Synthesis of Glycerol Nucleic Acid (GNA) Phosphoramidite Monomers and Oligonucleotide Polymers

Su Zhang¹,² and John C. Chaput¹,²

¹Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona
²The Biodesign Institute at Arizona State University, Tempe, Arizona

ABSTRACT

This unit describes a straightforward method for preparing glycerol nucleic acid (GNA) phosphoramidite monomers and oligonucleotide polymers using standard cyanoethyl phosphoramidite chemistry. GNA is an unnatural nucleic acid analog composed of an acyclic three-carbon sugar-phosphate backbone that contains one stereogenic center per repeating unit. GNA has attracted significant attention as a nucleic acid derivative due to its unique ability to form stable Watson-Crick anti-parallel duplex structures with thermal and thermodynamic stabilities rivaling those of natural DNA and RNA. The chemical simplicity of this nucleic acid structure provides access to enantiomerically pure forms of right- and left-handed helical structures that can be used as unnatural building blocks in DNA nanotechnology. Curr. Protoc. Nucleic Acid Chem. 42:4.40.1-4.40.18. © 2010 by John Wiley & Sons, Inc.

Keywords: glycerol nucleic acid (GNA) • phosphoramidite • oligonucleotide • chemical synthesis • solid-phase synthesis • thermal stability • nanotechnology

INTRODUCTION

Acyclic oligonucleotides are experiencing a tremendous resurgence in basic and applied research due to their unique structural and biophysical properties (for a review, see Zhang et al., 2010). This unit contains procedures that describe the chemical synthesis of one type of acyclic nucleic acid polymer commonly referred to as glycerol nucleic acid or GNA. The chemical synthesis and purification of glycerol nucleoside analogs bearing adenine (A), cytosine (C), guanine (G), and thymine (T) as the bases, and of oligonucleotides thereof (Fig. 4.40.1), are described in detail. Unless otherwise stated, all of the procedures start from \((R, +)-\)glycidol and yield \((S, -)\)-GNA. The same chemistry can also be applied to \((S, -)-\)glycidol to produce \((R, +)\)-GNA. Commercially available glycidol or tritylated glycidol is used to obtain the 2,3-dihydroxypropyl derivatives of each nucleobase via an epoxide ring opening reaction (see Basic Protocol 1). The glycerol nucleosides are then converted to their corresponding nucleotide phosphoramidites (see Basic Protocols 2 to 5), which can then be used as building blocks to synthesize GNA oligonucleotides (see Basic Protocol 6).

NOTE: For phosphoramidite synthesis (see Basic Protocols 2 to 5), the reaction progress is monitored by TLC and should be stopped as soon as the starting material is consumed.

SYNTHESIS OF ENANTIOMERICALLY PURE DIMETHOXYTRITYL-\(O-(S)\)-GLYCIDOL

2,3-Dihydroxypropyl derivatives of nucleobases have been synthesized by several different methods. The current approach is based on a modified version of Acevedo’s procedure (Acevedo and Andrews, 1996), and involves a direct ring opening of the stable glycidol intermediate by nucleophilic attack of one of the four natural nucleobases. In this
Figure 4.40.1 Chemical structures of GNA, DNA, and RNA.

![Chemical structures of GNA, DNA, and RNA.](image)

Figure 4.40.2 Preparation of (S)-DMT-O-glycidol (S.2). Abbreviations: DMT-Cl, 4,4′-dimethoxytritylchloride; Et₃N, triethylamine; DCM, dichloromethane.

protocol, pure (R)-(+-)glycidol is tritylated using 4,4′-dimethoxytritylchloride (DMT-Cl) in dichloromethane as illustrated in Figure 4.40.2. The tritylated glycidol is used to make the A, C, and T glycerol nucleoside phosphoramidites (see Basic Protocols 2, 3, and 5, respectively). Synthesis of the G glycerol nucleoside phosphoramidite requires an alternative strategy (see Basic Protocol 4). (R)-(+-)Glycidol substrates provide (S)-GNA phosphoramidite monomers that can be used to make GNA oligonucleotides with the natural right-handed stereoconfiguration.

**Materials**

- (R)-(+-)Glycidol
- Dichloromethane (DCM)
- Triethylamine (Et₃N), 99.5%
- 4,4′-Dimethoxytritylchloride (DMT-Cl), 95%
- Argon source
- Saturated aqueous sodium bicarbonate solution (sat. aq. NaHCO₃)
- Brine (sat. aq. NaCl)
- Sodium sulfate (Na₂SO₄)
- Silica gel (60 Å, 230 to 400 mesh; Whatman)
- Hexanes
- Ethyl acetate (AcOEt)
- 100-mL round-bottomed flasks
- Magnetic stir plate and stir bar
- Büchner funnels
- 200-mL separatory funnels
- Filter paper
- Gas balloon
1. To a 100-mL round-bottomed flask equipped with a magnetic stir bar, add:

- 780 mg (10.6 mmol) of (R)-(+)-glycidol
- 24 mL of DCM (freshly distilled over calcium hydride)
- 3.8 mL of Et₃N
- 4.54 g (13.4 mmol) of DMT-Cl.

2. Stir the reaction overnight at room temperature under an argon atmosphere.

3. Remove the precipitate by vacuum filtration and wash the residue with DCM.

4. Wash the filtrate sequentially with 50 mL each of sat. aq. NaHCO₃, water, and brine.

5. Dry the organic layer over Na₂SO₄ 10 min and filter.

6. Evaporate to dryness using a rotary evaporator equipped with a vacuum pump and cooling trap.

7. Purify the oily residue by column chromatography (APPENDIX 3E) on 20 g of silica gel in a 5 × 25–cm column. Deactivate the column with 97:3 (v/v) hexanes/Et₃N and elute the column using a step-wise gradient of 99:1 (v/v) hexanes/Et₃N to 18:1:1 (v/v/v) hexanes/AcOEt/Et₃N.

8. Determine products by TLC. Combine the product fractions and evaporate to dryness.

The resulting product, DMT-O-(S)-glycidol (S.2), should be obtained in a 90% yield (3.56 g, 9.47 mmol) as colorless oil. TLC (hexanes/AcOEt 10:1): Rf = 0.12. ¹H NMR (300 MHz, CDCl₃): δ = 2.61 (dd, J = 2.4, 5.1 Hz, 1 H), 2.76 (m, 1 H), 3.12 (m, 2 H), 3.29 (m, 1 H), 3.79 (s, 6 H), 6.83 (m, 4 H), 7.05-7.40 (m, 7 H), 7.45 (m, 2 H).

**SYNTHESIS OF 2′-O-(2-CYANOETHOXY)(DIISOPROPYLAMINO)-PHOSPHINO-3′-O-(4,4′-DIMETHOXYTRIPHENYL)METHYL-N⁶-BENZOYL-(S)-9-(2,3-DIHYDROXYPROPYL)ADENINE**

This protocol describes the synthesis of N⁶-benzyol-protected (S)-GNA adenosine nucleoside phosphoramidite S.5 from S.2 (Fig. 4.40.3).

**Materials**

- Adenine
- 60% sodium hydride in mineral oil (NaH)
- Dimethylformamide (DMF), anhydrous
- Argon source
- Benzoyl chloride (BzCl)
- DMT-O-(S)-glycidol (S.2; Basic Protocol 1)
- Ethyl acetate (AcOEt)
- Silica gel (60 A, 230 to 400 mesh)
- Dichloromethane (DCM)
- Triethylamine (Et₃N)
- Methanol (MeOH)
- Pyridine, anhydrous
Trimethylsilyl chloride (TMS-Cl)
Ammonium hydroxide (concentrated NH₄OH)
Hexanes
Diisopropylethylamine, redistilled (DIPEA)
Chloro(2-cyanoethoxy)-(diisopropylamino)phosphine
50- and 100-mL round-bottomed flasks
Magnetic stir plate and stir bar
Graham condenser
Büchner funnel
Filter paper
Rotary evaporator equipped with a vacuum pump
6.4 × 45–cm and 1.3 × 30–cm chromatography columns
TLC plate, EMD silica gel 60 F₂₅₄
254-nm UV lamp
Additional reagents and equipment for TLC (APPENDIX 3D) and column chromatography (APPENDIX 3E)

Prepare S.3
1. Add 0.71 g (5.25 mmol) of adenine and 43 mg (1.07 mmol) of 60% NaH in mineral oil in 8 mL of DMF to a 50-mL round-bottomed flask.
2. Stir reaction 2 hr at room temperature under an argon atmosphere.
3. Dissolve 1.86 g (4.95 mmol) of S.2 in 13.5 mL of DMF in a 50-mL round-bottomed flask.
4. Add the solution from step 3 into the 50-mL round-bottomed flask from step 1, attach a condenser to the flask, and stir the reaction mixture 15 hr at 105°C under an argon atmosphere.
5. Allow the solution to cool to room temperature and remove the precipitate by vacuum filtration. Wash the residue with 50 mL AcOEt.

6. Evaporate the organic solvent from step 5 to dryness using a rotary evaporator equipped with a vacuum pump and a cooling trap.

7. Purify the residue by column chromatography (APPENDIX 3E) on 50 g of silica gel in a 6.4 × 45–cm column. Deactivate the column with 97:3 DCM/Et₃N and elute the product using a step-wise gradient of 35:1:0.01 to 25:1:0.01 (v/v) DCM/MeOH/Et₃N.

8. Collect fractions containing the product, determined by TLC, and evaporate to dryness.

   The resulting product, 3′-O-(4,4′-dimethoxytriphenyl)methyl-(S)-9-(2,3-dihydroxypropyl) adenine (S.3), should be obtained in a 48% yield (1.21 g, 2.38 mmol) as a colorless foam.

   TLC (AcOEt/MeOH 25:1): \( R_f = 0.19 \). 1H NMR (300 MHz, CDCl₃): \( \delta = 3.02 \) (dd, J = 6.3, 9.6 Hz, 1H), 3.25 (dd, J = 5.7, 9.3 Hz, 1H), 3.78 (s, 6H), 4.18 (m, 1H), 4.29 (dd, J = 6.6, 14.4 Hz, 1H), 4.40 (dd, J = 2.4, 14.2 Hz, 1H), 5.75 (br s, 2H), 6.80 (m, 4H), 7.20–7.30 (m, 7H), 7.37–7.40 (m, 2H), 7.72 (s, 1H), 8.26 (s, 1H).

   Prepare S.4

9. Dissolve 1.69 g (3.29 mmol) of S.3 in 25 mL of anhydrous pyridine in a 100-mL round-bottom flask and add 1.7 mL (13.55 mmol) of TMS-Cl to the solution.

10. Stir the reaction for 2 hr at room temperature under an argon atmosphere.

11. Cool the mixture in a water/ice bath to 0°C and add in a drop-wise fashion 0.59 mL (5.1 mmol) of BzCl.

12. Continue stirring the reaction for 30 min at 0°C, then an additional 2 hr at room temperature.

13. Cool the solution in a water/ice bath to 0°C and add 4 mL of water to quench the reaction.

14. Keep the reaction at 0°C and add 8 mL of concentrated NH₄OH by injection after 15 min.

15. Stir the reaction in the ice bath for an additional 50 min.

16. Evaporate the solvent to dryness using a rotary evaporator equipped with a vacuum pump and a cooling trap.

17. Re-suspend the residue in 50 mL DCM and adsorb the mixture onto silica gel (1:1.2 w/w sample/silica gel ratio).

18. Purify the sample from step 17 by column chromatography (deactivate the column with 97:3 hexanes/Et₃N and elute using 1:2:0.01 hexanes/AcOEt/Et₃N, then 100:1 AcOEt/Et₃N, and finally 25:1:0.01 AcOEt/MeOH/Et₃N).

19. Collect fractions containing the product, determined by TLC, and evaporate to dryness.

   The resulting product, 3′-O-(4,4′-dimethoxytriphenyl)methyl-N⁶-benzoyl-(S)-9-(2,3-dihydroxypropyl)adenine (S.4), should be obtained in a 78% yield (1.6 g, 2.57 mmol) as a colorless foam. TLC (AcOEt/MeOH 25:1): \( R_f = 0.41 \). 1H NMR (500 MHz, CDCl₃): \( \delta = 3.14 \) (dd, J = 6.0, 9.5 Hz, 1H), 3.21 (dd, J = 6.0, 9.5 Hz, 1H), 3.78 (s, 6H), 4.19 (m, 1H), 4.34 (dd, J = 6.1, 14.2 Hz, 1H), 4.48 (dd, J = 2.8, 14.2 Hz, 1H), 6.81 (d, J = 9.0 Hz, 4H), 7.21 (t, J = 7.2 Hz, 1H), 7.25–7.27 (m, 6H), 7.39 (d, J = 7.0 Hz, 2H), 7.53 (t, J = 7.8 Hz, 2H), 7.61 (t, J = 7.5 Hz, 1H), 8.00 (s, 1H), 8.04 (d, J = 7.5 Hz, 2H), 8.74 (s, 1H), 9.03 (br s, 1H).
Prepare S.5

20. Dry 1.20 g (1.95 mmol) of S.4 overnight under vacuum of 0.05 mmHg in a 100-mL round-bottomed flask.

21. Dissolve S.4 in 2.1 mL (11.2 mmol) of DIPEA in 33 mL of DCM (freshly distilled over CaH₂) under an argon atmosphere.

22. In a drop-wise fashion, add 0.56 mL (2.53 mmol) of chloro(2-cyanoethoxy)-(diiisopropylamino)phosphine to the reaction mixture.

23. After 2 hr, add an additional 0.06 mL (0.25 mmol) of chloro(2-cyanoethoxy)-(diiisopropylamino)phosphine (1/10 the volume from step 22) to the reaction mixture.

24. Stir the mixture at room temperature under an argon atmosphere for a total of 4 hr.

25. Evaporate the solvent using a rotary evaporator and purify the residue by column chromatography. Deactivate the column with 97:3 hexanes/Et₃N and elute the product using a step-wise gradient of 1:2:0.01 hexanes/AcOEt/Et₃N to 1:2:0.1:0.01 hexanes/AcOEt/MeOH/Et₃N.

26. Combine the product fractions, determined by TLC, and evaporate to dryness using a rotary evaporator.

The resulting product, 2′-O-(2-cyanoethoxy)(diisopropylamino)phosphino-3′-O-(4,4′-dimethoxytriphenyl)methyl-N6-benzoyl-(S)-9-(2,3-dihydroxypropyl)adenine (S.5) should be obtained in an 82% yield (1.30 g, 1.60 mmol) as a colorless foam. TLC (hexanes/AcOEt/MeOH 16:32:1): Rf = 0.18, 0.34. ¹H NMR (500 MHz, CDCl₃): δ=1.00 (d, J=6.5 Hz, 6H), 1.06 (d, J=7.0 Hz, 6H), 1.10 (d, J=7.0 Hz, 12H), 2.39 (t, J=6.2 Hz, 2H), 2.47 (q, J=6.2 Hz, 2H), 3.15 (dd, J=6.0, 10.0 Hz, 1H), 3.21-3.26 (m, 2H), 3.30 (dd, J=4.0, 12.5 Hz, 1H), 3.45-3.69 (m, 8H), 3.78 (s, 6H), 3.79 (s, 6H), 4.37 (m, 2H), 4.47-4.58 (m, 4H), 6.82 (m, 8H), 7.17-7.33 (m, 14H), 7.45 (m, 4H), 7.60 (m, 2H), 8.04 (m, 4H), 8.05 (s, 1H), 8.06 (s, 1H), 8.78 (s, 1H), 8.79 (s, 1H), 9.03 (br, 2H). ³¹P NMR (202 MHz, CDCl₃): δ=148.98, 149.59.

**BASIC PROTOCOL 3**

SYNTHESIS OF 2′-O-(2-CYANOETHOXY)(DIISOPROPYLAMINO)-PHOSPHINO-3′-O-(4,4′-DIMETHOXYTRIPHENYL)METHYL-N⁴-BENZOYL-(S)-1-(2,3-DIHYDROXYPROPYL)CYTOSINE

This protocol describes the synthesis of N⁴-benzoyl-protected (S)-GNA cytidine nucleoside phosphoramidite S.7 from S.2 (Fig. 4.40.4).

![Figure 4.40.4](image-url)  
**Figure 4.40.4** Preparation of S.7. Abbreviations: NaH, sodium hydride; DMF, dimethylformamide; DIPEA, diisopropylethylamine; DCM, dichloromethane.

4.40.6
**Synthesis of Modified Oligonucleotides and Conjugates**

### Materials

- N^4^-benzoyl-protected cytosine
- 60% sodium hydride in mineral oil (NaH)
- Dimethylformamide (DMF), anhydrous
- Argon source
- DMT-O-(S)-glycidol (S.2; Basic Protocol 2)
- Ethyl acetate (AcOEt)
- Silica gel (60 Å, 230 to 400 mesh)
- Hexanes
- Triethylamine (Et₃N)
- Acetone
- Diisopropylethylamine (DIPEA)
- Dichloromethane (DCM)
- Chloro(2-cyanoethoxy)-(diisopropylamino)phosphine

### Prepare S.6

1. Add 1.94 g (8.99 mmol) of N^4^-benzoyl-protected cytosine and 73 mg (1.82 mmol) of 60% NaH in mineral oil in 18 mL of DMF in a 50-mL round-bottomed flask.
2. Stir the reaction for 1 hr at room temperature under an argon atmosphere using a gas balloon.
3. Dissolve 3.02 g (8.03 mmol) of S.2 in 19 mL of DMF in a 50-mL round-bottomed flask.
4. Add the solution from step 3 to the 50-mL round-bottomed flask from step 1, attach a condenser to the flask, and stir the mixture 20 hr at 110°C.
5. Cool the reaction mixture to room temperature and remove the precipitate by vacuum filtration. Wash the residue with 100 mL AcOEt.
6. Evaporate the organic solvent from step 5 to dryness using a rotary evaporator equipped with a vacuum pump and a cooling trap.
7. Purify the residue by column chromatography (APPENDIX 3E) on 80 g of silica gel in a 6.4 × 45–cm column. Deactivate the column with 100 mL of 97:3 hexanes/Et₃N and elute using 3:2:0.01 hexanes/acetone/Et₃N.
8. Collect the product fractions, determined by TLC, and evaporate to dryness.

The resulting product, 3′-O-(4,4′-dimethoxytriphenyl)methyl-N^4^-benzoyl-(S)-1-(2,3-dihydroxypropyl)cytosine (S.6), should be obtained in a 46% yield (2.2 g, 3.70 mmol) as a light-yellow foam. TLC (DCM/MeOH 25:1): R_f = 0.21. ^1H NMR (300 MHz, CDCl₃):
SYNTHESIS OF 2′-O-(2-CYANOETHOXY)(DIISOPROPYLAMINO)-PHOSPHINO-3′-O-(4,4′-DIMETHOXYPHENYL)METHYL-N2-ISOBUTYRYL-(S)-9-(2,3-DIHYDROXYPROPYL)GUANINE

This protocol describes the synthesis of N2-isobutyryl-protected (S)-GNA guanosine nucleoside phosphoramidite S.12 from S.1 (Fig. 4.40.5).

Materials

- Dimethylformamide (DMF), anhydrous
- R-(-)-glycidol (S.1)
- 2-Amino-6-chloropurine
- Potassium carbonate (K2CO3)
- Methanol (MeOH)
- Silica gel (60 A, 230 to 400 mesh)
- Ethyl acetate (AcOEt)
- 1 N hydrochloric acid solution (HCl)
- Ammonium hydroxide (concentrated NH4OH)
- Pyridine, anhydrous
- Argon source
- Trimethylsilyl chloride (TMS-Cl)
- Isobutyryl chloride (i-PrOCl)
- DMT-Cl
- Hexanes

\[ \delta = 3.12 \text{ (dd, } J=5.7, 9.6 \text{ Hz, } 1H), 3.26 \text{ (dd, } J=5.6, 9.4 \text{ Hz, } 1H), 3.78 \text{ (s, } 6H), 3.84 \text{ (dd, } J=6.8, 13.8 \text{ Hz, } 1H), 4.22 \text{ (m, } 1H), 4.36 \text{ (dd, } J=2.7, 13.8 \text{ Hz, } 1H), 6.84 \text{ (m, } 4H), 7.20-7.64 \text{ (m, } 14H), 7.89 \text{ (s, } 1H), 7.90 \text{ (s, } 1H), 8.67 \text{ (s br, } 1H). \]

**Prepare S.7**

9. Dry 1.0 g (1.7 mmol) of S.6 overnight under vacuum of 0.05 mmHg in a 100-mL round-bottomed flask.

10. Dissolve S.6 from step 9 and 1.9 mL (10.2 mmol) of DIPEA in 29 mL of DCM (freshly distilled over CaH2) under an argon atmosphere.

11. Add 0.5 mL (2.2 mmol) of chloro(2-cyanoethoxy)-(diisopropylamino)phosphine to the reaction mixture in a drop-wise fashion.

12. After 2 hr, add an additional 0.05 mL (0.22 mmol) of chloro(2-cyanoethoxy)-(diisopropylamino)phosphine (1/10 the volume from step 11) to the reaction mixture.

13. Allow the reaction to stir for an additional 1 hr for a total of 3 hr at room temperature under an argon atmosphere.

14. Evaporate the solvent and purify the residue by column chromatography. Deactivate the column with 100 mL of 97:3 hexanes/Et3N and elute using 2:1:0.01 hexanes/acetone/Et3N.

15. Combine the product fractions, determined by TLC, and evaporate to dryness.

The resulting product, 2′-O-(2-cyanoethoxy)(diisopropylamino)phosphino-3′-O-(4,4′-dimethoxytriphenyl)methyl-N4-Benzoyl-(S)-1-(2,3-dihydroxypropyl)cytosine (S.7) should be obtained in a 76% yield (1.02 g, 1.29 mmol) as a light-yellow foam. TLC (hexanes/acetone/Et3N 2:1:0.01): Rf = 0.21. 1H NMR (500 MHz, CDCl3): \( \delta = 1.12-1.16 \) (m, 16H), 1.27 (t, \( J = 7.2 \) Hz, 8H), 2.43 (t, \( J = 6.4 \) Hz, 2H), 2.55-2.77 (m, 3H), 3.15 (dd, \( J = 3.0, 10.0 \) Hz, 1H), 3.22 (dd, \( J = 4.5, 10.0 \) Hz, 1H), 3.28 (dd, \( J = 4.0, 10.0 \) Hz, 1H), 3.42-3.90 (m, 10H), 3.78 (s, 6H), 3.79 (s, 6H), 4.31-4.45 (m, 4H), 6.82-6.85 (m, 8H), 7.19-7.36 (m, 16H), 7.45-7.54 (m, 8H), 7.58 (m, 4H), 7.90 (d, \( J = 7.5 \) Hz, 4H), 8.60 (br, 2H). 31P NMR (202 MHz, CDCl3): \( \delta = 149.12, 149.18. \)
Figure 4.40.5 Preparation of S.12. Abbreviations: K₂CO₃, potassium carbonate; DMF, dimethylformamide; HCl, hydrogen chloride; TMS-Cl, trimethylsilyl chloride; i-PrOCl, isobutyryl chloride; Py, pyridine; DMT-Cl, 4,4′-dimethoxytritylchloride; DIPEA, diisopropylethylamine; THF, tetrahydrofuran.

Triethylamine (Et₃N)
Diisopropylethylamine (DIPEA)
Tetrahydrofuran (THF)
Chloro(2-cyanoethoxy)-(diisopropylamino)phosphine
Pentane
25-, 50-, 100-, and 250-mL round-bottomed flasks
Graham condenser
Magnetic stir plate and stir bar
Büchner funnel
Filter paper
Rotary evaporator equipped with a vacuum pump
1.3 × 30–cm and 6.4 × 45–cm chromatography columns
TLC plate, EMD silica gel 60 F₂₅₄
UV lamp, 254 nm
Balloon

Additional reagents and equipment for TLC (APPENDIX 3D) and column chromatography (APPENDIX 3E)

Prepare S.8

1. To 45 mL of DMF in a 100-mL round-bottomed flask, add:
   - 0.96 mL (14.5 mmol) of R-(+)-glycidol
   - 2.4 g (14.2 mmol) of 2-amino-6-chloropurine
   - 0.33 g (2.4 mmol) of K₂CO₃.
2. Attach a condenser to the flask and stir the reaction 14 hr at 90°C.

3. Immediately remove the precipitates by vacuum filtration before the reaction mixture cools to room temperature. Wash the residue with 50 mL MeOH.

4. Remove the solvent using a rotary evaporator equipped with a vacuum pump and a cooling trap.

5. Resuspend the residue in 100 mL MeOH and adsorb the mixture onto silica gel (1:1.2 w/w sample/silica gel ratio).

6. Separate the mixture from step 5 by column chromatography using a step-wise gradient of 10:1 to 20:3 (v/v) AcOEt/MeOH.

7. Collect the product fractions, determined by TLC, and evaporate to dryness.

   The resulting product, (S)-9-(2,3-dihydroxypropyl)-2-amino-6-chloropurine (S.8) should be obtained in a 32% yield (1.16 g, 4.77 mmol) as a white solid. TLC (AcOEt/MeOH 20:3): RF = 0.40. 1H NMR (500 MHz, DMSO-d6): δ = 3.30 (m, 1H), 3.40 (m, 1H), 3.81 (m, 1H), 4.18 (dd, J = 8.8, 13.8 Hz, 1H), 4.81 (t, J = 5.5 Hz, 1H), 5.09 (d, J = 5.5 Hz, 1H), 6.88 (s br, 2H), 8.01 (s, 1H).

8. Dissolve 1.84 g (7.54 mmol) of S.8 in 66 mL of 1 N HCl in a 250-mL round-bottomed flask.

9. Stir the reaction 3 hr at 85°C and cool the mixture to room temperature.

10. Adjust the pH of the solution from step 8 using concentrated NH₄OH to pH 9.0. The white product precipitates out of the solution.

   The resulting product, (S)-9-(2,3-dihydroxypropyl)-guanine (S.9) should be obtained in a 70% yield (1.20 g, 5.28 mmol) as a white solid. TLC (AcOEt/MeOH 20:3): RF = 0.04. 1H NMR (500 MHz, DMSO-d6): δ = 3.28 (m, 1H), 3.40 (m, 1H), 3.76 (m, 1H), 3.80 (dd, J = 8.0, 13.5 Hz, 1H), 4.08 (dd, J = 3.5, 13.5 Hz, 1H), 4.78 (t, J = 5.5 Hz, 1H), 5.06 (d, J = 5.0 Hz, 1H), 6.46 (s, 2H), 7.59 (s, 1H), 10.54 (s, 1H).

11. Collect the product, determined by TLC, with vacuum filtration and remove the solvent under high vacuum.

   The resulting product, (S)-9-(2,3-dihydroxypropyl)-guanine (S.9) should be obtained in a 70% yield (1.20 g, 5.28 mmol) as a white solid. TLC (AcOEt/MeOH 20:3): RF = 0.04. 1H NMR (500 MHz, DMSO-d6): δ = 3.28 (m, 1H), 3.40 (m, 1H), 3.76 (m, 1H), 3.80 (dd, J = 8.0, 13.5 Hz, 1H), 4.08 (dd, J = 3.5, 13.5 Hz, 1H), 4.78 (t, J = 5.5 Hz, 1H), 5.06 (d, J = 5.0 Hz, 1H), 6.46 (s, 2H), 7.59 (s, 1H), 10.54 (s, 1H).

12. Add 350 mg (1.55 mmol) of S.9 in 10 mL of anhydrous pyridine to a 50-mL round-bottomed flask in a water-ice bath at 0°C under an argon atmosphere.

13. Add 1.5 mL (11.8 mmol) of TMS-Cl to the solution and stir the reaction 30 min at 0°C and then an additional 2 hr at room temperature.

14. Cool the mixture in a water/ice bath to 0°C and add 1.3 mL (7.75 mmol) of i-PrOCl in a drop-wise fashion to the reaction mixture.

15. Stir the reaction 30 min at 0°C and then an additional 4 hr at room temperature.

16. Cool the solution to 0°C in a water/ice bath and quench the reaction by adding 2 mL of water to the solution, and stir for an additional 15 min.

17. Add 2 mL of concentrated NH₄OH and incubate for 50 min at 0°C.

18. Evaporate the solvent to dryness using a rotary evaporator equipped with a vacuum pump and a cooling trap.

19. Resuspend the residue in 50 mL MeOH, adsorb the mixture onto silica gel (1:1.2 w/w sample/silica gel ratio).
20. Purify the sample by column chromatography using a step-wise gradient of 200 mL of 20:3 to 40:9 (v/v) AcOEt/MeOH.

21. Collect the product fractions, determined by TLC, and evaporate to dryness.

The resulting product, \(N^2\)-isobutyryl-(S)-9-(2,3-dihydroxypropyl)-guanine (S.10), should be obtained in a 68\% yield (283 mg, 0.96 mmol) as a white solid. TLC (AcOEt/MeOH 20:3): \(R_f = 0.15\). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)): \(\delta = 1.08\) (d, \(J = 7.2\) Hz, 6H), 2.75 (sept, \(J = 7.1\) Hz, 1H), 3.32 (m, 1H), 3.38 (m, 1H), 3.78 (m, 1H), 4.18 (dd, \(J = 3.3, 13.8\) Hz, 1H), 4.80 (t, \(J = 5.4\) Hz, 1H), 5.07 (d, \(J = 5.7\) Hz, 1H), 7.86 (s, 1H), 11.65 (s br, 1H), 12.02 (s br, 1H).

Prepare S.11

22. Dissolve 780 mg (2.65 mmol) of S.10 in 11.5 mL of anhydrous pyridine in a 50-mL round-bottomed flask.

23. Add 1.1 g (3.24 mmol) of DMT-Cl to the solution from step 22.

24. Stir the reaction 3 hr at room temperature under an argon atmosphere.

25. Remove the solvent using a rotary evaporator equipped with a vacuum pump and a cooling trap.

26. Purify the residue from step 25 by column chromatography. Deactivate the column with 97:3 hexanes/Et\(_3\)N and elute using the gradient elution as follows:

- a. 75 mL of 1:2:0.01 hexanes/AcOEt/Et\(_3\)N
- b. 300 mL of 100:1 AcOEt/Et\(_3\)N
- c. elute product with 25:1:0.01 AcOEt/MeOH/Et\(_3\)N.

27. Collect the product fractions, determined by TLC, and evaporate to dryness.

The resulting product, 3′-O-(4,4′-dimethoxytriphenyl)methyl-N^2-isobutyryl-(S)-9-(2,3-dihydroxypropyl)-guanine (S.11), should be obtained in an 80\% yield (127 mg, 2.12 mmol) as a white solid. TLC (AcOEt/MeOH 50:1): \(R_f = 0.10\). \(^1\)H NMR (500 MHz, CDCl\(_3\)): \(\delta = 1.27\) (t, \(J = 7.0\) Hz, 6H), 2.62 (sept, \(J = 6.8\) Hz, 1H), 3.18 (dd, \(J = 5.8, 9.8\) Hz, 1H), 3.27 (dd, \(J = 5.0, 9.5\) Hz, 1H), 3.80 (s, 6H), 4.03 (dd, \(J = 8.0, 14.0\) Hz, 1H), 4.25 (dd, \(J = 2.5, 14.0\) Hz, 1H), 4.36 (m, 1H), 4.81 (br, 1H), 6.83 (d, \(J = 8.5\) Hz, 4H), 7.22 (t, \(J = 7.2\) Hz, 1H), 7.26-7.34 (m, 6H), 7.45 (d, \(J = 7.0\) Hz, 2H), 7.55 (s, 1H), 8.43 (br, 1H), 11.79 (br, 1H).

Prepare S.12

28. Dry 200 mg (0.33 mmol) of S.11 overnight under vacuum of 0.05 mmHg in a 25-mL round-bottomed flask.

29. Dissolve S.11 from step 28 and 0.11 mL (0.6 mmol) of DIPEA in 3.5 mL of THF (freshly distilled over sodium) under an argon atmosphere.

30. Add 0.1 mL (0.45 mmol) of chloro(2-cyanoethoxy)-(diisopropylamino)phosphine to the reaction mixture in a drop-wise fashion in a water/ice bath at 0°C. Stir the reaction at room temperature.

31. After 2 hr, add an additional 0.01 mL (0.05 mmol) of chloro(2-cyanoethoxy)-(diisopropylamino)phosphine (1/10 the volume from step 30) to the reaction mixture.

32. Allow the reaction to stir overnight at room temperature under an argon atmosphere.

33. Evaporate the solvent and dissolve the residue in a minimal amount of DCM.
34. Add the solution from step 33 to 100 mL of vigorously stirring pentane. Collect the white precipitate by vacuum filtration.

35. Purify the residue by column chromatography. Deactivate the column with 97:3 hexanes/Et3N and elute with a step-wise gradient of 50 mL of 45:45:1 DCM/AcOEt/Et3N and elute with 100:1 AcOEt/Et3N.

36. Combine the product fractions, determined by TLC, and evaporate to dryness. The resulting product, 2′-O-(2-cyanoethoxy)(diisopropylamino)phosphino-3′-O-(4,4′-dimethoxytriphenyl)methyl-N2-isobutyryl-(S)-9-(2,3-dihydroxypropyl)-guanine (S.11), should be obtained in a 60% yield (158 mg, 0.2 mmol) as a white foam. TLC (AcOEt/MeOH 50:1); Rf = 0.32, 0.42. 1H NMR (500 MHz, CDCl3): δ = 1.06 (t, J = 7.0 Hz, 2H), 1.10-1.15 (m, 17H), 1.20-1.29 (m, 17H), 2.47 (t, J = 6.0 Hz, 1H), 2.52-2.65 (m, 4H), 3.02 (dd, J = 4.0, 9.5 Hz, 1H), 3.08 (dd, J = 5.2, 9.8 Hz, 1H), 3.13-3.23 (m, 2H), 3.46-3.82 (m, 10H), 3.78 (s, 6H), 3.79 (s, 6H), 4.26-4.38 (m, 5H), 4.53 (m, 1H), 6.80 (m, 8H), 7.18-7.33 (m, 14H), 7.42-7.48 (m, 4H), 7.49 (s, 1H), 7.55 (s, 1H), 8.38 (br, 1H), 8.70 (br, 1H). 31P NMR (202 MHz, CDCl3): δ = 148.20, 148.28.

**BASIC PROTOCOL 5**

SYNTHESIS OF 2′-O-(2-CYANOETHOXY)(DIISOPROPYLAMINO)-PHOSPHINO-3′-O-(4,4′-DIMETHOXYTRIPHENYL)METHYL-(S)-1-(2,3-DIHYDOXYPROPYL)THYMINE

This protocol describes the synthesis of (S)-GNA thymidene nucleoside phosphoramidite monomer S.14 from S.2 (Fig. 4.40.6).

**Materials**

Thymine

60% sodium hydride in mineral oil (NaH)

Dimethylformamide (DMF), anhydrous

Argon source

DMT-O-(S)-glycidol (S.2; Basic Protocol 1)

Ethyl acetate (AcOEt)

Silica gel (60 Å, 230-400 mesh)

Diisopropylethylamine (DIPEA)

Dichloromethane (DCM)

Chloro(2-cyanoethoxy)-(diisopropylamino)phosphine

Hexanes

Triethylamine (Et3N)

Methanol (MeOH)

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Figure 4.40.6 Preparation of S.14. Abbreviations: NaH, sodium hydride; DMF, dimethylformamide; DIPEA, diisopropylethylamine; DCM, dichloromethane.
25- and 50-mL round-bottomed flasks
Magnetic stir plate and stir bar
Gas balloon
Rotary evaporator equipped with a vacuum pump
G.3 × 30–cm and 6.4 × 45–cm chromatography columns
TLC plate, EMD silica gel 60 F254
200-mL separatory funnel
UV lamp, 254 nm
Büchner funnel
Filter paper

Additional reagents and equipment for TLC (APPENDIX 3D) and column chromatography (APPENDIX 3E)

Prepare S.13

1. Add 0.95 g (7.54 mmol) of thymine and 64 mg (1.60 mmol) of 60% NaH in mineral oil in 12 mL DMF to a 50-mL round-bottomed flask.
2. Stir the mixture 2 hr at room temperature under an argon atmosphere.
3. Dissolve 2.68 g (7.13 mmol) of S.2 in 15 mL of DMF in a 50-mL round-bottomed flask.
4. Add the solution from step 3 to the 50-mL round-bottomed flask from step 1, attach a condenser to the flask, and stir the mixture 20 hr at 110°C under an argon atmosphere.
5. Cool the reaction mixture to room temperature and remove the precipitate by vacuum filtration. Wash the residue with 50 mL AcOEt.
6. Evaporate the organic solvent from step 5 to dryness using a rotary evaporator equipped with a vacuum pump and a cooling trap.
7. Purify the residue by column chromatography (APPENDIX 3E) on 60 g of silica gel in a 6.4 × 45–cm column. Deactivate the column with 200 mL of 97:3 DCM/Et3N and elute using 50:1:0.01 DCM/MeOH/Et3N.
8. Collect the product fractions, determined by TLC, and evaporate to dryness.

The resulting product, 3′-O-(4,4′-dimethoxytriphenyl)methyl (S)-1-(2,3-dihydroxypropyl)thymine (S.13), should be obtained in a 54% yield (1.95 g, 3.88 mmol) as a colorless foam. TLC (DCM/MeOH 25:1): Rf = 0.14. 1H NMR (400 MHz, CDCl3): δ = 1.73 (s, 3H), 3.07 (d, J = 5.6 Hz, 2H), 3.55 (dd, J = 7.2, 14.0 Hz, 1H), 3.68 (s, 6H), 3.89-3.96 (m, 2H), 6.73 (m, 4H), 6.94 (d, J = 5.6 Hz, 2H), 7.10-7.22 (m, 7H), 7.30 (m, 2H).

Prepare S.14

9. Dry 1.1 g (2.2 mmol) of S.13 under vacuum of 0.05 mmHg in a 100-mL round-bottomed flask overnight.
10. Dissolve S.13 from step 9 and 2.2 mL (11.7 mmol) of DIPEA in 36 mL of DCM (freshly distilled over CaH2) under an argon atmosphere.
11. Add 0.62 mL (2.8 mmol) of chloro(2-cyanoethoxy)-(diisopropylamino)phosphine to the reaction mixture in a drop-wise fashion.
12. After 2 hr, add an additional 0.06 mL (0.3 mmol) of chloro(2-cyanoethoxy)-(diisopropylamino)phosphine (1/10 of the amount from step 11) to the reaction mixture.
13. Stir the mixture for an additional 1 hr for a total of 3 hr at room temperature under an argon atmosphere.

14. Evaporate the solvent and purify the residue by column chromatography. Deactivate the column with 200 mL of 97:3 hexanes/Et$_3$N and elute using 1:2:0.01 hexanes/AcOEt/Et$_3$N.

15. Combine the product fractions, determined by TLC, and evaporate to dryness.

The resulting product, 2'-O-(2-cyanoethoxy)(diisopropylamino)phosphino-3'-O-(4,4'-dimethoxytriphenyl)methyl-(S)-1-(2,3-dihydroxypropyl)thymine (S.14), should be obtained in a 78% yield (1.21 g, 1.72 mmol) as a colorless foam. TLC (hexanes/AcOEt/Et$_3$N 1:2:0.01): $R_f = 0.33, 0.42$. $^1$H NMR (300 MHz, CDCl$_3$): $\delta = 1.12$-$1.18$ (m, 24H), 1.84 (m, 6H), 2.41 (t, $J = 6.3$ Hz, 2H), 2.59 (t, $J = 6.3$ Hz, 2H), 3.16-3.32 (m, 4H), 3.49-$3.88$ (m, 10H), 3.78 (s, 6H), 3.79 (s, 6H), 4.03-4.23 (m, 4H), 6.83 (m, 8H), 7.05 (m, 2H), 7.18-7.36 (m, 14H), 7.44-7.46 (m, 4H). $^{31}$P NMR (121 MHz, CDCl$_3$): $\delta = 148.76, 148.95.$

SYNTHESIS, ISOLATION, AND CHARACTERIZATION OF GLYCEROL NUCLEIC ACID (GNA) OLIGONUCLEOTIDES

The glycerol nucleoside phosphoramidites are completely stable under standard oligonucleotide synthesis conditions on a DNA synthesizer. An Applied Biosystems 3400 DNA synthesizer has been used successfully on both a 0.2- and 1.0-μmol scale synthesis. Optimal yields were achieved by extending the coupling time of the GNA phosphoramidite monomers to 300 sec (5 min). Because the free hydroxyl termini of GNA nucleotides are subject to decomposition during the base-deprotection step (Tsai et al., 2007), the authors recommend capping the 5'- and 3'-ends of each GNA sequence with a single DNA residue, typically T (Zhang et al., 2008). Several techniques have been reported for the isolation and purification of sequences containing GNA nucleotides, such as C18 reversed-phase HPLC and denaturing polyacrylamide gel electrophoresis (PAGE). This protocol describes the synthesis of GNA oligonucleotides from GNA monomer phosphoramidite precursors.

Materials

- GNA phosphoramidite monomers bearing nucleobases A, C, G, and T (S.5, S.7, S.12, and S.14, respectively; Basic Protocols 2, 3, 4, and 5, respectively)
- Anhydrous acetonitrile (MeCN; Applied Biosystems)
- Standard 5'-O-(4,4'-dimethoxytrityl)phosphoramidites (Glen Research)
- Ammonium hydroxide (concentrated NH$_4$OH)
- n-Butanol
- Nanopure water
- Ethanol
- 3-Hydroxypicolinic acid solution
- 25-mL round-bottomed flasks
- 4-A molecular sieves (freshly activated by heating 3 hr at 300°C)
- 0.45-μm disposable syringe filter
- Applied Biosystems 3400 DNA synthesizer
- 2-mL screw-capped microcentrifuge tubes (Eppendorf)
- 15-mL screw-capped centrifuge tubes (Falcon)
- Spectrophotometer
- MALDI-TOF mass spectrometer

Additional reagents and equipments for automated solid-phase oligodeoxyribonucleotide synthesis (APPENDIX 3C), isolation and characterization of synthetic nucleic acids (UNITS 10.1 & 10.4)
**Synthesize GNA oligonucleotides (0.2 μmol scale synthesis)**

Steps 1 through 3 are carried out under argon atmosphere. All the glassware, syringes and needles are pre-dried in a drying oven and then cooled to room temperature in a desiccator for later use.

1. Dissolve each GNA phosphoramidite (pre-dried under high vacuum for 48 hr) in 3 mL anhydrous MeCN in a 25-mL round-bottomed flask to make ~0.05 M solutions:
   - 150 mg of **S.5** (A)
   - 150 mg of **S.7** (C)
   - 150 mg of **S.12** (G)
   - 130 mg of **S.14** (T).

2. Dry the phosphoramidite solutions from step 1 over freshly activated 4-Å molecular sieves overnight at room temperature.

3. Filter the amidite solutions using 0.45-μm disposable syringe filters directly into clean, dry bottles, and immediately place the bottles on the corresponding amidite ports of the DNA synthesizer.

4. Start the automated solid-phase oligonucleotide synthesis from CPG-column pre-charged with a deoxynucleotide monomer or the universal support.

5. After completing the synthesis, remove the column and dry for 2 min using vacuum pipeline.

6. Open the CPG-column container and transfer the CPG beads into a 2-mL screw-capped microcentrifuge tube.

**Deprotect and purify GNA oligonucleotides**

7. Add 1 mL of concentrated NH₄OH, seal the tube, and incubate 48 hr at 55°C.

8. Place the tube on ice and cool for at least 10 min prior to opening.

9. Transfer the supernatant into a 15-mL tube and add 10 mL of n-butanol.

10. Place the tube 5 min on ice.

11. Centrifuge 5 min at 3220 × g (4000 rpm), 4°C, discard the supernatant and collect the oligonucleotide (white precipitate).

12. Resuspend the oligonucleotide pellet in 500 μl of nanopure water.

13. Purify the crude product by denaturing PAGE and desalt the oligonucleotide by ethanol precipitation *(UNIT 10.4)*.

14. Quantify the oligonucleotide by UV absorbance using Beer’s law at 254 nm.

**Check molecular weight**

15. Check the molecular weight of the desired GNA oligonucleotide by MALDI-TOF mass spectroscopy using aqueous saturated 3-hydroxypicolinic acid solution as matrix *(UNIT 10.1)*. Prepare matrix just before spotting the sample onto a MALDI-TOF mass spectrometry gold plate.

   See Figure 4.40.7 for sample MALDI-TOF mass spectroscopy results.
COMMENTARY

Background Information

Synthesis
The “spring-loaded” epoxide ring-opening procedure was first reported by Acevedo and Andrews (1996) to increase the structural diversity of acyclic libraries aimed at creating therapeutic agents to cellular and viral targets. This unit presents a modified version of this synthetic strategy. GNA nucleoside derivatives can also be synthesized using a nucleobase substitution approach with a tosyalted isopropylidene-glycerol (Holý, 1975). However, this approach requires more synthetic steps and results in a lower overall yield. Another approach is to use modified base-protecting groups (N-dimethylformamidine group for A and G, acetamide for C; Schlegel and Meggers, 2009). Both of these protecting groups are compatible with faster oligonucleotide deprotection conditions, which can be advantageous for minimizing GNA decomposition during the final deprotection step in ammonium hydroxide.

Properties of glycerol nucleic acid
GNA has an acyclic backbone composed of a three-carbon sugar connected by phosphodiester linkages. The repeating backbone unit contains one stereocenter, which results in two enantiomers, namely (S)- and (R)-GNA. Both (S)- and (R)-GNA form antiparallel duplex structures with complementary GNA strands of the same stereoconfiguration, and GNA duplexes are generally more stable than natural DNA and RNA (Zhang et al., 2005, 2006; Yang et al., 2007). For this reason, GNA represents the simplest solution to a stable nucleic acid structure based on phosphodiester bonds. Cross-pairing between complementary strands of GNA and RNA results in the formation of a dramatically weaker duplex than pure GNA or RNA alone. Cross-pairing between complementary sequences of GNA and DNA has not been observed, suggesting that the GNA helix is incompatible with the standard B-form helix of natural DNA (Zhang et al., 2005; Yang et al., 2007). This is also indicated by the fact that the incorporation of GNA residues into DNA strands strongly destabilizes duplex formation. Cross-pairing between (R)- and (S)-GNA is also highly destabilizing (Schlegel et al., 2007).

The X-ray crystal structure of a self-complementary (S)-GNA double helix was recently solved to a resolution limit of 1.3 Å (Schlegel et al., 2008). The three-dimensional structure revealed that (S)-GNA adopts a right-handed helix that is significantly different from the standard A- and B-form helical structures observed for natural RNA and DNA, respectively. A striking feature of the GNA duplex is the large average slide (3.4 Å) between adjacent base pairs due to a large backbone-base inclination of the duplex (42° to 50° in GNA versus almost 0° in the B-form DNA). This structural change results in extensive interstrand base stacking and significantly less intrastrand base stacking common to standard A- and B-form helical structures. Another unusual feature is the formation of a loosely wrapped helical ribbon around the central helix axis, which replaces the major groove by a
convex surface and leaves just one large minor groove (Schlegel et al., 2008, 2009, 2010).

**GNA application**

GNA, which is a structurally simplified artificial oligonucleotide, is capable of forming stable duplex structures by Watson-Crick base-pairing rules. This property indicates that GNA represents an interesting synthetic material for constructing programmable nucleic acid nanostructures. Toward this goal, the synthesis of a nanostructure composed entirely of GNA has been described. The GNA nanostructure, a 4-helix junction (4HJ) (Zhang et al., 2008), mimics a fixed-sequence DNA Holliday junction described previously by Seeman and co-workers (Kallenbach et al., 1983). Because the GNA backbone contains only one stereocenter per repeating unit, it was possible to synthesize two mirror-image nanostructures using (S)- and (R)-GNA. A major finding was that the GNA 4HJ was significantly more stable than the earlier 4HJ composed entirely of DNA ($T_m$ 73°C for GNA versus 37°C for DNA). This feature coupled with the ability to construct left- and right-handed nanostructures provides new opportunities for building highly stable nanostructures with topologies that are not readily available to DNA.

**Critical Parameters**

For all reactions using anhydrous solvents, the glassware must be pre-dried in a drying oven (80°C). To obtain optimal separations and to reduce the purification time, the authors recommend using flash column chromatography for all purification steps. For phosphoramidite synthesis, the reaction progress is monitored by TLC and should be stopped as soon as the starting material is consumed. The phosphitylation reaction affords two diastereomeric phosphoramidite products, which are generally visualized as two spots on the TLC plate. However, phosphoramidites bearing cytosine migrate on the TLC plate as one spot, even though the products are actually two isomers. The GNA phosphoramidite building blocks must be dried under high vacuum in a desiccator for 24 hr to give a well-formed solid before they can be used for solid-phase synthesis.

**Troubleshooting**

The phosphoramidites containing the nucleobase, guanine, will decompose when purified on a silica gel column (Zhang et al., 2006). In this protocol, a modified method is described for column chromatography purification that affords the desired intact compound verified by $^1$H and $^{31}$P NMR characterization.

**Anticipated Results**

The protocols described in this unit are useful for preparing GNA oligonucleotides. The GNA phosphoramidite monomers can be synthesized on a multi-gram scale. The oligonucleotide synthesis is similar to the standard DNA phosphoramidite protocol with the noted exception of elongated coupling times. The average stepwise yield is >95%, which is sufficient to produce GNA oligonucleotides in high yield.

**Time Considerations**

Each step in the chemical synthesis can be accomplished in 1 to 2 working days (including purification). The time for oligonucleotide synthesis varies slightly from the standard phosphoramidite method by longer coupling times. The deprotection, isolation, and the analysis of the final synthetic oligonucleotide are accomplished in 2 to 3 days.

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**Literature Cited**


