Deoxyribo- and Ribonucleoside *H*-Phosphonates

This unit includes a collection of four synthetic methods for nucleoside *H*-phosphonate building blocks and a variant on one of these syntheses. All the methods are quite efficient and experimentally simple, and they use readily available reagents. Depending on reactivity and reaction conditions, different methods may be preferred for preparation of different building blocks.

The phosphonylation of protected nucleosides with the in situ–generated tris-(1-imidazolyl)phosphine (from phosphorus trichloride and imidazole), followed by hydrolysis of the formed nucleoside diimidazolyl phosphite intermediate, is described in Basic Protocol 1. This method (or its variants with other azoles) is probably the most widely used and can be recommended as a general basic method applicable for the preparation of both deoxyribo- and ribonucleoside 3′-*H*-phosphonates. Imidazole from the first published procedure (Garegg et al., 1986c) is preferred over the other azoles (e.g., triazole; Froehler et al., 1986) because it is less expensive and forms the least reactive species. Since tris-(1-imidazolyl)phosphine is a trifunctional reagent, it should be used in excess in the phosphonylation reaction to avoid the formation of symmetrical *H*-phosphonate diesters. There are several variants of this protocol that differ in the kind of azoles, external bases, or solvents used, including procedures for ribonucleoside building blocks that combine 2′-O-protection and 3′-O-phosphonylation. The Alternate Protocol describes one of these procedures (also see Commentary).

In Basic Protocol 2, pyridinium *H*-pyrophosphonate, generated in situ from phosphorous acid and a condensing agent, is used. This one-step reaction is most likely carried out by a nucleophilic attack of nucleoside on a phosphorus center of *H*-pyrophosphonate. Usually 5 mol eq in excess of the nucleoside is used to speed up the reaction. The chemical nature of a condensing agent is of minor importance for the final yield of the *H*-phosphonate monoesters. Phosphonylation of ribonucleosides protected with 2′-*O*-*tert*-butyldimethylsilyl (2′-*O*-TBDMS) occurs very slowly with this reagent, which makes it less suitable for use in preparation of ribonucleoside building blocks.

Basic Protocol 3 describes a convenient approach for the preparation of nucleoside *H*-phosphonate monoesters based on transesterification of diphenyl *H*-phosphonate with protected nucleosides. Diphenyl *H*-phosphonate is relatively inexpensive, commercially available, stable, and easy to handle. It also gives high yields of nucleoside *H*-phosphonates. This is the newest of the presented methods and thus the least used, but some clear advantages of the method could make it a generally preferred choice. It should, however, be noted that synthesis of ribonucleoside building blocks with this method requires more basic work-up conditions, which may prevent its use with N-protections that are quite base labile.

Basic Protocol 4 makes use of 2-chloro-4*H*-1,3,2-benzo-dioxaphosphinan-4-one (salicylchlorophosphite) as the phosphonylating reagent. The reaction with nucleosides is usually fast and quantitatively produces a phosphite intermediate that is converted into the *H*-phosphonate monoester upon hydrolysis. Since salicylchlorophosphite is practically a monofunctional phosphonylating agent, it can be used in nearly stoichiometric amounts. The formation of symmetrical dinucleoside *H*-phosphonate diesters is usually negligible, but removal of byproducts from the reagent can sometimes be a problem.

Contributed by Jacek Stawinski and Roger Strömberg

Current Protocols in Nucleic Acid Chemistry (2001) 2.6.1-2.6.15

NOTE: These reactions should be carried out under strictly anhydrous conditions, using anhydrous solvents and reagents. All glassware should be dried in an oven prior to use. The nucleosidic component should be rendered anhydrous by consecutive evaporation of added pyridine or acetonitrile depending on solvent used for the reaction. All connections to atmospheric pressure should be through a drying tower containing a desiccant.

BASIC PROTOCOL 1

SYNTHESIS OF PROTECTED DEOXYRIBONUCLEOSIDE OR RIBONUCLEOSIDE 3′-*H*-PHOSPHONATES USING THE PHOSPHORUS TRICHLORIDE/IMIDAZOLE/TRIETHYLAMINE REAGENT

This is a general-purpose protocol, applicable to both ribo- and deoxyribonucleosides bearing standard protecting groups. This reaction is shown in Figure 2.6.1.

Materials

Imidazole, made anhydrous by repeated evaporation of added dry acetonitrile
Dichloromethane (99.9+%; HPLC grade), stored over 3A molecular sieves
Acetone (for dry ice/acetone bath)
Phosphorus trichloride, freshly distilled
Triethylamine, distilled, stored over calcium hydride
Protected deoxyribo- or ribonucleoside (see Chapter 2), dried by repeated evaporation of added pyridine
9:1 (v/v) chloroform/methanol
2 M TEAB, pH 7.5 (see recipe)
Sodium sulfate
5 × 25–cm glass chromatography column, packed with Merck silica gel 60, 230 to 400 mesh ASTM (e.g., EM Science) or matrix silica, 35 to 70 μm (Millipore) in chloroform (bed height, 15 to 20 cm)
Methanol
Chloroform
0.5:1:8.5 or 1:2:7 (v/v/v) concentrated ammonia/water/isopropanol
1:1 (v/v) hexane/diethyl ether

TLC silica-gel plates (e.g., Merck silica gel 60 F254, EM Science)
Rotary evaporator connected to water aspirator

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

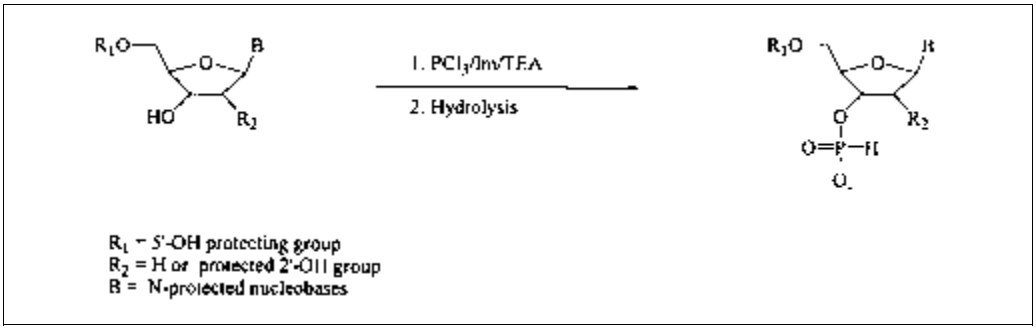

Figure 2.6.1 Synthesis of nucleoside 3′-*H*-phosphonates using PCl_3/Im/TEA reagent system.

Prepare phosphorus trichloride/imidazole/triethylamine reagent

1. Dissolve 5.8 g imidazole (86 mmol) in 300 mL dichloromethane and cool solution to approximately −10°C in a dry ice/acetone bath.

   Dry acetonitrile and tetrahydrofuran can be used as alternative solvents, but with dichloromethane the work-up procedure is more convenient.

2. While stirring, add dropwise 2.45 mL phosphorus trichloride (28 mmol) and then 12.5 mL triethylamine (90 mmol) mixed with 10 mL dichloromethane. Stir 15 to 30 min at 0° to −10°C and then cool to −78°C in dry ice/acetone bath.

Prepare 3′-H-phosphonates

3. Add a protected deoxyribo- or ribonucleoside (8 mmol) dissolved in 200 mL dichloromethane dropwise over ~30 min while stirring at −78°C. Stir 1 hr at −78°C.

   Steps 1 to 3 can be somewhat simplified by cooling the mixture with an ice/water bath instead; however, this is not recommended for guanosine and deoxyguanosine derivatives because substantial amounts of side products, and thus a lower yield, can be produced following subsequent reactions (presumably with the lactam system).

4. Monitor reactions for disappearance of the starting nucleoside and the formation of a baseline material by TLC (*APPENDIX 3D*) on silica-gel plates using 9:1 chloroform/methanol as the solvent.

5. When the reaction is complete (as determined by TLC analysis), pour the cold mixture directly onto 300 mL of 2 M TEAB and wash the organic layer with an additional 300 mL of 2 M TEAB.

   With acetonitrile as solvent, 20 mL of 0.5 M TEAB should be added to the cold mixture. The mixture is then concentrated under reduced pressure (at 30° to 40°C) and the residue is dissolved in 300 mL dichloromethane and washed twice with 150 mL of 2 M TEAB.

6. Separate the organic layer, dry it over ~20 g sodium sulfate, and evaporate solvent under reduced pressure using a rotary evaporator connected to a water aspirator.

Purify 3′-H-phosphonates

7. Purify crude product by short-column silica-gel chromatography (*APPENDIX 3E*) on a 5 × 25–cm glass chromatography column using a stepwise gradient of methanol in chloroform to elute products.

   For example, a stepwise gradient of 0% to 12% methanol in chloroform containing 0.1% triethylamine can be used.

   Unwanted isomers of the ribonucleoside H-phosphonates, that is 3′-TBDMS (or 2-ClBz) 2′-H-phosphonates, if present, are readily removed by silica-gel chromatography.

8. Check eluate fractions for *H*-phosphonate purity by TLC using 0.5:1:8.5 or 1:2:7 (v/v/v) concentrated ammonia/water/isopropanol as the solvent.

   The polarity, and thus the mobility on TLC, of an H-phosphonate will be affected by its protecting group. The latter solvent is probably better for more polar compounds.

   For compounds bearing more base-labile N-protection, the use of 3:7 or 2:8 (v/v) isopropanol/1 M TEAB can be advantageous.

9. Combine fractions containing the desired product and concentrate them to a white foam using the rotary evaporator. Store at <4°C.

   A yield of 70% to 95% 3′-H-phosphonate monoesters (triethylammonium salts), based on the staring quantity of nucleoside, is expected.

Occasionally, DBUH$^+$ salts of H-phosphonate monoesters can be preferred to TEAH$^+$ salts. The transformation can be effected by washing a solution of nucleoside H-phosphonate monoester (triethylammonium salt) in dichloromethane with 0.2 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) bicarbonate buffer (pH 8.5).

10. *Optional:* Dissolve foam in a small amount of dichloromethane and add it dropwise while stirring vigorously to 100 to 150 mL of 1:1 hexane/diethyl ether per mmol product. Store at <4°C.

The nucleoside H-phosphonate is stored as a microcrystalline solid. Although stable for several months at room temperature, it should be stored in the freezer to prevent decomposition due to residual solvents. Under such conditions, no decomposition is seen even after several months.

ALTERNATE PROTOCOL

SYNTHESIS OF RIBONUCLEOSIDE 3′-*H*-PHOSPHONATES VIA IN SITU 2′-*O*-2-CHLOROBENZOYLATION FOLLOWED BY PHOSPHONYLATION

This procedure is specific for ribonucleosides bearing a 2′-*O*-chlorobenzoyl group and permits simultaneous 2′-O-benzoylation and 3′-O-*H*-phosphorylation as a "one pot" reaction.

Additional Materials *(also see Basic Protocol 1)*

Protected ribonucleoside with free 2′- and 3′-hydroxyls (see Chapter 2), dried by repeated evaporation of added pyridine
Pyridine (HPLC grade; e.g., LabScan), dried by and stored over 4A molecular sieves
2-Chlorobenzoyl chloride

1. Dissolve a protected ribonucleoside with free 2′- and 3′-hydroxyls (8 mmol) in 95 mL dichloromethane and add 5 mL pyridine. Cool to −78°C in a dry ice/acetone bath while stirring.
2. Mix 1.18 mL 2-chlorobenzoyl chloride (8.8 mmol) and 10 mL dichloromethane, and add dropwise over a 15-min period. Incubate 30 min at −78°C with stirring.
3. Prepare phosphorus trichloride/imidazole/triethylamine reagent as described (see Basic Protocol 1, steps 1 and 2).
4. Add phosphorus trichloride/imidazole/triethylamine reagent dropwise over 15 to 30 min to nucleoside mixture while stirring at −78°C. Stir 1 hr at −78°C.
5. Continue with synthesis and purification as described (see Basic Protocol 1, steps 4 to 10).

A yield of 40% to 70%, based on the starting quantity of nucleoside, is expected.

BASIC PROTOCOL 2

SYNTHESIS OF PROTECTED DEOXYRIBONUCLEOSIDE 3′-*H*-PHOSPHONATES USING *H*-PYROPHOSPHONATE

This procedure is suitable for phosphorylation of hydroxyl groups in deoxyribonucleosides or 5′-hydroxyl groups in ribonucleosides. This reaction is shown in Figure 2.6.2.

Materials

Protected deoxyribonucleoside (see Chapter 2), dried by repeated evaporation of added pyridine
2 M phosphorous acid (H_3PO_3) solution (see recipe)

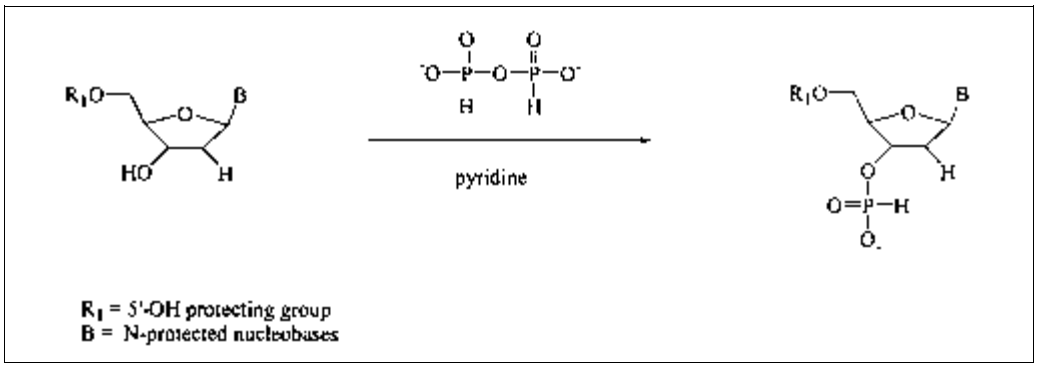

Figure 2.6.2 Synthesis of nucleoside 3′-*H*-phosphonates using *H*-pyrophosphonate.

Condensing agent (select one):
5,5-Dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane (NEPCl; see recipe)
Pivaloyl chloride (PV·Cl), commercial grade (e.g., Aldrich), distilled before use (store <2 months at −20°C)
9:1 (v/v) chloroform/methanol
1 M and 2 M TEAB, pH 7.5 (see recipe)
Dichloromethane (HPLC grade), stored over 3A molecular sieves
Sodium sulfate
4 × 25–cm glass chromatography column, packed with Merck silica gel 60, 230 to 400 mesh ASTM (e.g., EM Science) or Matrix silica, 35 to 70 µm (Millipore) in chloroform (bed height, 10 to 15 cm)
Methanol
Chloroform
0.5:1:8.5 or 1:2:7 (v/v/v) concentrated ammonia/water/isopropanol
1:1 (v/v) hexane/diethyl ether

TLC silica-gel plates (e.g., Merck silica gel 60 F254, EM Science)
Rotary evaporator connected to water aspirator

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

1. Dissolve protected deoxyribonucleoside (2 mmol) in 10 mL of 2 M H_3PO_3 solution (20 mmol).
2. Stir reaction mixture and slowly add 2.0 g NEPCl (11 mmol) or 1.35 mL PV·Cl (11 mmol). Stir ~3 hr.

   The reaction can be left overnight at 20°C (room temperature).

   The specified amount of a condensing agent should not be exceeded, otherwise more reactive species can be generated from phosphorous acid, which may result in a significant formation of symmetrical H-phosphonate diesters.

   Instead of generating H-pyrophosphonate in situ from phosphorous acid and a condensing agent, a 1 M stock solution of this phosphonylating reagent can be prepared by adding 0.5 mol eq PV·Cl or NEPCl to 2 M H_3PO_3 in pyridine. The stock solution of pyridinium H-pyrophosphonate in pyridine is stable for several months at room temperature.
3. Monitor reactions for disappearance of the starting nucleoside and the formation of a baseline material by TLC (*APPENDIX 3D*) on silica-gel plates using 9:1 chloroform/methanol as the solvent.

4. When the reaction is complete (as determined by TLC analysis), add 2 mL of 1 M TEAB and partition reaction mixture between 75 mL dichloromethane and 50 mL of 2 M TEAB.

5. Separate the organic layer, dry it over ~10 g sodium sulfate, and evaporate solvent under reduced pressure using a rotary evaporator connected to a water aspirator.

6. Purify crude product by short-column silica-gel chromatography (*APPENDIX 3E*) on a 4 × 25–cm glass chromatography column using a stepwise gradient of methanol in chloroform to elute products.

   For example, a stepwise gradient of 0% to 12% methanol in chloroform containing 0.1% triethylamine can be used.

   Unwanted isomers of the ribonucleoside H-phosphonates, that is 3′-TBDMS (or 2-ClBz) 2′-H-phosphonates, if present, are readily removed by silica-gel chromatography.

7. Check eluate fractions for *H*-phosphonate purity by TLC using 0.5:1:8.5 or 1:2:7 (v/v/v) concentrated ammonia/water/isopropanol as the solvents.

   The polarity, and thus the mobility on TLC, of an H-phosphonate will be affected by its protecting group. The latter solvent is probably better for more polar compounds.

   For compounds bearing more base-labile N-protection, the use of 3:7 or 2:8 (v/v) isopropanol/1M TEAB can be advantageous.

8. Combine fractions containing the desired product and concentrate them to a white foam using the rotary evaporator. Store at <4°C.

   A yield of 85% to 95% 3′-H-phosphonate monoesters (triethylammonium salts), based on the starting quantity of nucleoside, is expected.

   Occasionally, $DBUH^+$ salts of H-phosphonate monoesters can be preferred to $TEAH^+$ salts. The transformation can be effected by washing a solution of nucleoside H-phosphonate monoester (triethylammonium salt) in dichloromethane with 0.2 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) bicarbonate buffer (pH 8.5).

   When NEPCl is used to generate H-pyrophosphonate from phosphorous acid, the isolated nucleoside H-phosphonate monoesters can be occasionally contaminated by hydrolysis products of the condensing agent. If this is the case (as shown by ^{31}P NMR), the product should be dissolved in 20 mL dichloromethane and washed twice with 5 mL of 0.05 M TEAB buffer.

9. *Optional:* Dissolve the foam in a small amount of dichloromethane and add it dropwise while stirring vigorously to 100 to 150 mL of 1:1 hexane/diethyl ether per mmol product. Store at <4°C.

   The nucleoside H-phosphonate is stored as a microcrystalline solid. Although stable for several months at room temperature, it should be stored in the freezer to prevent decomposition due to residual solvents. Under such conditions, no decomposition is seen even after several months.

BASIC PROTOCOL 3

SYNTHESIS OF PROTECTED NUCLEOSIDE 3′-*H*-PHOSPHONATES USING DIPHENYL *H*-PHOSPHONATE

This is a general-purpose procedure, applicable to both ribo- and deoxyribonucleosides. One should exercise some caution when base-sensitive protecting groups are present in the substrates (see Commentary). This reaction is shown in Figure 2.6.3.

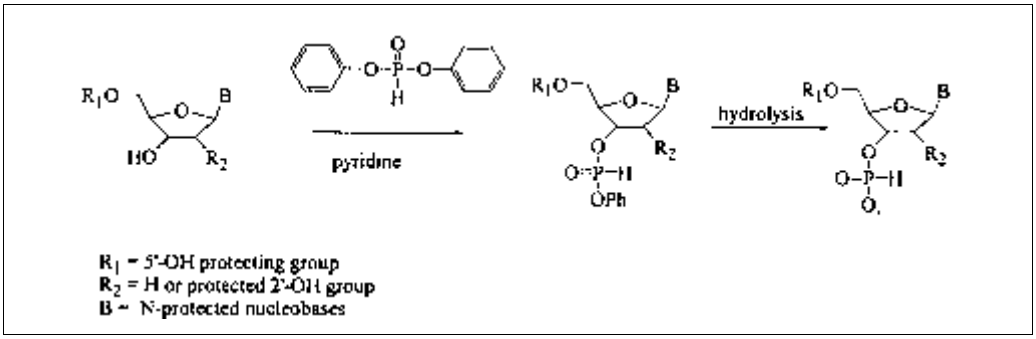

Figure 2.6.3 Synthesis of nucleoside 3′-*H*-phosphonates using diphenyl *H*-phosphonate.

Materials

Protected deoxyribo- or ribonucleoside (see Chapter 2), dried by repeated evaporation of added pyridine
Pyridine (HPLC grade; e.g., Labscan), dried by and stored over 4A molecular sieves
Diphenyl *H*-phosphonate, commercial grade (e.g., Aldrich)
9:1 (v/v) chloroform/methanol
Triethylamine, distilled, stored over calcium hydride
5% (w/v) sodium bicarbonate
Dichloromethane, distilled
Sodium sulfate
4 × 25–cm glass chromatography column, packed with Merck silica gel 60, 230 to 400 mesh ASTM (e.g., EM Science) or Matrix chloroform (bed height, 10 to 15 cm)
Methanol
Chloroform
0.5:1:8.5 or 1:2:7 (v/v/v) concentrated ammonia/water/isopropanol
1:1 (v/v) hexane/diethyl ether

TLC silica-gel plates (e.g., Merck silica gel 60 F254, EM Science)
Rotary evaporator connected to water aspirator

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

1. Add a protected deoxyribo- or ribonucleoside (1 mmol) to 5 mL pyridine and stir. Add 1.34 mL diphenyl *H*-phosphonate (7 mmol) in one portion. Stir ~15 min.

   An excess of phosphonylating agents is necessary to avoid the formation of symmetrical dinucleoside H-phosphonate diesters.

   Diphenyl H-phosphonate may be listed by some suppliers as diphenyl phosphite.

   In the instance of 2′-O-protected ribonucleosides, the amount of diphenyl H-phosphonate can be reduced to 3 mol eq.

2. Monitor reactions for disappearance of the starting nucleoside and the formation of a baseline material by TLC (*APPENDIX 3D*) on silica-gel plates using 9:1 chloroform/methanol as the solvent.

3. When the reaction is complete (as determined by TLC analysis), add 1 mL water and 1 mL triethylamine to quench the reaction, and let mixture stand 15 min.

4. Use a rotary evaporator connected to a water aspirator to evaporate solvents, and partition the residue between 20 mL dichloromethane and 20 mL of 5% sodium bicarbonate.

5. Extract organic phase twice with 20 mL of 5% sodium bicarbonate, dry it over ~10 g sodium sulfate, and evaporate the solvent to produce an oil.

   Most phenol and phenyl H-phosphonates formed during the reaction and from hydrolysis of diphenyl H-phosphonate are removed by the aqueous sodium bicarbonate extraction.

   Crude reaction products (after extraction with aqueous sodium bicarbonate) can be precipitated from dichloromethane into 1:1 (v/v) hexane/diethyl ether to produce nucleoside H-phosphonates of >95% purity, without chromatography.

6. Purify crude product by short-column silica-gel chromatography (*APPENDIX 3E*) on a 4 × 25–cm glass chromatography column using a stepwise gradient of methanol in chloroform to elute products.

   For example, a stepwise gradient of 0% to 12% methanol in chloroform containing 0.1% triethylamine can be used.

   Unwanted isomers of the ribonucleoside H-phosphonates, that is 3′-TBDMS (or 2-ClBz) 2′-H-phosphonates, if present, are readily removed by silica-gel chromatography.

7. Check eluate fractions for *H*-phosphonate purity by TLC using 0.5:1:8.5 or 1:2:7 concentrated ammonia/water/isopropanol.

   The polarity, and thus the mobility on TLC, of an H-phosphonate will be affected by its protecting group. The latter solvent is probably better for more polar compounds.

   For compounds bearing more base-labile N-protection, the use of 3:7 or 2:8 (v/v) isopropanol/1M TEAB can be advantageous.

8. Combine fractions containing the desired product and concentrate them in the rotary evaporator to a white foam. Store at <4°C.

   A yield of 75% to 90% 3′-H-phosphonate monoesters (triethylammonium salts), based on the starting quantity of nucleoside, is expected.

9. *Optional:* Dissolve the foam in a small amount of dichloromethane and add it dropwise while stirring vigorously to 100 to 150 mL of 1:1 hexane/diethyl ether per mmol product. Store at <4°C.

   The nucleoside H-phosphonate is stored as a microcrystalline solid. Although stable for several months at room temperature, it should be stored in the freezer to prevent decomposition due to residual solvents. Under such conditions, no decomposition is seen even after several months.

BASIC PROTOCOL 4

SYNTHESIS OF PROTECTED NUCLEOSIDE 3′-*H*-PHOSPHONATES USING 2-CHLORO-4*H*-1,3,2-BENZO-DIOXAPHOSPHINAN-4-ONE

This is a general-purpose protocol applicable to both ribo- and deoxyribonucleotides. This reaction is shown in Figure 2.6.4.

Materials

Protected deoxyribo- or ribonucleoside (see Chapter 2), dried by repeated evaporation of added pyridine
2:1 (v/v) dichloromethane/pyridine
1.25 M 2-chloro-4*H*-1,3,2-benzo-dioxaphosphinan-4-one solution (see recipe)
9:1 (v/v) chloroform/methanol
1 M TEAB, pH 7.5 (see recipe)

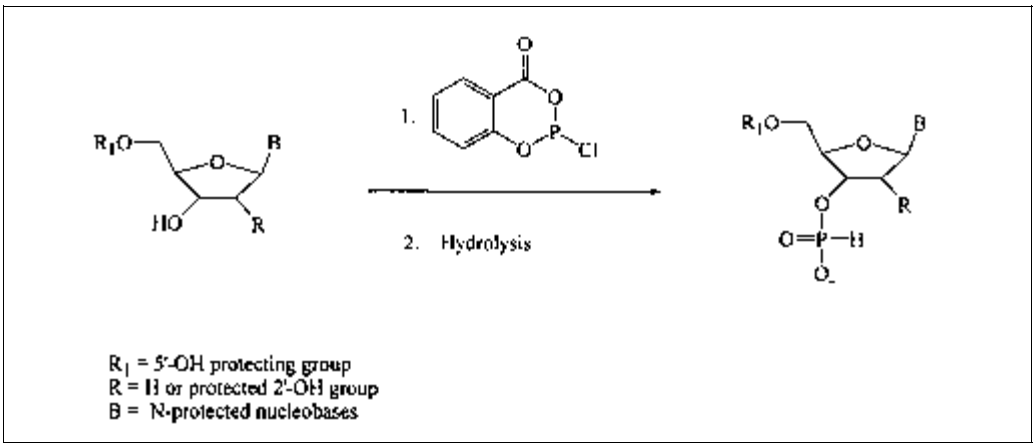

Figure 2.6.4 Synthesis of nucleoside 3′-*H*-phosphonates using salicylchlorophosphite.

Dichloromethane (99%; HPLC grade), stored over 3A molecular sieves
Sodium sulfate
5 × 25–cm glass chromatography column, packed with Merck silica gel 60, 230 to 400 mesh ASTM (e.g., EM Science) or Matrix silica, 35 to 70 µm (Millipore) in chloroform (bed height, 10 to 15 cm)
Methanol
Chloroform
0.5:1:8.5 or 1:2:7 (v/v/v) concentrated ammonia/water/isopropanol
1:1 (v/v) hexane/diethyl ether

TLC silica-gel plates (e.g., Merck silica gel 60 F254, EM Science)
Rotary evaporator connected to water aspirator

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

1. Dissolve a protected deoxyribo- or ribonucleoside (1 mmol) in 20 mL of 2:1 dichloromethane/pyridine while stirring and cool solution with an ice/water bath.
2. Add 1 mL of 1.25 M 2-chloro-4*H*-1,3,2-benzo-dioxaphosphinan-4-one solution in one portion, while stirring. Remove from bath and stir 15 min.
3. Monitor reactions for disappearance of the starting nucleoside and the formation of a baseline material by TLC (*APPENDIX 3D*) on silica-gel plates using 9:1 chloroform/methanol as the solvent.
4. When the reaction is complete (as determined by TLC analysis), add 100 mL of 1 M TEAB to quench reaction and extract the aqueous phase three times with 50 mL dichloromethane. Collect and pool organic phases.
5. Dry organic phase over ~10 g sodium sulfate, and evaporate solvent under reduced pressure using a rotary evaporator connected to a water aspirator.
6. Purify crude product by short-column silica-gel chromatography (*APPENDIX 3E*) on a 5 × 25–cm glass chromatography column using a stepwise gradient of methanol in chloroform to elute products.

   For example, a stepwise gradient of 0% to 12% methanol in chloroform containing 0.1% triethylamine can be used.

Unwanted isomers of the ribonucleoside H-phosphonates, that is 3′-TBDMS (or 2-ClBz) 2′-H-phosphonates, if present, are readily removed by silica-gel chromatography.

7. Check eluate fractions for *H*-phosphonate purity by TLC using 0.5:1:8.5 or 1:2:7 concentrated ammonia/water/isopropanol as the solvents.

   The polarity, and thus the mobility on TLC, of an H-phosphonate will be affected by its protecting group. The latter solvent is probably better for more polar compounds.

   For compounds bearing more base-labile N-protection, the use of 3:7 or 2:8 (v/v) isopropanol/1M TEAB can be advantageous.

   With nucleoside H-phosphonate monoesters produced in this way, it can sometimes be troublesome to remove contaminants resulting from the decomposition products of the phosphonylating reagent (these are visible as fluorescent spots on TLC plates under a UV lamp). In such cases, the samples should be rechromatographed.

8. Combine fractions containing the desired product and concentrate them in the rotary evaporator to a white foam. Store at <4°C.

   A yield of 80% to 90% 3′-H-phosphonate monoesters (triethylammonium salts), based on the starting quantity of nucleoside, is expected.

   Occasionally, $DBUH^+$ salts of H-phosphonate monoesters can be preferred to $TEAH^+$ salts. The transformation can be effected by washing a solution of nucleoside H-phosphonate monoester (triethylammonium salt) in dichloromethane with 0.2 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) bicarbonate buffer (pH 8.5).

9. *Optional:* Dissolve the foam in a small amount of dichloromethane and add it dropwise while stirring vigorously to 100 to 150 mL of 1:1 hexane/diethyl ether per mmol product. Store at <4°C.

   The nucleoside H-phosphonate is stored as a microcrystalline solid. Although stable for several months at room temperature, it should be stored in the freezer to prevent decomposition due to residual solvents. Under such conditions, no decomposition is seen even after several months.

REAGENTS AND SOLUTIONS

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

2-Chloro-4H-1,3,2-benzo-dioxaphosphinan-4-one solution, 1.25 M

Prepare crystalline 2-chloro-4*H*-1,3,2-benzo-dioxaphosphinan-4-one (salicylchlorophosphite) as described (Young, 1952) or purchase commercially (e.g., Aldrich). Dissolve 2.52 g in 6 mL dioxane and adjust final volume to 10 mL. Prepare fresh. Store crystalline product several months at 4°C.

Salicylchlorophosphite was previously called 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one and may still be listed as such by some suppliers.

5,5-Dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane (NEPCl)

Prepare this crystalline reagent as described (McConnell and Coover, 1959). Store several months at room temperature.

Phosphorous acid (H_3PO_3) solution, 2 M

Evaporate 1.62 g H_3PO_3 (commercial grade, e.g., Aldrich) from dry pyridine (HPLC grade, dried and stored over 4A molecular sieves) and dissolve residue in 8 mL dry pyridine. Adjust final volume to 10 mL. Stored several months at room temperature.

Phosphorous acid in pyridine is prepared according to Stawinski and Thelin (1990a).

Triethylammonium bicarbonate (TEAB), 2 M, pH 7.5

Bubble CO_2 through a suspension of 280 mL triethylamine (2 mol) in 700 mL water until the pH of the solution reaches 7.5. Adjust final volume to 1 L. Store buffer up to a few weeks at 4°C and check pH before use. Dilute as needed to appropriate molarity.

COMMENTARY

Background Information

The methods for the preparation of *H*-phosphonate monoesters of natural products can be divided into four major types. They are based on: (1) esterification of phosphorous acid in the presence of various condensing reagents (Hata and Sekine, 1974; Holy et al., 1965; Schofield and Todd, 1961; Sekine and Hata, 1975); (2) reactions of phosphorus trichloride (or reactive species derived thereof) with alcohols (Holy and Sorm, 1966; Honjo et al., 1966; Sekine et al., 1979); (3) transesterification of trialkyl or triaryl phosphites with appropriate hydroxylic components (Holy and Smrt, 1966); and (4) transesterification of β-substituted dialkyl *H*-phosphonate diesters with alcohols, followed by hydrolysis of the produced mixed diesters (Gibbs and Larsen, 1984; Takaku et al., 1988). Most of these methods, unfortunately, suffer from variable yields and are often incompatible with common protecting groups used in oligonucleotide synthesis.

This unit describes four variants of the above general types for the preparation of nucleoside *H*-phosphonate monoesters: Basic Protocol 2 is a type 1 reaction; Basic Protocol 1, the Alternate Protocol, and Basic Protocol 4 are type 2 reactions; and Basic Protocol 3 is related to the type 4 reaction. These methods consistently give high yields of the desired products (75% to 90%), and taken together they provide an arsenal of phosphonylation (phosphitylation) procedures that are compatible with most common protecting groups used in oligodeoxyribo- and oligoribonucleotide synthesis.

Phosphonylation using phosphorus trichloride

Phosphonylation of protected nucleosides using the phosphorus trichloride/imidazole/triethylamine reagent system (Basic Protocol 1) is a mild and efficient method (with yields of 75% to 90%) for the preparation of protected deoxyribo- (Garegg et al., 1986a,c) and ribonucleoside 3′-*H*-phosphonates (Garegg et al., 1986b; Stawinski et al., 1988; Rozners et al., 1995b, 1998) as well as for arabinonucleoside *H*-phosphonates (Rozners et al., 1995a). A popular variant of this procedure involves the replacement of imidazole by 1,2,4-triazole (Froehler, 1993; Froehler et al., 1986). Gaffney and Jones (1988) also reported that using *N*-methylmorpholine instead of triethylamine as an acid scavenger offers some advantages.

Acetonitrile and dichloromethane are commonly used as solvents in this procedure. The phosphonylating reagent is generated in situ immediately prior to synthesis and the reaction is frequently carried out in a dry ice/acetone bath (or a similar bath well below 0°C), which is particularly beneficial with guanine-containing nucleosides as these are prone to side reactions. (The side products that can be formed are readily removed, but they will lower the yields.) The reagent is probably made less suitable for a large-scale production of building blocks, because of the use of a sub-zero cold bath.

A convenient procedure for one-flask 2′-hydroxyl protection (2-chlorobenzoylation) and subsequent phosphonylation using this reagent (Alternate Protocol) has also been developed (Rozners et al., 1990, 1992, 1995b, 1998) and used for the preparation of formacetal-linked (Rozners and Strömberg, 1997) as well as amide-linked (D. Katkevica, E. Rozners, E. Bizdena, and R. Strömberg, unpub. observ.) dimeric *H*-phosphonate building blocks. Another recent procedure for preparation of purine-containing ribonucleoside building blocks is based on the reaction of tris(triazolyl)phosphine with 2′,3′-unprotected purine ribonucleosides to produce, after hydrolysis, an isomeric mixture of 2′- and 3′-*H*-phosphonates. Upon silylation with TBDMS·Cl, this mixture gives a remarkable selectivity of 85% to 90% for 2′-O-silylation (Zhang et al., 1997).

Phosphonylation using phosphorous acid esterification

Direct condensation of phosphorous acid with appropriate alcohols in the presence of an arene sulfonyl derivative (Sekine and Hata, 1975) to produce *H*-phosphonate monoesters is of little synthetic value due to the concomitant formation of *H*-phosphonate diesters (Sekine et al., 1988) and oxidation of *H*-phosphonate monoesters (Garegg et al.,

1987). However, by using a limited amount of a condensing agent such as pivaloyl chloride or 5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane (McConnell and Coover, 1959; Basic Protocol 2), it is possible to convert phosphorous acid into its *H*-pyrophosphonate (Stawinski and Thelin, 1990b), which in turn may act as a mild phosphonylating agent (Stawinski and Thelin, 1990a). The procedure is experimentally simple and reliable, and produces deoxyribonucleoside 3′-*H*-phosphonate in yields of 86% to 92% (Stawinski and Thelin, 1990a).

Pyridinium *H*-pyrophosphonate can also be prepared and stored for several months at room temperature as a stock solution in pyridine. Reaction mixtures containing a nucleoside and 5 mol eq of pyrophosphonate can be left overnight without the danger of side reactions with heterocyclic base residues because of this reagent's moderate reactivity (Stawinski and Thelin, 1990a). However, this reagent is rather slow to react with sterically hindered hydroxyl functions such as the 3′-hydroxyl group in 2′-*O*-TBDMS ribonucleosides (Stawinski and Thelin, 1990a), and thus cannot be recommended for preparation of ribonucleoside *H*-phosphonate building blocks. The mild reaction conditions, experimental simplicity, and inexpensive starting materials make this method a strong candidate for large-scale preparations of deoxyribonucleotide *H*-phosphonates.

Phosphonylation by transesterification

Transesterification of diphenyl *H*-phosphonate with protected nucleosides (Basic Protocol 3) is a most convenient approach for the preparation of *H*-phosphonate building blocks. Diphenyl *H*-phosphonate is an inexpensive and commercially available reagent that is stable and easy to handle. *H*-Phosphonate monoesters are synthesized with a purity usually >95% even without column chromatography (Jankowska et al., 1994). No side reactions involving the heterocyclic bases were detected. The same observation has also been reported for nucleosides with unprotected nucleobases (Wada et al., 1997). Although the reagent in pyridine undergoes disproportionation (Kers et al., 1996), this process is much slower than the transesterification reaction, and the side products formed (phenyl *H*-phosphonate and triphenyl phosphite) are easily removed during subsequent purification steps.

Diphenyl *H*-phosphonate usually has to be used in a 3 to 7 mol eq excess of the nucleoside to minimize the formation of symmetrical *H*-phosphonate diesters. However, by introducing some changes into the protocol (Jankowska et al., 1994), it is possible to reduce significantly the excess of diphenyl *H*-phosphonate. The method has been used for the preparation of both deoxyribo- and ribonucleoside *H*-phosphonates. It may, however, pose some problems when applied to ribonucleosides carrying more base-labile protection (e.g., *N*-phenoxyacetyl protection), due to the relatively basic conditions required for hydrolysis of 2′-*O*-TBDMS-protected nucleoside 3′-(phenyl *H*-phosphonate).

Phosphonylation using salicylchlorophosphite

Nucleoside *H*-phosphonate monoesters are also easily accessible via the salicylchlorophosphite method (Basic Protocol 4), which utilizes 2-chloro-4*H*-1,3,2-benzo-dioxaphosphinan-4-one as the phosphonylating reagent (Marugg et al., 1986). Salicylchlorophosphite is crystalline, stable, readily prepared (Young, 1952), and also commercially available. Although the phosphonylation is virtually quantitative, some amounts of the hydroxylic component (~5%) are occasionally regenerated during hydrolysis of the phosphite intermediate (Froehler, 1993). The most serious inconvenience of the method is that it often can be difficult to separate the nucleoside *H*-phosphonate from hydrolysis products of the phosphonylating reagent.

Some other synthetic methods that have been used for preparation of nucleoside *H*-phosphonate monoesters are: (1) phosphonylation of nucleosides with 2-cyanoethyl phosphordiamidite, hydrolysis to the *H*-phosphonate diester, and β-elimination of the 2-cyanoethyl group (Garegg et al., 1985); (2) phosphonylation of nucleosides with 2-cyanoethyl *H*-phosphonate, followed by β-elimination (Szabó et al., 1995); (3) condensation of aryl *H*-phosphonates with nucleosides, followed by hydrolysis (Ozola et al., 1996); (4) anionic debenzylation of nucleoside benzyl *H*-phosphonates (Hall et al., 1957); (5) oxidative phosphonylation with phosphinic acid in the presence of mesitylenedisulfonyl chloride (Sekine et al., 1988); (6) reaction of nucleoside bis-(*N*,*N*-di-isopropylamino)chlorophosphine, followed by acidolysis (Marugg et al., 1986); and (7) condensation of phosphorous acid with nucleosides promoted by triphosgene (Bhongle and Tang, 1995).

Critical Parameters

For these protocols, anhydrous reaction conditions must be used, especially if the phosphonylating reagent is present in almost stoichiometric amounts. Solvents should be stored over molecular sieves (or be freshly distilled). All glassware should be dried in an oven prior to use. The air inlets of apparatuses used in syntheses should be connected to atmospheric pressure through a drying tower. All nucleosides should be anhydrous, which is usually most conveniently accomplished by repeated evaporation of added pyridine.

The purity of the produced compounds should be checked by NMR spectroscopy (^{31}P and ^{1}H), but TLC is also most convenient, especially for the detection of minor amounts of impurities. For example, the contamination of ribonucleoside *H*-phosphonate building blocks with the 3′-O-protected 2′-*H*-phosphonate isomers can be conveniently detected by TLC analysis, as these separate well in ammonia/water/isopropanol systems (and usually also in isopropanol/TEAB).

In the phosphorus trichloride/imidazole method, the slow addition of nucleoside (see Basic Protocol 1, step 3) is important to reduce the formation of symmetrical *H*-phosphonate diesters. It is also important to note that a low temperature is required to avoid side reactions on guanine-containing nucleosides. Although the procedure is rather insensitive to the kind of azole used, there is a higher risk of formation of the symmetrical *H*-phosphonate esters using triazole (or tetrazole) rather than imidazole due to the higher reactivity of the former reagents. Imidazole is also less costly. If overly concentrated solutions or too small an excess of reagent is used, or if the nucleoside solution is added too quickly, smaller or larger amounts of symmetrical *H*-phosphonate diesters can be formed. These can be easily hydrolyzed during subsequent purification (giving monoester product and nucleoside), but their formation will lower the yield of the *H*-phosphonate monoesters.

For the pyrophosphonate method, pyridine seems to be an optimal solvent. Strong bases (e.g., triethylamine) or basic nucleophilic catalysts (e.g., *N*-methylimidazole) should be avoided, because they usually slow down the phosphonylation. When NEPCl is used to generate *H*-pyrophosphonate, the resulting *H*-phosphonate monoesters should be checked for impurities arising from the condensing agent (e.g., by ^{31}P NMR). During the phosphonylation of nucleosides, two species are formed from the condensing agent: 5,5-dimethyl-2-oxo-2-hydroxy-1,3,2-dioxaphosphinane and its pyrophosphate. The first product is usually completely removed during the extraction step. The second product is easy to remove during chromatography, but may slowly hydrolyze on silica gel, releasing 5,5-dimethyl-2-oxo-2-hydroxy-1,3,2-dioxaphosphinane.

Transesterification of diphenyl *H*-phosphonate also works best when pyridine is used as solvent and base. The reaction in neutral solvents in the presence of a strong base (e.g., triethylamine) may fail due to rapid disproportionation of the phosphonylation reagent under such conditions.

Formation of symmetrical *H*-phosphonates is usually not detected when salicylchlorophosphite is used as a phosphonylating reagent. The purity of nucleoside *H*-phosphonates prepared by this method should be carefully checked, however, for the presence of fluorescent spots visible on TLC plates under UV light. These are due to contamination by hydrolysis products of the phosphonylating reagent, which may be nontrivial to remove by silica-gel chromatography.

Anticipated Results

All methods described in this unit afford, after silica-gel column chromatography, nucleoside *H*-phosphonate monoesters of comparable quality and in comparable yields, but they vary in their suitability for the preparation of different nucleoside derivatives. All methods can be scaled up to ~25-mmol reactions. A 1-mmol scale generates 0.5 to 1 g product and a 25-mmol scale generates 15 to 25 g. Compounds prepared by any of these methods are suitable for solid-phase as well as solution synthesis of oligonucleotides and their analogs. For deoxyribonucleoside *H*-phosphonates bearing quite stable *N*-acyl protection, all methods can produce good results. For ribonucleoside *H*-phosphonates, the pyrophosphonate method seems to be too slow, although it may be preferred in large-scale preparation of deoxyribonucleoside building blocks (another possible choice for this being the diphenyl *H*-phosphonate method). For use with ribonucleosides carrying more base-labile N-protection, the phosphorus trichloride/imidazole method is still preferred, with the salicylchlorophosphite approach being the second alternative.

Time Considerations

None of the procedures described in this unit are very time consuming. The whole synthesis procedure including workup usually does not take more than a few hours. The pyrophosphonate method is the slowest one, but in this instance the reaction mixtures can conveniently be left overnight. Together with chromatographic purification, it will take from half a day to a day, depending on method, to obtain the purified nucleoside *H*-phosphonates. The method that probably involves the least manual effort and technical skill is the diphenyl *H*-phosphonate procedure.

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Key References

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Provide primary sources with complete experimental details for Basic Protocol 1.

Stawinski and Thelin, 1990a. See above.

Provides primary sources with complete experimental details for Basic Protocol 2.

Jankowska et al., 1994. See above.

Provides primary sources with complete experimental details for Basic Protocol 3.

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Provides primary sources with complete experimental details for Basic Protocol 4.

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Provide general aspects of the underlying chemistry in the context of nucleotide and oligonucleotide synthesis.

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