Phosphate Prodrugs for Amines Utilizing a Fast Intramolecular Hydroxy Amide Lactonization

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A novel phosphate prodrug system for amines, amino acids, peptides, and peptide mimetics, which utilizes a fast hydroxy amide lactonization of a 3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic amide system, was developed. Prodrugs of five model amine/amino acids, including *p*-anisidine, GlyOMe, PheOMe, LysOMe, and Asp-α-OMe, were synthesized. The syntheses of these model phosphate prodrugs were accomplished by coupling the amine or the protected amino acids with 3-[2′-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic acid using coupling agents such as bis(2-oxo-3-oxazolidinyl)phosphinic chloride and 1-(3-dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride, followed by hydrogenolysis. These phosphate prodrugs were evaluated as substrates for the human placental alkaline phosphatase (AP). The structural features of the amine/amino acids attached to the carboxylic acid group of the promoiety were not found to significantly affect the substrate activity for AP, as evidenced by the small variations observed in the Michaelis−Menten parameters (K_m and V_{max}) of the phosphate prodrugs. Results obtained from this study suggest that such a phosphate prodrug system may be applied to a variety of structurally diverse amine-containing drugs.

Introduction

Phosphate prodrugs have been successfully utilized to overcome a variety of drug delivery problems that might otherwise have compromised the therapeutic utilities of the parent drugs.[1−8] Structurally, they consist of a phosphate group directly attached to the drug molecule in the form of phosphomonoesters or phosphoramidates or indirectly attached with a chemical linker known as a promoiety. The ionic nature of the phosphate group in these prodrugs may significantly improve the solubility and dissolution rate of poorly soluble drugs. In the presence of alkaline phosphatase (AP), an enzyme widely distributed in a variety of tissues such as liver, kidney tubules, intestinal epithelium, etc., phosphomonoesters and phosphoramidates were shown to undergo hydrolysis to release the parent alcohol or amine and inorganic phosphate.[9−11] In addition, certain tumors could be artificially enriched in AP with the aid of monoclonal antibodies; thus, phosphate derivatives of amine-containing anticancer agents with a variety of structural features may be used for cellular targeting.[12,13]

For example, a phosphate prodrug of the antiseizure drug phenytoin (recently approved by the FDA) was shown to enhance the aqueous solubility and dissolution rate as well as the oral bioavailability of this drug due to the presence of AP in the small intestine, especially in the jejunum.[14−16]

The major prerequisites for the success of a phosphate prodrug approach are adequate aqueous chemical stability and the complete *in vivo* enzymatic conversion of the prodrug to the active drug species. Unfortunately, not all phosphomonoesters and phosphoramidates are both chemically stable and good substrates for AP. Phosphomonoesters of sterically hindered secondary and tertiary alcohols suffer from slow rates of bioconversion,[17,18] and many phosphoramidates suffer from poor chemical stability.[19] For example, phosphorylation at the secondary hydroxyl group of the poorly soluble anticancer agent Taxol significantly improved its solubility. However, AP-mediated bioconversion of this prodrug was extremely slow.[20]

Recently, through either rational drug design or combinatorial chemistry approaches, peptide and peptide mimetics have emerged as a new generation of therapeutic agents. However, the therapeutic utility of these

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(1) Sinkula, A. A.; Yalkowsky, S. H. *J. Pharm. Sci.* **1975**, *64*, 181−210.
(2) Stella, V. J.; Charman, W. N. A.; Naringrekar, V. H. *Drugs* **1985**, *29*, 455−473.
(3) Hare, L. E.; Yeh, K. C.; Ditzler, C. A.; McMahon, F. G.; Duggan, D. E. *Clin. Pharmacol. Ther.* **1975**, *18*, 330−337.
(4) Gunnarsson, P. O.; Andersson, S. B.; Johansson, S. A.; Nilsson, T.; Plym-Forshell, G. *Eur. J. Clin. Pharmacol.* **1984**, *26*, 113−119.
(5) Mollmann, H.; Schmidt, E. W.; Salomon, V.; Derendorf, H. *Eur. J. Clin. Pharmacol.* **1985**, *29*, 85−89.
(6) Gerber, N.; Mays, D. C.; Donn, K. H.; Laddu, A.; Guthrie, R. M.; Turlapaty, P.; Quon, C. Y.; Rivenburg, W. K. *J. Clin. Pharmacol.* **1988**, *28*, 1023−1032.
(7) Mollmann, H.; Rohdewald, P.; Barth, J.; Verho, M.; Derendorf, H. *Biopharm. Drug Dispos.* **1989**, *10*, 453−464.
(8) Plaisance, K. I.; Drusano, G. L.; Forrest, A.; Townsend, R. J.; Standiford, H. C. *Antimicrob. Agents Chemother.* **1989**, *33*, 618−620.
(9) McComb, R. B.; Bowers, G. N., Jr.; Posen, S. *Alkaline Phosphatase*; Plenum Press: New York, 1979.
(10) Price, N. C.; Stevens, L. *Fundamentals of Enzymology*, 2nd ed.; Oxford University Press: New York, 1989; pp 458−459.
(11) Snyder, S. L.; Wilson, I. B. *Biochemistry* **1972**, *9*, 1616−1623.
(12) Senter, P. D.; Saulnier, M. G.; Schreiber, G. J.; Hirschberg, D. L.; Brown, J. P.; Hellstrom, I.; Hellstrom, K. E. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4842−4846.
(13) Senter, P. D.; Wallace, P. M.; Svensson, H. P.; Vrudhula, V. M.; Kerr, D. E.; Hellstrom, I.; Hellstrom, K. E. *Bioconjuguate Chem.* **1993**, *4*, 3−9.
(14) TenHoor, C. N.; Stewart, B. H. *Pharm. Res.* **1995**, *12*, 1806−1809.
(15) Varia, S. A.; Schuller, S.; Sloan, K. B.; Stella, V. J. *J. Pharm. Sci.* **1984**, *73*, 1068−1073.
(16) Varia, S. A.; Stella, V. J. *J. Pharm. Sci.* **1984**, *73*, 1087−1090.
(17) Safadi, M.; Oliyai, R.; Stella, V. J. *Pharm. Res.* **1993**, *10*, 1350−1355.
(18) Kearney, A. S.; Stella, V. J. *Pharm. Res.* **1992**, *9*, 497−503.
(19) Bruice, T. C.; Bencovic, S. *Bioorganic Mechanisms*; W. A. Benjamin, Inc: New York, 1966; Vol. 2, pp 71−84.
(20) Vyas, D. M.; Wong, H.; Crosswell, A. R.; Casazza, A. M.; Knipe, J. O.; Mamber, S. W.; Doyle, T. W. *Bioorg. Med. Chem. Lett* **1993**, *3*, 1357.

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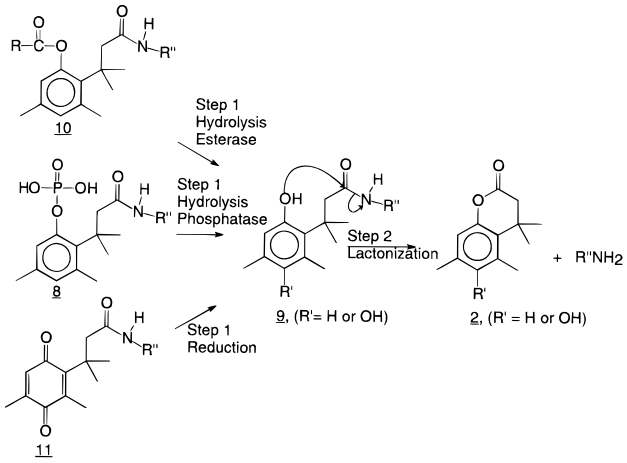
Scheme 1



compounds is often limited by poor solubility or drug delivery problems.[21] Thus, new approaches to accommodate the phosphate group into peptide and peptide mimetics in order to enhance their solubility and improve their delivery are needed.

Recently, Amsberry and co-workers[22−24] and Carpino *et al.*[25] have reported the use of the phenolic propionic acids **9** (R′ = H or OH) shown in Scheme 1 as promoieties for amines and alcohols. Derivatization of the phenolic group of **9** (R′ = H) with a carboxylic acid yields **10**, which is an esterase sensitive prodrug.[23] Oxidation of **9** (R′ = OH) to the quinone **11** yields a redox-sensitive prodrug.[24,25]

Upon hydrolysis of the carboxylic acid in **10** or reduction of the quinone in **11**, the hydroxyamides **9** (R′ = H) and **9** (R′ = OH), respectively, are generated. These hydroxyamides rapidly lactonize ($t_{1/2}$ = 1 min at pH = 7.4) to release equimolar amounts of the amine-containing drug and the inactive lactone **2**.[22] The reason for the fast lactonization is the trimethyl substitution, "trimethyl lock", which for steric reasons leads to a productive "cissoid" conformation favorable to lactonization.[26,27]

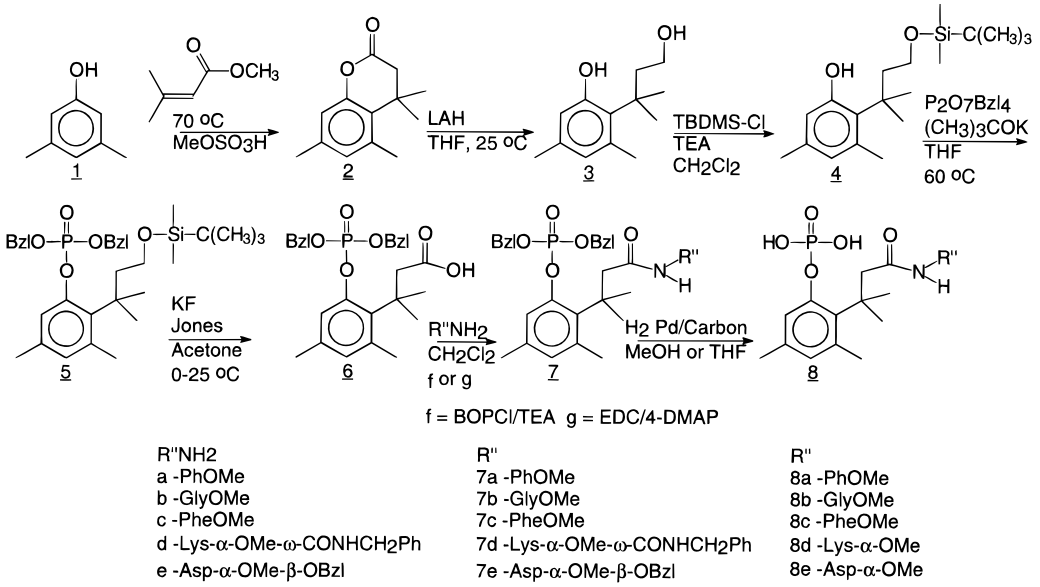
In a recent communication, Ueda *et al.*[28] reported the use of a phosphate ester of 3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic acid to derivatize a secondary alcohol on Taxol. This Taxol prodrug exhibited substantially better solubility than Taxol itself, and it was hydrolyzed to the parent drug by AP. In order to extend the utility of this promoiety to improve the solubility of amine-containing drugs and/or target amine-containing drugs to tissues rich in AP, we synthesized a series of model phosphate derivatives **8a**−**e** (Scheme 2) and evaluated their substrate activity for human placental AP. The model amine-containing compounds used in this study included anisidine, a neutral aromatic amine, two neutral amino acid methyl esters, GlyOMe and PheOMe, and two charge-bearing amino acid methyl esters, LysOMe and AspOMe (Scheme 2). We report here that all five phosphate prodrugs were converted equimolarly to the corresponding amine or amino acid by AP, and the structure of the amides did not significantly affect the rate of bioconversion from the phosphate prodrugs to the parent amine/amino acids. These results suggest that this promoiety may be used for a variety of amine-containing drugs with different structural features.

Results and Discussion

Synthesis. The synthesis of the model phosphate prodrugs **8a**−**e** was accomplished as shown in Scheme 2. Starting from the commercially available 3,5-dimethylphenol (**1**) and methyl 3,3-dimethylacrylate in methanesulfonic acid the lactone **2** was prepared. The use of methanesulfonic acid as both the solvent and the catalyst was reported earlier by Carpino *et al.*[25] for the synthesis of a similar lactone. It was found to afford a higher yield and easier product isolation compared to the method published earlier by our laboratory.[22] Efforts to open the lactone **2** to its corresponding hydroxy acid, followed by subsequent phosphorylation of the phenolic hydroxyl group and attachment of the amine/amino acids to the carboxylic acid, were met with little success. Thus, the syntheses of the model prodrugs were accomplished by reduction of lactone **2** to the diol **3** with lithium aluminum hydride (LAH) using a modification of a procedure reported earlier by our laboratory.[23] The use of a saturated aqueous solution of NH_4Cl to quench the unreacted LAH simplified the product isolation by enabling removal of the lithium aluminum salts by simple filtration over the filter agent Celite. Selective protection of the alkylhydroxyl group of the diol **3** as a *tert*-butyldimethyl silyl ether with *tert*-butyldimethylsilyl chloride (TBDMSCl) in CH_2Cl_2 under basic conditions yielded the intermediate compound **4**. Phosphorylation of the phenolic hydroxyl group of **4** was accomplished using tetrabenzyl pyrophosphate. Efforts to synthesize **5** with other reagents, such as bis(2,2,2-trichloroethyl) phosphorochloridate or phosphorus oxychloride, were unsuccessful. Complete reviews on phosphorylation have appeared elsewhere.[29,30] Difficulty in phosphorylating the phenolic hydroxyl group of **4** with diphenyl phosphorochloridate was also observed by Ueda *et al.*[28] The carboxylic acid **6** was synthesized after *in situ* deprotection of the silyl ether and subsequent oxidation using potassium fluoride and Jones reagent in acetone. Potassium fluoride in conjunction with the Jones reagent has been used previously to synthesize ketones from their corresponding silyl-protected secondary alcohols.[31] This synthetic procedure for obtaining the carboxylic acid **6** was simpler and more efficient than the previous synthetic procedures used to form other derivatives of the phenolic propionic acids from their corresponding alcohols.[23] The use of pyridinium chlorochromate to oxidize the alcohol to the aldehyde followed by further oxidation with $KMnO_4$ to the desirable final product is no longer necessary;[23] thus, three steps are reduced to one with relatively high yield (72%). Ueda *et al.*[28] also reported the use of Jones reagent for the synthesis of carboxylic acid **6** after deprotection and isolation of the corresponding alcohol of **5** followed by subsequent oxidation with the Jones reagent.

(21) Oliyai, R. *Adv. Drug. Delivery Rev.* **1996**, *19*, 275−286.
(22) Amsberry, K. L.; Borchardt, R. T. *J. Org. Chem.* **1990**, *55*, 5867−5877.
(23) Amsberry, K. L.; Gerstenberger, A. E.; Borchardt, R. T. *Pharm. Res.* **1991**, *8*, 455−461.
(24) Amsberry, K. L.; Borchardt, R. T. *Pharm. Res.* **1991**, *8*, 323−330.
(25) Carpino, L. A.; Triolo, S. A.; Berglund, R. A. *J. Org. Chem.* **1989**, *54*, 3303−3310.
(26) Milstien, S.; Cohen, L. A. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *67*, 1143−1147.
(27) Wang, B.; Nicolaou, M.; Liu, S.; Borchardt, R. T. *Bioorg. Chem.* **1996**, *24*, 39−49.
(28) Ueda, Y.; Mikkilineni, A. B.; Knipe, J. O.; Rose, W. C.; Casazza, A. M.; Vyas, D. M. *Bioorg. Med. Chem. Lett* **1993**, *3*, 1761−1766.
(29) Slotin, L. A. *Synthesis* **1977**, 737−752.
(30) Lemmen, P.; Richter, W.; Werner, B.; Karl, R.; Stumpf, R.; Ugi, I. *Synthesis* **1993**, 1−10.
(31) Muzart, J. *Synthesis* **1993**, 11−26.

Scheme 2



R"NH2	R"	R"
a -PhOMe	7a -PhOMe	8a -PhOMe
b -GlyOMe	7b -GlyOMe	8b -GlyOMe
c -PheOMe	7c -PheOMe	8c -PheOMe
d -Lys-α-OMe-ω-CONHCH$_2$Ph	7d -Lys-α-OMe-ω-CONHCH$_2$Ph	8d -Lys-α-OMe
e -Asp-α-OMe-β-OBzl	7e -Asp-α-OMe-β-OBzl	8e -Asp-α-OMe

Coupling the amine/amino acids (a−e, Scheme 2) with the carboxylic acid **6** was accomplished using either 1-(3-dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) plus 4-(dimethylamino)pyridine (4-DMAP) or bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOPCl) as coupling reagents. In both cases, the byproducts, 1-(3-dimethylamino)propyl)-3-ethyl urea (EDU) and the bis-(2-oxo-3-oxazolidinyl)phosphoric acid formed after the coupling, can be removed by washing the reaction milieu with H_2O. The desired model prodrugs **8a**−**e** were obtained after hydrogenolytic debenzylation of **7a**−**e** in either THF or CH_3OH. The amino acid methyl esters (b−e, Scheme 2) were carefully chosen so that the functional groups were benzyl protected and hydrogenolysis afforded the final products in one step. Model prodrugs **8d**−**e** were obtained initially as colorless oils; however, after addition of H_2O, freezing, and subsequent lyophilization, the final products were obtained as amorphous solids.

Biological Evaluation. The phosphate monoesters of the phenolic propionic amides **8a**−**e** synthesized in this study were examined for their ability to release the model amine/amino acids in the presence of human placenta AP. This conversion process includes two steps: a rate-determining dephosphorylation step catalyzed by AP to generate the intermediate hydroxy amide **9a**−**e** (R′ = H) (Schemes 1 and 2) followed by a fast intramolecular lactonization step to release the model amine/amino acids and the lactone **2** (R = H). As shown in Scheme 1, the amount of lactone formed upon hydrolysis of the prodrug should be equal to the original amount of the prodrug. Figure 1 shows a representative time course for the bioconversion from prodrug **8a** to lactone **2** (R′ = H). As expected, an equimolar amount of lactone **2** (R′ = H) was formed from **8a**. This bioconversion process was followed by HPLC as shown in Figure 2.

To assess the effects of the structural features of the amides on the AP-mediated conversion of prodrugs to the model amine/amino acids, the five model prodrugs (**8a**−**e**) were evaluated as substrates for human placenta AP. Their Michaelis−Menten kinetic parameters (K_m and V_{max}) are provided in Table 1. For compounds **8a**−**d** there were no significant differences in either K_m or V_{max} values. In contrast, compound **8e** showed slightly larger K_m and V_{max} values than the other model compounds.

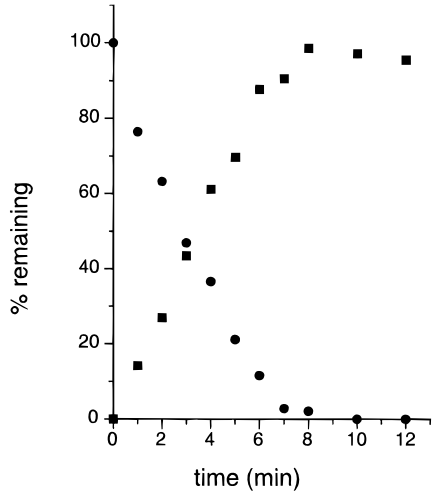


Figure 1. Time profile for the enzymatic degradation of the prodrug **8a** (●) and the appearance of the lactone **2** (■) (Scheme 2): pH = 7.4, 100 mM MOPS buffer, 37 °C, 50 μM of **8a**, and 32.9 μg or AP. One millimolar $ZnCl_2$ and 1 mM $MgCl_2$ were added to the buffer solutions. The samples were analyzed by HPLC.

This may be attributed to the basicity of the ω-amine nitrogen. It has been suggested that as the acidity of the substrate increases the K_m value decreases, and in this case, the basic amino group of lysine may contribute to such an increase in K_m.[9,32]

One reason for choosing the phosphate esters of the phenolic propionic acid as a prodrug strategy for amines is that phenolic phosphates are regarded as good substrates for AP. Previous studies suggested that the rate of the AP-mediated release of the parent alcohol or phenol may depend on the pK_a value of the hydroxyl leaving group.[33,34] The relatively lower pK_a values of phenols compared to other aliphatic alcohols may be one reason that aromatic phosphates were found to be good substrates for AP.[33,34] An important criterion for a prodrug system that aims to deliver a variety of amine-containing drugs of wide structural variation, including functional

(32) Walker, P. G.; King, E. J. *Biochem. J.* **1950**, *47*, 93−95.
(33) Varia, S. A.; Schuller, S.; Stella, V. J. *J. Pharm. Sci.* **1984**, *73*, 1074−1080.
(34) Hall, A. H.; Williams, A. *Biochemistry* **1986**, *25*, 4784−4790.

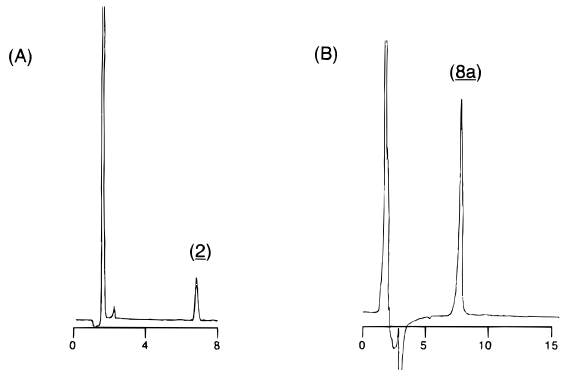


Figure 2. HPLC chromatograms of the lactone **2** (A) and the model prodrug **8a** (B): (A) column, 15 cm C18 Hypersil; mobile phase, 60% aqueous acetonitrile; UV detection, $\lambda = 225$ nm; flow rate, 1 mL/min; (B) column, 15 cm C18 Hypersil; mobile phase, 40% acetonitrile in 20 mM aqueous phosphate buffer pH = 3, 20 mM TBA phosphate; UV detection, $\lambda = 240$ nm; flow rate 1 mL/min.

Table 1. Michaelis−Menten Parameters for Compounds 8a−8e and Phenol Phosphate

model prodrug	K_m[a] (mM)	V_{max}[a] ((nmol/min)/mg of protein)
8a	16.5	175.7
8b	15.6	183.3
8c	22.3	170.9
8d[b]	21.3	156.7
8e	74.3	264.2
phenol phosphate	4.6	202.5

[a] K_m and V_{max} values were obtained after curve fitting the initial rate data for **8a−8e** and phenol phosphate to the Michaelis−Menten equation. [b] Performed in 50 mM MOPS and quenched with 2 M acetic acid in acetonitrile.

groups, charge, size, and steric hindrance, is to be equally cleavable by AP regardless of the structural features. On the basis of data generated in this study, prodrugs **8a−e** have similar Michaelis−Menten parameters when tested as substrates for human placental AP (Table 1). This system was found particularly useful for amines since their release does not depend on direct hydrolysis of the amide bond but, rather, on the enzymatic lability of the phenolic ester bond.

To further probe the structural contribution to the AP kinetic parameters (K_m and V_{max}), the Michaelis−Menten parameters for phenol phosphate were also determined. As shown in Table 1, the K_m value (4 μM) of phenol phosphate was lower than the K_m values (15.6−74.3 μM) observed for prodrugs **8a−e**, suggesting that the high degree of methylation and the restricted conformation of the phenolic propionic amide system may slightly restrict binding to the active site of the enzyme. However, phenol phosphate has a V_{max} value (202.5 (nmol/min)/mg of protein) similar to the V_{max} values (156.7−264.2 (nmol/min)/mg of protein) for the prodrugs **8a−e**, suggesting that the enzyme may catalyze the dephosphorylation reaction equally well, regardless of the structural features in the molecule.

A major advantage of the phenolic propionic acid promoiety is that by applying different derivatization methods to the phenolic hydroxyl group, one may circumvent different barriers to drug delivery, including solubility, permeability, targeting, etc. For example, we have shown previously that ester prodrugs of the phenolic propionic amide type could significantly reduce the hydrophilicity of amines with high pK_a values, including peptides,[23] and could possibly enhance their transport through biological membranes.[35] A phosphate ester of this prodrug system was recently reported to improve the solubility of Taxol and to exhibit phosphatase-mediated bioconversion to the anticancer agent Taxol.[28]

Conclusions

The applicability of the phosphate phenolic propionic amide system shown in Scheme 1 was examined. Five model compounds were synthesized and shown to be substrates for human placental AP. The small variations in the Michaelis−Menten parameters (K_m and V_{max}) among the prodrugs for AP suggest that this system may be used for amine drugs with a wide variety of structural features. Future studies will be focused on *in vivo* bioconversion of model prodrugs of this phenolic propionic amide system.

Experimental Section

General. ^{1}H NMR spectra were recorded on a Varian XL 300 (300 MHz). Chemical shifts are expressed in parts per million (δ) relative to residual solvents as internal standards. Mass spectral analyses were conducted by The University of Kansas Mass Spectral Laboratory, and elemental analyses were determined by The University of Kansas Elemental Analysis Laboratory, Lawrence, KS. HPLC analyses were conducted using a Shimadzu 10A system equipped with a UV spectrophotometric detector. All materials used for the synthesis of the described compounds were purchased from Aldrich Chemical Co., Milwaukee, WI, except bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOPCl) which was purchased from TCI America, Portland, OR, and the protected amino acids were purchased from Bachem California, Torrance, CA. HPLC solvents and 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer were purchased from Fisher Scientific, Pittsburgh, PA, and alkaline phosphatase (P 3895, type XXIV) was purchased from Sigma Chemical Co., St. Louis, MO. All materials were used as received.

General Methods. 4,4,5,7-Tetramethyl-3,4,-dihydrocoumarin (2). 3,5-Dimethylphenol (**1**) (5.00 g, 41.00 mmol) was added to methanesulfonic acid (5 mL) followed by addition of methyl 3,3-dimethylacrylate (5.14 g, 45.03 mmol). The mixture was brought to 70 °C using a thermostated oil bath and was allowed to react for 12 h with constant stirring. The reaction mixture was then diluted with H_2O (1 L) and extracted three times with 200 mL portions of EtOAc. The extracts were combined and washed three times with 5% aqueous $NaHCO_3$ (200 mL × 3) and saturated aqueous NaCl solution (100 mL) and dried over anhydrous $MgSO_4$. Solvent removal by rotary evaporation and recrystallization from EtOAc−hexane afforded the pure lactone **2** in 87% yield. Structural data for this compound are described elsewhere.[22]

3-(2′-Hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (3). The synthesis of 3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (**3**) was performed by LAH reduction of **2**, as previously described,[23] with a minor modification. Instead of using aqueous HCl to quench and dissolve the lithium/aluminum salts, a saturated solution of NH_4Cl was used. The insoluble white precipitate (lithium/aluminum salts) was removed by filtration over Celite and the resulting solution was mixed with H_2O (100 mL) and extracted with Et_2O (200 mL × 3). The ethereal extracts were combined and dried over $MgSO_4$. Solvent removal by rotary evaporation and recrystallization in EtOAc/hexane afforded the analytically pure, white crystalline product **3** in 90% yield.

1-*O*-*tert*-Butyldimethylsilyl-3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (4). The synthesis and structural data for 1-*O*-*tert*-butyldimethylsilyl-3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (**4**) have been previously reported by this laboratory and appeared elsewhere.[23]

(35) Pauletti, G. M.; Gangwar, S.; Wang, B.; Siahaan, T. J.; Vander Velde, D. G.; Borchardt, R. T. *Pharm. Res.* **1995**, *12*, S-208.

1-*O*-*tert*-Butyldimethylsilyl-3-[2′-(dibenzylphosphono)-oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropanol (5). 1-*O*-*tert*-Butyldimethylsilyl-3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (**4**) (2.74 g, 8.5 mmol) was added to 200 mL of dry THF followed by addition of solid potassium *tert*-butoxide (1.04 g, 9.30 mmol). The mixture was heated to 60 °C in an oil bath for approximately 5 min, becoming pale yellow in color. To this warm reaction mixture was added the solid tetrabenzyl pyrophosphate, which was synthesized based on the method of Khorana and Todd[36] and recrystallized from benzene/hexane. Approximately 10 min after the addition of the tetrabenzyl pyrophosphate, an insoluble white precipitate formed. The reaction mixture was kept under continuous stirring at 60 °C for 45 min and then allowed to cool to room temperature. To the reaction milieu was added hexane (200 mL), and the insoluble white precipitate was removed by filtration. After removal of all solvents by rotary evaporation, a viscous residue was obtained. The desired product was eluted from a silica gel column with 20% EtOAc in hexane and isolated as a green-yellow oil (4.29 g, 87%): ^{1}H NMR ($CDCl_3$) δ 7.32 (10 H, s), 7.10 (1 H, s), 6.71 (1 H, s), 5.11 (4 H, d, J = 8.13 Hz), 3.48 (2 H, t, J = 7.26 Hz), 2.50 (3 H, s), 2.17 (3 H, s), 2.09 (2 H, t, J = 7.26 Hz), 1.53 (6 H, s), 0.83 (9 H, s), 0.05 (6 H, s); MS (FAB) m/z 583 ($M^+ + 1$). Anal. Calcd for $C_{33}H_{47}O_5PSi$: C, 68.01; H, 8.13. Found: C, 68.00; H, 8.50.

3-[2′-(Dibenzylphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Acid (6). 1-*O*-(*tert*-Butyldimethylsilyl)-3-[2′-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropanol (**5**) (4.3g 7.39 mmol) was dissolved in 60 mL of acetone followed by addition of solid KF (0.43 g, 29.6 mmol). The above mixture was placed in an ice bath, and Jones reagent (6.1 mL), whose preparation was previously described,[37] was added dropwise over a period of 20 min under continuous stirring. The reaction mixture was stirred for 2 h; after that time, 2-propanol (2.7 mL) was added to quench the residual Jones reagent, and the reaction was allowed to continue for an additional 20 min. The reaction mixture was further concentrated under reduced pressure rotary evaporation, and EtOAc (100 mL) and H_2O (100 mL) were added. The organic layer was separated, and the aqueous layer was extracted with EtOAc (100 mL × 3). The organic extracts were then combined and washed with saturated NaCl aqueous solution (100 mL × 2) and dried over anhydrous $MgSO_4$. Removal of the solvents by rotary evaporation afforded a yellow-green oil which, upon recrystallization from Et_2O and hexane, afforded the desired white, solid product (2.56 g, 72% yield): ^{1}H NMR ($CDCl_3$) δ 7.33 (10 H, m), 6.99 (1 H, s), 6.73 (1 H, s), 5.07−5.13 (4 H, m), 2.84 (2 H, s), 2.50 (3 H, s), 2.13 (3 H, s), 1.61 (6 H, s); MS (FAB) m/z 483 ($M^+ + 1$). Anal. Calcd for $C_{27}H_{31}O_6P$: C, 67.22; H, 6.43. Found: C, 67.30; H,6.38.

General Method I for Preparation of Amides. All glassware was flame dried, and the reaction was kept under argon atmosphere at all times. To anhydrous CH_2Cl_2 (20 mL) were added solid 3-[2-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic acid (**6**) (200 mg, 0.415 mmol), triethylamine (TEA) (126 mg, 1.25 mmol) and the amine or the protected amino acid. This was mixed and cooled to 0 °C in an ice bath. BOPCl (159 mg, 0.62 mmol) was added all at once to the solid form. The reaction mixture was kept under continuous stirring and an argon atmosphere for 12 h while it was gradually allowed to reach ambient temperature. Solvent was removed under reduced pressure rotary evaporation, and the obtained residue was mixed with EtOAc (50 mL) and 5% aqueous citric acid solution (50 mL). The organic layer was separated and the aqueous layer was washed with EtOAc (50 mL × 3). The EtOAc extracts were combined and concentrated under reduced pressure rotary evaporation. The desired product was eluted from a silica gel column.

General Method II for Preparation of Amides. All glassware was flame dried, and the reaction was kept under argon atmosphere at all times. To anhydrous CH_2Cl_2 (50 mL) was added 3-[2-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic acid (**6**) (200 mg, 0.415 mmol), and the mixture was dissolved and cooled to 0 °C. To the cooled solution were added 4-DMAP (2 mg, 0.016 mmol) and EDC (119 mg, 0.62 mmol), and the reaction mixture was kept under continuous stirring for 15 min. The amine or the protected amino acid in the solid form was then added at once, and the reaction mixture was stirred for 24 h while it was gradually allowed to reach ambient temperature. The solvent was then removed under reduced pressure rotary evaporation, and the desired product was eluted from a silica gel column.

***p*-Anisidine-3-[2′-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Amide (7a).** Compound **7a** was prepared and purified according to the general method II for the preparation of amides as described above. To prepare compound **7a** the amine used was *p*-anisidine (56 mg, 0.456 mmol). The desired product was eluted from a silica gel column with EtOAc/hexane (80:20) and recrystallized from EtOAC/hexane (165 mg, 67%): ^{1}H NMR ($CDCl_3$) δ 8.30 (1 H, s), 7.36 (10 H, s), 7.18 (2 H, d, J = 8.88 Hz), 6.94 (1 H, s), 6.73 (2 H, d, J = 9.06 Hz), 6.68 (1 H, s), 5.18−5.12 (4 H, m), 3.74 (3 H, s), 2.68 (2 H, s), 2.45 (3 H, s), 2.11 (3 H, s), 1.67 (6 H, s); MS (FAB) m/z 588 ($M^+ + 1$). Anal. Calcd for $C_{34}H_{38}NO_6P$: C, 69.50; H, 6.47; N, 2.39. Found: C, 69.19; H, 6.80; N, 2.10.

GlyOMe-3-[2′-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Amide (7b). Compound **7b** was prepared and purified according to the general method I for the preparation of amides as described above. To prepare compound **7b** the amino acid used was GlyOMe (58 mg, 0.46 mmol). The desired white, crystalline solid product was obtained after elution from a silica gel column with EtOAc/hexane (80:20) (85 mg, 37%): ^{1}H NMR ($CDCl_3$) δ 7.34 (10 H, m), 7.03 (1 H, s), 6.72 (1 H, s), 6.54 (1 H, t, J = 5.58 Hz), 5.20−5.08 (4 H, m), 3.67 (2 H, d, J = 5.46 Hz), 3.62 (3 H, s), 2.67 (2 H, s), 2.49 (3 H, s), 2.15 (3 H, s), 1.62 (6 H, s); MS (FAB) m/z 554 ($M^+ + 1$). Anal. Calcd for $C_{30}H_{36}NO_7P$: C, 65.10; H, 6.51; N, 2.53. Found: C, 65.21; H, 6.89; N, 2.40.

L-PheOMe-3-[2′-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Amide (7c). Compound **7c** was prepared and purified according to the general method I for the preparation of amides as described above. To prepare compound **7c** the amino acid used was L-PheOMe (99 mg, 0.46 mmol). The desired product, a yellow oil, was obtained after elution from a silica gel column with EtOAc/hexane (80:20) (222 mg, 83%): ^{1}H NMR ($CDCl_3$) δ 7.36−7.25 (10 H, m), 7.19−7.17 (3 H, m), 7.07 (1 H, s), 6.93−6.90 (2 H, m), 6.70 (1 H, s), 6.53 (1 H, d, J = 7.71 Hz), 5.13−5.00 (4 H, m), 4.63 (1 H, q, J = 6.84 Hz), 3.56 (3 H, s), 2.87−2.70 (2 H, m), 2.61 (2 H, s), 2.47 (3 H, s), 2.15 (3 H, s), 1.59 (3 H, s), 1.58 (3 H, s); MS (FAB) m/z 644 ($M^+ + 1$). Anal. Calcd for $C_{37}H_{42}NO_7P$: C, 69.05; H, 6.53; N, 2.18. Found: C, 69.00; H, 6.80; N, 2.40.

Lys-ϵ-CBZ-α-OMe-3-[2′-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Amide (7d). Compound **7d** was prepared and purified according to the general method I for the preparation of amides as described above. To prepare compound **7d** the amino acid used was *N*-ϵ-CBZ-L-LysOMe (153 mg, 0.46 mmol). The desired product was obtained after elution from a silica gel column with EtOAc/hexane (50:50) (251 mg, 81%): ^{1}H NMR ($CDCl_3$) δ 7.41−7.28 (15 H, m), 6.98 (1 H, s), 6.77 (1 H, d, J = 7.32 Hz), 6.71 (1 H, s), 5.29 (1 H, m), 5.18−5.02 (6 H, m), 4.25−4.21 (1 H, m), 3.61 (3 H, s), 3.14−3.03 (2 H, m), 2.73 (1 H, d, J = 13.23 Hz), 2.47 (3 H, s), 2.46 (1 H, d, J = 13.14 Hz), 2.11 (3 H, s), 1.67 (3 H, s), 1.56 (3 H, s), 1.30−1.47 (4 H, m), 1.10−0.90 (2 H, m); MS (FAB) m/z 759 ($M^+ + 1$). Anal. Calcd for $C_{42}H_{51}N_2O_9P$: C, 66.49; H, 6.72; N, 3.69. Found: C, 66.09; H, 7.10; N, 3.98.

Asp-α-OMe-*β*-benzyl-3-[2′-(dibenzylphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Amide (7e). Compound **7e** was prepared and purified according to the general method I for the preparation of amides as described above. To prepare compound **7e** the amino acid used was Asp-α-OMe-*β*-Obzl (164 mg, 0.50 mmol). The synthesis of this protected amino acid is described later in this section. The desired product was eluted from a silica gel column with EtOAc/hexane gradient from 30−40% (218 mg, 75%): ^{1}H NMR ($CDCl_3$) δ 7.37−7.28 (15 H, m), 7.03 (1 H, S), 6.88 (1 H, d, J = 7.83 Hz), 6.69 (1 H, s), 5.20−5.06 (4 H, m), 5.05 (2 H, s), 4.72−4.66 (1 H, m), 3.55 (3 H, s), 2.72−2.51 (4 H, m), 2.48 (3 H, s), 2.11 (3 H, s), 1.63 (3 H, s), 1.59 (3 H, s); MS (FAB) m/z 702 ($M^+ + 1$).

(36) Khorana, H. G.; Todd, A. R. *J. Chem. Soc.* **1953**, 2257−2260.
(37) Fieser, L. F.; Fieser, M. *Reagents for Organic Synthesis*; John Wiley and Sons, Inc: New York, 1967; Vol. 1, p 142.

Anal. Calcd for $C_{39}H_{44}NO_9P$: C, 66.76; H, 6.28; N, 1.99. Found: C, 66.93; H, 6.10; N, 1.90.

General Procedure for Hydrogenation. Compounds **7a**−**e** were dissolved in either THF or CH_3OH. Ten percent Pd catalyst on activated carbon was added to the solution, and the reaction was placed under H_2 atmosphere. The reaction was kept under vigorous stirring for 12 h. The insoluble catalyst was removed by filtration, followed by solvent removal under reduced pressure rotary evaporation. The final solid product was obtained by either trituration in an anhydrous Et_2O and hexane mixture or lyophilization from a frozen aqueous solution.

***p*-Anisidine-3-[2′-(dihydroxyphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Amide (8a).** Compound **7a** (150 mg, 0.255 mmol) was dissolved in THF (20 mL) and treated according to the general procedure for Hydrogenation described above. The final product was obtained as a white amorphous powder after vigorous trituration in anhydrous Et_2O and hexane (80 mg, 77%): ^{1}H NMR (DMSO) δ 9.64 (1 H, s), 7.30−7.27 (2 H, m), 7.05 (1 H, S), 6.78 (2 H, d, J = 9.12 Hz), 6.62 (1 H, s), 3.68(3 H, s), 2.74 (2 H, s), 2.40 (3 H, s), 2.14 (3 H, m), 1.62 (6 H, s); MS (FAB) m/z 408 ($M^+ + 1$). Anal. Calcd for $C_{20}H_{26}NO_6P$: C, 58.96; H, 6.39; N, 3.44. Found: C, 58.48; H, 6.10; N, 3.24.

GlyOMe-3-[2′-(dihydroxyphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Amide (8b). Compound **7b** (85 mg, 0.153 mmol) was dissolved in CH_3OH (10 mL) and treated according to the general procedure for hydrogenation described above. The final product was obtained as a white amorphous powder (38 mg, 67%): ^{1}H NMR (CD_3OD) δ 7.16 (1 H, s), 6.69 (1 H, s), 3.77 (2 H, s), 3.65 (3 H, s), 2.83 (2 H, s), 2.49 (3 H, s), 2.21 (3 H, s), 1.66 (6 H, s); MS (FAB) m/z 374 ($M^+ + 1$). Anal. Calcd for $C_{16}H_{24}NO_7P$: C, 51.47; H, 6.43; N, 3.75. Found: C, 51.25; H, 6.57; N, 3.88.

L-PheOMe-3-[2′-(dihydroxyphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Amide (8c). Compound **7c** (160 mg, 0.25 mmol) was dissolved in THF (20 mL) and treated according to the general procedure for hydrogenenation described above. The final product was obtained as a white amorphous powder by trituration in Et_2O/hexane (96 mg, 83%): ^{1}H NMR (CD_3OD) δ 7.28−7.09 (6 H, m), 6.68 (1 H, s), 4.53−4.48 (1 H, m), 3.59 (3 H, s), 3.02−2.95 (1 H, m), 2.81−2.70 (3 H, m), 2.39 (3 H, s), 2.21 (3 H, s), 1.57 (3 H, s), 1.54 (3 H, s); MS (FAB) m/z 464 ($M^+ + 1$); HRMS (FAB) m/z 464.1823 ($M^+ + 1$, Calcd 464.1838). Anal. Calcd for $C_{23}H_{30}NO_7P \times 0.5\ H_2O$: C, 58.48; H, 6.56; N, 2.97. Found: C, 58.50; H, 6.76; N, 2.85.

Lys-ϵ-CBZ-α-OMe-3-[2′-(dihydroxyphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Amide (8d). Compound **7d** (120 mg, 0.16 mmol) was dissolved in CH_3OH (20 mL) and treated according to the general procedure for hydrogenation described above. The final product was obtained as a white amorphous solid after the obtained residue was dissolved in H_2O and subsequently lyophilized (43 mg, 80%):^{1}H NMR (D_2O) δ 7.14 (1 H, s), 6.74 (1 H, s), 4.07−405 (1 H, m), 3.63 (3 H, s), 3.02 (1 H, d, J = 13.2 Hz), 2.85 (2 H, t, J = 6.65 Hz), 2.60 (1 H, d, J = 13.08 Hz), 2.41 (3 H, s), 2.19 (3 H, s), 1.62 (3 H, s), 1.57 (3 H, s), 1.49−1.40 (4 H, m), 1.02−0.88 (2 H, m); MS (FAB) m/z 445 ($M^+ + 1$); HRMS (FAB) m/z 445.2120 ($M^+ + 1$, Calcd 445.2104). Anal. Calcd for $C_{20}H_{33}N_2O_7P \times 1\ H_2O$: C, 51.95; H, 7.14; N, 6.06. Found: C, 52.10; H, 7.40; N, 5.80.

Asp-α-OMe-β-benzyl-3-[2′-(dihydroxyphosphono)oxy-4′,6′-dimethylphenyl]-3,3-dimethylpropionic Amide (8e). Compound **7e** (160 mg, 0.23 mmol) was dissolved in THF (20 mL) and treated according to the general procedure for hydrogenation described above. The final product was obtained as a white amorphous powder after the obtained residue was dissolved in H_2O and subsequently lyophilized (60 mg, 61%):^{1}H NMR (D_2O) δ 7.11 (1 H, s), 6.72 (1 H, s), 4.59 (1 H, m), 3.62 (3 H, s), 2.94 (1 H, d, J = 14.28 Hz), 2.69 (1 H, d, J = 13.47 Hz), 2.54 (2 H, m), 2.42 (3 H, s), 2.18 (3 H, s), 1.60 (3 H, s), 1.59 (3 H, s); MS (FAB) m/z 432 ($M^+ + 1$). Anal. Calcd for $C_{18}H_{26}NO_9P$: C, 50.12; H, 6.03; N, 3.24. Found: C, 49.98; H, 6.30; N, 3.00.

***t*-Boc-Asp-β-Bzl-α-OMe.** *t*-Boc-Asp-β-Bzl (2 g, 6.2 mmol) was dissolved in CH_2Cl_2 (50 mL) and allowed to cool to 0 °C in an ice bath. EDC (1.55 g, 8.06 mmol) was added, and the reaction mixture was stirred for 15 min. CH_3OH (396 mg, 12.4 mmol) was then added to the mixture, and stirring was continued for 12 h. Solvent was removed under reduced pressure rotary evaporation, and EtOAc (50 mL) and H_2O (50 mL) were added to the residue. The organic layer was separated and further washed with a saturated aqueous NaCl solution (20 mL) and dried over $MgSO_4$. The desired product was recrystallized from EtOAc and hexane (1.34 g, 64%): ^{1}H NMR ($CDCl_3$) δ 7.35 (5 H, m), 5.49 (1 H, d, J = 8.31 Hz), 5.17−5.09 (2 H, m), 4.61−4.50 (1 H, m), 3.70 (3 H, s), 3.09−3.01 (1 H, m), 3.08−2.83 (1 H, m), 1.44 (9 H, s); MS (FAB) m/z 338 ($M^+ + 1$). Anal. Calcd for $C_{17}H_{23}NO_6$: C, 60.53; H, 6.83; N, 4.15. Found: C, 60.50; H, 6.98; N, 4.00.

Asp-α-OMe-β-Bzl. *t*-Boc-Asp-β-Bzl-α-OMe (500 mg, 1.48 mmol) was dissolved in 50 mL of CH_2Cl_2 and 1 mL of TEA. The reaction mixture was stirred for a period of 2 h. After all solvents were removed under reduced pressure rotary evaporation, the oily residue was suspended in 50 mL of 1 M HCl in Et_2O solution. After a few minutes, white crystals of the final product precipitated out. The final product was obtained as a crystalline white solid (303 mg, 86% yield): ^{1}H NMR ($CDCl_3$) δ 7.30 (5 H, m), 5.16 (2 H, s), 4.61 (1 H, m), 3.66 (3 H, s), 3.42−3.23 (2 H, s); MS (FAB) m/z 238 ($M^+ + 1$); HRMS (FAB) m/z 238.1077 ($M^+ + 1$, Calcd 238.1079).

Kinetics of Enzyme-Mediated Hydrolysis of Prodrug 8a. The kinetics of enzymatic hydrolysis of prodrug **8a** were determined by measuring the rate of formation of the lactone **2** after incubating a solution of **8a** (45 μmol) with human placenta AP (32.9 μg) in 100 mM MOPS buffer containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$, pH 7.4 (2.24 mL) at 37 °C. At specific time intervals, aliquots of the reaction mixture (200 μL) were removed and quenched by addition of 1 M $HClO_4$ solution in CH_3CN (200 μL). The quenched mixture was analyzed for both the disappearance of **8a** and appearance of the lactone **2** using an ODS hypersil HPLC column (C18, 150 × 24 mm). For **8a**, the column was equilibrated and eluted with 40% CH_3CN in 20 mM aqueous phosphate buffer, pH 3.0, containing 20 mM tetrabutylammonium (TBA) phosphate, the flow rate was 1 mL/min. For **2**, the quenched mixture was analyzed using the same column and flow rate, but the column was equilibrated and eluted with 60% CH_3CN in H_2O. Representative chromatograms are shown in Figure 2.

Michaelis−Menten Kinetics. For determination of Michaelis−Menten constants, various concentrations of substrates **8a**−**d** were incubated with AP (1.95 μg for **8a**, **8b**, and **8d**; 2.51 μg for **8c**) in 100 mM MOPS buffer, pH 7.4, in a total volume of 220 μL for 3 min at 25 °C. Each reaction was quenched with 1 M $HClO_4$ solution in CH_3CN (220 μL). AP solution was first prepared in H_2O containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$ and then diluted with 100 mM MOPS buffer, pH 7.4, to the desired enzyme concentration. The initial rates of formation of the lactone **2** were measured by HPLC as described above, except that the analysis of **2** was monitored at 225 nm to improve detection limits. It should be noted that since **8e** was not stable in MOPS buffer, pH 7.4, for prolonged periods of time, solutions of this phosphate prodrug at various concentrations were prepared in H_2O (100 μL) and the reaction was initiated by mixing the substrate solution with an equal volume of enzyme solution (1.78 μg in AP) in MOPS buffer (100 mM, pH 7.4). After incubation for 3 min, the reaction was quenched by addition of 2 M acetic acid in CH_3CN (200 μL) to avoid degradation prior to HPLC sampling. The Michaelis−Menten parameters, K_m and V_{max} values, were obtained by fitting the data to the Michaelis−Menten equation using a computer program.

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