Synthesis of Threose Nucleic Acid (TNA) Triphosphates and Oligonucleotides by Polymerase-Mediated Primer Extension

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ABSTRACT

This unit describes the chemical synthesis of α-L-threofuranosyl nucleic acid (TNA) triphosphates for thymidine (T), guanosine (G), cytidine (C), and the diaminopurine (D) analog of adenosine and their incorporation into TNA oligonucleotides by enzyme-mediated polymerization of a DNA primer-template complex. Starting from suitably protected threofuranosyl nucleosides, TNA triphosphates are synthesized in a single-pot reaction and purified by ion-exchange and HPLC chromatography. Purified TNA triphosphates are diluted into stock solutions and used as substrates for the synthesis of TNA oligonucleotides. Oligonucleotide synthesis is accomplished using Therminator DNA polymerase, a commercial variant of the 9°N DNA polymerase bearing the A485L mutation. *Curr. Protoc. Nucleic Acid Chem.* 52:4.54.1-4.54.17. © 2013 by John Wiley & Sons, Inc.

Keywords: alternative nucleic acids • threose nucleic acid (TNA) • triphosphates • oligonucleotide • polymerase-mediated primer extension

INTRODUCTION

α-L-Threofuranosyl nucleic acid (TNA; Fig. 4.54.1) is an unnatural genetic polymer developed by Eschenmoser as a potentially natural alternative to RNA (Eschenmoser, 1999; Schöning et al., 2000, 2002; Hall, 2004). TNA has a chemical structure in which the natural five-carbon ribose sugar, found in RNA is replaced with an unnatural four-carbon threose sugar, and phosphodiester linkages are connected at the 2′ and 3′ vicinal positions in a quasi-diaxial orientation (Wilds et al., 2002). This chemical modification leads to a five-atom backbone repeat unit, which is one atom shorter than the backbone repeat unit found in natural DNA and RNA (Orgel, 2000). Despite this difference, TNA is capable of forming stable anti-parallel duplex structures in a self-pairing mode, and can also base pair opposite complementary strands of DNA and RNA in a cross-pairing mode (Schöning et al., 2000, 2002, Yang et al., 2007). NMR and X-ray crystallography studies reveal that TNA adopts a helical structure that is similar to the A-form DNA and RNA, which accounts for its ability to recognize complementary strands of DNA and RNA (Wilds et al., 2002; Pallan et al., 2003; Ebert et al., 2008). Because of its chemical simplicity, TNA has been examined as a possible progenitor candidate of RNA in the context of a hypothetical RNA world. Model non-enzymatic polymerization experiments designed to examine how genetic information can be transferred in the absence of protein enzymes reveal that cytosine-rich TNA strands can template-direct the synthesis of short oligoguanosine products by extending a DNA primer annealed to a TNA template with activated rGMP monomers (Heuberger and Switzer, 2006). Similar template-copying studies have also been carried-out using DNA polymerases and TNA triphosphates with the goal of developing the methodology necessary to evolve functional TNA molecules in the laboratory. Early work in this area led to the discovery that certain DNA polymerases can copy short stretches of DNA on a TNA template

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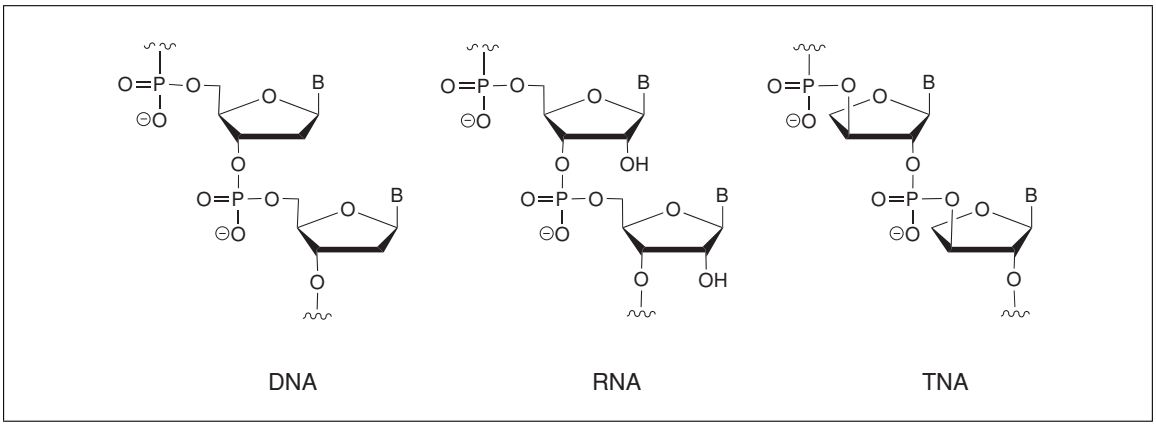

Figure 4.54.1 Chemical structures of DNA, RNA, and TNA.

(Chaput et al., 2003), while other polymerases can copy limited stretches of TNA on a DNA template (Chaput and Szostak, 2003; Kempeneers et al., 2003). Because the activity observed in these assays was insufficient to support in vitro selection, additional enzymes were screened for enhanced efficiency. The most active enzyme discovered to date is Therminator DNA polymerase, an engineered variant of 9°N DNA polymerase containing the A485L mutation (Horhota et al., 2005; Ichida et al., 2005a). Under optimal conditions, Therminator can transcribe a single-stranded DNA template or library of DNA templates into TNA with high efficiency and fidelity (Ichida et al., 2005a,b). Using DNA display, a strategy that covalently links artificial genetic polymers to their encoding DNA templates, a functional TNA aptamer was evolved to bind human thrombin with high affinity and specificity (Yu et al., 2012). This remarkable accomplishment demonstrated that the chemical problem of ligand binding is not limited to the natural polymers of DNA and RNA and suggests that it may be possible to evolve TNA enzymes or "threozymes" from large pools of random sequences. This unit contributes to that broader goal by describing the synthesis of tNTPs (see Basic Protocols 1 through 4) and conditions that lead to efficient polymerase-mediated synthesis of TNA oligonucleotides (see Basic Protocol 5).

BASIC PROTOCOL 1 SYNTHESIS OF (α-L-THREOFURANOSYL)THYMINE-3′-TRIPHOSPHATE (2)

The synthetic strategy for TNA nucleoside triphosphate (tNTP) construction is based on P^{III}-chemistry developed by Ludwig and Eckstein (1989) for the synthesis of modified nucleoside triphosphates. In this reaction, activated nucleoside phosphite triester is reacted with pyrophosphate to give a cyclic metaphosphite intermediate, which is subsequently oxidized and hydrolyzed to yield the desired nucleoside triphosphate. To synthesize the target nucleoside triphosphate **2**, the nucleoside **1** is used as an advanced intermediate, which itself is synthesized from L-ascorbic acid in seven steps (Fig. 4.54.2; see *UNIT 4.51*).

Materials

1-{2′-*O*-[(4″,4‴-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}thymine
Pyridine, freshly distilled
Phosphorous pentoxide (P_2O_5)
Argon balloons
1,4-Dioxane, freshly distilled
2-Chloro-4H-1,3,2-benzodioxaphosphorin-4-one
Tributylammonium pyrophosphate
N,N-Dimethyformamide, freshly distilled (DMF)

Tributylamine, freshly distilled
1% I_2 solution in 98:2 (v/v) pyridine/water
5% aq. Na_2SO_3
50% aq. acetic acid
Diethyl ether (Et_2O)
1 M aq. triethylammonium bicarbonate buffer, pH 8.0 (TEAB)
100 mM aq. triethylammonium acetate buffer, pH 7.0 (TEAA)
Methanol (HPLC-grade MeOH)
Acetone, HPLC grade
Sodium perchlorate ($NaClO_4$)

10- and 50-mL round-bottom flasks, oven-dried
50-mL separatory funnel
Rotary evaporator equipped with a vacuum pump
Magnetic stir plate and stir bar
Vacuum desiccator
Lyophilizer
GE Pharmacia ÄKTA FPLC system
Customized DEAE Sephadex anion-exchange column (Essential Life Solutions)
ZORBAX C-18 reversed-phase HPLC column (Agilent Technologies)
Agilent 1100 HPLC system

Additional reagents and equipment for HPLC (*UNIT 10.5*)

Perform phosphorylation

1. Add 50 mg (95 μmol) of 1-{2′-*O*-[(4″,4‴-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}thymine to an oven-dried 10-mL one-neck, round-bottom flask.
2. Add 2 mL of anhydrous pyridine to the flask and evaporate to dryness with a rotary evaporator equipped with a vacuum pump. Repeat an additional two times.
3. Place a magnetic stir bar into the reaction flask containing the starting material and dry the contents over P_2O_5 in a desiccator under high vacuum for 1 hr.

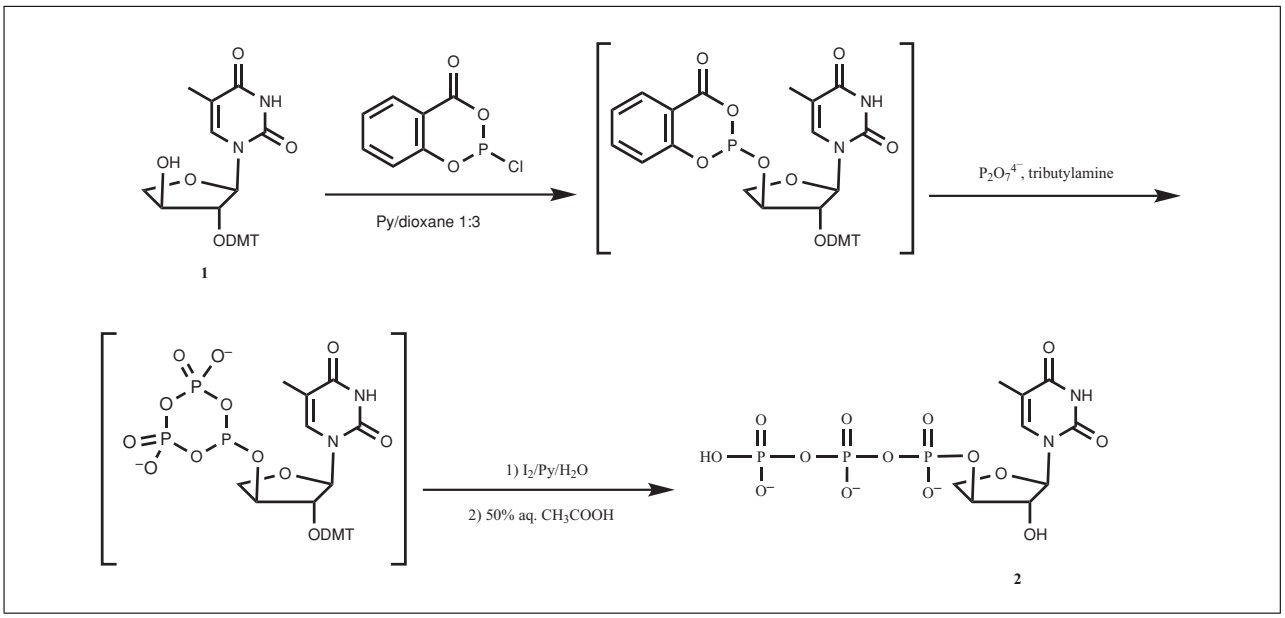

Figure 4.54.2 Preparation of (α-L-threofuranosyl)thymine-3′-triphosphate 2. Abbreviations: DMT, 4,4′-dimethoxytrityl; Py, pyridine.

4. Introduce an argon atmosphere into the flask and maintain it throughout the reaction. Dissolve compound **1** in 100 μL of freshly distilled pyridine and 300 μL of freshly distilled dioxane.

5. Under an argon atmosphere, add a 95 μL solution of 1 M 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in freshly distilled dioxane (1 eq.) into the reaction.

6. After 10 min, add a well-vortexed mixture of 300 μL of 0.5 M tributylammonium pyrophosphate in freshly distilled DMF (1.5 eq.) and 100 μL of freshly distilled tributylamine.

7. Stir the reaction mixture for another 15 min, and add 2.4 mL of 1% I_2 solution in 98:2 (v/v) pyridine/water (1 eq.) to the reaction mixture.

8. After 10 min, add 0.8 mL of 5% aq. Na_2SO_3 to quench excess iodine.

9. Remove the solvent under reduced pressure.

Perform deprotection

10. Resuspend the residue in 20 mL of 50% acetic acid and allow reaction to stir 1 hr at room temperature.

11. Remove the solvent under reduced pressure and resuspend residue in 20 mL of water.

12. Wash the aqueous layer two times, each time with 10 mL of Et_2O using a 50-mL separatory funnel, and lyophilize the aqueous residue to dryness.

13. Dissolve the crude product in 20 mL of 0.5 M TEAB buffer and purify the mixture on an FPLC system using a DEAE anion-exchange column with a linear gradient from 0 to 1 M TEAB (pH 8.0), over 300 min.

14. Collect the eluted peaks manually when A_{260} crosses a threshold of 500 milli-absorbance units (mAU) and check fractions by mass spectrometric analysis. Collect the product-containing fractions and lyophilize the sample to dryness.

15. Purify the residue using a ZORBAX C-18 reversed-phase HPLC column on an Agilent 1100 HPLC system. Elute the product with 95% of 100 mM TEAA (pH 7.0) and a linear gradient from 5% to 100% methanol over 40 min.

16. Collect the eluted peaks using a fraction collector when A_{260} crosses a threshold of 300 milli-absorbance units (mAU) and check fractions by mass spectrometric analysis. Collect the product-containing fractions and remove the TEAA buffer by lyophilization.

17. Dissolve the residue in MeOH to make a solution of ~0.05 M triethylammonium nucleoside triphosphate. Determine the concentration of the tNTP solution by UV quantification.

18. Add 5 vol of an acetone solution of $NaClO_4$ (15 eq.) to the solution in step 17.

19. Collect the precipitated triphosphate in sodium salt form by centrifuging 5 min at 3220 × *g*, 4°C, and washing resulting white pellet three times with 1 mL of acetone each time, followed by centrifuging 5 min at 3220 × *g*, 4°C. Dry the product under high vacuum.

*The resulting product, (α-L-threofuranosyl)thymine-3′-triphosphate (**2**), is obtained in 20% yield (9 mg, 19 μmol) as a white powder. ^{31}P-NMR (161.8 MHz, D_2O): δ=–10.3 (d, J = 20), −12.4 (d, J = 20), −22.8 (t, J = 19.7). MALDI-TOF MS (neg.) 466.97 (calc. 466.97).*

SYNTHESIS OF (α-L-THREOFURANOSYL)-2,6-DIAMINOPURINE-3′-TRIPHOSPHATE (5)

To synthesize the target nucleoside triphosphate **5**, the compound **3** is used as the key intermediate, which itself is synthesized from L-ascorbic acid in seven steps (see *UNIT 4.51*; Fig. 4.54.3). The exocyclic protecting group is first removed through mild alkaline hydrolysis. Phosphorylation of the resulting nucleoside (**4**) provides the desired triphosphate.

Materials

N^2,N^6-Dibenzoyl-9-{2′-*O*-[(4″,4‴-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}-2,6-diaminopurine
8 M methylamine in ethanol
12 M methylamine in water
Dichloromethane (CH_2Cl_2)
Triethylamine (Et_3N)
Methanol, HPLC grade
Pyridine, freshly distilled
Phosphorous pentoxide (P_2O_5)
Argon balloons
N,N-dimethyformamide (freshly distilled DMF)
2-Chloro-4H-1,3,2-benzodioxaphosphorin-4-one
1,4-Dioxane, freshly distilled
Tributylammonium pyrophosphate
Tributylamine, freshly distilled
1% I_2 solution in 98:2 pyridine/water
5% aq. Na_2SO_3
50% aq. acetic acid
Diethyl ether (Et_2O)
1 M aq. triethylammonium bicarbonate buffer, pH 8.0 (TEAB)
100 mM aq. triethylammonium acetate buffer, pH 7.0 (TEAA)
Acetone, HPLC grade
Sodium perchlorate ($NaClO_4$)

Magnetic stir plate and stir bar
10- and 50-mL round-bottom flasks
Rotary evaporator equipped with a vacuum pump
6.4 × 45-cm chromatography column
50-mL separatory funnel
Vacuum desiccators
Lyophilizer
Customized DEAE Sephadex anion-exchange column (Essential Life Solutions)
GE Pharmacia ÄKTA FPLC system
ZORBAX C-18 reversed-phase HPLC column (Agilent Technologies)
Agilent 1100 HPLC system

Additional reagents and equipment for column chromatography (*APPENDIX 3E*), and HPLC (*UNIT 10.5*)

Perform deprotection

1. Dissolve 1.5 g (1.9 mmol) of N^2,N^6-dibenzoyl-9-{2′-*O*-[(4″,4‴-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}-2,6-diaminopurine in 100 mL of 1:1 (v/v) 8 M methylamine in ethanol/12 M methylamine in water.
2. Stir the reaction mixture 3 hr at 50°C.

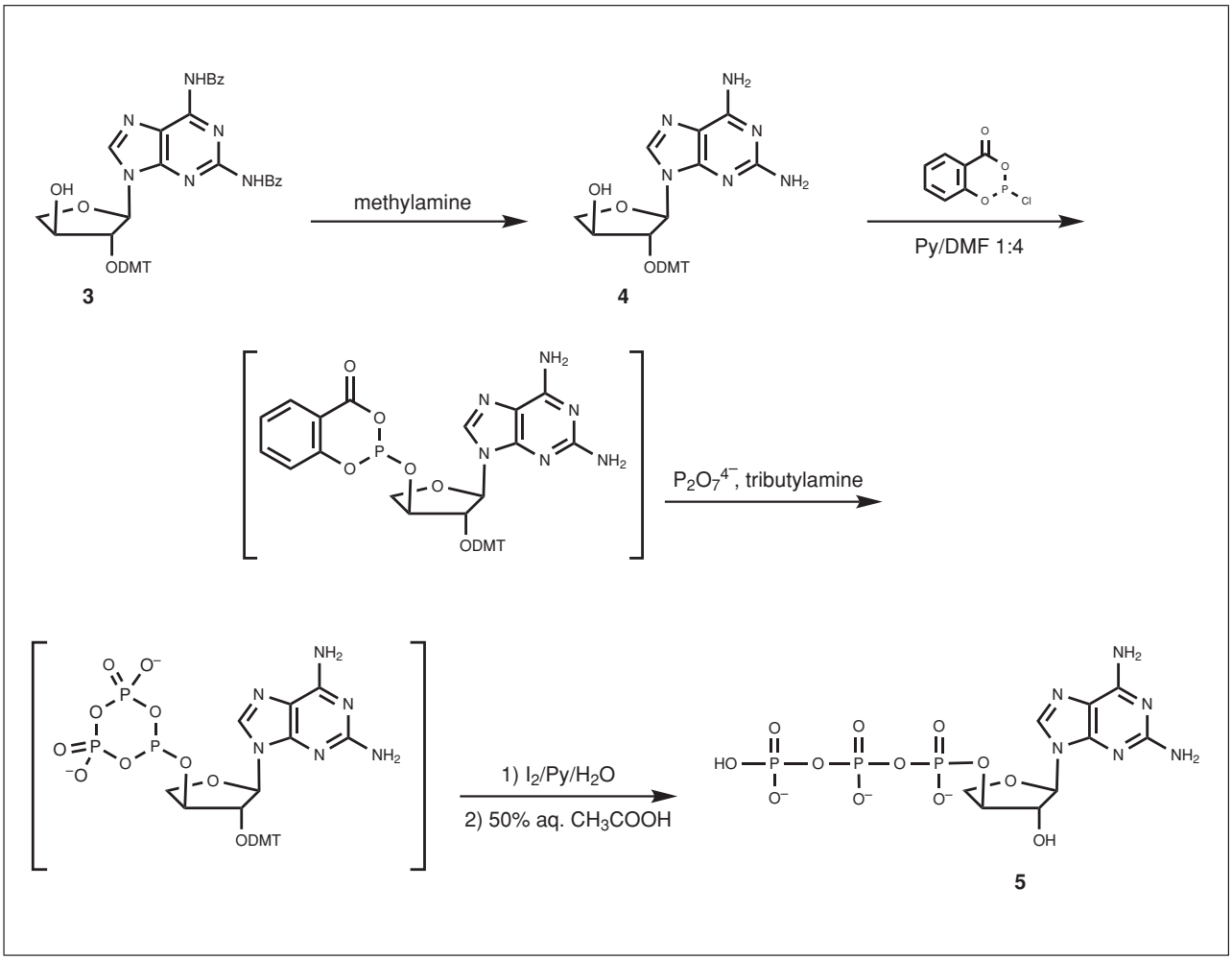

Figure 4.54.3 Preparation of (α-L-threofuranosyl)-2,6-diaminopurine-3′-triphosphate **5**. Abbreviations: DMT, 4,4′-dimethoxytrityl; Py, pyridine; DMF, dimethylformamide.

3. Remove the solvent under reduced pressure using a rotary evaporator equipped with a vacuum pump and purify the residue by silica column chromatography in a 6.4 × 45-cm column. Deactivate the column with 100:1 CH_2Cl_2/Et_3N and elute product using a stepwise gradient from 100:1 CH_2Cl_2/Et_3N to 100:4:1 CH_2Cl_2/MeOH/Et_3N.

*The resulting product, 9-{2′-O-[(4″,4‴-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}-2,6-diaminopurine (**4**), is obtained in 95% yield (1.0 g, 1.8 mmol) as a white powder. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 3.77 (2s, 6H), 4.05 (d, J = 9.6, 1H), 4.17 (dd, J = 9.6, 3.6, 1H), 4.27 (m, 1H), 4.67 (d, J = 5.6, 1H), 5.21 (d, J = 1.2, 1H), 5.42 (br, 2H), 6.72 (br, 2H), 6.72-6.79 (m, 4H), 7.22-7.41 (m, 9H), 7.58 (s, 1H).*

Perform phosphorylation

4. Add 53 mg (95 μmol) of 1-{2′-O-[(4″,4‴-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}-2,6-diaminopurine to an oven-dried 10-mL one-neck, round-bottom flask.

5. Add 2 mL of anhydrous pyridine to the flask and evaporate to dryness with a rotary evaporator equipped with a vacuum pump. Repeat this step two additional times.

6. Place a magnetic stir bar into the reaction flask containing the starting material and dry it over P_2O_5 in a desiccator under high vacuum for 1 hr.

7. Introduce an argon atmosphere into the flask and maintain it throughout the reaction. Dissolve compound **4** in 160 μL of freshly distilled pyridine and 640 μL of freshly distilled DMF.

8. Under an argon atmosphere, add a 104 μL solution of 1 M 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in freshly distilled dioxane (1.1 eq.) into the reaction.

9. After 10 min, add a well-vortexed mixture of 285 μL of 0.5 M tributylammonium pyrophosphate in freshly distilled DMF (1.5 eq.) and 95 μL of freshly distilled tributylamine.

10. Stir the reaction mixture for an additional 15 min, and add 2.4 mL of 1% I_2 solution in 98:2 pyridine/water (1 eq.) to the reaction mixture.

11. After 10 min, add 0.8 mL of 5% aq. Na_2SO_3 to quench the excess iodine.

12. Remove the solvent under reduced pressure.

13. Resuspend the residue in 20 mL of 50% acetic acid and allow the reaction to stir for 90 min at 0°C in an ice-water bath.

14. Remove the solvent under reduced pressure and resuspend the residue in 20 mL of water.

15. Wash the aqueous layer two times, each time with 10 mL of Et_2O using a 50-mL separatory funnel and lyophilize the aqueous residue to dryness.

16. Redissolve the crude product in 20 mL of 0.5 M TEAB buffer and purify the mixture on an FPLC system using a DEAE anion-exchange column with a linear gradient from 0 to 1 M TEAB, pH 8.0, over 300 min.

17. Collect the eluted peaks manually when A_{260} crosses a threshold of 500 milli-absorbance units (mAU) and check fractions by mass spectrometric analysis. Collect the product-containing fractions and lyophilize the sample to dryness.

18. Purify the residue using a ZORBAX C-18 reversed-phase HPLC column on an Agilent 1100 HPLC system. Elute the product with 95% of 100 mM TEAA, pH 7.0, and a linear gradient from 5% to 100% methanol over 40 min.

19. Collect the eluted peaks using a fraction collector when A_{260} crosses a threshold of 300 milli-absorbance units (mAU) and check fractions by mass spectrometric analysis. Collect the product-containing fractions and remove the TEAA buffer by lyophilization.

20. Dissolve the residue in MeOH to make a solution of ~0.05 M triethylammonium nucleoside triphosphate.

21. Add 5 vol of an acetone solution of $NaClO_4$ (15 eq.) to the solution in step 20.

22. Collect the precipitated triphosphate in sodium salt form by centrifuging 5 min at 3220 × *g*, 4°C, and washing the resulting white pellet three times with 1 mL acetone each time, followed by centrifuging 5 min at 3220 × *g*, 4°C. Dry the product under high vacuum.

*The resulting product, (α-L-threofuranosyl)-2,6-diaminopurine-3′-triphosphate (**5**), is obtained in 15% yield (7 mg, 14.2 μmol) as a white powder. ^{31}P-NMR (161.8 MHz, D_2O): δ = -9.12 (d, J = 20.0), −12.1 (d, J = 20.0), −22.6 (t, J = 18.4). MALDI-TOF MS (neg.) 491.16 (calc. 491.16).*

BASIC PROTOCOL 3

SYNTHESIS OF (α-L-THREOFURANOSYL)GUANINE-3′-TRIPHOSPHATE (8)

To synthesize the target nucleoside triphosphate **8**, compound **6** is used as the key intermediate, which itself is synthesized from L-ascorbic acid in seven steps (see *UNIT 4.51*). The exocyclic protecting group is first removed through mild alkaline hydrolysis. Phosphorylation of the resulting nucleoside (**7**) provides the desired triphosphate. See Figure 4.54.4.

Materials

N^2-Acetyl-O^6-diphenylcarbamoyl-9-{2′-*O*-[(4″,4‴-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}-guanine
8 M methylamine in ethanol
12 M methylamine in water
Dichloromethane (CH_2Cl_2)
Triethylamine (Et_3N)
Methanol (HPLC-grade MeOH)
Pyridine, freshly distilled
Phosphorous pentoxide (P_2O_5)
Argon balloons
N,N-Dimethyformamide, freshly distilled (DMF)
2-Chloro-4H-1,3,2-benzodioxaphosphorin-4-one
1,4-Dioxane, freshly distilled
Tributylammonium pyrophosphate
Tributylamine, freshly distilled
1% I_2 solution in 98:2 pyridine/water
5% aq. Na_2SO_3
50% aq. acetic acid
Diethyl ether (Et_2O)
1 M aq. triethylammonium bicarbonate buffer, pH 8.0 (TEAB)
100 mM aq. triethylammonium acetate buffer, pH 7.0 (TEAA)
Acetone, HPLC grade
Sodium perchlorate ($NaClO_4$)

Magnetic stir plate and stir bar
10- and 50-mL round-bottom flasks
Rotary evaporator equipped with a vacuum pump
6.4 × 45-cm chromatography column
50-mL separatory funnel
Vacuum desiccator
Lyophilizer
Customized DEAE Sephadex anion-exchange column (Essential Life Solutions)
GE Pharmacia ÄKTA FPLC system
ZORBAX C-18 reversed-phase HPLC column (Agilent Technologies)
Agilent 1100 HPLC system

Additional reagents and equipment for thin layer chromatography (TLC) (*APPENDIX 3D*), column chromatography (*APPENDIX 3E*), and HPLC (*UNIT 10.5*)

Perform deprotection

1. Dissolve 1.5 g (1.9 mmol) of N^2-acetyl-O^6-diphenylcarbamoyl-9-{2′-*O*-[(4″,4‴-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}-guanine in 100 mL of 1:1 8 M methylamine in ethanol/12 M methylamine in water.
2. Stir the reaction mixture 3 hr at 50°C.

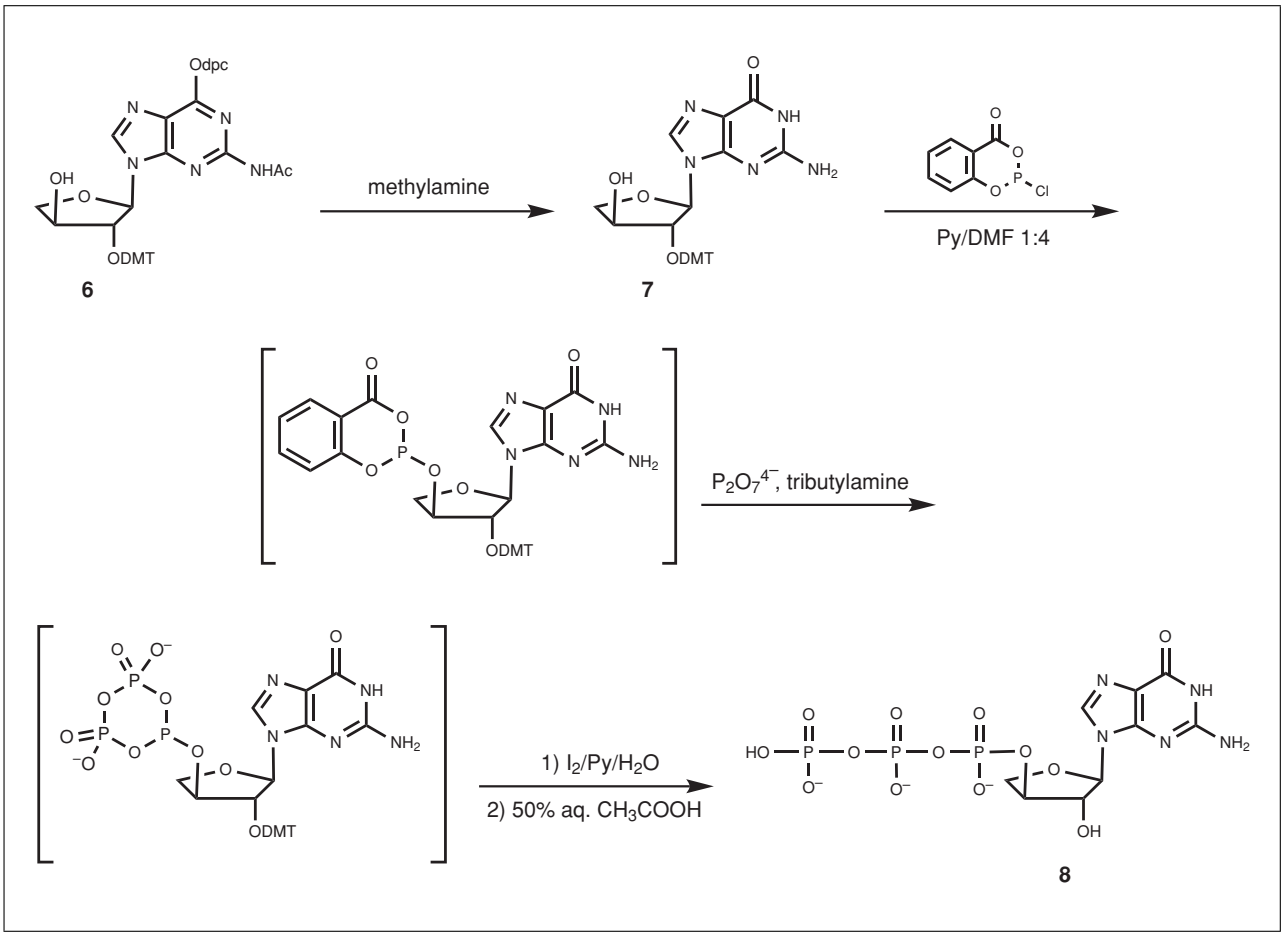

Figure 4.54.4 Preparation of (α-L-threofuranosyl)guanine-3′-triphosphate **8**. Abbreviations: DMT, 4,4′-dimethoxytrityl; Py, pyridine; DMF, dimethylformamide.

3. Remove the solvent under reduced pressure using a rotary evaporator equipped with a vacuum pump and purify the residue by column chromatography in a 6.4 × 45–cm column. Deactivate the column with 100:1 CH_2Cl_2/Et_3N and elute the product using a stepwise gradient from 100:1 CH_2Cl_2/Et_3N to 100:4:1 CH_2Cl_2/MeOH/Et_3N.

*The resulting product, 9-{2′-O-[(4″,4‴-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}guanine (**7**), is obtained in 95% yield (1.0 g, 1.8 mmol) as a white powder. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 3.71 (2s, 6H), 4.05 (d, J = 9.5, 1H), 4.14 (m, 2H), 4.64 (s, 1H), 5.34 (s, 1H), 6.59 (br, 2H), 6.71-6.76 (m, 4H), 7.10 (br, 1H), 7.17-7.41 (m, 10H).*

Perform phosphorylation

4. Add 65 mg (117 μmol) of 9-{2′-*O*-[(4″,4‴-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}guanine to an oven-dried 10-mL one-neck, round-bottom flask.
5. Add 2 mL anhydrous pyridine to the flask and evaporate to dryness with a rotary evaporator equipped with a vacuum pump. Repeat this procedure two times.
6. Place a magnetic stir bar into the reaction flask containing the starting material and dry it over P_2O_5 in a desiccator under high vacuum for 1 hr.
7. Introduce an argon atmosphere into the flask and maintain it throughout the reaction. Dissolve compound **7** in 195 μL of freshly distilled pyridine and 780 μL of freshly distilled DMF.
8. Under an argon atmosphere, add a 129 μL solution of 1 M 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in freshly distilled dioxane (1.1 eq.) into the reaction.

9. After 10 min, add a well-vortexed mixture of 351 μL of 0.5 M tributylammonium pyrophosphate in freshly distilled DMF (1.5 eq.) and 117 μL of freshly distilled tributylamine.
10. Stir the reaction mixture for an additional 15 min, and add 3.0 mL of 1% I_2 solution in 98:2 pyridine/water (1 eq.) to the reaction mixture.
11. After 10 min, add 1.0 mL of 5% aq. Na_2SO_3 to quench excess iodine.
12. Remove the solvent under reduced pressure using a rotary rvaporator equipped with a vacuum pump.
13. Resuspend the residue in 20 mL of 50% acetic acid and allow the reaction to stir for 40 min at room temperature.
14. Remove the solvent under reduced pressure and resuspend the residue in 20 mL of water.
15. Wash the aqueous layer two times, each time with 10 mL of Et_2O using a 50-mL separatory funnel and lyophilize the aqueous residue to dryness.
16. Dissolve the crude product in 20 mL of 0.5 M TEAB buffer and purify the mixture on an FPLC system using a DEAE anion-exchange column with a linear gradient from 0 to 1 M TEAB, pH 8.0, over 300 min.
17. Collect the eluted peaks manually when A_{260} crosses a threshold of 500 milli-absorbance units (mAU) and check fractions by mass spectrometric analysis. Collect the product-containing fractions and lyophilize the sample to dryness.
18. Purify the residue using a ZORBAX C-18 reversed-phase HPLC column on an Agilent 1100 HPLC system. Elute the product with 95% of 100 mM TEAA, pH 7.0 and a linear gradient from 5% to 100% methanol over 40 min.
19. Collect the eluted peaks using a fraction collector when A_{260} crosses a threshold of 300 milli-absorbance units (mAU) and check fractions by mass spectrometric analysis. Collect the product-containing fractions and remove the TEAA buffer by lyophilization.
20. Dissolve the residue in MeOH to make a solution of ~0.05 M triethylammonium nucleoside triphosphate.
21. Add 5 vol of an acetone solution of $NaClO_4$ (15 eq.) to the solution in step 20.
22. Collect the precipitated triphosphate in sodium salt form by centrifuging 5 min at 3220 × *g*, 4°C and washing the resulting white pellet three times with 1 mL acetone each time, followed by centrifuging 5 min at 3220 × *g*, 4°C. Dry the product under high vacuum.

*The resulting product, (α-L-threofuranosyl)-guanine-3′-triphosphate (**8**), is obtained in 21% yield (12 mg, 24 μmol) as a white powder. ^{31}P-NMR (161.8 MHz, D_2O): δ = –8.57 (d, J = 20.0), −12.2 (d, J = 20.1), −22.6 (t, J = 20.0). MALDI-TOF MS (neg.) 492.16 (calc. 492.15).*

SYNTHESIS OF (α-L-THREOFURANOSYL)CYTOSINE-3′-TRIPHOSPHATE

BASIC PROTOCOL 4

Because tritylation of the protected threofuranosyl cytosine leads to the 3′-O-DMT isomer as the dominant product, an alternative protection-deprotection strategy is used for the synthesis of tCTP (Zou et al., 2005). Using this approach, the undesired 3′-O-DMT isomer (**9**) is prepared and treated with acetic anhydride to protect the 2′-OH as a 2′-O-acetyl group. The 3′-O-DMT group is then removed under mild acidic conditions and the

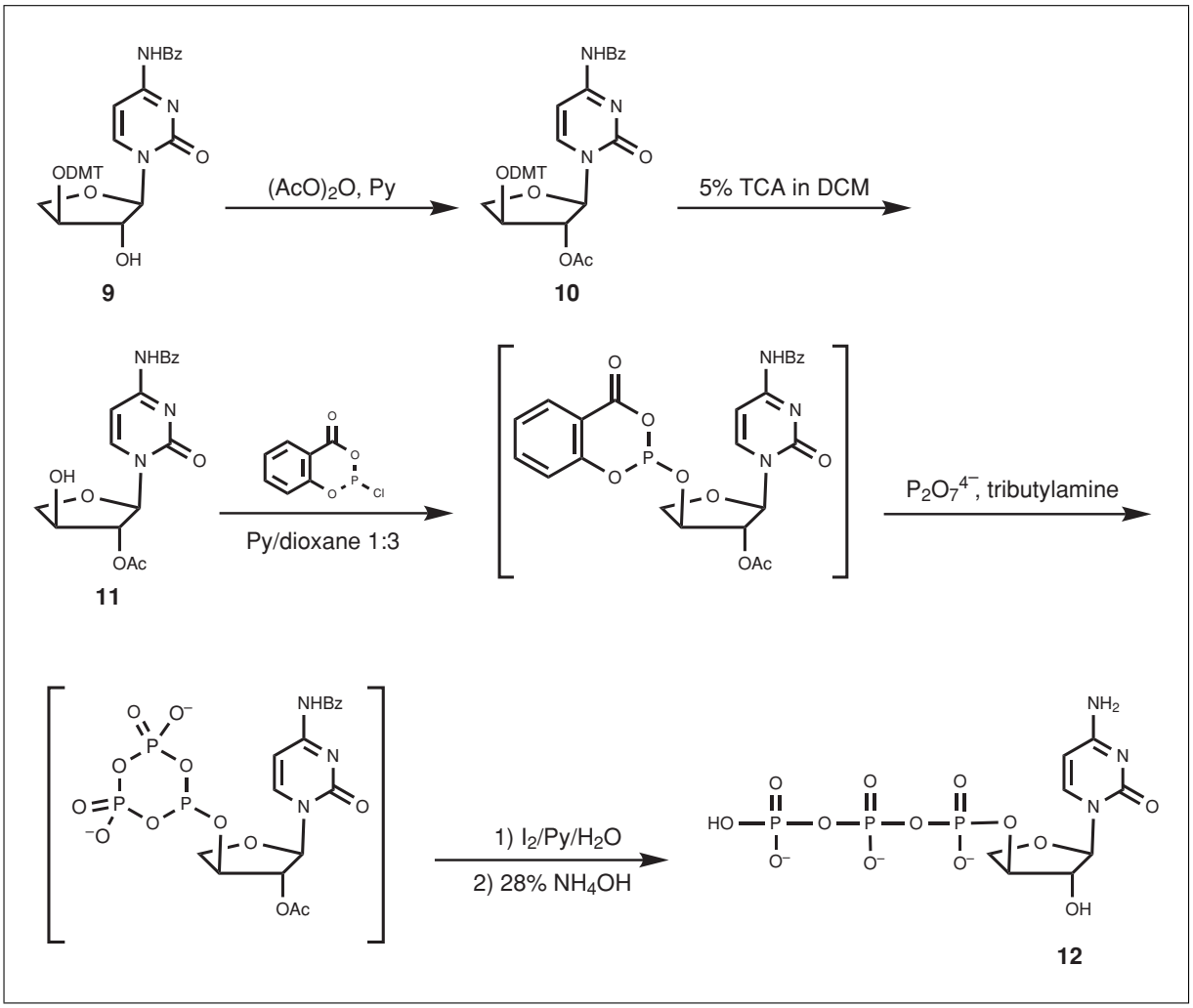

Figure 4.54.5 Preparation of (α-L-threofuranosyl)cytosine-3′-triphosphate 12. Abbreviations: DMT, 4,4′-dimethoxytrityl; Py, pyridine; $(AcO)_2O$, acetic anhydride; DCM, dichloromethane; TCA, trichloroacetic acid.

free 3′-OH is phosphorylated as described previously. The 2′-O-acetyl group is removed with concentrated ammonium hydroxide to furnish the target nucleoside triphosphate (**12**). See Figure 4.54.5.

Materials

N^4-Benzoyl-1-{3′-*O*-[(4″,4‴-dimethoxytriphenyl)methyl]-α-L-threofuranosyl}cytosine **(9)**
Pyridine, freshly distilled
Acetic anhydride
Argon balloons
Methanol (HPLC-grade MeOH)
Dichloromethane (CH_2Cl_2)
Saturated aqueous sodium bicarbonate solution (sat. aq. $NaHCO_3$)
Brine (sat. aq. NaCl)
Sodium sulfate (Na_2SO_4)
Hexanes
Triethylamine (Et_3N)
Trichloroacetic acid (TCA)
SiO_2

Phosphorous pentoxide (P_2O_5)
1,4-Dioxane, freshly distilled
2-Chloro-4H-1,3,2-benzodioxaphosphorin-4-one
Tributylammonium pyrophosphate
N,N-Dimethyformamide, freshly distilled (DMF)
Tributylamine, freshly distilled
1% I_2 solution in 98:2 pyridine/water
5% aq. Na_2SO_3
Concentrated (28%) ammonium hydroxide (NH_4OH)
Diethyl ether (Et_2O)
1 M aq. triethylammonium bicarbonate buffer, pH 8.0 (TEAB)
100 mM aq. triethylammonium acetate buffer, pH 7.0 (TEAA)
Acetone, HPLC grade
Sodium perchlorate ($NaClO_4$)

Magnetic stir plate and stir bar
10- and 50-mL round-bottom flasks, oven dried
Rotary evaporator equipped with a vacuum pump
50- and 250-ml separatory funnels
Vacuum desiccator
Lyophilizer
Customized DEAE Sephadex anion-exchange column (Essential Life Solutions)
GE Pharmacia ÄKTA FPLC system
ZORBAX C-18 reversed-phase HPLC column (Agilent Technologies)
Agilent 1100 HPLC system

Additional reagents and equipment for thin layer chromatography (TLC; *APPENDIX 3D*), column chromatography (*APPENDIX 3E*), and HPLC (*UNIT 10.5*)

Perform acetylation

1. Co-evaporate 3.0 g (4.8 mmol) of compound **9** with anhydrous pyridine two times and re-dissolve the residue in 90 mL of anhydrous pyridine.

2. Add 0.83 mL of acetic anhydride to the solution in step 1 and stir the reaction mixture for 12 hr at room temperature under an argon atmosphere.

3. Quench the reaction with 6 mL of MeOH and remove the solvent under reduced pressure using a rotary evaporator equipped with a vacuum pump.

4. Dissolve the residue in 80 mL CH_2Cl_2 and wash the mixture subsequently with 50 mL sat. aq. $NaHCO_3$, 50 mL water, and 50 mL brine using a 250-mL separatory funnel and dry it over Na_2SO_4.

5. Remove the organic solvent under reduced pressure and purify the residue by column chromatography. Deactivate the column with 99:1 hexanes/Et_3N. Elute the column using a step-wise gradient of 1:1:0.025 hexanes/CH_2Cl_2/Et_3N to 1:0.025 CH_2Cl_2/Et_3N.

   *The resulting product, N^4-Benzoyl-1-{2′-O-acetyl-3′-O-[(4′′,4′′′-dimethoxytriphenyl) methyl]-α-L-threofuranosyl}cytosine (**10**) is obtained in 94% yield (3.0 g, 4.4 mmol) as a colorless foam. TLC (CH_2Cl_2/MeOH 25:1): R_f 0.32. ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 2.08 (s, 3H), 3.04 (d, J = 10.4, 1H), 3.60 (dd, J = 10.4, 3.6, 1H), 3.76-3.77 (2s, 6H), 4.24 (d, J = 3.6, 1H), 5.57 (s, 1H), 5.95 (s, 1H), 6.80-6.85 (m, 4H), 7.22-7.65 (m, 13H), 7.92-7.96 (m, 3H), 8.80 (br, s, 1H).*

Perform detritylation

6. Treat 2.0 g (3.0 mmol) of **10** with 100 mL of 3% TCA in CH_2Cl_2 for 3 min at room temperature.

7. Add ~2 to 3 mL MeOH dropwise to quench the reaction until the solution changes color from orange to clear.
8. Neutralize the acidic solution in step 2 with 40 mL of Et_3N, and remove the solvent immediately under reduced pressure.
9. Adsorb the residue onto SiO_2 and purify the product by column chromatography. Elute the column using 50:1 CH_2Cl_2/MeOH.

*The resulting product, N^4-benzoyl-1-{2′-O-acetyl-α-L-threofuranosyl}cytosine (**11**) is obtained in 93% yield (1.0 g, 2.8 mmol) as a white powder. TLC (CH_2Cl_2/MeOH 25:1): R_f 0.23. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 2.10 (s, 3H), 4.16 (m, 2H), 4.25 (d, J = 9.2, 1H), 5.10 (s, 1H), 5.70 (d, J = 2.4, 1H), 5.79 (s, 1H), 7.36 (d, J = 7.2, 1H), 7.50 (m, 2H), 7.62 (m, 1H), 7.99 (m, 2H), 8.13 (d, J = 8.0, 1H), 11.24 (s, 1H).*

Perform phosphorylation

10. Add 50 mg (139 μmol) of 11 to an oven-dried 10-mL one-neck, round-bottom flask.
11. Add 2 mL of anhydrous pyridine to the flask and evaporate to dryness with a rotary evaporator equipped with a vacuum pump. Repeat this step two additional times.
12. Place a magnetic stir bar into the reaction flask containing the starting material and dry it over P_2O_5 in a desiccator under high vacuum for 1 hr.
13. Introduce an argon atmosphere into the flask and maintain it throughout the reaction. Dissolve compound **11** in 150 μL of freshly distilled pyridine and 450 μL of freshly distilled dioxane.
14. Under an argon atmosphere, add a 139 μL solution of 1 M 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in freshly distilled dioxane (1.0 eq.) to the reaction.
15. After 10 min, add a well-vortexed mixture of 420 μL of 0.5 M tributylammonium pyrophosphate in freshly distilled DMF (1.5 eq.) and 140 μL of freshly distilled tributylamine.
16. Stir the reaction mixture for an additional 15 min, and add 1.8 mL of 1% I_2 solution in 98:2 pyridine/water (1 eq.) to the reaction mixture.
17. After 10 min, add 0.6 mL of 5% aq. Na_2SO_3 to quench the excess iodine.
18. Remove the solvent under reduced pressure.
19. Resuspend the residue in 20 mL of 28% aq. NH_4OH and allow the reaction to stir for 3 hr at 50°C.
20. Remove the solvent under reduced pressure using a rotary evaporator equipped with a vacuum pump and resuspend the residue in 20 mL of water.
21. Wash the aqueous layer two times, each time with 10 mL Et_2O using a 50-mL separatory funnel and lyophilize the aqueous residue to dryness.
22. Dissolve the crude product in 20 mL of 0.5 M TEAB buffer and purify the mixture on an FPLC system using a DEAE anion-exchange column with a linear gradient from 0 to 1 M TEAB, pH 8.0, over 300 min.
23. Collect the eluted peaks manually when A_{260} crosses a threshold of 500 milli-absorbance units (mAU) and check fractions by mass spectrometric analysis. Collect the product-containing fractions and lyophilize the sample to dryness.
24. Purify the residue using a ZORBAX C-18 reversed-phase HPLC column on an Agilent 1100 HPLC system. Elute the product with 95% of 100 mM TEAA, pH 7.0 and a linear gradient from 5% to 100% methanol over 40 min.

25. Collect the eluted peaks using a fraction collector when A_{260} crossed a threshold of 300 milli-absorbance units (mAU) and check fractions by mass spectrometric analysis. Collect the product-containing fractions and remove the TEAA buffer by lyophilization.

26. Dissolve the residue in MeOH to make a solution of ~0.05 M triethylammonium nucleoside triphosphate.

27. Add 5 vol of an acetone solution of $NaClO_4$ (15 eq.) to the solution in step 26.

28. Collect the precipitated triphosphate in sodium salt form by centrifuging 5 min at 3220 × *g*, 4°C and washing the resulting white pellet three times, each time with 1 mL acetone, followed by centrifuging 5 min at 3220 × *g*, 4°C. Dry the product under high vacuum.

*The resulting product, (α-L-threofuranosyl)cytosine-3′-triphosphate (**12**), is obtained in 12% yield (8 mg, 17 μmol) as a white powder. ^{31}P-NMR (161.8 MHz, D_2O): δ = −10.4 (d, J = 19.6), −12.6 (d, J = 20.0), −22.9 (t, J = 19.6). MALDI-TOF MS (neg.) 452.11 (calc. 452.12).*

BASIC PROTOCOL 5

ENZYMATIC SYNTHESIS OF THREOSE NUCLEIC ACID (TNA) OLIGONUCLEOTIDES

This protocol describes the enzyme-mediated synthesis of 3′,2′-TNA oligonucleotides using tNTPs as substrates and Therminator DNA polymerase as a DNA-dependent TNA polymerase. Relative to solid-phase synthesis, which is limited to the synthesis of relatively short TNA oligonucleotides (typically ≤12-mers) due to low reactivity of the 3′-hydroxyl group toward the activated phosphite, Therminator-mediated TNA synthesis provides access to long strands of TNA via an enzymatic process that proceeds with high efficiency and high fidelity.

Materials

5 mM (α-L-threofuranosyl)thymine-3′-triphosphate (tTTP)
5 mM (α-L-threofuranosyl)-2,6-diaminopurine-3′-triphosphate (tDTP)
5 mM (α-L-threofuranosyl)guanine-3′-triphosphate (tGTP)
5 mM (α-L-threofuranosyl)cytosine-3′-triphosphate (tCTP)
$MnCl_2 \cdot 4H_2O$ (mol. wt. 197.91 g/mol, Fisher)
Dithiothreitol (DTT, mol. wt. 154.3 g/mol, BioRad)
5 μM DNA primer
10 μM DNA template
10× ThermoPol reaction buffer (New England BioLabs)
10 mg/mL BSA (New England BioLabs)
Therminator DNA polymerase (2000 U/mL, New England BioLabs)

Microcentrifuge tubes
95°C heating block
55°C water bath
UV spectrophotometer

Additional reagents and equipment for denaturing PAGE and ethanol precipitation (*UNIT 10.4*)

1. Prepare a tNTP working stock solution (0.2 mM each) by combining 10 μL of each tNTP solution (5 mM) together with 210 μL water.

2. Prepare a fresh $MnCl_2$ solution (50 mM) by dissolving 0.01 g $MnCl_2 \cdot 4H_2O$ into 1011 μL water.

Table 4.54.1 Reagents for Therminator-Mediated TNA Transcription Reaction

Reagent	Stock concentration	Volume (μL)	Final concentration
DNA primer	5 μM	1	0.5 μM
DNA template	10 μM	1	1 μM
ThermoPol reaction buffer	10×	1	1×
Water		1.05	
BSA	10 mg/mL	0.1	100 μg/mL
$MnCl_2$	50 mM	0.25	1.25 mM
DTT	100 mM	0.1	1 mM
Therminator DNA polymerase	2 U/μL	0.5	0.1 U/μL
tNTPs	200 μM	5	100 μM
Total volume		10	

3. Prepare a fresh DTT solution (100 mM) by dissolving 0.01 g DTT into 648 μL water.
4. Combine 1 μL DNA primer, 1 μL DNA template, 1 μL ThermoPol reaction buffer, and 1.05 μL water in an appropriately sized microcentrifuge tube.
5. Anneal DNA primer-template complex by heating the solution 5 min at 95°C and cooling on ice for at least 10 min.
6. Combine 0.1 μL BSA, 0.25 μL $MnCl_2$, 0.1 μL DTT, and 0.5 μL Therminator DNA polymerase in an appropriately sized microcentrifuge tube.
7. Add the entire solution from step 6 to the annealed primer-template complex.
8. Add 5 μL of the tNTP working stock solution to the reaction mixture. See Table 4.54.1 for reagents for Therminator-mediated TNA transcription reactions.
9. Incubate the reaction 1 to 2 hr at 55°C.
10. Purify the crude product by denaturing PAGE and desalt the oligonucleotide by ethanol precipitation (*UNIT 10.4*).
11. Quantify the oligonucleotide by UV absorbance using Beer's law at 254 nm.

COMMENTARY

Background Information

Polymerase-mediated synthesis of TNA and in vitro evolution of synthetic TNA polymers

The chemical simplicity of threose relative to ribose, coupled with the ability for TNA to exchange genetic information with itself and RNA, has raised significant interest in TNA as a potentially natural progenitor of RNA in a hypothetical pre-RNA, world. This hypothesis is supported by the ability for TNA to serve as a template in the non-enzymatic synthesis of RNA from activated ribonucleotide monomers (Heuberger and Switzer, 2006) and the presence of threose on the surfaces of carbonaceous meteorites, which suggests that this simple sugar can be generated spontaneously in the absence of life (Cooper et al., 2001). Motivated by the desire to investigate the functional properties of TNA, several laboratories have explored the polymerase-mediated synthesis of TNA on DNA templates (Chaput et al., 2003; Chaput and Szostak, 2003; Kempeneers et al., 2003; Horhota et al., 2005; Ichida et al., 2005a,b). The most active polymerase discovered thus far is the Therminator DNA polymerase, an engineered variant of 9°N DNA polymerase bearing the A485L mutation. The authors have optimized the reaction

conditions for Therminator-mediated synthesis of TNA on DNA templates and used these conditions to select a functional TNA aptamer that binds to human thrombin with high affinity and specificity (Yu et al., 2012). Pinheiro et al. (2012) performed a compartmentalized self-tagging (CST) selection to develop a novel polymerase, called RT 521, that could transfer information back and forth between DNA and several alternative genetic polymers, including TNA. Collectively, these results have expanded the scope of Darwinian evolution from natural DNA and RNA to synthetic information-carrying polymers. This work led to the emergence of a new field of science called synthetic genetics that aims to develop functional molecules with novel physical and chemical properties that are of potential interest in material science and molecular therapeutics (Joyce, 2012).

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 cooled under an Ar (g) atmosphere. The anhydrous solvents used in phosphorylation should be freshly distilled and stored over newly activated molecular sieves to maximize the yield of the reaction. It is recommended to extend the deprotection time for removing trityl or acetyl groups at the 2′-OH position to compensate for the reduced reactivity of secondary hydroxyl groups.

Primer extension assays conducted using defined combinations of dNTP and tNTP substrates revealed that tCTP is incorporated into the growing strand less efficiently than the other three TNA triphosphates. This problem can be mitigated using shorter DNA templates or templates with reduced G-content (Yu et al., 2012).

Optimal yield of TNA oligonucleotides is achieved by adding the reagents in the order given in Basic Protocol 5 and using freshly prepared solutions of DTT and $MnCl_2$.

Anticipated Results

The protocols described in this unit are useful for preparing long strands of TNA oligonucleotides. The enzyme-mediated polymerization can be performed on the milliliter scale with high efficiency and fidelity under conditions described in Basic Protocol 5. The average yield is >70%, which is sufficient to produce TNA oligonucleotides for in vitro selection and other biochemistry assays.

Time Considerations

Most of the steps in the chemical synthesis can be accomplished in 1 to 2 working days (including purification). The liquid chromatography steps require slightly more time due to the removal of large volumes of aqueous solution generated during the chromatographic separation. TNA oligonucleotide synthesis and purification can be accomplished in 1 day.

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