Phosphinate Inhibitors of the D-Glutamic Acid-Adding Enzyme of Peptidoglycan Biosynthesis

Martin E. Tanner,*,† Sabine Vaganay,‡ Jean van Heijenoort,‡ and Didier Blanot‡

Department of Chemistry, University of British Columbia, Vancouver, British Columbia, V6T 1Z1, Canada and URA 1131 du CNRS, Université de Paris-Sud, 91405 Orsay, France

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We report the synthesis and initial evaluation of the first effective inhibitors of the D-glutamic acid-adding enzyme (UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase or MurD). This enzyme plays a key role in bacterial peptidoglycan biosynthesis and is therefore a target for antibiotic design. Phosphinic acid **3** is a dipeptide analog linked to uridine diphosphate by a hydrophobic spacer. It is a good inhibitor of the enzyme ($IC_{50} = 0.68\ \mu M$) as it closely resembles the tetrahedral intermediate that is presumed to form in the ligation reaction. Compound **4** lacks the terminal UMP group, and compound **5** lacks both the linker and UDP functionalities. These are less effective inhibitors of the enzyme with IC_{50} values of 29 μM and >1 mM, respectively. Preincubation of the enzyme in the presence of inhibitor **3** and ATP does not result in irreversible inhibition or in the formation of a slowly decomplexing species, suggesting that the phosphinic acid is not phosphorylated in the active site.

Introduction

Many antibiotics, such as penicillins, cephalosporins, vancomycin, and D-cycloserine, are known to interfere with bacterial peptidoglycan construction.[1] Peptidoglycan is a network of polysaccharides that are cross-linked by D-amino acid-containing polypeptides. It serves to impart rigidity to the cell wall and protects bacteria from osmotic lysis. The UDP-*N*-acetylmuramic acid (UDP-MurNAc)[2] pentapeptide **1** (Figure 1) is a common precursor in the biosynthesis of peptidoglycan in both Gram-positive and Gram-negative bacteria, and therefore enzymes involved in its biosynthesis serve as attractive targets for the design of broad spectrum antibiotics.[3] Compound **1** is biosynthesized from UDP-MurNAc to which is sequentially added L-Ala, D-Glu, *meso*-Dap (or L-Lys), and finally the dipeptide D-Ala-D-Ala by a series of ATP-dependent amino acid ligases. In this paper we describe the syntheses of, and initial studies on, the first examples of effective mechanism-based inhibitors of the second of these ligases, the D-glutamic acid-adding enzyme (Figure 1).

ATP-dependent amide-forming enzymes are thought to operate by catalyzing an initial phosphorylation of the acid carboxylate (Figure 2).[4−7] The resulting acyl phosphate is subsequently attacked by the amine to produce a tetrahedral intermediate which ultimately collapses to the amide and inorganic phosphate. In several cases it has been found that appropriately substituted phosphinic acids act as slow-binding inactivators of these enzymes.[6a,8,9] The remarkable feature of this inhibition is that the enzyme promotes the transfer of the γ-phosphate of ATP onto the phosphinate anion to produce ADP and a phosphorylated inhibitor. This process has been confirmed by X-ray diffraction analysis of the phosphorylated

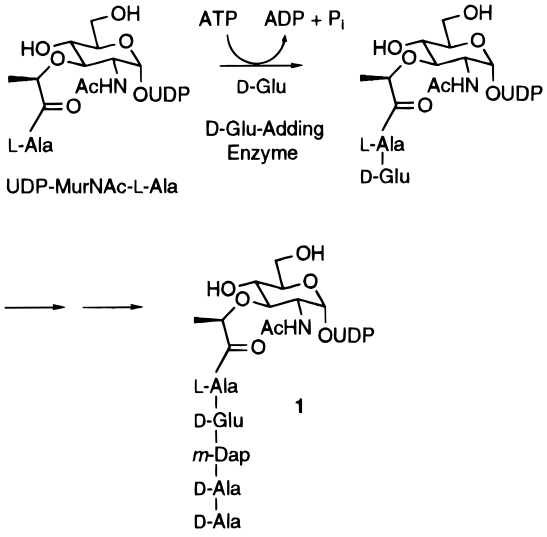

Figure 1. The structure of UDP-MurNAc pentapeptide **1** and the reaction catalyzed by the D-glutamic acid-adding enzyme.

†University of British Columbia.
‡Université de Paris-Sud.
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(1) Gale, E. F.; Cundliffe, E.; Reynolds, P. E.; Richmond, M. H.; Waring, M. J. *The Molecular basis of Antibiotic Action*, 2nd ed.; John Wiley and Sons: London, 1981.

(2) Abbreviations: UDP = uridine diphosphate; UMP = uridine monophosphate; *meso*-Dap = *meso*-diaminopimelic acid; TEA = triethylamine; HOBt = 1-hydroxybenzotriazole; DCI = desorption chemical ionization; LSI = liquid secondary ion.

(3) van Heijenoort, J. In *Bacterial Cell Wall*; Ghuysen, J.-M., Hakenbeck, R., Eds.; Elsevier Science B.V.: Amsterdam, 1994; pp 39−54.

(4) Glutamine synthetase: (a) Meek, T. D.; Johnson, K. A.; Villafranca, J. J. *Biochemistry* **1982**, *21*, 2158. (b) Meek, T. D.; Villafranca, J. J. *Biochemistry* **1980**, *19*, 5513. (c) Midelfort, C. F.; Rose, I. A. *J. Biol. Chem.* **1976**, *251*, 5881. (d) Meister, A. In *The Enzymes*; 3rd ed., Boyer, P. D., Ed.; Academic Press: New York, 1974; Vol. 10, pp 699−754.

(5) D-Ala-D-Ala ligase: (a) Shi, Y.; Walsh, C. T. *Biochemistry* **1995**, *34*, 2768. (b) Fan, C.; Moews, P. C.; Walsh, C. T.; Knox, J. R. *Science* **1994**, *266*, 439. (c) Wright, G. D.; Walsh, C. T. *Acc. Chem. Res.* **1992**, *25*, 468. (d) Mullins, L. S.; Zawadzke, L. E.; Walsh, C. T.; Raushel, F. M. *J. Biol. Chem.* **1990**, *265*, 8993.

(6) Glutathione synthetase: (a) Hiratake, J.; Kato, H.; Oda, J. *J. Am. Chem. Soc.* **1994**, *116*, 12059. (b) Kato, H.; Tanaka, T.; Yamaguchi, H.; Hara, T.; Nishioka, T.; Katsube, Y.; Oda, J. *Biochemistry* **1994**, *33*, 4995. (c) Meister, A. In *The Enzymes*; 3rd ed., Boyer, P. D., Ed.; Academic Press: New York, 1974; Vol. 10, pp 671−697.

(7) The multienzyme systems involved in microbial peptide biosyntheses often utilize an alternative thiotemplate mechanism. An initial formation of an aminoacyl adenylate is followed by the formation of a thioester and then an amide. For leading references see: Stein, T.; Vater, J.; Kruft, V.; Wittmann-Liebold, B.; Franke, P.; Panico, M.; Mc Dowell, R.; Morris, H. R. *FEBS Lett.* **1994**, *340*, 39. Preliminary studies indicate that the D-glutamic acid-adding enzyme is not following such a mechanism.[11]

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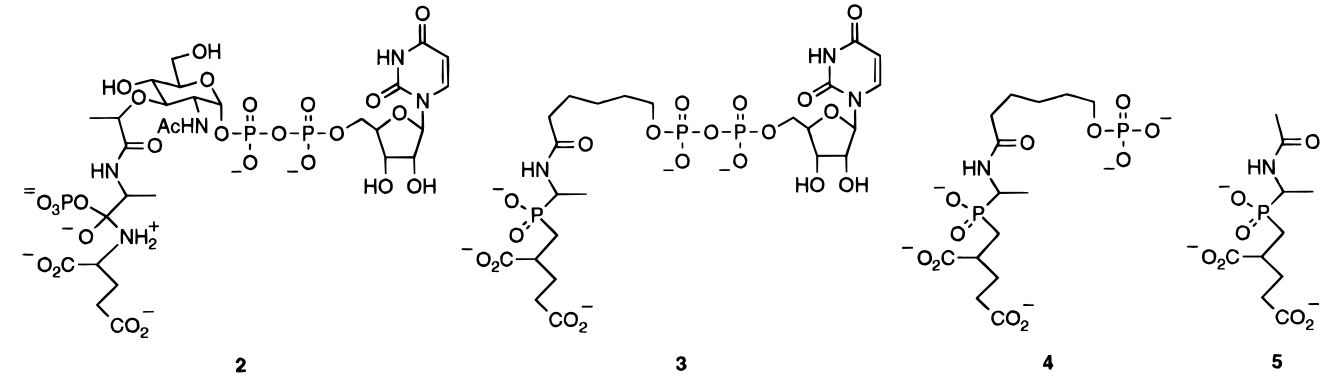
Chart 1



2 **3** **4** **5**

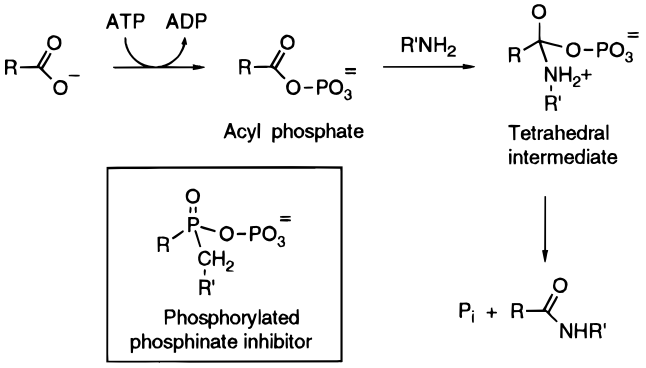


Figure 2. The proposed mechanism followed by ATP-dependent amide-forming enzymes and the general structure of a phosphorylated phosphinate inhibitor.

inhibitor−enzyme complex in the cases of D-Ala-D-Ala ligase[5b] and glutathione synthetase.[6a] The resulting phosphoryl phosphinate moiety closely mimics the tetrahedral intermediate formed in the normal reaction pathway and is tightly bound by the enzyme. In the case of D-Ala-D-Ala ligase, an enzyme-inhibitor decomplexation half-life of 17 days was measured, which renders this noncovalent inhibition effectively irreversible.[9a]

The D-glutamic acid-adding enzyme (UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase or MurD) catalyzes the addition of D-glutamate to the free carboxylate of UDP-MurNAc-L-Ala (Figure 1).[10] Although relatively few mechanistic studies[11] have been performed with this enzyme, and no effective inhibitors are known,[12] it is reasonable to assume that the mechanism outlined in Figure 2 is followed. This would mean that the tetrahedral intermediate **2** is formed in the enzyme active site and that analogs of this intermediate would therefore inhibit the enzyme. With this goal in mind, inhibitor **3** was designed. Inhibitor **3** contains a phosphinic acid that has a tetrahedral geometry at the "dipeptide" center, which could be enzymatically phosphorylated to give a close analog of intermediate **2**. It also retains the charged UDP moiety which is likely to be important for binding. The muramoyl residue has been replaced by a hydrophobic linker of appropriate length for ease of synthesis. The truncated inhibitors **4** and **5** are also of interest in that they can provide information on the importance of the UDP and the linker/phosphate groups to the observed inhibition. We have synthesized compounds **3**-**5** as a mixture of diastereomers about the "peptide" chiral centers and tested them as inhibitors of the purified D-glutamic acid-adding enzyme from *Escherichia coli*.

Results

Compounds **3**-**5** were synthesized from the known phosphonous methyl ester **6** (Scheme 1).[9c,13] Treatment of racemic **6** with sodium methoxide and dimethyl 2-methylenepentanedioate[14] provided a good yield of compound **7** as a mixture of diastereomers. Hydrogenolysis of the carbobenzyloxy protecting group provided the free amine which was unstable to heat or prolonged storage at room temperature and was used directly in subsequent reactions. Acylation of the amine with acetyl chloride gave compound **8** which was deprotected under basic conditions to afford compound **5**. Compound **5** prepared in this fashion was found to be an equimolar mixture of the four stereoisomers possible from the two "peptide" chiral centers (two pairs of enantiomers) as indicated by the presence of two phosphinate ^{31}P NMR signals of approximately equal intensity.

Hydrogenation of **7** followed by a DCC/1-hydroxybenzotriazole-mediated coupling of the resulting amine and acid **9** provided compound **10** in good yield. Deprotection of **10** was brought about by hydrogenation over PtO_2 at 50 psi followed by treatment with NaOH. This produced compound **4** which was purified by size exclusion chromatography. Compound **3** was prepared using the standard Khorana coupling conditions[15] with the trioctylammonium salt of **4** and uridine 5′-monophosphomorpholidate in pyridine and was purified by preparative

(8) Phosphinothricin: (a) Abell, L. M.; Villafranca, J. J. *Biochemistry* **1991**, *30*, 6135. (b) Colanduoni, J. A.; Villafranca, J. J. *Bioorg. Chem.* **1986**, *14*, 163. (c) Bayer, E.; Gugel, K. H.; Hagele, K.; Hagenmaier, H.; Jessipow, S.; Konig, W. A.; Zahner, H. *Helv. Chim. Acta* **1972**, *55*, 224.

(9) D-Ala-D-Ala ligase inhibition: (a) McDermott, A. E.; Creuzet, F.; Griffin, R. G.; Zawadzke, L. E.; Ye, Q. Z.; Walsh, C. T. *Biochemistry* **1990**, *29*, 5767. (b) Duncan, K.; Walsh, C. T. *Biochemistry* **1988**, *27*, 3709. (c) Parsons, W. H.; Patchett, A. A.; Bull, H. G.; Schoen, W. R.; Taub, D.; Davidson, J.; Combs, P. L.; Springer, J. P.; Gadebusch, H.; Weissberger, B.; Valiant, M. E.; Mellin, T. N.; Busch, R. D. *J. Med. Chem.* **1988**, *31*, 1772. See also references 5a and 5b.

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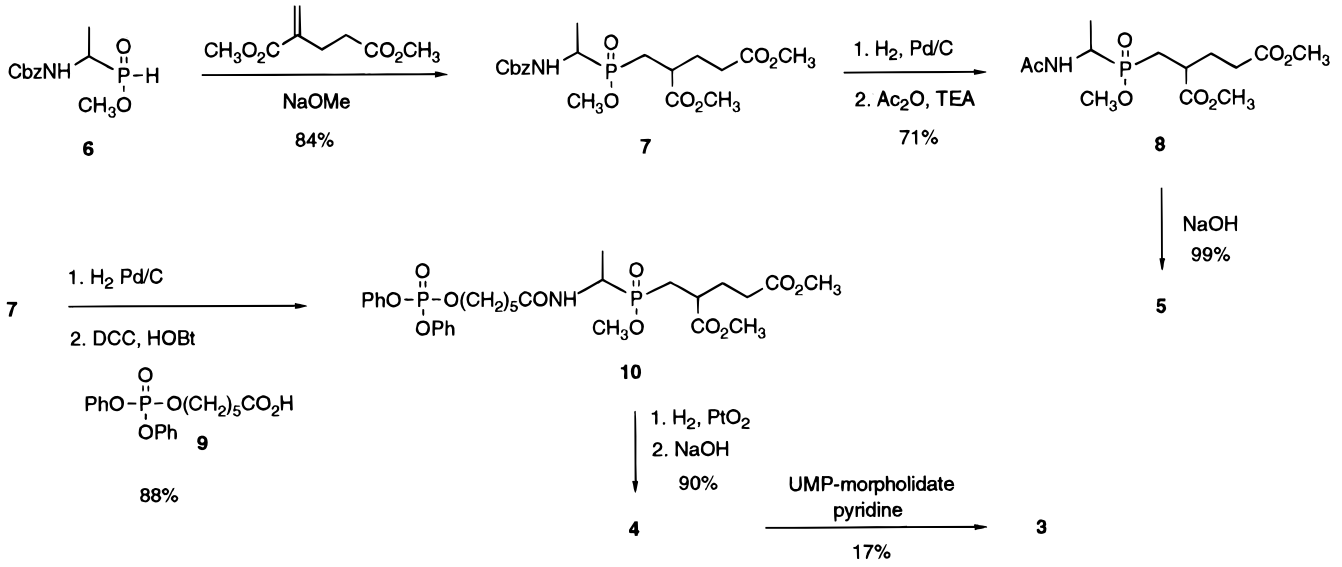
(15) Roseman, S.; Distler, J. J.; Moffatt, J. G.; Khorana, H. G. *J. Am. Chem. Soc.* **1961**, *83*, 659.

Scheme 1



layer chromatography followed by size exclusion chromatography. It is interesting to note that the phosphate coupling reaction can be successfully carried out in the presence of several carboxylates and a phosphinate functionality. Compounds **3** and **4** were also isolated as equimolar mixtures of the four possible stereoisomers as indicated by the phosphinate signals in their ^{31}P NMR spectra. Attempts to separate the diastereomers by reverse-phase HPLC were unsuccessful.[16]

Compounds **3**-**5** were tested for their ability to inhibit the addition of D-[^{14}C]Glu to UDP-MurNAc-L-Ala catalyzed by recombinant *E. coli* D-glutamic acid-adding enzyme. The concentration of inhibitor necessary to reduce the rate of addition by one-half (IC_{50} value) was determined in the presence of 25 μM UDP-MurNAc-L-Ala (K_m = 7.5 μM) and 25 μM D-Glu (K_m = 55 μM). Compound **3** was found to be a very good inhibitor of the enzyme with an IC_{50} = 0.68 μM. Compound **4** was a weaker inhibitor with an IC_{50} = 29 μM. Compound **5** was a poor inhibitor of the enzyme (IC_{50} > 1 mM), and the addition of UDP-*N*-acetylglucosamine (1 mM) did not significantly increase the inhibitory activity (IC_{50} $\approx$ 1 mM). Preincubation of the enzyme and **3**, with and without added ATP, followed by dilution into an assay mixture and kinetic analysis, showed no signs of irreversible inhibition or slow decomplexation rates.

Discussion

The observation that compound **3** is a good inhibitor of the D-glutamic acid-adding enzyme is consistent with the notion that the enzyme will strongly bind species containing a tetrahedral geometry and an oxyanion between the two amino acid units (as in the putative intermediate **2**). It also indicates that the muramoyl sugar is not a crucial recognition element for the enzyme and that hydrophobic linkers are accommodated in its place. Since the sample consisted of an equimolar mixture of four stereoisomers, and since the one most closely mimicking the L-Ala-D-Glu stereochemistry would presumably be bound the strongest, the actual IC_{50} for the best stereoisomer may be as much as four-fold lower than that observed. The absence of any irreversible inhibition or slow decomplexation rates indicates that **3** is behaving differently than other reported phosphinate inhibitors and is possibly not being phosphorylated in the active site. An ATP/**3** complex may closely resemble an ADP/**2** complex and account for the observed inhibition.

Compound **4** was also a good inhibitor of the enzyme but needed to be present at concentrations 43-fold higher than **3** in order to show comparable inhibition. This indicates that it is important, but not crucial, to retain the terminal UMP functionality when designing inhibitors of this enzyme. This also agrees well with the observation that 1-phospho-MurNAc-L-Ala is a substrate for the enzyme and has a K_m value 36-fold higher than the natural substrate, UDP-MurNAc-L-Ala.[10b] Compound **5** was a poor inhibitor of the enzyme since it lacked both the hydrophobic spacer and the UDP functionality. Attempts at increasing the potency of **5** by including UDP-*N*-acetylglucosamine in the medium (which should be accommodated in the active site as a coinhibitor without any steric problems) met with limited success. This is likely due to the entropic costs of binding two separate ligands, and points out the importance of maintaining a covalent bond between the "sugar" and "peptide" portions when designing inhibitors of this enzyme.[17] Analogs of **3** that contain a muramoyl sugar in place of the lipophilic spacer will likely act as very potent inhibitors of, and may be phosphorylated by, the D-glutamic acid-adding enzyme.

Experimental Section

General Procedures. Chemicals were purchased from Aldrich Chemical Co. or Sigma Chemical Co. UDP-MurNAc-L-Ala was prepared according to Michaud et al.[10b] D-[^{14}C]Glu was obtained after treatment of D,L-[^{14}C]Glu (NEN, Du Pont de Nemours, Les Ulis, France; 2 GBq/mmol) with L-glutamate decarboxylase and purification by paper rheophoresis. Ion exchange and gel chromatographic supports were from Bio-Rad Laboratories. Silica gel (230-400 mesh) was from BDH Inc. and preparative layer chromatography plates (2 mm, silica gel 60 F_{254}) were from Merck. Pyridine, CH_2Cl_2, and TEA were distilled under N_2 from CaH_2. MeOH was distilled under

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(17) Jencks W. P. *Catalysis in Chemistry and Enzymology*; Dover Publications, Inc.: New York, 1975; pp 615−806.

N_2 from magnesium methoxide. DMF was dried over 4 Å molecular sieves and vacuum degassed before use. All reactions performed in organic solvents were carried out under 1 atm of argon unless otherwise specified. All compounds were prepared for microanalysis by heating at 70 °C (0.1 mmHg) for 48 h, except compound **3** which was dried at rt (0.1 mmHg) for 48 h. Microanalyses were carried out in the microanalytical laboratory at the University of British Columbia by Mr. Peter Borda.

Methyl [1-[(Benzyloxycarbonyl)amino]ethyl](2,4-dicarbomethoxybutyl)phosphinate (7). To a solution of methyl [1-[(benzyloxycarbonyl)amino]ethyl]phosphinate (**6**) (1.78 g, 6.9 mmol) in 15 mL of dry MeOH at 0 °C was added a solution of sodium methoxide in MeOH (3.8 mL of a 2 M solution). The reaction was stirred for 5 min and neat dimethyl 2-methylenepentanedioate (1.1 mL, 6.9 mmol) was added dropwise. The reaction was stirred at 0 °C for 15 min and then allowed to warm to rt and stirred for an additional 4 h. The reaction was quenched with 1 M HCl (150 mL) and extracted twice with CH_2Cl_2. The combined organic extracts were dried over $MgSO_4$, and the solvent was removed *in vacuo*. The residue was purified by silica gel chromatography (MeCN−EtOAc 1:9 v/v) to give 2.5 g (84%) of **8** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.34 (m, 5H), 5.34−5.08 (3 brd, 1H), 5.10 (s, 2H), 4.05 (brm, 1H), 3.68 (m, 9H), 2.83 (brm, 1H), 2.35−1.17 (m, 6H), 1.39−1.27 (m, 3H); ^{31}P NMR ($CDCl_3$) δ 52.84 (s), 52.03 (s); DCI-MS (NH_3) 430 (M + H$^+$, 100%); Anal. Calcd for $C_{19}H_{28}NO_8P$: C, 53.14; H, 6.57; N, 3.26. Found: C, 52.83; H, 6.66; N, 3.40.

Methyl [1-Acetamidoethyl](2,4-dicarbomethoxybutyl)phosphinate (8). A solution of methyl [1-[(benzyloxycarbonyl)amino]ethyl](2,4 dicarbomethoxybutyl)phosphinate (**7**) (363 mg, 0.85 mmol) in 5 mL of MeOH containing 5% Pd/C (70 mg) was stirred under 1 atm of hydrogen at room temperature for 11 h. The mixture was filtered, and the solvent was removed under reduced pressure to give 245 mg of a colorless oil. This was dissolved in 10 mL of dry CH_2Cl_2, and triethylamine (0.5 mL) and acetyl chloride (0.070 mL, 0.98 mmol) were added. The reaction was stirred for 2 h at rt, and the solvent was removed *in vacuo*. The residue was taken up in minimal EtOAc and filtered to remove the insoluble salts. This solution was loaded directly onto a silica gel column and eluted with MeOH−EtOAc (1:19 v/v). This procedure afforded 200 mg (71%) of **8** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 6.47−6.05 (2 brd, 1H), 4.42 (m, 1H), 3.76−3.65 (m, 9H), 2.83 (brm, 1H), 2.36−2.16 (m, 3H), 2.02 (s, 3H), 2.01−1.80 (m, 3H), 1.39−1.26 (m, 3H); ^{31}P NMR ($CDCl_3$) δ 53.38 (s), 52.54 (s), 52.45 (s), 52.37 (s); DCI-MS (NH_3) 338 (M + H$^+$, 100%); Anal. Calcd for $C_{13}H_{24}NO_7P$: C, 46.29; H, 7.17; N, 4.15. Found: C, 46.29; H, 7.09; N, 4.03.

(1-Acetamidoethyl)(2,4-dicarboxybutyl)phosphinic acid (5). A solution of methyl (1-acetamidoethyl)(2,4 dicarbomethoxybutyl)phosphinate (**8**) (320 mg, 0.95 mmol) in 10 mL of 0.5 M NaOH was stirred at rt for 3 h. The solution was neutralized by the addition of AG 50W-X8 resin (H$^+$ form, 20−50 mesh) and filtered. The pH was then readjusted to 10 with 2 M NaOH, and the solution was applied to a column (10 mL) of AG 1-X8 (formate form, 100-200 mesh). The column was washed with water (50 mL) and 0.5 M formic acid (50 mL), and the product was eluted with 4 M formic acid (80 mL). The latter fraction was evaporated to dryness *in vacuo* to yield 280 mg (99%) of the free acid **5** as a hygroscopic foam. For use in inhibition studies the trisodium salt was prepared by dissolving the free acid in 15 mL of water and adjusting the pH to 7.5 with 0.5 M NaOH. The volume was reduced to 5 mL under reduced pressure, and 10 mL of ethanol and 2 mL of acetone were added. The sample was cooled at −20 °C overnight, and the resulting oil was obtained by decanting the solvent. The oil was dissolved in 5 mL of water and lyophilized to dryness to yield the salt as a white solid: ^{1}H NMR of free acid (D_2O) δ 4.11 (m, 1H), 2.71 (brm, 1H), 2.39 (t, 2H, J = 7.3 Hz), 2.12−1.75 (m, 4H), 1.96 (s, 3H), 1.23 (dd, 3H, J = 7.4, 14.5 Hz); ^{31}P NMR of free acid (D_2O) δ 48.67 (s), 48.38 (s). Anal. Calcd for $C_{10}H_{18}NO_7P$ (free acid): C, 40.68; H, 6.15; N, 4.74. Found: C, 41.00; H, 6.26; N, 4.65. +LSIMS of trisodium salt (thioglycerol/H_2O matrix) 340 (M − Na$^+$ + 2H$^+$, 100%), 362 (M + H$^+$, 80%), 318 (M − 2Na$^+$ + 3H$^+$, 70%), 384 (M + Na$^+$, 70%); Anal. Calcd for $C_{10}H_{15}NO_7PNa_3 \cdot H_2O$: C, 31.68; H, 4.52; N, 3.69. Found: C, 31.95; H, 4.19; N, 3.75.

5-Carboxypentyl Diphenyl Phosphate (9). To a solution of 6-hydroxyhexanoic acid[18] (3.3 g, 25 mmol) in 50 mL of dry pyridine was slowly added diphenyl phosphorochloridate (6.7 g, 25 mmol). The mixture was stirred at rt for 12 h, and the solvent was removed *in vacuo*. The residue was dissolved in CH_2Cl_2 and extracted twice with 1 M HCl. The organic layer was dried over $MgSO_4$, and the solvent was removed *in vacuo*. The residue was purified by silica gel chromatography (MeOH−CH_2Cl_2 1:19 v/v) to give 4.6 g (50%) of **9** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.4−7.1 (m, 10H), 4.23 (dt, 2H, J = 7.5 Hz, J = 6.4 Hz), 2.29 (t, 2H, J = 7.2 Hz), 1.64 (m, 4H), 1.38 (m, 2H); ^{31}P NMR ($CDCl_3$) δ −12.02 (s); DCI-MS (NH_3) 365 (M + H$^+$, 100%); Anal. Calcd for $C_{18}H_{21}O_6P$: C, 59.34; H, 5.81. Found: C, 58.97; H, 5.90.

Methyl [1-[6-[(Diphenoxyphosphinyl)oxy]hexanamido]ethyl](2,4-dicarbomethoxybutyl)phosphinate (10). A solution of methyl [1-[(benzyloxycarbonyl)amino]ethyl](2,4 dicarbomethoxybutyl)phosphinate **7** (1.04 g, 2.4 mmol) in 10 mL of MeOH containing 5% Pd/C (200 mg) was stirred under 1 atm of hydrogen at rt for 11 h. The mixture was filtered, and the solvent was removed *in vacuo* to give 702 mg of the amine as a colorless oil. In a separate flask, a solution of 5-carboxypentyl diphenyl phosphate (**9**) (0.862 g, 2.4 mmol) in CH_2Cl_2/DMF (4:1 v/v, 12.5 mL) was prepared. To this solution was added 1-hydroxybenzotriazole•H_2O (0.351 g, 2.6 mmol) and dicyclohexylcarbodiimide (0.513 g, 2.5 mmol). The mixture was stirred at rt for 1 h during which time a white precipitate formed. A solution of the amine in DMF (15 mL) was then added, and the mixture was allowed to stir for 24 h at rt. The reaction was filtered and the filtrate was partitioned between water (120 mL) and CH_2Cl_2 (120 mL). The organic layer was back-extracted with water (120 mL) and then dried over $MgSO_4$. The solvent was removed *in vacuo*, and the residue was dissolved in EtOAc (5 mL) and applied to a column (100 mL) of silica gel. The column was washed with EtOAc (300 mL) and the product was eluted with MeOH−EtOAc (2:23 v/v) to give 1.34 g (88%) of **10** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.37−7.13 (m, 10H), 6.24−5.85 (4 brd, 1H), 4.43 (m, 1H), 4.23 (dt, 2H, J = 7.6 Hz, J = 6.6 Hz), 3.72−3.60 (m, 9H), 2.82 (brm, 1H), 2.30 (t, 2H, J = 7.2 Hz), 2.15 (m, 3H), 1.98−1.58 (m, 7H), 1.41−1.23 (m, 5H); ^{31}P NMR ($CDCl_3$) δ 53.10 (s), 52.40 (s), 52.32 (s), −11.96 (s); DCI-MS (NH_3) 642 (M + H$^+$, 100%); Anal. Calcd for $C_{29}H_{41}NO_{11}P_2$: C, 54.29; H, 6.44; N, 2.18. Found: C, 54.05; H, 6.38; N, 2.20.

[1-(6-Phosphonohexanamido)ethyl](2,4-dicarboxybutyl)phosphinate Trisodium Salt (4). A solution of compound **10** (0.635 g, 0.99 mmol) in 15 mL of MeOH containing PtO_2 monohydrate (160 mg) was shaken under 50 psi of hydrogen at rt for 20 h. The mixture was filtered, and the solvent was removed *in vacuo* to give 513 mg of the phosphate as a colorless oil. This was dissolved in 0.5 M NaOH (20 mL) and stirred at rt for 3 h. The solution was brought to pH 5 by the addition of AG 50W-X8 resin (H$^+$ form, 20−50 mesh) and filtered. The pH was adjusted to 7 with 0.5 M NaOH, and the volume was reduced to 10 mL under reduced pressure. To this solution was added 10 mL of ethanol and 10 mL of acetone. The sample was cooled at −20 °C overnight, and the resulting oil was obtained by decanting the solvent. The oil was dissolved in water (6 mL) and applied, in three separate portions, to a column (250 mL) of Bio-Gel P-2 (fine). The product was eluted with water, and the fastest moving fractions were lyophilized to dryness to give 457 mg (90%) of **4** (trisodium salt) as a white solid: ^{1}H NMR (D_2O) δ 3.95 (m, 1H), 3.80 (dt, 2H, J = 6.6 Hz, J = 6.6 Hz), 2.54 (brm, 1H), 2.25 (t, 2H, J = 7.2 Hz), 2.17 (t, 2H, J = 8.2 Hz), 1.86 (m, 2H), 1.59 (m, 6H) 1.34 (m, 2H) 1.18 (dd, 3H, J = 7.3, J = 13.2 Hz); ^{31}P NMR (D_2O) δ 39.64 (s), 39.53 (s), 0.79 (s); +LSIMS (thioglycerol/H_2O matrix) 536 (M + Na$^+$, 100%), 558 (M +

(18) This compound was prepared by refluxing a solution of caprolactone in 4 N NaOH for 1 h, washing with CH_2Cl_2, acidifying with HCl, and extracting with ether. The organic phase was removed *in vacuo*, and the resulting clear oil was used without further purification.

$2Na^+ - H^+$, 95%), 514 ($M + H^+$, 50%), 580 ($M + 3Na^+ - 2H^+$, 45%). Anal. Calcd for $C_{14}H_{24}NO_{11}P_2Na_3 \cdot 0.5H_2O$: C, 32.20; H, 4.82; N, 2.68. Found: C, 32.24; H, 4.56; N, 2.57.

[1-[(6-Uridinediphospho)hexanamido]ethyl](2,4-dicarboxybutyl)phosphinate Pentasodium Salt (3). A solution of compound **4** (0.192 g, 0.37 mmol) in 10 mL of water was acidified by the addition of AG 50W-X8 resin (H^+ form, 20−50 mesh) and filtered. Pyridine (6 mL) was added, and the volume was decreased to 5 mL under reduced pressure. A solution of trioctylamine (0.820 mL, 1.9 mmol) in 10 mL of pyridine was added, and the solvent was removed under reduced pressure. Three successive additions of pyridine (15 mL) followed by evaporation to dryness under reduced pressure were used to remove the residual water. In a separate flask a sample of uridine 5′-monophosphomorpholidate (0.308 g, 0.45 mmol) was dried by three successive additions of pyridine (10 mL) followed by evaporation under reduced pressure. The morpholidate was dissolved in 15 mL of pyridine and added to the trioctylammonium salt of **4**. The reaction was stirred at rt for 48 h. An additional sample of uridine 5′-monophosphomorpholidate (0.150 g, 0.22 mmol) was dried as before and added to the reaction in 5 mL of pyridine. The reaction was stirred for an additional 24 h, and the volume was decreased to 3 mL under reduced pressure. Preparative layer chromatography (2-isopropanol−water−ammonium hydroxide, 7:3:1 v/v) and extraction (2-isopropanol−water, 4:1 v/v) of the slowest band visible under UV light afforded a mixture containing **3**, **4**, and uridine 5'-monophosphate. This mixture was applied to a column (250 mL) of Bio-Gel P-2 (fine) and eluted with water. The fastest moving UV active fractions were collected, concentrated under reduced pressure, passed through a column (5 mL) of AG 50W-X8 resin (Na^+ form, 20−50 mesh) and a column (5 mL) of Chelex 100 (Na+ form, 50−100 mesh), and then lyophilized to dryness. This afforded 60 mg of **3** (17% yield calculated as pentasodium salt pentahydrate) containing a 15% impurity of **4** as a white solid. Selected fractions (20 mg) containing <5% of **4** were further purified on a second column (250 mL) of Bio-Gel P-2 (fine) followed by treatment with AG 50W-X8 resin and Chelex 100 to afford 11 mg of pure **3** as a white solid: ^{1}H NMR (D_2O) δ 7.94 (d, 1H, J = 8.1 Hz), 5.95 (d, 1H, J = 2.9 Hz), 5.93 (d, 1H, J = 8.1 Hz), 4.33 (d, 2H, J = 3.6 Hz), 4.24 (m, 1H), 4.18 (m, 2H), 3.91 (m, 1H), 3.88 (dt, 2H, J = 6.5 Hz, J = 6.6 Hz), 2.43 (brm, 1H), 2.24 (t, 2H, J = 7.4 Hz), 2.14 (t, 2H, J = 8.6 Hz), 1.67 (m, 2H), 1.59 (m, 6H) 1.34 (m, 2H) 1.18 (dd, 3H, J = 7.7, 13.7 Hz); ^{31}P NMR (D_2O) δ 39.90 (s), 39.62 (s), −10.57 (d, J = 21.3 Hz), −11.29 (d, J = 21.1 Hz); +LSIMS (thioglycerol/H_2O matrix) 864 ($M + H^+$, 100%), 842 ($M - Na^+ + 2H^+$, 80%), 886 ($M + Na^+$, 70%), 908 ($M + 2Na^+ - H^+$, 35%) 820 ($M - 2Na^+ + 3H^+$, 35%); HR+LSIMS Calcd for $C_{23}H_{34}N_3O_{19}P_3Na_5$ 864.0488. Found: 864.0505; Anal. Calcd for $C_{23}H_{33}N_3O_{19}P_3Na_5 \cdot 5H_2O$: C, 28.97; H, 4.55; N, 4.41. Found: C, 29.10; H, 4.65; N, 4.14.

Inhibition Studies. The enzyme used in this study was purified from overproducing strain *E. coli* JM83 (pMLD58) as described elsewhere.[11] The concentrations of stock solutions of **3** were calculated from A_{262} measurements using ϵ = 9890 M^{-1} cm^{-1}. The D-glutamic acid-adding activity was assayed by following the appearance of UDP-MurNAc-L-Ala-D-[^{14}C]Glu in a mixture containing, in a final volume of 50 μL, 0.1 M Tris/HCl, pH 8.6, 5 mM $MgCl_2$, 5 mM ATP, 25 μM UDP-MurNAc-L-Ala, 25 μM D-[^{14}C]Glu (0.88 KBq), enzyme, and inhibitor. The mixture was incubated for 30 min at 37 °C, and the reaction was stopped by the addition of 10 μL glacial acetic acid. The radioactive substrate and product were separated by HPLC and quantitated by on-line scintillation counting as previously described.[12a] Preincubation experiments were carried out for 10 min in the presence of 3 mM **3** and 5 mM ATP. The samples were then diluted 25 fold and assayed directly.

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