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Synthesis of Peptidomimetic Conjugates of Acyclic Nucleoside Phosphonates

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Abstract

Cyclic nucleoside phosphonates connected through a P-O-C linkage to a promoiety represent a class of prodrugs designed to overcome the low oral bioavailability of parent antiviral acyclic nucleoside phosphonates. In our prodrug approach, a non-toxic promoiety such as an amino acid or dipeptide is conjugated to the cyclic form of the parent drug by esterification of the phosphonic acid moiety by an alcoholic amino acid side chain (Ser, Tyr, and analogues) or through a glycol linker. For the biological evaluation and investigation of the pharmacokinetic profiles of these modified nucleoside phosphonates, a reliable synthetic procedure that allows preparation of sufficient amount of potential prodrugs is needed. This unit describes a method for generating peptidomimetic conjugates of two potent antiviral acyclic nucleoside phosphonates: 1-[(2*S*)-3-hydroxy-2-phosphonomethoxypropyl]cytosine ((*S*)-HPMPC, and 9-[(2*S*)-3-hydroxy-2-phosphonomethoxypropyl]adenine ((*S*)-HPMPA). Two alternate strategies allowing synthesizing selected amino acid, dipeptide, or ethylene glycol-linked amino acid prodrugs of (*S*)-HPMPC and (*S*)-HPMPA in solution and using a solid-phase approach are presented.

INTRODUCTION

This unit describes a method for generating peptidomimetic conjugates of two potent antiviral acyclic nucleoside phosphonates (ANPs): 1-[(2*S*)-3-hydroxy-2-phosphonomethoxypropyl]cytosine ((*S*)-HPMPC, also known as cidofovir) and 9-[(2*S*)-3-hydroxy-2-phosphonomethoxypropyl]adenine ((*S*)-HPMPA), which, due to their ionic charge, have very poor oral bioavailability and hence are only effective when delivered intravenously (De Clercq and Holý, 2005; Holý, 2003). We previously reported our efforts to improve the oral absorption of these nucleoside phosphonate analogs by formation of ester conjugates (Eriksson et al., 2007, 2008; Krylov et al., 2009; McKenna et al., 2009; Peterson and McKenna, 2009). In these prodrugs, one negative charge at phosphorus is masked by the formation of an internal phosphonate ester bond with the hydroxymethylene functionality of the nucleoside, leading to the conversion into the corresponding cyclic nucleoside phosphonate derivatives (CNP). The remaining negative charge is masked by conjugation of an amino acid, a dipeptide, or an ethylene glycol-linked amino acid to the parent drug by esterification of the phosphonic acid moiety with an alcoholic amino acid side chain. Two different approaches for synthesizing these ANP conjugates are described: (1) the Basic Protocol, in which a Boc-protected amino acid, dipeptide or ethylene glycol-linked amino acid is coupled using DIEA-PyBOP in DMF in solution with either (*S*)-HPMPC or (*S*)-HPMPA, followed by standard deprotection with TFA; (2) the Alternate Protocol, elaborated for (*S*)-HPMPA prodrugs, in which solid support chemistry is used, the Boc protecting group being essentially replaced by a resin. The latter method has the advantage of avoiding exposure of the intermediate conjugates to silica gel during purification, while also facilitating scale-up of the synthesis. **CAUTION:** Carry out all

operations involving organic solvents and reagents in a well-ventilated fume hood. Wear appropriate protective clothing and glasses.

NOTE: All glassware should be oven dried, and all reactions should be performed under anhydrous conditions.

BASIC PROTOCOL

SYNTHESIS OF AMINO ACID, DIPEPTIDE AND ETHYLENE GLYCOL-LINKED AMINO ACID CYCLIC HPMPA AND HPMPA CONJUGATES

This methodology, utilizing PyBOP to couple the amino acid (**S.3a,b**), the dipeptide (**S.3c**) or the ethylene glycol-linked amino acid (**S.3d**) with (*S*)-HPMPC or (*S*)-HPMPA in the presence of DIEA in DMF at 40°C (Figure 15.4.1), was previously applied in our laboratory (Eriksson *et al.*, 2007, 2008). The reaction is conveniently monitored by ³¹P-NMR and stopped when cyclic HPMPA (or cyclic HPMPA), which is first formed as an intermediate during the reaction *in situ*, is no longer detected. The coupling procedure produces both possible diastereoisomeric conjugates, in different ratios depending on the particular coupling partners. Deprotection of the Boc-protected intermediate ester conjugates (**S.4a–d**) is achieved with TFA in dichloromethane at rt, followed by purification using HPLC (**S.5a**), column chromatography (**S.5b**) or preparative TLC (**S.5c,d**), depending on the stability of the compound purified. The individual diastereoisomers can be isolated, if desired, by an HPLC separation procedure reported elsewhere (Eriksson *et al.*, 2008).

Materials

1-[(2*S*)-3-Hydroxy-2-phosphonomethoxypropyl]cytosine (Cidofovir, CDV, (*S*)-HPMPC; Sapala Organics Private Limited, India) (Eriksson *et al.*, 2008; Holý, 2003)

9-[(2*S*)-3-Hydroxy-2-phosphonomethoxypropyl]adenine ((*S*)-HPMPA; Sapala Organics Private Limited, India) (Holý, 2003)

N,N-Dimethylformamide (DMF; EMD), anhydrous

N-Ethyl-diisopropylamine, (DIEA; Alfa Aesar), freshly distilled over KOH

Amino acids or dipeptide:

N-tert butoxycarbonyl-(*L*)-serine methyl ester (**S.3a**; Aldrich)

N-tert butoxycarbonyl-(*L*)-tyrosine methyl ester (**S.3b**; Aldrich)

N-tert butoxycarbonyl-(*L*)-valine-(*L*)-serine methyl ester (**S.3c**; see Support Protocol)

N-tert butoxycarbonyl-(*L*)-valine ethylene glycol (**S.3d**; see Support Protocol)

Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; Aldrich)

Diethylether (Et₂O), ACS reagent grade

Dichloromethane (CH₂Cl₂), ACS reagent grade

Acetone, ACS reagent grade

Methanol (MeOH), ACS reagent grade

Trifluoroacetic acid (TFA; EMD), anhydrous

Acetonitrile (CH₃CN), ACS reagent grade

Dry nitrogen (N₂) or Argon (Ar)
 Silica Gel (60–200 mesh; EMD)
 500-, 100-, 50-, 25-mL round bottom flasks
 40 °C oil bath
 Molecular sieves 4Å
 Rotary evaporator equipped with a vacuum pump
 Silica gel thin-layer chromatography (TLC) plates (Merck)
 Preparative TLC chamber
 KMnO₄ spray solution
 Glass flash chromatography columns: 2.5 × 30 cm and 1.5 × 25 cm
 C18 HPLC column (5μM, 80 Å pore size, 21.2 × 150 mm)
 Test tubes
 Glass filter funnels
 254 nm UV lamp

Synthesize S.4a–d

- 1 Under a N₂ or Ar atmosphere, place 0.30 g (1.08 mmol) (*S*)-HPMPC or 0.33 g (1.08 mmol) (*S*)-HPMPA, 15 mL anhydrous DMF and 1.95 mL (10.8 mmol) DIEA in a 100-mL round bottom flask containing a magnetic stir bar.
- 2 Warm the reaction flask using a heat gun to facilitate the dissolution of the (*S*)-HPMPC- or (*S*)-HPMPA-DIEA salt. Remove the volatiles using a rotary evaporator under vacuum. Repeat the process of addition and evaporation of DMF and DIEA three times.

NOTE: The (*S*)-HPMPA-DIEA salt is more soluble in DMF than the (*S*)-HPMPC-DIEA salt.

- 3 Add to the residue 15 mL anhydrous DMF, 1.95 mL (10.8 mmol) DIEA, (1.62 mmol) amino acid or ethylene glycol amino acid or dipeptide **S.3a–d** (listed below), and 1.12 g (2.16 mmol) PyBOP under the N₂ or Ar atmosphere at room temperature. While stirring, heat the mixture to 40 °C during 2 hrs.

For **S.4a** 0.35 g *N*-tert-butoxycarbonyl-(*L*)-serine methyl ester (**S.3a**)

For **S.4b** 0.47 g *N*-tert-butoxycarbonyl-(*L*)-tyrosine methyl ester (**S.3b**)

For **S.4c** 0.51 g *N*-tert-butoxycarbonyl-(*L*)-valine-(*L*)-serine methyl ester (**S.3c**)

For **S.4d** 0.42 mg *N*-tert-butoxycarbonyl-(*L*)-valine ethylene glycol (**S.3d**)

Note: The solution turns from a pale yellow to a dark brown color.

- 4 After 2 hrs, monitor the reaction by ³¹P NMR using a D₂O capillary as an internal standard and to achieve lock. Add additional portions of PyBOP if cyclic HPMPC (or cyclic HPMPA) (singlet at ~6 ppm) is still present and excess PyBOP (singlet at ~30 ppm) is not observed in the mixture.
- 5 Once completion of the reaction is confirmed by ³¹P NMR, evaporate the DMF and DIEA using a rotary evaporator under reduced pressure (oil pump).

The following steps should be performed the same day.

The crude reaction mixture should not be stored after step 5.

- 6 Wash the brown residue with diethyl ether (45 mL $\times$ 3) until the ether solution is clear.
- 7 Dissolve the crude product in the minimum amount of CH₂Cl₂ and carefully place the solution on top of a 2.5 cm $\times$ 30 cm column of silica gel (60–200 mesh) equilibrated with CH₂Cl₂. Elute the first 200 mL with CH₂Cl₂, change to 6:3 (v/v) CH₂Cl₂/acetone (300 mL) and then to 6:3:1 (v/v/v) CH₂Cl₂/acetone/MeOH to elute the desired product.
- 8 Combine fractions containing the pure product, as determined by TLC using 9:1 (v/v) CH₂Cl₂/MeOH as the elution solvent and visualization by the UV light and/or a KMnO₄ spray reagent. Evaporate the solvents using a rotary evaporator under reduced pressure (water pump).
- 9 Dry under vacuum for 2 hrs (oil pump).
- 10 Characterize the product **S.4a–d** by ¹H and ³¹P NMR.

All the compounds contain a small amount of hydroxybenzotriazole (HOBt), which does not affect the next step and can be easily removed during the final purification.

³¹P NMR spectra documented below were obtained with proton decoupling Methyl-(2S)-3-[[[(5S)-5-[(4-amino-2-oxo-2H-pyrimidin-1-yl)methyl]-2-oxo-1,4,2λ⁵-dioxaphosphinan-2-yl]oxy]-2-[[[(tert-butoxy)carbonyl]amino]propanoate (**S.4a**). Yield 53%. Mixture of two diastereoisomers in ratio 1:1.6. ¹H NMR (400 MHz, CD₃OD): δ 7.59–7.53 (m, 1H), 5.90–5.87 (m, 1H), 4.57–3.73 (m, 10H), 3.80 (s, 1.86H), 3.78 (s, 1.14H), 1.49 (s, 5.58H), 1.47 (s, 3.42H). ³¹P NMR (162 MHz, CD₃OD): δ 14.10 (0.38P), 12.94 (0.62P).

Methyl-(2S)-3-(4-[[[(5S)-5-[(6-amino-9H-purin-9-yl)methyl]-2-oxo-1,4,2λ⁵-dioxaphosphinan-2-yl]oxy]phenyl]-2-[[[(tert-butoxy)carbonyl]amino]propanoate (**S.4b**). Yield 73%. Mixture of two diastereoisomers in ratio 4:1. ¹H NMR (400 MHz, CD₃OD): δ 8.25 (s, 1H), 8.17 (s, 0.80H), 8.15 (s, 0.20H), 7.28–7.04 (m, 4H), 4.76 (ddd, J = 11.8, 11.8, 2.6 Hz, 0.80H); 4.62–4.07 (m, 7.20H), 3.73 (s, 0.60H), 3.72 (s, 2.40H), 3.15 (dd, J = 14.0, 5.3 Hz, 1H), 2.91 (dd, J = 14.0, 9.2 Hz, 1H), 1.41 (s, 1.8H), 1.39 (s, 7.2H). ³¹P NMR (162 MHz, CD₃OD) δ: 9.84 (0.80P), 8.61 (0.20P).

Methyl-(2S)-3-[[[(5S)-5-[(4-amino-2-oxo-1,2-dihydropyrimidin-1-yl)methyl]-2-oxo-1,4,2λ⁵-dioxaphosphinan-2-yl]oxy]-2-[(2S)-2-[[[(tert-butoxy)carbonyl]amino]-3-methylbutanamido]propanoate (**S.4c**) Yield 73%. Mixture of two diastereoisomers in ratio 1:2.35. ¹H NMR (400 MHz, CD₃OD): δ 7.64 (d, J = 7.1 Hz, 0.3H), 7.58 (d, J = 7.4 Hz, 0.7H), 5.93 (m, 1H), 5.11 (m, 1H), 4.78–3.87 (m, 10H), 3.87 (s, 2.1H), 3.84 (s, 0.9H), 2.33–2.21 (m, 1H), 1.43 (2s, 9H), 1.15–1.07 (m, 6H). ³¹P NMR (202 MHz, CD₃OD): ³¹P NMR (162 MHz, CD₃OD): δ 13.88 (0.3P), 12.82 (0.70P).

2-[[[(5S)-5-[(4-amino-2-oxo-1,2-dihydropyrimidin-1-yl)methyl]-2-oxo-1,4,2λ⁵-dioxaphosphinan-2-yl]oxy]ethyl (2S)-2-[[[(tert-butoxy)carbonyl]amino]-3-methylbutanoate (**S.4d**). Yield 29%. Mixture of two diastereoisomers in ratio 1:2.2. ¹H NMR (400 MHz, CDCl₃): δ 7.72–7.60 (m, 1H), 5.73 (2d, J = 7.2 Hz, 1H), 4.43–3.52 (m, 12H), 2.02 (m, 1H), 1.33 (2s, 9H), 0.84 (m, 6H). ³¹P NMR (162 MHz, CD₃OD): δ 15.02 (0.69P), 13.84 (0.31P).

Synthesize S.5a–d

- 11** Dissolve 0.10 g **S.4a–d** in 2 mL CH₂Cl₂ in a 25-mL round bottom flask containing a magnetic stir bar, add 2 mL TFA at room temperature and stir the reaction mixture overnight.
- 12** Remove the volatiles using a rotary evaporator under reduced pressure (water pump).
For **S.5a** purify the crude product by HPLC.
- 13a** Dissolve the residue in 0.5 mL H₂O containing 0.1 % TFA and purify it on a C18 HPLC column (5μM, 80 Å pore size, 21.2 × 150 mm) using a mobile phase of H₂O containing 0.1% TFA and 4.5% CH₃CN with a flow rate of 7.0 mL/min, and UV detection at 274 nm.
- 14a** Collect fractions that contain the product as determined by UV detection at 274 nm.
- 15a** Concentrate using a rotary evaporator under reduced pressure (oil pump).
For **S.5b** purify the crude by silica gel column chromatography.
- 13b** Dissolve the crude product in the minimum amount of 100:5:0.5 (v/v) CH₂Cl₂/MeOH/TFA and carefully place the solution on top of a 1.5 cm × 25 cm column packed with silica gel (60–200 mesh) in 100:0.5 (v/v) CH₂Cl₂/TFA. Elute the first 100 mL with 100:0.5 (v/v) CH₂Cl₂/TFA, then switch to 100:15:0.5 (v/v) CH₂Cl₂/MeOH/TFA to elute the desired product.
- 14b** Combine fractions containing the pure product, as determined by TLC using 8:2 (v/v) CH₂Cl₂/MeOH visualized by UV light and/or KMnO₄ spray reagent.
- 15b** Evaporate the solvents using a rotary evaporator under reduced pressure (water pump).
For **S.5c** and **S.5d** purify the crude product by preparative thin layer chromatography on silica gel.
- 13c** Dissolve the crude product in the minimum amount of 6:1 (v/v) CH₂Cl₂/MeOH solution and apply the sample on a TLC plate (Silica Gel 20 × 20 cm, 1000 microns) about 1.5 cm from the bottom edge and allow the solvent to evaporate. Place the TLC plate in a separation chamber containing 200 mL 6:1 (v/v) CH₂Cl₂/MeOH. When the solvent reaches 1.5 cm from the upper edge remove the TLC plate and allow the solvents to evaporate.
- 14c** Scrape off the backing material of the desired band, visualized using the UV light, and extract it with minimal MeOH. Filter the silica off using a glass filter funnel, wash it using a small amount of MeOH and concentrate the filtrate using a rotary evaporator under reduced pressure (water pump).
- 15c** Re-dissolve the crude product in a minimum amount of cold (0 °C) MeOH and centrifuge the solution at 6000 g/min for 10 min. Collect the supernatant and remove the volatiles using a rotary evaporator under reduced pressure (water pump).
- 16** Add a few drops of MeOH to **S.5a–d** and precipitate the product as a white solid with diethyl ether.
- 17** Collect the solid product by filtration through a glass filter funnel.
- 18** Dry the product under vacuum for two hours (oil pump).

- 19** Characterize compounds **S.5a–d** by ^1H , ^{31}P NMR and HRMS. Determine the content of the active compound present in the salt obtained by UV spectroscopy using the extinction coefficients of HPMPC ($\epsilon = 9,000$ at 272 nm, pH = 7.0) for **S.5a,c,d** or HPMPA ($\epsilon = 14,191$ at 260 nm, pH = 7.0) for **S.5b** respectively. Adjust the content for compound **S.5b** using the UV absorption of tyrosine ($\epsilon = 1,335$ at 274 nm and 709 at 260 nm).

Methyl-(2S)-2-amino-3-[[[(5S)-5-[(4-amino-2-oxo-2H-pyrimidin-1-yl)-methyl]-2-oxo-1,4,2- λ^5 -dioxaphosphinan-2-yl]oxy]propanoate (**S.5a**). Yield 62%. TFA salt; mixture of two diastereoisomers in ratio 1:2.2. ^1H NMR (400 MHz, CD_3OD): δ 7.84 (d, $J = 7.6$ Hz, 0.31H), 7.79 (d, $J = 7.2$ Hz, 0.69H), 6.04 (d, $J = 7.6$ Hz, 0.31H), 6.03 (d, $J = 7.6$ Hz, 0.69H), 4.67–4.03 (m, 9H), 3.96 (dd, $J = 14.4$, 7.6 Hz, 0.31H), 3.89 (s, 2.07H), 3.86 (s, 0.93H), 3.76 (dd, $J = 14.8$, 7.6 Hz, 0.69H). ^{31}P NMR (162 MHz, CD_3OD): δ 14.11 (0.31P), 12.97 (0.69P). HRMS: m/z calcd 363.1064 ($\text{M} + \text{H}$) $^+$, found 363.1065 ($\text{M} + \text{H}$) $^+$.

Methyl-(2S)-2-amino-3-[[[(5S)-5-[(6-amino-9H-purin-9-yl)methyl]-2-oxo-1,4,2- λ^5 -dioxaphosphinan-2-yl]oxy]phenyl]propanoate (**S.5b**). Yield 74%. TFA salt; mixture of two diastereoisomers in ratio 5.4:1. ^1H NMR (400 MHz, CD_3OD): δ 8.41 (s, 0.84H), 8.40 (s, 0.16H), 8.34 (s, 0.84H), 8.30 (s, 0.16H), 7.34–7.24 (m, 4H), 4.78 (ddd, $J = 12.1$, 12.1, 3.0 Hz, 0.84H), 4.69 (dd, $J = 15.0$, 8.3 Hz, 0.84H), 4.61–4.34 (m, 5.32H), 4.24 (dd, $J = 14.8$, 4.3 Hz, 0.84H), 4.12 (dd, $J = 15.3$, 1.3 Hz, 0.16), 3.86 (s, 0.48H), 3.85 (s, 2.52H), 3.29 (dd, $J = 14.5$, 6.2 Hz, 1H), 3.18 (dd, $J = 14.5$, 7.4 Hz, 1H). ^{31}P NMR (202.5 MHz, CD_3OD): δ 10.51 (0.84P), 8.92 (0.16P). HRMS: m/z calcd 463.1489 ($\text{M} + \text{H}$) $^+$, found 463.1499 ($\text{M} + \text{H}$) $^+$.

Methyl-(2S)-3-[[[(5S)-5-[(4-amino-2-oxopyrimidin-1-yl)methyl]-2-oxo-1,4,2- λ^5 -dioxaphosphinan-2-yl]oxy]-2-[(2S)-2-amino-3-methylbutanamido]propanoate (**S.5c**). Yield 50%. TFA salt; mixture of two diastereoisomers in ratio 1:2.3. ^1H NMR (400 MHz, CD_3OD): δ 7.63 (d, $J = 7.1$ Hz, 0.3H), 7.57 (d, $J = 7.4$ Hz, 0.7H), 5.92 (d, $J = 7.4$ Hz, 1H), 5.10 (m, 1H), 4.78 (m, 1H), 4.61–3.81 (m, 10H), 3.85 (s, 2.1H), 3.83 (s, 0.9H), 2.33–2.21 (m, 1H), 1.31 (2d, $J = 6.2$ Hz, 6H), 1.15–1.07 (m, 6H). ^{31}P NMR (202 MHz, CD_3OD): δ 14.34 (0.70P), 15.29 (0.3P). HRMS: m/z calcd 490.2067 ($\text{M} + \text{H}$) $^+$, found 490.2058 ($\text{M} + \text{H}$) $^+$.

2-[[[(5S)-5-[(4-amino-2-oxo-1,2-dihydropyrimidin-1-yl)methyl]-2-oxo-1,4,2- λ^5 -dioxaphosphinan-2-yl]oxy]ethyl]-(2S)-2-amino-3-methylbutanoate (**S.5d**). Yield 7.8%. TFA salt; mixture of two diastereoisomers in ratio 1:2. ^1H NMR (400 MHz, CD_3OD): δ 7.62–7.54 (2d, 1H, $J = 7.1$ Hz), 5.85 (m, 1H), 4.43–3.67 (m, 12H), 2.21 (m, 1H), 0.98 (m, 6H). ^{31}P NMR (162 MHz, CD_3OD): δ 15.23 (0.66P), 13.87 (0.33P).

ALTERNATE PROTOCOL

SOLID PHASE SYNTHESIS OF AMINO ACID cHPMPA CONJUGATES

This protocol describes the alternate synthesis of peptidomimetic prodrugs of cHPMPA using solid support chemistry (Figure 15.4.2), wherein the Boc protecting group of an amino acid is replaced with a resin. *O*-TBDMS-protected serine alkyl esters (**S.6a–c**) are immobilized by reaction with a tritylchloride polystyrene (TCP) resin. After removal of the TBDMS protecting group with TBAF, coupling to the drug is accomplished in a similar way to the Basic Protocol procedure using PyBOP to cyclize HPMPA and conjugate it to the immobilized amino acid. The final immobilized products (**S.7a–c**) are cleaved from the resin using TFA and converted into corresponding chloride salts with 0.1N solution of HCl in methanol.

Additional Materials (also see Basic Protocol)

O-Tetrabutyltrimethylsilyl-(*L*)-serine methyl ester (**S.6a**, see Support Protocol)

O-Tetrabutyltrimethylsilyl-*(D)*-serine methyl ester (**S.6b**, see Support Protocol)

O-Tetrabutyltrimethylsilyl-*(L)*-serine isopropyl ester (**S.6c**, see Support Protocol)

Trityl chloride polystyrene (TCP) resin (100–200 mesh, 1% cross-linked, typical loading 1.0–1.8 mmol/g; Aldrich)

Tetrabutylammonium fluoride trihydrate (TBAF; Aldrich)

Tetrahydrofuran (THF), ACS reagent grade

0.1N HCl in methanol

Desiccator

Synthesize **S.7a–c**

- 1 Suspend 0.40 g trityl chloride polystyrene (TCP) resin in 10 mL CH₂Cl₂, add (2.6 mmol) *O*-TBDMS serine alkyl ester hydrochloride salt **6a–c** and 1.0 mL (10.4 mmol) DIEA and stir the resulting solution overnight.

For S.7a 0.70 g *O*-tetrabutyltrimethylsilyl-*(L)*-serine methyl ester hydrochloride **S.6a**
For S.7b 0.70 g *O*-tetrabutyltrimethylsilyl-*(D)*-serine methyl ester hydrochloride **S.6b**
For S.7c 0.77 g *O*-tetrabutyltrimethylsilyl-*(L)*-serine isopropyl ester hydrochloride **S.6c**
- 2 Remove the liquid by filtration on a glass filter and wash the resin with 150 mL CH₂Cl₂.
- 3 Suspend the resin in 10 mL THF and add 0.60 g (2.3 mmol) TBAF, stir the suspension for 5 hr at room temperature.
- 4 Remove the liquid by filtration on a glass filter and wash the resin with 50 mL THF, followed by 100 mL CH₂Cl₂.
- 5 Dry the resin under reduced pressure in a desiccator before using for the coupling reaction.
- 6 Dissolve 0.18 g (0.58 mmol) *(S)*-HPMPA in 10 mL DMF, add 2.0 mL (20.8 mmol) DIEA, 0.91 g (1.75 mmol) PyBOP and stir the solution for 1 hr at room temperature.
- 7 Add the TCP resin containing the amino acid alkyl ester (**6a–c**) and shake the reaction mixture overnight at 38 °C. Monitor the reaction by ³¹P NMR using a D₂O capillary. Add additional portions of PyBOP if cyclic HPMPA (singlet at ~6 ppm) is still present and PyBOP reagent (singlet at ~30 ppm) is missing in the mixture. Once based on ³¹P NMR no cyclic HPMPA is detected the reaction is completed.
- 8 Remove the liquid by filtration, wash the resin with 60 mL DMF and 200 mL CH₂Cl₂.
- 9 Add 12 mL 10:2 (v/v) CH₂Cl₂/TFA to the resin and stir the mixture for 18 hr.
- 10 Filter off the resin and evaporate the volatiles using a rotary evaporator.
- 11 Re-dissolve the residue in 20 mL MeOH and add 20 mL 0.2N HCl in methanol at –20 °C. Evaporate the volatiles using a rotary evaporator under reduced pressure (water pump) and repeat the same process three times.
- 12 Add few drops of MeOH to **S.7a–c** and precipitate the product as white solid with diethyl ether.
- 13 Collect the solid by filtration through a glass filter.

14 Dry under vacuum for two hours (oil pump).

15 Characterize the compound by ^1H , ^{31}P NMR and HRMS. Determine the content of the active compound in its salt by UV spectroscopy using the extinction coefficients of HPMPA ($\epsilon = 14,191$ at 260 nm, pH = 7).

Methyl-(2S)-2-amino-3-[[[(5S)-5-[(6-amino-9H-purin-9-yl)methyl]-2-oxo-1,4,2 λ^5 -dioxaphosphinan-2-yl]oxy]propanoate (**S.7a**). Yield 29%. HCl salts; mixture of two diastereoisomers in ratio 1:2.3 ^1H NMR (400 MHz, CD_3OD) δ : 8.39 (s, 1H), 8.31 (s, 0.6H), 8.28 (s, 0.4H), 4.65-4.42 (m, 6H), 4.37-4.25 (m, 3H), 4.12-4.01 (m, 1H), 3.82 (s, 3H). ^{31}P NMR (202 MHz, CD_3OD): δ 14.17 (0.69P), 13.04 (0.31P). HRMS: m/z calcd 387.1176 ($\text{M} + \text{H}$) $^+$, found: 387.1181. ($\text{M} + \text{H}$) $^+$.

Methyl-(2R)-2-amino-3-[[[(5S)-5-[(6-amino-9H-purin-9-yl)methyl]-2-oxo-1,4,2 λ^5 -dioxaphosphinan-2-yl]oxy]propanoate (**S.7b**). Yield 26%. HCl salt; mixture of two diastereoisomers in ratio 1:2 ^1H NMR (400 MHz, CD_3OD) δ : 8.37 and 8.38 (2s, 1H), 8.28 (s, 0.6H), 8.23 (s, 0.4H), 4.75-4.29 (m, 9H), 4.13-4.01 (m, 1H), 3.92 and 3.90 (2s, 3H). ^{31}P NMR (202 MHz, CD_3OD): δ 14.30 (0.67P), 12.93 (0.33P). HRMS: m/z calcd 387.1176 ($\text{M} + \text{H}$) $^+$, found: 387.1181 ($\text{M} + \text{H}$) $^+$.

Isopropyl-(2R)-2-amino-3-[[[(5S)-5-[(6-amino-9H-purin-9-yl)methyl]-2-oxo-1,4,2 λ^5 -dioxaphosphinan-2-yl]oxy]propanoate (**S.7c**) Yield 30%. HCl salt; mixture of two diastereoisomers in ratio 1:1.3. ^1H NMR (400 MHz, CD_3OD) δ : 8.47 (s, 1H), 8.41 (s, 0.6H), 8.36 (s, 0.4H), 5.20-5.13 (m, 1H), 4.76-4.32 (m, 9H), 4.20-4.04 (m, 1H), 1.39-1.30 (m, 6H). ^{31}P NMR (202 MHz, CD_3OD): δ 13.85 (0.56P), 12.94 (0.44P). HRMS: m/z calcd 415.1489 ($\text{M} + \text{H}$) $^+$, found: 415.1495 ($\text{M} + \text{H}$) $^+$.

SUPPORT PROTOCOL

SYNTHESIS OF THE PEPTIDOMIMETIC PROMOIETY

The promoiety largely dictates the stability and the activation pathway of the compound, which are of crucial importance to prodrug design. This protocol describes the synthesis of the promoiety: *N*-Boc-(*L*)-valine-(*L*)-serine methyl ester (**S.3c**), *N*-Boc-(*L*)-valine ethylene glycol (**S.3d**), *O*-tetrabutyltrimethylsilyl-(*L*)-serine methyl ester (**S.6a**), *O*-tetrabutyltrimethylsilyl-(*D*)-serine methyl ester (**S.6b**), *O*-tetrabutyltrimethylsilyl-(*L*)-serine isopropyl ester (**S.6c**). See Figure 15.4.3.

Additional materials (also see Basic Protocol)

N-*tert*-Butoxycarbonyl-(*L*)-valine hydrochloride (Fluka)

(*L*)-Serine methyl ester hydrochloride (Aldrich)

(*D*)-Serine methyl ester hydrochloride (Aldrich)

(*L*)-Serine isopropyl ester hydrochloride (Aurora Building Blocks)

Triethylamine (Et_3N ; Aldrich), freshly distilled over KOH

Ethan-1,2-diol (ethylene glycol)

1-Hydroxybenzotriazole hydrate (HOBt; Aldrich)

3-(Ethyliminomethyleneamino)-*N,N*-dimethyl-propan-1-amine hydrochloride (EDC; Aldrich)

tert-Butyldimethylsilyl chloride (TBDMSCl; Aldrich)

Imidazole (Aldrich)

Citric acid (Baker Analyzed)

Sodium bicarbonate (NaHCO₃; Mallinckrodt Chemicals)

Sodium chloride (NaCl, EMD)

Sodium sulfate (Na₂SO₄; EMD), anhydrous

Separatory funnel 250 mL

Synthesize S.3c,d

1a Dissolve 0.70 g (3.2 mmol) *N*-Boc-(*L*)-valine hydrochloride in 30 mL anhydrous CH₂Cl₂, cool the reaction mixture to 0 °C and add:

For S.3c 0.381 g (3.2 mmol) (*L*)-serine methyl ester

For S.3d 0.72 mL (12.9 mmol) ethylene glycol

2a Add 0.65 g (4.8 mmol) HOBt hydrate and 2.23 mL (16 mmol) Et₃N and stir the resulting mixture at 0 °C for 15 min. Add 766 mg (4 mmol) EDC·HCl and stir the reaction mixture overnight at room temperature.

3a Add an additional 30 mL CH₂Cl₂ and wash the organic layer sequentially with 25 mL of 1.6 M citric acid solution, 25 mL saturated NaHCO₃ solution, and 20 mL saturated NaCl solution. Use separating funnel to separate organic layer.

4a Dry the organic layer over anhydrous Na₂SO₄, filter by gravity filtration, and then evaporate the solvent using a rotary evaporator under reduced pressure (water pump).

5a Dry under vacuum for 2 hrs (oil pump). Characterize the compound by ¹H NMR.

Methyl-(2*S*)-2-[(2*S*)-2-[[*tert*-butoxy]carbonyl]amino]-3-methylbutanamido]-3-hydroxypropanoate (**S.3c**). Yield 76%. ¹H NMR (400 MHz, CDCl₃) δ 7.28 (bs, 1H), 5.43 (bs, 1H), 4.63-4.61 (m, 1H), 3.96-3.78 (m, 4H), 1.36 (s, 9H), 3.70 (s, 3H), 0.92 (d, *J* = 6.6 Hz), 0.89 (d, *J* = 6.6 Hz).

2-Hydroxyethyl-(2*S*)-2-[[*tert*-butoxy]carbonyl]amino]-3-methylbutanoate (**S.3d**) Yield 48%. ¹H NMR (CD₃OD): δ 4.13-4.03 (m, 2H), 3.94 (d, *J* = 5.8 Hz, 1H), 3.65-3.62 (t, *J* = 5.0 Hz, 2H), 2.07-1.95 (m, 1H), 1.34 (s, 9H), 0.85 (d, *J* = 5.8 Hz, 3H), 0.82 (d, *J* = 6.8 Hz, 3H).

Synthesize S. 6a–c

1b Dissolve 0.72 g (4.8 mmol) *tert*-butylchlorodimethylsilane and (2.3 mmol) amino acid alkyl ester in 20 mL anhydrous CH₂Cl₂ and cool the mixture to 0 °C before addition of 0.48 g (7.1 mmol) imidazole. Stir the reaction mixture overnight at room temperature.

For S.6a 0.36 g (*L*)-serine methyl ester

For S.6b 0.36 g (*D*)-serine methyl ester

For S.6c 0.42 g (*L*)-serine isopropyl ester

2b Add an additional 100 mL CH₂Cl₂ to the reaction mixture, and wash with 25 mL 1.6M citric acid solution and 25 mL saturated NaHCO₃ solution. Use a separating funnel to separate the two layers.

3b Extract water phases twice with 30 mL CH₂Cl₂. Combine organic phases, dry the over anhydrous Na₂SO₄, filter by gravity filtration, and then evaporate the solvent using a rotary evaporator under reduced pressure (water pump).

- 4b** Collect the solid on a glass filter and wash it with 20 mL hexane to remove the TBDMS-OH.
- 5b** Dry under vacuum for two hours (oil pump).
- 6b** Characterize the compound by ^1H NMR.

Methyl (2S)-2-amino-3-[(tert-butyldimethylsilyl)oxy]propanoate hydrochloride (**S.6a**) Yield 69%. ^1H NMR (400 MHz, CDCl_3) δ : 8.72 (brs, 3H), 4.32 (m, 1H), 4.26 (dd, J = 10.7, 3.0 Hz, 1H), 4.15 (dd, J = 10.7, 3.0 Hz, 1H), 3.83 (s, 3H), 0.88 (s, 9H), 0.11 (s, 3H), 0.08 (s, 3H).

Methyl (2R)-2-amino-3-[(tert-butyldimethylsilyl)oxy]propanoate hydrochloride (**S.6b**) Yield 72%. ^1H NMR (400 MHz, CDCl_3) δ : 8.78 (brs, 3H), 4.32 (t, J = 3.0 Hz, 1H), 4.25 (dd, J = 10.7, 3.0 Hz, 1H), 4.14 (dd, J = 10.7, 3.0 Hz, 1H), 3.82 (s, 3H), 0.87 (s, 9H), 0.10 (s, 3H), 0.07 (s, 3H).

Isopropyl (2S)-2-amino-3-[(tert-butyldimethylsilyl)oxy]propanoate hydrochloride (**S. 6c**). Yield 64%. ^1H NMR (400 MHz, CDCl_3) δ : 8.72 (brs, 3H), 5.11 (m, J = 6.7 Hz, 1H), 4.25 (dd, J = 10.7, 2.6 Hz, 1H), 4.20 (t, J = 2.6 Hz, 1H), 4.13 (dd, J = 10.7, 2.6 Hz, 1H), 1.29 (d, J = 6.4 Hz, 6H), 0.88 (s, 9H), 0.12 (s, 3H), 0.08 (s, 3H).

COMMENTARY

Background Information

Among the various structural classes of compounds investigated for the treatment of viral infections, acyclic nucleoside phosphonates have proved to be among the most effective broad-spectrum anti-viral agents to date (De Clercq and Holý, 2005). 1-[(2S)-3-Hydroxy-2-phosphonomethoxypropyl]cytosine (HPMPC, cidofovir, Vistide) was approved by the FDA for treatment of cytomegalovirus retinitis in AIDS patients, and 9-[(2S)-3-hydroxy-2-phosphonomethoxypropyl]adenine (HPMPA) belongs to the family of 3-hydroxy-2-(phosphonomethoxypropyl) (HPMP) ANPs that are especially active against various DNA-virus infections, in particular those caused by pox viruses (De Clercq and Holý, 2005; De Clercq, 2007). Because the phosphonic acid group of ANPs ionizes at physiological pH, resulting in a drug molecule largely impermeable to cellular membranes, ANPs are very poorly absorbed orally and must be administered intravenously. This limits their usefulness, and has prompted different research groups to explore different prodrug strategies. Development of phosphonate prodrugs has previously focused on acyloxyalkyl (pivaloyloxymethyl, POM), alkyloxyalkyl, S-acylthioethyl (SATE, aryl, cyclic 1-aryl-1,3-propanyl and cyclosaligenyl phosphonate esters), and various phosphonate amidates bearing mostly amino acids (Ariza, 2005; Peterson and McKenna, 2009; Hostetler, 2009). However, only a few of these prodrugs have so far proceeded to clinical studies.

Addition of *L*-valine to acyclovir (to form valacyclovir)(Soul-Lawton, 1995) as well as other successful examples have demonstrated enhancement of oral bioavailability and stimulated interest in amino acids or dipeptides as promoieties. To address the limitation of ANPs McKenna and coworkers recently explored the potential of biologically benign amino acids and peptides conjugated to the cyclic form of HPMPC or HPMPA, reported to have decreased nephrotoxicity (Mendel *et al.*, 1997), via a phosphonate ester with the amino acid (Ser, Thr or Tyr) side chain hydroxyl group (Eriksson *et al.*, 2007, 2008; McKenna *et al.*, 2009; Peterson and McKenna, 2009). Conjugation of CNPs to an amino acid *via* the side chain hydroxyl group leaves both the amino and carboxylic functions of the amino acid free for the formation of di- and higher peptide moieties or other modifications. In contrast to other prodrug approaches, our strategy involves the incorporation of a non-toxic promoiety,

also offering the advantage of versatility in “tuning” the transport and activating pharmacology by changing the promoiety at one or more of its multiple functionalizable sites (e.g. NH₂, CO₂H, α -C and P stereochemistry).

Synthesis of amino acid, dipeptide, or ethylene-glycol-linked amino acid ANPs derivatives

In our initial studies we reported the synthesis of several phosphono ester dipeptide prodrugs of cHPMPC with the dipeptide attached *via* the side chain hydroxyl group of an X-(L)-SerOMe (Eriksson *et al.*, 2008). In an alternative approach, we communicated the synthesis and biological evaluation of ethylene glycol-linked amino acid prodrugs of cHPMPC (Eriksson *et al.*, 2007). In an effort to expand the repertoire of cHPMPC prodrugs and establish SAR, a number of single amino acid promoieties were investigated (McKenna *et al.*, 2009). Most of the investigated compounds demonstrated biological activity similar to the parent drug. An eight-fold increase in oral bioavailability compared to the parent drug was observed for the (L)-Val-(L)-SerOMe cHPMPC prodrug in a rat model (Eriksson *et al.*, 2008).

The Basic Protocol summarizes the variations in synthetic approaches to the aforementioned three types of compounds. Utilizing a general PyBOP coupling procedure allows “one-pot” synthesis of *N*-Boc-protected conjugates from HPMPC or HPMPA and corresponding *N*-Boc-protected promoiety avoiding isolation of intermediate cyclic HPMPC or HPMPA. The deprotection step of *N*-Boc-protected conjugates using TFA in methylene chloride generates the final products in form of TFA salts. Conversion to HCl or other alternative salts for pharmacological reasons may be readily effected but is not covered in this protocol.

The Alternate approach reported here represents a solid-phase extension of peptidomimetic prodrug approach to HPMPA and was developed to facilitate scaled up synthesis of prodrugs for *in-vivo* testing, primarily to avoid a chromatographic purification step. As the solid support, an acid-labile tritylchloride polystyrene (TCP) resin was chosen. Before attaching the amino acid alkyl ester to the TCP-resin, the amino acid side chain hydroxyl group is protected by a *tert*-butyl dimethylsilyl (TBDMS) group to avoid side reactions. TBDMSO-protected amino acid alkyl esters can be conveniently synthesized using modified literature procedures (Novachek *et al.*, 1996) involving the amino acid alkyl ester and *tert*-butyl dimethylsilyl chloride (TBDMSCl) in dichloromethane, and attached to the solid-support under mild conditions in good yields. After capping unreacted sites with methanol, the TBDMS protecting group is removed using TBAF in THF. Conjugation of the drug is accomplished using PyBOP as in the solution-phase approach. At this step, the usage of a solid-phase allows all the side products to be removed away by washing, leaving the prodrug bound to the TCP-resin, from which it can be cleaved using TFA in dichloromethane.

Although TFA in the product salt is not necessarily incompatible with *vitro* biological tests and is convenient for purification using TLC or column chromatography (HCl salts of similar compounds cannot be purified using these methods), they can be easily converted to the respective hydrochloride salts by treatment with 0.1N HCl methanolic solution at -20°C as demonstrated for compounds prepared using Alternate Protocol. Although the synthesis of other peptidomimetic derivatives of ANPs is beyond the scope of this unit, this protocols can be readily adapted to the other HPMP-based ANPs and various amino acid/dipeptide promoieties.

Critical Parameters and Troubleshooting

Phosphonate-peptidomimetic conjugates of acyclic nucleoside phosphonates are sensitive to moisture; consequently they should be handled under dry argon or nitrogen atmosphere and stored at -20°C .

In addition to knowledge of basic organic chemistry laboratory skills such as TLC, solvent evaporation, extraction, distillation and column chromatography, basic knowledge of practical ^1H and ^{31}P NMR spectroscopy is required. Techniques such as preparative HPLC and UV spectroscopy are essential for purification and determination of the purity of the compounds.

Both Basic and Alternate Protocols utilize PyBOP as a coupling reagent, which reacts with any moisture traces in the system. The procedure of repetitive addition and evaporation of DIEA and DMF together with the solution of the solubility issue assists in drying the ANP. The final amount of equivalents of PyBOP added depends on how anhydrous the conditions of the experiment are.

The final products, initially produced as TFA salts, are stable under acidic conditions, but may hydrolyze at basic pH with various half lives depending on the promoiety. Therefore, the final purification step should be performed using TFA buffer as an eluent in HPLC or with addition of 0.5% TFA to the mobile phase for column chromatography purification. TLC separation is easier to perform on small amounts of the product, however, it has usually given lower yields than the other two purification methods.

Anticipated Results

The protocols described here for exemplary peptidomimetic promoieties and two antiviral drugs can be applied to other ANP analogs and a wide range of promoieties. The yields of intermediates and final products that can be expected are stated in the protocols for each reaction step and vary from good to moderate. The yields of the final products obtained by the Basic Protocol depend highly on the stability of the compound and method of purification chosen. Utilization of the solid-phase approach offers the possibility of automating the synthesis, however we have not implemented this thus far.

Time Considerations

The synthesis of peptidomimetic prodrugs of acyclic nucleoside phosphonates can be accomplished in 3–4 days starting from ANP and corresponding *N*-Boc-protected or immobilized on the resin promoiety. The preparation of the promoieties that are not commercially available requires 2–3 days.

Acknowledgments

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Figure 15.4.2.

Synthesis of peptidomimetic cHPMPA conjugates (**7a-c**) from (*S*)-HPMPA (**S.2**) and amino acid (**S.6a-c**) on solid support.

Figure 3.
 Synthesis of the peptidomimetics promoieties **S.3c,d** and **S.6a–c**.