Synthesis of Threose Nucleic Acid (TNA) Phosphoramidite Monomers and Oligonucleotide Polymers

Su Zhang[1] and John C. Chaput[1]

[1]Center for Evolutionary Medicine and Informatics, The Biodesign Institute and Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona

ABSTRACT

This unit describes the preparation of dimethoxytrityl (DMTr)−protected α-L-threofuranosyl nucleic acid (TNA) phosphoramidite monomers for A, C, G, T, and diaminopurine, as well as their incorporation into TNA oligonucleotides by solid-phase synthesis. Starting from commercially available L-ascorbic acid, the protected threofuranosyl sugar is obtained in four steps. Vorbrüggen-Hilbert-Johnson glycosylation affords the desired threofuranosyl nucleosides, which are converted to their corresponding DMTr-protected phosphoramidite nucleosides in four additional steps. Phosphoramidite monomers are then used to construct TNA oligonucleotides by solid-phase synthesis using a standard DNA synthesizer. *Curr. Protoc. Nucleic Acid Chem.* 50:4.51.1-4.51.26.

Keywords: alternative nucleic acids (ANA) • threose nucleic acid (TNA) • phosphoramidite • oligonucleotide • chemical synthesis • solid-phase synthesis

INTRODUCTION

α-L-Threofuranosyl nucleic acid (TNA; Fig. 4.51.1) is an alternative nucleic acid system with a four-carbon threose sugar in place of the natural five-carbon ribose sugar found in RNA. TNA is capable of forming stable duplex structures via antiparallel Watson-Crick base-pairing with complementary strands of TNA as well as DNA or RNA (Schöning et al., 2000, 2002). This property is unusual, considering that TNA has a backbone repeat unit that is one atom shorter than natural DNA and RNA. The solution NMR structure of a self-complementary octamer reveals that TNA forms a right-handed double helix that closely approximates the canonical structure of A-type DNA and RNA (Ebert et al., 2008).

The ability for TNA to exchange information with RNA, coupled with the relative chemical simplicity of threose compared to ribose, has generated considerable interest

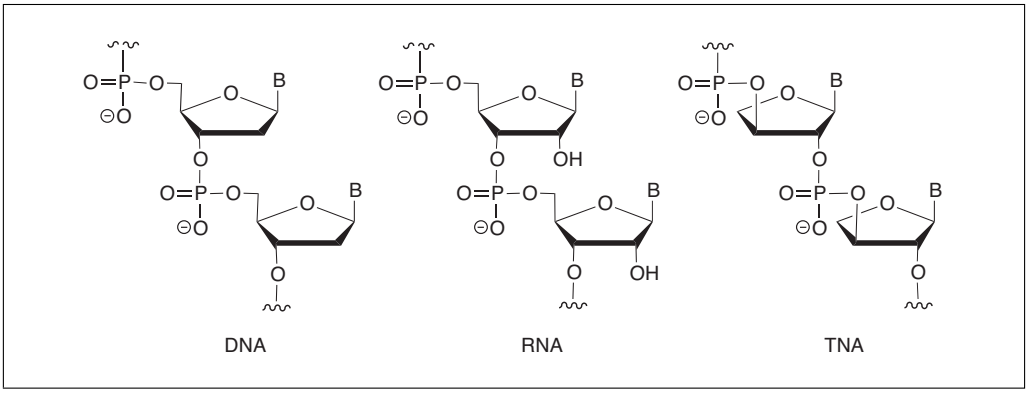

Figure 4.51.1 Chemical structures of DNA, RNA, and TNA.

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in TNA as a possible progenitor of RNA (Orgel, 2000; Yang et al., 2007). In addition to informational base-pairing and chemical simplicity, early genetic systems would have needed the ability to fold into three-dimensional shapes that can perform desired functions such as ligand binding and catalysis. To study the possibility that TNA was involved in such early systems, many polymerases were screened for the ability to copy a DNA template into TNA and to copy a TNA template back into DNA (Chaput and Szostak, 2003; Chaput et al., 2003). These experiments led to the discovery that Therminator DNA polymerase, an engineered variant of the thermophilic 9°N DNA polymerase, functions as an efficient DNA-dependent TNA polymerase (Horhota et al., 2005; Ichida et al., 2005a,b). More recently, in vitro selection was recently used to evolve TNA molecules that fold into tertiary structures with discrete ligand-binding functions (Yu et al., 2012). This demonstration provided the first experimental evidence that TNA could have served as an ancestral genetic system during an early stage of life. Additional experiments of this type have the potential to shed new light on the fitness of alternative genetic systems and their possible contribution to the origins and early evolution of life on Earth.

To this end, it is essential to understand basic questions about the structure and function of synthetic TNA oligonucleotides that can only be accessed by solid-phase synthesis. This unit contributes to the broader goal by describing the synthesis of TNA phosphoramidite monomers of adenosine, guanosine, cytidine, thymidine, and diaminopurine (see Basic Protocols 2 to 6), as well as the solid-phase synthesis and purification of TNA oligonucleotides (see Basic Protocol 7).

BASIC PROTOCOL 1

SYNTHESIS OF PROTECTED L-THREOFURANOSE, 1-*O*-ACETYL-2,3-DI-*O*-BENZOYL-L-THREOFURANOSE, AND 1,2,3-TRI-*O*-BENZOYL-L-THREOFURANOSE

Although the synthesis of L-threose has been reported by several different groups (Schöning et al., 2002, and references therein), most of these methods are unsuitable for large-scale preparation. The present protocol is based on Eschenmoser's procedure (Schöning et al., 2000, 2002) and is illustrated in Figure 4.51.2. The synthesis is initiated from the oxidative degradation of L-ascorbic acid (**S.1**) to afford L-threonic acid (**S.2**). A one-pot lactonization and in situ benzoylation affords 2,3-di-*O*-benzoyl-L-threonolactone (**S.3**). The lactone is reduced with diisobutylaluminium hydride (DIBAL-H) to furnish the corresponding 2,3-di-*O*-benzoylated lactol (**S.4**), which is acetylated to give 1-*O*-acetyl-2,3-di-*O*-benzoyl-L-threofuranose (**S.5**). **S.5** is used to prepare the threofuranosyl nucleosides for C, G, T, and diaminopurine (D) in Basic Protocols 2 to 5. The benzoylated lactol 1,2,3-tri-*O*-benzoyl-L-threofuranose (**S.6**) is used to make the threose A nucleoside in Basic Protocol 6.

Materials

L-Ascorbic acid (**S.1**)
Ultra pure water (H_2O)
Calcium carbonate ($CaCO_3$)
Hydrogen peroxide (H_2O_2)
Activated charcoal
Methanol (MeOH)
Dowex 50WX4-50 resin, acidic form
Acetonitrile (MeCN), anhydrous
p-Toluenesulfonic acid (TsOH)
Argon gas
Pyridine, anhydrous
Benzoyl chloride (BzCl)
Dichloromethane (DCM)

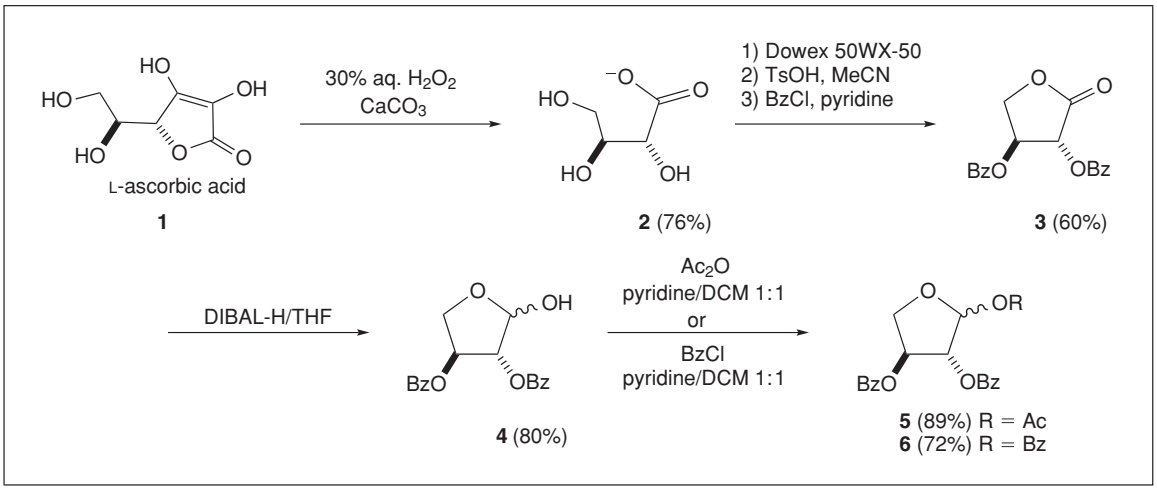

Figure 4.51.2 Preparation of protected L-threofuranoses **S.5** and **S.6**. Abbreviations: TsOH, *p*-toluenesulfonic acid; BzCl, benzoyl chloride; DIBAL-H, diisobutylaluminium hydride; DCM, dichloromethane.

1 M aq. HCl
Saturated aqueous sodium bicarbonate solution (sat. aq. $NaHCO_3$)
Brine (sat. aq. NaCl)
Magnesium sulfate ($MgSO_4$)
Hexanes
Ethyl acetate (EtOAc)
Tetrahydrofuran (THF), anhydrous
1.5 M diisobutylaluminium hydride (DIBAL-H) in toluene
Saturated aqueous sodium potassium tartrate
Acetic anhydride (Ac_2O)
4-Dimethylaminopyridine (DMAP)
Silica gel (60 Å, 230-400 mesh, Whatman)
Ethanol (EtOH)

250-, 500-, 1000-, and 2000-mL round-bottom flasks
Dropping funnels
Quantofix peroxide test sticks (Sigma-Aldrich)
Filter paper
Büchner funnel
250-, 500-, 1000-, and 2000-mL separatory funnels
Rotary evaporator equipped with a vacuum pump
Gas balloon
Allihn condenser (reflux condenser)
Thin-layer chromatography (TLC) plate, EMD silica gel 60 F_{254}
UV lamp, 254 nm

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

Synthesize threonate

1. Dissolve 88 g (0.5 mol) L-ascorbic acid in 700 mL H_2O in a 2000-mL round-bottom flask and slowly add 100 g (1 mol) $CaCO_3$ with stirring.
2. Cool the mixture to 15°C and then add 200 mL of 30% aq. H_2O_2 over a period of 3 hr using a dropping funnel.
3. Warm the reaction to room temperature and stir for 18 hr.

4. Add 20 g activated charcoal and increase the temperature to 70°C until peroxide is no longer detected using a peroxide test stick.
5. Filter the hot reaction mixture and wash the filter cake with water.
6. Concentrate the filtrate to ~500 mL under reduced pressure.
7. Add 200 mL MeOH dropwise over 5 hr at room temperature using a dropping funnel.

   The product gradually precipitates out of the solution during this process.

8. Stir the mixture for an additional 5 hr at room temperature.
9. Add another 200 mL MeOH and isolate the precipitate by vacuum filtration. Wash with cold MeOH.
10. Collect the filtrate and repeat steps 5-9 to obtain additional precipitate.
11. Dry the product under reduced pressure to a constant weight.

    *Calcium-L-threonate monohydrate (**S.2**) is obtained in 76% yield (64 g, 195 mmol) as a white powder. ^{1}H-NMR (300 MHz, D_2O): δ 3.63 (dd, J = 11.6, 7.8, 1H), 3.70 (dd, J = 11.4, 5.4, 1H), 3.98 (ddd, J = 12.6, 5.4, 2.1, 1H), 4.04 (d, J = 2.1, 1H).*

Perform lactonization

12. Dissolve 30 g (91.3 mmol) **S.2** in 640 mL water in a 2000-mL round-bottom flask at 40°C.
13. Add 190 mL Dowex 50WX4-50 resin (acidic form) and stir at 70°C for 30 min.
14. Remove the resin by vacuum filtration and wash with water. Concentrate the filtrate to dryness under reduced pressure to a constant weight at 50°C.
15. Resuspend the residue in 200 mL MeCN, co-evaporate twice with 200 mL MeCN, and resuspend the residue in 320 mL anhydrous MeCN.
16. Add 0.64 g (3.2 mmol) TsOH and reflux the reaction for 24 hr under an argon atmosphere.
17. Allow the reaction to cool to room temperature. Filter the solution and evaporate the solvent to dryness under reduced pressure.
18. Dissolve the residue in 128 mL pyridine and cool the solution to 0°C in an ice-water bath.
19. Add 26.4 mL (228 mmol) BzCl dropwise to the solution. Remove the ice-water bath and stir at room temperature for 16 hr.
20. Dilute the reaction mixture with 250 mL DCM in a 100-mL separatory funnel and wash the organic solvent sequentially with:

    160 mL ice-cold 1 M aq. HCl
    200 mL water
    200 mL sat. aq. $NaHCO_3$
    130 mL brine.

21. Dry the organic phase over 3.0 g $MgSO_4$ and evaporate the solvent to dryness under reduced pressure.
22. Crystallize the residue from 480 mL of 6:1 (v/v) hexanes/EtOAc.

    *2,3-Di-O-benzoyl-L-threonolactone (**S.3**) is obtained in 60% yield (17.9 g, 54.8 mmol) as colorless needles. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 4.38 (m, 1H), 5.02 (m, 1H), 5.81 (m, 2H), 7.45-8.12 (m, 10H).*

Perform reduction

23. Dissolve 20 g (60 mmol) **S.3** in 250 mL anhydrous THF in a 1000-mL round-bottom flask at −78°C under an argon atmosphere.

24. Add 75 mL (225 mmol) of 1.5 M DIBAL-H in toluene dropwise using a dropping funnel and then stir the reaction at −78°C until **S.3** is completely consumed. Monitor the reaction by TLC.

25. Add 10 mL MeOH over 3 min to quench the reaction, then allow the mixture to warm to room temperature.

26. Dilute the mixture with 400 mL EtOAc and 200 mL sat. aq. sodium potassium tartrate. Stir vigorously at room temperature for 3 hr.

27. Separate the organic phase using a 2000-mL separatory funnel, wash it with 280 mL brine, and dry it over 4.8 g $MgSO_4$.

28. Remove the solvent under reduced pressure to afford both diastereomers of the product without further purification.

    *2,3-Di-O-benzoyl-L-threofuranose (**S.4**) is obtained in 60% yield (12.0 g, 36 mmol) as a colorless oil. TLC (hexanes/EtOAc 2:1): R_f 0.37. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 3.97 (dd, J = 8.1, 3.0, 1H), 4.26 (dd, J = 10.2, 4.0), 4.60 (m, 2H), 5.47 (t, J = 4.0, 1H), 5.53 (m, 2H), 5.57 (s, 1H), 5.75 (m, 1H), 5.85 (d, J = 4.0, 1H), 7.42-7.47 (m, 8H), 7.53-7.59 (m, 4H), 8.01-8.10 (m, 8H).*

    *This crude product is used in separate reactions to prepare **S.5** and **S.6**.*

Perform acetylation

29. Dissolve crude **S.4** (19.7 g) in 200 mL of 1:1 (v/v) DCM/pyridine in a 500-mL round-bottom flask.

30. Add 6.8 mL (62 mmol) Ac_2O and 50 mg (0.4 mmol) DMAP, and then stir the reaction at room temperature for 18 hr.

31. Pour the reaction mixture into an ice-cold stirring solution of 1:1 (v/v) sat. $NaHCO_3$/DCM and then continue stirring at 0°C for 1 hr.

32. Separate the organic phase using a 500-mL separatory funnel and wash it sequentially with:

    200 mL sat. $NaHCO_3$
    200 mL water
    200 mL brine.

33. Dry the organic phase over $MgSO_4$ and evaporate to dryness.

34. Purify the residue by silica gel column chromatography, eluting with 2:1 (v/v) hexanes/EtOAc to afford both diastereomers of the product.

    *1-O-Acetyl-2,3-di-O-benzoyl-L-threofuranose (**S.5**) is obtained in 89% yield (19.6 g, 53.4 mmol) as a colorless oil. TLC (hexanes/EtOAc 2:1): R_f 0.43. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 2.04 (s, 3H), 2.16 (s, 3H), 4.05 (dd, J = 10.4, 3.6, 1H), 4.22 (dd, J = 10.6, 3.8, 1H), 4.62 (dd, J = 10.8, 6.4, 1H), 4.67 (dd, J = 10.4, 6.0, 1H), 5.56 (m, 1H), 5.65 (s, 1H), 5.70 (t, J = 4.6, 1H), 5.76 (m, 1H), 6.38 (s, 1H), 6.64 (d, J = 4.4, 1H), 7.26-7.50 (m, 8H), 7.58-7.64 (m, 4H), 8.05-8.10 (m, 8H).*

Perform benzoylation

35. Dissolve 4.1 g (12.5 mmol) **S.4** in 42 mL of 1:1 (v/v) DCM/pyridine in a 250-mL round-bottom flask.

36. Add 1.7 mL (14 mmol) BzCl at 0°C in an ice-water bath.

37. Remove the ice-water bath and stir the reaction at room temperature for 18 hr.

38. Cool the reaction mixture to 0°C and then add 16 mL of 0.1 M aq. HCl.

39. Separate the organic phase using a 250-mL separatory funnel and wash sequentially with:

    40 mL sat. $NaHCO_3$
    30 mL water
    30 mL brine.

40. Dry the organic phase over 1.6 g $MgSO_4$, then recrystallize the residue from 200 mL EtOH to afford the β-isomer.

41. Concentrate the ethanolic mother liquor and run the residue through a silica gel chromatography column using 2:1 (v/v) hexanes/EtOAc to yield the α-isomer.

*1,2,3-Tri-O-benzoyl-L-threofuranose (**S.6**) is obtained in a combined 72% yield (3.9 g, 9 mmol). ^{1}H-NMR α-isomer (300 MHz, $CDCl_3$): δ = 4.15 (dd, J = 11.6, 3.0, 1H), 4.70 (dd, J = 11.8, 6.0, 1H), 5.84 (m, 2H), 6.86 (d, J = 4.0, 1H), 7.38-7.55 (m, 9H), 7.95-7.20 (m, 6H). β-isomer (300 MHz, $CDCl_3$): δ = 4.32 (dd, J = 10.5, 3.0, 1H); 4.74 (dd, J = 10.7, 5.9, 1H), 5.64 (dd, J = 5.1, 3.0, 1H), 5.82 (s, 1H), 6.64 (s, 1H), 7.40-7.51 (m, 6H), 7.56-7.65 (m, 3H), 8.06-8.14 (m, 6H).*

BASIC PROTOCOL 2 SYNTHESIS OF THE PROTECTED TNA CYTIDINE NUCLEOSIDE PHOSPHORAMIDITE

This protocol describes the synthesis of the N^4-benzoylated TNA cytidine nucleoside phosphoramidite **S.10** from N^4-benzoylcytosine and **S.5** (Fig. 4.51.3).

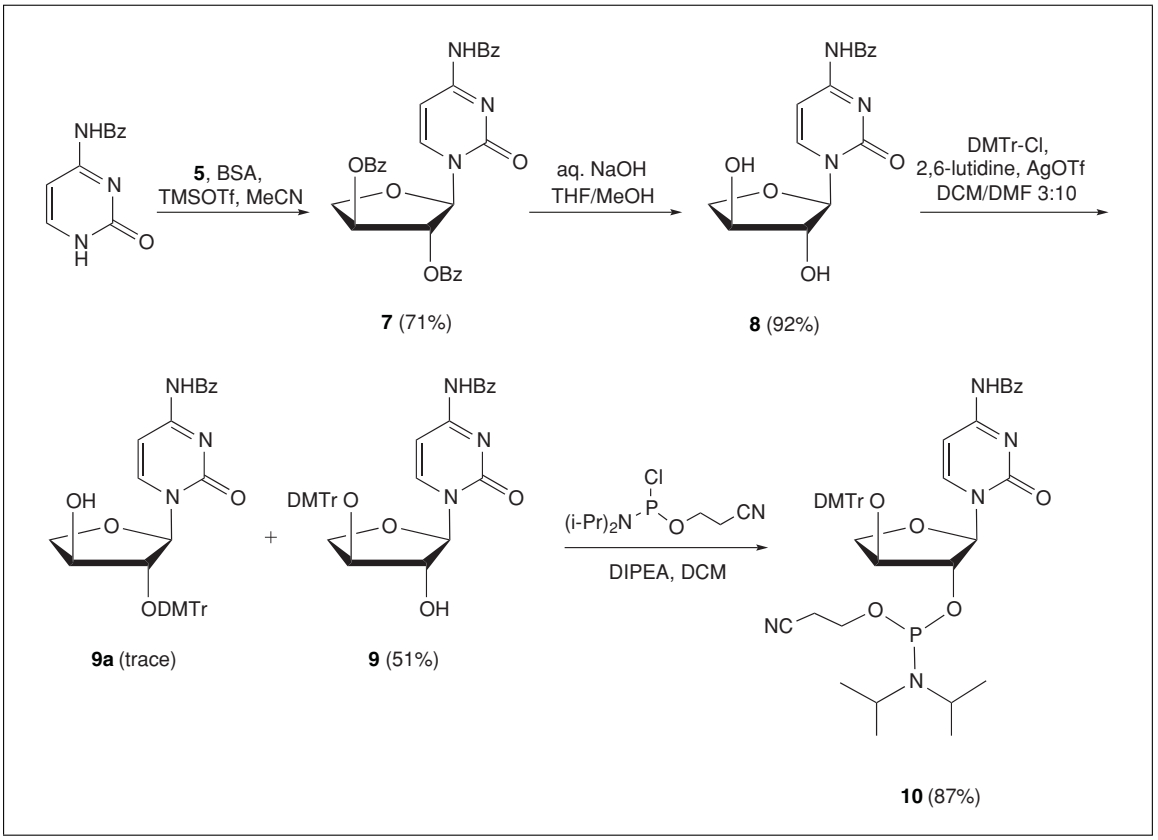

Figure 4.51.3 Preparation of cytidine TNA nucleoside phosphoramidite **S.10**. Abbreviations: BSA, *N,O*-bis(trimethylsilyl)acetamide; TMSOTf, trimethylsilyl trifluoromethanesulfonate; THF, tetrahydrofuran; DMTr-Cl, 4,4′-dimethoxytriphenylmethyl chloride; AgOTf, silver trifluoromethanesulfonate; DCM, dichloromethane; DMF, dimethylformamide; DIPEA, diisopropylethylamine.

Materials

1-*O*-Acetyl-2,3-di-*O*-benzoyl-L-threofuranose (**S.5**; see Basic Protocol 1)
Acetonitrile (MeCN), anhydrous
N,*O*-Bis(trimethylsilyl)acetamide (BSA)
N^4-Benzoylcytosine (C^{Bz})
Trimethylsilyl trifluoromethanesulfonate (TMSOTf)
Ethyl acetate (EtOAc)
Saturated aqueous sodium bicarbonate solution (sat. aq. $NaHCO_3$)
Brine (sat. aq. NaCl)
Magnesium sulfate ($MgSO_4$)
Silica gel (60 Å, 230-400 mesh, Whatman)
Hexanes
Dichloromethane (DCM), anhydrous
Tetrahydrofuran (THF)
Methanol (MeOH)
Sodium hydroxide (NaOH)
0.1 M aq. HCl
Toluene
N,*N*-Dimethylformamide (DMF), anhydrous
4,4′-Dimethoxytriphenylmethyl chloride (DMTr-Cl)
2,6-Lutidine
Silver trifluoromethanesulfonate (AgOTf)
Ultra pure water (H_2O)
Sodium sulfate (Na_2SO_4)
Triethylamine (Et_3N)
N,*N*-Diisopropylethylamine (DIPEA)
Chloro(2-cyanoethoxy)(diisopropylamino)phosphine
Argon gas
Benzene

50-, 100-, and 250-mL round-bottom flasks
Allihn condenser (reflux condenser)
Rotary evaporator equipped with a vacuum pump
Filter paper
Büchner funnel
250-mL separatory funnel
Gas balloon
Thin-layer chromatography (TLC) plate, EMD silica gel 60 F_{254}
UV lamp, 254 nm

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

Perform glycosylation

1. Dissolve 2.4 g (6.5 mmol) **S.5** in 19 mL anhydrous MeCN in a 100-mL round-bottom flask.
2. Add 4 mL (16.2 mmol) BSA and 1.45 g (6.8 mmol) N^4-benzoylcytosine. Attach a condenser to the flask and stir at 70°C for 1 hr.
3. Add 3.5 mL (19.4 mmol) TMSOTf and stir the reaction at 70°C for an additional ~1 hr until **S.5** is completely consumed. Monitor the reaction by TLC.
4. Cool the reaction to 24°C and dilute with 60 mL EtOAc. Pour the mixture into 76 mL sat. $NaHCO_3$ and stir for 15 min.

5. Isolate the product by vacuum filtration using a Büchner funnel, and wash with 50 mL cold EtOAc.

6. Wash the organic phase of the filtrate with 100 mL brine using a 250-mL separatory funnel and dry it over 2.0 g $MgSO_4$. Evaporate the organic solvent under reduced pressure.

7. Purify the residue by silica gel column chromatography, eluting the product with a step-wise gradient of 4:1 (v/v) hexanes/EtOAc to 3:1 (v/v) DCM/EtOAc.

*N^4-Benzoyl-1-(2′,3′-di-O-benzoyl-α-L-threofuranosyl)cytosine (**S.7**) is obtained in a combined 71% yield (2.4 g, 4.6 mmol) as a white solid. TLC (DCM/EtOAc 1:1): R_f 0.48. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 4.57 (d, J = 11.2, 1H), 4.61 (dd, J = 11.2, 3.6, 1H), 5.61 (d, J = 3.2, 1H), 5.84 (s, 1H), 6.30 (s, 1H), 7.40-7.66 (m, 10H), 7.87 (m, 2H), 7.92 (d, J = 7.6, 1H), 8.03 (d, J = 7.2, 1H), 8.09 (m, 2H), 8.75 (br, s, 1H).*

Perform debenzoylation

8. Suspend 2.6 g (5.1 mmol) **S.7** in 83 mL of 5:4:1 (v/v/v) THF/MeOH/H_2O in a 250-mL round-bottom flask and cool the mixture to 0°C in an ice-water bath.

9. Add 8.3 mL of 2 N NaOH and stir the reaction at 0°C for 30 min.

10. Neutralize by adding 8.3 mL of 2 N HCl and evaporate the solvent to dryness under reduced pressure.

11. Resuspend the residue in 20 mL MeOH and co-evaporate it with 30 mL toluene.

12. Resuspend the residue in 50 mL MeOH and adsorb the mixture onto silica gel at a sample/silica gel ratio of 1:1.2 (w/w).

13. Purify the residue by column chromatography, eluting the product with a step-wise gradient of 25:1 to 10:1 (v/v) DCM/MeOH.

*N^4-Benzoyl-1-(α-L-threofuranosyl)cytosine (**S.8**) is obtained in 92% yield (1.5 g, 4.7 mmol). TLC (DCM/MeOH 25:1): R_f 0.02. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 4.03 (s, 1H), 4.08 (s, 1H), 4.19 (m, 2H), 5.66 (s, 1H), 7.30 (d, J = 7.2, 1H), 7.52 (m, 2H), 7.63 (m, 1H), 8.00 (m, 2H), 8.11 (d, J = 7.6, 1H).*

Perform tritylation

14. Dissolve 1.5 g (4.7 mmol) **S.8** in 9 mL anhydrous DCM and 30 mL anhydrous DMF in a 100-mL round-bottom flask.

15. Add 2.2 g (6.3 mmol) DMTr-Cl, 2.8 mL (24 mmol) 2,6-lutidine, and 1.44 g (0.6 mmol) AgOTf. Stir at room temperature for 5 hr.

16. Add another 0.15 g (0.45 mmol) DMTr-Cl and 0.14 g (0.6 mmol) AgOTf, and stir the reaction at room temperature overnight.

17. Quench the reaction with 6 mL MeOH and then add 1.5 mL H_2O.

18. Dilute the mixture with 150 mL DCM, then filter the mixture and wash the residue with 50 mL DCM.

19. Wash the combined filtrate sequentially with:

100 mL ice-cold 0.1 M aq. HCl (2×)
100 mL water (2×)
100 mL sat. aq. $NaHCO_3$
100 mL brine.

20. Dry the solution over 1.7 g Na_2SO_4 and remove the organic solvent under reduced pressure.

21. Purify the residue by column chromatography. Deactivate the column with 97:3 (v/v) DCM/Et_3N and elute using a step-wise gradient of 99:1 (v/v) DCM/Et_3N to 98:1:1 (v/v/v) DCM/MeOH/Et_3N.

*N^4-Benzoyl-1-{3′-O-[(4′,4′′-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}cytosine (**S.9**) is obtained in 51.3% yield (1.5 g, 2.4 mmol). TLC (DCM/MeOH 16:1): R_f 0.26. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 3.37 (d, J = 9.6, 1H), 3.62 (dd, J = 10.0, 3.2, 1H), 3.77 (2s, 6H), 3.95 (br, s, 1H), 4.26 (m, 2H), 5.64 (s, 1H), 6.80-6.84 (m, 4H), 7.20-7.36 (m, 9H), 7.53-7.66 (m, 4H), 7.91 (1H), 7.92 (d, J = 7.6, 1H), 8.05 (d, J = 7.6, 1H), 8.76 (br, s, 1H).*

Perform phosphitylation

22. Dissolve 400 mg (0.68 mmol) **S.9** in 3.2 mL anhydrous DCM and 0.67 mL (3.8 mmol) DIPEA in a 50-mL round-bottom flask at room temperature under an argon atmosphere.

23. Add 180 µl (0.68 mmol) chloro(2-cyanoethoxy)(diisopropylamino)phosphine in a dropwise manner and then stir at room temperature for 1 hr.

24. Add an additional 20 µl (0.07 mmol) chloro(2-cyanoethoxy) (diisopropylamino) phosphine and stir at room temperature for 18 hr.

25. Quench the reaction with 1 mL MeOH and evaporate to dryness under reduced pressure.

26. Purify the residue by column chromatography. Deactivate the column with 97:3 (v/v) DCM/Et_3N, load the sample with 800 µL benzene, and elute the product using 59:39:2 (v/v/v) hexanes/EtOAc/Et_3N.

*N^4-Benzoyl-1-{2′-O-[(2-cyanoethoxy)(diisopropylamino)phosphino]-3′-O-[(4′,4′′-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}cytosine (**S.10**) is obtained in 87% yield (466 mg, 0.59 mmol) as a mixture of diastereomers. TLC (DCM/MeOH 50:1): R_f 0.11, 0.17. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.06-1.22 (m, 24H), 2.33, 2.68 (2m, 4H), 3.13 (d, J = 10, 1H), 3.22 (d, J = 9.6, 1H), 3.46-3.52 (m, 4H), 3.68, 3.69 (2s, 12H), 3.78-3.83 (m, 4H), 3.98-4.06 (m, 4H), 4.17 (d, J = 3.6, 1H), 4.47 (d, J = 8.4, 1H), 4.65 (d, J = 9.2, 1H), 5.79 (s, 1H), 5.81 (s, 1H), 6.72-6.76 (m, 8H), 7.09-7.19 (m, 14H), 7.45-7.58 (m, 8H), 7.84-7.85 (m, 6H), 7.91 (d, J = 7.6, 1H), 7.96 (d, J = 7.2, 1H), 8.58 (br, 2H). ^{31}P-NMR (161 MHz, $CDCl_3$): δ = 150.60, 152.58.*

SYNTHESIS OF THE PROTECTED TNA DIAMINOPURINE NUCLEOSIDE PHOSPHORAMIDITE

BASIC PROTOCOL 3

This protocol describes the synthesis of N^2,N^6-dibenzoylated TNA diaminopurine nucleoside phosphoramidite **S.14** from N^2,N^6-dibenzoyl-2,6-diaminopurine and **S.5** (Fig. 4.51.4).

Materials

N^2,N^6-Dibenzoyl-2,6-diaminopurine ($D^{(Bz)2}$)
Acetonitrile (MeCN), anhydrous
Argon gas
N,*O*-Bis(trimethylsilyl)acetamide (BSA)
1-*O*-Acetyl-2,3-di-*O*-benzoyl-L-threofuranose (**S.5**; see Basic Protocol 1)
Trimethylsilyl trifluoromethanesulfonate (TMSOTf)
Ethyl acetate (EtOAc)
Saturated aqueous sodium bicarbonate solution (sat. aq. $NaHCO_3$)
Brine (sat. aq. NaCl)

Magnesium sulfate ($MgSO_4$)
Silica gel (60 Å, 230-400 mesh, Whatman)
Hexanes
Tetrahydrofuran (THF)
Methanol (MeOH)
Ultra pure water (H_2O)
Sodium hydroxide (NaOH)
Ammonium chloride (NH_4Cl)
Dichloromethane (DCM)
N,*N*-Dimethylformamide (DMF)
4,4′-Dimethoxytriphenylmethyl chloride (DMTr-Cl)
2,6-Lutidine
4Å molecular sieves (freshly activated by heating at 300°C for at least 3 hr)
Silver trifluoromethanesulfonate (AgOTf)
0.1 M aq. HCl
Sodium sulfate (Na_2SO_4)
Triethylamine (Et_3N)
Benzene
N,*N*-Diisopropylethylamine (DIPEA)
Chloro(2-cyanoethoxy)(diisopropylamino)phosphine

50-, 100-, and 500-mL round-bottom flasks
Gas balloon
Rotary evaporator equipped with a vacuum pump
Filter paper
Büchner funnel
250-mL separatory funnel
Allihn condenser (reflux condenser)
Thin layer chromatography (TLC) plate, EMD silica gel 60 F_{254}
UV lamp, 254 nm

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

Perform glycosylation

1. Suspend 3.2 g (8.9 mmol) N^2,N^6-dibenzoyl-2,6-diaminopurine in 30 mL anhydrous MeCN in a 100-mL round-bottom flask. Stir the mixture under an argon atmosphere at 65°C.
2. Immediately add 5.9 mL (23.8 mmol) BSA and stir the mixture at 65°C for 30 min until a clear solution is formed.
3. Add 2.86 g (7.7 mmol) **S.5** in 25.5 mL anhydrous MeCN to the clear solution.
4. Add 2 mL (10 mmol) TMSOTf and stir the reaction at 65°C for 3 hr.
5. Cool the mixture to room temperature and dilute with 190 mL EtOAc. Wash the reaction mixture with 250 mL sat. aq. $NaHCO_3$.
6. Separate and filter the organic phase, and then wash with 200 mL sat. aq. $NaHCO_3$ followed by 200 mL brine.
7. Dry over 2.0 g $MgSO_4$ and then evaporate to dryness under reduced pressure.
8. Purify the residue by column chromatography, eluting with a step-wise gradient of 3:2 to 1:3 (v/v) hexanes/EtOAc.

*N^2,N^6-Dibenzoyl-9-(2′,3′-di-O-benzoyl-α-L-threofuranosyl)-2,6-diaminopurine (**S.11**) is obtained in 35% yield (1.8 g, 2.7 mmol) as a white foam. TLC (hexanes/EtOAc 1:3): R_f*

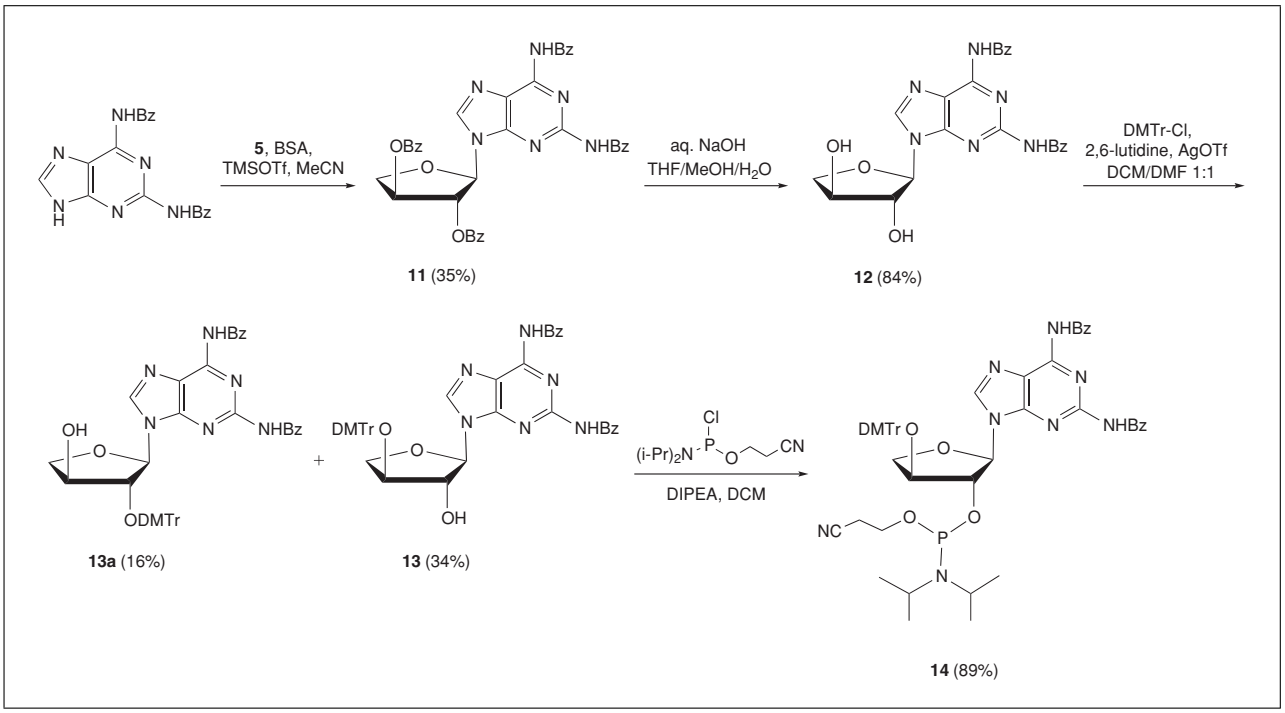

Figure 4.51.4 Preparation of diaminopurine TNA nucleoside phosphoramidite **S.14**. Abbreviations: BSA, *N,O*-bis(trimethylsilyl)acetamide; TMSOTf, trimethylsilyl trifluoromethanesulfonate; THF, tetrahydrofuran; DMTr-Cl, 4,4′-dimethoxytriphenylmethyl chloride; AgOTf, silver trifluoromethanesulfonate; DCM, dichloromethane; DMF, dimethylformamide; DIPEA, diisopropylethylamine.

0.48. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 4.64 (dd, J = 10.0, 6.4, 1H), 4.82 (dd, J = 9.6, 4.8, 1H), 5.89 (m, 1H), 6.49 (m, 2H), 7.33-8.10 (m, 20H), 8.61 (s, 1H), 10.99 (s, 1H), 11.27 (s, 1H).

Perform debenzoylation

9. Dissolve 1.9 g (2.8 mmol) **S.11** in 160 mL ice-cold 5:4:1 (v/v/v) THF/MeOH/H_2O in a 500-mL round-bottom flask.

10. Slowly add 11 mL (22.1 mmol) of 2 N aq. NaOH over 10 min, then stir at 0°C for an additional 5 min.

11. Quench the reaction with 1.25 (23.5 mmol) solid NH_4Cl. Keep stirring at 0°C for another 30 min.

12. Remove the solvent under reduced pressure and co-evaporate the residue twice with 50 mL MeOH.

13. Adsorb the residue onto 3.4 g silica gel and purify the product by column chromatography, eluting with a step-wise gradient of 100:1 to 40:1 (v/v) DCM/MeOH.

*N^2,N^6-Dibenzoyl-9-(α-L-threofuranosyl)-2,6-diaminopurine (**S.12**) is obtained in 84% yield (1.1 g, 2.4 mmol) as a white powder. TLC (DCM/MeOH 20:1): R_f 0.26. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 4.07 (dd, J = 9.2, 2.4, 1H), 4.14 (dd, J = 9.2, 3.6, 1H), 4.18 (m, 1H), 4.41 (m, 1H), 5.61 (br, s, 1H), 5.90 (d, J = 4.0, 1H), 5.95 (br, s, 1H), 7.46-7.59 (m, 6H), 7.98-8.05 (m, 4H), 8.05 (s, 1H), 11.00 (br, s, 2H).*

Perform tritylation

14. Dissolve 3.44 g (7.5 mmol) **S.12** in 63 mL of 1:1 (v/v) DCM/DMF in a 500-mL round-bottom flask at room temperature in a water bath under an argon atmosphere.

15. Add 3.46 g (10.2 mmol) DMTr-Cl with stirring, followed by 5.1 mL (44.2 mmol) 2,6-lutidine and then ~3.5 g activated molecular sieves.

16. Add 2.94 g (11.4 mmol) AgOTf in four portions over a period of 10 min at room temperature, then stir at room temperature for 4 hr.

17. Add another 457 mg (1.4 mmol) DMTr-Cl and 330 mg (1.3 mmol) AgOTf and stir for an additional 20 hr.

18. Quench the reaction by adding 2.5 mL MeOH.

19. Dilute with 290 mL DCM and filter the resulting mixture.

20. Cool the filtrate to 0°C and wash it with:

    290 mL cold 0.1 M aq. HCl (2×)
    200 mL sat. aq. $NaHCO_3$
    200 mL brine.

21. Dry the organic phase over 3.0 g Na_2SO_4 and then evaporate to dryness under reduced pressure.

22. Purify the residue by column chromatography. Deactivate the column with 97:3 (v/v) DCM/Et_3N and elute the product using a step-wise gradient of 100:100:25:25:2:1 to 100:100:25:25:2:4 (v/v/v/v/v/v) hexanes/benzene/DCM/EtOAc/Et_3N/MeOH and finally 35:1 (v/v) DCM/Et_3N plus 1% Et_3N.

    *N^2,N^6-Dibenzoyl-9-{3′-O-[(4′,4″-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}-2,6-diaminopurine (**S.13**) and its 2′-O-regioisomer N^2,N^6-dibenzoyl-9-{2′-O-[(4′,4″-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}-2,6-diaminopurine (**S.13a**) are obtained in 33.7% yield (2.0 g, 2.5 mmol) and 16% yield (0.9 g, 1.2 mmol), respectively. TLC (benzene/EtOAc/DCM/MeOH 8:1:1:0.2): R_f 0.40 (**S.13**), 0.35 (**S.13a**). ^{1}H-NMR **S.13** (400 MHz, $CDCl_3$): δ = 3.05 (dd, J = 9.4, 6.2, 1H), 3.36 (dd, J = 9.2, 6.2, 1H), 3.77 (2s, 6H), 4.44 (m, 1H), 4.76 (m, 1H), 5.79 (d, J = 4.4, 1H), 6.80-6.85 (m, 4H), 7.18-7.64 (m, 15H), 7.99-8.06 (m, 5H), 9.43 (br, s, 1H), 9.64 (br, s, 1H). **S.13a** (500 MHz, DMSO-d_6): δ = 3.62 (2s, 6H), 3.76 (m, 1H), 3.97 (d, J = 7.6, 1H), 4.08 (dd, J = 7.6, 2.4, 1H), 4.44 (s, 1H), 5.36 (d, J = 2.8, 1H), 5.92 (s, 1H), 6.70-6.77 (m, 4H), 7.14-7.65 (m, 16H), 7.98-8.20 (m, 4H), 11.01 (s, 1H), 11.19 (s, 1H).*

Perform phosphitylation

23. Dissolve 400 mg (0.52 mmol) **S.13** in 3.8 mL anhydrous DCM and 0.34 mL DIPEA in a 50-mL round-bottom flask at room temperature under an argon atmosphere.

24. Add 840 µl (3.7 mmol) chloro (2-cyanoethoxy) (diisopropylamino) phosphine in a dropwise manner and stir the reaction at room temperature for 1 hr.

25. Add an additional 84 µl (0.4 mmol) chloro (2-cyanoethoxy) (diisopropylamino) phosphine and stir at room temperature for an additional 1 hr.

26. Evaporate the solvent to dryness under reduced pressure.

27. Purify the residue by column chromatography. Deactivate the column with 97:3 (v/v) DCM/Et_3N, load the sample with 800 µL benzene, and elute the product using 10:1:1:0.2:0.2 (v/v/v/v/v) benzene/DCM/EtOAc/Et_3N/MeOH.

    *N^2,N^6-Dibenzoyl-9-{2′-O-[(2-cyanoethoxy)(diisopropylamino)phosphino]-3′-O-[(4′,4″-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}-2,6-diaminopurine (**S.14**) is obtained in 89% yield (450 mg, 0.46 mmol) as a mixture of two diastereoisomers. TLC (DCM/MeOH 50:1): R_f 0.21. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.08-1.30 (m, 24H), 2.39 (m, 2H), 2.60 (m, 2H), 3.36-3.39 (m, 1H), 3.54-3.90 (m, 8H), 3.72 (2s, 12H), 4.27 (m, 2H), 4.35 (m, 1H), 4.71 (d, J = 8.0, 2H), 5.16 (d, J = 9.6, 1H), 6.12 (s, 1H), 6.22 (s, 2H),6.78 (m, 8H), 7.12-7.35 (m, 17H), 7.46-7.50 (m, 13H), 7.94-8.04 (m, 8H), 8.26, 8.31 (2s, 2H), 8.90, 9.11, 9.23 (3br, 4H). ^{31}P-NMR (161 MHz, $CDCl_3$): δ = 150.5, 151.9. MALDI-TOF MS (pos. 4-hydroxybenzylidenemalononitrile): 985.63 (M+Na^+).*

SYNTHESIS OF THE PROTECTED TNA GUANOSINE NUCLEOSIDE PHOSPHORAMIDITE

This protocol describes the synthesis of base-protected TNA guanosine nucleoside phosphoramidite **S.18** from N^2-acetyl-O^6-diphenylcarbamoylguanine and **S.5** (Fig. 4.51.5).

Materials

N^2-Acetyl-O^6-diphenylcarbamoylguanine (G^{PAC})
1,2-Dichloroethane (DCE), anhydrous
N,*O*-Bis(trimethylsilyl)acetamide (BSA)
Argon gas
Toluene, anhydrous
Trimethylsilyl trifluoromethanesulfonate (TMSOTf)
1-*O*-Acetyl-2,3-di-*O*-benzoyl-L-threofuranose (**S.5**; see Basic Protocol 1)
Ethyl acetate (EtOAc)
Saturated aqueous sodium bicarbonate solution (sat. aq. $NaHCO_3$)
Brine (sat. aq. NaCl)
Magnesium sulfate ($MgSO_4$)
Silica gel (60 Å, 230-400 mesh, Whatman)
Dichloromethane (DCM), anhydrous
Triethylamine (Et_3N)
Methanol (MeOH)
Tetrahydrofuran (THF)
Ultra pure water (H_2O)
Sodium hydroxide (NaOH)
Ammonium chloride (NH_4Cl)
N,*N*-Dimethylformamide (DMF)
2,6-Lutidine
4,4′-Dimethoxytriphenylmethyl chloride (DMTr-Cl)
N,*N*-Diisopropylethylamine (DIPEA)
Chloro(2-cyanoethoxy)(diisopropylamino)phosphine
Pentane

25-, 100-, 250-, and 500-mL round-bottom flasks
Allihn condenser
Gas balloon
Rotary evaporator equipped with a vacuum pump
Filter paper
Büchner funnel
Thin layer chromatography (TLC) plate, EMD silica gel 60 F_{254}
UV lamp, 254 nm

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

Perform glycosylation

1. Suspend 3.2 g (8.3 mmol) N^2-acetyl-O^6-diphenylcarbamoylguanine in 77 mL anhydrous DCE in a 250-mL round-bottom flask and attach a condenser to the flask.
2. Add 4 mL (16.8 mmol) BSA and stir under an argon atmosphere at 70°C for 1 hr.
3. Evaporate the solvent to dryness and dissolve the residue in 35 mL anhydrous toluene.
4. Add 4 mL (16.8 mmol) TMSOTf.
5. Add a solution of 2.8 g (7.6 mmol) **S.5** in 35 mL anhydrous toluene and stir at 70°C for 2.5 hr.

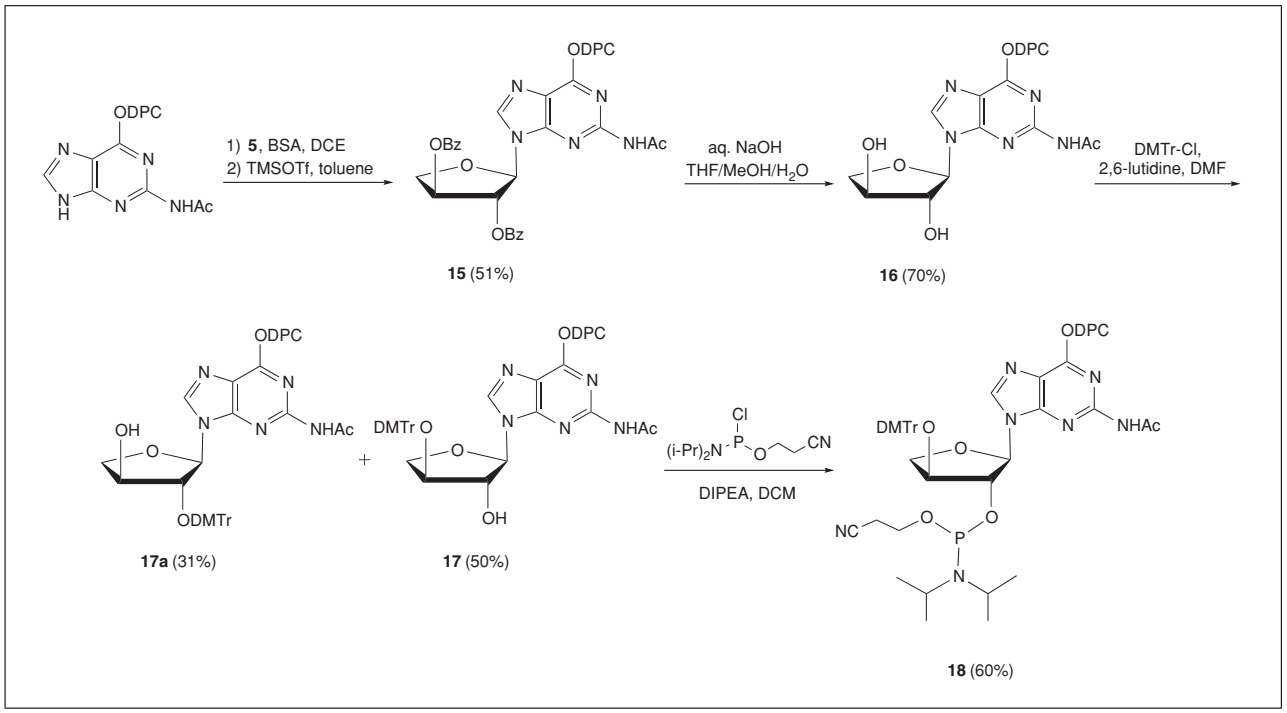

Figure 4.51.5 Preparation of guanosine TNA nucleoside phosphoramidite **S.18**. Abbreviations: DPC, diphenylcarbamoyl; BSA, *N,O*-bis(trimethylsilyl)acetamide; DCE, 1,2-dichloroethane; TMSOTf, trimethylsilyl trifluoromethanesulfonate; THF, tetrahydrofuran; DMTr-Cl, 4,4′-dimethoxytriphenylmethyl chloride; DMF, dimethylformamide; DCM, dichloromethane; DIPEA, diisopropylethylamine.

6. Cool the reaction to room temperature, then dilute with 70 mL EtOAc.
7. Wash with 100 mL sat. aq. $NaHCO_3$ followed by 100 mL brine.
8. Dry over $MgSO_4$ and remove the organic solvent under reduced pressure.
9. Purify the residue by column chromatography, eluting with a step-wise gradient of 18:1 (v/v) DCM/EtOAc plus 1% Et_3N to 13:1:0.05 (v/v/v) DCM/EtOAc/MeOH plus 1% Et_3N.

*N^2-Acetyl-O^6-diphenylcarbamoyl-9-(2′,3′-di-O-benzoyl-α-L-threofuranosyl)guanine (**S.15**) is obtained in 51% yield (2.74 g, 4.2 mmol) as a colorless foam. TLC (hexanes/EtOAc 1:3): R_f 0.27. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 2.48 (s, 3H), 4.57 (dd, J = 11.0, 1.8, 1H), 4.66 (dd, J = 11.0, 4.6, 1H), 5.74 (dd, J = 2.8, 1.6, 1H), 6.21 (s, 1H), 6.32 (d, J = 1.6, 1H), 7.34-8.09 (m, 20H), 8.23 (s, 1H).*

Perform debenzoylation

10. Dissolve 2.74 g (3.9 mmol) **S.15** in 230 mL ice-cold 5:4:1 (v/v/v) THF/MeOH/H_2O in a 500-mL round-bottom flask.
11. Slowly add 16 mL of 2 N aq. NaOH over 5 min under an argon atmosphere, then stir at 0°C for an additional 15 min.
12. Quench the reaction with 1.8 (34 mmol) solid NH_4Cl. Continue stirring the reaction at 0°C for 30 min.
13. Remove the solvent under reduced pressure.
14. Adsorb the residue onto 3.2 g silica gel and purify by column chromatography, eluting the product with 15:1 (v/v) DCM/MeOH.

*N^2-Acetyl-O^6-diphenylcarbamoyl-9-(α-L-threofuranosyl)guanine (**S.16**) is obtained in 70% yield (1.34 g, 2.7 mmol) as a white powder. TLC (DCM/MeOH 5:1): R_f 0.68. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 4.08-4.16 (m, 3H), 4.42 (m, 1H), 5.53 (d, J = 3.2,*

1H), 5.88 (d, J = 4.4, 1H), 5.92 (d, J = 1.6, 1H), 7.29-7.48 (m, 10H), 8.52 (s, 1H), 10.73 (br, s, 1H).

Perform tritylation

15. Dissolve 1.0 g (2.0 mmol) **S.16** in 30 mL anhydrous DMF in a 100-mL round-bottom flask at room temperature under an argon atmosphere.

16. Add 0.94 mL (8.2 mmol) 2,6-lutidine and 1.4 g (4.1 mmol) DMTr-Cl with stirring, then continue stirring at room temperature for 24 hr.

17. Add an additional 350 mg (1.0 mmol) DMTr-Cl and stir at room temperature for an additional 6 hr.

18. Quench the reaction by adding 8.5 mL MeOH.

19. Dilute with 25 mL DCM and wash the organic solvent with 40 mL sat. aq. $NaHCO_3$ and 40 mL brine.

20. Dry the organic phase over 1.5 g $MgSO_4$ and evaporate the solvent to dryness under reduced pressure.

21. Purify the residue by column chromatography. Deactivate the column with 97:3 (v/v) DCM/Et_3N and elute the product using a step-wise gradient of 99:1 (v/v) DCM/Et_3N to 98:1:1 (v/v/v) DCM/MeOH/Et_3N.

    *N^2-Acetyl-O^6-diphenylcarbamoyl-9-{3′-O-[(4′,4′′-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}guanine (**S.17**) and its 2′-O-regioisomer N^2-acetyl-O^6-diphenylcarbamoyl-9-{2′-O-[(4′,4′′-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}guanine (**S.17a**) are obtained in 50% yield (0.79 g, 1 mmol) and 31% yield (0.5 g, 0.6 mmol), respectively. TLC (DCM/MeOH 25:1) R_f 0.36 (**S.17**), 0.52 (**S.17a**). ^{1}H-NMR **S.17** (500 MHz, DMSO-d_6): δ = 2.20 (s, 3H), 3.44 (dd, J = 7.4, 4.4, 1H), 3.58 (dd, J = 7.6, 2.8, 1H), 3.68 (2s, 6H), 4.13 (m, 1H), 4.44 (m, 1H), 5.78 (m, 2H), 6.79-6.81 (m, 4H), 7.10-7.48 (m, 19H), 8.49 (s, 1H), 10.68 (s, 1H). **S.17a** (500 MHz, DMSO-d_6): δ = 2.16 (s, 3H), 3.52 (s, 3H), 3.56 (s, 3H), 3.92 (dd, J = 8.0, 2.8, 1H), 4.12 (m, 2H), 4.53 (s, 1H), 5.42 (d, J = 7.2, 1H), 5.62 (s, 1H), 6.61-6.70 (m, 4H), 7.13-7.47 (m, 19H), 8.17 (s, 1H), 10.73 (s, 1H).*

Perform phosphitylation

22. Dissolve 200 mg (0.25 mmol) **S.17** and 0.27 mL (2.5 mmol) DIPEA in 6.2 mL anhydrous DCM in a 25-mL round-bottom flask.

23. Add 140 µl (0.62 mmol) chloro(2-cyanoethoxy)(diisopropylamino)phosphine in a dropwise manner and stir the reaction at room temperature for 45 min.

24. Add an extra 15 µl (0.07 mmol) chloro (2-cyanoethoxy) (diisopropylamino) phosphine and stir at room temperature for an additional 1 hr.

25. Quench the reaction with 1 mL MeOH and evaporate to dryness under reduced pressure.

26. Purify the residue by column chromatography. Deactivate the column with 97:3 (v/v) DCM/Et_3N and elute the product using 99:1 (v/v) DCM/Et_3N to give a syrup.

27. Dissolve the syrup in 2 mL DCM and add slowly to 90 mL of rapidly stirred pentane. Collect the white precipitate by filtration.

    *N^2-Acetyl-O^6-diphenylcarbamoyl-9-{2′-O-[(2-cyanoethoxy) (diisopropylamino) phosphino]-3′-O-[(4′,4′′-dimethoxytriphenyl)methyl]-α-L−threofuranosyl}guanine (**S.18**) is obtained in 60% yield (150 mg, 0.15 mmol) as a mixture of diastereomers. TLC (DCM/MeOH 50:1): R_f 0.24, 0.33. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.06-1.15 (m, 24H), 2.12-2.44 (m, 4H), 2.51, 2.60 (2s, 6H), 3.51-3.68 (m, 10H), 3.69-3.72 (4s, 12H), 4.30, 4.42 (m, 2H), 4.70 (d, J = 8.4, 1H), 4.79 (d, J = 8.0, 1H), 5.96, 6.03 (2s, 2H), 6.74-7.44 (m, 46H), 7.90, 8.02 (2br, 2H), 8.24, 8.25 (2s, 2H). ^{31}P-NMR (161 MHz, $CDCl_3$): 150.99, 151.34.*

BASIC PROTOCOL 5

SYNTHESIS OF THE TNA THYMIDINE NUCLEOSIDE PHOSPHORAMIDITE

This protocol describes the synthesis of TNA thymidine nucleoside phosphoramidite **S.22** from thymine and **S.5** (Fig. 4.51.6).

Materials

1-*O*-Acetyl-2,3-di-*O*-benzoyl-L-threofuranose (**S.5**; see Basic Protocol 1)
Thymine
Acetonitrile, anhydrous (MeCN)
N,*O*-Bis(trimethylsilyl)acetamide (BSA)
Argon gas
Trimethylsilyl trifluoromethanesulfonate (TMSOTf)
Saturated aqueous sodium bicarbonate solution (sat. aq. $NaHCO_3$)
Ethyl acetate (EtOAc)
Brine (sat. aq. NaCl)
Magnesium sulfate ($MgSO_4$)
Silica gel (60 Å, 230-400 mesh, Whatman)
Dichloromethane (DCM), anhydrous
2 M NH_3 in MeOH
Ultra pure water (H_2O)
N,*N*-Dimethylformamide (DMF), anhydrous
4,4′-Dimethoxytriphenylmethyl chloride (DMTr-Cl)
2,6-Lutidine
Silver trifluoromethanesulfonate (AgOTf)
Methanol (MeOH)
0.1 M aq. HCl
Sodium sulfate (Na_2SO_4)
Triethylamine (Et_3N)
N,*N*-Diisopropylethylamine (DIPEA)
Chloro(2-cyanoethoxy)(diisopropylamino)phosphine
Hexanes

50-, 100-, and 500-mL round-bottom flasks
Allihn condenser (reflux condenser)
Gas balloon
250-mL separatory funnel
Rotary evaporator equipped with a vacuum pump
Filter paper
Büchner funnel
Thin layer chromatography (TLC) plate, EMD silica gel 60 F_{254}
UV lamp, 254 nm

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

Perform glycosylation

1. Suspend 4.4 g (12 mmol) **S.5** and 1.5 g (12 mmol) thymine in 35 mL anhydrous MeCN in a 100-mL round-bottom flask.
2. Attach a condenser to the flask and heat the mixture to 60°C.
3. Add 5.8 mL (24 mmol) BSA and stir the reaction at 60°C for 1 hr under an argon atmosphere.

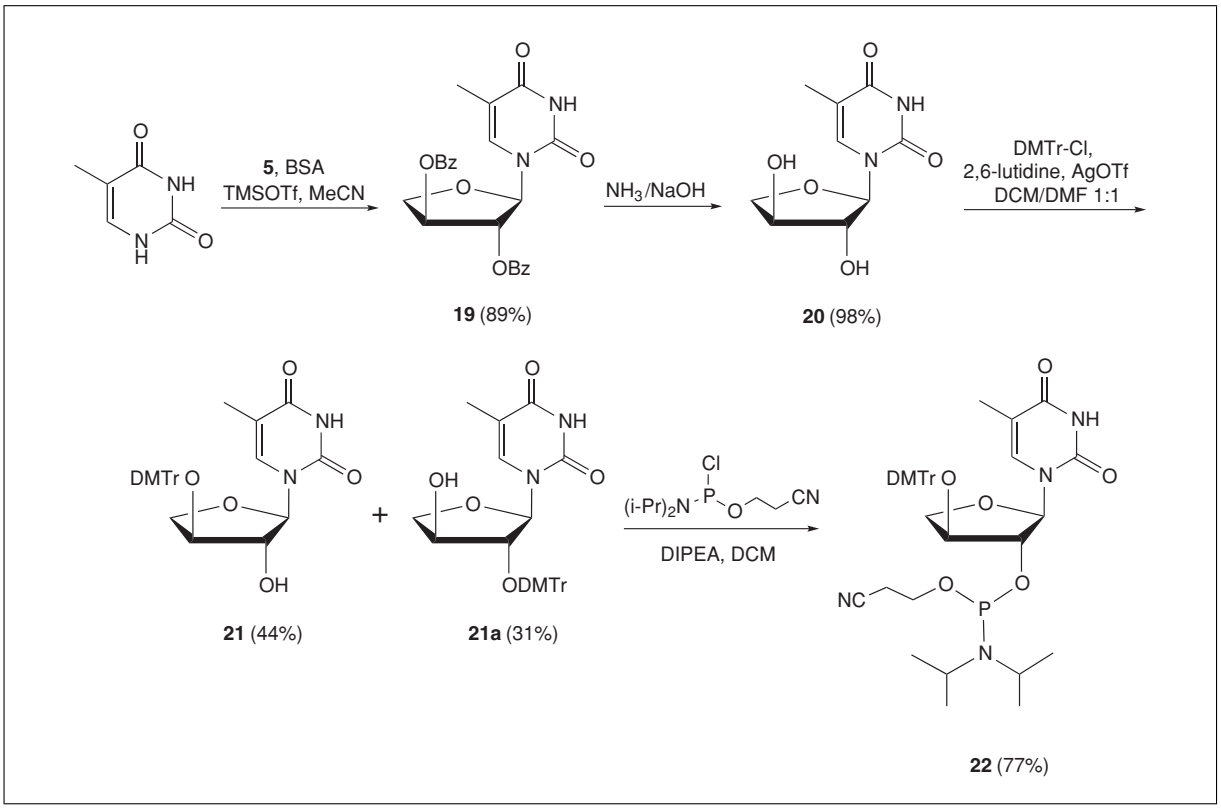

Figure 4.51.6 Preparation of thymidine TNA nucleoside phosphoramidite **S.22**. Abbreviations: BSA, *N,O*-bis (trimethylsilyl) acetamide; TMSOTf, trimethylsilyl trifluoromethanesulfonate; DMTr-Cl, 4,4′-dimethoxytriphenylmethyl chloride; AgOTf, silver trifluoromethanesulfonate; DCM, dichloromethane; DMF, dimethylformamide; DIPEA, diisopropylethylamine.

4. Add 6.4 mL (36 mmol) TMSOTf and stir the reaction at 60°C for an additional ~3 hr until **S.5** is completely consumed. Monitor the reaction by TLC.
5. Cool the reaction to 24°C and then pour into an ice-cold stirred mixture of 120 mL sat. aq. $NaHCO_3$ and 150 mL EtOAc.
6. Separate the organic phase and wash with 120 mL brine.
7. Dry over 3.0 g $MgSO_4$ and evaporate the organic solvent under reduced pressure.
8. Purify the residue by silica gel column chromatography, eluting the product with 7:1 (v/v) DCM/EtOAc.

*1-(2′,3′-Di-O-benzoyl-α-L-threofuranosyl)thymine (**S.19**) is obtained in 89% yield (2.4 g, 4.6 mmol) as a colorless foam. TLC (hexanes/EtOAc 1:1): R_f 0.41. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.88 (s, 3H), 4.48 (m, 2H), 5.60 (t, 1H), 5.67 (s, 1H), 6.21 (m, 1H), 7.27-8.09 (m, 11H), 8.25 (br, s, 1H).*

Perform debenzoylation

9. Suspend 4.67 g (11.1 mmol) **S.19** in 220 mL of 2 M NH_3 in MeOH in a 500-mL round-bottom flask and stir the reaction at room temperature for ~18 hr.
10. Concentrate the solution under reduced pressure and co-evaporate the residue three times with 30 mL water.
11. Dissolve the residue in 50 mL water and wash the solution with 50 mL DCM.
12. Remove the solvent under reduced pressure to give the product.

*1-(α-L-Threofuranosyl)thymine (**S.20**) is obtained in 98% yield (2.5 g, 10.9 mmol). ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 1.74 (s, 3H), 3.32 (br, s, 2H), 4.00 (m, 4H), 5.64 (m, 1H), 7.56 (m, 1H), 11.27 (br, s, 1H).*

Perform tritylation

13. Dissolve 2.5 g (11 mmol) **S.20** in 150 mL of 1:1 (v/v) anhydrous DCM/DMF in a 500-mL round-bottom flask at room temperature under an argon atmosphere.

14. Add 4.4 g (13 mmol) DMTr-Cl with stirring, then add 6.4 mL (55 mmol) of 2,6-lutidine and 3.1 g (12 mmol) AgOTf. Stir at room temperature overnight under an argon atmosphere.

15. Quench the reaction by adding 15.2 mL MeOH. Dilute with 500 mL DCM and then filter the resulting mixture.

16. Wash the filtrate sequentially with:

    200 mL ice-cold 0.1 M aq. HCl (2×)
    200 mL water (2×)
    200 mL sat. aq. $NaHCO_3$ (2×)
    300 mL brine.

17. Dry the organic phase over 2.5 g Na_2SO_4 and evaporate to dryness under reduced pressure.

18. Purify the residue by column chromatography. Deactivate the column with 97:3 (v/v) DCM/Et_3N and elute the product using a step-wise gradient of 100:1 (v/v) DCM/Et_3N to 98:1:1 (v/v/v) DCM/MeOH/Et_3N.

    *1-{3′-O-[(4′,4″-Dimethoxytriphenyl)methyl]-α-L-threofuranosyl}thymine (**S.21**) and its 2′-O-regioisomer 1-{2′-O-[(4′,4″-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}thymine (**S.21a**) are obtained in 44% yield (2.55 g, 4.8 mmol) and 31% yield (1.79 g, 3.4 mmol), respectively. TLC (DCM/MeOH 25:1): R_f 0.33 (**S.21**), 0.43 (**S.21a**). ^{1}H-NMR **S.21** (400 MHz, DMSO-d_6): δ = 1.68 (s, 3H), 3.56-3.57 (m, 3H), 3.60 (s, 3H), 3.73 (s, 3H), 3.99 (d, J = 1.6, 1H), 5.46 (d, J = 1.6, 1H), 5.58 (d, J = 4.4, 1H), 6.85-6.88 (m, 4H), 7.20-7.50 (m, 10H), 11.28 (s, 1H). **S.21a** (400 MHz, DMSO-d_6): δ = 1.66 (s, 3H), 3.32 (s, 3H), 3.34 (s, 3H), 3.71 (d, J = 2.8, 1H), 3.82 (m, 2H), 3.92 (d, J = 1.6, 1H), 5.10 (d, J = 2.8, 1H), 5.91 (d, J = 1.6, 1H), 6.80-7.20 (m, 4H), 7.21-7.39 (m, 10H), 11.33 (s, 1H).*

Perform phosphitylation

19. Dissolve 330 mg (0.63 mmol) **S.21** and 0.43 mL (2.5 mmol) DIPEA in 4.0 mL anhydrous DCM in a 50-mL round-bottom flask.

20. Add 150 µl (0.7 mmol) chloro(2-cyanoethoxy)(diisopropylamino)phosphine in a dropwise manner and stir at room temperature for 1 hr.

21. Add an extra 15 µl (0.07 mmol) chloro (2-cyanoethoxy) (diisopropylamino) phosphine and stir at room temperature overnight.

22. Evaporate to dryness under reduced pressure.

23. Purify the residue by column chromatography. Deactivate the column with 97:3 (v/v) DCM/Et_3N and elute the product using a step-wise gradient of 80:18:2 to 50:48:2 (v/v/v) hexanes/EtOAc/Et_3N.

    *1-{2′-O-[(2-Cyanoethoxy)(diisopropylamino)phosphino]-3′-O-[(4′,4″-dimethoxytriphenyl)methyl]-α-L−threofuranosyl}thymine (**S.22**) is obtained in 77% yield (400 mg, 0.48 mmol) as a mixture of diastereomers. TLC (DCM/MeOH 25:2): R_f 0.26, 0.36. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.03, 1.13 (m, 24H), 1.79 (s, 6H), 2.64 (m, 4H), 3.13 (m, 2H), 3.55-3.80 (m, 18H), 4.25 (d, J = 3.3, 1H), 4.47 (d, J = 9.3, 1H), 5.77 (br, 2H), 6.81-6.86 (m, 8H), 7.22-7.40 (m, 20H). ^{31}P-NMR (202 MHz, $CDCl_3$): 153.4, 154.4.*

SYNTHESIS OF THE PROTECTED TNA ADENOSINE NUCLEOSIDE PHOSPHORAMIDITE

This protocol describes the synthesis of N^6-benzoylated TNA adenosine nucleoside phosphoramidite **S.26** from N^6-benzoyladenine and **S.6** (Fig. 4.51.7).

Materials

1,2,3-Tri-*O*-benzoyl-L-threofuranose (**S.6**; see Basic Protocol 1)
Acetonitrile (MeCN), anhydrous
N^6-Benzoyladenine (A^{Bz})
N,*O*-Bis(trimethylsilyl)acetamide (BSA)
Tin (IV) chloride ($SnCl_4$)
Argon gas
Ethyl acetate (EtOAc)
Saturated aqueous sodium bicarbonate solution (sat. aq. $NaHCO_3$)
Magnesium sulfate ($MgSO_4$)
Silica gel (60 Å, 230-400 mesh, Whatman)
Dichloromethane (DCM), anhydrous
Tetrahydrofuran (THF)
Methanol (MeOH)
Ultra pure water (H_2O)
Sodium hydroxide (NaOH)
2 N and 0.1 M aq. HCl
Toluene
Ethanol (EtOH)
N,*N*-Dimethylformamide (DMF), anhydrous
4,4′-Dimethoxytriphenylmethyl chloride (DMTr-Cl)
2,6-Lutidine
Silver trifluoromethanesulfonate (AgOTf)
Brine (sat. aq. NaCl)
Sodium sulfate (Na_2SO_4)
Triethylamine (Et_3N)
N,*N*-Diisopropylethylamine (DIPEA)
Chloro(2-cyanoethoxy)(diisopropylamino)phosphine
Hexanes

10-, 100-, and 250-mL round-bottom flasks
Allihn condenser
Gas balloon
250-mL separatory funnel
Rotary evaporator equipped with a vacuum pump
Filter paper
Büchner funnel
Thin layer chromatography (TLC) plate, EMD silica gel 60 F_{254}
UV lamp, 254 nm

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

Perform glycosylation

1. Dissolve 1.33 g (3.1 mmol) **S.6** in 26 mL anhydrous MeCN in a 100-mL round-bottom flask and attach a condenser to the flask.
2. Add 830 mg (3.5 mmol) N^6-benzoyladenine and 1.5 mL (6.2 mmol) BSA. Stir the reaction at 65°C for ~30 min until a clear solution is formed.

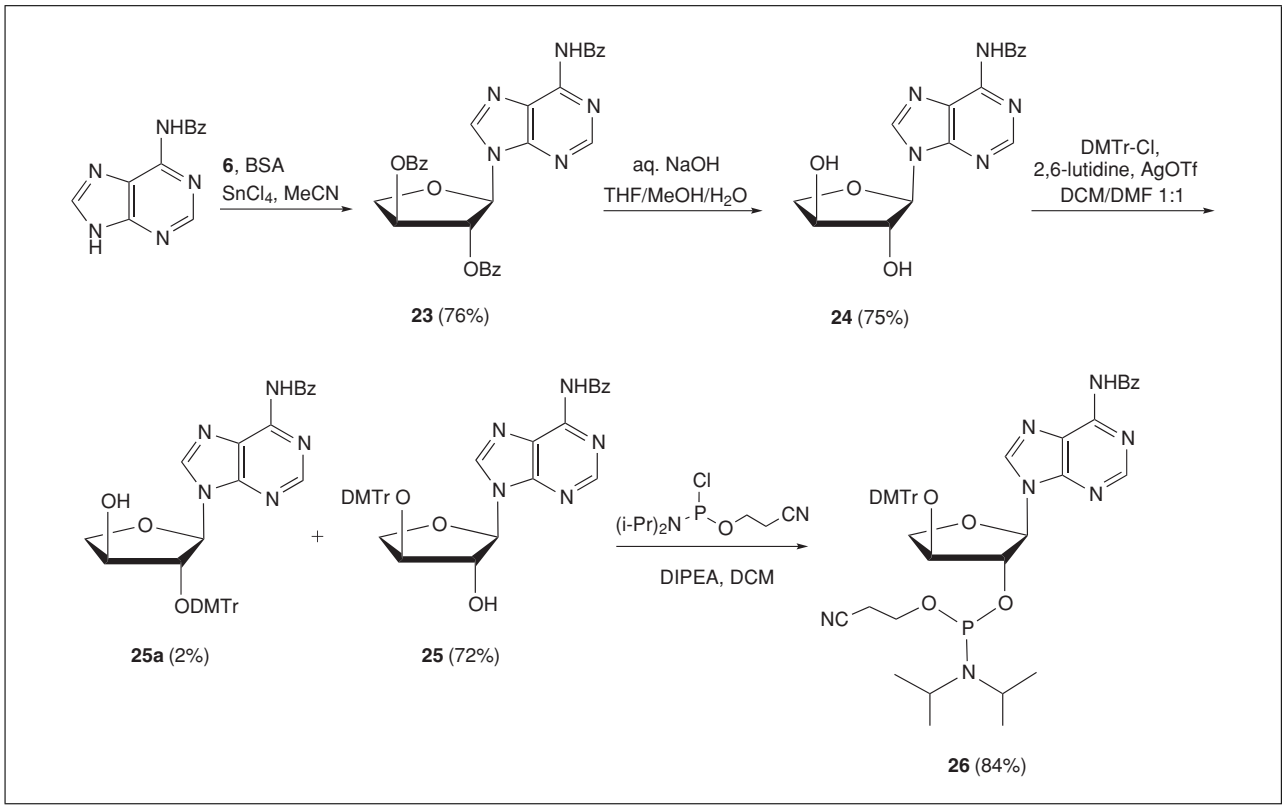

Figure 4.51.7 Preparation of adenosine TNA nucleoside phosphoramidite **S.26**. Abbreviations: BSA, *N,O*-bis (trimethylsilyl) acetamide; THF, tetrahydrofuran; DMTr-Cl, 4,4′-dimethoxytriphenylmethyl chloride; AgOTf, silver trifluoromethanesulfonate; DCM, dichloromethane; DMF, dimethylformamide; DIPEA, diisopropylethylamine.

3. Add 1.1 mL (5 mmol) $SnCl_4$ in three portions over 2 min. Stir the reaction at 65°C for 1 hr under an argon atmosphere.
4. Cool the reaction to room temperature and pour into 400 mL of ice-cold 1:1 (v/v) EtOAc and sat. aq. $NaHCO_3$.
5. Separate the organic phase, extract the aqueous phase with 150 mL EtOAc, and combine the organic phases.
6. Wash the combined organic phase with 300 mL sat. aq. $NaHCO_3$, dry over 1.8 g $MgSO_4$, and evaporate to dryness under reduced pressure.
7. Purify the residue by column chromatography, eluting the product with 5:1 (v/v) DCM/EtOAc.

   *N^6-Benzoyl-9-(2′,3′-di-O-benzoyl-α-L-threofuranosyl)adenine (**S.23**) is obtained in 76% yield (1.29 g, 2.3 mmol) as a colorless foam. TLC (DCM/EtOAc 4:1): R_f 0.13. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 4.58 (dd, J = 11.0, 2.2, 1H), 4.68 (dd, J = 10.8, 4.8, 1H), 5.77 (t, J = 2.2, 1H), 6.30 (dd, J = 2.0, 0.5, 1H), 6.47 (d, J = 2.0, 1H), 7.43-7.67 (m, 9H), 7.86-7.88 (m, 2H), 8.03-8.11 (m, 4H),8.33 (s, 1H), 8.79 (s, 1H), 9.02 (br, 1H).*

Perform debenzoylation

8. Dissolve 1.28 g (2.3 mmol) **S.22** in 45 mL of 5:4:1 (v/v/v) THF/MeOH/H_2O in a 250-mL round-bottom flask in an ice-water bath at 0°C.
9. Slowly add 3 mL of 2 N aq. NaOH and then stir the reaction at 0°C for 20 min.
10. Neutralize the solution by adding 3 mL of 2 N aq. HCl.
11. Evaporate the filtrate to dryness under reduced pressure.

12. Dissolve the residue in 20 mL MeOH and co-evaporate from 20 mL toluene.

13. Purify the residue by column chromatography, eluting the product using a step-wise gradient from 1:1 (v/v) DCM/EtOAc to 12:1 (v/v) DCM/EtOH.

*N^6-Benzoyl-9-(α-L-threofuranosyl)adenine (**S.24**) is obtained in 76% yield (600 mg, 1.8 mmol) as a colorless crystal. TLC (DCM/EtOAc 1:1): R_f 0.03. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 4.17 (m, 3H), 4.44 (t, J = 2.0, 1H), 5.58 (d, J = 2.8, 1H), 5.95 (d, J = 4.4, 1H), 6.06 (d, J = 1.6, 1H), 7.56 (m, 2H), 7.65 (m, 1H), 8.05 (m, 2H), 8.55 (s, 1H), 8.76 (s, 1H), 11.18 (br, 1H).*

Perform tritylation

14. Dissolve 500 mg (1.45 mmol) **S.24** in 9 mL of 1:1 (v/v) anhydrous DCM/DMF in a 100-mL round-bottom flask.

15. Cool the solution to 10°-15°C.

16. Add 650 mg (1.9 mmol) DMTr-Cl, 0.9 mL (7.3 mmol) 2,6-lutidine, and 440 mg (1.7 mmol) AgOTf, and stir the reaction at 10°C for 5 hr.

17. Add 65 mg (0.2 mmol) DMTr-Cl and 44 mg (0.2 mmol) AgOTf, and stir at room temperature overnight.

18. Quench the reaction by adding 1.5 mL of 1:4 (v/v) MeOH/H_2O.

19. Dilute the mixture with 75 mL DCM, then filter the mixture, and wash the residue with 75 mL DCM.

20. Wash the combined filtrate using a 250-mL separatory funnel sequentially with:

60 mL ice-cold 0.1 M HCl (2×)
60 mL water (2×)
100 mL sat. aq. $NaHCO_3$
100 mL brine.

21. Dry the organic solvent over 1.0 g Na_2SO_4 and evaporate to dryness under reduced pressure.

22. Purify the residue by column chromatography. Deactivate the column with 97:3 (v/v) DCM/Et_3N and elute the product using a step-wise gradient of 99:1 (v/v) DCM//Et_3N to 98:1:1 (v/v/v) DCM/MeOH/Et_3N.

*N^6-Benzoyl-9-{3′-O-[(4′,4′′-dimethoxytriphenyl)methyl]-α-L−threofuranosyl}adenine (**S.25**) and its 2′-O-regioisomer N^6-benzoyl-9-{2′-O-[(4′,4′′-dimethoxytriphenyl)methyl]-α−L-threofuranosyl}adenine (**S.25a**) are obtained in 72% yield (679 mg, 1.05 mmol) and 2% yield (25 mg, 0.04 mmol), respectively. TLC (DCM/MeOH 20:1): R_f 0.22 (**S.25**), 0.38 (**S.25a**). ^{1}H-NMR **S.25** (400 MHz, $CDCl_3$): δ = 3.76 (2s, 6H), 3.80 (m, 2H), 4.23 (m, 1H), 4.33 (m, 1H), 5.92 (d, J = 2.0, 1H), 6.79 (m, 4H), 7.18-7.35 (m, 9H), 7.50-7.63 (m, 3H), 8.03 (d, J = 7.6, 2H), 8.31 (s, 1H), 8.78 (s, 1H), 9.13 (br, 1H). **S.25a** (400 MHz, $CDCl_3$): δ = 3.77 (2s, 6H), 4.09 (dd, J = 10.0, 1.2, 1H), 4.26 (dd, J = 10.0, 4.0, 1H), 4.37 (m, 1H), 4.76 (s, 1H), 5.48 (d, J = 1.5, 1H), 6.15 (br, 1H), 6.74 (m, 4H), 7.25-7.42 (m, 10H), 7.37 (s, 1H), 7.52-7.63 (m, 3H), 8.01 (m, 2H), 8.72 (s, 1H), 9.05 (br, 1H).*

Perform phosphitylation

23. Dissolve 200 mg (0.31 mmol) **S.25** and 0.3 mL DIPEA in 2.0 mL anhydrous DCM in a 10-mL round-bottom flask.

24. Add 80 µl (0.4 mmol) chloro(2-cyanoethoxy)(diisopropylamino)phosphine in a dropwise manner and stir the reaction at room temperature for 1 hr.

25. Add an additional 10 µl (0.04 mmol) chloro (2-cyanoethoxy) (diisopropylamino) phosphine and stir at room temperature overnight.

26. Quench the reaction by adding 2 drops of MeOH and evaporate to dryness under reduced pressure.

27. Purify the residue by column chromatography. Deactivate the column with 97:3 (v/v) DCM/Et_3N and elute the product using 19:80:1 (v/v/v) hexanes/DCM/Et_3N.

*N^6-Benzoyl-9-{2′-O-[(2-cyanoethoxy)(diisopropylamino)phosphino]-3′-O-[(4′,4″-dimethoxytriphenyl)methyl]-α-L−threofuranosyl}adenine (**S.26**) is obtained in 84% yield (220 mg, 0.26 mmol) as a mixture of diastereomers. TLC (DCM/MeOH 50:1): R_f 0.20, 0.25. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.10-1.26 (m, 24H), 2.35-2.44 (m, 2H), 2.53-2.60 (m, 2H), 3.44 (m, 2H), 3.55-3.89 (m, 32H), 4.26 (m, 1H), 4.36 (m, 1H), 4.50 (m, 1H), 4.98 (m, 1H), 6.16 (s, 1H), 6.23 (s, 1H), 6.77-6.81 (m, 8H), 7.08-7.28 (m, 18H), 7.52-7.62 (m, 6H), 8.02-8.05 (m, 4H), 8.34 (s, 1H), 8.39 (s, 1H), 8.80 (s, 1H), 8.81 (s, 1H), 9.00 (br, 2H). ^{31}P-NMR (121 MHz, $CDCl_3$): 151.70, 152.70.*

BASIC PROTOCOL 7

SYNTHESIS, ISOLATION, AND CHARACTERIZATION OF THREOSE NUCLEIC ACID (TNA) OLIGONUCLEOTIDES

The threofuranosyl 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 a 1.0-μmol scale. In some cases, alternative reagents are used to increase the reactivity of phosphoramidite coupling. These include the use of (1) 0.1 M solutions of threofuranosyl nucleoside phosphoramidites in MeCN; (2) a detritylation solution consisting of 6% dichloroacetic acid in 1,2-dichloroethane; and (3) a coupling solution of 0.35 M (ethylthio)-1*H*-tetrazole. Modifications to the synthesis cycle are employed to optimize the chemistry required for automated synthesis (Fig. 4.51.8). Several techniques have been reported for isolating and purifying sequences containing TNA nucleotides, such as C18 reversed-phase HPLC (Schöning et al., 2002) and denaturing polyacrylamide gel electrophoresis (PAGE; Yang et al., 2007).

Materials

TNA phosphoramidite monomers (see Basic Protocols 2 to 6), pre-dried under high vacuum
Acetonitrile (MeCN), anhydrous (Applied Biosystems)
Argon gas (ultra high purity)
4Å molecular sieves (freshly activated by heating at 300°C for at least 3 hr)
Detritylation solution: 6% dichloroacetic acid in 1,2-dichloroethane (DCE)
Coupling solution: 0.35 M (ethylthio)-1*H*-tetrazole
Concentrated ammonium hydroxide (conc. NH_4OH)
n-Butanol
Nanopure water

25-mL round-bottom flasks
0.45-μm disposable syringe filters
CPG-column precharged with Universal Support II (Glen Research)
2-mL screw-cap microcentrifuge tube
15-mL screw-cap conical centrifuge tube (Falcon)

Additional reagents and equipments for automated solid-phase oligonucleotide synthesis (*APPENDIX 3C*) and isolation and characterization of synthetic nucleic acids (*UNITS 10.1* & *10.4*)

NOTE: All glassware, syringes, and needles should be pre-dried in a drying oven and cooled to room temperature in a desiccator prior to use. All TNA phosphoramidites should be dried under high vacuum for 48 hr prior to use.

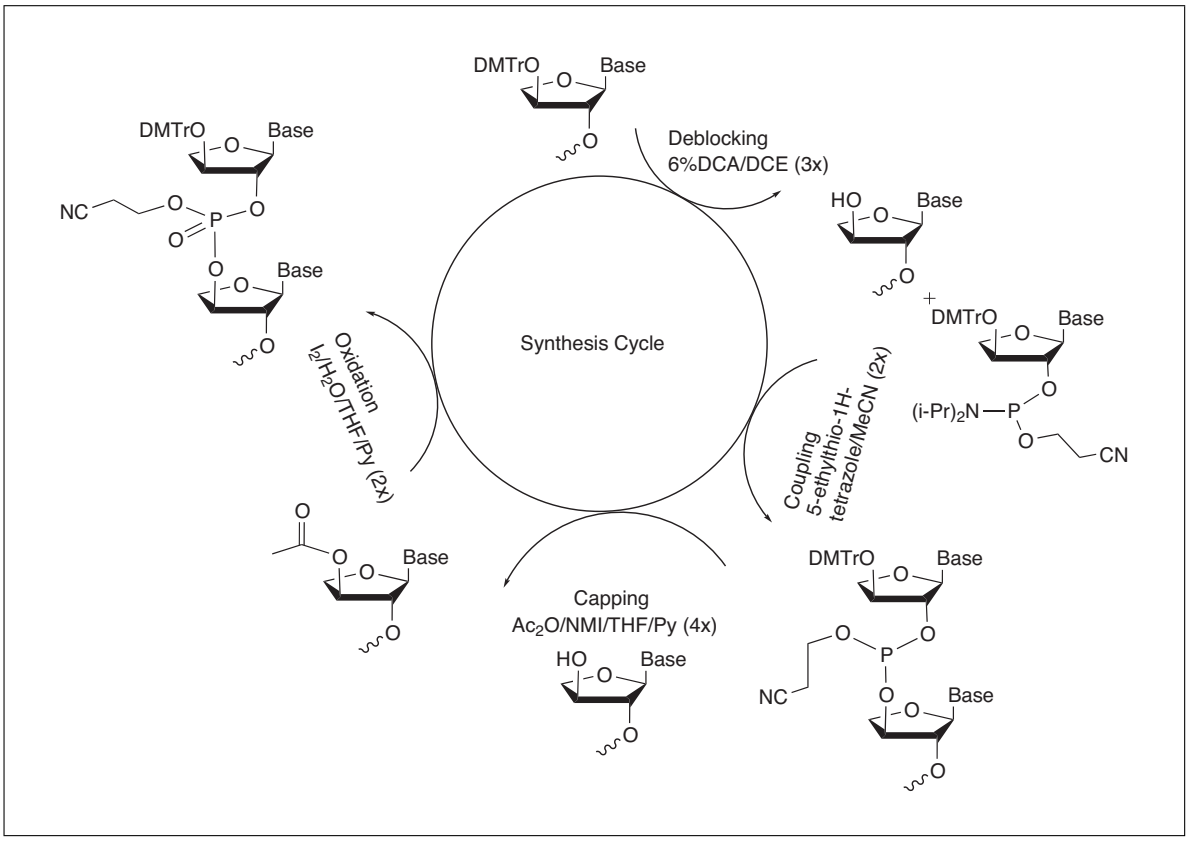

Figure 4.51.8 Synthesis cycle for automated solid-phase TNA synthesis. Abbreviations: DMTr, 4,4′-dimethoxytriphenylmethyl; DCA, dichloroacetic acid; DCE, 1,2-dichloroethane; NMI, *N*-methylimidazole; THF, tetrahydrofuran; Py, pyridine.

Synthesize TNA oligonucleotides (0.2 µmol scale)

1. Dissolve each pre-dried TNA phosphoramidite in anhydrous MeCN in a 25-mL round-bottom flask to make an approximate 0.1 M solution:

   *For instance, dissolve 300 mg **S.22** in 4.1 mL MeCN (for T) or 310 mg **S.26** in 3.7 mL MeCN (for A).*

   Steps 1-3 are carried out under argon atmosphere.

2. Dry phosphoramidite solutions over freshly activated molecular sieves overnight at room temperature.

3. Filter the 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. Carry out automated solid-phase oligonucleotide synthesis (*APPENDIX 3C*) using the modified cycle (see Fig. 4.51.8) on a CPG-column precharged with a universal support.

5. After synthesis, remove the column and dry for 2 min using a vacuum line.

6. Open the synthesis column and transfer the CPG beads to a 2-mL screw-cap micro-centrifuge tube.

Deprotect TNA oligonucleotides

7. Add 1 mL conc. NH_4OH to the support, seal the tube, and incubate at 55°C for 12 hr.

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

9. Transfer the supernatant to a 15-mL Falcon tube and add 10 mL *n*-butanol. Place the tube on ice for 5 min.

10. Centrifuge for 5 min at 3220 × *g*, 4°C, discard the supernatant, and collect the oligonucleotide (a white precipitate).

11. Resuspend the oligonucleotide pellet in 500 μL nanopure water.

Purify and characterize TNA oligonucleotides

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

13. Quantify the oligonucleotide by UV absorbance at 254 nm using Beer's law.

14. Check the molecular weight of the desired TNA oligonucleotide by MALDI-TOF-MS (*UNIT 10.1*) using aqueous saturated 3-hydroxypicolinc acid solution as the matrix.

    The matrix should be prepared just before the sample is spotted onto the gold MALDI-TOF-MS plate.

COMMENTARY

Background Information

A systematic exploration of molecules that are structurally similar to RNA (Eschenmoser, 1999, 2004) led to the discovery of threose nucleic acids (TNA), an alternative genetic system that uses a four-carbon threose sugar in place of the natural five-carbon ribose. Despite having a backbone repeat unit that is one atom shorter than RNA or DNA, TNA can form stable antiparallel duplex structures by Watson-Crick base-pairing with complementary strands of itself (self-pairing mode) as well as DNA or RNA (cross-pairing mode; Schöning et al., 2000, 2002). Thermodynamic studies indicate that TNA pairs more strongly with RNA than with DNA, and that RNA serves as a better template than DNA for TNA ligation (Wu et al., 2002). These observations were recently explained using solution NMR and X-ray crystallography to show that TNA adopts a duplex structure that closely resembles the helical structure of natural A-form RNA and DNA (Wilds et al., 2002; Pallan et al., 2003; Ebert et al., 2008).

The ability for TNA to undergo intersystem base-pairing with RNA, coupled with the chemical simplicity of threose relative to ribose, has raised significant interest in TNA as a potential natural progenitor of RNA. The possible existence of a hypothetical TNA world is supported by the ability of TNA to serve as a template in the non-enzymatic synthesis of RNA from activated ribonucleotides (Heuberger and Switzer, 2006), and by the existence of threose on meteorites, which suggests that it can appear spontaneously in the absence of life (Cooper et al., 2001). Intrigued by the possibility of evolving TNA receptors and enzymes, several groups have explored the template-directed synthesis of TNA by DNA polymerases (Chaput and Szostak, 2003; Chaput et al., 2003; Kempeneers et al., 2003; Horhota et al., 2005; Ichida et al., 2005a,b). These findings eventually led to the evolution of a TNA aptamer from a pool of random TNA polymers (Yu et al., 2012), which demonstrates that TNA can fold into shapes with sophisticated chemical functions. Possible genetic information transfer between TNA and glycerol nucleic acid (GNA; *UNIT 4.40*), an alternative genetic system that is structurally related to TNA, has provided additional constraints on early genetic polymers and the role of genetic takeover during the origin and evolution of life on Earth (Yang et al., 2007).

The preparation of DMTr-protected TNA phosphoramidites described in this unit was adapted from the original protocol described by Eschenmoser and colleagues (Schöning et al. 2000, 2002). In some cases, the synthetic procedures were revised or optimized to improve compound yield and purity. For example, silica gel chromatography was used in place of crystallization, as we were unable to crystallize some compounds using the conditions described in the original synthesis. These changes reduce the time required for purification.

Critical Parameters and Troubleshooting

For reactions that require anhydrous solvents, glassware should be dried overnight in a drying oven (80°C) or flame-dried, and then cooled under an Ar (g) atmosphere.

The protected threofuranose sugar undergoes glycosylation (Vorbrüggen and Bennua, 1981) to afford exclusively L-threofuranosyl nucleosides. Tritylation of the resulting TNA nucleosides proceeds with low regioselectivity between the 2′ and 3′ hydroxyl positions, requiring careful chromatographic separation to isolate the 3′-tritylated nucleosides from the unwanted 2′ variants.

To reduce purification time and achieve optimal compound purity, silica gel flash column chromatography is recommended for all purification steps. For synthesis of TNA phosphoramidites, 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 can be seen as separate spots on a TLC plate.

After silica gel purification, the guanosine phosphoramidite is isolated in syrup form and requires pentane precipitation to convert the compound to a solid form that can be dried and used for solid-phase synthesis.

Assignment of the constitutional 2′- and 3′-*O*-DMTr isomers was based on homo decoupling and/or ^{1}H,^{1}H-COSY-NMR spectroscopy by correlating the H-C(2′) or H-C(3′) to the OH atom.

Protected threose sugars should be stored at 4°C under an argon atmosphere. TNA phosphoramidites should be dried extensively before being stored at −80°C under an argon atmosphere. TNA phosphoramidite building blocks should be dried under high vacuum as a well-foamed solid for at least 24 hr prior to use as a substrate for solid-phase synthesis.

Anticipated Results

The protocols described in this unit are useful for preparing short TNA oligonucleotides. TNA 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 step-wise yield is >95%, which is sufficient to produce TNA oligonucleotides of dodecamer length or less in high yield.

Time Considerations

Each step in the chemical synthesis can be accomplished in 1-2 working days (including purification). The time for oligonucleotide synthesis varies slightly from the standard phosphoramidite method as longer coupling times and reagent delivery steps are required to synthesize TNA oligonucleotides in high yield. The deprotection, isolation, and the analysis of the final synthetic oligonucleotide can be accomplished in 2-3 days.

Acknowledgments

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