Synthesis and Polymerase Incorporation of 5′-Amino-2′,5′-Dideoxy-5′-*N*-Triphosphate Nucleotides

This unit describes procedures for synthesizing 5′-amino-2′,5′-dideoxy analogs of four common 2′-deoxynucleosides (A, C, G, and T) and corresponding nucleotides, and the efficient incorporation of the latter into DNA (Wolfe et al., 2002). Since the 5′-amino functional group is much more reactive than the native 5′-hydroxyl group, these analogs can be exploited for various applications where differential chemical reactivity between natural and modified nucleotides is desirable. Unlike modifications on diverse heterocyclic structures, the 5′-modification on the sugar moiety is common to all nucleotides. Consequently, the preparation of these analogs is more likely to be generalizable among all four bases, and the products are more likely to exhibit similar traits in chemical reactivity and substrate specificity.

Through the intermediacy of 5′-azido nucleosides (N_3-dNs), 5′-amino nucleosides (NH_2-dNs) are conveniently prepared through robust chemical reactions such as tosylation, azide exchange, and the Staudinger reaction. Efficient conversion of NH_2-dNs to corresponding NH_2-dNTPs is achieved using an elegant one-step reaction with trisodium trimetaphosphate (TMP), which was first reported in the 1970s for the preparation of NH_2-dTTP (Letsinger et al., 1976a and b). By adding tris(hydroxymethyl)aminomethane (Tris) to neutralize protons generated during the reaction, the yield and stability of NH_2-dNTPs have been significantly improved.

Basic Protocols 1 and 2 describe detailed procedures for displacing the 5′-hydroxyl group of pyrimidines and purines, respectively, with a 5′-azido group. Tosylation at the 5′-hydroxyl followed by azide exchange is applied for purines (Fig. 13.3.1; see Basic Protocol 1), whereas a one-step reaction using carbon tetrabromide, triphenylphosphine, and lithium azide is utilized for pyrimidines (Fig. 13.3.2; see Basic Protocol 2). Because it is necessary to use protected dA, dC, and dG for these reactions, an additional hydrolysis step is required to unmask the exocyclic amino groups. The reduction of N_3-dNs to generate NH_2-dNs is achieved by the Staudinger reaction (Mungal et al., 1975), and is described in Basic Protocol 3 (Fig. 13.3.3). Efficient conversion of these nucleosides to corresponding NH_2-dNTPs is accomplished through a one-step reaction with TMP (Fig. 13.3.3) in the presence of Tris base (see Basic Protocol 4).

Basic Protocol 5 outlines procedures for template-directed polymerase incorporation of NH_2-dNTPs. Each NH_2-dNTP participates in a DNA replication reaction in place of the corresponding dNTP, either completely or partially, through its exclusive or supplementary use. Mild acid treatment of the resulting DNA generates polynucleotide fragments that arise from specific cleavage at each modified nucleotide, providing a sequence ladder for each base when analyzed by polyacrylamide gel electrophoresis.

Preparation methods for all four different NH_2-dNTPs are described in the protocols. If only one analog is needed for a desired application, NH_2-dTTP should be the first choice because NH_2-dT is commercially available; hence, Basic Protocols 1 and 2 can be skipped. Among the remaining analogs, pyrimidine NH_2-dC is somewhat more convenient to prepare than the purine analogs NH_2-dA and NH_2-dG.

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BASIC PROTOCOL 1

PREPARATION OF 5′-AZIDO-2′,5′-DIDEOXYPURINES

This protocol provides streamlined procedures to prepare N_3-dA and N_3-dG (Fig. 13.3.1). The synthetic route involves three commonly used chemical transformations and has been previously applied for synthesizing derivatives of N_3-dA and N_3-dG (Mag and Engels, 1989). Starting materials N^4-benzoyl-dA and N^2-isobutyryl-dG are tosylated at the 5′-hydroxyl group, then reacted with lithium azide to afford 5′-azido derivatives. Treatment with ammonia removes the protecting groups on the exocyclic amines of N_3-dA and N_3-dG. N_3-dG is purified from the isobutyramide side product using solvent partitioning alone; however, purification of N_3-dA from benzoylamide requires column chromatography.

CAUTION: All chemical reactions should be carried out in a fume hood to avoid exposure to toxic vapors.

NOTE: In order to achieve satisfactory results, anhydrous reagents should be used and experiments should be performed under an inert atmosphere.

Materials

Dry nitrogen (N_2) or argon (Ar)
N^6-Benzoyl-2′-deoxyadenosine (N^6-Bz-dA; **S.1**)
Pyridine, anhydrous
p-Toluenesulfonyl chloride (TsCl)
N^2-Isobutyryl-2′-deoxyguanosine (N^2-*i*-Bu-dG; **S.5**)
Ethyl acetate (EtOAc)
Saturated aqueous sodium bicarbonate ($NaHCO_3$)
Saturated aqueous sodium chloride (brine)
Sodium sulfate (Na_2SO_4), anhydrous
Dichloromethane (CH_2Cl_2)
Silica gel 60, 230 to 400 mesh
Methanol (MeOH)
Lithium azide (LiN_3), concentrated to dryness from a 20% solution in dH_2O
Dimethyl sulfoxide (DMSO), anhydrous
Ammonium hydroxide (NH_4OH), concentrated aqueous solution
Chloroform ($CHCl_3$)
Dry ice

250-, 100-, 50-, and 25-mL round-bottom flasks, oven dried
Inert atmosphere/vacuum manifold with dry ice/2-propanol trap
Balloons
Tubing adaptor
10-mL syringes
18-G needles
Rotary evaporator with a built-in dry ice/2-propanol trap, attached to a vacuum pump
250-mL separatory funnels
250-mL Erlenmeyer flasks
Fritted funnels
250-mL glass chromatography columns
Test tubes
Thin-layer chromatography (TLC) plates, 0.25-mm silica gel 60F-254 on glass plates
UV light source
60° and 50°C oil baths
Vacuum pump

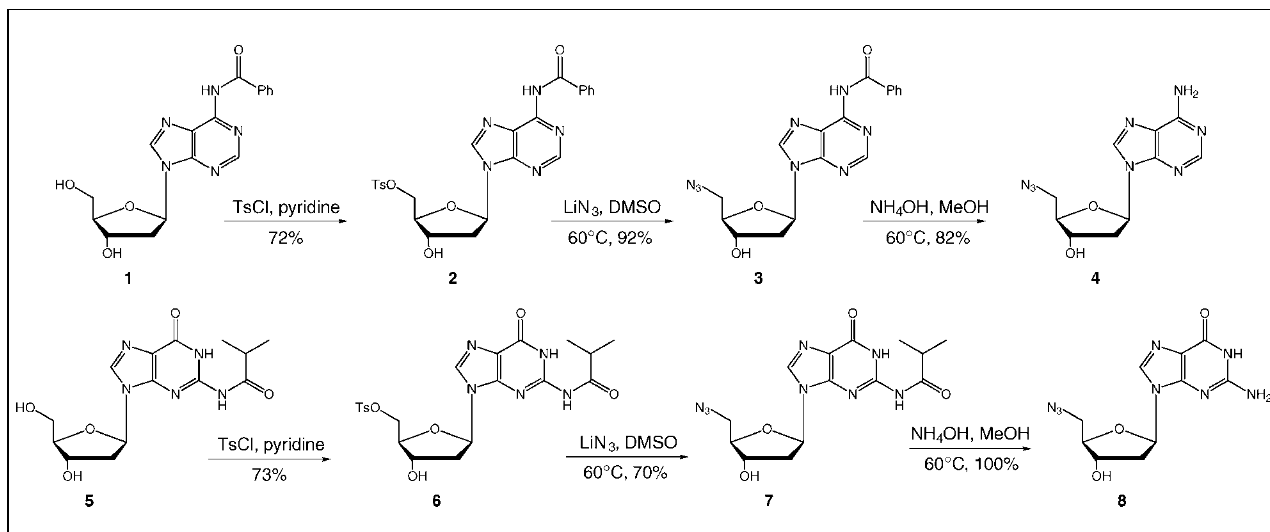

Figure 13.3.1 Preparation of 5′-azido-dA and 5′-azido-dG.

10-mL screw-capped micro-vials (e.g., Accuform vials, Kimble)
Lyophilizer
Additional reagents and equipment for column chromatography (*APPENDIX 3E*) and TLC (*APPENDIX 3D*)

5′-Tosylate nucleoside

For adenosine derivative

1a. Under a N_2 or Ar atmosphere, add 994 mg (2.8 mmol) of N^6-Bz-dA (**S.1**) and 20 mL anhydrous pyridine to an oven-dried 100-mL round-bottom flask containing a $^3/_4$-in. magnetic stir bar. Place the flask in an ice water bath on top of a magnetic stir plate.

A N_2 or Ar atmosphere can be provided through a tubing adaptor that connects the flask to a balloon filled with N_2 or Ar, or an inert atmosphere/vacuum manifold attached to a regulated N_2 or Ar tank.

2a. In a separate flask dissolve 802 mg (4.2 mmol, 1.5 eq.) TsCl in 10 mL anhydrous pyridine under N_2 or Ar.

For guanosine derivative

1b. Under N_2 or Ar atmosphere, add 777 mg (2.30 mmol) of N^2-*i*-Bu-dG (**S.5**) and 20 mL anhydrous pyridine in an oven-dried 100-mL round-bottom flask containing a $^3/_4$-in. magnetic stir bar. Place the flask in an ice water bath on top of a magnetic stir plate.

2b. In a separate flask dissolve 574 mg (3.01 mmol) TsCl in 5 mL anhydrous pyridine under N_2 or Ar.

3. Transfer the TsCl solution to a 10-mL syringe through an 18-G needle and slowly add it to the cooled, stirring nucleoside in pyridine under N_2 or Ar. Allow reaction to proceed 1 hr on ice.

Work up product

4. Remove the ice-water bath and transfer the flask to a 4°C refrigerator or cold room; continue stirring overnight under N_2 or Ar provided through a balloon.

5. Place the reaction flask back into an ice bath, add 3 mL of dH_2O to the mixture, and continue to stir on ice for 1 hr.

6. Concentrate the solution to near dryness on a rotary evaporator.

7. To the residue, add 150 mL of EtOAc and 30 mL of dH_2O. Stir the mixture using a magnetic stir plate to partition the products between organic and aqueous phases.

8. Transfer the biphasic mixture to a 250-mL separatory funnel to separate the organic and aqueous layers.

9. Collect the EtOAc (top) layer and wash it sequentially in the separatory funnel as follows: two times with 30 mL $NaHCO_3$, once with 30 mL dH_2O, and two times with 30 mL brine.

10. Collect the resulting EtOAc solution in a 250-mL Erlenmeyer flask containing ~15 g of anhydrous Na_2SO_4 and allow it to dry for 30 min.

11. Remove the drying agent by filtration using a fritted funnel.

12. Concentrate the EtOAc solution on a rotary evaporator to afford a white solid residue.

13. Dissolve the residue in ~2 mL of CH_2Cl_2 and load it onto a 100-mL chromatography column filled with 40 g of silica gel (*APPENDIX 3E*). Elute the column using CH_2Cl_2 followed by 5:95 (v/v) $MeOH/CH_2Cl_2$ and collect fractions in test tubes.

14. Analyze all fractions by TLC (*APPENDIX 3D*) on silica gel plates. Develop TLC plates with 10:90 (v/v) $MeOH/CH_2Cl_2$ and analyze them under UV illumination.

15. Combine fractions that contain the desired product ($R_f \approx 0.5$ for dA; $R_f \approx 0.44$ for dG) and concentrate to dryness using a rotary evaporator to afford the product as a light yellow or off-white foam.

    *5′-TsO-N^6-Bz-dA (**S.2**): yield 1092 mg (72%). ^{1}H NMR (DMSO-d_6): δ 2.32 (3H, s, Me), 2.37 (1H, ddd, J = 4.4, 6.7, 13.5 Hz, 2′a), 2.85 (1H, td, J = 6.5, 13.6 Hz, 2′b), 4.01 (1H, m, 4′), 4.22 (1H, dd, J = 6.8, 10.7 Hz, 5′a), 4.31 (1H, dd, J = 3.6, 10.7 Hz, 5′b), 4.47 (1H, m, 3′), 5.57 (1H, d, J = 6.6 Hz, 3′OH), 6.42 (1H, t, J = 6.6 Hz, 1′), 7.30 (2H, d, J = 8.1 Hz, Ts), 7.55 (2H, m, Ph), 7.63 (3H, m, Ts & Ph), 8.05 (2H, d, J = 7.4 Hz, Ph), 8.54 (1H, s, 2), 8.66 (1H, s, 8), 11.24 (1H, s, NH).*

    *5′-TsO-N^2-i-Bu-dG (**S.6**): yield 822 mg (73%). ^{1}H NMR (DMSO-d_6): δ 1.11 (3H, d, J = 6.8 Hz, Me), d 1.12 (3H, d, J = 6.8 Hz, Me), 2.29 (1H, m, C<u>H</u>Me$_2$), 2.35 (3H, s, Me(Ts)), 2.63 (1H, td, J = 6.5, 13.3 Hz, 2′a), 2.74 (1H, m, 2′b), 3.96 (1H, m, 4′), 4.16 (1H, dd, J = 6.8, 10.8 Hz, 5′a), 4.24 (1H, dd, J = 3.3, 10.8 Hz, 5′b), 4.37 (1H, m, 3′), 5.50 (1H, d, J = 6.6 Hz, 3′OH), 6.17 (1H, t, J = 6.6 Hz, 1′), 7.32 (2H, d, J = 8.1 Hz, Ts), 7.66 (2H, d, J = 8.2 Hz, Ts), 8.06 (1H, s, 8), 11.56 (1H, s, NH), 12.04 (1H, d, J = 5.0 Hz, NH).*

Convert to 5′-azido derivative

16a. *For dA:* Transfer 1063 mg (2.1 mmol) 5′-TsO-N^6-Bz-dA to a 50-mL round-bottom flask containing a magnetic stir bar, 514 mg (10.5 mmol, 5 eq.) LiN_3, and 11 mL DMSO.

16b. *For dG:* Transfer 811 mg (1.65 mmol) 5′-TsO-N^2-*i*-Bu-dG to a 25-mL round-bottom flask containing a magnetic stir bar, 411 mg (8.39 mmol, 5 eq.) LiN_3, and 8 mL DMSO.

17. Place the flask in a 60°C oil bath that is heated on top of a magnetic stir plate, and stir the reaction mixture for 5 hr while the oil bath temperature is maintained at ~60°C.

18. Concentrate the reaction mixture to ~5 mL using a vacuum pump while heating the flask in a 50°C oil bath.

19. Dissolve the residual material in 100 mL of EtOAc. Wash the solution with 20 mL $NaHCO_3$ followed by 20 mL brine using a 250-mL separatory funnel.

20. Transfer the EtOAc solution to a 250-mL Erlenmeyer flask containing ~10 g of anhydrous Na_2SO_4 and allow it to dry for 30 min.
21. Collect the organic solution by filtration through a fritted funnel.
22. Concentrate the EtOAc solution on a rotary evaporator to afford a pale yellow foam product.
23. Dissolve the crude product in ~2 mL of CH_2Cl_2 and apply it to a 100-mL chromatography column filled with 40 g of silica gel. Elute the column with CH_2Cl_2 followed by 5:95 (v/v) MeOH/CH_2Cl_2.
24. Analyze all fractions by TLC on silica gel, developing the TLC plates with 10:90 (v/v) MeOH/CH_2Cl_2.
25. Collect the fractions containing the desired product ($R_f \approx 0.4$ for dA; $R_f \approx 0.37$ for dG) and concentrate to dryness.

    *5′-N$_3$-N^6-Bz-dA (**S.3**): yield 764 mg (92%). ^{1}H NMR (DMSO-d$_6$): δ 2.40 (1H, ddd, J = 4.1, 6.6, 13.4 Hz, 2′a), 2.97 (1H, td, J = 6.6, 13.3 Hz, 2′b), 3.53 (1H, dd, J = 3.9, 13.1 Hz, 5′a), 3.65 (1H, dd, J = 7.0, 13.0 Hz, 5′b), 4.01 (1H, m, 4′), 4.48 (1H, m, 3′), 5.54 (1H, d, J = 3.7 Hz, 3′OH), 6.51 (1H, t, J = 6.7 Hz, 1′), 7.54 (2H, m, Ph), 7.64 (1H, t, J = 7.3 Hz, Ph), 8.03 (2H, d, J = 7.5 Hz, Ph), 8.69 (1H, s, 2), 8.76 (1H, s, 8), 11.20 (1H, s, NH).*

    *5′-N$_3$-N^2-i-Bu-dG (**S.7**): yield 419 mg (70%). ^{1}H NMR (DMSO-d$_6$): δ 1.11 (6H, d, J = 6.8 Hz, Me), 2.31 (1H, ddd, J =3.7, 6.1, 13.3 Hz, 2′a), 2.75 (2H, m, C<u>H</u>Me$_2$ & 2′b), 3.50 (1H, dd, J = 4.2, 13.1 Hz, 5′a), 3.58 (1H, dd, J = 6.8, 13.1 Hz, 5′b), 3.94 (1H, m, 4′), 4.34 (1H, m, 3′), 5.48 (1H, d, J = 3.7 Hz, 3′OH), 6.24 (1H, t, J = 6.8 Hz, 1′), 8.24 (1H, s, 8), 11.64 (1H, s, NH), 12.07 (1H, d, J = 4.7 Hz, NH).*

Deprotect exocyclic amine

26a. *For dA:* Transfer 599 mg (1.6 mmol) of 5′-N$_3$-*N*^{6}-Bz-dA to a 10-mL screw-capped micro-vial containing a magnetic stir bar and add 4 mL of MeOH to dissolve the solid.

26b. *For dG:* Transfer 409 mg (1.13 mmol) of 5′-N$_3$-*N*^{6}-*i*-Bu-dG to a 10-mL screw-capped micro-vial containing a magnetic stir bar and add 4 mL of MeOH to dissolve the solid.

27. Transfer 2 mL (half) of the resulting solution to a second 10-mL screw-capped micro-vial containing a magnetic stir bar.
28. Add 3 mL of concentrated NH_4OH to each micro-vial, place the vials in a ~60°C oil bath on top of a magnetic stir plate, and stir overnight (13 to 15 hr) at ~60°C.
29. Cool the vials on ice. Transfer the reaction mixtures to a round-bottom flask and concentrate to dryness using a rotary evaporator to afford a light yellow or off-white solid residue.
30. Dissolve the crude product in 60 mL of dH_2O, and extract the resulting aqueous solution five times with 5 mL $CHCl_3$ using a separatory funnel. Collect the aqueous (top) layer and save the $CHCl_3$ extracts.
31. Back extract the combined $CHCl_3$ solution two times with 5 mL dH_2O using the separatory funnel. Collect the aqueous solutions.

For N$_3$-dA

32a. Combine all the aqueous solutions in a 250-mL round-bottom flask and freeze on dry ice. Concentrate to dryness using a lyophilizer.

33a. Dissolve the crude N_3-dA product in CH_2Cl_2 and purify on a chromatography column containing 40 g of silica gel. Elute the column with 2:98 to 10:90 (v/v) $MeOH/CH_2Cl_2$.

34a. Analyze all fractions by TLC, developing the silica gel plates with 10:90 (v/v) $MeOH/CH_2Cl_2$.

35a. Collect the fractions containing the desired product ($R_f \approx 0.2$) and concentrate it to dryness to afford the product as light yellow foam.

*5′-Azido-2′,5′-dideoxyadenosine (N_3-dA; **S.4**): yield 350 mg (82%). ^1H NMR (DMSO-d_6): δ 2.30 (1H, ddd, J = 3.7, 6.4, 13.3 Hz, 2′a), 2.92 (1H, td, J = 6.7, 13.4 Hz, 2′b), 3.48 (1H, ddd, J = 3.9, 12.9 Hz, 5′a), 3.65 (1H, ddd, J = 7.3, 12.9 Hz, 5′b), 3.96 (1H, m, 4′), 4.41 (1H, m, 3′), 5.48 (1H, d, J = 4.0 Hz, 3′OH), 6.37 (1H, t, J = 6.9 Hz, 1′), 7.29 (2H, s, NH_2), 8.14 (1H, s, 2), 8.33 (1H, s, 8).*

For N_3-dG

32b. Combine all the aqueous solutions in a 250-mL round-bottom flask and freeze on dry ice. Concentrate to dryness using a lyophilizer to afford the product as a white solid. Use this material to prepare NH_2-dG without further purification.

*5′-Azido-2′,5′-dideoxyguanosine (N_3-dG; **S.8**): yield 340 mg (100%). ^1H NMR (DMSO-d_6): δ 2.22 (1H, ddd, J = 3.2, 6.1, 13.2 Hz, 2′a), 2.70 (1H, td, J = 6.3, 13.5 Hz, 2′b), 3.46 (1H, dd, J = 4.2, 13.0 Hz, 5′a), 3.61 (1H, dd, J = 7.2, 13.0 Hz, 5′b), 3.91 (1H, m, 4′), 4.29 (1H, m, 3′), 5.44 (1H, br, 3′OH), 6.14 (1H, t, J = 6.9 Hz, 1′), 6.53 (2H, brs, NH_2), 7.90 (1H, s, 8), 9.48 (1H, brs, NH).*

BASIC PROTOCOL 2

PREPARATION OF 5′-AZIDO-2′,5′-DIDEOXYPYRIMIDINES

N_3-dC is prepared based on a one-step azidation procedure (Fig. 13.3.2) that utilizes a mixture of triphenylphosphine, carbon tetrabromide, and lithium azide (Yamamoto et al., 1980). This same procedure is applicable for the preparation of N_3-dT, although the latter is commercially available. An extra aqueous ammonia deprotection step is used in the synthesis of N_3-dC to remove the exocyclic amino protection that is required during the azidation procedure.

CAUTION: All chemical reactions should be carried out in a fume hood to avoid toxic vapors.

NOTE: In order to achieve satisfactory results, anhydrous reagents should be used and experiments should be performed under an inert atmosphere.

Materials

N^4-Benzoyl-2′-deoxycytidine (N^4-Bz-dC; **S.9**)

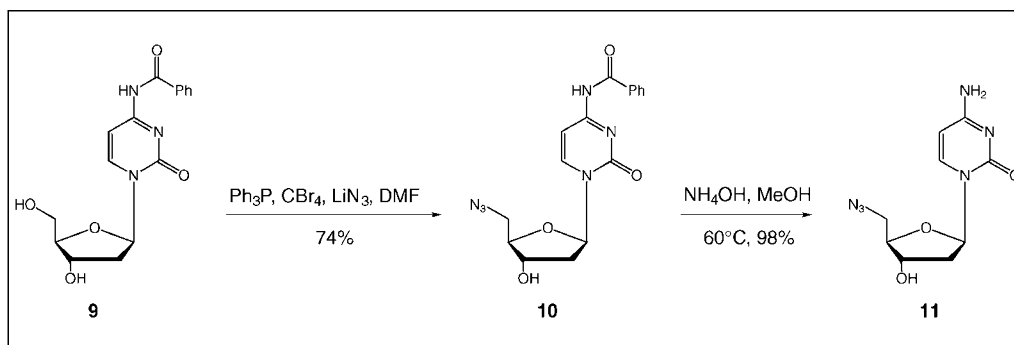

Figure 13.3.2 Preparation of 5′-azido-dC.

Dimethylformamide (DMF), anhydrous
Lithium azide (LiN_3), concentrated to dryness from a 20% solution in dH_2O
Triphenylphosphine (Ph_3P)
Dry nitrogen (N_2) or argon (Ar)
Carbon tetrabromide (CBr_4)
Silica gel 60, 230 to 400 mesh
Chloroform ($CHCl_3$)
Methanol (MeOH)
Dichloromethane (CH_2Cl_2)
Pyridine, anhydrous
Ammonium hydroxide (NH_4OH), concentrated aqueous solution
Ethyl acetate (EtOAc)
Diethyl ether (Et_2O)
Dry ice

25-, 100-, and 250-mL round-bottom flasks, oven dried
Rotary evaporator with dry ice/2-propanol trap, attached to a vacuum pump
Vacuum pump
Inert atmosphere/vacuum manifold with dry ice/2-propanol trap
Tubing adaptor
Balloons
250-mL glass chromatography column
Thin-layer chromatography (TLC) plates, 0.25-mm silica gel 60F-254 on glass
UV light source
10-mL screw-capped micro-vials (e.g., Accuform vials, Kimble)
60°C oil bath
100-mL separatory funnel
Lyophilizer

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

Convert to 5′-azido derivative

1. Mix 603 mg (1.82 mmol) N^4-Bz-dC (**S.9**) and 5 mL of anhydrous DMF in a 25-mL round-bottom flask and concentrate the solution to dryness using a rotary evaporator.

2. Add a ½-in. magnetic stir bar to the flask and attach the flask to a vacuum pump through a vacuum manifold to remove residual solvent.

3. Add 272 mg LiN_3 (5.56 mmol, 3 eq.), 621 mg Ph_3P (2.37 mmol, 1.3 eq.), and 10 mL anhydrous DMF to the flask under a N_2 or Ar atmosphere. Stir the mixture on a magnetic stir plate for a few minutes to afford a slightly cloudy solution (LiN_3 does not dissolve completely).

   A N_2 or Ar atmosphere can be provided through a tubing adaptor that connects the flask to a balloon filled with N_2 or Ar, or an inert atmosphere/vacuum manifold attached to a regulated N_2 or Ar tank.

4. Add 789 mg (2.34 mmol, 1.3 eq.) CBr_4 to the solution and stir the mixture overnight (19 hr) at room temperature.

5. Remove DMF using a rotary evaporator to afford an orange residue.

6. Purify the residue using a 250-mL chromatography column containing 40 g of silica (*APPENDIX 3E*). Elute the column with $CHCl_3$ followed by 4:100 (v/v) $MeOH/CHCl_3$.

7. Analyze all fractions by TLC (*APPENDIX 3D*) on silica gel plates. Develop TLC plates with 10:90 (v/v) $MeOH/CH_2Cl_2$.

8. Collect the fractions containing the major product ($R_f \approx 0.4$) and concentrate to dryness to afford the product as a white foam.

*5′-N$_3$-N^4-Bz-dC (**S.10**): yield 477 mg (74%). ^{1}H NMR (DMSO-d$_6$): δ 2.19 (1H, td, J = 6.4, 13.2 Hz, 2′a), 2.29 (1H, ddd, J = 4.5, 6.2, 13.2 Hz, 2′b), 3.60 (1H, dd, J = 4.0, 13.2 Hz, 5′a), 3.67 (1H, dd, J = 6.4, 13.2 Hz, 5′b), 3.94 (1H, m, 4′), 4.17 (1H, m, 3′), 5.48 (1H, d, J = 4.3 Hz, 3′OH), 6.19 (1H, t, J = 6.4 Hz, 1′), 7.39 (1H, brs, 5), 7.50 (2H, m, Ph), 7.61 (1H, t, J = 7.3 Hz, Ph), 7.99 (2H, d, J = 7.5 Hz, Ph), 8.18 (1H, d, J = 7.2 Hz, 6), 11.29 (1H, s, NH).*

Deprotect exocyclic amine

9. Transfer 454 mg (1.27 mmol) 5′-N$_3$-*N*^{4}-Bz-dC to a 10-mL screw-capped micro-vial containing a magnetic stir bar. Add 5 mL pyridine and 5 mL concentrated NH_4OH.

10. Stir the resulting mixture to dissolve the solids, and then transfer 5 mL of the solution to another 10-mL screw-capped micro-vial containing a magnetic stir bar.

11. Place both vials in a ∼60°C oil bath heated on top of a magnetic stir plate and stir at ∼60°C for 8.5 hr.

12. Cool the vials on ice, transfer the reaction mixture to a 100-mL round-bottom flask, and concentrate it to dryness using a rotary evaporator.

13. To the residue, add 30 mL dH_2O and 3 mL of EtOAc, then partition the resulting mixture in a 100-mL separatory funnel.

14. Collect the aqueous solution and extract it three times with 3 mL Et_2O followed by two times with 5 mL of 1:2 (v/v) EtOAc/Et_2O.

15. Freeze the aqueous solution on dry ice and concentrate it using a lyophilizer to afford the product as a light yellow solid.

*5′-Azido-2′,5′-dideoxycytosine (N$_3$-dC; **S.11**): yield 316 mg (98%). ^{1}H NMR (DMSO-d$_6$): δ 2.07 (2H, m, 2′), 3.51 (1H, dd, J = 4.4, 13.1 Hz, 5′a), 3.56 (1H, dd, J = 6.2, 13.1 Hz, 5′b), 3.83 (1H, m, 4′), 4.12 (1H, m, 3′), 5.38 (1H, d, J = 4.3 Hz, 3′OH), 5.73 (1H, d, J = 7.4 Hz, 5), 6.21 (1H, t, J = 6.8 Hz, 1′), 7.16 (1H, brs, NH), 7.20 (1H, brs, NH), 7.60 (1H, d, J = 7.4 Hz, 6).*

BASIC PROTOCOL 3

PREPARATION OF 5′-AMINO-2′,5′-DIDEOXYNUCLEOSIDES

This protocol describes a common procedure useful for the preparation of all four 5′-amino-2′,5′-dideoxynucleosides (NH_2-dNs) from corresponding 5′-azido derivatives (Fig. 13.3.3). Treatment with triphenylphosphine (Ph_3P) followed by hydrolysis with aqueous ammonia quantitatively converts 5′-azido nucleosides to 5′-amino nucleosides (Wolfe et al., 2002). The protocol uses the preparation of NH_2-dG as an example, but is also applicable for the synthesis of NH_2-dA and NH_2-dC. Specific experimental conditions for each of these analogs are listed in Table 13.3.1. NH_2-dT is commercially available and is not described here (interested readers are referred to Tetzlaff et al., 1998).

Materials

N_3-dA, N_3-dG, and N_3-dC (see Basic Protocols 1 and 2)
Pyridine, anhydrous
Triphenylphosphine (Ph_3P)
Dry nitrogen (N_2) or argon (Ar)
Ammonium hydroxide (NH_4OH), concentrated aqueous solution
Ethyl acetate (EtOAc)

25-mL round-bottom flask
Rotary evaporator with dry ice/2-propanol trap, attached to a vacuum pump

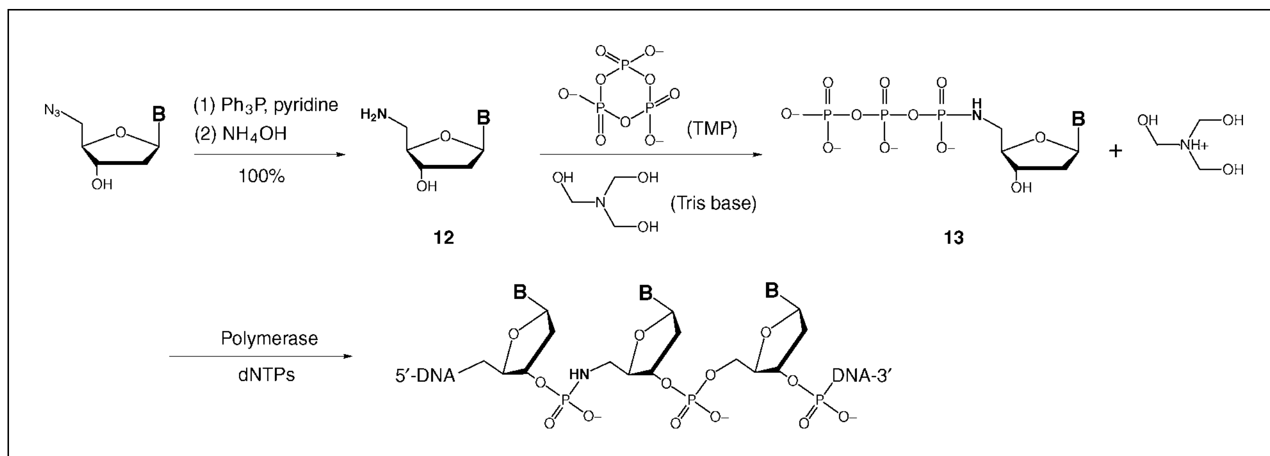

Figure 13.3.3 Preparation of 5′-amino-dNs, 5′-amino-dNTPs, and an oligonucleotide containing a single P-N linkage.

Table 13.3.1 Synthesis Conditions Used for Each NH_2-dN[a]

	N_3-dN (step 1)	Ph_3P/Pyr (step 3)	NH_4OH (steps 4 to 5)	H_2O (step 6)
NH_2-dA	0.12 mmol	0.38 mmol/1 mL/7 hr	0.2 mL/15 hr RT/1 hr 55°C	5 mL
NH_2-dC	0.31 mmol	0.93 mmol/2 mL/7 hr	0.2 mL/15 hr RT	5 mL
NH_2-dG	0.14 mmol	0.43 mmol/1 mL/5 hr	0.3 mL/17 hr RT/1 hr 55°C	6 mL

[a]Pyr, pyridine; RT, room temperature.

Inert atmosphere/vacuum manifold with dry ice/2-propanol trap
Tubing adaptor
Balloon
55°C oil bath
Fritted funnel
Lyophilizer

1. Place 40 mg (0.14 mmol) N_3-dG (**S.8**) in a 25-mL round-bottom flask (also see Table 13.3.1).

2. Add 2 mL anhydrous pyridine and concentrate it to dryness on a rotary evaporator. Repeat.

3. Add 3 molar equiv. (111 mg, 0.43 mmol) Ph_3P, 1 mL anhydrous pyridine, and a $^1/_2$-in. magnetic stir bar to the flask. Under a N_2 or Ar atmosphere, stir the reaction mixture using a magnetic stir plate 5 hr at room temperature.

   A N_2 or Ar atmosphere can be provided through a tubing adaptor that connects the flask to a balloon filled with N_2 or Ar, or an inert atmosphere/vacuum manifold attached to a regulated N_2 or Ar tank.

4. Add 0.3 mL concentrated NH_4OH and stir overnight (17 hr) at room temperature.

5. Place the flask in a 55°C oil bath on top of a magnetic stir plate and continue to stir for 1 hr.

6. Cool the flask to room temperature and add 6 mL dH_2O. Remove the resulting precipitate using a fritted funnel and rinse with 1 mL dH_2O.

7. Collect the filtrate (aqueous solution) and extract it with 10 mL EtOAc.

8. Concentrate the resulting aqueous solution to dryness using a lyophilizer to afford a crude product which can be used in Basic Protocol 4 without further purification.

*5′-Amino-2′,5′-dideoxyguanosine (NH_2-dG; **S.12**): yield 50 mg (100%) as pale yellow solid. ^1H NMR (DMSO-d_6): δ 2.19 (1H, m, 2′a), 2.57 (1H, td, J = 6.6, 13.4 Hz, 2′b), 2.82 (2H, brs, 5′), 3.79 (1H, brs, 4′), 4.37 (1H, brs, 3′), 5.76 (3H, brs, NH_2, & 3′OH), 6.11 (1H, td, J = 6.9 Hz, 1′), 6.90 (2H, brs, NH_2), 7.88 (1H, s, 8).*

*5′-Amino-2′,5′-dideoxyadenosine (NH_2-dA; **S.12**): yield 63 mg (>100%) as white solid. ^1H NMR (DMSO-d_6): δ 2.23 (1H, ddd, J = 3.3, 6.1, 13.1 Hz, 2′a), 2.80 (3H, m, 2′b & 5′), 3.82 (1H, m, 4′), 4.42 (1H, m, 3′), 5.70 (3H, br, NH_2 & 3′OH), 6.32 (1H, t, J = 6.6 Hz, 1′), 7.29 (2H, brs, NH_2), 8.14 (1H, s, 2), 8.35 (1H, s, 8).*

*5′-Amino-2′,5′-dideoxycytosine (NH_2-dC; **S.12**): yield 104 mg (>100%) as light brown foam. ^1H NMR (DMSO-d_6): δ 1.95 (1H, td, J = 6.6, 13.4 Hz, 2′a), 2.09 (1H, ddd, J = 3.9, 5.7, 13.2 Hz, 2′b), 2.75 (2H, brs, 5′), 3.69 (1H, m, 4′), 4.15 (1H, m, 3′), 5.14 (3H, br, NH_2 & OH), 5.73 (1H, d, J = 7.3 Hz, 5), 6.14 (1H, t, J = 6.7 Hz, 1′), 7.31 (2H, br, NH_2), 7.72 (1H, d, J = 7.3 Hz, 6).*

Yields >100% are likely due to the incomplete removal of water.

BASIC PROTOCOL 4

PREPARATION AND HPLC ANALYSIS OF 5′-AMINO-2′,5′-DIDEOXY-5′-*N*-TRIPHOSPHATE NUCLEOTIDES

This protocol describes a common procedure for synthesizing NH_2-dNTPs from corresponding NH_2-dNs. As shown in Figure 13.3.3, the procedure utilizes a facile reaction between primary amines and trisodium trimetaphosphate (TMP). This straightforward approach was previously applied for the preparation of NH_2-dTTP with some success. However, the conversion yield was moderate and the product was unstable, which was likely caused by the inherently low stability of NH_2-dTTP (Letsinger et al., 1976b). By adding a commonly used, sterically hindered primary amine (Tris) to the reaction mixtures, all four NH_2-dNTPs have been prepared in dramatically improved yields (Wolfe et al., 2002). This protocol uses the preparation of NH_2-dTTP as an example, and the reaction conditions and yields for other analogs are summarized in Table 13.3.2.

Materials

NH_2-dA, NH_2-dC, NH_2-dG (see Basic Protocol 3), and NH_2-dT (Sigma)
Trisodium trimetaphosphate (TMP)
0.5 M aqueous Tris base
HPLC buffers A and B (see recipes)

1.5-mL microcentrifuge tubes
Vortex mixer
C18 column (Waters Nova-pak; 3.9 × 150 mm, 4 μm)

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

Table 13.3.2 Reagents and Conditions Used for the Synthesis of Each NH_2-dNTP

	Nucleoside[a] (step 1)	TMP (step 1)	0.5 M Tris base (step 2)	Reaction time (step 3)	Conversion yield by HPLC (step 5)
NH_2-dATP	47 μmol	234 μmol	468 μL	7 days	85.6%
NH_2-dCTP	86 μmol	429 μmol	859 μL	5 days	78.9%
NH_2-dGTP	86 μmol	430 μmol	860 μL	5 days	83.2%
NH_2-dTTP	112 μmol	560 μmol	1120 μL	5 days	91.5%

[a]For NH_2-dA, NH_2-dC, and NH_2-dG, the starting material quantities are based on the assumption that N_3-dN was quantitatively converted to NH_2-dN using Basic Protocol 3.

1. Place 27.0 mg (112 μmol) of NH_2-dT and 171.4 mg (560 μmol, 5 molar equiv.) TMP in a 1.5-mL microcentrifuge tube (also see Table 13.3.2).

2. Add 1120 μL of 0.5 M aqueous Tris base (560 μmol, 5 molar equiv.) to the tube and dissolve the solids using a vortex mixer.

3. Allow the resulting solution to stand 5 days at room temperature to generate NH_2-dTTP.

   The 100 mM NH_2-dNTP/500 mM Tris solutions (based on 100% conversion) are used directly for experiments described in Basic Protocol 5. The following steps for HPLC analysis are optional. HPLC analysis is not necessary for application described in this unit and is recommended only when the knowledge of a specific conversion yield is deemed useful. The product is stable at room temperature and can be stored for weeks without losing activity. However, to avoid water loss and slow oxidation of Tris base, the mixture should be stored at –20°C for a longer term (up to 1 year).

   ^{31}P NMR spectra were recorded on a Bruker DPX-400 NMP spectrometer using 80% H_3PO_4 as an external standard. A doublet around –1 ppm was observed for all four triphosphate samples, corresponding to a phosphoramidate (Pα-N) resonance. In all spectra the most prominent peak was at –21 ppm, corresponding to excess TMP. Due to excess TMP and its breakdown products, Pβ and Pγ were not assigned. ^{31}P NMR (D_2O): NH_2-dATP, –0.99 ppm (d, J = 20.8 Hz); NH_2-dCTP, –0.94 ppm (d, J = 19.5 Hz); NH_2-dGTP, –1.00 ppm (d, J = 20.8 Hz); NH_2-dTTP, –0.97 ppm (d, J = 20.1 Hz).

4. Monitor the reaction mixture using an HPLC system equipped with a C18 column (*UNIT 10.5*). Elute the column with a mobile phase of HPLC buffers A and B at a flow rate of 1.00 mL/min. Vary the buffer gradient from 98% A to 100% B to achieve separation of NH_2-dT from NH_2-dTTP:

0 to 8 min	98% A to 70% A
8 to 9 min	70% A to 0% A
9 to 10 min	0% A
10 to 11 min	0% A to 98% A
11 to 15 min	98%A.

5. Compare peak areas corresponding to NH_2-dT (retention time ≈ 4.0 min) and NH_2-dTTP (retention time ≈ 10.7 min) in the HPLC chromatograms to provide the yield of conversion (91.5%).

   Retention times for NH_2-dA and NH_2-dATP: ≈5.5 min and ≈14 min, respectively; for NH_2-dC and NH_2-dCTP: ≈2.0 min and ≈5.0 min, respectively; for NH_2-dG and NH_2-dGTP ≈3.0 min and ≈10.5 min, respectively.

POLYMERASE INCORPORATION OF NH_2-dNTPS

BASIC PROTOCOL 5

This protocol describes procedures for incorporating each NH_2-dNTP into DNA through a DNA polymerase–catalyzed template-directed primer extension reaction. The procedure is similar to routine primer extension reactions, with the exceptions that NH_2-dNTP is used to replace or supplement its naturally occurring counterpart to afford complete or statistical analog substitution in the DNA product, respectively, and that an alkaline pH 9.5 buffer is used to improve the NH_2-dNTP stability. Among the many commercially available polymerases tested, Klenow polymerase (exo$^-$) provides the most efficient NH_2-dNTP incorporation. In this protocol, NH_2-dNTPs are used in higher concentrations than those of native dNTPs to compensate for their reduced incorporation efficiency compared to dNTPs. Mild acid treatment of the substituted DNA product generates fragments that correspond to site-specific cleavage at each modified nucleotide, which provides direct evidence of successful NH_2-dNTP incorporation as well as the sequence of the original DNA template (Wolfe et al., 2002).

Materials

20 μM oligonucleotide primer
1.67 μM [γ-^{32}P]ATP (6000 Ci/mmol; Perkin-Elmer)
10 U/μL T4 polynucleotide kinase and 10× buffer (New England Biolabs)
TE buffer, pH 8.0 (*APPENDIX 2A*)
Single-stranded DNA templates: ∼100 or 500 nucleotides in length (for complete or partial NH_2-dNTP substitution, respectively)
200 mM $MgCl_2$
500 mM sodium acetate (NaOAc)
10× polymerase extension buffers A and B (see recipes)
100 mM NH_2-dNTP solution(s) in 0.5 M Tris (see Basic Protocol 4)
2′-Deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP)
5 U/μL Klenow fragment of DNA polymerase (exo$^-$; New England Biolabs)
1% and 10% (v/v) acetic acid
Formamide loading buffer (Life Technologies)
Denaturing polyacrylamide gel mix and buffer solutions (National Diagnostics)
1× TBE buffer (*APPENDIX 2A*)
10 mM EDTA, pH 8 (*APPENDIX 2A*)

0.5-mL microcentrifuge tubes
Heat block at 37° and 90°C
Sephadex G-50 columns, preswollen in dH_2O
Speedvac evaporator

Additional reagents and equipment for polyacrylamide gel electrophoresis (PAGE; *UNIT 10.4*)

Label and anneal oligonucleotide primer

1. Label a primer using T4 polynucleotide kinase and [γ-^{32}P]ATP. In a 0.5-mL microcentrifuge tube mix the following:

   1 μL 20 μM oligonucleotide primer
   1 μL 1.67 μM [γ-^{32}P]ATP (6000 Ci/mmol)
   1 μL 10× T4 polynucleotide kinase buffer
   6 μL dH_2O
   1 μL 10 U/μL T4 polynucleotide kinase.

2. Incubate the mixture for 45 to 60 min at 37°C.

3. Add 10 μL TE buffer and purify the solution using a Sephadex G-50 column to afford an ∼1 μM primer solution (assuming 100% recovery).

Perform primer extension with complete NH_2-dNTP substitution

4a. In a 0.5-mL microcentrifuge tube mix the following:

   2 μL ∼1 μM labeled primer
   10 μL ∼0.24 μM ssDNA template (∼100 nt in length)
   2 μL 200 mM $MgCl_2$
   2 μL 500 mM of NaOAc
   4 μL dH_2O.

5a. Heat the solution in a heat block 2 min at 90°C, then allow the heat block to cool down to room temperature to afford a solution containing ∼0.1 M DNA duplex in 20 mM $MgCl_2$/50 mM NaOAc.

6a. In a 0.5-mL microcentrifuge tube mix the following:

1 μL annealed DNA duplex solution
1 μL 10× polymerase extension buffer A
1 μL 100 mM DTT
0.4 μL 100 mM NH_2-dATP/0.5 M Tris (or other NH_2-dNTP)
1 μL 1 mM each dCTP/dGTP/dTTP (or any 3 dNTPs that complement the NH_2-dNTP)
4.6 μL dH_2O
1 μL 5 U/μL Klenow (exo^-) DNA polymerase.

The resulting mixture contains 45 mM Tris·Cl, pH 9.5, 10 mM DTT, 22 mM $MgCl_2$, 5 mM NaOAc, 4 mM NH_2-dNTP, 0.1 mM each of the other three dNTPs, and 5 U Klenow (exo^-) polymerase.

7a. Incubate 1 hr at 37°C.

8a. Add 30 μL TE buffer and purify using Sephadex G-50 columns to afford ∼50 μL of modified DNA solution. Store up to 2 weeks at −20°C.

Prepare G-50 columns using G-50 resin preswollen in dH_2O. G-50 resin that has been pre-equilibrated in Tris buffers may alter the amount of acetic acid needed in step 9a.

Perform site-specific cleavage at NH_2-dN for complete substitution

9a. In a 0.5-mL microcentrifuge tube mix 8 μL of each modified DNA solution with 2 μL of 1% acetic acid and incubate 30 min at 37° to 40°C.

10a. Add 100 μL dH_2O to each sample, and concentrate the resulting solution to dryness on a Speedvac evaporator.

11a. Dissolve each sample in dH_2O. Adjust volume depending on the radioactivity of the samples.

12a. Transfer 2 μL of each solution to another microcentrifuge tube and add 2 μL formamide loading buffer.

13a. Analyze the samples by electrophoresing on a 12% denaturing polyacrylamide gel using standard procedures (*UNIT 10.4*) and 1× TBE buffer.

Perform primer extension with partial (statistical) NH_2-dNTP substitution

4b. Anneal labeled primer to a ssDNA template as in steps 4a and 5a, but use a template that is ∼500 nt in length.

5b. In a 0.5-mL microcentrifuge tube mix the following:

1 μL annealed DNA duplex solution
1 μL 10× polymerase extension buffer B
0.4 μL 100 mM NH_2-dATP/0.5 M Tris (or other NH_2-dNTP)
4 μL 0.1 mM dATP (or 2.6 μL dCTP, 1.6 μL dGTP, 4.6 μL dTTP)
2 μL 2 mM each dCTP/dGTP/dTTP (or any 3 dNTPs that complement the NH_2-dNTP)
dH_2O to 9 μL
1 μL 5 U/μL Klenow (exo^-) DNA polymerase.

The resulting mixture contains 50 mM Tris·Cl, pH 9.5, 5 mM DTT, 22 mM $MgCl_2$, 5 mM NaOAc, 4 mM NH_2-dNTP, 0.016 to 0.046 mM of the corresponding dNTP (0.04 mM dATP, 0.026 mM dCTP, 0.016 mM dGTP, 0.046 mM TTP), 0.4 mM each of the other three dNTPs, and 5 U of Klenow (exo^-) polymerase.

6b. Incubate 1 hr at 37°C.

7b. Add 1 μL of 1 mM (each) dATP/dCTP/dGTP/dTTP and incubate for an additional 15 min at 37°C.

8b. Add 20 μL TE buffer and store the resulting solutions up to 2 weeks at −20°C.

Perform site-specific cleavage at NH_2-dN for partial substitution

9b. In a 0.5-mL microcentrifuge tube mix the following:

2 μL modified DNA solution
1 μL 10 mM EDTA, pH 8
6 μL dH_2O
1 μL 10% acetic acid.

10b. Incubate 10 min at 37°C.

11b. Proceed with analyis as in steps 10a to 13a.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

HPLC buffer A

Add 10 mL of 1 M Tris·Cl, pH 9 (*APPENDIX 2A*), and 50 mL of 2 M TEAA buffer (see recipe) to ∼900 mL dH_2O in a 1-L graduated cylinder. Add trace amounts of triethylamine and/or acetic acid to adjust the pH to 9 (measure using pH paper). Bring volume to 1 L with dH_2O (final 10 mM Tris/100 mM TEAA). Store up to several weeks at room temperature.

HPLC buffer B

Mix 750 mL HPLC buffer A (see recipe) and 250 mL MeOH. Store up to several weeks at room temperature.

Polymerase extension buffer A, 10×

0.25 M Tris·Cl, pH 9.5
0.2 M $MgCl_2$
Store up to several months at −20°C

Polymerase extension buffer B, 10×

0.3 M Tris·Cl, pH 9.5
0.2 M $MgCl_2$
50 mM dithiothreitol (DTT)
Store up to several months at −20°C

TEAA (triethylammonium acetate) buffer, 2 M

Chill triethylamine and acetic acid on ice. In a fume hood, mix 278.8 mL of triethylamine and 114.4 mL of acetic acid in a 1-L graduated cylinder. Add dH_2O to 1 L. Store up to several months at room temperature.

COMMENTARY

Background Information

Due to their notably increased chemical reactivity and limited structural deviation from naturally occurring nucleosides, 5′-amino-2′,5′-dideoxy nucleosides (NH_2-dNs) and nucleotides (NH_2-dNTPs) have been exploited for various applications. For example, derivatives of NH_2-dT have been used for model studies on DNA replication (Luo et al., 1998), template-directed chemical amplification (Zhan and Lynn, 1997), mechanistic studies on polymerases and reverse transcriptases (Lutz et al., 1997), and the construction of combinatorial peptide-DNA hybrids (Bergmann and Bannwarth, 1995) and their libraries (Koppitz et al., 1998; Tetzlaff et al., 1998). In addition, NH_2-dTTP and NH_2-dCTP have been used to incorporate acid-labile phosphoramidate (P-N) linkers into DNA for high-throughput detection of single nucleotide polymorphisms using matrix-assisted fragmentation (Shchepinov et al., 2001), while NH_2-dTTP has been applied in a dinucleotide DNA cleavage method developed for discovery of single nucleotide polymorphisms (Wolfe et al., 2003).

Most of the aforementioned applications have been demonstrated utilizing NH_2-dT or its triphosphate NH_2-dTTP, perhaps because NH_2-dT is the only commercially available analog among the commonly used nucleosides and nucleotides. The ready availability of all four NH_2-dNs and NH_2-dNTPs will help expand the use of this class of compounds. The synthetic strategy described here takes advantage of common chemical features of all NH_2-dNTPs and utilizes synthetic methods including tosylation (also described in *UNIT 13.2*), azide exchange, and the Staudinger reaction. Because this approach does not require special equipment such as a Parr hydrogenation apparatus, these compounds can be conveniently made in many minimally equipped chemistry laboratories.

Besides polymerase incorporation of NH_2-dNTPs, P-N linkers can also be introduced into DNA through solid-phase oligonucleotide synthesis using 5′-NH_2-modified phosphoramidate building blocks (Mag and Engels, 1989; Shchepinov et al., 2001). This approach provides an opportunity to place P-N linkers at any specific position within an oligonucleotide, which is of importance for applications where predetermined localization of P-N bonds is desirable. However, solid-phase synthesis is not compatible with applications where limited DNA sequence information is available, as is the case for DNA sequence or polymorphism discovery (Wolfe et al., 2002, 2003). In addition, due to the lower efficiency of chemical coupling relative to enzymatic elongation, oligonucleotides generated by solid-phase synthesis have a very short length limit (up to ∼150 bases) in comparison to replication reactions catalyzed by polymerases (up to several kilobases). For example, the authors have generated a 7.2-kb long NH_2-dT-containing polymerization product using Klenow (exo^-) polymerase and the M13 plasmid as template DNA (Wolfe et al., 2002). Furthermore, solid-phase synthesis requires access to an automated DNA synthesizer, as well as 5′-NH_2-modified phosphoramidate building blocks that are not available commercially.

Critical Parameters and Troubleshooting

For chemical reactions described in Basic Protocols 1 through 3, it is critical to avoid moisture in all reaction mixtures. In the authors' experience, sealed bottles of anhydrous solvents obtained directly from vendors are sufficiently dry for these reactions. Starting material (nucleosides) should be routinely co-evaporated with anhydrous solvents followed by vacuum drying before use.

In Basic Protocol 4, using a freshly prepared 0.5 M Tris base solution will ensure sufficient buffering capacity. The NH_2-dNTP products should be stored and used as prepared; isolation or dilution is not recommended because they may reduce the amount of 5′-*N*-triphosphate nucleotides (Letsinger et al., 1976b). Although the concentration of the NH_2-dNTP from individual attempted reactions may vary slightly, the overall reproducibility of this reaction is very high. HPLC analysis can provide information on the actual amount of the desired product, but is not required.

In Basic Protocol 5, the authors routinely use Klenow (exo^-) polymerase from New England Biolabs. Polymerases from other vendors may also be applicable, but reaction conditions may need to be optimized since other polymerase products may have different enzymatic activities. Because the purity and concentration of each nucleotide may affect its polymerase incorporation efficiency, slight modifications of the dNTP/NH_2-dNTP ratios may be necessary for statistical incorporation

experiments to achieve even incorporation of NH_2-dNTPs.

The efficiency of acid cleavage reactions may be very sensitive to the amount of Tris in each DNA sample and the actual concentration of acetic acid used. Minor adjustments in acid concentration and/or reaction time may be necessary to accommodate these variations.

Anticipated Results

For Basic Protocols 1 through 3, the yields of chemical reactions are generally very high, although the recovered yield of purified products may be variable and considerably lower, depending on the scale or the method of purification.

Basic Protocol 4 is highly reproducible and the reaction mixtures can be directly used for downstream enzymatic reactions.

Polymerase incorporation of one of the NH_2-dNTPs as complete replacement of its naturally occurring counterpart is very reproducible, as long as the Klenow (exo^-) polymerase is fresh and relatively short DNA templates are used. Acid cleavage of these products may need to be optimized to achieve even cleavage bands.

Statistical incorporation of NH_2-dNTPs and subsequent acid cleavage provide a sequencing ladder from long DNA templates. Using harsher cleavage conditions than described (i.e., higher concentration of acetic acid, longer reaction time, or higher temperature) will enrich shorter DNA fragments. Insufficient cleavage will enrich longer DNA fragments and the full-length extension product.

Time Considerations

The approximate time scale for the sequence of reactions and procedures described in this unit is 1 to 2 days for tosylation; 2 to 3 days for azidation and deprotection; 1 day for Ph_3P reduction and hydrolysis; 5 to 7 days for triphosphate formation (mostly hands-off time); 2 days for annealing of primer to template, followed by polymerase incorporation of NH_2dNTPs and subsequent chemical cleavage reactions; and 1 to 2 days for PAGE analysis of the products.

A major uncertainty in time consumption is related to column chromatography. The optimum solvent composition may depend on column diameter, quality of silica gel, purity of eluting solvents, and rate of elution. If removal of impurity is not completely successful in the first attempt, another column chromatography purification may be necessary.

Since HPLC analysis of NH_2-dNTPs requires instrumentation and is time consuming, users may choose to skip this procedure. In the authors' experience, the preparation of NH_2-dNTPs from NH_2-dNs has been very reproducible.

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