Synthesis and Purification of Oligonucleotide N3′→P5′ Phosphoramidates and their Phosphodiester and Phosphorothioate Chimeras

Oligonucleotide N3′→P5′ phosphoramidates (pnODNs), wherein each 3′-oxygen is replaced by a 3′-amine in the 2′-deoxyribose ring (Figure 4.7.1), have shown significant therapeutic potential as antisense and antigene agents via duplex and triplex formation, respectively. Their binding properties also make them good candidates for oligonucleotide-based diagnostics and research tools. This unit describes the solid-phase synthesis of pnODNs (see Basic Protocol 1 and Alternate Protocol 1) using a method based on a phosphoramidite amine-exchange reaction wherein the key step is an exchange of a solid support–bound 3′-amino oligonucleotide for the amino group of a phosphoramidite monomer. Purification of these analogs is performed by either ion-exchange chromatography (IEC; see Basic Protocol 2) or reversed-phase high-performance liquid chromatography (RP-HPLC; see Alternate Protocol 2). The unit also describes the synthesis of required monomers and application of the method to preparation of phosphodiester- and phosphorothioate-containing chimeras of pnODNs.

SOLID-PHASE CHAIN ASSEMBLY OF OLIGONUCLEOTIDE N3′→P5′ PHOSPHORAMIDATES FOR IEC PURIFICATION

BASIC PROTOCOL 1

The pnODN syntheses are performed in the 5′→3′ direction using a 3′-tritylamino nucleoside bound to aminopropyl controlled-pore glass (CPG) by a succinyl linker as the solid support (Figure 4.7.2). The trityl group was chosen as the amino-protecting group because it provides the requisite stability to the coupling, oxidation, and capping reagents, and is quantitatively removed by a relatively short treatment with dichloroacetic acid in dichloromethane. The resulting 3′-ammonium dichloroacetate salt is then coupled to 3′-tritylamino-5′-*O*-[(*cis*-2,6-dimethylpiperidino)(2-cyanoethoxyl)]phosphinyl-2′,3′-dideoxynucleoside monomer (Figure 4.7.3; **S.1**) in the presence of 1*H*-tetrazole. After neutralization, the resulting internucleotide phosphoramidite is oxidized to the stable, protected phosphoramidate. If phosphodiester or phosphorothioate chimeras are desired, the 3′-*O*-(4,4′-dimethoxytrityl)-5′-*O*-[(*cis*-2,6-dimethylpiperidino)(2-cyanoethoxy)]phosphinyl-2′-deoxyribonucleoside monomers (Figure 4.7.3; **S.2**) are used for those linkages, followed by either oxidation or sulfurization, respectively. A mixture of isobutyric anhydride and *N*-methylimidazole is used to cap any unreacted 3′-amines

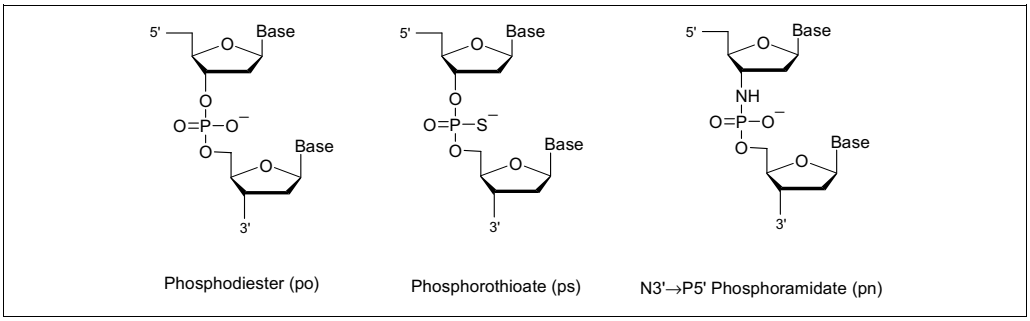

Figure 4.7.1 Structures of phosphodiester, phosphorothioate, and phosphoramidate linkages.

Contributed by Karen L. Fearon and Jeffrey S. Nelson
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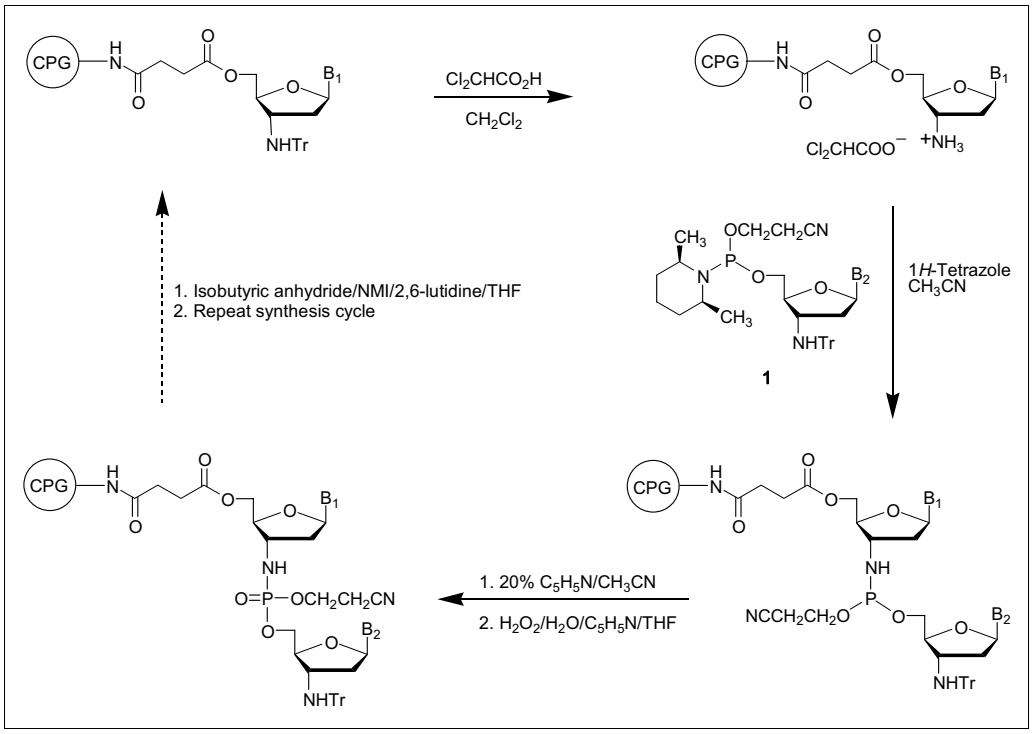


Figure 4.7.2 Synthetic steps in the preparation of pnODNs by the amine-exchange method. CPG, controlled-pore glass; Tr, triphenylmethyl; NMI, *N*-methylimidazole; THF, tetrahydrofuran.

because less hindered electrophiles, such as acetic anhydride, react with the tritylamino group of the oligonucleotide product.

The method of purification needs to be decided on and planned for during the setup of the solid-phase synthesis. RP-HPLC purification of pnODNs using the hydrophobicity of the trityl group (Tr) is problematic because once the cyanoethyl groups are removed during ammonolysis, the phosphoramidate linkages are no longer stable to the acidic, post-RP-HPLC detritylation conditions. A new purification method requiring nonacidic conditions was therefore developed; however, it is only applicable to compounds terminating with a 3′-hydroxyl group. If a 3′-amine is desired, then IEC purification must be used. Chimera-containing phosphorothioate linkages are best purified by RP-HPLC because the phosphorothioate groups cause a severe loss of resolution during IEC. This

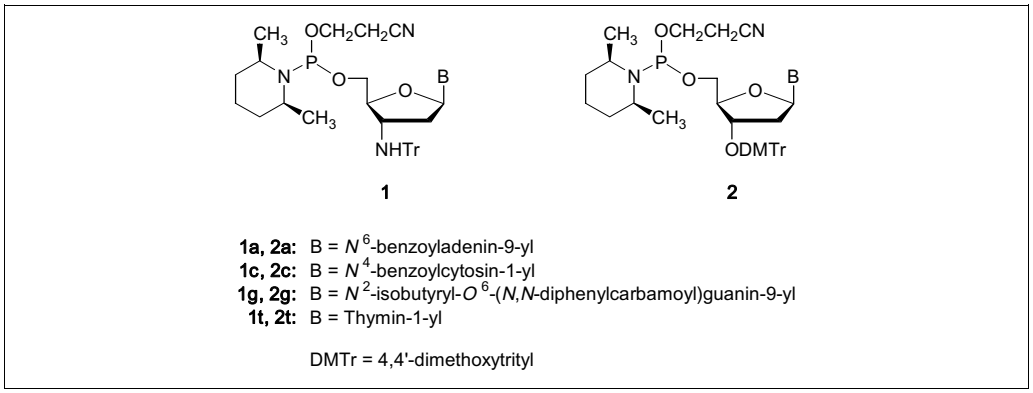


Figure 4.7.3 Structures of key phosphoramidite monomers.

protocol describes synthesis requiring IEC purification, whereas Alternate Protocol 1 describes synthesis requiring RP-HPLC purification.

Materials

3% (v/v) dichloroacetic acid (Cl_2CHCO_2H) in dichloromethane (CH_2Cl_2; see recipe)
Acetonitrile (CH_3CN; ≤0.001% H_2O)
0.1 M phosphoramidite monomer solutions (see recipe):
  0.1 M 3′-tritylamino-5′-*O*-[(*cis*-2,6-dimethylpiperidino)(2-cyanoethoxy)]phosphinyl-2′,3′-dideoxynucleoside monomers (A^{Bz}, C^{Bz}, $G^{i\text{-}Bu,DPC}$, T; **S.1**; see Support Protocol 7)
  0.1 M 3′-*O*-(4,4′-dimethoxytrityl)-5′-*O*-[(*cis*-2,6-dimethylpiperidino)(2-cyanoethoxy)]phosphinyl-2′-deoxyribonucleoside monomers (A^{Bz}, C^{Bz}, $G^{i\text{-}Bu,DPC}$, T; **S.2**; see Support Protocol 7; for phosphodiester or phosphorothioate chimera)
0.167 M 1*H*-tetrazole in CH_3CN (see recipe)
20% (v/v) pyridine in CH_3CN (see recipe)
1.5:3.5:20:75 (v/v/v/v) H_2O_2/H_2O/pyridine/THF (see recipe)
0.2 M *S*-Tetra (Stec et al., 1993) in pyridine, prepared under argon, *or* 3*H*-1,2-benzodithiol-3-one-1,1-dioxide in acetonitrile (Beaucage reagent; Glen Research)
1:1:8 (v/v/v) isobutyric anhydride/2,6-lutidine/THF (see recipe)
16.5% (v/v) *N*-methylimidazole (NMI)/THF (see recipe)
3′-Tritylamino-2′,3′-dideoxynucleoside-5′-*O*-hemisuccinate conjugated to aminopropyl-controlled-pore glass (CPG; A^{Bz}, C^{Bz}, $G^{i\text{-}Bu,DPC}$, or T; see Support Protocol 6)
Concentrated aqueous ammonia

Column-mode DNA synthesizer capable of 1-µmol-scale syntheses (e.g., 392 or 394, PE Biosystems) with at least four monomer positions (preferably eight for synthesis of chimeras)
Empty 1-µmol synthesis columns
Desiccator with vacuum
4-mL glass screw-cap vials
Heat block or oven set at 58°C

Additional reagents and equipment for automated DNA synthesis (see manufacturer's instructions and *APPENDIX 3C*)

NOTE: This chemistry is extremely water sensitive. Oven-dry all bottles and syringes used for transferring solvents and solutions. Dissolve solids using a manifold or firestone valve to maintain an argon atmosphere. Perform all transfers under argon.

Input oligonucleotide sequence

1. Enter the desired sequence into the automated synthesizer in backwards order.

   The pnODN syntheses are performed in the 5′-to-3′ direction instead of the 3′-to-5′ direction, which is standard for commercially available synthesizers. For instance, if the desired sequence is 5′-GGACCCTCCTCCGGAGCC$_{OH}$-3′, then the synthesizer is programmed as follows: 5′-cCGAGGCCTCCTCCCAGG-3′, where lowercase letters represent 3′-O-dimethoxytrityl (3′-O-DMTr) monomers and uppercase letters represent 3′-trityl-amino monomers. If a pn/po/pn chimera of sequence 5′-GGACCCpTpCpCpTpCpCGGAGCC$_{OH}$-3′ is desired, where p is a phosphodiester linkage, then the synthesizer should be programmed as follows: 5′-cCGAGGCctcctcCCAGG-3′.

2. For synthesis of a pn/ps/pn chimera, program each flank to end with the first residue of the subsequent flank.

   If this is overlooked, the attaching residues at the pn/ps and ps/pn junctions will contain a thioamidate and a phosphodiester linkage, respectively. For instance, if the desired sequence is 5′-GGACCsCsTsCsCsTsCsCsGGAGCC$_{OH}$-3′, where s is a phosphorothioate linkage and the other linkages are phosphoramidate linkages, then the synthesizer should be programmed and run three times as follows:

   5′-cCAGG-3′ with oxidation

   5′-Gcctcctcc-3′ with sulfurization

   5′-cCGAGG-3′ with oxidation.

   This assumes that the synthesizer used requires the inclusion of the base already on the support when programming the sequence.

Program synthesizer

3. Program the synthesizer to perform the following cycle:

   a. 3% Cl_2CHCO_2H in CH_2Cl_2 for 60 sec for tritylamino or 90 sec for *O*-DMTr, then CH_3CN wash (six times with 0.5 mL).

   b. Phosphoramidite monomer solution (**S.1** or **S.2;** 0.1 M; 15 eq) + 1*H*-tetrazole (0.167 M; 65 eq) in CH_3CN for 5 min.

   c. Neutralize and wash with 20% pyridine in CH_3CN (six times with 0.4 mL).

   d. 1.5:3.5:20:75 H_2O_2/H_2O/pyridine/THF (0.65 mL; 2 min) *or,* for a phosphorothioate linkage, 0.2 M (50 eq) *S*-Tetra in pyridine (220 sec), followed by CH_3CN washes (six times with 0.5 mL).

   e. 1:1:8 isobutyric anhydride/2,6-lutidine/THF (0.5 mL) + 16.5% NMI/THF(0.5 mL) for 2 min, then CH_3CN washes (six times with 0.5 mL).

   f. Repeat steps a to e.

   g. Repeat step a ("trityl-off").

   The trityl-off step is not performed when RP-HPLC purification of ODN is required.

   Phosphoramidite/tetrazole deliveries should be modeled after the instrument manufacturer's protocol for delivering these reagents during phosphodiester synthesis. Modifications may need to be made to accommodate differences between flow synthesizers that only deliver reagents (e.g., PE Biosystems) and those that can recirculate phosphoramidite monomers (e.g., Amersham Pharmacia Biotech). The above details refer to the former (PE-ABI) synthesizer.

   N,N-Diisopropylamino phosphoramidite monomers can be used in place of cis-2,6-dimethylpiperidino phosphoramidite monomers with the following changes. A couple-oxidize-couple-oxidize protocol (Nelson et al., 1997) must be used for the couplings to 3′-amino groups (i.e., repeat steps b through d before proceeding to step e for capping). Couplings to 3′-hydroxyls require only a single coupling. Also, the concentration of tetrazole in acetonitrile must be increased to 0.5 M because N,N-diisopropylamino phosphoramidites require more tetrazole for activation than cis-2,6-dimethylpiperidino-phosphoramidites (Hirschbein et al., 1998). 3′-O-(4,4′-Dimethoxytrityl)-5′-O[(N,N-diisopropylamino)(2-cyanoethoxy)]phosphinyl-2′-deoxyribonucleoside monomers (A^{Bz}, C^{Bz}, $G^{i\text{-}Bu}$, T) are available from Glen Research.

   Using a vortexing-mode synthesizer (e.g., PE Biosystems 390Z) on the 10-μmol scale, 3.6 eq of monomer and 9 eq of 1H-tetrazole is sufficient for complete coupling.

   Commercially available 3H-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent) in acetonitrile can be used in place of S-Tetra/pyridine for sulfurization, if desired. Use the vendor's protocol for this reagent.

4. Calculate the amount of monomer necessary for the desired syntheses by multiplying the number of couplings of each type of base by 15 µmol/coupling. Add 100 µmol to the total for each base in order to have enough solution to prime the lines and to cover the bottom of the bottle.

Prepare and add reagents

5. Dissolve the monomers to a concentration of 0.1 M in CH_3CN as described (see Reagents and Solutions).

   Due to the stability and expense of the monomers, dissolve only as much monomer as will be used in 1 week.

   If using N,N-diisopropylamino phosphoramidites, 30 µmol of monomer is used per coupling to 3′-amino groups because the couple-oxidize-couple-oxidize protocol must be performed.

6. Formulate a sufficient amount of each auxiliary reagent to complete the desired syntheses.

   Consumptions on a per-coupling basis are typically 0.4 mL for the tetrazole solution, 0.65 mL for the hydrogen peroxide formulation, 0.5 mL each for the capping reagents, and 2.4 mL for the 20% pyridine/CH_3CN solution.

7. Load the reagents on the instrument, keeping them under argon at all times.

   a. Place the four 3′-tritylamino-5′-*O*-[(*cis*-2,6-dimethylpiperidino)(2-cyanoethoxyl)]phosphinyl-2′,3′-dideoxynucleoside monomers (A^{Bz}, C^{Bz}, $G^{i\text{-Bu,DPC}}$, T) (**S.1**) in monomer positions 1 to 4 and, for chimeras, place the four 3′-*O*-(4,4′-dimethoxytrityl)-5′-*O*-[(*cis*-2,6-dimethylpiperidino)(2-cyanoethoxy)]phosphinyl-2′-deoxyribonucleoside monomers (A^{Bz}, C^{Bz}, $G^{i\text{-Bu,DPC}}$, T) (**S.2**) in monomer positions 5 to 8.

   b. Place the hydrogen peroxide solution in the oxidation position.

   c. Place the *S*-Tetra or Beaucage reagent in the sulfurization position.

   d. Place the capping agents in their usual positions on the instrument.

   e. Place 20% pyridine/CH_3CN in the position usually used for concentrated aqueous ammonia or, if available, an extra position on the synthesizer.

8. Weigh 1 µmol of 3′-tritylamino-2′,3′-dideoxynucleoside-5′-*O*-hemisuccinate-CPG corresponding to the 5′-terminal base. Place the CPG in an empty 1-µmol synthesis column and check the column thoroughly for leaks on the synthesizer by passing acetonitrile through it.

Run synthesizer

9. Prime all of the lines with reagent and start the synthesizer according to the manufacturer's guidelines.

10. At the end of the synthesis, remove the CPG column from the synthesizer and dry the CPG in a desiccator under vacuum for 15 min.

11. Carefully open the column and transfer the CPG to a 4-mL glass screw-cap vial.

12. Add 1 mL concentrated aqueous ammonia and deprotect the oligonucleotide for 8 to 12 hr at 58°C.

13. Store the deprotected pnODN in the ammonia solution at −20°C until purification (maximum of 4 weeks).

ALTERNATE PROTOCOL 1

SOLID-PHASE CHAIN ASSEMBLY FOR RP-HPLC PURIFICATION

Additional Materials *(also see Basic Protocol 1)*

0.1 M 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-3′-*O*-[(*N*,*N*-diisopropylamino)(2-cyanoethoxy)]phosphinyl uridine (see recipe)
0.5 M 1*H*-tetrazole in CH_3CN
3:1 (v/v) concentrated aqueous ammonia/ethanol

1. Program, set up, and run the synthesizer as described (see Basic Protocol 1, steps 1 through 9), with the following modifications:

   a. Ensure that the terminal coupling is with a 3′-*O*-DMTr-protected deoxyribonucleoside (**S.2**) in order to ultimately have a terminal 3′-hydroxyl group on the oligonucleotide. Also, do not remove the DMTr group at the end of the synthesis (step 3g).

   b. Place 0.1 M 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-3′-*O*-[(*N*,*N*-diisopropylamino)(2-cyanoethoxy)]phosphinyl uridine solution on the instrument in one of the monomer positions. Also, replace the 0.167 M 1*H*-tetrazole solution with 0.5 M 1*H*-tetrazole solution.

   It is important to use 0.5 M 1H-tetrazole for this coupling because the uridine monomer is an N,N-diisopropylamino phosphoramidite, which requires more tetrazole for activation than the cis-2,6-dimethylpiperidino phosphoramidites.

   c. Program the synthesizer to couple the uridine monomer to the 3′ terminus of the pnODN or chimera (see Basic Protocol 1), but extend the coupling time to 10 min (step 3b). Once again, make sure not to remove the DMTr group at the end of the synthesis.

2. At the end of the synthesis, dry the CPG-column and deprotect the oligonucleotide as described (see Basic Protocol 1, steps 10 to 12), but use 1 mL of 3:1 (v/v) concentrated aqueous ammonia/ethanol in step 12.

   Do not heat >18 hr.

   The deprotected pnODN can be stored in the ammonia solution at −20°C until purification for a maximum of 1 week, although it is best to remove the ammonia and purify the crude oligonucleotide as soon as possible after synthesis.

BASIC PROTOCOL 2

IEC PURIFICATION, ISOLATION, AND ANALYSIS

The IEC purification method is used for pnODNs synthesized with a terminal 3′-amine or 3′-hydroxyl following removal of the trityl protecting group. Preparative IEC is able to separate failure sequences from the full-length product (n-mer). However, the resolution between the n-1 failure sequence and the product is not great; therefore, the main product peak must be fractioned and analyzed by analytical IEC or capillary gel electrophoresis (CGE) in order to decide which fractions to combine.

Materials

Deprotected oligonucleotide solution (see Basic Protocol 1), 4°C
Buffer A: 0.01 M aqueous NaOH/0.01 M NaCl, pH 12
Buffer B: 0.01 M aqueous NaOH/1.5 M NaCl, pH 12
Concentrated aqueous ammonia, 4°C
0.5 M aqueous NaOH solution
100% ethanol

UV/visible spectrometer

Analytical IEC column (preferably a 4 × 250–mm Dionex PA-100 NucleoPac column)
HPLC or FPLC system compatible with high pH buffer systems equipped with a UV detector, data collection system, and a 1- or 2-mL sample injection loop
3-mL disposable syringe with luer lock
0.45-µm filter that fits the end of a luer lock syringe
Speedvac evaporator (Savant)
Preparative IEC column (preferably a Pharmacia MonoQ 10/10 column)
Sample holder with 1.5-mL centrifuge tubes *or* fraction collector
Sephadex G-25 column (e.g., Pharmacia NAP-10; optional)

Analyze quality of crude oligonucleotide

1. Dilute 10 µL cold, deprotected oligonucleotide solution in 990 µL water and scan from 200 to 400 nm using a UV spectrometer. Determine the absorbance at 260 nm and multiply by 100 (dilution factor) to determine the concentration of crude oligonucleotide in the 1 mL sample.

   To prevent loss of sample and to improve the accuracy of the dilution, make sure the ammonia solution is cold before opening the vial.

2. Dilute 0.5 OD_{260} units oligonucleotide into 0.5 mL water to serve as an analytical IEC sample.

3. Preequilibrate the analytical IEC column for ≥10 min with buffer A at a flow rate of 1 mL/min.

4. Program an HPLC or FPLC to run a gradient of 0% to 50% buffer B versus buffer A over 40 min at a flow rate of 1 mL/min and inject the 0.5-mL ODN sample. Monitor the run at 260 nm.

   Reequilibrate the column for ≥10 min with buffer A before performing a second run.

Prepare sample and IEC purify

5. Filter the CPG away from the remaining cold ammonia solution using a 3-mL disposable syringe with an attached 0.45-µm filter.

6. Wash the CPG twice with 0.5 mL cold, concentrated aqueous ammonia.

7. Add 10 µL of 0.5 M aqueous NaOH solution, and concentrate in a Speedvac evaporator to ~0.5 mL.

   Do not completely dry the sample.

8. Filter the concentrated oligonucleotide again using a new 3-mL disposable syringe with an attached 0.45-µm filter and wash with 0.3 mL water.

9. Preequilibrate the preparative IEC column for ≥15 min with buffer A.

   Run a "blank" gradient if the column has not been used recently, and between purifications of samples with different sequences.

10. Program the HPLC to run a gradient ramping at 1%/min of buffer B versus buffer A at a flow rate of 1 mL/min. Use the analytical IEC (step 4) to determine the approximate percent of buffer B that will be needed to elute the sample.

11. Prepare a sample holder with at least ten 1.5-mL centrifuge tubes or use a fraction collector.

    This is necessary to obtain the highest level of purity; preparative IEC does not have the resolution of analytical IEC.

12. Inject the entire crude ODN sample from the 1-µmol synthesis and monitor elution at 260 nm. Collect ~0.5-mL fractions during the elution of the product peak. Store the fractions at 4°C until they have been analyzed and are ready for desalting.

Analyze and desalt

13. Analyze a small amount (i.e., ~0.1 OD_{260}) of the fractions by analytical IEC.
14. Combine the fractions that are ≥85% pure and concentrate in a Speedvac evaporator to a volume of ~1 mL.

    Do not let the samples evaporate completely in the Speedvac evaporator; NaOH can potentially degrade pnODNs.

15. Precipitate the pnODN with 2.5 mL of 100% ethanol and cool for ≥30 min at −20°C.
16. Centrifuge for 2 min at 3000 × *g*, 4°C, and carefully remove the supernatant.
17. Dissolve the pellet in 1 mL of deionized water and repeat ethanol precipitation (steps 15 and 16) two more times to desalt the sample.

    Alternatively, desalt the sample on a Sephadex G-25 column using the manufacturer's protocol.

18. Dissolve pellet in 1 mL of water and measure the OD_{260} as in step 1 to determine the yield of pnODN.
19. Inject a 0.2 OD_{260} sample on the analytical IEC column to determine the purity of the product.

    Alternatively, determine the purity using capillary gel electrophoresis (CGE) or polyacrylamide gel electrophoresis (PAGE; APPENDIX 3B).

20. Concentrate the pnODN to dryness and store up to 1 year at −20°C.

ALTERNATE PROTOCOL 2

REVERSED-PHASE HPLC PURIFICATION, ISOLATION, AND ANALYSIS

The RP-HPLC method developed for the pnODNs and their chimeras relies on the 3′-addition of a commercially available RNA monomer, 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-*tert*-butyldimethylsilyl-3′-*O*-[(*N*,*N*-diisopropylamino)(2-cyanoethoxy)]phosphinyl uridine, to the terminal 3′-OH via 1*H*-tetrazole activation, followed by oxidation to a 3′→3′ phosphodiester linkage (Figure 4.7.4). The DMTr group is retained at the end of the synthesis to enable hydrophobic purification. After the RP-HPLC purification, the 3′-terminal uridine phosphodiester is cleaved from the oligonucleotide product by treatment with fluoride and base.

Additional Materials *(also see Basic Protocol 2)*

Deprotected oligonucleotide solution (see Alternate Protocol 1), 4°C
Buffer C: acetonitrile
Buffer D (see recipe): 0.1 M TEAB/2% acetonitrile, pH 8
3:1 (v/v) concentrated aqueous ammonia/ethanol
1 M TEAB buffer, pH 8 (see recipe)
Acetonitrile
1 M aqueous NaF (0.45-µm filtered)

Analytical RP-HPLC column (e.g., Polymer Laboratories 0.46 × 15–cm PLRP-S column)
HPLC system compatible with reversed-phase buffers and solvents, equipped with a UV detector, data collection system, and a 2-mL sample injection loop

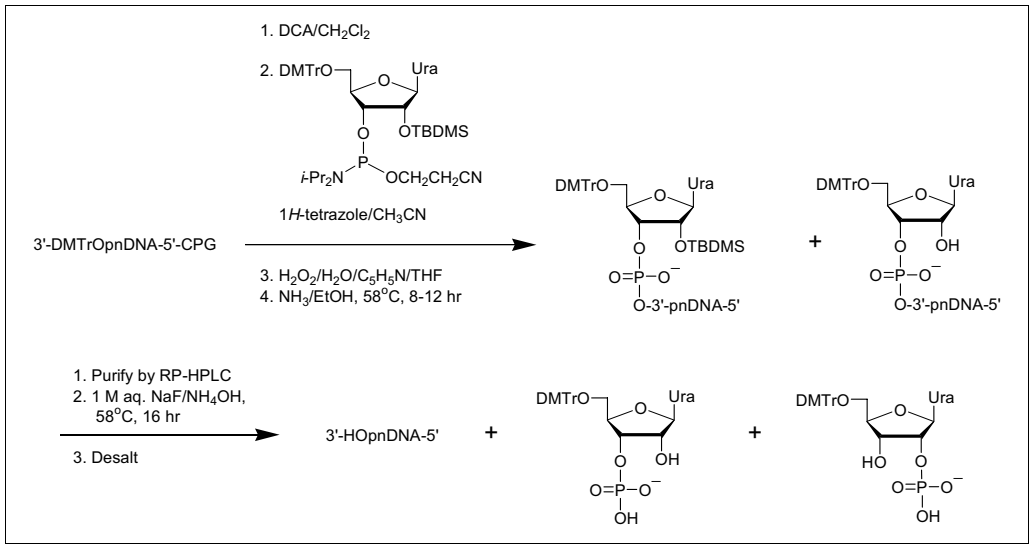


Figure 4.7.4 Method facilitating the purification of oligonucleotide phosphoramidates by RP-HPLC. DCA, dichloroacetic acid; Ura, uracil-1-yl; TBDMS, *tert*-butyldimethylsilyl; *i*-Pr, isopropyl.

Semipreparative RP-HPLC column (e.g., Polymer Labs 0.8 × 30–cm PLRP-S column)
Heat block or oven set at 58°C

Analyze quality of crude oligonucleotide

1. *Optional:* Follow the procedure for measurement of the crude OD_{260} and analysis by analytical IEC (see Basic Protocol 2, steps 1 to 4).
2. Dilute a second 0.5 OD_{260} units of ODN sample into 0.5 mL water for analysis by analytical RP-HPLC.
3. Preequilibrate the analytical RP-HPLC column for ≥10 min with 5% buffer C versus buffer D at a flow rate of 1 mL/min.
4. Program the HPLC to run a gradient of 5% to 40% buffer C versus buffer D over 40 min, followed by holding at 40% buffer C for 10 min at 1 mL/min.
5. Inject the 0.5-mL ODN sample and monitor the run at 260 nm.

   Reequilibrate the column for ≥10 min with 5% buffer C versus buffer D before performing a second run.

   Typical analytical IEC and RP-HPLC chromatograms of a crude pnODN containing a hydrophobic 3′-terminal uridine phosphodiester are shown in Figure 4.7.5.

   There are two product peaks in the RP-HPLC chromatogram because of partial loss of the tert-butyldimethylsilyl (TBDMS) group from uridine. Add the two peaks together to determine the amount of ODN product present. The byproduct peak generated from diphenylcarbamoyl (DPC) deprotection of G is observed near the product at 260 nm.

Prepare ODN sample and RP-HPLC purify

6. Filter the CPG away from the remaining cold ammonia solution using a 3-mL syringe attached to a 0.45-µm filter.
7. Wash the CPG twice with 0.5 mL of 3:1 concentrated aqueous ammonia/ethanol.
8. Concentrate in a Speedvac evaporator to ~0.5 mL.

   Do not completely dry the sample.

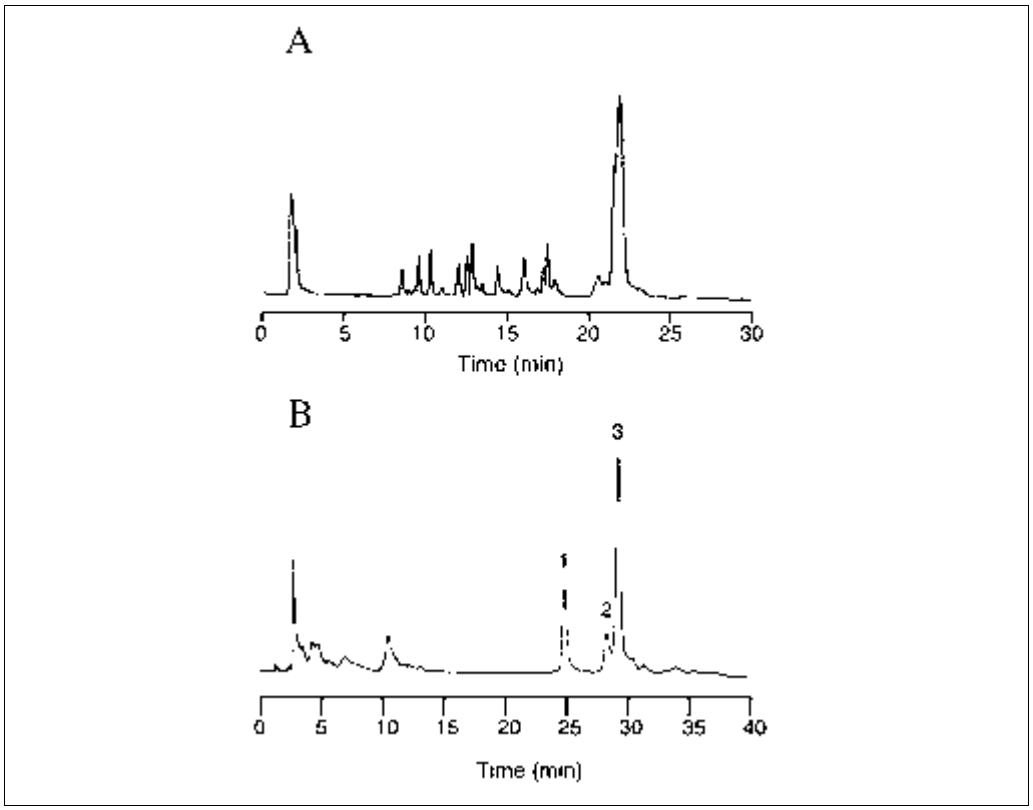


Figure 4.7.5 (**A**) Analytical IEC chromatogram (40.1% pure) and (**B**) RP-HPLC chromatogram (42.9% pure) of the phosphoramidate oligonucleotide, 5′-CCCTCCTCCGGAGCCpU^{DMTr} where p is a (3′,3′)-phosphodiester linkage. Two ODN product peaks are seen in the RP-HPLC because some of the TBDMS group on the 3′-terminal uridine is removed prematurely by ammonia treatment and/or subsequent workup. Peak 1, product containing uridine with 5′-*O*-DMTr but not 2′-*O*-TBDMS; peak 2, byproduct generated from diphenylcarbamoyl (DPC) deprotection of G; peak 3, product containing uridine with 5′-*O*-DMTr and 2′-*O*-TBDMS.

Do not add 0.5 M aqueous NaOH to this sample as the hydrophobic 3′-terminal uridine phosphodiester will cleave prematurely.

9. Filter the concentrated oligonucleotide again using a new 3-mL syringe attached to a 0.45-µm filter and wash twice with 0.3 mL of water.

10. Add 0.2 mL of 1 M TEAB buffer, pH 8, and 25 µL acetonitrile.

The sample, once concentrated and buffered, should be purified within 12 hr. Occasionally the byproduct produced from DPC deprotection of G continues to precipitate out after filtration, especially if the sample is frozen; refilter the solution just prior to purification, if necessary, to prevent clogging of the column.

11. Preequilibrate a semipreparative RP-HPLC column for ≥15 min in 5% buffer C versus buffer D at a flow rate of 2 mL/min.

Run a "blank" gradient if the column has not been used recently, and between purifications of samples with different sequences.

TEAB (buffer D) is used because, unlike the more commonly used triethylammonium acetate (TEAA), it remains basic during the post-RP-HPLC concentration and enables the isolation of pure pnODN without accompanying acid-mediated degradation.

12. Program the HPLC to run a gradient of 5% to 40% buffer C versus buffer D over 40 min, followed by a hold for 10 min at 40% buffer C at a flow rate of 2 mL/min.

13. Inject 75 to 120 OD_{260} crude pnODN and collect both product peaks. Monitor the chromatography at 296 nm for preparative runs.

    The byproduct peak generated from DPC deprotection of G is not observed at 296 nm; the higher wavelength is used to attenuate the peak height.

    In general, there are some impurities just prior to the products, as well as a backside shoulder; both of these should be avoided during collection of the major fractions. It is usually best to collect only to approximately half the highest UV reading on the backside of the peak because this region contains more short-mer impurities. An example of a semipreparative RP-HPLC chromatogram and the fractionation of the peaks is shown in Figure 4.7.6.

14. Combine the two product peaks, concentrate in the Speedvac evaporator until the sample can be transferred to a 4-mL screw-cap vial, and then concentrate the sample to dryness.

    It is not necessary to add NaOH to the fractions; the TEAB buffer will stay basic during the concentration.

Remove 3′-terminal uridine phosphodiester, desalt, and analyze

15. Add 200 µL concentrated aqueous ammonia and 200 µL of 1 M aqueous NaF to the dry pnODN, vortex the mixture until the pnODN is dissolved, and heat the sample for 12 to 16 hr at 58°C.

    The fluoride removes the TBDMS group and the base causes intramolecular cleavage of the uridine phosphodiester function.

16. Cool the pnODN solution and check a small aliquot (0.2 OD_{260}) by analytical IEC for complete cleavage.

    The retention time of the cleaved product is shorter than that of the uridine-containing product, and the two early-eluting uridine byproducts (see Fig. 4.7.4) are present.

17. Concentrate the solution to ~200 µL in the Speedvac evaporator and precipitate the pnODN with 0.6 mL of 100% ethanol to remove the bulk of NaF. Freeze the sample for ≥30 min at –20°C.

18. Centrifuge for 2 min at 10,000 × *g*, room temperature, and carefully remove the supernatant.

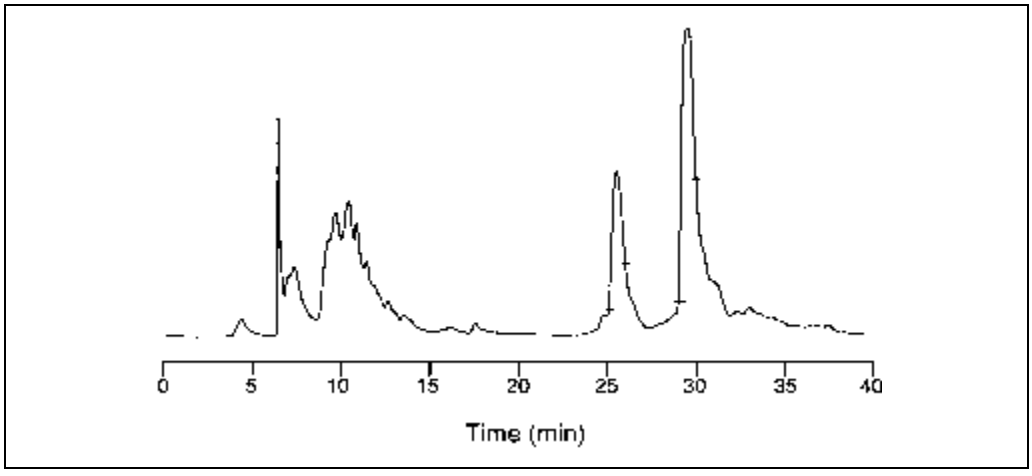


Figure 4.7.6 Semipreparative RP-HPLC chromatogram of a pnODN with the sequence 5′-CCCTCCTCCGGAGCCpU^{DMTr} where p is a (3′,3′)-phosphodiester linkage.

19. Dissolve the product in 1 mL of water and desalt the sample on a Sephadex G-25 column using the manufacturer's protocol.

    The Sephadex G-25 column is necessary in this case because ethanol precipitation does not remove the uridine byproducts.

20. Measure the OD units at 260 nm to determine the yield of pnODN. Inject 0.2 OD_{260} units on an analytical IEC column to determine the purity of the product.

    Alternatively, determine ODN purity by CGE or PAGE (APPENDIX 3B).

21. Concentrate the pnODN to dryness in the Speedvac evaporator and store up to 1 year at −20°C.

SUPPORT PROTOCOL 1

SYNTHESIS OF 3′-TRITYLAMINO-2′,3′-DEOXYTHYMIDINE

The synthesis of 3′-tritylamino-2′,3′-deoxythymidine (**S.5**) from thymidine is shown in Figure 4.7.7. The 3′-azido-5′-*O*-(4-methoxybenzoyl)-2′,3′-deoxythymidine (**S.3**) is prepared as previously reported (Czernecki and Valéry, 1991).

Materials

Thymidine
N,*N*-Dimethylformamide (DMF)
Triphenylphosphine
p-Anisic acid
Diisopropylazodicarboxylate (DIAD)
Diethyl ether, 5°C
Lithium azide (LiN_3)
Ethyl acetate
Saturated aqueous NaCl
Sodium sulfate (Na_2SO_4, anhydrous)
8:2 (v/v) ethyl acetate/hexane
1:1 (v/v) ethanol/dichloromethane (CH_2Cl_2)
Hydrogen
10% Pd/C catalyst (Aldrich)
Pyridine (anhydrous)
Triethylamine
Trityl chloride
Chromatography-grade silica gel, 70-230 mesh 60 Å (Aldrich)
0.5% to 5% (v/v) triethylamine in 2% (v/v) methanol/CH_2Cl_2
2% to 5% (v/v) methanol/CH_2Cl_2
5:95, 1:9, and 2:8 (v/v) methanol/CH_2Cl_2
57:43 (v/v) 1,4-dioxane/methanol
2 M aqueous NaOH (*APPENDIX 2A*)
Dowex 50W-X8 cation-exchange resin (pyridinium H^+ form; see recipe)
Saturated aqueous $NaHCO_3$
1:1 (v/v) ethyl ether/hexane

Heating mantle, variac, and temperature controller
Mechanical overhead stirrer (Fisher)
TLC plates (e.g., 0.2-mm-thick precoated Merck silica gel 60 F254 plates)
Rotary evaporator
Parr shaker-type hydrogenator able to hold pressures up to 60 psi

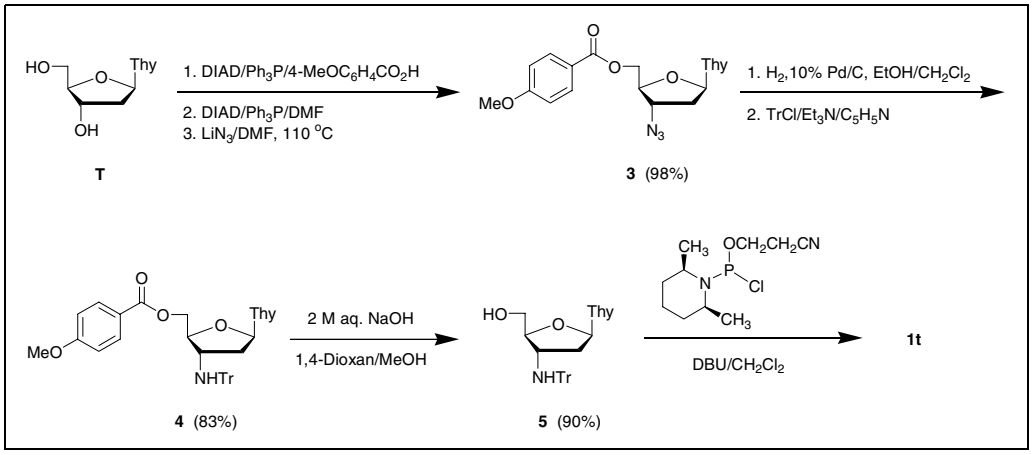


Figure 4.7.7 Synthetic steps in the preparation of 3′-tritylamino-2′,3′-dideoxythymidine (**S.5**) from thymidine. Thy, thymin-1-yl; DIAD, diisopropylazodicarboxylate; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene.

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

Synthesize 3′-azido-5′-O-(4-methoxybenzoyl)-2′,3′-deoxythymidine (S.3)

1. Dissolve 141.0 g (582.2 mmol) thymidine in 1125 mL DMF in a 3-liter round-bottom flask.
2. Add 183.2 g (698.4 mmol) triphenylphosphine and 106.3 g (698.4 mmol) *p*-anisic acid.
3. Add 137.5 mL (698.4 mmol) diisopropylazodicarboxylate, diluted in 150 mL DMF, over 35 min using an additional funnel.
4. After 40 min, add another 183.2 g (698.4 mmol) triphenylphosphine all at once, and another 141.3 g (698.4 mmol) diisopropylazodicarboxylate, dissolved in 150 mL DMF, over a 40-min period using an additional funnel.
5. Stir the resultant mixture for an additional 65 min.
6. Quench the reaction with 10 mL of water and concentrate the solution to a volume of ~800 mL using a rotary evaporator equipped with a vacuum pump.
7. Precipitate the product by pouring it into 6000 mL cold diethyl ether, 5°C, with rapid stirring.
8. Filter the solid using a Büchner funnel and house vacuum, wash with 2000 mL cold diethyl ether, and dry in vacuo to give semipure 2,3′-anhydro-5′-*O*-(4-methoxybenzoyl)-2′,3′-deoxythymidine.

   Crude yield = 110% (230.2 g).

9. Using a mechanical stirrer, dissolve 230.0 g (642.5 mmol) of 2,3′-anhydro-5′-*O*-(4-methoxybenzoyl)-2′,3′-deoxythymidine in 1200 mL DMF, add 47.2 g (963.8 mmol) lithium azide, and heat the mixture for 48 hr at 100° to 110°C.
10. Concentrate the solution on the rotary evaporator using a vacuum pump, dissolve the residue in 3000 mL ethyl acetate, and wash two times with 1000 mL water and three times with 600 mL saturated aqueous NaCl.

11. Dry the organic solution over anhydrous Na_2SO_4, filter, and concentrate on the rotary evaporator with a vacuum line to afford 3′-azido-5′-*O*-(4-methoxybenzoyl)-2′,3′-deoxythymidine (**S.3**) as an amber foam.

12. Perform TLC analysis (*APPENDIX 3D*) on 0.2-mm-thick precoated Merck silica gel 60 F254 plates to confirm the purity of the product. Elute with 8:2 (v/v) ethyl acetate/hexane.

Crude yield = 97.9% (228.9 g, 570.0 mmol). R_f (8:2 ethyl acetate/hexane) = 0.51. ^{1}H NMR ($CDCl_3$/TMS): δ 9.36 (1H, br s), 7.98 (2H, d, J = 8.7 Hz), 7.22 (1H, s), 6.95 (2H, d, J = 8.7 Hz), 6.18 (1H, t, J = 6.5 Hz), 4.65 (1H, dd, J = 12.3, 3.3 Hz), 4.53 (1H, dd, J = 12.3, 3.6 Hz), 4.35 (1H, m), 4.21 (1H, dt, J = 3.6, 2.5 Hz), 3.87 (3H, s), 2.54 (1H, m), 2.35 (1H, m), 1.71 (3H, s).

Synthesize 5′-O-(4-methoxybenzoyl)-3′-tritylamino-2′,3′-deoxythymidine (S.4)

13. Dissolve 10.0 g (24.9 mmol) of **S.3** in 500 mL of 1:1 (v/v) ethanol/CH_2Cl_2 and reduce via hydrogenation (60 psi H_2) in the presence of 1 g of 10% Pd/C catalyst for 16 hr.

14. Remove the catalyst by vacuum filtration and evaporate the solvent in vacuo.

Yield = 92% (8.6 g, 22.9 mmol) of the corresponding 3′-amine, which is taken directly to the next reaction.

15. Dry 8.6 g (22.9 mmol) of 3′-amino-5′-*O*-(4-methoxybenzoyl)-2′,3′-deoxythymidine by azeotropic removal of water two times with 50 mL pyridine, evaporating to dryness on a rotary evaporator each time. Dissolve in 50 mL anhydrous pyridine.

16. Add 6.17 mL (48.1 mmol) triethylamine and 7.0 g (25.2 mmol) trityl chloride. Stir this mixture for 2 hr at room temperature. Perform TLC analysis (see step 12), eluting with 5:95 (v/v) methanol/CH_2Cl_2, to determine if the reaction is complete

17. If the reaction is complete, go to step 18, otherwise add an additional 1.9 g (6.9 mmol) trityl chloride, if necessary, and continue stirring for an additional 2 hr.

18. Remove the solvents using a rotary evaporator equipped with a vacuum pump and purify the crude product (**S.4**) by gravity on a silica gel column (*APPENDIX 3E*) preequilibrated with 0.5% triethylamine in 2% methanol/CH_2Cl_2. Obtain product by eluting with 2% to 5% methanol/CH_2Cl_2.

For acid-sensitive (trityl-containing and phosphoramidite) compounds, the column should be packed and equilibrated with 0.5% triethylamine.

19. Perform TLC analysis (step 12) to confirm the purity of the product. Elute with 5:95 (v/v) methanol/CH_2Cl_2.

Yield = 90% (12.7 g, 20.6 mmol). R_f (5:95 methanol/CH_2Cl_2) = 0.45. ^{1}H NMR ($CDCl_3$/TMS): δ 8.26 (1H, br s, exchanges with D_2O), 7.84, 6.90 (4H, AB, J = 8.86 Hz), 7.55 (6H, d, J = 7.45 Hz), 7.29 (6H, t, J = 7.56 Hz), 7.21 (3H, t, J = 7.27 Hz), 7.02 (1H, s), 6.08 (1H, t, J = 6.19 Hz), 4.59 (1H, dd, J = 12.35, 2.35 Hz), 4.29 (1H, dd, J = 12.43, 3.94 Hz), 3.98 (1H, m), 3.88 (3H, s), 3.41 (1H, m), 1.97 (1H, br, exchanges with D_2O), 1.65-1.75 (1H, m), 1.58 (3H, s), 1.30-1.40 (1H, m). HRMS (FAB$^+$): calcd for [M + Cs]$^+$, 750.1580, observed 750.1559.

Synthesize 3′-tritylamino-2′,3′-deoxythymidine (S.5)

20. Remove the 5′-*O*-anisoyl protecting group by dissolving 30.1 g (48.7 mmol) of **S.4** in 150 mL of 57:43 (v/v) 1,4-dioxane/methanol, and then adding 73.1 mL (146.2 mmol) of 2 M aqueous NaOH. Stir the solution for 1.5 hr at room temperature.

21. Neutralize with ~150 g of Dowex 50W-X8 cation-exchange resin (dry pyridinium H^+ form, 1.6 meq/g).

22. Once the pH is neutral (~10 min), filter the resin, wash three times with 40 mL of 2:8 methanol/CH_2Cl_2, and concentrate the crude product on the rotary evaporator using a vacuum line.

23. Dissolve the residue in 500 mL ethyl acetate and extract two times with 250 mL saturated aqueous $NaHCO_3$, once with 250 mL water, and once with 250 mL saturated aqueous NaCl.

24. Dry the organic phase over anhydrous Na_2SO_4 and filter.

25. Remove the solvents using a rotary evaporator with a vacuum line and dissolve the resulting foam in 300 mL of 5:95 methanol/CH_2Cl_2.

26. Add this solution slowly to 1250 mL of a rapidly stirring mixture of 1:1 (v/v) diethyl ether/hexane to precipitate the pure 3′-tritylamino-2′,3′-deoxythymidine (**S.5**).

27. Perform TLC analysis (step 12) to confirm the purity of the product. Elute with 1:9 (v/v) methanol/CH_2Cl_2.

Yield = 90% (21.2 g, 43.8 mmol). R_f (1:9 methanol/CH_2Cl_2) = 0.50. ^{1}H NMR ($CDCl_3$/TMS): δ 8.30 (1H, br s, exchanges with D_2O), 7.52 (6H, d, J = 7.46 Hz), 7.29 (6H, t, J = 7.55 Hz), 7.21 (3H, t, J = 7.25 Hz), 7.16 (1H, s), 6.01 (1H, t, J = 6.38 Hz), 3.85 (1H, d, J = 11.71 Hz), 3.74 (1H, m), 3.65 (1H, dd, J = 11.99, 2.59), 3.34 (1H, q, J = 6.54 Hz), 1.80-2.00 (1H, br, exchanges with D_2O), 1.83 (3H, s), 1.45-1.55 (1H, m), 1.30-1.40 (1H, m). HRMS (FAB$^+$): calcd for [M + Cs]$^+$, 616.1212, observed 616.1226.

SYNTHESIS OF N^4-BENZOYL-3′-TRITYLAMINO-2′,3′-DIDEOXYCYTIDINE

SUPPORT PROTOCOL 2

The synthesis of N^4-benzoyl-3′-tritylamino-2′,3′-deoxycytidine (**S.9**) is shown in Figure 4.7.8. The C monomer is more readily and efficiently synthesized by the dU→dC route, rather than by lithium azide ring opening of a 2,3′-anhydro-2′-deoxycytidine derivative (Reese and Skone, 1984; Nelson et al., 1997).

Additional Materials *(also see Support Protocol 1)*

2′-Deoxyuridine
4-Dimethylaminopyridine
tert-Butyldimethylsilyl (TBDMS) chloride
2:1 (v/v) ethanol/CH_2Cl_2
1,2,4-Triazole
Phosphorus oxychloride ($POCl_3$)
1,4-Dioxane
Benzoyl chloride
Concentrated aqueous ammonia (28%), 4°C
Tetrahydrofuran (THF)
1 M tetra-*n*-butylammonium fluoride (TBAF) in THF
1% (v/v) triethylamine in 30% to 50% (v/v) ethyl acetate/hexane

Synthesize 3′-azido-5′-O-(tert-butyldimethylsilyl)-2′,3′-dideoxyuridine (S.6)

1. Dry 11.4 g (50 mmol) of 2′-deoxyuridine two times thoroughly via co-evaporation with 100 mL anhydrous DMF in vacuo.

2. Add 100 mL DMF followed by 8.36 mL (60 mmol) triethylamine, 0.31 g (2.5 mmol) of 4-dimethylaminopyridine, and 8.29 g (55.0 mmol) *tert*-butyldimethylsilyl chloride. Stir the reaction mixture for 1 hr at room temperature.

3. Dilute with 600 mL CH_2Cl_2 and extract three times with 200 mL water and once with 200 mL saturated aqueous NaCl.

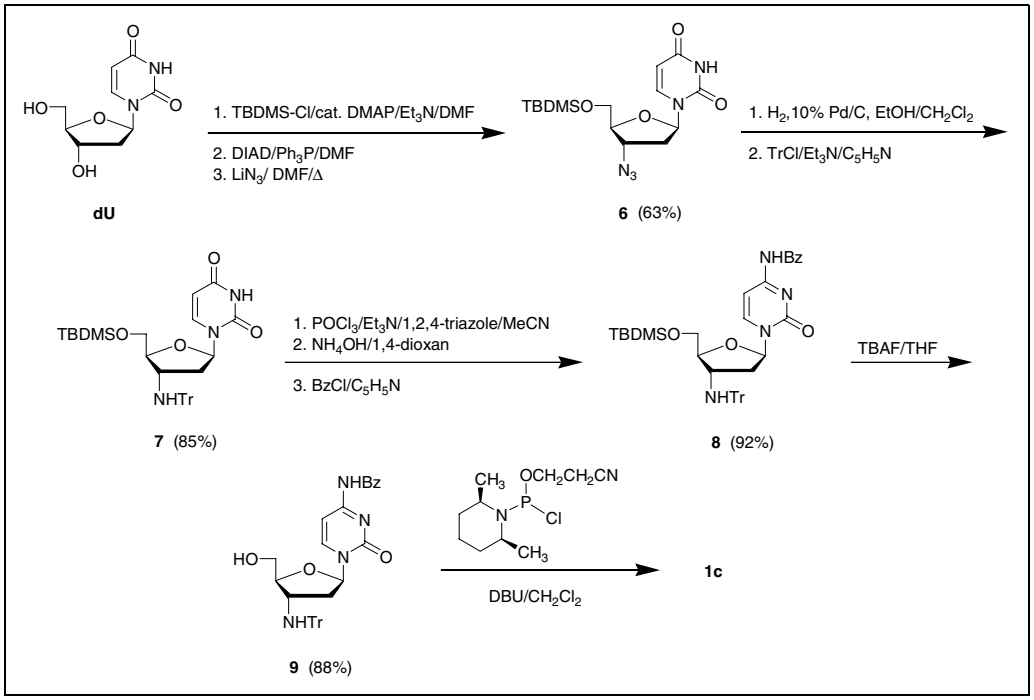


Figure 4.7.8 Synthetic steps in the preparation of N^4-benzoyl-3′-tritylamino-2′,3′-dideoxycytidine (**S.9**) from 2′-deoxyuridine. DMAP, 4-dimethylaminopyridine; Bz, benzoyl; TBAF, tetra-*n*-butylammonium fluoride.

4. Dry the organic layer over anhydrous Na_2SO_4, vacuum filter, and concentrate on a rotary evaporator using a vacuum pump.

5. Purify the resulting residue by gravity column chromatography (*APPENDIX 3E*) on silica gel with 2% to 10% methanol/CH_2Cl_2 to afford 5′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyuridine.

   Yield = 80% (13.7 g, 40.0 mmol).

6. Dissolve 13.7 g (40.0 mmol) of 5′-protected nucleoside and 16.8 g (64.0 mmol) triphenylphosphine in 100 mL DMF. While stirring, add 12.6 mL (64.0 mmol) diisopropylazodicarboxylate in 20 mL DMF.

7. Stir 2 hr at room temperature, concentrate the reaction mixture on a rotary evaporator using a vacuum pump to ~30 mL, and pour into 1200 mL diethyl ether.

   The desired product precipitates out after ~10 min of rapid stirring.

8. Place the resulting mixture overnight at 4°C.

9. Collect the precipitate by vacuum filtration, wash two times with 300 mL cold diethyl ether, and dry in vacuo to afford 2,3′-anhydro-5′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyuridine as a white solid.

   Yield = 90% (11.7 g, 36.0 mmol).

10. React 33.8 g (104.2 mmol) of 2,3′-anhydro-5′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyuridine with 7.65 g (156.3 mmol) LiN_3 in 300 mL DMF for 48 hr at 95° to 100°C.

11. Cool the resulting brown homogeneous mixture to room temperature, and concentrate to an oil using a rotary evaporator and vacuum pump.

12. Dissolve the residue in 800 mL ethyl acetate and extract with 200 mL water.

13. Extract the aqueous layer twice more with 75 mL ethyl acetate and combine the organics. Wash three times with 250 mL water and once with 250 mL saturated aqueous NaCl.

14. Dry the ethyl acetate solution over anhydrous Na_2SO_4, vacuum filter, and concentrate on a rotary evaporator with a vacuum line to afford 3′-azido-5′-*O*-(*tert*-butyldimethylsilyl)-2′,3′-dideoxyuridine (**S.6**) as a brownish foam. Proceed directly to hydrogenation.

15. Perform TLC analysis (*APPENDIX 3D*) on 0.2-mm-thick precoated Merck silica gel 60 F254 plates to confirm the purity of the product. Elute with 8:92 (v/v) methanol/CH_2Cl_2.

Yield = 87% (33.2 g, 90.3 mmol). R_f(8:92 methanol/CH_2Cl_2) = 0.57. ^{1}H NMR ($CDCl_3$/TMS): δ 8.87 (1H, br s, exchanges with D_2O), 7.91 (1H, d, J = 8.10 Hz), 6.23 (1H, t, J = 5.88 Hz), 5.71 (1H, d, J = 8.18 Hz), 4.25 (1H, q, J = 5.91 Hz), 3.95-4.05 (2H, m), 3.83 (1H, dd, J = 11.40, 1.68 Hz), 2.45-2.55 (1H, m), 2.25-2.35 (1H, m), 0.95 (9H, s), 0.15 (3H, s), 0.14 (3H, s). HRMS (FAB$^+$): calcd for [M + H]$^+$, 368.1754, observed 368.1747.

Synthesize 5′-O-(tert-butyldimethylsilyl)-3′-tritylamino-2′,3′-dideoxyuridine (S.7)

16. Dissolve 33.2 g (90.3 mmol) of crude **S.6** in 300 mL of 2:1 (v/v) ethanol/CH_2Cl_2 and reduce via hydrogenation (60 psi H_2) in the presence of 3.0 g of 10% Pd/C catalyst for 18 hr.

17. Remove the catalyst by vacuum filtration and evaporate the solvent on a rotary evaporator using a vacuum line to afford the corresponding 3′-amine. Proceed directly to the next reaction.

Yield = 99.4% (30.4 g, 89.8 mmol).

18. Azeotrope 30.4 g (89.8 mmol) of 3′-amino-5′-*O*-(*tert*-butyldimethylsilyl)-2′,3′-dideoxyuridine two times with 300 mL pyridine and dissolve the solid in a mixture of 600 mL CH_2Cl_2 and 70 mL anhydrous pyridine.

19. Add 25.0 mL (179.6 mmol) triethylamine and 30.0 g (125.7 mmol) trityl chloride to this solution and stir for 2 hr at room temperature.

20. Purify (see Support Protocol 1, step 18) to afford 5′-*O*-(*tert*-butyldimethylsilyl)-3′-tritylamino-2′,3′-dideoxyuridine (**S.7**).

21. Perform TLC analysis (step 15) to confirm the purity of the product. Elute with 8:2 (v/v) ethyl acetate/hexane.

Yield = 85% (44.3 g, 75.9 mmol). R_f (8:2 ethyl acetate/hexane) = 0.58. ^{1}H NMR ($CDCl_3$/TMS): δ 8.24 (1H, br s, exchanges with D_2O), 7.73 (1H, d, J = 8.25 Hz), 7.52 (6H, d, J = 7.78 Hz), 7.31 (6H, m), 7.23 (3H, t, J = 7.23 Hz), 6.21 (1H, t, J = 6.69 Hz), 5.60 (1H, d, J = 8.17 Hz), 3.84 (1H, m), 3.76 (1H, dd, J = 11.34, 2.00 Hz), 3.48 (1H, dd, J = 11.37, 2.27 Hz), 3.32 (1H, m), 2.07 (1H, br, exchanges with D_2O), 1.60-1.70 (1H, m), 1.45-1.55 (1H, m), 0.84 (9H, s), 0.01 (3H, s), –0.05 (3H, s). HRMS (FAB$^+$): calcd for [M + Na]$^+$, 606.2764, observed 606.2751.

Synthesize N^4-benzoyl-5′-O-(tert-butyldimethylsilyl)-3′-tritylamino-2′,3′-dideoxycytidine (S.8)

22. Add 22.5 mL (161.1 mmol) triethylamine dropwise over a period of 10 min to a stirring mixture of 11.1 g (161.1 mmol) of 1,2,4-triazole and 3.5 mL (37.1 mmol) phosphorus oxychloride in 125 mL anhydrous CH_3CN at 0°C.

23. To this cold stirring mixture, add 9.4 g (16.1 mmol) of **S.7** as a solution in 50 mL acetonitrile. Stir 2 hr at room temperature.

24. Quench the reaction with 30 mL triethylamine and 10 mL water.

25. Remove the solvents using a rotary evaporator and vacuum line.

26. Dissolve the resulting brown solid in 250 mL CH_2Cl_2. Extract three times with 150 mL saturated aqueous $NaHCO_3$ and once with 150 mL saturated aqueous NaCl.

27. Dry the organic solution over anhydrous Na_2SO_4, vacuum filter, and concentrate on a rotary evaporator using a vacuum line to afford 4-(1,2,4-triazol-1-yl)-5′-*O*-(*tert*-butyldimethylsilyl)-3′-tritylamino-2′,3′-dideoxyuridine as an orange solid.

    Crude yield = 100% (10.2 g, 16.1 mmol).

28. Dissolve this crude material in 200 mL of 1,4-dioxane and add 50 mL concentrated NH_4OH, 4°C.

29. Stir the reaction mixture for 4 hr at room temperature and concentrate on a rotary evaporator using a vacuum pump to afford 5′-*O*-(*tert*-butyldimethylsilyl)-3′-tritylamino-2′,3′-dideoxycytidine as a beige solid.

    Crude yield = 100% (9.4 g, 16.1 mmol).

30. Azeotrope the crude material two times with 200 mL anhydrous pyridine.

31. Dissolve in 200 mL pyridine, cool externally in a 0°C ice bath, and add 2.2 mL (19.3 mmol) benzoyl chloride.

32. Allow the reaction to slowly warm to room temperature and stir an additional 16 hr at room temperature.

33. Cool the reaction mixture externally to 0°C and quench with 40 mL water. Stir 5 min.

34. Add 40 mL concentrated aqueous ammonia, 4°C, and stir the reaction mixture for an additional 15 min at 0°C.

35. Remove the solvents using a rotary evaporator and vacuum pump, dissolve the residue in 125 mL CH_2Cl_2, and extract three times with 75 mL saturated aqueous $NaHCO_3$.

36. Dry the organic phase over Na_2SO_4, vacuum filter, and evaporate the solvents using a rotary evaporator and vacuum line.

37. Purify the crude material (see Support Protocol 1, step 18) to afford N^4-benzoyl-5′-*O*-(*tert*-butyldimethylsilyl)-3′-tritylamino-2′,3′-dideoxycytidine (**S.8**).

38. Perform TLC analysis (step 15) to confirm the purity of the product. Elute with 5:95 (v/v) methanol/CH_2Cl_2.

    Yield = 92% (10.2 g, 14.8 mmol). R_f (5:95 methanol/CH_2Cl_2) = 0.71. ^{1}H NMR ($CDCl_3$ / TMS): δ 8.70 (1H, d, J = 7.36 Hz, exchanges with D_2O), 8.27 (1H, d, J = 7.36 Hz), 7.91 (2H, d, J = 7.46 Hz), 7.62 (1H, t, J = 7.20 Hz), 7.50-7.60 (8H, m; with 6H, d, J = 7.74 Hz at 7.52), 7.42 (1H, br d, J = 7.41 Hz), 7.30 (6H, t, J = 7.39 Hz), 7.22 (3H, t, J = 7.39 Hz), 6.26 (1H, t, J = 6.26 Hz), 3.80 (1H, br m), 3.77 (1H, br d, J = 11.39 Hz), 3.49 (1H, dd, J = 11.24, 2.33 Hz), 3.30 (1H, m), 1.90-2.10 (2H, br m; 1H exchanges in D_2O), 1.52 (1H, dt, J = 13.57, 6.76 Hz), 0.86 (9H, s), 0.04 (3H, s), –0.01 (3H, s). HRMS (FAB$^+$): calcd for [M + Cs]$^+$, 819.2343, observed 819.2366.

Synthesize N^4-benzoyl-3′-tritylamino-2′,3′-dideoxycytidine (S.9)

39. Remove the 5′-TBDMS protecting group by dissolving 2.0 g (2.85 mmol) **S.8** in 15 mL THF and reacting it with 15 mL of 1 M TBAF in THF for 16 hr.

40. Concentrate the reaction mixture to a syrup on the rotary evaporator using a vacuum line and dissolve the residue in 25 mL CH_2Cl_2. Extract four times with 25 mL water and once with 25 mL saturated aqueous NaCl.

41. Dry the organic layer over Na_2SO_4, vacuum filter, and remove the solvent using the rotary evaporator and vacuum line.

42. Purify the crude product on a silica gel column preequilibrated with 1% triethylamine in 30% ethyl acetate/hexane, and elute with 30% to 50% ethyl acetate/hexane to afford N^4-benzoyl-3′-tritylamino-2′,3′-dideoxycytidine (**S.9**).

43. Perform TLC analysis (step 15) to confirm the purity of the product. Elute with 5:95 (v/v) methanol/CH_2Cl_2.

Yield = 88% (1.4 g, 2.50 mmol). R_f (5:95 methanol/CH_2Cl_2) = 0.55. ^{1}H NMR ($CDCl_3$/TMS): δ 8.65 (1H, br s, exchanges with D_2O), 8.19 (1H, d, J = 7.36 Hz), 7.87 (2H, d, J = 7.57 Hz), 7.62 (1H, t, J = 7.37 Hz), 7.47-7.57 (9H, m), 7.30 (6H, t, J = 7.50 Hz), 7.23 (3H, t, J = 7.24 Hz), 6.07 (1H, dd, J = 6.66, 4.31), 3.91 (1H, d, J = 12.00 Hz), 3.79 (1H, m), 3.73 (1H, d, J = 12.10 Hz), 3.30 (1H, q, J = 6.38 Hz), 1.80-2.00 (2H, m, 1 br H exchanges in D_2O), 1.40 (1H, ddd, J = 13.89, 7.03, 4.41 Hz). HRMS (FAB$^+$): calcd for [M + Na]$^+$, 595.2321, observed 595.2310.

SYNTHESIS OF N^2-ISOBUTYRYL-O^6-(*N*,*N*-DIPHENYLCARBAMOYL)-3′-TRITYLAMINO-2′,3′-DIDEOXYGUANOSINE

SUPPORT PROTOCOL 3

The synthesis of N^2-isobutyryl-O^6-(*N*,*N*-diphenylcarbamoyl)-3′-tritylamino-2′,3′-dideoxyguanosine (**S.13**) is depicted in Figure 4.7.9. The 3′-*O*-benzoyl-N^2-isobutyryl- 2′-deoxyxyloguanosine (**S.10**) is synthesized as previously reported (Nishino et al., 1986; Herdewijn and Van Aerschot, 1989).

Additional Materials *(also see Support Protocol 1)*

N^2-Isobutyryl-2′-deoxyguanosine
Benzoyl chloride
Trifluoromethanesulfonic anhydride
4-Dimethylaminopyridine
tert-Butyldimethylsilyl chloride
1:1 (v/v) methanol/1,4-dioxane
1 M aqueous HCl
Diethylazodicarboxylate
Argon
N,*N*-Diisopropylethylamine
N,*N*-Diphenylcarbamyl chloride
Triethylamine trihydrofluoride
Toluene
7:3 and 6:4 (v/v) ethyl acetate/hexane
2-liter large-mouth Erlenmeyer flask

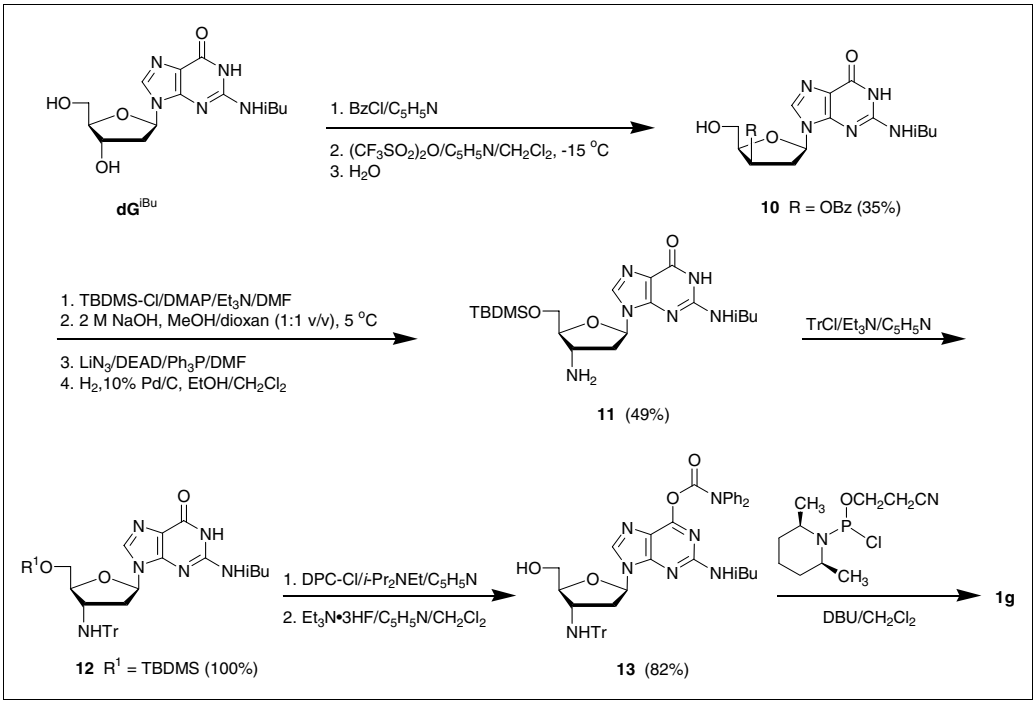


Figure 4.7.9 Synthetic steps in the preparation of N^2-isobutyryl-O^6-(*N,N*-diphenylcarbamoyl)-3′-tritylamino-2′,3′-dideoxyguanosine (**S.13**) from N^2-isobutyryl-2′-deoxyguanosine. DEAD, diethylazodicarboxylate; iBu, isobutyryl; DPC-Cl, *N,N*-diphenylcarbamyl chloride.

Synthesize 3′-O-benzoyl-N^2-isobutyryl-2′-deoxyxyloguanosine (S.10)

1. Azeotrope 119.1 g (353.1 mmol) N^2-isobutyryl-2′-deoxyguanosine two times with 600 mL pyridine and then add 1500 mL pyridine.

   The solid does not completely dissolve.

2. Add 45.1 mL (388.4 mmol) benzoyl chloride, dissolved in 250 mL pyridine, dropwise over 5 hr using an additional funnel, and then continue stirring for another 11 to 16 hr.

3. Quench the reaction with 20 mL water, then remove the solvent using a rotary evaporator and vacuum pump.

4. Dissolve the solid in 900 mL CH_2Cl_2, wash with 400 mL water, and then transfer the organic phase to a 2-liter large-mouth Erlenmeyer flask.

5. Add 400 mL water and stir vigorously to precipitate the white solid product.

6. Vacuum filter the solid and wash three times with 100 mL of water.

7. Dry the solid using a vacuum pump to obtain the 5′-*O*-benzoyl-N^2-isobutyryl-2′-deoxyguanosine.

   Yield = 91.6% (142.7 g, 323.4 mmol). R_f (1:9 methanol/CH_2Cl_2) = 0.27.

8. Azeotrope 142.7 g (323.4 mmol) of 5′-*O*-benzoyl-N^2-isobutyryl-2′-deoxyguanosine three times with 400 mL pyridine, and then add 240 mL pyridine and 2200 mL CH_2Cl_2.

9. Cool the mixture to −15°C and add, with stirring, 92.5 mL (549.7 mmol) trifluoromethanesulfonic anhydride dissolved in 500 mL CH_2Cl_2, dropwise over 1 hr, while maintaining the temperature between −10° and −15°C.

10. Perform TLC analysis (*APPENDIX 3D*) on 0.2-mm-thick precoated Merck silica gel 60 F254 plates to confirm that the reaction is complete. Elute with 1:9 (v/v) methanol/CH_2Cl_2 (R_f = 0.6).

11. When the reaction is complete (after ~1.5 hr), slowly quench the reaction with 150 mL water, maintaining the reaction temperature below 10°C.

12. Allow the solution to warm to room temperature and continue stirring for an additional 16 hr to complete the inversion.

13. Wash the organic layer two times with 1000 mL water and once with 1000 mL saturated aqueous NaCl.

14. Dry the organic solution over anhydrous Na_2SO_4, vacuum filter, and concentrate on the rotary evaporator and vacuum line to a brown solid.

15. Dissolve the solid in 550 mL CH_2Cl_2, stir for 1 hr, and then cool overnight at 4°C.

16. Vacuum filter the white solid, wash with 750 mL cold CH_2Cl_2, and then dry using a vacuum pump to afford 3′-*O*-benzoyl-N^2-isobutyryl-2′-deoxyxyloguanosine (**S.10**).

17. Concentrate the mother liquor using a rotary evaporator and vacuum line and repeat the recrystallization to obtain a second crop of pure product.

18. Perform TLC analysis (step 10) to confirm the purity of the product.

Combined yield = 38.6% (55.1 g, 124.9 mmol). R_f (1:9 methanol/CH_2Cl_2) = 0.27. ^{1}H NMR (DMSO-d_6): δ 12.04 (1H, s), 11.70 (1H, s), 8.16 (1H, s), 7.84 (2H, d, J = 7.79 Hz), 7.67 (1H, t, J = 7.54 Hz), 7.52 (2H, t, J = 7.69 Hz), 6.25 (1H, dd, J = 7.55, 2.00 Hz), 5.69 (1H, t, J = 4.3 Hz), 4.96 (1H, t, J = 5.49 Hz), 4.32 (1H, dt, J = 7.79, 5.88 Hz), 3.77 (2H, m), 3.00 (1H, m), 2.76 (2H, m), 1.11 (6H, d, J = 6.76 Hz).

*The product (**S.10**) coelutes by TLC with the 5′-O-benzoyl-N^2-isobutyryl-2′-deoxyguanosine but can be distinguished by ^{1}H NMR spectroscopy.*

Synthesize 3′-amino-5′-O-(tert-butyldimethylsilyl)-N^2-isobutyryl-2′,3′-dideoxyguanosine (S.11)

19. To a stirring solution of 4.86 g (11.0 mmol) **S.10** in 20 mL DMF, add 3.4 mL (24.2 mmol) triethylamine, 54 mg (0.44 mmol) 4-dimethylaminopyridine, and 3.31 g (22.0 mmol) *tert*-butyldimethylsilyl chloride.

20. Stir the reaction for 2 hr at room temperature.

21. Add 10 mL methanol and, after stirring an additional 5 min, concentrate the reaction mixture on a rotary evaporator using a high vacuum.

22. Dissolve the residue in 150 mL CH_2Cl_2 and wash three times with 40 mL water and once with 60 mL saturated aqueous NaCl.

23. Dry the organic layer over Na_2SO_4, vacuum filter, and concentrate with a rotary evaporator using a vacuum line to afford 6.40 g (>100% crude yield) of 3′-*O*-benzoyl-5′-*O*-(*tert*-butyldimethylsilyl)-N^2-isobutyryl-2′-deoxyxyloguanosine.

24. Dissolve the crude material in 100 mL 1:1 methanol/1,4-dioxane and cool to 5°C.

25. Add 44.0 mL (87.9 mmol) prechilled (5°C) 2 M aqueous NaOH and stir the reaction mixture for 15 to 20 min in an ice bath.

Monitor this reaction carefully and neutralize the hydroxide as soon as possible in order to avoid loss of the isobutyryl group.

26. Neutralize the reaction with 97 mL of 1 M aqueous HCl to pH 6 to 7.

27. Remove the ice bath and concentrate the reaction mixture to ~50 mL on the rotary evaporator with a high vacuum.

28. Extract three times with 75 mL CH_2Cl_2.

29. Wash the combined organics three times with 50 mL saturated aqueous $NaHCO_3$ and two times with 50 mL saturated aqueous NaCl.

30. Dry the organic solution over Na_2SO_4, vacuum filter, and concentrate with a rotary evaporator using a vacuum line to afford 5′-*O*-(*tert*-butyldimethylsilyl)-N^2-isobutyryl-2′-deoxyxyloguanosine. Proceed to the next reaction without further purification.

Yield = 82% (4.1 g, 9.1 mmol).

31. To 47.3 g (104.7 mmol) crude 5′-*O*-(*tert*-butyldimethylsilyl)-N^2-isobutyryl-2′-deoxyxyloguanosine dissolved in 1000 mL anhydrous DMF, add 15.4 g (314.1 mmol) LiN_3 and 41.2 g (157.1 mmol) triphenylphosphine.

32. Add 24.7 mL (157.1 mmol) diethylazodicarboxylate and stir the reaction mixture for 5 hr at room temperature under argon.

33. Quench the reaction with 20 mL water and concentrate the reaction mixture on the rotary evaporator using a vacuum pump.

34. Dissolve the residue in 1500 mL ethyl acetate.

35. Wash three times with 1000 mL water and once with 1000 mL saturated aqueous NaCl.

36. Dry the organic solution over Na_2SO_4, vacuum filter, and concentrate using a rotary evaporator and vacuum line. Proceed directly to hydrogenation and purification of the 3′-amine.

37. Dissolve ≤104.7 mmol crude azide in 1600 mL of 1:1 (v/v) ethanol/CH_2Cl_2 and hydrogenate (60 psi H_2) in the presence of 2.5 g of 10% Pd/C catalyst for 16 hr at room temperature.

38. Remove the catalyst by vacuum filtration and evaporate the solvent using a rotary evaporator and vacuum line to afford the crude 3′-amine.

39. Purify by gravity on a silica gel column (*APPENDIX 3E*) using 2% to 6% methanol/CH_2Cl_2 and then 1% triethylamine/6% methanol/CH_2Cl_2 to afford 3′-amino-5′-*O*-(*tert*-butyldimethylsilyl)-N^2-isobutyryl-2′,3′-dideoxyguanosine (**S.11**) as an off-white foam.

40. Perform TLC analysis (step 10) to confirm the purity of the product.

Yield = 60% (28.2 g, 63.2 mmol). R_f (1:9 methanol/CH_2Cl_2) = 0.14. ^{1}H NMR ($CDCl_3$/TMS): δ 8.01 (1H, s), 6.17 (1H, dd, J = 6.77, 3.98 Hz), 3.80-3.90 (4H, mm), 2.83 (1H, septet, J = 6.80 Hz), 2.59 (1H, ddd, J = 13.26, 6.16, 4.03 Hz), 2.33 (1H, dt, J = 13.19, 6.79 Hz), 1.26 (6H, dd, J = 6.86, 2.79 Hz), 0.88 (9H, s), 0.07 (3H, s), 0.06 (3H, s). HRMS (FAB$^+$): calcd for [M + H]$^+$, 451.2489, observed 451.2480.

Synthesize 5′-O-(tert-butyldimethylsilyl)-N^2-isobutyryl-3′-tritylamino-2′,3′-dideoxyguanosine (S.12)

41. Dissolve 28.5 g (63.2 mmol) **S.11** in 500 mL pyridine, add 17.6 mL (126.4 mmol) triethylamine and 28.2 g (101.1 mmol) trityl chloride, and stir for 16 hr at room temperature.

42. Concentrate the reaction product to a solid using a rotary evaporator and vacuum pump.

43. Purify crude product (see Support Protocol 1, step 18) to afford 5′-*O*-(*tert*-butyldimethylsilyl)-N^2-isobutyryl-3′-tritylamino-2′,3′-dideoxyguanosine (**S.12**).

44. Perform TLC analysis (step 10) to confirm the purity of the product.

Yield = 100% (43.8 g, 63.2 mmol). R_f (1:9 methanol/CH_2Cl_2) = 0.72. ^{1}H NMR ($CDCl_3$ / TMS): δ 11.90 (1H, br s, exchanges with D_2O), 8.01 (1H, br s, exchanges with D_2O), 7.58 (1H, s), 7.56 (6H, d, J = 7.39 Hz), 7.31 (6H, t, J = 7.58 Hz), 7.23 (3H, t, J = 7.28 Hz), 6.00 (1H, dd, J = 6.86, 4.63 Hz), 3.88 (1H, dt, J = 5.91, 3.01 Hz), 3.75 (2H, ABX, J_{AB} = 11.25 Hz), 3.52 (1H, m), 2.57 (1H, septet, J = 6.91 Hz), 2.00- 2.10 (1H, br s, exchanges with D_2O), 1.72 (1H, dt, J = 13.63, 6.89 Hz), 1.59 (1H, ddd, J = 13.73, 6.69, 4.87 Hz), 1.28 (6H, dd, J = 6.90, 3.22 Hz), 0.81 (9H, s), –0.03 (3H, s), –0.04 (3H, s). HRMS (FAB$^+$): calcd for [M + Cs]$^+$, 825.2561, observed 825.2540.

Synthesize O^6-(N,N-diphenylcarbamoyl)-N^2-isobutyryl-3′-tritylamino-2′,3′-dideoxyguanosine (S.13)

45. Dissolve 30.3 g (43.7 mmol) **S.12** in 90 mL anhydrous pyridine.

46. Add 11.4 mL (65.6 mmol) *N,N*-diisopropylethylamine and 11.1 g (48.1 mmol) *N,N*-diphenylcarbamyl chloride under argon and stir for 1.5 hr at room temperature.

47. Concentrate the intensely red/purple reaction mixture in vacuo (see step 42).

48. Dissolve the residue in 600 mL CH_2Cl_2, extract two times with 400 mL water and once with 400 mL saturated aqueous NaCl.

49. Dry the CH_2Cl_2 solution over Na_2SO_4, vacuum filter, and concentrate in vacuo (see step 36) to afford impure 5′-*O*-(*tert*-butyldimethylsilyl)-O^6-(*N,N*-diphenylcarbamoyl)-N^2-isobutyryl-3′-tritylamino-2′,3′-dideoxyguanosine.

This product is generally taken on directly to desilylation, although it can also be purified on silica.

50. Perform TLC analysis (step 10) using 6:4 (v/v) ethyl acetate/hexane.

Crude yield > 100% (43.8 g). R_f (6:4 ethyl acetate/hexane) = 0.58. ^{1}H NMR ($CDCl_3$/TMS): δ 8.03 (1H, s), 7.90 (1H, br s, exchanges with D_2O), 7.55 (6H, d, J = 7.63 Hz), 7.24-7.50 (16H, mm), 7.21 (3H, t, J = 7.22 Hz), 6.29 (1H, t, J = 6.07 Hz), 3.89 (1H, m), 3.75 (2H, ABX, J_{AB} = 11.25 Hz), 3.49 (1H, br m), 3.01 (1H, br m), 2.77 (1H, septet, J = 6.78 Hz), 2.00-2.10 (br s, exchanges with D_2O), 1.65-1.75 (2H, m), 1.28 (6H, d, J = 6.64 Hz), 0.83 (9H, s), –0.01 (3H, s), –0.02 (3H, s). HRMS (FAB$^+$): calcd for [M + Cs]$^+$, 1020.3245, observed 1020.3281.

51. Dissolve ~43.7 mmol crude 5′-*O*-(*tert*-butyldimethylsilyl)-O^6-(*N,N*-diphenylcarbamoyl)-N^2-isobutyryl-3′-tritylamino-2′,3′-dideoxyguanosine in 200 mL CH_2Cl_2 and 25 mL pyridine.

52. Add 49.8 mL (305.8 mmol) triethylamine trihydrofluoride, followed by a 25-mL CH_2Cl_2 rinse, and stir the reaction mixture for 20 hr under argon at room temperature.

53. Dilute the reaction mixture with 600 mL CH_2Cl_2 and extract two times with 400 mL water.

54. Back-extract the first aqueous layer with 50 mL CH_2Cl_2.

55. Dry the combined organics over Na_2SO_4, vacuum filter, and concentrate in vacuo (see step 36).

56. Dissolve the residue in 100 mL CH_2Cl_2 and azeotrope three times with 50 mL toluene to remove traces of pyridine using a rotary evaporator and vacuum pump.

57. Purify on silica gel, using a column packed in 2% triethylamine in 7:3 (v/v) ethyl acetate/hexane and eluting with 7:3 ethyl acetate/hexane, to afford O^6-(*N*,*N*-diphenylcarbamoyl)-N^2-isobutyryl-3′-tritylamino-2′,3′-dideoxyguanosine (**S.13**).

58. Confirm the purity of the product by TLC analysis (step 10), eluting with 6:4 (v/v) ethyl acetate/hexane.

Yield = 82% (27.6 g, 35.7 mmol). R_f (6:4 ethyl acetate/hexane) = 0.20. ^1H NMR ($CDCl_3$ / TMS): δ 7.95 (1H, s), 7.84 (1H, br s, exchanges with D_2O), 7.55 (6H, d, J = 7.85 Hz), 7.25-7.45 (16H, mm), 7.21 (3H, t, J = 7.25 Hz), 6.15 (1H, t, J = 6.31 Hz), 3.77-3.87 (2H, br m), 3.69 (1H, m), 3.62 (1H, m), 3.19 (1H, m), 2.80 (1H, septet, J = 6.86 Hz), 1.92-2.05 (2H, mm, 1H exchanges with D_2O), 1.65 (1H, m), 1.24 (6H, d, J = 6.86 Hz). HRMS (FAB^+): calcd for $[M + Cs]^+$, 906.2380, observed 906.2350.

Do not use 1 M tetra-n-butylammonium fluoride in THF to remove the TBDMS group because the O^6-(N,N-diphenylcarbamoyl) group is not stable to this reagent.

SUPPORT PROTOCOL 4

SYNTHESIS OF N^6-BENZOYL-3′-TRITYLAMINO- 2′,3′-DIDEOXYADENOSINE

The synthesis of N^6-benzoyl-3′-tritylamino-2′,3′-dideoxyadenosine (**S.18**) from adenosine is illustrated in Figure 4.7.10. The 5′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyxyloadenosine is synthesized by slightly modified literature procedures (Wagner et al., 1974; Hansske and Robins, 1983), which is more efficient than the inversion route that is used for the pyroration of **S.10**.

Additional Materials *(also see Support Protocol 1)*

(−)-Adenosine
Dibutyltin oxide
p-Toluenesulfonyl chloride
tert-Butyldimethylsilyl chloride
1 M lithium triethyl borohydride in THF
Ammonium chloride
Benzoyl chloride
7:10 (v/v) methanol/1,4-dioxane
Pyridinium hydrochloride
Diethylazodicarboxylate
Argon
4:6 (v/v) ethyl acetate/hexane
Tetrahydrofuran (THF)
1.0 M tetrabutylammonium fluoride (TBAF) in THF

Synthesize 2′-O-(p-toluenesulfonyl)adenosine (S.14)

1. Gently reflux 25.0 g (93.5 mmol) (−)-adenosine and 23.3 g (93.5 mmol) dibutyltin oxide in 1000 mL methanol for 2 hr, until the cloudy suspension becomes clear.

2. Cool the solution to 4°C, then cautiously add 53.5 g (281 mmol) *p*-toluenesulfonyl chloride and 39.1 mL (281 mmol) triethylamine, keeping the reaction temperature at 4°C.

3. Stir the resulting cloudy suspension overnight at room temperature.

4. Vacuum filter the white solid, wash two times with 100 mL cold methanol, and dry using a vacuum pump to afford 2′-*O*-*p*-toluenesulfonyladenosine (**S.14**).

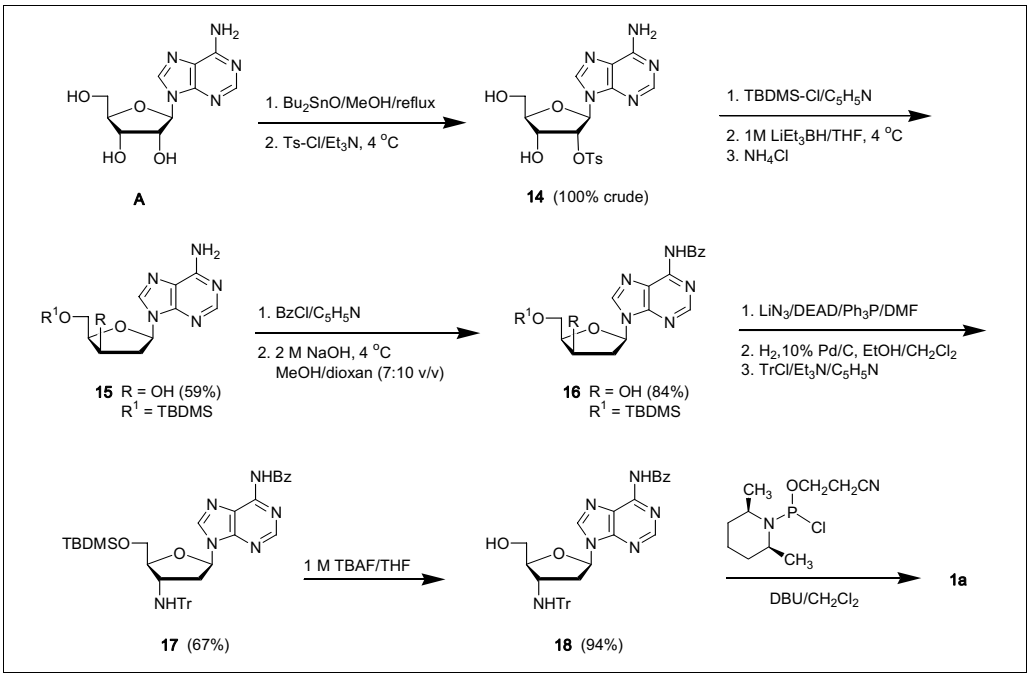


Figure 4.7.10 Synthetic steps in the preparation of N^6-benzoyl-3′-tritylamino-2′,3′-dideoxyadenosine (**S.18**) from adenosine. Bu, butyl; Ts, *p*-toluenesulfonyl.

Crude yield = 114% (45 g). ^{1}H NMR (DMSO-d_6): δ 8.19 (1H, s), 8.02 (1H, s), 7.37 (2H, s), 7.42 (2H, d), 7.03 (2H, d), 6.11 (1H, d), 6.03 (1H, d), 5.75 (1H, t), 5.49 (1H, dd), 4.39 (1H, ddd), 4.07 (1H, br d), 3.62 (2H, m), 2.25 (3 H, s).

Synthesize 5′-O-(tert-butyldimethylsilyl)-2′-deoxyxyloadenosine (S.15)

5. Dissolve 45 g (< 93.5 mmol) **S.14** in 1000 mL pyridine, add 28.2 g (187 mmol) *tert*-butyldimethylsilyl chloride, and stir overnight at room temperature.
6. Quench the reaction with 100 mL methanol, concentrate on a rotary evaporator using a vacuum pump, and azeotrope three times with 25 mL toluene.
7. Dissolve the solid in 200 mL methanol, stir for 2 hr to desilylate the N^6-amino group, and concentrate with a rotary evaporator and vacuum line.
8. Purify the reaction product by gravity on a silica gel column (*APPENDIX 3E*) using 3% to 4% methanol/CH_2Cl_2 to give 5′-*O*-(*tert*-butyldimethylsilyl)-2′-*O*-(*p*-toluenesulfonyl)adenosine.

   Yield = 75.6% (37.9 g, 70.7 mmol). TLC (1:9 methanol/CH_2Cl_2) R_f = 0.51.

9. Dissolve 37.9 g (70.7 mmol) 5′-*O*-(*tert*-butyldimethylsilyl)-2′-*O*-(*p*-toluenesulfonyl)adenosine in 100 mL anhydrous THF and cool to 4°C.
10. Add dropwise 283 mL (283 mmol) prechilled 1.0 M lithium triethyl borohydride in THF.
11. Stir the solution for 30 min at 4°C and then overnight at room temperature.
12. Cool the solution to 4°C, carefully quench with 11.3 g (212 mmol) NH_4Cl, and concentrate on the rotary evaporator with a vacuum line.
13. Dissolve the residue first in 50 mL methanol and then 500 mL diethyl ether.
14. Wash with 100 mL saturated aqueous NaCl, and concentrate to a foam (see step 12).

15. Purify on silica (2% to 4% methanol/CH_2Cl_2) to afford 5′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyxyloadenosine (**S.15**).

Yield = 77.5% (20.1 g, 54.8 mmol). TLC (1:9 methanol/CH_2Cl_2) R_f = 0.43. ^{1}H NMR ($CDCl_3$/TMS): δ 8.35 (1H, s), 7.98 (1H, s), 7.08 (1H, d, J = 9.34 Hz), 6.14 (1H, dd, J = 9.36, 2.68 Hz), 5.98 (2H, br s), 4.47 (1H, m), 4.10 (1H, m), 3.97 (2H, m), 2.88 (1H, ddd, J = 15.47, 9.40, 6.32 Hz), 2.56 (1H, dd, J = 15.35, 2.76 Hz), 0.88 (9H, s), 0.063 (3H, s), 0.060 (3H, s).

Make sure the reaction is fully quenched before concentrating the solution because lithium triethyl borohydride is pyrophoric.

Other solvents, such as ethyl acetate or CH_2Cl_2, should not be used for the extraction as they lead to severe emulsions.

Synthesize N^6-benzoyl-5′-O-(tert-butyldimethylsilyl)-2′-deoxyxyloadenosine (S.16)

16. Dissolve 5.0 g (13.6 mmol) **S.15** in 25 mL pyridine, add 3.28 mL (27.4 mmol) benzoyl chloride, and stir for 2 hr at room temperature.

17. Quench the reaction with 1 mL water and concentrate on a rotary evaporator with vacuum pump.

18. Dissolve the residue in 80 mL of 7:10 (v/v) methanol/1,4-dioxane, cool to 4 °C.

19. Add 34 mL (68 mmol) prechilled 2.0 M aqueous NaOH and stir for 5 min to selectively remove the 3′-benzoyl group.

Monitor the hydrolysis carefully and neutralize the hydroxide as soon as possible in order to avoid loss of the N^6-benzoyl group.

20. Neutralize the solution to pH 7 with 4.0 g (35 mmol) pyridinium hydrochloride and concentrate on a rotary evaporator with vacuum pump.

21. Dissolve the residue in 100 mL CH_2Cl_2, extract two times with 50 mL saturated aqueous $NaHCO_3$ and two times with 50 mL saturated aqueous NaCl, and concentrate in vacuo (see step 7).

22. Purify on silica (2% methanol/CH_2Cl_2) to give N^6-benzoyl-5′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyxyloadenosine (**S.16**).

Yield = 84.6% (5.4 g, 11.5 mmol). TLC (5:95 methanol/CH_2Cl_2) R_f = 0.38. ^{1}H NMR ($CDCl_3$/TMS): δ 9.00 (1H, br s), 8.82 (1H, s), 8.32 (1H, s), 8.02 (2H, d, J = 7.26 Hz), 7.63 (1H, t, J = 7.41 Hz), 7.54 (2H, t, J = 7.56 Hz), 6.31 (1H, dd, J = 9.06, 2.44 Hz), 5.95 (1H, d, J = 7.63 Hz), 4.55 (1H, m), 4.11 (1H, pseudo q, J = 6.21 Hz), 4.02 (2H, dd, J = 8.08, 2.68 Hz), 2.90 (1H, ddd, J = 15.22, 9.12, 5.93 Hz), 2.58 (1H, dd, J = 15.33, 2.47 Hz), 0.88 (9H, s), 0.07 (3H, s), 0.06 (3H, s).

Synthesize N^6-benzoyl-5′-O-(tert-butyldimethylsilyl)-3′-tritylamino-2′,3′-dideoxyadenosine (S.17)

23. Dissolve 17.6 g (37.5 mmol) **S.16** in 375 mL anhydrous DMF and add 5.5 g (113.0 mmol) LiN_3 and 14.8 g (56.3 mmol) triphenylphosphine.

24. Add 8.9 mL (56.3 mmol) diethylazodicarboxylate and stir the reaction mixture for 6 hr under argon at room temperature.

25. Quench the reaction with 10 mL water and concentrate in a rotary evaporator with a vacuum pump.

26. Dissolve the residue in 500 mL ethyl acetate, and wash three times with 300 mL water and once with 300 mL saturated aqueous NaCl.

27. Dry the ethyl acetate solution over Na_2SO_4, filter, and concentrate with a rotary evaporator and vacuum line.

This crude (triphenylphosphine oxide–contaminated) 3′-azido-N^6-benzoyl-5′-O-(tert-butyldimethylsilyl)-2′,3′-dideoxyadenosine (18.8 g) is taken on directly to hydrogenation and purified as the 3′-amine.

28. Dissolve 18.8 g of the crude azide in 250 mL of 1:1 (v/v) ethanol/CH_2Cl_2 and reduce by hydrogenation (60 psi H_2) in the presence of 1.0 g of 10% Pd/C catalyst for 16 hr at room temperature.

29. Remove the catalyst by vacuum filtration, and evaporate the solvent in a rotary evaporator with vacuum line to afford the crude 3′-amine.

30. Purify on silica (preequilibrate with 2% methanol/CH_2Cl_2 and elute with 2% to 6% methanol/CH_2Cl_2 and then 1% triethylamine/6% methanol/CH_2Cl_2) to afford 3′-amino-N^6-benzoyl-5′-*O*-(*tert*-butyldimethylsilyl)-2′,3′-dideoxyadenosine as an off-white foam.

31. Perform TLC analysis (*APPENDIX 3D*) on 0.2-mm-thick precoated Merck silica gel 60 F254 plates to confirm the purity of the product. Elute with 8:92 (v/v) methanol/CH_2Cl_2.

Yield = 68% (12.0 g, 25.6 mmol). R_f (8:92 methanol/CH_2Cl_2) = 0.30. ^{1}H NMR ($CDCl_3$/TMS): δ 8.95 (1H, br s, exchanges with D_2O), 8.81 (1H, s), 8.40 (1H, s), 8.02 (2H, d, J = 7.23 Hz), 7.62 (1H, t, J = 7.43 Hz), 7.54 (2H, t, J = 7.48), 6.49 (1H, dd, J = 6.81, 3.68 Hz), 3.80-3.98 (4H, mm), 2.76 (1H, ddd, J = 13.27, 6.42, 3.69), 2.39 (1H, dt, J = 13.43, 6.93), 0.92 (9H, s), 0.11 (3H, s), 0.00 (3H, s). HRMS (FAB$^+$): calcd for [M + Cs]$^+$, 601.1360, observed 601.1373.

32. Protect 29.5 g (63.0 mmol) of 3′-amino-N^6-benzoyl-5′-*O*-(tert-butyldimethylsilyl)-2′,3′-dideoxyadenosine by reacting with 12.9 mL (94.5 mmol) triethylamine and 21.1 g (75.6 mmol) trityl chloride in 350 mL CH_2Cl_2 for 16 hr at room temperature.

33. Dilute the reaction mixture with an additional 150 mL CH_2Cl_2.

34. Extract once with 400 mL water, three times with 300 mL saturated aqueous $NaHCO_3$, and two times with 300 mL saturated aqueous NaCl.

35. Concentrate to a glassy foam with a rotary evaporator and vacuum line.

36. Purify the crude product on a silica gel column preequilibrated with 1% triethylamine in 4:6 (v/v) ethyl acetate/hexane, and elute with 4:6 ethyl acetate/hexane to afford N^6-benzoyl-5′-*O*-(*tert*-butyldimethylsilyl)-3′-tritylamino-2′,3′-dideoxyadenosine (**S.17**).

37. Perform TLC analysis (step 33) using 5:95 (v/v) methanol/CH_2Cl_2 as the eluent.

Yield = 98% (44.1 g, 62.1 mmol). R_f (5:95 methanol/CH_2Cl_2) = 0.48. ^{1}H NMR ($CDCl_3$/TMS): δ 8.99 (1H, br s, exchanges with D_2O), 8.76 (1H, s), 8.12 (1H, s), 8.00 (2H, d, J = 7.29 Hz), 7.60 (1H, t, J = 7.41 Hz), 7.54 (6H, d, J = 7.44 Hz), 7.51 (2H, t, J = 7.28 Hz), 7.28 (6H, t, J = 7.56 Hz), 7.20 (2H, t, J = 7.22 Hz), 6.36 (1H, t, J = 5.96 Hz), 3.90 (1H, m), 3.82 (1H, dd, J = 11.28, 2.74 Hz), 3.67 (1H, dd, J = 11.26, 3.10 Hz), 3.50 (1H, br m), 2.00-2.10 (1H, br s, exchanges with D_2O), 1.68-1.83 (2H, mm), 0.82 (9H, s), –0.02 (3H, s), –0.03 (3H, s). HRMS (FAB$^+$): calcd for [M + Cs]$^+$, 843.2455, observed 843.2477.

Synthesize N^6-benzoyl-3′-tritylamino-2′,3′-dideoxyadenosine (S.18)

38. Remove the 5′-TBDMS protecting group by dissolving 43.7 g (61.5 mmol) **S.17** in 123.0 mL THF and reacting with 123.0 mL (123.0 mmol) of 1.0 M TBAF in THF for 24 hr, room temperature.

39. Concentrate the reaction mixture with vacuum line and dissolve the residue in 400 mL ethyl acetate.

40. Extract three times with 250 mL water and two times with 250 mL saturated aqueous NaCl.

41. Dry the organic layer over Na_2SO_4, vacuum filter, and remove the solvent with a rotary evaporator and vacuum line.

42. Purify the crude product on a silica gel column preequilibrated with 2% triethylamine in 8:2 (v/v) ethyl acetate/hexane, and elute with 8:2 ethyl acetate/hexane to 100% ethyl acetate to afford N^6-benzoyl-3′-tritylamino-2′,3′-dideoxyadenosine (**S.18**).

43. Perform TLC analysis (step 33) using 5:95 (v/v) methanol/CH_2Cl_2 as the eluent.

Yield = 94% (34.5 g, 57.9 mmol). R_f (5:95 methanol/CH_2Cl_2) = 0.40. ^1H NMR ($CDCl_3$/TMS): δ 9.06 (1H, br s, exchanges with D_2O), 8.68 (1H, s), 8.02 (1H, s), 8.01 (2H, d, J = 7.33 Hz), 7.60 (1H, t, J = 7.47 Hz), 7.53 (6H, d, J = 7.38 Hz), 7.51 (2H, t, J = 7.20 Hz), 7.29 (6H, t, J = 7.58 Hz), 7.21 (3H, t, J = 7.25 Hz), 6.24 (1H, dd, J = 7.56, 6.30 Hz), 4.85 (1H, dd, J = 9.87, 3.19 Hz, exchanges with D_2O), 3 65-3.82 (3H, mm), 3.37 (1H, t, J = 10.16 Hz), 2.38 (1H, dt, J = 13.46, 7.02 Hz), 2.00-2.20 (1H, br s, exchanges with D_2O), 1.75 (1H, ddd, J = 13.28, 5.96, 2.97 Hz). HRMS (FAB$^+$): calcd for [M + Na]$^+$, 619.2434, observed 619.2421.

SUPPORT PROTOCOL 5

SYNTHESIS OF 3′-*O*-(4,4′-DIMETHOXYTRITYL)-PROTECTED DEOXYRIBONUCLEOSIDES

An approach to the synthesis of 3′-*O*-(4,4′-dimethoxytrityl)-protected deoxyribonucleosides (**S.20**) is presented in Figure 4.7.11. These nucleosides are necessary for the synthesis of the phosphodiester or phosphorothioate portion of chimeric oligonucleotides.

Additional Materials *(also see Support Protocol 1)*

N^6-Benzoyl-2′-deoxyadenosine
N^4-Benzoyl-2′-deoxycytidine
N^2-Isobutyryl-2′-deoxyguanosine
Thymidine
4-Dimethylaminopyridine
tert-Butyldimethylsilyl chloride
Tetrahydrofuran (THF)
1 M tetrabutylammonium fluoride (TBAF) in THF
N,*N*-Diisopropylethylamine

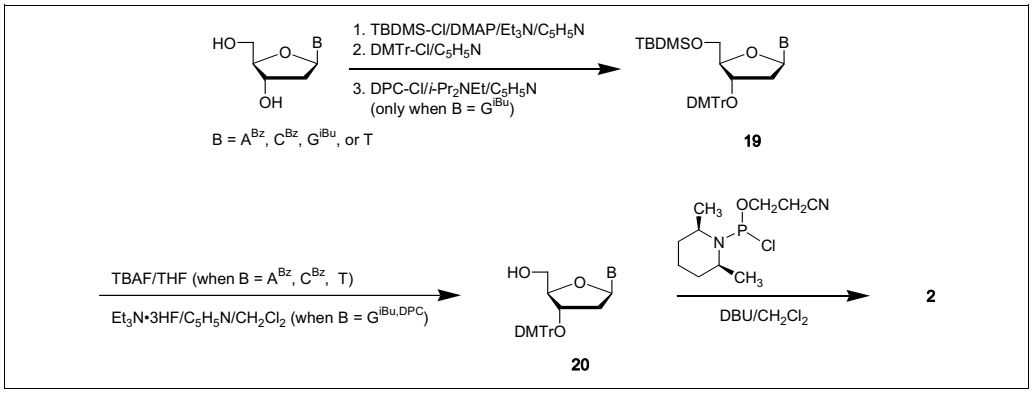


Figure 4.7.11 General approach to the synthesis of 3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyribonucleosides (**S.20**) from *N*-protected 2′-deoxyribonucleosides.

N,*N*-Diphenylcarbamyl chloride
9:1 (v/v) CH_2Cl_2/pyridine
Triethylamine trihydrofluoride
50% to 70% ethyl acetate/hexane

Prepare 5′-O-(tert-butyldimethylsilyl)-2′-deoxyribonucleosides (S.19)

1. Azeotrope the *N*-protected 2′-deoxyribonucleoside (dA^{Bz}, dC^{Bz}, $dG^{i\text{-}Bu}$, or T) two times from 10 mL/g pyridine and suspend in pyridine at 10 mL/g.
2. To this stirring mixture, add sequentially 0.1 eq 4-dimethylaminopyridine, 1.2 eq triethylamine, and 1.05 to 1.2 eq *tert*-butyldimethylsilyl chloride. Stir for 8 to 24 hr at room temperature.
3. Remove the pyridine using a rotary evaporator and vacuum pump.
4. Dissolve the residue in 15 mL/g CH_2Cl_2 and extract two times with 10 mL/g water and one time with 10 mL/g saturated aqueous NaCl.
5. Dry the organic solution over anhydrous Na_2SO_4, vacuum filter, and concentrate under reduced pressure to a solid that is used in the next reaction without further purification.

Prepare 5′-O-(tert-butyldimethylsilyl)-3′-O-(4,4′-dimethoxytrityl)-2′-deoxyribonucleosides

6. Azeotrope the 5′-*O*-(*tert*-butyldimethylsilyl)-protected 2′-deoxyribonucleoside two times from 10 mL/g pyridine and dissolve in pyridine at 10 mL/g.
7. While stirring this solution, add 1.2 to 1.3 eq 4,4′-dimethoxytrityl chloride. Stir the solution for 16 to 24 hr at room temperature.
8. Concentrate on a rotary evaporator using a vacuum pump.
9. Dissolve the residue in 15 mL/g CH_2Cl_2 and extract once each with 10 mL/g water, 10 mL/g saturated aqueous $NaHCO_3$, and 10 mL/g saturated aqueous NaCl.
10. Dry the CH_2Cl_2 solution over Na_2SO_4, vacuum filter, and concentrate under reduced pressure to a foam.

The product can be used directly in the next reaction (T) or purified on silica (dA^{Bz}, dC^{Bz}, and $dG^{i\text{-}Bu}$) using a gradient of 1% to 5% methanol in CH_2Cl_2.

Prepare 3′-O-(4,4′-dimethoxytrityl)-2′-deoxyribonucleosides (S.20)

For dA^{Bz}, dC^{Bz}, and T:

11a. Remove the 5′-TBDMS protecting group by dissolving the 5′-(*tert*-butyldimethylsilyl)-3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyribonucleoside in THF at 3 mL/g and reacting it with 1 M (2.0 eq) TBAF in THF for 16 to 24 hr, room temperature.

12a. Concentrate the solution under reduced pressure.

13a. Dissolve the residue in 15 mL/g CH_2Cl_2, and extract two times with 10 mL/g water and one time with 10 mL/g saturated aqueous NaCl.

14a. Dry the organic layer over Na_2SO_4, vacuum filter, and evaporate on a rotary evaporator with pump.

15a. Purify the crude 3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyribonucleosides (**S.20**) (see Support Protocol 1, step 18).

16a. Perform TLC analysis (*APPENDIX 3D*) on 0.2-mm-thick precoated Merck silica gel 60 F254 plates to confirm the purity of the product. Elute with 1:9 (v/v) methanol/CH_2Cl_2.

*N^6-Benzoyl-3′-O-(4,4′-dimethoxytrityl)-2′-deoxyadenosine (**S.20**). Yield from dA^{Bz} = 56.7% (55.4 g). R_f (1:9 methanol/CH_2Cl_2) = 0.68. ^{1}H NMR ($CDCl_3$/TMS): δ 9.14 (1H, br s, exchanges with D_2O), 8.72 (1H, s), 8.06 (1H, s), 8.02 (2H, d, J = 7.43 Hz), 7.62 (1H, t, J = 7.33 Hz), 7.53 (2H, d, J = 7.74 Hz), 7.50 (2H, d, J = 7.54 Hz), 7.39 (4H, d, J = 8.81 Hz), 7.34 (2H, t, J = 7.54 Hz), 7.26 (2H, t, J = 7.95 Hz), 6.87 (4H, dd, J = 8.91, 2.43 Hz), 6.37 (1H, dd, J = 9.95, 5.26 Hz), 5.79 (1H, br d, J = 10.38 Hz, exchanges with D_2O), 4.66 (1H, d, J = 5.32 Hz), 4.08 (1H, s), 3.81 (6H, s), 3.76 (1H, d, J = 12.78 Hz), 3.35 (1H, t, J = 11.86 Hz), 2.73 (1H, ddd, J = 13.21, 10.10, 7.99 Hz), 1.76 (1H, dd, J = 13.31, 5.30 Hz). HRMS (FAB$^+$): calcd for $[M + H]^+$, 658.2666; found, 658.2666.*

*N^4-Benzoyl-3′-O-(4,4′-dimethoxytrityl)-2′-deoxycytidine (**S.20**). Yield from dC^{Bz} = 74.7% (70.0 g) including additional mixed fractions that were purified further by precipitation from CH_2Cl_2 into a 20× volume of 3:1 hexane/diethyl ether over 1.5 hr. R_f (1:9 methanol/CH_2Cl_2) = 0.66. ^{1}H NMR ($CDCl_3$/TMS): δ 8.66 (1H, br s, exchanges with D_2O), 8.09 (1H, d, J = 7.35 Hz), 7.87 (2H, d, J = 7.43 Hz), 7.62 (1H, t, J = 7.36 Hz), 7.53 (2H, d, J = 7.80 Hz), 7.48 (2H, d, J = 7.63 Hz), 7.37 (4H, d, J = 8.86 Hz), 7.32 (2H, t, J = 7.53 Hz), 7.25 (1H, t, J = 7.17 Hz), 6.85 (4H, d, J = 8.76 Hz), 6.25 (1H, dd, J = 7.63, 6.12 Hz), 4.36-4.43 (1H, br m), 3.94 (1H, d, J = 2.19 Hz), 3.81 (6H, s), 3.66 (1H, br d, J = 11.86 Hz), 3.26 (1H, br d, J = 11.90 Hz), 2.48 (1H, br s, exchanges with D_2O), 2.22 (1H, dd, J = 13.13, 5.20 Hz), 2.08 (1H, quintet, J = 6.94 Hz). HRMS (FAB$^+$): calcd for $[M + Na]^+$, 656.2373; found, 656.2383.*

*3′-O-(4,4′-Dimethoxytrityl)-thymidine (**S.20**). Yield from T = 81.8% (45.2 g). R_f (1:9 methanol/CH_2Cl_2) = 0.56. ^{1}H NMR ($CDCl_3$/TMS): δ 8.61 (1H, br s, exchanges with D_2O), 7.46 (2H, d, J = 7.47 Hz), 7.36 (4H, d, J = 8.83 Hz), 7.32 (2H, t, J = 7.94 Hz), 7.25 (1H, t, J = 7.43 Hz), 6.86 (4H, d, J = 7.39 Hz), 6.15 (1H, dd, J = 8.87, 5.76 Hz), 4.38 (1H, d, J = 6.20 Hz), 3.99 (1H, d, J = 2.13 Hz), 3.81 (6H, s), 3.68 (1H, br d, J = 11.79 Hz), 3.30-3.37 (1H, br m), 2.47-2.55 (1H, br m, exchanges with D_2O), 1.95 (1H, ddd, J = 13.98, 8.42, 6.00 Hz), 1.87 (3H, s), 1.67-1.74 (1H, m). HRMS (FAB$^+$): calcd for $[M + Na]^+$, 567.2107; found, 567.2111.*

For $dG^{i\text{-}Bu,DPC}$:

11b. Prepare a stirring solution of 101.9 g (135.2 mmol) 5′-*O*-(*tert*-butyldimethylsilyl)-3′-*O*-(4,4′-dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine in 300 mL pyridine, add 26.6 g (206.1 mmol) *N*,*N*-diisopropylethylamine and 41.4 g (178.7 mmol) *N*,*N*-diphenylcarbamyl chloride.

12b. Stir the dark solution for 2 hr and then concentrate on a rotary evaporator with a vacuum pump.

13b. Dissolve the residue in 600 mL CH_2Cl_2 and extract two times with 250 mL water and once with 250 mL saturated aqueous NaCl.

14b. Dry the organic solution over Na_2SO_4, vacuum filter, and concentrate to a purple-colored foam with a vacuum line on a rotary evaporator.

15b. Dissolve the crude nucleoside in 800 mL of a 9:1 (v/v) CH_2Cl_2/pyridine solution, then add 155.0 g (961.5 mmol) triethylamine trihydrofluoride and react for 16 hr at room temperature.

16b. Remove the solvents with a rotary evaporator and vacuum pump, dissolve the residue in 600 mL CH_2Cl_2, and wash two times with 250 mL water and 250 mL saturated aqueous NaCl.

17b. Dry the organic solution over Na_2SO_4, vacuum filter, and concentrate to a dark red foam with a vacuum line on a rotary evaporator.

18b. Purify the crude product by gravity on a silica gel column (*APPENDIX 3E*) preequilibrated with 1% Et_3N/50% ethyl acetate/hexane and elute with 50% to 70% ethyl acetate/hexane to afford the protected 2′-deoxyguanosine (**S.20**).

19b. Perform TLC analysis (step 16a), eluting with 75:25 (v/v) ethyl acetate/hexane.

Yield from $dG^{i\text{-}Bu}$ = 35.6% (43.3 g). R_f (75:25 ethyl acetate/hexane) = 0.38. ^1H NMR ($CDCl_3$/TMS): δ 8.00 (1H, s), 7.91 (1H, br s, exchanges with D_2O), 7.49 (2H, d, J = 7.75 Hz), 7.20-7.45 (19H, mm with 4H, d, J = 8.93 Hz at 7.38), 6.86 (4H, dd, J = 8.82, 2.09 Hz), 6.26 (1H, dd, J = 9.80, 5.12 Hz), 4.65 (1H, d, J = 5.25 Hz), 4.35 (1H, dd, J = 10.35, 3.26 Hz, exchanges with D_2O), 4.04 (1H, s), 3.80 (6H, s), 3.73 (1H, br d, J = 11.48 Hz), 3.39 (1H, br t, J = 11.54 Hz), 2.64-2.80 (2H, m), 1.68 (1H, dd, J = 13.18, 5.16 Hz), 1.24 (6H, d, J = 6.93 Hz). HRMS (FAB^+): calcd for $[M + Na]^+$, 857.3275; found, 857.3270.

Do not use 1 M tetra-n-butylammonium fluoride in THF to remove the TBDMS group because the O^6-(N,N-diphenylcarbamoyl) group is not stable to this reagent.

SYNTHESIS OF 3′-AMINONUCLEOSIDE-CONTAINING SOLID SUPPORT

SUPPORT PROTOCOL 6

Additional Materials *(also see Support Protocol 1)*

3′-Tritylamino-2′,3′-dideoxynucleosides (**S.5**, **S.9**, **S.13**, or **S.18**; see Support Protocols 1 to 4)
4-Dimethylaminopyridine
Succinic anhydride
10% (v/v) aqueous citric acid, cold
1-Hydroxybenzotriazole
1:1 (v/v) 1-methyl-2-pyrrolidinone (anhydrous)/dimethyl sulfoxide (anhydrous)
N,*N*-Diisopropylethylamine
2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Aminopropyl-conjugated controlled-pore glass (aminopropyl-CPG; Sigma)
1:1:8 (v/v/v) acetic anhydride/2,6-lutidine/THF
16.5% (v/v) *N*-methylimidazole in THF (see recipe)
Mechanical shaker

Synthesize 3′-tritylamino-2′,3′-dideoxynucleoside-5′-O-hemisuccinates

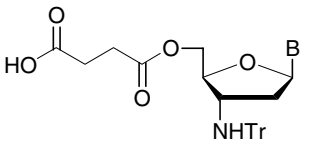
1. To a solution of 1.5 mmol of 3′-tritylamino-2′,3′-dideoxynucleoside (**S.5**, **S.9**, **S.13**, or **S.18**;) in 5 mL CH_2Cl_2, add 0.22 g (1.8 mmol) 4-dimethylaminopyridine and 0.18 g (1.8 mmol) succinic anhydride. Stir for 1 hr at room temperature.
2. Quench the reaction with 0.6 mL methanol and dilute with 30 mL CH_2Cl_2.
3. Extract once each with 20 mL cold 10% aqueous citric acid, 20 mL water, and 20 mL saturated aqueous NaCl.
4. Dry the organic layer over Na_2SO_4, vacuum filter, and concentrate the product (**S.21**; Figure 4.7.12) to a foam on a rotary evaporator using a vacuum line.

N^6-Benzoyl-3′-tritylamino-2′,3′-dideoxyadenosine-5′-O-hemisuccinate: 100% yield (1.15 g).

N^4-Benzoyl-3′-tritylamino-2′,3′-dideoxycytidine-5′-O-hemisuccinate: 76% yield (0.77 g).

3′-Tritylamino-2′,3′-deoxythymidine-5′-O-hemisuccinate: 94% yield (0.82 g).

O^6-(N,N-Diphenylcarbamoyl)-N^2-isobutyryl-3′-tritylamino-2′,3′-dideoxyguanosine-5′-O-hemisuccinate: 78% yield (1.02 g).



21a B = A^{Bz}
21c B = C^{Bz}
21g B = $G^{iBu,DPC}$
21t B = T

Figure 4.7.12 Structures of *N*-protected-3′-tritylamino-2′,3′-dideoxyribonucleoside-5′-*O*-hemisuccinates.

Conjugate 3′-tritylamino-2′,3′-dideoxynucleoside-5′-O-hemisuccinates to CPG

5. To a solution of 1 mmol 3′-tritylamino-2′,3′-dideoxynucleoside-5′-*O*-hemisuccinate and 0.13 g (0.95 mmol) of 1-hydroxybenzotriazole in 10 mL of 1:1 (v/v) 1-methyl-2-pyrrolidinone/DMSO, add 0.35 mL (2.0 mmol) *N*,*N*-diisopropylethylamine and 0.36 g (0.95 mmol) 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.
6. Stir the solution 5 min, add 10.0 g aminopropyl-CPG, and put on a shaker for 6 hr, room temperature.
7. Vacuum filter the CPG and wash successively with 20 mL each of DMF, methanol, and ethyl ether.
8. Prepare a 1:1 (v/v) mixture of 1:1:8 acetic anhydride/2,6-lutidine/THF and 16.5% *N*-methylimidazole/THF, add this mixture to the support on the funnel, and let stand for 30 min to cap any unreacted amino groups on the CPG.
9. Vacuum filter the CPG and wash successively with 20 mL each of acetonitrile, methanol, and diethyl ether.
10. Dry the CPG using a vacuum pump.

The nucleoside loadings, determined by trityl assay at 432 nm in 20% TFA/$CHCl_3$ using a molar extinction coefficient of 40.7 μmol^{-1} cm^{-1}, were 38.6 μmol/g for A, 33.6 μmol/g for C, 29.0 μmol/g for T, and 39.0 μmol/g for G.

For larger scales, use an overhead stirrer instead of a shaker. Do not use a magnetic stir bar because the CPG will be crushed.

SUPPORT PROTOCOL 7

PHOSPHORAMIDITE SYNTHESIS

The preparation of nucleoside 5′-*O*-*cis*-(2,6-dimethylpiperidinyl)-2-cyanoethylphosphoramidite monomers is described below. While either *cis*-2,6-dimethylpiperidino phosphoramidites or *N*,*N*-diisopropylamino phosphoramidites can be prepared and used for the preparation of pnODNs, the former are preferred because they allow the use of significantly lower equivalents per coupling (Fearon et al., 1998).

Additional Materials *(also see Support Protocols 1 to 5)*

3′-Tritylamino-2′,3′-dideoxynucleosides (see Support Protocols 1 to 4) *or*
3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyribonucleosides (see Support Protocol 5)
Phosphorus trichloride
3-Hydroxypropionitrile
10% (w/v) aqueous KOH
1:4 (v/v) toluene/hexane

cis-2,6-Dimethylpiperidine
1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), distilled from CaH_2 before use

Synthesize 2-cyanoethylphosphorodichloridite

1. To a solution of 500 mL (5.73 mol) phosphorus trichloride in 250 mL acetonitrile, add dropwise at room temperature, with stirring and bubbling argon, a solution of 47 mL (0.69 mol) 3-hydroxypropionitrile in 250 mL acetonitrile.
2. Stir the solution 15 min at room temperature, with absorption of evolving HCl into a solution of 10% aqueous KOH.
3. Concentrate on a rotary evaporator using a pump and vacuum filter, under argon, into a distillation flask.
4. Distill the product under reduced pressure.

The 2-cyanoethylphosphorodichloridite distills as a colorless liquid at 78° to 80°C at 1.0 mmHg. Yield = 75.7% (88.5 g). ^{31}P NMR ($CDCl_3$): δ 180.3.

Synthesize 2-cyanoethyl-cis-(2,6-dimethylpiperidinyl)chlorophosphoramidite

5. To a solution of 35.0 g (203.6 mmol) 2-cyanoethylphosphorodichloridite in 300 mL of 1:4 toluene/hexane, add 55 mL (408.1 mmol) *cis*-2,6-dimethylpiperidine dropwise, with stirring at 4°C.
6. Stir the reaction for 2 hr at room temperature.
7. Vacuum filter and wash the solid with 40 mL of 1:4 toluene/hexane under argon.
8. Concentrate the filtrate using a rotary evaporator and vacuum pump.
9. To the resultant oil, add 5 mL CH_2Cl_2 and 300 mL hexane, and crystallize the product overnight at 4°C.
10. Filter the 2-cyanoethyl-*cis*-(2,6-dimethylpiperidinyl)chlorophosphoramidite under argon.
11. Crush with a spatula, wash with 100 mL of 100:3 (v/v) hexane/CH_2Cl_2, and dry using a rotary evaporator and vacuum pump.
12. Concentrate the mother liquor (step 3) and recrystallize again (step 9) to obtain a second crop of pale yellow 2-cyanoethyl-*cis*-(2,6-dimethylpiperidinyl)chlorophosphoramidite.

Combined yield = 76.5% (38.8 g). ^{31}P NMR ($CDCl_3$): δ 172.7.

Synthesize nucleoside 5′-O-[cis-(2,6-dimethylpiperidinyl)(2-cyanoethyl)]phosphoramidite monomers (S.1 and S.2)

13. Azeotrope 10.0 mmol of 3′-tritylamino-2′,3′-dideoxynucleoside (**S.5**, **S.9**, **S.13**, or **S.18**) or 3′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyribonucleoside (**S.20**) two times from 50 mL CH_3CN.
14. Dissolve azeotroped nucleoside in 30 mL CH_2Cl_2 and then add 3.0 mL (20.0 mmol) DBU.
15. With stirring, add a solution of 3.0 g (12.0 mmol) 2-cyanoethyl-*cis*-(2,6-dimethylpiperidinyl)chlorophosphoramidite in 8 mL CH_2Cl_2, under an argon atmosphere. Stir the reaction mixture for 15 min at ambient temperature.
16. Check the reaction by TLC.

In order to obtain accurate TLC of the product, pre-wet the TLC plate by immersing it in 10% triethylamine/CH_2Cl_2, quickly let it dry, then immediately spot the sample, and elute with 5:70:25 Et_3N/ethyl acetate/hexane.

17. To avoid decomposition, desalt the crude reaction by loading the mixture directly onto a silica gel column (*APPENDIX 3E*) preequilibrated in 5% triethylamine/CH_2Cl_2 and quickly elute it in the same solvent system.

    These phosphoramidites are not stable to an aqueous workup after the reaction.

18. Remove solvents under reduced pressure and purify the crude product on a silica gel column preequilibrated with 0.5% to 5% triethylamine in 2% methanol/CH_2Cl_2. Elute as indicated below.

    N^6-*Benzoyl-3′-tritylamino-5′-O-[(cis-2,6-dimethylpiperidino)(2-cyanoethoxy)]-phosphinyl-2′,3′-dideoxyadenosine (**S.1**)*. Purify on silica (60% to 70% ethyl acetate/hexane containing 3% triethylamine). Yield = 83.1% (6.72 g). ^{31}P NMR (CD_3CN): δ 148.82, 149.16. R_f = 0.45.

    N^4-*Benzoyl-3′-tritylamino-5′-O-[(cis-2,6-dimethylpiperidino)(2-cyanoethoxy)]phosphinyl-2′,3′-dideoxycytidine (**S.1**)*. Purify on silica (70% ethyl acetate/hexane containing 3% triethylamine). Yield = 82.7% (6.50 g). ^{31}P NMR (CD_3CN): δ 149.31, 149.68. R_f = 0.43.

    O^6-*(N,N-Diphenylcarbamoyl)-N^2-isobutyryl-3′-tritylamino-5′-O-[(cis-2,6-dimethylpiperidino)(2-cyanoethoxy)]phosphinyl-2′,3′-dideoxyguanosine (**S.1**)*. Purify compound on silica (60% ethyl acetate/hexane containing 3% triethylamine). Yield = 76.9% (7.58 g). ^{31}P NMR (CD_3CN): δ 148.93, 149.50. R_f = 0.57.

    3′-*Tritylamino-5′-O-[(cis-2,6-dimethylpiperidino)(2-cyanoethoxy)]phosphinyl-2′,3′-dideoxythymidine (**S.1**)*. Purify compound on silica (50% ethyl acetate/hexane containing 3% triethylamine). Yield = 79.4% (5.52 g). ^{31}P NMR (CD_3CN): δ 149.13, 149.49. R_f = 0.60.

    N^6-*Benzoyl-3′-O-(4,4′-dimethoxytrityl)-5′-O-[(cis-2,6-dimethylpiperidino)(2-cyanoethoxy)]phosphinyl-2′-deoxyadenosine (**S.2**)*. Purify compound on silica (60% to 70% ethyl acetate/hexane containing 3% triethylamine). Yield= 76.7% (6.67 g). ^{31}P NMR (CD_3CN): δ 149.26, 149.39. R_f = 0.47.

    N^4-*Benzoyl-3′-O-(4,4′-dimethoxytrityl)-5′-O-[(cis-2,6-dimethylpiperidino)(2-cyanoethoxy)]phosphinyl-2′-deoxycytidine (**S.2**)*. Purify compound on silica (60% to 75% ethyl acetate/hexane containing 3% triethylamine). Yield = 74.4% (6.29 g). ^{31}P NMR (CD_3CN): δ 149.37, 149.76. R_f = 0.43.

    O^6-*(N,N-diphenylcarbamoyl)-N^2-isobutyryl-3′-O-(4,4′-Dimethoxytrityl)-5′-O-[(cis-2,6-dimethylpiperidino)(2-cyanoethoxy)]phosphinyl-2′-deoxyguanosine (**S.2**)*. Purify compound on silica (50% ethyl acetate/hexane containing 3% triethylamine). Yield = 71.0% (7.43 g). ^{31}P NMR (CD_3CN): δ 149.32, 149.51. R_f = 0.60.

    3′-*O-(4,4′-Dimethoxytrityl)-5′-O-[(cis-2,6-dimethylpiperidino)(2-cyanoethoxy)]phosphinyl-2′-deoxythymidine (**S.2**)*. Purify compound on silica (60% ethyl acetate/hexane containing 3% triethylamine). Yield = 74.1% (5.61 g). ^{31}P NMR (CD_3CN): δ 149.24, 149.65. R_f = 0.60.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. The acetonitrile used for all formulations must contain ≤0.001% water. Oven-dry all bottles and syringes. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffer D: 0.1 M TEAB/2% acetonitrile, pH 8

Dilute 100 mL of 1 M TEAB buffer, pH 8 (see recipe), in 880 mL water and add 20 mL acetonitrile. Check that the pH is 8.0 and correct with triethylamine or dry ice, if necessary. Filter through a 0.2-µm filter before use on the HPLC. Store up to 6 months at 4°C.

Dichloroacetic acid in CH_2Cl_2, 3% (v/v)

Dissolve 12 mL of dichloroacetic acid in 388 mL of CH_2Cl_2. Store up to 1 week at room temperature.

This solution should only be kept for about 1 week due to its potential to generate HCl, which is extremely detrimental to the pnODN synthesis. Also, only use bottles of DCA <6 months old.

5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-3′-O-[(N,N-diisopropyl-amino)(2-cyanoethoxy)]phosphinyl uridine, 0.1 M

Dissolve 86 mg of uridine phosphoramidite (Glen Research) per milliliter of acetonitrile in an oven-dried bottle under argon.

This solution can be used on the synthesizer for 1 week if kept under argon. Also remember that this monomer requires 0.5 M 1H-tetrazole in acetonitrile for activation.

cis-2,6-Dimethylpiperidinyl phosphoramidite monomer solutions, 0.1 M

Weigh solid nucleoside 5′-*O*-[*cis*-(2,6-dimethylpiperidinyl)(2-cyanoethyl)]phosphoramidite monomer (see Support Protocol 7) and transfer it to a monomer bottle. Cap the bottle with a rubber septum, use vacuum to evacuate the bottle, and refill it with argon. Using a syringe, dissolve the monomer in the appropriate amount of acetonitrile under argon. Use the following amounts of monomer per milliliter acetonitrile for 0.1 M solutions: **S.1:** 80.8 mg A; 78.4 mg C; 98.5 mg G; 69.5 mg T; **S.2:** 85.7 mg A; 83.3 mg C; 104.6 mg G; 75.6 mg T. Store monomer solutions up to 5 days at room temperature under argon.

Store the solid monomers in desiccated bags at −20°C and allow to warm up to room temperature before opening.

Dowex 50W-X8 cation-exchange resin (pyridinium H^+ form)

Suspend Dowex 50W-X8 cation-exchange resin (sulfonic acid form; Janssen Pharmaceutica) in pyridine and let cool to room temperature. Filter the resin and wash three times with methanol. Store the filtered resin in a sealed bottle until use.

H_2O_2/H_2O/pyridine/THF, 1.5:3.5:20:75 (v/v/v/v)

Dissolve 5 mL of 30% aqueous H_2O_2 (v/v; Aldrich) in 20 mL pyridine and 75 mL tetrahydrofuran.

The water in the formulation comes from the aqueous hydrogen peroxide solution. This solution is stable for 3 weeks on the synthesizer, but should be disposed of within a few days of removing it from the synthesizer due to its potential to form explosive peroxides.

Isobutyric anhydride/2,6-lutidine/THF, 1:1:8 (v/v/v)

Dissolve 20 mL isobutyric anhydride and 20 mL of 2,6-lutidine in 160 mL anhydrous tetrahydrofuran under argon.

This solution is stable on the instrument for 2 months as long as it is kept dry and under argon.

N-Methylimidazole in THF, 16.5% (v/v)

This solution can be bought directly from PE Biosystems or made by dissolving 33 mL of *N*-methylimidazole in 167 mL anhydrous tetrahydrofuran.

This solution is stable on the instrument for 2 months as long as it is kept dry and under argon.

Pyridine in CH_3CN, 20% (v/v)

Dissolve 40 mL pyridine, previously dried over chunks of CaH_2 for at least 24 hr, in 160 mL acetonitrile under argon.

This formulation must be kept dry in order to prevent hydrolysis of the internucleotide phosphoramidite during synthesis; it can be kept on the instrument for 1 month under argon.

Tetrazole in CH_3CN, 0.167 M

Under argon, add 50 mL of a 0.5 M solution of tetrazole in acetonitrile (PE Biosystems) to 100 mL acetonitrile.

This solution is stable on the instrument for 2 months as long as it is kept dry and under argon.

Triethylammonium bicarbonate (TEAB) buffer, 1 M, pH 8

Add 139 mL of triethylamine to ~800 mL of water. In a hood, slowly add dry ice to the stirring solution until the pH measures 8.0. Add enough water to make the final volume 1 L. Correct the pH to 8.0 with either triethylamine or dry ice, if necessary. Store the solution up to 6 months at 4°C and check the pH before use.

Triethylamine will be immiscible in water at first. The solution gets very cold and CO_2 is given off during the dissolution of dry ice.

COMMENTARY

Background Information

Oligonucleotides are widely used in research and diagnostics and are rapidly gaining acceptance as potential therapeutic agents. Antisense oligonucleotides, which are complementary to selected sequences of mRNA associated with a disease, control gene expression either by sterically blocking translation or processing of the RNA or by the irreversible cleavage of the target RNA by endogenous RNase H (Uhlman and Peyman, 1990). Alternatively, antigene therapeutics are oligonucleotides that prevent gene transcription by forming a triple-helical structure with targeted polypurine:polypyrimidine sites in double-stranded (ds) DNA (Thuong and Hélène, 1993). Many oligonucleotide analogs have been investigated in an attempt to improve properties such as resistance to nuclease degradation, binding strength to RNA and/or dsDNA, cellular uptake, and pharmacokinetic parameters (Uhlman and Peyman, 1990). Unfortunately, phosphorothioates, and the majority of other first-generation analogs that possess increased resistance to nuclease degradation, have a decreased binding affinity for their RNA or dsDNA targets. Additionally, phosphorothioate ODNs may exhibit nonspecific effects, presumably due to adventitious protein binding (Stein and Cheng, 1993). The fully modified oligonucleotide N3′→P5′ phosphoramidates (pnODNs), on the other hand, possess an increased stability to nucleases relative to both phosphodiester and phosphorothioate ODNs, the ability to tightly and sequence-specifically bind to both RNA and dsDNA, and have shown minimal sequence-independent protein binding (Gryaznov and Chen, 1994; Chen et al., 1995; Gryaznov et al., 1995). These improved properties have translated well in both cell culture and in vivo antisense studies where pnODNs have demonstrated efficacy at 10% of the comparably effective dose of phosphorothioate ODNs (Gryaznov et al., 1996; Heidenreich et al., 1997; Skorski et al., 1997). In addition, a pnODN sequence showed no toxicity in preliminary in vivo studies in mice up to dosages of 150 mg/kg administered intravenously six times over a period of two weeks, whereas kidney and liver toxicity were seen for the isosequential phosphorothioate ODN at the same (and lower) dose (Zon, pers. comm.).

Consistent with most oligonucleotide analogs other than phosphorothioates, uniformly

modified pnODNs do not activate RNase H (DeDionisio and Gryaznov, 1995; Heidenreich et al., 1997). However, in many systems, RNase H activity has been necessary in order to obtain an "antisense" effect and is generally thought to increase the activity of such compounds. Although pnODNs have demonstrated significant RNase H–independent activity, the efficacy of chimeric ODNs that possess an RNase H-active core of 6 to 8 phosphorothioate linkages flanked by pnODN linkages is under investigation.

The significantly increased and sequence-specific binding affinity of uniformly modified pnODNs for dsDNA renders these "antigene" compounds potentially useful for inhibition of gene expression via triplex helix formation. Hélène and coworkers (Escudé et al., 1996; Giovannangeli et al., 1997) have demonstrated that pnODNs work best when they are designed to form triple-helical DNA by Hoogsteen hydrogen bonding to the duplex with the third strand in a parallel orientation with respect to the polypurine target sequence. Using a eukaryotic transcription assay, they showed that a pnODN can arrest transcription at a specific triplex site in the type 1 human immunodeficiency virus *nef* gene under the control of a cytomegalovirus promoter.

The synthetic method described herein (McCurdy et al., 1997; Nelson et al., 1997; Fearon et al., 1998), based on a phosphoramidite amine-exchange reaction, is 3 to 6 times more efficient than the oxidative phosphorylation-based method previously reported by Gryzanov and Chen (1994) and Chen et al. (1995). An advantage of the described method is that the monomer is activated and used in excess; thus, a small amount of inadvertent hydrolysis only wastes monomer and does not necessarily decrease the step yield of the coupling. Also, because the method is based on phosphoramidite chemistry, the synthesis of chimeras possessing any combination of phosphoramidate, phosphodiester, and/or phosphorothioate linkages at predefined positions is easily performed on a commercially available synthesizer with no instrument modifications. Fluorescent and other specialty phosphoramidites are also easily used in conjunction with this chemistry.

This amine-exchange method was modified for use on a vortexing-mode synthesizer (e.g., PE Biosystems 390Z; 10 µmol-scale) and only ≤3.6 eq of the *cis*-2,6-dimethylpiperidinyl phosphoramidite monomers and 9 eq of 1*H*-tetrazole were necessary to achieve optimal coupling yields (Fearon et al., 1998). Even lower stoichiometric requirements may be possible using an activator other than 1*H*-tetrazole.

Compound Characterization

Analytical IEC and/or capillary gel electrophoresis (CGE) is generally used to determine the chain-length purity of pnODNs and their chimeras (see Fig. 4.7.13). CGE was performed on a Beckman P/ACE 5510 system with 10% Microgel Capillaries (0.1 × 500 mm) in 35 mM Tris-borate buffer, pH 9.0, in the presence of 15% ethylene glycol, with a 5 sec injection at 10 kV and a running voltage of 25 kV. Alternatively, 20% polyacrylamide gel electrophoresis followed by visualization with Stains-All and densitometric scanning can also be used. If further characterization is desired, electrospray ionization mass spectrometry can be used to determine the molecular weight of the oligonucleotide synthesized; however, sodium counterions must first be exchanged for ammonium ions (Stultz and Marsters, 1991). In the case of chimeras, the number of phosphoramidate, phosphodiester, and/or phosphorothioate linkages can be determined by ^{31}P-NMR spectroscopy in D_2O.

Critical Parameters and Troubleshooting

The phosphoramidite monomer, 1*H*-tetrazole, and 20% pyridine in acetonitrile solutions all must be kept rigorously anhydrous; the method is extremely sensitive to water. Additionally, the 5′-phosphoramidite monomers must have a purity of ≥99% by ^{31}P-NMR spectroscopy in order to afford optimal coupling efficiency. The 2-cyanoethyl-protected phosphoramidate linkages are reasonably stable to acidic conditions; however, older formulations of 3% dichloroacetic acid in CH_2Cl_2 can degrade the protected phosphoramidate linkages and cause an increase in failure sequences. In general, only enough 3% dichloroacetic acid solution is formulated for a one week period. For the same reason, bottles of dichloroacetic acid from the vendor are generally used or discarded within 6 months of purchase.

RP-HPLC using either the 3′-terminal tritylamino group or 3′-terminal *O*-DMTr group alone must be avoided because the use of acetic acid during post-HPLC detritylation severely degrades the pnODN. For this reason, the DMTr uridine incorporation method should be used for RP-HPLC purification and subsequent deprotection or IEC of the "trityl-off" pnODN should be performed. Do not add any base other

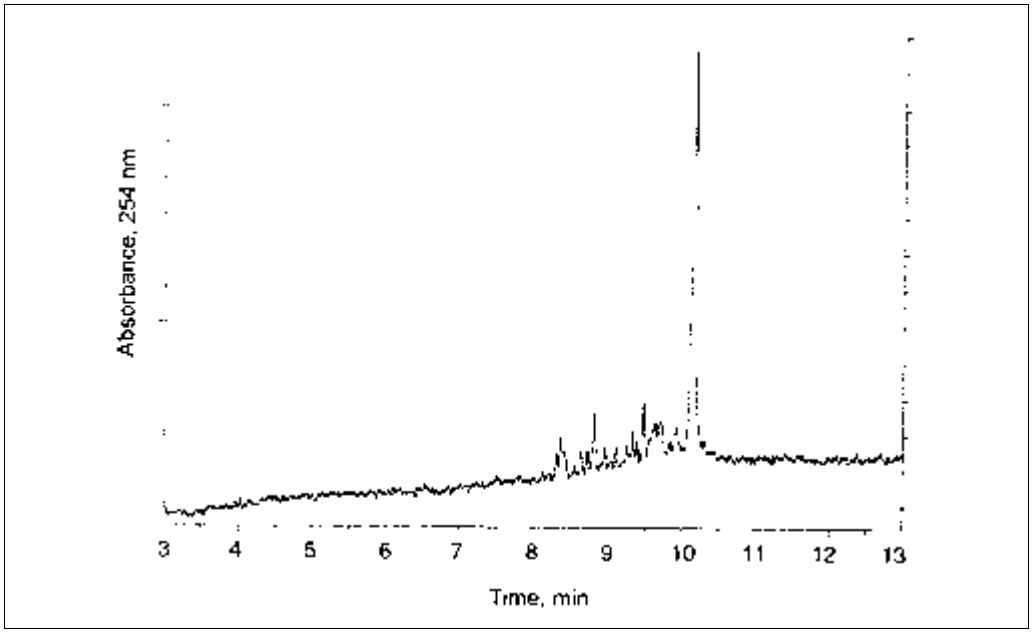


Figure 4.7.13 Analytical CGE of a crude pnODN with the sequence 5′-ATGACTGAGTACAAAC$_{OH}$-3′.

than concentrated aqueous ammonia to the uridine-containing pnODNs to prevent premature cleavage of the phosphodiester function. It is best to remove the ammonia and RP-HPLC purify the pnODN as soon as possible.

The uridine RP-HPLC purification method should be used for pnODN/phosphorothioate ODN chimeras because the phosphorothioate portion of the molecule greatly reduces the resolution of the IEC. Additionally, avoid exposure of these chimeras to aqueous NaOH solutions, which can result in small amounts of inadvertent conversion of the phosphorothioate linkages to phosphodiester linkages. Also avoid concentrating the uridine-containing oligonucleotide to dryness before purification.

Isolated pnODNs and their chimeras should be stored frozen (–20°C) as lyophilized powders or aqueous solutions in deionized water or buffer at pH ≥ 7.4. During purification and analysis, working solutions of pnODNs should be stored at 4°C. If the pH of the solution is <7.0, there is potential for the pnODN to degrade.

Anticipated Results

The isolated yield of pnODNs and their chimeras depends on both length, sequence, and number of phosphoramidate, phosphorothioate, and phosphodiester linkages, but is generally 15 to 30 OD_{260} units for pnODNs and 20 to 35 OD_{260} units for chimeras 12 to 25 nt in length with a purity of ≥85%. Occasionally, certain sequences require a second IEC purification after RP-HPLC to obtain purities ≥85%. Figure 4.7.14 shows a representative IEC and CE for the purified pnODN with the sequence 5′-CCCTCCTCCGGAGCC$_{OH}$-3′. Table 4.7.1 shows the yields and purities of several pnODNs and chimeras synthesized by this method.

Time Considerations

The formulation of reagents and set-up of the synthesizer generally take about 3 to 4 hr. The cycle time to introduce each nucleotide is 22 min, which is multiplied by the length of the oligonucleotide being synthesized to determine the overall synthesis time. Deprotection is usually performed overnight in an oven equipped with a timer set to turn off after 8 to 12 hr. Analysis of the quality of the synthesis, sample preparation, and either IEC or RP-HPLC purification take about 3 hr per sample; however for IEC purification, the analysis of the resulting fractions can take an additional 6 to 10 hr. For RP-HPLC, there is an additional 16 hr treatment with fluoride and base to cleave the uridine phosphodiester linkage. The work-up and analysis steps take another 4 to 8 hr, depending on the method used for desalting. Thus, a sample can be synthesized, purified, worked-up, and analyzed in 4 to 5 days. Using the advantages of automation and a multi-column

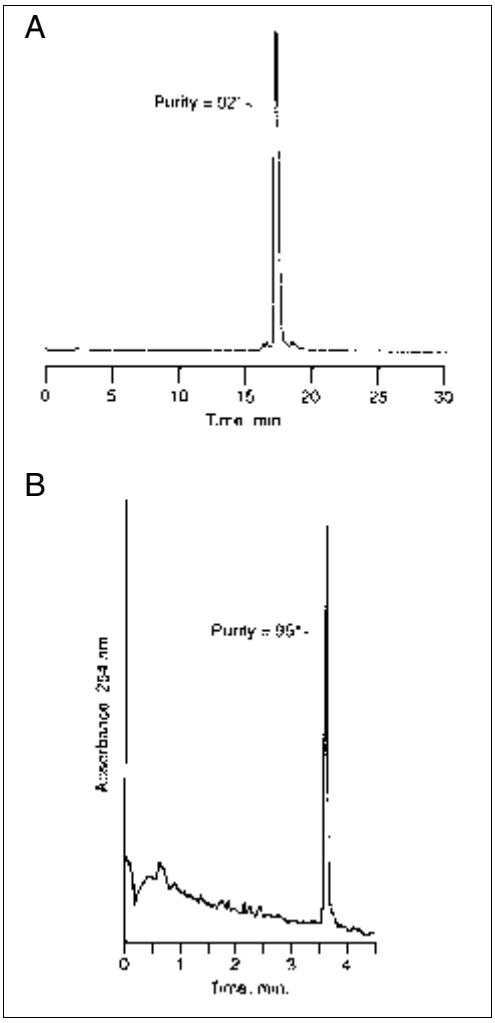


Figure 4.7.14 (**A**) Analytical IEC chromatogram and (**B**) CGE electropherogram of the RP-HPLC purified and deprotected phosphoramidate oligonucleotide, 5′-CCCTCCTCCGGAGCC$_{OH}$-3′.

instrument, the rather long turn-around time can be overcome by multiplexing the synthesis and purification steps. Generally, about 3 to 4 pnODNs can be synthesized and purified per week by one chemist, assuming an autosampler is utilized for fraction analysis.

The synthesis of the monomers is a challenging task that will take a skilled organic chemist approximately 2 to 3 months for the four 3′-tritylamino nucleoside monomers and 1 month for the four 3′-*O*-DMTr nucleoside monomers. Alternatively, the 3′-*O*-DMTr-nucleoside-5′-*O*-(*N*,*N*,-diisopropylamino)phosphoramidite monomers and the 3′-tritylamino-nucleoside-5′-*O*-(*N*,*N*-diisopropylamino)phosphoramidite monomers are available from Glen Research and Annovis, respectively, and can be used in place of the *cis*-(2,6-dimethylpiperidino) phosphoramidite monomers, as long as the couple-oxidize-couple-oxidize protocol is used for the couplings to 3′-amines. These monomers also require 0.5 M 1*H*-tetrazole in acetonitrile for activation.

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Table 4.7.1 Yields and Purities of Phosphoramidate Oligonucleotides and Chimera Synthesized by the Amine-Exchange Method

Sequence[a]	Purification method	OD_{260} by CGE	% Purity
$CCCTCCTCCGGAGCC_{OH}$	RP-HPLC	20	95%
$AGAGATTTTTACACC_{OH}$	IEC	29	98%
$CCAGAGTCACACAACA_{OH}$	IEC	15	93%
$CAGATCGTCCATGGTC_{OH}$	IEC	17	99+%
$CAGATpCpGpTpCpCpApTGGTC_{OH}$	IEC	28	85%
$GGACCsCsTsCsCsTsCsCsGGAGCC_{OH}$	RP-HPLC	26	97%
$TTGCCCACAsCsCsGsAsCsGsGsCGCCCACCA_{FAM}$	RP-HPLC	29	70%[b]

[a]The sequences are reported in the 5′→3′ direction. The linkages are phosphoramidate unless noted by "s" or "p," which are phosphorothioate or phosphodiester linkages, respectively. CGE, capillary gel electrophoresis; FAM, fluorescein; IEC, ion-exchange chromatography; RP-HPLC, reversed-phase high-pressure liquid chromatography.

[b]Polyacrylamide gel electrophoresis of this compound, followed by staining with Stains-All and densitometric scanning, indicated a purity of 90%.

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Key References

Nelson et al., 1997. See above.

This reference details the couple-oxidize-couple-oxidize amine-exchange method for synthesis of pnODNs, as well as the synthesis of the 3′-trityl-amino protected nucleosides.

Fearon et al., 1998. See above.

This reference describes the optimized amine-exchange method for the synthesis of pnODNs and the synthesis of the 3′-O-DMTr-protected nucleosides and the cis-2,6-dimethylpiperidinyl phosphoramidite monomers.

It also demonstrates the synthesis of pnODNs using low equivalents of monomer and 1H-tetrazole and describes the equilibrium and oxidation steps in detail.

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