4H), 3.543 (t, 2H), 3.779 (s, 6H), 3.914, 3.943 (d, 4H), 6.817-7.365 (m, 13H).

*N-[3-O-(2-Cyanoethyl-N,N-diisopropylphosphoramidite)propyl]-N′-[3-(4,4′-dimethoxytrityloxy)propyl]-2,2′-oxydiacetamide (**S.13**).* ^{31}P NMR: 148 (referenced to external 85% H_3PO_4).

Critical Parameters

Anyone with a moderate amount of experience in chemical synthesis should not find any difficulties in the synthesis of any of these endcaps. The reactions used to generate the DMTr and phosphoramidite derivatives are extremely sensitive to moisture and must be performed under anhydrous conditions. Additionally, the DMTr group is acid labile, and thus care must be taken to avoid any exposure to acid. As a precaution, small amounts of triethylamine can be used during chromatography.

The tritylation reaction in each protocol yields a mixture of unreacted, monotritylated, and detritylated products. The ratio of reactants was chosen to maximize the yield of the monotritylated product. The reactant (untritylated), monotritylated, and ditritylated products can be distinguished by TLC. The ditrylated product runs at higher R_f, while the reactant migrates more slowly than the monotritylated products.

The phosphoramidite derivative also requires careful handling, and exposure to air or acids must be avoided. It is preferable to synthesize the phosphoramidite derivative of the required endcap just before oligonucleotide synthesis.

Anticipated Results

Yields of endcapped oligonucleotides should generally be >90% using standard automated synthesis procedures (see, e.g., *APPENDIX 3C*). The coupling time for the endcap phosphoramidite, however, should be increased to 15 min.

Time Considerations

The phosphoramidite derivatives of the naphthalene diimide and terthiophene hydrophobic endcaps can each be prepared in 3 days including the time required for purification. The hydrophilic oxydiacetamide endcap synthesis requires significantly longer times and can be completed in 7 to 8 days.

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