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Serine Peptide Phosphoester Prodrugs of Cyclic Cidofovir: Synthesis, Transport, and Antiviral Activity

Ulrika Eriksson,[†,‡] Larryn W. Peterson,[†] Boris A. Kashemirov,[†] John M. Hilfinger,[§] John C. Drach,[‡,||] Katherine Z. Borysko,[||] Julie M. Breitenbach,[||] Jae Seung Kim,[§] Stefanie Mitchell,[§] Paul Kijek,[§] and Charles E. McKenna*[,†]

Department of Chemistry, University of Southern California, Los Angeles, California 90089-0744, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109-1065, School of Dentistry, University of Michigan, Ann Arbor, Michigan 48109-1078, and TSRL, Inc., Ann Arbor, Michigan 48108

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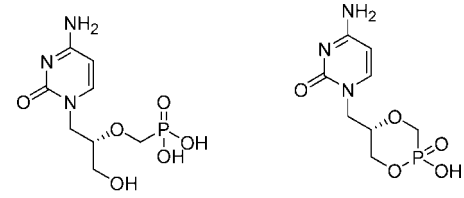
Abstract: Cidofovir (HPMPC, **1**), a broad-spectrum antiviral agent, is currently used to treat AIDS-related human cytomegalovirus (HCMV) retinitis and has recognized therapeutic potential for orthopox virus infections, but is limited by its low oral bioavailability. Cyclic cidofovir (**2**) displays decreased nephrotoxicity compared to **1**, while also exhibiting potent antiviral activity. Here we describe in detail the synthesis and evaluation as prodrugs of four cHPMPC dipeptide conjugates in which the free POH of **2** is esterified by the Ser side chain alcohol group of an X-L-Ser(OMe) dipeptide: **3** (X = L-Ala), **4** (X = L-Val), **5** (X = L-Leu), and **6** (X = L-Phe). Perfusion studies in the rat establish that the mesenteric permeability to **4** is more than 20-fold greater than to **1**, and the bioavailability of **4** is increased 6-fold relative to **1** in an *in vivo* murine model. In gastrointestinal and liver homogenates, the cHPMPC prodrugs are rapidly hydrolyzed to **2**. Prodrugs **3**, **4**, and **5** are nontoxic at 100 μM in HFF and KB cells and in cell-based plaque reduction assays had IC_{50} values of 0.1−0.5 μM for HCMV and 10 μM for two orthopox viruses (vaccinia and cowpox). The enhanced transport properties of **3**−**6**, conferred by incorporation of a biologically benign dipeptide moiety, and the facile cleavage of the Ser−O−P linkage suggest that these prodrugs represent a promising new approach to enhancing the bioavailability of **2**.

Keywords: Cyclic cidofovir; oral bioavailability; prodrug; antiviral activity; drug delivery

Introduction

Cidofovir (HPMPC, **1**, Chart 1), a potent acyclic phosphonate nucleotide analogue of cytosine, is clinically used for treatment of AIDS-related cytomegalovirus retinitis.[2] Cidofovir has broad-spectrum antiviral activity against many DNA viruses and has shown therapeutic potential against herpes simplex virus, varcella-zoster virus, Epstein−Barr virus, and human herpes virus types 6, 7, and 8 as well as adeno-, papilloma-, polyoma-, and poxvirus infections.[3,4] Recently, concern has grown about potential accidental or deliberate reintroduction of variola virus, the etiological agent of smallpox.[5] Although cidofovir has shown potent activity against orthopox viruses,[4,6–9] its low oral bioavailability of less than 5% necessitates intravenous administration, limiting

Chart 1. Structures of HPMPC (**1**) and cHPMPC (**2**)



* To whom correspondence should be addressed. Mailing address: University of Southern California, Department of Chemistry, University Park, Los Angeles, CA 90089-0744. Tel: +1 213 740 7007. Fax: +1 213 740 0930. E-mail: mckenna@usc.edu..

[†] Department of Chemistry, University of Southern California.

[‡] College of Pharmacy, University of Michigan.

[§] TSRL, Inc.

[||] School of Dentistry, University of Michigan.

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its therapeutic scope.[10,11] Cidofovir is actively transported into renal proximal tubular cells more rapidly than it is secreted and may accumulate to toxic levels, causing renal insufficiency.[10,12,13] Therefore, the development of an orally available form of cidofovir or a less toxic, effective analogue is highly desirable.

Cidofovir's poor oral bioavailability can be attributed to its phosphonic group ($-P(O)(OH)_2$), which ionizes under physiological conditions. A number of alternative prodrug strategies for enhancing the oral delivery of **1** by incorporating various phosphonate anion masking groups have been documented.[14–22]

Cyclic cidofovir (cHPMPC, **2**, Chart 1) is known to undergo biotransformation to **1** when exposed to endogenous cCMP phosphodiesterase.[23,24] Although cidofovir has been associated with severe kidney toxicity,[25] **2** has been reported to be less nephrotoxic, while also exhibiting potent antiviral activity.[23] However, **2** also has low oral bioavailability,[10,11] indicating the need to mask the residual phosphonate negative charge present at physiological pH.

We recently reported the synthesis and biological evaluation of ethylene glycol-linked amino acid prodrugs of **2**.[26,27] These derivatives were found to be stable under mildly acidic

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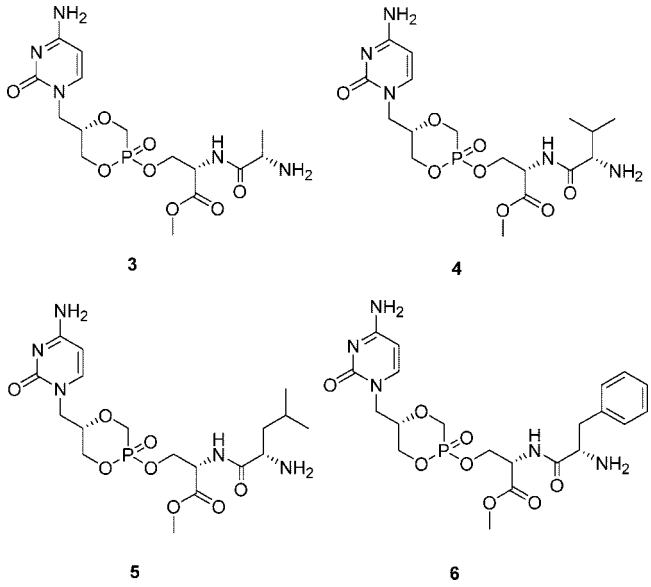
Chart 2. Structures of **3**−**6**



conditions and slowly hydrolyzed at neutral pH. When exposed to cellular and tissue homogenates, they were rapidly activated to **2**. These prodrugs were active against human cytomegalovirus (HCMV), exhibiting an IC_{50} value of 0.68 μM, 4-fold more potent than ganciclovir, the positive control. However, they did not show enhanced oral bioavailability when evaluated in a murine model.[26]

In an alternative approach, we communicated the synthesis of serine peptide phosphoester prodrugs of **2** with the dipeptide attached via the side chain hydroxyl group of an X-L-Ser(OMe) moiety: **3** (X = L-Ala), **4** (X = L-Val), **5** (X = L-Leu), and **6** (X = L-Phe) (Chart 2).[28] By attaching a biologically benign dipeptide promoiety via a Ser side chain phosphoester linkage, we create an analogue of a naturally occurring phosphoserine bond that should be readily cleaved by endogenous phosphatases. Preliminary *in vitro* single pass perfusion assay data indicated that **3**−**6** exhibited enhanced oral bioavailability, relative to **1** and **2** in a murine model.[28] In this article, we present a detailed account of the synthesis and characterization of **3**−**6** together with studies of their pH-dependent stability to hydrolysis, activation in cellular and tissue homogenates, *in vitro* and *in situ* perfusion, HCMV and poxvirus inhibition using a plaque reduction assay, and cellular toxicity data.

Experimental Section

General Methods. ^{1}H and ^{31}P NMR spectra were obtained on 250 MHz Bruker AC, 360 MHz Bruker AM, 400 MHz Varian, or 500 MHz Bruker spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) relative to internal $CHCl_3$ (δ 7.24, ^{1}H) or external 85% H_3PO_4 (δ 0.00 ^{31}P NMR). ^{31}P NMR spectra were proton-decoupled, and ^{1}H coupling constants (J values) are quoted in Hz. The following NMR abbreviations are used: s (singlet), d (doublet), m (unresolved multiplet), dd (doublet of doublets), br (broad signal). The elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. HR-MS spectra were recorded at the UCR High Resolution Mass Spectrometry Facility, Riverside, CA, with the assistance of Dr. Ron New. The HPLC systems used were a Rainan Dynamax model SD-200 with a Rainan Dynamax absorbance detector model UV-DII and a Varian ProStar with a Shimadzu SPD-10A detector. The LC−MS/MS system was a Micromass Quattro II with a HP 1100 LC component. All reagents were purchased from commercial sources and used as obtained, unless specified otherwise.

General Method for Dipeptide Synthesis. L-Serine methyl ester hydrochloride (500 mg, 3.2 mmol, 1 equiv) and 1 equiv (3.2 mmol) of the Boc-protected amino acid (for **7**, 608 mg of Boc-L-alanine; for **8**, 697 mg of Boc-L-valine; for **9**, 742 mg of Boc-L-leucine; for **10**, 853 mg of Boc-L-phenylalanine) were dissolved in 30 mL of dry CH_2Cl_2. The reaction mixture was cooled to 0 °C before addition of HOBt hydrate (4.8 mmol, 1.5 equiv) and TEA (16 mmol, 5 equiv). The reaction mixture was kept at 0 °C for 15 min before EDC·HCl (4.0 mmol, 1.25 equiv) or DCC (3.9 mmol, 1.2 equiv) was added. The reaction mixture was stirred at room temperature overnight. An additional 30 mL of CH_2Cl_2 was added, and the organic layer was washed successively with citric acid (1.6 M, 25 mL), saturated $NaHCO_3$ (25 mL), and saturated NaCl (20 mL). The organic phase was dried over Na_2SO_4 and concentrated under vacuum.

Methyl (2*S*)-2-({(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-propanoyl}amino)-3-hydroxypropanoate (7) was obtained as white crystals, 506 mg (54%). ^{1}H NMR (CD_3OD): δ 1.22 (3H, d, J = 7.25), 1.34 (9H, s), 3.63 (3H, s), 3.65−3.84 (2H, dd, J = 11.3), 3.97−4.06 (1H, m), 4.41 (1H, m), 8.00 (1H, br). The ^{1}H chemical shift values are similar to previously reported values for the same compound obtained using a standard DCC−HOBt procedure.[29]

Methyl (2*S*)-2-({(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-methylbutanoyl}amino)-3-hydroxypropanoate (8) was obtained similarly as white crystals, 667 mg (66%). ^{1}H NMR (CD_3OD): δ 0.92 (3H, d, J = 6.5), 0.97 (3H, d, J = 6.5) 1.41 (9H, s), 2.13 (1H, m), 3.68 (3H, s), 3.73−4.18 (3H, m), 4.56 (1H, m), 5.93 (1H, br), 7.49 (1H, br). The ^{1}H NMR chemical shift values are similar to those previously reported for the same compound prepared using ethyl chloroformate in THF.[30]

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Methyl (2*S*)-2-({(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-pentanoyl}amino)-3-hydroxypropanoate (9) was obtained similarly as white crystals, 470 mg (44%). ^{1}H NMR (C_3D_6O): δ 0.90 (3H, d, $J = 6.4$), δ 0.93 (3H, d, $J = 6.4$), δ 1.40 (9H, s), δ 1.47−1.85 (3H, m), δ 3.68 (3H, s), δ 3.76−3.90 (2H, m), δ 4.10−4.25 (2H, m).

Methyl (2*S*)-2-({(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-phenylpropanoyl}amino)-3-hydroxypropanoate (10) was obtained similarly as white crystals, 1.16 g (98%). ^{1}H NMR ($CDCl_3$): δ 1.38 (9H, s), 3.65 (2H, m), 3.73 (3H, s), 3.89 (2H, m), 4.30 (1H, m), 4.54 (1H, m), 6.77 (1H, br), 7.2 (5H, m). The ^{1}H NMR chemical shift values match those previously reported for the same compound prepared using standard DCC-HOBt methods.[31]

General Method for Condensations Utilizing PyBOP. To 300 mg of **1**[28,32] (0.95 mmol), were added 4 mL of dry DMF and 0.5 mL of DIEA. The reaction flask was warmed by a heat gun to facilitate the dissolution of the HPMPC−DIEA salt. The solvent was then removed under vacuum. To the residue were added 8 mL of anhydrous DMF, 450 μL of DIEA, the relevant dipeptide (1.5 equiv), and PyBOP (990 mg, 2 equiv), and the reaction mixture was stirred under N_2 at 40 °C for 2 h. The reaction was monitored by ^{31}P NMR, and additional portions of PyBOP were added as necessary. Solvent was removed under vacuum, and the product was purified by silica gel column chromatography [DCM, DCM:acetone (2:1), and DCM:acetone:CH_3OH (6:3:1)]. The Boc-protected derivatives (**11**, **12**, **13**, and **14**) were recovered in 49, 61, 59, and 56% yield, respectively.

General Method for Boc-Deprotections. TFA (2 mL) was added to a solution of the Boc-protected derivatives (**11**, **12**, **13**, and **14** dissolved in 4 mL of dry DCM). After stirring for 3 h at room temperature, the solvent was removed under vacuum. Purification of **3**, **4**, **5**, and **6** was conducted by preparative TLC (1 mm silica gel on 20 × 20 cm glass support) using DCM:CH_3OH (5:1) as the mobile phase. The desired products were found in the lower band, cut out, and extracted using 100 mL of methanol. Solutions of **3**, **4**, **5**, and **6** in methanol were concentrated, and the products were precipitated by the addition of Et_2O and collected by filtration. The white crystals obtained were carefully dried in vacuo. The final products (**3**, **4**, **5**, and **6**) were analyzed by ^{1}H NMR and ^{31}P NMR, HR-MS, and HPLC.

Methyl (*S*)-3-[(*S*)-5-(4-Amino-2-oxo-2*H*-pyrimidin-1-ylmethyl)-2-oxido-1,4,2-dioxaphosphinan-2-yloxy]-2-((*S*)-2-amino-propionylamino)propanoate (3). Overall yield: 9.1%. ^{1}H NMR (CD_3OD): δ 1.41−1.45 (3H, m), 3.66 and 3.69 (3H, 2s), 3.72−4.46 (9H, m), 5.83−5.87 (1H, m), 7.56−7.65 (1 H, 2d, $J = 7.3$). ^{31}P NMR (CD_3OD): δ 14.0 (s) and 15.2 (s). HR-MS: *m*/*z* calcd 434.1435, found 434.145 $(M + H)^+$. Anal. ($C_{19}H_{26}F_6N_5O_{12}P$): C, H, N.

Methyl (*S*)-2-((*S*)-2-Amino-3-methyl-butyrylamino)-3-[(*S*)-5-(4-amino-2-oxo-2*H*-pyrimidin-1-ylmethyl)-2-oxido-1,4,2-dioxaphosphinan-2-yloxy]propanoate (4). Overall yield: 30.0%. ^{1}H NMR (CD_3OD): δ 0.92−1.04 (6H, m), 2.06−2.20 (1H, m), 3.66 and 3.69 (3H, 2s), 3.71−4.50 (11H, m), 5.78−5.86 (1H, m), 7.50−7.60 (1H, 2d, $J = 7.7$). ^{31}P NMR (CD_3OD) δ 14.09 (s) and 15.31 (s). HRMS: *m*/*z* calcd 462.1748, found 462.175 $(M + H)^+$.

Methyl (*S*)-2-((*S*)-2-Amino-4-methyl-pentanoylamino)-3-[(*S*)-5-(4-amino-2-oxo-2*H*-pyrimidin-1-ylmethyl)-2-oxido-1,4,2-dioxaphosphinan-2-yloxy]propanoate (5). Overall yield: 14.8%. ^{1}H NMR (CD_3OD): δ 0.89−0.94 (6H, m), 1.55−1.72 (3H, m), 3.67 and 3.69 (3H, 2s), 3.7−4.51 (11H, m), 5.79−5.83 (1H, m), 7.48−7.56 (1H, m). ^{31}P NMR (CD_3OD): δ 14.14 (s) and 15.24 (s). HR-MS: *m*/*z* calcd 476.1905, found 476.192 $(M + H)^+$.

Methyl (*S*)-3-[(*S*)-5-(4-Amino-2-oxo-2*H*-pyrimidin-1-ylmethyl)-2-oxido-1,4,2-dioxaphosphinan-2-yloxy]-2-((*S*)-2-amino-3-phenyl-propionylamino)propanoate (6). Overall yield: 12.0%. ^{1}H NMR (CD_3OD): δ 2.89−4.51 (13H, m), 3.66 and 3.68 (3H, 2s), 5.79−5.86 (1H, m), 7.15−7.30 (5H, m), 7.48−7.56 (1H, 2d, $J = 7.1$). ^{31}P NMR (CD_3OD): δ 14.04 (s) and 15.28 (s). HR-MS: *m*/*z* calcd 510.1748, found 510.178 $(M + H)^+$.

Separation of the Diastereomers of 4. Approximately 4.5 mg of solid **4** was dissolved in 0.5 mL of buffer solution (0.1 N TEAA buffer pH 5.0 containing 5% acetonitrile). The diastereomers were initially separated on a Beckman Coulter Ultraprep C-18 column (5 μm, 80 Å pore size, 21.2 × 150 mm) using a mobile phase of 0.1 N TEAA buffer pH 5.0 containing 5% acetonitrile with a flow rate of 8.0 mL/min, and UV detection at 274 nm. Each diastereomer was collected separately and subsequently concentrated under vacuum while monitoring the pH. When necessary, the pH was adjusted to 4.0−4.5 using dilute acetic acid. After buffer and solvent were removed from diastereomer S (slow), the residue was redissolved in a small amount of 0.1% TFA solution containing 9% acetonitrile. The sample was filtered using an Nanosep 30K Omega filter and purified on a Dynamax C-18 column (5 μm, 100 Å pore size, 21.4 × 250 mm) eluted with 0.1% TFA solution containing 9% acetonitrile at a flow rate of 7.0 mL/min using UV detection at 274 nm. Following the second column purification, the volatiles were removed under reduced pressure. When the amount of solution remaining was approximately 1 mL, it was transferred to a vial and lyophilized to yield a white powder. Diastereomer F (fast) was purified in the same manner and was also obtained as a white powder.

Diastereomer S (4S). ^{1}H NMR (D_2O): δ 0.89 and 0.90 (6H, 2d, $J = 6.9$), 2.10 (1H, m), 3.64 (3H, s), 3.60−4.53 (11H, m), 5.97 (1H, d, $J = 7.9$), 7.60 (1H, d, $J = 7.9$). ^{31}P NMR (D_2O): δ 14.48.

Diastereomer F (4F). ^{1}H NMR (D_2O): δ 0.87 and 0.89 (6H, 2d, $J = 6.0$), 2.09 (1H, m), 3.62 (3H, s), 3.72−4.51 (11H, m), 5.97 (1H, d, $J = 7.9$), 7.63 (1H, d, $J = 7.9$). ^{31}P NMR (D_2O): δ 15.81.

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Stability and Hydrolysis Assays. The stability of the prodrugs was tested in aqueous solution at a pH of 3.0, 4.0, 5.0, 6.0, 6.5, 7.0 and 7.5 and in tissue and cell homogenates. For the aqueous solution assays, 2× prodrug solutions were mixed with the appropriate 2× buffer and incubated at 37 °C in a shaking water bath. Aliquots were removed at 0, 1, 2, and 3 h and were immediately combined with an equal volume of 10% TFA to minimize degradation prior to HPLC analysis. For homogenate stability assays, the test solution consisted of 600 μL of 0.1 M phosphate buffer at pH 6.5 to which was added 0.5−0.7 mg of total protein from the tissue or cell homogenates. Samples of 250 μL were taken at time intervals of 0, 5, 15, 30, and 60 min. The samples were immediately added to an equivalent volume of ice cold 10% TFA solution to precipitate the protein and to acidify the sample. Analysis was performed using HPLC.

Cell Culture Homogenates. 6.0×10^5 Caco-2 cells or HFF cells were seeded onto a 6-well plate. After 6 days, the cells were washed with 2 mL of uptake buffer twice and collected with 500 μL of uptake buffer. The cells were treated with Triton X100 (0.2%) and vortexed. The cell homogenates were stored at −80 °C until needed.

Intestinal and Liver Homogenates. Liver and intestinal segments were obtained from Sprague−Dawley rats. The tissue sample was placed into a 50 mL conical tube into which 15 mL of 0.1 M phosphate buffer at pH 6.5 was added. The tissue was homogenized with an Omni International GLH homogenizer at a setting of 6 for 10 min. Aliquots of homogenate (1 mL) were transferred to microcentrifuge tubes, centrifuged at 14000*g* for 15 min, and stored at −80 °C until use. Total protein content was determined using a protein assay kit (BioRad).

Perfusate Preparation. To collect intestinal perfusate from rats, male Sprague−Dawley rats, 8 to 10 weeks old, weighing 250 to 350 g were fasted 15 to 18 h with water given *ad libitum*. Anesthesia was administered by intramuscular injection of ketamine/xylazine (40 mg/kg and 80 mg/kg, respectively) or by 2−5% isofluorane. Jejunal intestinal segments were exposed through a midline, abdominal incision, and an approximately 20 cm segment of the jejunum was cannulated. The intestinal segments were perfused with 10 mM MES (pH 6.5) containing 135 mM NaCl, and 5 mM KCl at a flow rate of 0.5 mL/min at 37 °C using a constant infusion pump (Harvard Apparatus, South Natick, MA). The collected perfusate was stored at −80 °C until used for *ex vivo* stability studies.

***In Situ* Perfusion Methodology.** To determine the intestinal permeability of the prodrugs, the *in situ* single pass perfusion model was used.[33–38] Male albino Sprague−Dawley rats, 9 to 10 weeks old and weighing 250 to 350 g, were fasted for 18 h with free access to water. The rats were anesthetized with 2−5% isofluorane. The abdomen was opened by a 4 to 5 cm midline incision, and a 10 cm jejunal segment was cannulated on two ends. Jejunal perfusion was initiated with 135 mM NaCl, 5 mM KCl, and 0.01% PEG 4000 at a flow rate of 0.2 mL/min containing test compound and ^{14}C-PEG 4000 as a nonabsorbable marker for measuring water flux. After steady state was reached within the perfused intestinal segment (30 min), samples were taken at 10 min intervals for 1 h. Carbon-14 (PEG-4000) levels were assayed by scintillation counting, and test compounds and internal permeability standards were analyzed by LC−MS/MS. Samples were frozen and stored at −80 °C until analysis.

The effective permeability (P_{eff}) determined from the *in situ* perfusion is calculated from eq 1.

$$P_{eff} = -\frac{Q \ln\left(\frac{C'_{out}}{C'_{in}}\right)}{2 \times 60 \times \pi RL} \quad (1)$$

where Q is the perfusion buffer flow rate, C'_{out} is the outlet concentration of the compound that has been adjusted for water transport after passing through the intestinal segment, C'_{in} is the inlet or starting concentration of the compound, R is the radius of the intestinal segment (set to 0.2 cm), and L is the length of the intestinal segment. To correct for water transport, a nonabsorbed radioactive tracer, ^{14}C-PEG-4000, is included in the perfusion buffer.

In certain instances, plasma was drawn through a portal vein cannula to determine the amount of material entering the portal system during the perfusion. This additional experimental step can aid in determining the true intestinal permeability of unstable compounds. Aliquots of plasma (0.5 mL) are withdrawn from the portal vein at regular intervals throughout the experimental time frame. The plasma samples are subjected to SPE and LC−MS/MS analysis as described below. From the plasma concentration of compound, the mesenteric permeability can be determined by substitution into eq 2.

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$$\text{Flux} = A \times P_{\text{e-mes}} \times C_i \quad (2)$$

Flux is equal to the portal blood flow (estimated at 1 mL/min in the rat) × the steady state mesenteric blood concentration of prodrug, A is the absorptive area of the intestine (estimated at 12.56 cm^2 for a 10 cm length of rat intestine, assuming a radius of 0.2 cm), $P_{\text{e-mes}}$ is the mesenteric permeability (cm/s), and C_i is the starting concentration of drug in the perfusate (μg/mL).

To directly compare uptake potential between **3**−**5** and **2**, a correction factor was applied to the data to account for the differences in total cidofovir for each sample. For example, all prodrugs were 2× TFA salts. Thus, 1.0 mg of **4** contained 0.33 mg of TFA, 0.29 mg of the dipeptide, and 0.38 mg of **2**. The correction factor for this compound was 1 mg/0.38 mg or 2.64. The correction factors for the remaining compounds are [**1** (2× hydrate) − 1.21], [**3** (2× TFA) − 2.53], and [**5** (2× TFA) − 2.69].

HPLC Analysis. Samples of 10 μL were injected onto a Synergi Hydro-RP reversed phase column (4 μm, 80 Å pore size, 4.6 × 250 mm). The compounds were eluted using an isocratic mobile phase consisting of 93:7 (17 mM phosphate buffer with 1 mM ion pairing agent (tetrabutylammonium dihydrogen phosphate):acetonitrile) at pH 7.3. The flow rate was 1 mL/min, and detection of the prodrugs and metabolites was at 274 nm.

Open Gut Injection. Male albino Sprague−Dawley rats, 9 to 10 weeks old and weighing 250 to 350 g, were fasted for 18 h with free access to water. The rats were anesthetized with an intramuscular injection of ketamine/xylazine/butorphanol (87 mg/kg, 6 mg/kg, and 0.2 mg/kg body weight, respectively) or by 2−5% isofluorane. To withdraw timed plasma samples, a catheter was placed in the jugular vein. The abdomen was opened by a 4 to 5 cm midline incision, and the duodenal segment was located. Drug sample in solution was injected directly into the duodenal segment, the intestine was placed back into the abdominal cavity, and the incision was covered with gauze. Plasma samples (~0.5 mL) were withdrawn over a 4 to 8 h period, and the systemic plasma concentrations of the injected drug and for the prodrugs, HPMPC and cHPMPC were determined simultaneously using LC−MS/MS.

Oral Gavage. Mice (CFW Swiss-Webster) 4 weeks old and weighing 25 g were fasted for 18 h with free access to water. Drug sample in solution was administered by gavage needle. Due to the sensitivity of the cHPMPC assay and the limited blood volume of the mouse, one mouse represented one time point in these experiments. Therefore, cohorts of mice were dosed with a given drug mixture, then groups of 3 mice within the cohort were sacrificed at the indicated times (e.g., 1, 2, 4, 8, 16, and 24 h) and blood was withdrawn by cardiac puncture. Experiments were run in triplicate; with a 6 point time course, 18 mice were dosed with the test solution. Systemic plasma concentrations of the administered prodrug, **2**, and **1** were determined using LC−MS/MS analysis.

LC−MS/MS Analysis. Samples for analysis were prepared on a cation exchange solid-phase cartridge (MCX, 30 mg/1 cc, Waters). A 250 μL aliquot of acidified rat plasma was loaded onto the activated SPE cartridge, washed with 1% TFA in methanol and 1.0 mL of methanol, and then eluted with 1 mL of 5% NH_4OH in methanol. The solvent was evaporated to dryness under vacuum at 30−35 °C, and the residue was reconstituted in 250 μL of mobile phase. The samples were transferred to microinserts for analysis by LC−MS/MS. A minimum recovery of 86% of **4** was confirmed by control experiments. Samples were separated on a C-8 column (5 μm, 50 mm × 2.2 mm) at a flow rate of 0.2 mL/min in mobile phase composed of 30:70 (acetonitrile: 0.1% formic acid in water). For the MS detector, electrospray positive ion mode was used in multiple reaction monitoring acquisition. For the MS detector, ESP (+) mode was used for acquisition, and the detector parameters were optimized such that all prodrugs, **1**, and **2** were analyzed under the same conditions. The data acquisition software was MassLynx (version 1.4). Calibration curves were constructed by weighted ($1/x$) least-squares regression of peak area versus concentrations of the calibration standards.

Propagation of Cells and Virus. The routine growth and passage of KB cells were performed in monolayer cultures using minimal essential medium (MEM) with either Hanks salts [MEM(H)] or Earle salts [MEM(E)] supplemented with 5% fetal bovine serum. Cells were routinely enumerated with a Coulter Counter model ZF equipped with 100 mm orifice. Viable cells were detected separately by means of trypan blue dye exclusion. KB cells were plated at 1×10^5 cells/well using 24-well cluster dishes. The routine growth and passage of primary human foreskin fibroblast (HFF) cells and methods for propagation and titration of virus have been previously described by Turk et al.[39] The plaque-purified P_0 isolate of the Towne strain of HCMV was a kind gift of Dr. Mark Stinski, University of Iowa, Iowa City, IA.

Vaccinia virus (VV) strain Copenhagen and cowpox virus (CV) strain Brighton stock pools were obtained from Dr. Earl R. Kern, University of Alabama at Birmingham. These pools were prepared in HFF cells and were diluted in our laboratory to provide working stocks. The Towne strain, plaque-purified isolate P_0 of HCMV was used as a reference virus in most studies.[39] All viruses were titered using monolayer cultures of HFF cells.[40] Following incubation of three days (poxviruses) or 10−12 days (HCMV), cells were fixed and stained with 0.1% crystal violet in 20% methanol and macroscopic plaques (poxviruses) or microscopic plaques (HCMV) enumerated.

Assays for Antiviral Activity. The effects of compounds on the replication of poxviruses and HCMV were

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measured using plaque reduction assays.[39,41] Briefly, for poxviruses, virus used was diluted in MEM containing 10% FBS to a desired concentration which would give ~50 plaques per well in 6-well cluster plates. After a 1 h incubation period, an equal amount of 1% agarose was added to an equal volume of each drug dilution (100 μM and ending with 0.03 μM in a methocel overlay). The drug−methocel mixture was added, and the plates were incubated for 3 days, after which cells were stained with 0.1% crystal violet in 20% methanol. Similar techniques were used for HCMV differing in that ~100 plaques were used per well in 24-well cluster plates and incubation was for approximately 10 days. Drug effects were calculated as a percentage of the reduction in plaque number in the presence of each drug concentration compared to the numbers obtained in the absence of drug. Cidofovir (HPMPC) and ganciclovir were used as positive controls in experiments with poxviruses and HCMV, respectively. The 50% inhibitory concentrations (IC_{50}) and corresponding 95% confidence intervals were calculated from the regression lines using the methods described by Goldstein.[42] Samples containing positive controls were used in all assays.

Cytotoxicity Assays. Effects of all compounds on HFF cells used in plaque reduction assays were scored visually for cytotoxicity. Cytotoxicity to KB cell growth was tested using a colorimetric assay. In HFF cells, cytopathology was estimated at 20- to 60-fold magnification in areas of the assay plate not affected with virus infection and scored on a zero to four plus basis. Cells were scored on the day of staining. In KB cells, the effect of compounds during two population doublings of KB cells was determined by crystal violet staining and spectrophotometric quantization of dye eluted from stained cells as described earlier.[40] Briefly, 96-well cluster dishes were plated with KB cells at 5000 cells per well. After overnight incubation at 37 °C, test compound was added in triplicate at eight concentrations. Plates were incubated at 37 °C for 48 h in a CO_2 incubator, rinsed, fixed with 95% ethanol, and stained with 0.1% crystal violet. Acidified ethanol was added and plates read at 570 nm in a spectrophotometer designed to read 96-well ELISA assay plates. Dose−response relationships were constructed by linearly regressing the percent inhibition of parameters derived in the preceding sections against log drug concentrations. The 50% inhibitory concentrations were calculated from the regression lines using the methods described by Goldstein.[42]

Results and Discussion

Chemistry. The synthesis of **3**−**6** required gram quantities of **1** as the starting marterial. Of several reported routes[32,43–48] to **1** from cytosine, that briefly described by Brodfuehrer *et al.*[32] offers advantages of economy and efficiency. The method begins with regiospecific alkylation of *N*-benzoyl cytosine with (*S*)-2-trityloxymethyloxirane and subsequent reaction of the benzoylated cytosine intermediate with diethyl tosylmethylphosphonate, detritylation with HCl, conversion to the phosphonic acid via bromotrimethylsilane (BTMS) silylation−dealkylation, and finally NH_4OH treatment to remove the benzoyl protecting group. In our preparation, this sequence was followed with the exception that cytosine was alkylated with (*S*)-2-trityloxymethyloxirane and protected with benzoic anhydride.

The dipeptide building blocks (**7**−**10**) were synthesized by standard methods using DCC or EDC as condensing agents.[49] There are several methods available for the intramolecular cyclization of **1** to **2**.[23,41,50] However, we discovered that in the presence of excess PyBOP, **1** is converted in one pot to the protected dipeptide conjugates of **2** (**11**−**14**; Scheme 1). The intramolecular cyclization and intermolecular conjugation reactions are easily monitored by ^{31}P NMR. As **1** disappears, the formation of **2**, which resonates at approximately δ 5 ppm, and the Boc-protected derivatives **11**−**14**, found at δ 14−15 ppm, are observed (reaction mixture run with a D_2O capillary). The reaction was allowed to proceed until both **1** and **2** had been consumed.

After deprotection with TFA in dichloromethane (Scheme 1), the target prodrugs **3**−**6** were purified by preparative TLC (mobile phase DCM:CH_3OH, 5:1). The bis(trifluoroacetic

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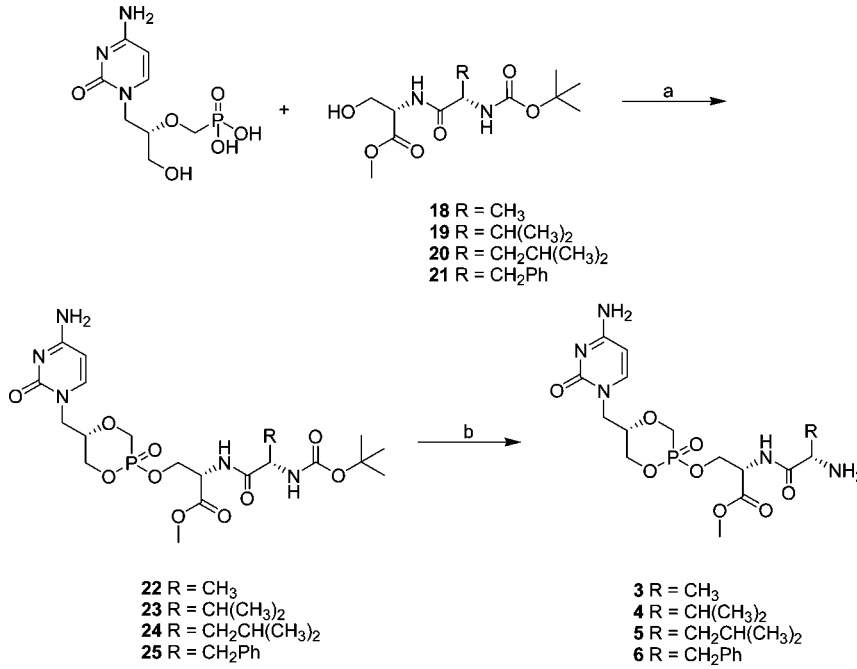
Scheme 1. Synthesis of Prodrugs **3**, **4**, **5**, and **6**



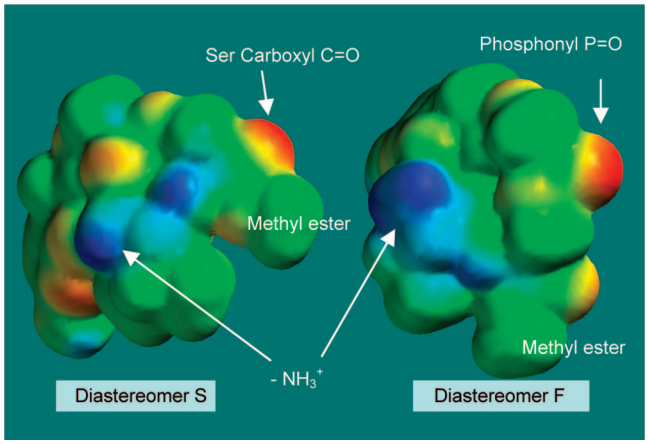


Figure 1. Electrostatic potential maps of diastereomer S (left) and diastereomer F (right) as calculated on Spartan '02.

acid) salts of **3**−**6** were obtained by precipitation with diethyl ether from a saturated methanol solution (overall isolated yields, not optimized, 10−30%). The structures were confirmed by ^{1}H and ^{31}P NMR and by HR-MS. Purity was verified by elemental analysis (**3**) or analytical HPLC (**4**−**6**).

It is of interest that when the X-L-Ser(OMe) dipeptide is conjugated with **2**, a new chiral center is formed at the phosphorus atom resulting in two diastereomers. They can be detected individually by ^{31}P NMR and separated by RP-HPLC, each isolated peak providing one of the two ^{31}P signals present in the mixture. The more rapidly eluting diastereomer (diastereomer F, Figure 1) is less stable in the 0.1 N triethylammonium acetate buffer when removing solvent under reduced pressure than the more slowly eluting diastereomer (diastereomer S, Figure 1). Geometry optimization using a 3-21G* basis set on Spartan '02 predicts a substantial difference in polarity between the two diastereomers (5.72 D vs 13.17 D). As the diastereomers are presumably separated on the C-18 HPLC column due to their differing hydrophobicity, the less hydrophobic compound is likely the *R* isomer, which is calculated to have a larger dipole moment than the *S* isomer (the slower eluting compound). It will be of future interest to determine whether this significant difference in ground state polarities is reflected in the relative rates of enzyme-mediated activation processes. However, in the present study, the cyclic cidofovir dipeptide conjugates were evaluated as the diastereomeric mixtures.

Table 1. Estimated Half-Lives of Cidofovir Prodrugs at 37 °C as a Function of pH[a]

	$t_{1/2}$ (h)				
	5[b]	6[b]	6.5[b]	7[b]	7.5[b]
3	st	36.7	10.6	3.3	1.4
4	st	9.1	12.1	1.5	nd
5	st	4.9	1.8	0.8	0.5

[a] st = stable and nd = not determined. [b] Buffer pH.

Stability to Hydrolysis. At pH 5 and 37 °C **3**−**5** are stable for at least 3 h. From pH 6 to 7.5, the prodrugs slowly hydrolyze with half-lives ($t_{1/2}$) ranging from 36.7 h (**3**) to 0.5 h (**5**) (as measured by a decrease in the prodrug concentration; Table 1). In solution, the chemical release of **2** from the dipeptide promoiety apparently occurred either directly or indirectly via two intermediates, which were tentatively identified as compounds wherein the dipeptide was cyclized or the methyl ester was hydrolyzed. Cleavage of the dipeptide moiety from these intermediates would then give **2**. In all cases, a small amount (less than 5%) of **1** was observed due to the opening of the cycle in **2**.

In several tissue and cellular homogenates, the stability of the prodrugs decreased dramatically as **3**−**5** underwent

Table 2. Estimated Half-Lives of Cidofovir Prodrugs in Cell and Tissue Homogenates at 37 °C, pH 6.5.

	$t_{1/2}$ (min)				
	Caco-2[a]	HFF[a]	liver[a]	intestine[a]	perfusate[a]
3	52.9	63.2	11.2	12.9	205.4
4	20.2	49.5	2.3	5.0	172.4
5	16.6	12.1	1.3	1.4	85.3

[a] Cell, tissue homogenate or biological fluid source.

Table 3. Summary of Uptake and Permeability Data of cHPMPC Prodrugs[a]

	Caco-2 permeability, cm/s × 10^4	*in situ* permeability (P_{eff}), cm/s × 10^4	mesenteric permeability ($P_{e\text{-}mes}$), cm/s × 10^5
1	0.001	0.000	0.052
2	nd	nd	0.003
3	0.0007	unstable	0.98
4	0.0005	unstable	1.18
5	nd	nd	0.48

[a] nd = not determined. The *in situ* permeability was determined either from the perfusate outflow (P_{eff}) or from the mesenteric plasma levels ($P_{e\text{-}mes}$) at 90 min (see Figure *3*).

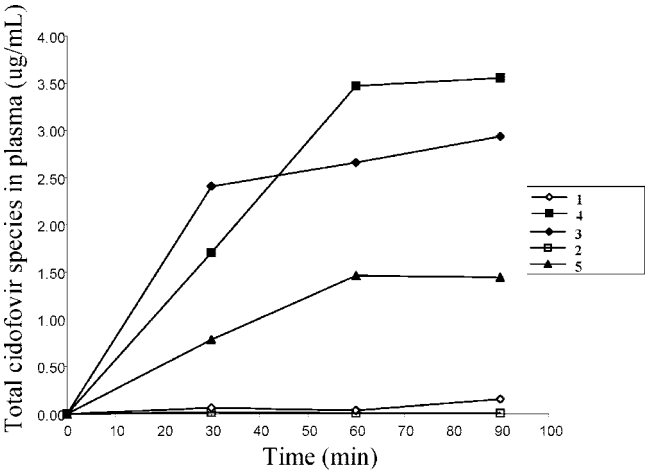


Figure 2. Prodrugs **3**−**5** show increased permeability over **1** or **2** in an *in situ* single pass perfusion assay. Plasma was sampled at the indicated times, and the total cidofovir content (prodrug and **1** or **2**) was determined using LC−MS/MS analysis. The plasma level of drug at 90 min was used to determine the permeability shown in Table 3.

rapid hydrolysis quantitatively to give **1** and **2**, particularly in liver extracts, indicating that the prodrugs could be activated *in vivo* by endogenous enzymes (Table 2). The enzymatic activation pathway of the prodrug to the parent drug appears to differ in some respects from the chemical activation pathway. Using LC−MS/MS analysis, it was observed that in the homogenates the peptide bond was cleaved to remove the N-terminal amino acid, which hypothetically activates the chemical hydrolysis of the serine residue to release **2** (a rate enhancement in the hydrolysis of amine-containing phosphoryl esters has been previously observed, attributed to the catalytic effects of the amino group).[51,52]

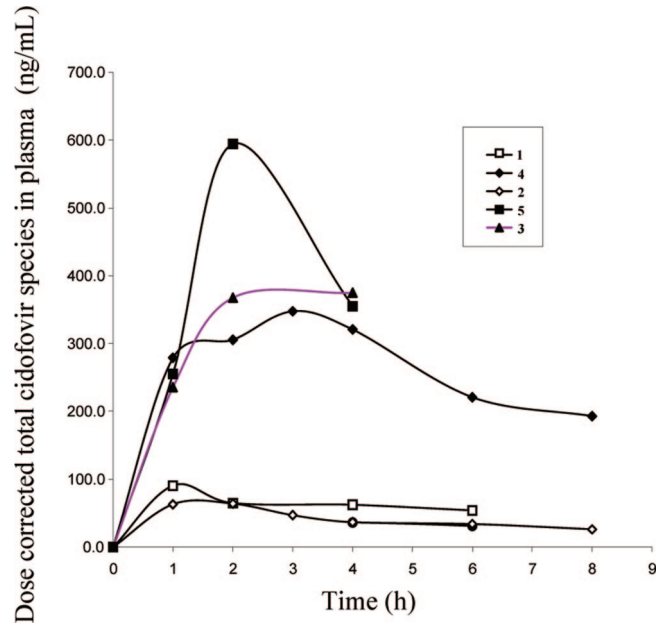


Figure 3. Dose corrected total cidofovir found in plasma after intestinal dosing of **3**−**5**. Dosing of the prodrug boosts the plasma levels of total cidofovir over **1** and **2** controls. Rats were dosed by direct injection of 3 mg (∼10 mg/kg) of prodrug into the intestine, and plasma was sampled over a time course of 8 h. Prodrug **4** showed a greater than 8-fold increase in total cidofovir levels over **2**. Prodrugs **3** and **5** showed a greater than 5-fold increase in absorption.

Table 4. PK Data from Selected cHPMPC Dipeptide Prodrugs

	dose normalized mean $AUC_{0-\infty}$ (ng·h/mL)	% bioavailability (based on total cHPMPC)	fold increase
1	432.3	3.1	
2	304.6	2.2	1.0
2-IV	472.2	100	
3	1655.3	11.7	5.3
4	2559.9	18.1	8.2
5	1675.7	11.8	5.4

To test the hypothesis that cleavage of the peptide bond releasing the N-terminal amino acid from the conjugate activates hydrolysis of the serine−drug ester link, **4** was incubated in a fractionated Caco-2 cell homogenate in the presence of a panel of protease inhibitors, which included 4-(2-aminoethyl)benzenesulfonyl-fluoride (AEBSF), aprotinin, leupeptin, pepstatin A, L-*trans*-epoxysuccinyl-leucyl amido(4-guanidio)-butane (E64), and bestatin. The conversion of prodrug to drug was monitored by HPLC. A

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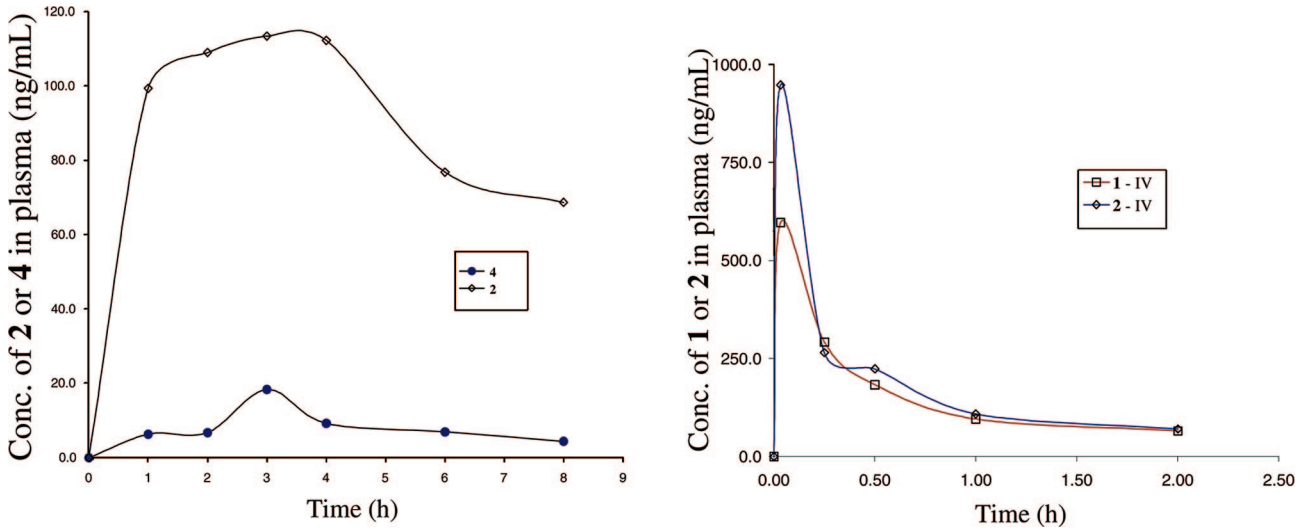

Figure 4. The left panel shows the drug species found in the plasma after intestinal dosing of **4**. The right panel shows plasma concentration time data for an intravenous dose of **1** and **2**. Both compounds have similar kinetics of disappearance. The AUC values from the IV dose of **2** were used to calculate the bioavailability of the prodrug compounds.

progression of intermediate peaks is observed during the course of the reaction, culminating in the appearance of **1** and **2**. Bestatin, a metallo aminopeptidase inhibitor and AEBSF, a serine protease inhibitor, increased the half-life of **4** from 16 ± 2 to 99 min and 56 min, respectively. The other inhibitors had little or no effect on the hydrolysis of **4**. In the presence of bestatin, which inhibits a wide range of aminopeptidases typically found in intestinal tissue, the rate of prodrug hydrolysis was reduced almost to that seen in buffer. This inhibition coincided with the disappearance of one of the intermediate peaks observed during hydrolysis in Caco-2 homogenate. The mass of this peak was 363 (positive ion mode), corresponding to the expected mass of **4** with the N-terminal Val deleted. These data confirm the key role of a peptidase in accelerating the release of **2** from the prodrug, as illustrated by the decrease in hydrolysis when the peptidase was inhibited, which resulted in a half-life similar to that observed in enzyme-free solution.

Transport Studies. The permeability of the prodrugs was tested using a modified *in situ* single pass perfusion technique, in which plasma is sampled from the mesenteric vein that drains the perfused intestinal segment. This independent assessment of the permeability is very useful for compounds that may be unstable during intestinal perfusion because the "traditional" single pass perfusion calculation assumes that the difference between the outflow and the starting concentration of the perfusion is a result of drug transport and does not account for prodrug instability. As seen in the hydrolysis analysis (Table 2), the dipeptide prodrugs of **2** are moderately unstable in intestinal perfusate, with half-lives of approximately 1 to 3 h. The dipeptide prodrugs **3**−**5** (as the diastereomeric mixtures), **1**, and **2** were individually perfused with a starting concentration of 400 μg/mL over a 90 min time course. Plasma samples were analyzed for total cidofovir content, which included the prodrug, intermediates, **2**, and **1**. The major species found

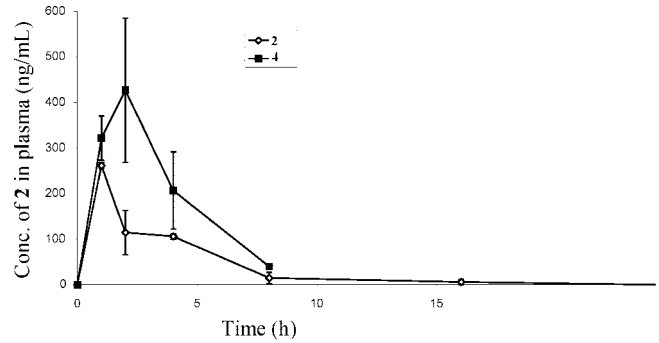

Figure 5. Drug plasma profile in mice after dosing with **2** or **4**. Mice were dosed by oral gavage. Prodrug **4** (■) showed an approximate 2-fold increase in AUC of total cidofovir compared with that seen after dosing with **2** (○).

in plasma was **2** (greater than 90%). The cHPMPC prodrugs showed enhanced permeability, which for **4** was more than 20 times greater than that seen with unmodified drug (Table 3 and Figure 2).

Intestinal Uptake Studies. The cHPMPC prodrugs **3**−**5** were tested for oral availability by direct injection of the drugs into the gastrointestinal tract of a rat at a level of 10 mg/kg (Table 4 and Figure 3). Oral uptake of the prodrugs was significantly enhanced over **2**. As expected from the stability results presented in Table 2, there was little intact prodrug in the plasma (Figure 4, left panel). However, when the prodrugs were dosed, the levels of **2** were significantly higher than when **2** was dosed alone. All compounds showed dose linearity up to the 3 mg dose (data not shown). Oral bioavailability was calculated from the ratio of the dose-normalized oral AUC divided by the intravenous AUC data (472 ng × h/mL) with an adjustment for the differences in the intravenous dose vs oral dose (3 mg oral vs 0.1 mg intravenous). Taking the correction factor (1 mg of the **4** contained ~0.38 mg of **2**) and the dose linearity into account, the total amount of cidofovir

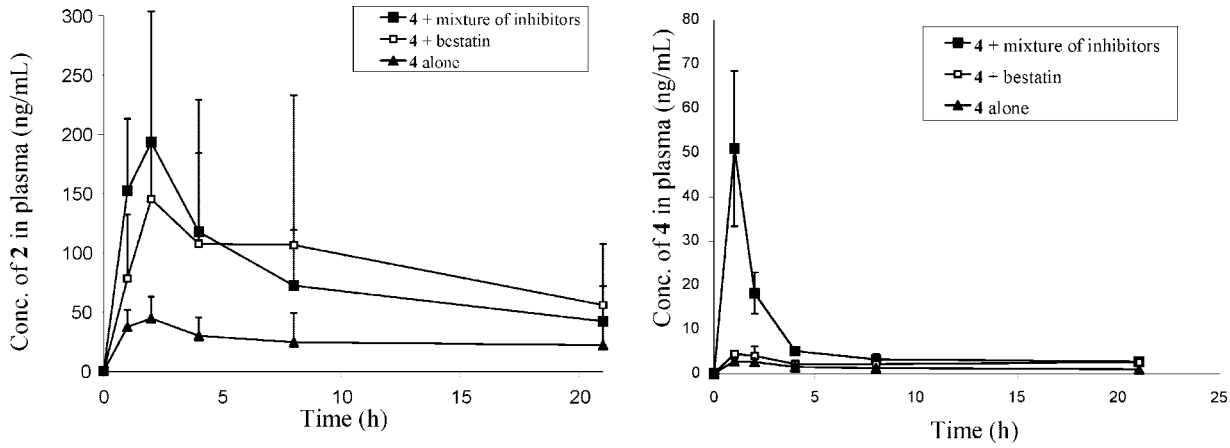

Figure 6. Plasma profiles of **2** after oral dosing of **4** to mice with and without protease inhibitors (left panel) and plasma profiles of **4** after oral dosing of **4** to mice with and without protease inhibitors (right panel). Mice were dosed with 0.3 mg of **4** with and without the protease inhibitors, which were dissolved in water. For each dosing cohort, groups of 3 mice were sacrificed at the indicated time points and their plasma was analyzed for **2** and prodrug by LC−MS/MS. These figures show a comparison of the plasma profiles of **2** or **4** after dosing with **4** alone (▲), **4** with 100 μM bestatin (□), or **4** with the mixture of inhibitors (■).

delivered (**2** + prodrug) $AUC_{0-\infty}$ for **4** was 8 times that of **2**. We estimate the total bioavailability derived from the oral administration of **4** in this experiment was 18.1% compared to 2.2% for the parent compound, **2**, and that overall the serine dipeptide phosphoester prodrugs show greater total cidofovir AUC values than **2**.

The transport of **4** was also investigated when the dipeptide prodrug was stabilized against enzymatic degradation in the gut by codosing with a panel of protease inhibitors. We first compare the absorption of **4** with that of **2** after oral dosing in mice (Figure 5). As seen in the figure, the plasma profile of total cidofovir after dosing with **4** gave a broader and more extensive release than **2**, but showed only a modest 2-fold increase over the parent drug when comparing AUC values.

We then repeated the oral dosing in mice and included protease inhibitors, testing three conditions; **4** alone, **4** in the presence of bestatin (100 μM), or **4** with a mixture of protease and peptidase inhibitors (Figure 6). We found that the inclusion of protease inhibitors greatly enhanced the absorption of the prodrug, boosting the level of **2** present in plasma over that seen by dosing with **4** alone and allowing for significant levels of the prodrug to be observed in plasma. The inhibitors used for the oral dosing included aprotinin (0.2 mg/mL), leupeptin (100 μM), bestatin (100 μM), amastatin (100 μM), and diprotin A (50 μM). As can be seen in Figure 6 (left panel), both bestatin and the mixture of inhibitors increased the plasma exposure of **2** by more than 3-fold compared to dosing **4** alone, as determined by AUC_{0-21h} calculations. Notably, the mixture of inhibitors, when codosed with **4**, led to significantly ($p < 0.05$) higher levels of the prodrug in plasma, with concentration maximum (C_{max}) values rising greater than 17-fold and AUC_{0-21h} showing a 5-fold increase (Figure 6, right panel). These data support the hypothesis that, with stabilization of the dipeptide structure against enzymatic cleavage, drug uptake *in vivo* after oral administration could be further enhanced.

Table 5. Antiviral Activity of **1**, **2**, and the cHPMPC Prodrugs

	IC_{50} (μM)		
	vaccinia	cowpox	HCMV
1	27	30	0.26
2	20	15	<0.1
3	100	80	0.25
4	100	>30	0.3
5	50	nd	0.48
control[a]	nd	nd	3

[a] Control for HCMV antiviral activity was ganciclovir.

Table 6. Cytotoxicity of HPMPC-Related Compounds

	IC_{50} (μM)	
	KB	HFF
1	>100	>100
2	>100	100
3	>100	>100
4	>100	>100
5	>100	100
control[a]	~2	nd

[a] Control for KB cytotoxicity was 2-acetylpyridine thiosemicarbazone.

Antiviral Activity and Cytotoxicity. Prodrugs **3**−**5** were evaluated for their antiviral activity against vaccinia virus (VV), cowpox virus (CV), and HCMV. HPMPC (**1**) and **2** were consistently more active against the poxviruses than their prodrugs, with IC_{50} values in the range of 10 to 30 μM for **1** and **2** compared to values of 30 to >100 μM for the prodrugs (Table 5). However, **3**−**5** demonstrated antiviral activity against HCMV showing IC_{50} values in the submicromolar range, 10-fold lower than ganciclovir, the positive control. When prodrugs **3**, **4**, and **5** were evaluated for cytotoxicity using KB and HFF cells, little or no toxicity at concentrations up to 100 μM was observed (Table 6).

1 and **2** showed antiviral activity against the orthopox viruses consistent with published values[53] and were more active against the poxviruses than **3**−**5** in cell assays. The reduced antiviral activity observed is attributed to the absence of prodrug-biotransformation in these *in vitro* cellular assays. The differences in activity against different viruses may reflect differences in the assays themselves. In particular, the HCMV assay requires incubation for 10 days, and for the CV and VV assays, the incubation times are 3 days. It is evident that the prodrugs are less potent in these cell based assays than the parent drug **2** due to inadequate activation and that the shorter incubation time in the poxvirus assays did not allow sufficient time for the prodrug to exert a therapeutic effect by conversion to **2**.

Conclusion

Four novel cidofovir prodrugs that incorporate X-Ser(OMe) dipeptide promoieties conjugated to the cyclic form of cidofovir through the Ser side chain hydroxyl group have been synthesized and evaluated for stability, transport, and antiviral activity against VV, CV, and HCMV. Stability experiments showed that in gastrointestinal and liver homogenates, **3**−**6** are rapidly hydrolyzed to the parent drug. Prodrugs **3**−**6** exhibited a 5- to 8-fold increase in levels of **2** in *in vivo* dosing experiments over those obtained from dosing with the parent drug **2**. The bioavailability of L-Val-L-Ser(OMe) cHPMPC (**4**) was increased 8-fold relative to **2** (18.1% versus 2.2%) in a rat model. The prodrugs showed little or no cytotoxicity up to 100 μM in KB and HFF cells. All prodrugs gave potent inhibition of HCMV replication, with IC_{50} values in the submicromolar range. It is clear that the biologically benign dipeptide moiety, strategically linked to **2** to mask its anionic properties, significantly enhances intestinal transport, creating the possibility of an orally bioavailable form of **2** with low toxicity. Further studies of these and related peptide prodrugs are in progress.

(53) Keith, K. A.; Wan, W. B.; Ciesla, S. L.; Beadle, J. R.; Hostetler, K. Y.; Kern, E. R. Inhibitory activity of alkoxyalkyl and alkyl esters of cidofovir and cyclic cidofovir against orthopoxvirus replication in vitro. *Antimicrob. Agents Chemother.* **2004**, *48* (5), 1869–71.

Abbreviations Used

HPMPC, cidofovir; cHPMPC, cyclic cidofovir; HFF, human foreskin fibroblast; HCMV, human cytomegalovirus; CV, cowpox virus; VV, vaccinia virus; BTMS, bromotrimethylsilane; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DMAP, *N*,*N*-dimethylaminopyridine; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; HOBt, 1-hydroxybenzotriazole; TEA, triethylamine; TEAA, triethylammonium acetate; TFA, trifluoroacetic acid.

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Supporting Information Available: ^{1}H and ^{31}P NMR spectra of intermediates and final products and purity data for the target compounds. HPLC conditions and traces for the target compounds and diastereomer separation. This material is available free of charge via the Internet at http://pubs.acs.org.

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