(3-Amino-2-oxoalkyl)phosphonic Acids and Their Anlogues as Novel Inhibitors of D-Alanine:D-alanine Ligase

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The dipeptide D-alanyl-D-alanine is an essential precursor of bacterial peptidoglycan; thus, blocking its formation is a possible target for the design of novel antibacterial agents. The synthesis of this dipeptide by bacterial D-alanine:D-alanine ligase requires ATP. In analogy with glutamine synthetase, we hypothesized a mechanism for this enzyme involving the intermediacy of D-alanyl phosphate. Several (3-amino-2-oxoalkyl)phosphonic acids and their analogues have been synthesized as possible inhibitory mimics of this proposed intermediate. The most active of them, (3(R)-amino-2-oxobutyl)phosphonic acid (**8a**) and the corresponding aza analogue (**22**), were effective ligase inhibitors although they had no significant antibacterial activity. The ligase inhibition of these compounds is consistent with an acyl phosphate displacement step in the mechanism of DAla-DAla ligase.

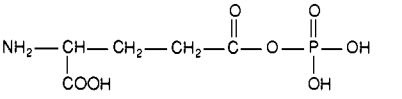
Many clinically useful antibacterial agents display their selective toxicity by interfering with the synthesis of peptidoglycan in bacteria.[1] Peptidoglycan consists of polysaccharide strands in parallel chains that are cross-linked by short peptides. The precursor unit of peptidoglycan, uridine 5′-diphosphate–*N*-acetylmuramyl-LAla-DGlu-Dap(Lys)–DAla–DAla (UDP-NAM-pentapeptide) (**1**), is assembled in the cytoplasm via a multienzyme pathway involving the addition of D-alanyl-D-alanine (**2**) to the uridine 5′-diphosphate–*N*-acetylmuramyl tripeptide by the DAla-DAla adding enzyme. The biosynthesis of D-alanyl-D-alanine in turn involves initial formation of D-alanine from L-alanine by alanine racemase followed by the coupling of two molecules of D-alanine by D-alanine:D-alanine ligase (synthetase) (EC 6.3.2.4)[1,2] (Figure 1).

The latter enzyme was selected as a target for enzyme-inhibitor design. The only antibiotic known to be a DAla-DAla ligase inhibitor is D-cycloserine, although it has multiple activities including alanine racemase inhibition and its reported K_i as an inhibitor of DAla-DAla ligase is a modest 2.5×10^{-4} M.[3] D-(1-Aminoethyl)phosphonic acid ($IC_{50} = 9.3 \times 10^{-4}$ M)[4] and peptides such as the product DAla-DAla ($IC_{50} = 6.6 \times 10^{-4}$ M)[5] have also been reported to show some inhibitory activity. Attempts to design more effective inhibitors have accelerated in recent years. Syntheses of several aminophosphonic and aminophosphonamidic acids were recently reported.[6] Also the synthesis and essentially irreversible inhibition of this enzyme by novel phosphinic acid analogues of DAla-DAla have been described.[7-9]

Design Considerations. The reaction catalyzed by DAla-DAla ligase can be summarized as shown below:[10,11]

$$2\ \text{DAla} + \text{ATP} \xrightarrow[Mg^{++}]{\text{DAla-DAla Ligase}} \text{DAla-DAla} + \text{ADP} + \text{Pi}$$

Specificity studies[12,13] have indicated that the enzyme has two separate binding sites for D-Alanine. The binding site for the N-terminal D-Ala, termed the donor site, is highly specific for this amino acid whereas the C-terminal D-Ala binding site, known as the acceptor site, is less specific and accepts a variety of D-amino acids. The mechanism (stoichiometry) of the ligase reaction is similar to that of glutamine synthetase in which the γ-carboxyl group of glutamic acid is activated by ATP forming an acyl phosphate intermediate **3**. Attack by enzyme-bound ammonia on the intermediate completes the formation of glutamine. Compelling evidence in favor of this acyl phosphate mediated mechanism for glutamine synthetase exists.[14] Moreover, the methylene isostere **4** of γ-glutamyl phosphate has been described as a good inhibitor of the enzyme ($K_i = 5 \times 10^{-4}$ M; ovine brain glutamine synthetase).[15]



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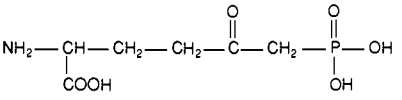

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Although the mechanism for DAla-DAla ligase has not been established, the acyl phosphate displacement reaction

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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 & Sons: New York, 1981; Chapter 3.
(2) Neuhaus, F. C.; Hammes, W. P. *Pharmacol. Ther.* **1981**, *14*, 265.
(3) (a) Neuhaus, F. C. In *Anbitiocis*; Gottlieb, D., Shaw, P. D., Eds.; Springer-Verlag: New York, 1967; Vol 1, pp 40–83. (b) Neuhaus, F. C.; Lynch, J. L. *Biochemistry* **1964**, *3*, 471.
(4) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Lambert, R. W.; Lloyd, W. J.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 696.
(5) Lacoste, A. M.; Poulsen, M.; Cassaigne, A.; Neuzil, E. *Curr. Microbiol.* **1979**, *2*, 113.
(6) (a) Vo-Quang, Y.; Carniato, D.; Vo-Quang, L.; Lacoste, E.; Neuzil, E.; Le Goffic, F. *J. Med. Chem.* **1986**, *29*, 148. (b) Vo-Quang, Y.; Garvey, A. M.; Simonneau, R.; Vo-Quang, L.; Lactose, A. M.; Le Goffic, F. *Tetrahedron Lett.* **1987**, *28*, 6167.
(7) (a) Walsh, C. T.; Daub, E.; Zawadzke, L.; Duncan, K. In *Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function*; Shockman, G. D.; Daneo-Moore, L., Actor, P., Salton, M., Higgins, M., Eds.; ASM: Washington, D.C., Chapter 54. (b) Duncan, K.; Walsh, C. T. *Biochemistry* **1988**, *27*, 3709–3714.
(8) Patchett, A. A. In *Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function*; Shockman, G. D., Daneo-Moore, L., Actor, P., Salton, M., Higgins, M., Eds.; ASM: Washington, D.C., Chapter 55.
(9) Parsons, W. H.; Patchett, A. A.; Bull, H. G.; Schoen, W. R.; Taub, D.; Davidson, J.; Combs, P.; Springer, J. P.; Gadebusch, H.; Weissberger, B.; Valiant, M. E.; Mellin, T. N.; Busch, R. D. *J. Med. Chem.* **1988**, *31*, 1772.
(10) Ito, E.; Strominger, J. L. *J. Biol. Chem.* **1960**, *235*, PC 5.
(11) Neuhaus, F. C. *Biochem. Biophys. Res. Commun.* **1960**, *3*, 401.
(12) Neuhaus, F. C. *J. Biol. Chem.* **1962**, *237*, 778.
(13) Neuhaus, F. C. *J. Biol.. Chem.* **1962**, *237*, 3128.
(14) Meister, A. In *Methods in Enzymology*; Meister, A., Ed.; Academic Press: New York, 1985; Vol. 113, pp 185–199.
(15) Wedler, F. C. *Arch. Biochem. Biophys.* **1980**, *202*, 482.

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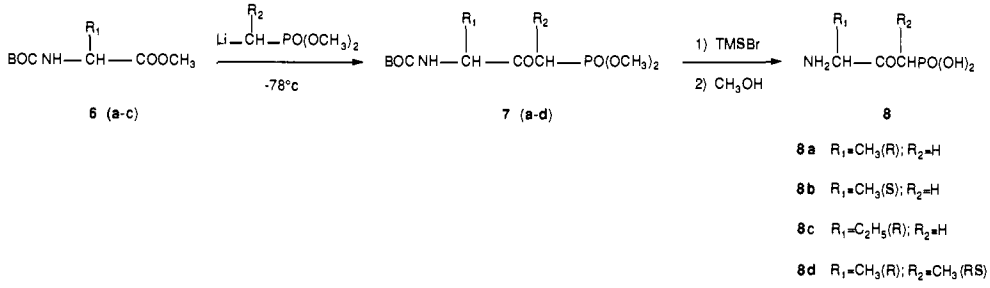
Scheme I



Scheme II

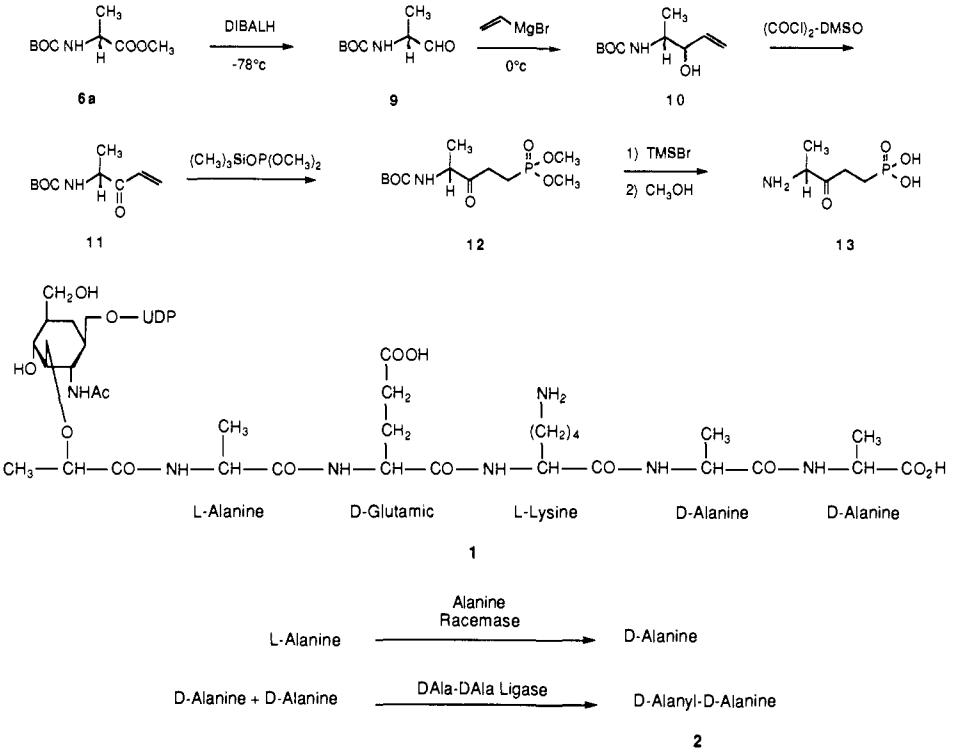


Figure 1. The structure of muramylpenta peptide precursor (1) of *Staphylococcus aureus* cell wall peptidoglycan and the synthesis of its DAla-DAla component (2).

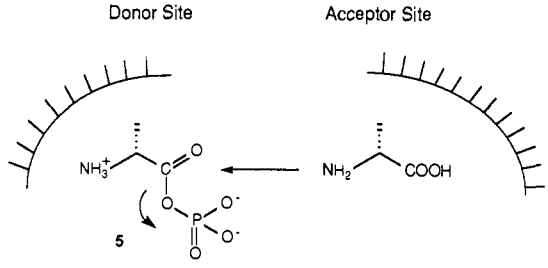
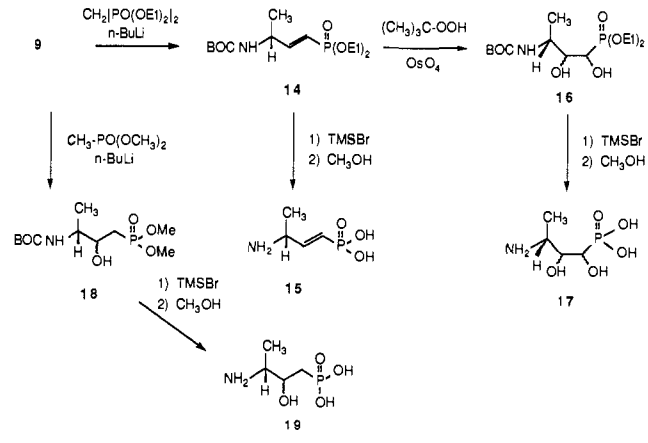


Figure 2. Proposed displacement reaction in the formation of DAla-DAla by DAla-DAla ligase.

which we show schematically in Figure 2 is a possibility. If so, the methylene isostere **8a** and related analogues of the presumed intermediate D-alanyl phosphate **5** might be expected to inhibit the enzyme, as was the case in the inhibition of glutamine synthetase by inhibitor **4**. We report below the synthesis and enzyme inhibitory properties of several of these compounds.

Chemistry. The general method[16] used for the synthesis of analogues related to 8 is outlined in Scheme I. In this approach, the methyl ester of an *N-tert*-butoxycarbonyl α-amino acid was reacted with 6 equiv[17] of the lithium salt of dimethyl methylphosphonate at −78 °C in THF to give the fully protected derivative of **7**. The keto phosphonate **7** was then treated sequentially with tri-

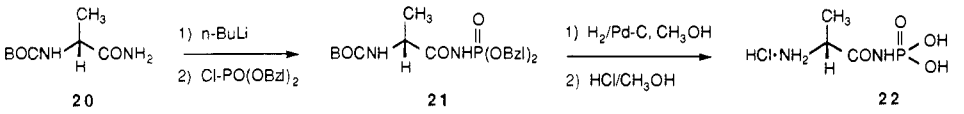
Scheme III



(16) Chakravarty, P. K.; Combs, P.; Roth, A.; Greenlee, W. J. *Tetrahedron Lett.* **1987**, *28*, 611.

(17) Yield of 2-oxoalkylphosphonates are generally lower when less than 6 equiv of lithium salt of dimethyl methylphosphonate is used.

Scheme IV



20 21 22

methylsilyl bromide and methanol at room temperature to give 8 in good yield (70–80%). In most cases the product 8 was found to have undergone less than 5% racemization as determined by reversed-phase HPLC analysis of the adduct with (*R*)-(+)-α-methylbenzyl isocyanate.

Inhibitor **13**, a homologue of inhibitor **8a**, was prepared as outlined in Scheme II. Aldehyde **9**, obtained from Boc-DAla-OMe (**6a**), was transformed into alcohol **10**, which on Swern oxidation[18] gave the corresponding unsaturated ketone **11**. 1,4-Addition of $Me_3SiOP(OMe)_2$ to **11**, using conditions described by Evans et al.[19] gave the protected phosphonate **12** in 65% yield along with 15% of the corresponding 1,2-addition product. Deprotection of **12** using TMSBr afforded the phosphonic acid **13**.

The phosphonic acids **15**, **17**, and **19** were prepared as shown in Scheme III. Olefination[20] of the aldehyde **9** under Horner–Emmons conditions gave the unsaturated phosphonate **14**, which was treated with Me_3COOH[21] and catalytic OsO_4 to give the corresponding dihydroxy phosphonate **16**. Deprotection of **14** and **16** gave the corresponding phosphonic acids **15** and **17**, respectively. Compound **19** was prepared by deprotection of hydroxyphosphonate **18**, which was obtained by the addition of the lithium salt of dimethyl methylphosphonate to aldehyde **9**.

The synthesis of aza analogue **22** was accomplished as outlined in Scheme IV. *N*-(*tert*-Butoxycarbonyl)-D-alanine amide (**20**) was deprotonated at −78 °C with *n*-butyllithium and phosphorylated with dibenzyl chlorophosphonate which was prepared in situ from dibenzyl phosphonate and *N*-chlorosuccinimide. Intermediate **21** was hydrogenated to remove the benzyl esters and then briefly exposed to HCl in methanol to provide compound **22** as the hydrochloride. Aza analogue **22** is unstable and was kept at 0 °C before assay.

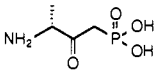
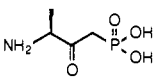
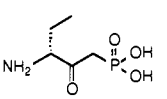
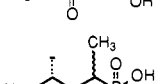
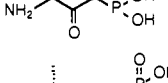
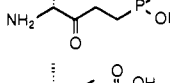
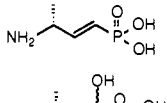
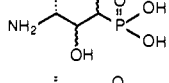
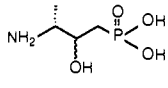
Results and Discussion

Table I lists the DAla-DAla ligase inhibitory properties of the (3-amino-2-oxoalkyl)phosphonic acids and their analogues which were prepared in this study. As expected, specific structural requirements for binding at the donor site of DAla-DAla ligase were observed. The substituent R is optimally a methyl group in the D configuration. Other alkyl groups, or a methyl group in the L configuration in that position, cause significant reduction of enzyme-inhibitory activity.

The methylene analogue of D-alanyl phosphate (**5**), (3-(R)-amino-2-oxobutyl)phosphonic acid (**8a**) ($K_i = 5.1 \times 10^{-4}$ M) and the corresponding aza analogue (**22**) ($K_i = 5.0 \times 10^{-5}$ M)[22] emerged as the best inhibitors of this series. Variations of the $COCH_2PO_3H_2$ part of the structure of **8a** generally reduce activity. The carbonyl group is required and several substitutions on the methylenephosphonate group weakened inhibitory potency. Presumably that aza analogue (**22**) is a better inhibitor than the methylene analogue (**8a**) since the polarity and bond length of the NH–P group better mimics the O–P bond of the proposed acyl phosphate intermediate (**5a**). Thus the SAR data of these compounds are consistent with their design as isosteres of D-alanyl phosphate. The inhibitory potencies of **8a** and **22** against DAla-DAla ligase are in the same range as is the inhibition of glutamine synthetase by inhibitor **4**. These results lend support to an acyl phosphate displacement mechanism for DAla-DAla ligase.

Compound **22** is a more potent inhibitor of the enzyme than is D-cycloserine. However, neither it nor compound **8a** displayed any significant Gram-positive or Gram-negative antibacterial activity at levels up to 256 μg/mL. These compounds, presumably, do not inhibit the additional enzymes known to be inhibited by cycloserine and are apparently either too weak or poorly transported into bacteria to confirm DAla–DAla ligase as a useful antibacterial target.

Table I. Inhibition of DAla-DAla Ligase by Analogues of (3(*R*)-Amino-2-oxobutyl)phosphonic Acid (**8a**)

inhibitor	structure	% of ligase inhibn (at 10^{-3} M)
8a		71, $K_i = 5.1 \times 10^{-4}$ M
8b		14
8c		26
8d		18
13		10
15		24
17		32
19		25
22		85, $K_i = 5.0 \times 10^{-5}$ M
23	D-cycloserine	($K_i = 2.5 \times 10^{-4}$ M)[3]

Experimental Section

General Methods. ^1H NMR spectra were recorded on a Varian XL300 pulsed Fourier transform instrument or a Varian T-60. Mass spectra were recorded on a Finnigan MAT731 mass spectrometer. Elemental analyses were within ±0.4% of the theoretical values. Thin-layer chromatography was carried out on silica gel MK6 (Whatman, 0.2 mm) glass-backed plates, and spots were

(18) Mancuso, A. J.; Huangard, S. L.; Swern, D. *J. Org. Chem.* **1978**, *43*, 2480.
(19) Evans, D.; Hurst, K. M.; Takacs, J. M. *J. Am. Chem. Soc.* **1978**, *100*, 3467.
(20) (a) Teulade, M. P.; Savignac, P.; Aboujaoude, E. E.; Leitje, S.; Collignon, N. *J. Organomet. Chem.* **1986**, *304*, 283. (b) Paulsen, H.; Bartsch, W. *Chem. Ber.* **1975**, *108*, 1732.
(21) Akashi, K.; Palermo, R. E.; Sharpless, K. B. *J. Org. Chem.* **1978**, *43*, 2063.
(22) D-Alanine amide, the decomposition product of inhibitor **22**, was found to be inactive against the ligase at 10^{-3} M.

visualized by UV, iodine vapor, or ninhydrin spray. Preparative chromatography was carried out with a medium-pressure system (MPLC) with Lobar LiChroprep Si60 (E. Merck, 40–63 mm) prepacked columns or flash column chromatography[23] using E. Merck silica gel (230–400 mesh). D- and L-amino acids and their derivatives were purchased from either Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO). Boc-DAla-OMe (**6a**) was prepared according to the procedures used to synthesize Boc-LAla-OMe.[24] Boc-DAla amide (**20**) was prepared according to the procedure used for the preparation of Cbz-LAla amide.[25] Dimethyl trimethylsilyl phosphite was prepared according to the published procedure.[26] All reactions were carried out under an atmosphere of dry N_2 unless specified otherwise.

General Procedure for the Synthesis of (3-Amino-2-oxoalkyl)phosphonic Acids (8). To a solution of dimethyl methylphosphonate (0.17 mol) in dry THF (150 mL) is added a solution of *n*-BuLi (0.17 mol) slowly at −78 °C, and the mixture was stirred for 1 h. A solution of *N*-*tert*-butoxycarbonyl α-amino acid methyl ester (**6a–c**) (0.0256 mol) in dry THF (100 mL) is then added and the stirring is continued at −78 °C (2 h) and then at −30 °C (1 h). The reaction is quenched with glacial AcOH (0.8 mL), and the mixture is poured into saturated $NaHCO_3$ (100 mL) and extracted with ethyl acetate (3 × 100 mL). The combined organic layer is washed with brine, dried ($MgSO_4$), filtered, and then concentrated in vacuo. The crude product, thus obtained, is purified by MPLH or flash chromatography to give the corresponding phosphonate **7a–d** as colorless oil. The phosphonate **7a–d** (0.0034 mol) is treated with trimethylsilyl bromide (3.6 mL, 0.0272 mol) in CH_2Cl_2 (7 mL) at 25 °C for 18 h. The foamlike residue, obtained after removal of the solvent, is stirred with MeOH (20 mL) for 2 h and evaporated to dryness. The residue is dissolved in MeOH (1 mL) and precipitated with dry ether to give pure **8a–d** as hygroscopic solids.

(3(*R*)-Amino-2-oxobutyl)phosphonic Acid (8a). A solution of **6a** (5.0 g, 0.0246 mol) was reacted with lithium salt of dimethyl methylphosphonate as described above to give the protected phosphonate **7a** (6.4 g, 87%) as a colorless viscous oil: R_f (EtOAc–hexane 2:1) 0.28; NMR ($CDCl_3$) δ 1.35 (d, J = 8 Hz, 3 H), 1.46 (s, 9 H), 3.15 (dd, J_1 = 14 Hz and J_2 = 20 Hz, 1 H), 3.24 (dd, J_1 = 14 Hz and J_2 = 20 Hz, 1 H), 3.80 (d, J = 12 Hz, 6 Hz), 4.24–4.38 (m, 1 H); MS (FAB) m/e 267 (MH^+). Anal. ($C_9H_{18}NPO_6$) C, H, N, P.

The phosphonate **7a** (1.0 g, 0.0034 mol) was deprotected with trimethylsilyl bromide (3.6 mL, 0.0272 mol) in CH_2Cl_2 (7 mL) to give the title compound **8a** (0.5 g, 88%) as a white solid: R_f (EtOAc–MeOH–H_2O 3:3:2) 0.31; NMR (D_2O) δ 1.25 (d, J = 8 Hz, 3 H), 3.0 (dd, J_1 = 12 Hz and J_2 = 20 Hz, 1 H), 3.3 (dd, J_1 = 12 Hz and J_2 = 20 Hz, 1 H); MS (FAB) m/e 168 (MH^+). Anal. ($C_4H_{10}NPO_4 \cdot H_2O$) C, H, N.

(3(*S*)-Amino-2-oxobutyl)phosphonic Acid (8b). The phosphonate **7b**, obtained in 78% from Boc-Ala-OMe, was deprotected to give the title compound **8b**: NMR (D_2O) similar to **8a**; MS (FAB) m/e 168 (MH^+). Anal. ($C_4H_{10}NPO_4 \cdot 2H_2O$) C, H, N.

Methyl *N*-(*tert*-Butoxycarbonyl)-D-2-aminobutyrate (6c). To a solution of D-2-aminobutyric acid (5.5 g, 0.0534 mol) in dry MeOH (20 mL) was added $SOCl_2$ (4.3 mL, 0.059 mol) at −10 °C, and the mixture was stirred for 17 h at 25 °C. After removal of the solvent, the residue was dissolved in dry MeOH (5 mL) and the ester hydrochloride was precipitated with dry ether. The solid was filtered and dried over P_2O_5 overnight. The methyl ester hydrochloride (2.5 g, 0.01628 mol) was mixed with Et_3N (2.5 mL, 0.0179 mol) and di-*tert*-butyl dicarbonate (3.7 mL, 0.01628 mol) in CH_3CN (30 mL) and stirred at 25 °C for 24 h. The solvent was removed and the residue was partitioned between 5% citric acid and EtOAc. The organic phase was washed with water and brine and then dried ($MgSO_4$). Removal of the solvent gave pure **6c** as a colorless oil (3.5 g): NMR ($CDCl_3$) δ 0.94 (t, J = 8 Hz, 3 H), 1.44 (s, 9 H), 1.60–1.84 (m, 2 H), 3.76 (s, 3 H), 4.22–4.36 (m, 1 H), 5.02–5.14 (m, 1 H); MS (FAB) m/e 218 (MH^+).

(3(*R*)-Amino-2-oxopentyl)phosphonic Acid (8c). The phosphonate **7c** was prepared from **6c** in 68% yield. NMR ($CDCl_3$) δ 0.92 (t, J = 8 Hz, 3 H), 1.44 (s, 9 H), 3.15 (dd, J_1 = 14 Hz and J_2 = 20 Hz, 1 H), 3.20 (dd, J_1 = 14 Hz and J_2 = 20 Hz, 1 H), 3.80 (dd, J = 12 Hz, 6 H), 4.24–4.38 (m, 1 H), 5.32–5.44 (m, 1 H); MS (FAB) m/e 310 (MH^+). Deprotection of **7c** (0.15 g) with TMSBr gave the titled compound **8c** (0.08 g, 91%) as an amorphous solid: NMR (D_2O) δ 1.0 (t, 3 H), 2.08 (m, 2 H), 3.10 (dd, J_1 = 12 Hz and J_2 = 20 Hz, 1 H), 3.43 (dd, J_1 = 12 Hz and J_2 = 20 Hz, 1 H), 4.4 (m, 1 H); MS (FAB) m/e 182 (MH^+). Anal. ($C_5H_{12}NPO_4 \cdot H_2O$) C, H, N.

(3(*R*)-Amino-1(*RS*)-methyl-2-oxobutyl)phosphonic Acid (8d). The phosphonate **7d** was obtained as an oil in 66% yield from **6a** and lithium salt of diethyl ethylphosphonate (prepared from diethyl ethylphosphonate and *n*-BuLi): R_f (ether–hexane 3:2) 0.31; NMR ($CDCl_3$) δ 1.05–1.65 (m, 21 H), 3.38 (q, 1 H), 3.76–4.75 (m, 5 H); MS (FAB) m/e 338 (MH^+). Anal. ($C_{14}H_{28}NPO_6$) C, H, N.

The title compound **8d** was obtained as a light brown foam after deprotection of **7d** (0.12 g): yield 0.052 g (81%); NMR (D_2O δ 1.2 (d, J = 7 Hz, 3 H), 1.54 (d, 3 H), 3.4 (q, 1 H), 4.5 (q, 1 H); MS (FAB) m/e 182 (MH^+). Anal. ($C_5H_{12}NPO_4 \cdot HCl \cdot H_2O$) C, H, N.

3(*R*)-[*N*-(*tert*-Butoxycarbonyl)amino]propanal (9). The ester **6a** (2.03 g, 0.01 mol) was dissolved in dry THF (25 mL) and cooled to −78 °C. A precooled (−78 °C) solution of diisobutylaluminum hydride (1 M in hexane, 24 mL) was slowly added over 15 min. After stirring for 20 min at that temperature, the reaction was quenched with the careful addition of MeOH (20 mL) and then a saturated solution of Rochelle salt (200 mL) was added. The mixture was stirred at 25 °C for 1 h and then extracted with ether (3 × 100 mL). The combined organic phase was washed with brine, dried ($MgSO_4$), and then concentrated in vacuo to give an oil. The oil was purified by flash chromatography using ether–hexane (1:1) to give the aldehyde **9** as a solid (1.6 g, 92%): mp 87–88 °C; $[\alpha]_D$ +28.53 (c = 2.45, CH_3OH); R_f (ether–hexane 1:1) 0.31; NMR ($CDCl_3$) δ 1.34 (d, J = 7 Hz, 3 H), 1.44 (s, 9 H), 4.02 (m, 1 H), 9.5 (s, 1 H); MS (EI) m/e 173 (M^+). Anal. ($C_8H_{15}NO_3$) C, H, N.

(4(*R*)-Amino-3-oxopentyl)phosphonic Acid (13). A solution of **9** (0.38 g, 0.0022 mol) in THF (5 mL) was added to a solution of vinylmagnesium bromide (1.3 M in THF, 5.3 mL) at 0 °C, and the mixture was stirred for 2 h. After stirring for an additional 1 h at 25 °C, a saturated solution of NH_4Cl (25 mL) was added to the mixture and extracted with EtOAc. The organic phase was washed with brine, dried ($MgSO_4$), and then evaporated to give an oil. The crude product was purified by flash chromatography using EtOAc–hexane (1:1), giving the desired alcohol **10** (0.33 g, 75%, as a mixture of diastereoisomers): NMR ($CDCl_3$), δ 1.126 (d, J = 7 Hz, 3 H), 1.43 (s, 9 H), 3.53 (m, 1 H), 4.01 (t, J = Hz, 1 H), 5.27 (m, 1 H), 5.87 (m, 2 H); MS (EI) m/e 201 (M^+).

To a solution of $(COCl)_2$ (0.29 mL) and DMSO (0.45 mL) in CH_2Cl_2 (8 mL) at −40 °C was added dropwise a solution of **10** (0.6 g, 0.00299 mol) in CH_2Cl_2 (10 mL). After stirring for 1 h at −40 °C, Et_3N (2.1 mL) was added, and the mixture was stirred for 6 h at 25 °C. Water was added and the organic layer was separated. The aqueous phase was extracted with CH_2Cl_2, and the combined organic phase was washed with brine and then dried ($MgSO_4$). The crude material obtained after removal of the solvent was purified by flash chromatography using EtOAc–hexane (1:11) to give the desired ketone **11** as an oil (which formed a waxlike solid in the cold, 0.45 g, 76%): NMR ($CDCl_3$) δ 1.13 (d, J = 7 Hz, 3 H), 1.44 (s, 9 H), 4.55 (t, J = 8 Hz, 1 H), 5.85 (m, 1 H), 6.4 (m, 2 H); MS (EI) m/e 199 (M^+).

A mixture of ketone **11** (0.3 g, 0.0015 mol) and dimethyl trimethylsilyl phosphite (0.3 g, 0.0075 mol) in dry THF (6 mL) was heated at 50 °C for 16 h. The mixture was concentrated in vacuo, and the residue was purified by flash chromatography using EtOAc–hexane (9:1) to give the keto phosphonate **12** as a clear oil (0.3 g, 65%): NMR ($CDCl_3$) δ 1.29 (d, J = 7 Hz, 3 H), 1.45 (s, 9 H), 1.75–2.25 (m, 2 H), 2.58–3.4 (m, 2 H), 3.70 (d, 6 H); MS (FAB) m/e 310 (MH^+). Anal. ($C_{12}H_{24}NPO_6$) C, H, N. The keto phosphonate **12** (0.2 g) was then deprotected with TMSBr, as described above, to give the phosphonic acid **13** (0.1 g, 83%).

(23) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.
(24) Ali, A.; Fahrenholz, F.; Weinstein, B. *Angew. Chem., Int. Ed. Engl.* **1972**, *11*, 289.
(25) Matsuo, H.; Kawazoe, Y.; Sato, M.; Ohnishi, M.; Tatsuno, T. *Chem. Pharm. Bull.* **1970**, *18*, 1788.
(26) Nesterov, L. V.; Krepysheva, N. E.; Sabirova, R. A.; Romanova, G. N. *J. Gen. Chem. USSR (Engl. Transl.)* **1971**, *41*, 2449.

NMR (CD_3OD) δ 1.58 (d, J = 7 Hz, 3 H), 1.8–2.35 (m, 2 H), 2.65–3.2 (m, 2 H), 4.28 (m, 1 H); MS (FAB) m/e 182 (MH^+). Anal. (as HCl salt) ($C_5H_{12}NPO_4 \cdot HCl \cdot 2H_2O$) C, H, N.

(3(*R*)-Amino-1-butenyl)phosphonic Acid (15). *n*-BuLi (1.6 M in hexane, 1.83 mL, 0.00292 mmol) was added at −78 °C to a solution of tetraethyl methelenediphosphonate (Fluka) (0.84 g, 0.00292 mol) in THF (8 mL) and stirred for 20 min. A solution of the aldehyde **9** (0.17 g, 0.001 mol) in THF (4 mL) was then added, and the mixture was stirred at 0 °C. After stirring for 5 h, the mixture was poured into saturated NH_4Cl and was extracted with EtOAc. The organic layer was washed with brine and then dried over $MgSO_4$. Removal of the solvent in vacuo and purification of the crude product by flash chromatography using EtOAc–hexane (1:1) gave the phosphonate **14** as an oil (0.22 g, 73%): R_f (EtOAc–hexane 1:1) 0.29; NMR ($CDCl_3$) δ 1.25 (d, J = 7 Hz, 3 H), 1.46 (s, 9 H), 4.46 (m, 1 H), 5.7 (m, 1 H), 6.7 (m, 1 H); MS (FAB) m/e 308 (MH^+), 615 (2 MH^+). Anal. ($C_{11}H_{22}NPO_6$) C, H, N.

The phosphonate **14** (0.42 g) was deprotected according to the method described above to give pure **15** as a white solid (after crystallization from MeOH–ether) (0.17 g, 85%). mp 269–270 °C dec; R_f (*n*-BuOH–AcOH–H_2O 4:1:1) 0.254; NMR (D_2O) δ 1.39 (dd J_1 = 5.2 Hz and J_2 = 9 Hz, 3 H), 4.05 (m, 1 H), 6.07 (t, J = 22.5 Hz, 1 H), 6.50 (t, J = 14 Hz, 1 H); MS (FAB) m/e 152 (MH^+). Anal. ($C_4H_{10}NPO_3$) C, H, N.

(3(*R*)-Amino-1(*RS*),2(*RS*)-dihydroxybutyl)phosphonic Acid (17). To a mixture of **14** (0.15 g, 0.0005 mol), $Et_4NOAc \cdot 4H_2O$ (0.33 g), *tert*-butyl hydroperoxide (0.4 mL), acetone (1.5 mL) and water (0.04 mL) was added a solution of OsO_4 (1% in hexane, 0.2 mL) at 0 °C, and the mixture was stirred at 25 °C for 24 h. The reaction was diluted with EtOAc (60 mL) and the organic phase was washed with 10% aqueous $NaHSO_3$, water, and brine and then dried ($MgSO_4$). Removal of the solvent in vacuo and purification of the crude material by MPLC using EtOAc–hexane (3:1) gave the product **16** as a clear oil (0.14 g, 82%): R_f (EtOAc) 0.49; NMR (CD_3OD) δ 1.18 (dd, 3 H), 1.33 (t, 6 H), 1.45 (s, 9 H), 3.55–3.85 (m, 2 H), 3.9–4.05 (m, 2 H), 4.2 (q, 4 H); MS (FAB) m/e 342 (MH^+).

The phosphonate **16** (0.1 g) was deprotected with trimethylsilyl bromide, as described before, and the crude material obtained was purified on a Dowex-50 (H^+) column using water as the eluent, to give pure **17** (0.045 g, 85%) as a white powder after freeze-drying. NMR (D_2O) δ 1.21 (dd, 3 H), 3.5–3.9 (m, 3 H); MS (FAB) m/e 187 (MH^+). Anal. ($C_4H_{12}NPO_5 \cdot 2H_2O$) C, H, N.

(3(*R*)-Amino-2(*RS*)-hydroxybutyl)phosphonic Acid (19). To a solution of dimethyl methylphosphonate (0.92 g, 0.0074 mol) and *n*-BuLi (2.2 M in hexane, 3.35 mL) in THF (10 mL) was added a solution of **9** (0.51 g, 0.0029 mol) in THF (9 mL) at −78 °C, and the mixture was slowly brought to 0 °C. After 1 h at that temperature, saturated NH_4Cl (20 mL) was added, and the mixture was extracted with EtOAc. The organic phase was washed with brine and then dried ($MgSO_4$) and evaporated to give an oil. The oil was purified by flash chromatography using EtOAc to give the protected product **18** as a clear oil (0.193 g, 24%): NMR ($CDCl_3$) δ 1.18 (dd, 3 H), 1.44 (s, 9 H), 1.7–2.4 (m, 2 H), 3.65 (m, 1 H), 3.78 (d, 6 H), 4.0 (m, 1 H); MS (FAB) m/e 269 (MH^+). The phosphonate **18** (0.15 g) was treated with TMSBr (1 mL) in CH_2Cl_2 (2 mL), as described before, to give the phosphonic acid **19** (0.078 g, 83%): NMR (D_2O) δ 1.12 (dd, 3 H), 1.65–2.2 (m, 2 H), 3.52 (m, 1 H), 3.98 (m, 1 H); MS (FAB) m/e 170 (MH^+). Anal. (as the HCl salt) ($C_4H_{12}NPO_4 \cdot HCl \cdot H_2O$) C, H, N.

(3(*R*)-Amino-1-aza-2-oxobutyl)phosphonic Acid (22). To a solution of *N*-(*tert*-butoxycarbonyl)-D-alanine amide (0.35 g, 0.0027 mol) in THF (5 mL) at −78 °C was added *n*-BuLi (2.6 M in hexanes, 1 mL). After stirring at −78 °C for 10 min, a solution of dibenzyl phosphorochloridate[27] (0.0054 mol) in benzene (10 mL) was slowly added dropwise. After 1 h the reaction mixture was quenched with H_2O and extracted twice with EtOAc. The organic fractions were combined, dried over Na_2SO_4, filtered through a thin pad of $MgSO_4$, and evaporated to an oil that was purified by chromatography (silica, 7:3, EtOAc–hexanes) to give compound **21** (0.75 g, 62%) as white solid: mp 96–97 °C; NMR ($CDCl_3$) δ 1.25 (d, 3 H), 1.4 (s, 9 H), 4.05–4.2 (m, 1 H), 4.85 (br s, 1 H), 5.2 (d, 4 H), 7.25–7.45 (m, 10 H); MS (FAB) m/e 449 (MH^+). Anal. ($C_{22}H_{29}N_2O_6P$) C, H, N.

A solution of compound **21** (0.3 g, 0.00067 mol) in EtOH with Pd/C (10%, 0.050 g) was hydrogenated (40 psi) for 24 h at which time it was evaporated in vacuo. This material was dissolved in a saturated solution of HCl in MeOH and stirred for 30 min at 25 °C. This reaction mixture was then evaporated in vacuo to give the title compound **22** (0.070 g, 62%) as its HCl salt. This compound was found to be labile[28] and so was stored at 0 °C and was assayed without delay: NMR (D_2O) δ 1.6 (d, 3 H), 4.0–4.4 (q, 1 H); MS (FAB) m/e 169 (MH^+).

D-Alanine:D-alanine Ligase. *Streptococcus faecalis* (ATCC 8043) was grown as described by Neuhaus.[12] Cell-free extracts were prepared by sonication followed by centrifugation at 27 000*g* for 30 min. Protein was precipitated with 50% ammonium sulphate. The precipitate was dissolved in 0.05 M Tris-Cl buffer (pH 7.0), dialyzed against 0.005 M Tris-Cl containing 0.0025 M glutathione, and stored in liquid nitrogen. Enzyme prepared in this way was used for the assay of inhibitors and had a specific activity of 0.38 units/mg of protein.

Enzyme Assay. The assay mixture contained 0.05 M Tris-Cl buffer (pH 7.9), 0.01 M KCl, 0.008 M $MgCl_2$, 0.005 M ATP, 0.005 M (^{14}C-1)-D-alanine, and 5 μL of enzyme (specific activity = 0.38 units/mg of protein). Assay volume was 0.1 mL. Inhibitors were preincubated with enzyme, $MgCl_2$, KCl, and buffer for 40 min at room temperature before addition of ATP and substrate. Samples were subsequently incubated at 37 °C for 30 min. Each sample (20 μL) was applied to the preabsorbent layer of high-resolution, prechanneled silica gel TLC plates to stop the reaction. Plates were developed in EtOH–NH_4OH–H_2O (11:1:8) for 2–3 h and the radioactive zones located and integrated with Berthold Linear Analyzer to determine IC_{50}s.

Registry No. **6a**, 91103-47-8; **6b**, 28875-17-4; **6c**, 112392-65-1; **7a**, 120962-15-4; **7b**, 112392-62-8; **7c**, 112392-60-6; (1*R*,3*R*)-**7d**, 120881-89-2; (1*S*,3*R*)-**7d**, 120881-90-5; **8a**, 112392-53-7; **8b**, 112392-54-8; **8c**, 112392-52-6; (1*R*,3*R*)-**8d**, 120881-91-6; (1*R*,3*R*)-**8c**·HCl, 120881-93-8; (1*S*,3*R*)-**8d**, 120881-92-7; (1*S*,3*R*)-**8d**·HCl, 120881-94-9; **9**, 82353-56-8; (3*R*,4*R*)-**10**, 120881-95-0; (3*S*,4*R*)-**10**, 120881-96-1; **11**, 120881-97-2; **12**, 120881-98-3; **13**, 120881-99-4; **13**·HCl, 120882-11-3; **14**, 120882-00-0; **15**, 120962-16-5; **16**, 120882-01-1; **17**, 120882-02-2; (2*R*,3*R*)-**18**, 120882-03-3; (2*S*,3*R*)-**18**, 120882-04-4; (2*R*,3*R*)-**19**, 120882-05-5; (2*R*,3*R*)-**19**·HCl, 120882-07-7; (2*S*,3*R*)-**19**, 120882-06-6; (2*S*,3*R*)-**19**·HCl, 120882-08-8; **20**, 78981-25-6; **21**, 120882-09-9; **22**, 120882-12-4; **22**·HCl, 120882-10-2; $MeP(O)(OMe)_2$, 756-79-6; (*R*)-$H_2NCH(CH_2CH_3)COOH$, 2623-91-8; (*R*)-$H_2NCH(CH_2CH_3)$-$COOMe \cdot HCl$, 85774-09-0; $EtP(O)(OEt)_2$, 78-38-6; $H_2C{=}CHMgBr$, 1826-67-1; $CH_2[P(O)(OEt)_2]_2$, 1660-94-2; $ClP(O)(OCH_2Ph)_2$, 538-37-4; EC 6.3.2.4, 9023-63-6.

(27) Kenner, G. W.; Todd, A. R.; Weymouth, F. J. *J. Chem. Soc.* **1952**, 3675.

(28) It was difficult to obtain good elemental analysis of **22** due to the unstable nature of the compound.