7: 21% yield; MS-FAB, m/e 627 ($M^+ + H$); NMR (D_2O) δ 3.70 (s, 3 H), 3.8–4.0 (d, 2 H), 4.2–4.6 (m, 5 H), 6.06 (d, 1 H), 6.62 (d, 2 H), 7.05 (d, 2 H), 8.15 (s, 1 H), 8.48 (s, 1 H). Anal. ($C_{18}H_{25}N_6O_{13}P_3 \cdot 2NH_3 \cdot 3H_2O$) C, H, N.

8: 20% yield; MS-FAB, m/e 592 ($M^+ + H$); NMR (D_2O) δ 2.00 (s, 3 H), 3.0–3.5 (m, 4 H), 4.2–4.6 (m, 5 H), 6.20 (d, 1 H), 8.44 (s, 1 H), 8.68 (s, 1 H). Anal. ($C_{14}H_{24}N_7O_{13}P_3 \cdot 2NH_3 \cdot 1.5H_2O$) C, H, N.

9: 61% yield; MS-FAB, m/e 711 ($M^+ + H$); NMR (D_2O) δ 2.00 (s, 3 H), 2.72 (t, 2 H), 4.2–4.6 (m, 6 H), 6.05 (d, 1 H), 6.98 (s, 4 H), 8.22 (s, 1 H), 8.52 (s, 1 H). Anal. ($C_{21}H_{29}N_8O_{14}P_3 \cdot 2NH_3 \cdot 4H_2O$) C, H, N.

10: 60% yield; MS-FAB m/e 791 ($M^+ + H$); NMR (D_2O) δ 2.00 (s, 3 H), 2.85 (m, 2 H), 4.3–4.6 (m, 5 H), 6.13 (d, 1 H), 6.98 (s, 4 H), 8.28 (s, 1 H), 8.60 (s, 1 H). Anal. ($C_{21}H_{30}N_8O_{17}P_4 \cdot 3NH_3 \cdot 4.5H_2O$) C, H, N.

Registry No. **5**, 115094-21-8; **6**, 115094-22-9; **7**, 115094-23-0; **8**, 115094-24-1; **9**, 115117-16-3; **10**, 115094-25-2; **11**, 115094-26-3; **12**, 115117-17-4; **13**, 115094-27-4; 14, 90290-56-5; **15**, 56-65-5; **15**·2Na, 987-65-5; **16**, 90290-56-5; **17**, 949-99-5; **18**, 33305-77-0; **19**, 66163-66-4; **20**, 81677-61-4; **21**, 61925-80-2; tyrosine-specific protein kinase, 80449-02-1; *p*-toluidine, 106-49-0; L-phenylalanine, 63-91-2; 4-nitro-*N*-*t*-BOC-phenylalanine amide, 66163-67-5.

Phosphinic Acid Inhibitors of D-Alanyl-D-alanine Ligase

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We report the synthesis of a series of phosphinic acid dipeptide analogues, $NH_2CH(R^1)PO(OH)CH_2CH(R^2)CO_2H$, related to DAla-DAla. The best of these compounds are potent, essentially irreversible inhibitors of DAla-DAla ligase, and their preferred stereochemistry was shown by chiral synthesis of (1(*S*)-aminoethyl)(2(*R*)-carboxy-1-*n*-propyl)phosphinic acid, **12b**, and by X-ray crystallography of its derivative benzyl [1(*S*)-[(benzyloxycarbonyl)-amino]ethyl](2(*R*)-carbomethoxy-1-propyl)phosphinate, **13**, to correspond to the stereochemical configuration of DAla-DAla at both centers. A mechanism for the inhibition of DAla-DAla ligase by these compounds is proposed to involve an ATP-dependent formation of phosphorylated inhibitor within the enzyme's active site. The antibacterial activities of these compounds are modest although their spectra include both Gram-positive and Gram-negative susceptible organisms. The best antibacterial activity was shown by (1(*S*)-aminoethyl)[2-carboxy-2(*R*)-(methylthio)-1-ethyl]phosphinic acid, **3e**, whose MIC's range from 4–128 μg/mL on nine of a panel of 11 bacterial organisms. Combination of one of the more active phosphinic acids **12b** with the alanine racemase inhibitor fluoro-D-alanine enhances the antibacterial spectrum of the latter on several strains of bacteria and inhibits fluoro-D-alanine's self-reversal, which normally occurs at concentrations several fold higher than its MIC level. This inhibition of fluoro-D-alanine self-reversal is consistent with an involvement of DAla-DAla ligase inhibition in the antibacterial activity of these compounds.

Most therapeutically useful antibacterial agents inhibit the biosynthesis or function of target structures present only in bacteria. One of these targets, peptidoglycan, is a cross-linked cell wall polymer that plays an essential role in protecting bacteria from lysis. Its assembly is blocked by numerous antibacterials that are enzyme inhibitors, including β-lactams, D-cycloserine, fluoro-D-alanine, alafosfalin, moenomycin, and fosfomycin.[1]

Peptidoglycan biosynthesis is initiated with the construction of uridine-5′-diphosphate–*N*-(acetylmuramyl)-LAla-DGlu-Dap(Lys)-DAla-DAla, which is synthesized via a multienzyme pathway culminating in the addition of D-alanyl-D-alanine to a uridine-5′-diphosphate–*N*-acetylmuramyl tripeptide. D-Alanyl-D-alanine is in turn synthesized by D-alanyl-D-alanine ligasae [D-alanyl-D-alanine synthetase (ADP); EC 6.3.2.4].[1,2] It seems quite probable that failure to assemble and incorporate DAla-DAla into the bacterial cell wall will result in bacterial lysis since racemase inhibitors such as fluoro-D-alanine by inhibiting DAla formation are antibacterial as are β-lactam antibiotics, which inhibit transpeptidation involving the DAla-DAla unit. D-Cycloserine, which is transported across the cell wall via the D-alanine/glycine transport system, is a fair inhibitor of this enzyme, and it is an effective antibiotic presumably because it also inhibits alanine racemase and D-amino acid transaminase as well.[2,26] However, apart from cycloserine analogues,[3] surprisingly little has been done in the synthesis of D-alanyl-D-alanine ligase inhibitors, and the ability to establish if ligase inhibition is lethal or not has never been fully accomplished. Some dipeptide analogues[4] of DAla-DAla are modest inhibitors of the ligase as is (1-aminoethyl)phosphonic acid.[5,6] We report here the synthesis and properties of a series of phosphinic acids, the most active of which are tight-binding inhibitors of the enzyme.

Design Considerations. The chemistry catalyzed by DAla-DAla ligase can be summarized as follows:[7,8]

$$2\text{DAla} + \text{ATP} \xrightarrow{Mg^{2+} + K^+} \text{DAla-DAla} + \text{ADP} + P_i$$

The stoichiometry of this reaction is similar to that of glutamine and γ-glutamylcysteine synthetases in which the γ-carboxyl group of glutamic acid is activated by ATP for

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‡ Merck Institute for Therapeutic Research.

(1) Gale, E. F.; Cundliffe, E.; Reynolds, P. E.; Richmond, M. H.; Waring, M. J. *The Molecular Basis of Antibiotic Action*, 2nd ed.; Wiley: New York, 1981; Chapter 3.
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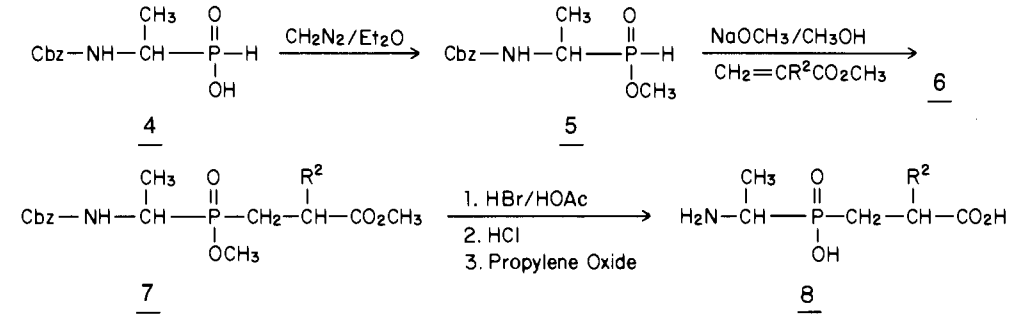
0022-2623/88/1831-1772$01.50/0

Scheme I



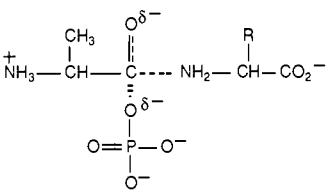
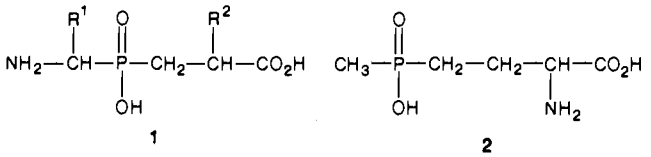


Figure 1. Proposed displacement reaction in the synthesis of D-alanyl dipeptides by DAla-DAla ligase.

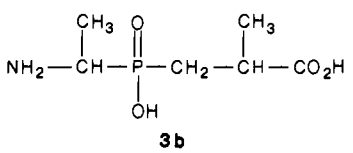
the acylation of ammonia and cysteine, respectively, via intermediacy of an acyl phosphate group.[9] We hypothesize a similar mechanism for DAla-DAla ligase as shown in Figure 1.

Specificity studies of DAla-DAla ligase have identified two binding sites for DAla. The N-terminal one is highly specific for this amino acid whereas the C-terminal site accepts a variety of D-amino acids. Thus the enzyme can synthesize mixed dipeptides such as DAla-DSer, DAla-DNva, etc.[10,11] Furthermore, DAla-DAla ligase is susceptible to product inhibition, and several of these mixed peptides are slightly more effective inhibitors than DAla-DAla itself. Neuhaus et al.[12] report the following K_i's: DAla-DAla (1.2×10^{-3} M), DAla-DNva (0.55×10^{-3} M), and DAla-D-α-Abu (0.66×10^{-3} M).

Assuming DAla-DAla ligase does share mechanistic similarities with glutamine synthetase as hypothesized in Figure 1, then it might be possible to design transition-state inhibitors of type 1. Analogy can also be drawn with the glutamine synthetase inhibitor phosphinothricin (**2**).[13]



We synthesized **3b**, and its good enzyme inhibition and modest antibacterial activity prompted the study of additional analogues, some of which are described below.



Chemistry. Scheme I outlines the general method used in synthesizing phosphinodipeptide analogues of type 1. The starting material [1-[(carbobenzyloxy)amino]ethyl]-phosphonous acid **4** was prepared by the method of Baylis et al.[14] This acid was esterified with diazomethane, and the product purified by chromatography on silica gel to yield ester **5**. Treatment of **5** with sodium methoxide in methanol and then with an acrylate methyl ester **6** afforded **7**, which was deprotected by using successive HBr/acetic acid and refluxing 6 N HCl treatments. Compounds of structure **8** were liberated from their salts with propylene oxide and were generally hygroscopic solids. The 2-alkylacrylates required in these syntheses that could not be purchased were prepared by the method of Atta-Ur-Rahman et al.[15] Methyl 2-(methylthio)acrylate, a good Michael acceptor,[16] was prepared according to the method of Gundermann and Schulze.[17] A smaller group of optically active analogues was synthesized by using the enantiomers of [[(carbobenzyloxy)amino]ethyl]phosphonous acid. Resolution was achieved by the procedure of Baylis et al.[14] Compound **3g** was made in a manner analogous to the methyl series only with (1-aminopropyl)-phosphonous acid, made also by the procedure of Baylis.

Stereochemical control at the remaining asymmetric center in **8** was achieved as summarized in Scheme II. The conversion of the *S* isomer of **5** into dehydro compound **9** via Michael addition to trimethyl phosphonoacrylate and subsequent reaction with formaldehyde is a new method for the preparation of α-substituted acrylates. Chiral reduction of **10** using homogeneous rhodium catalysis with chiral ligands was patterned after methods used in the stereoselective reduction of dehydroamino acid derivatives.[18-20] A number of conditions were explored to maximize the enantiomeric excess of the D,D-(*S,R*) over the D,L-(*S,S*) diastereomer. In the best of these, (-)-DIOP [2,3-*O*-isopropylidene-2,3-dihydroxy-1,4-bis(diphenylphosphino)butane] with $[RhCl(COD)]_2$ (COD = 1,5-cyclooctodiene) in benzene was added to **10** in methanol, and subsequent hydrogenation at 40 psi provided product **11** with a desired diastereomeric ratio of 5.2 to 1. Recrystallization afforded a diastereomer determined to be pure by 300-MHz NMR and HPLC. After removal of protecting groups, the stereochemistry of resulting compound **12b** was inferred from its enzyme inhibition to correspond to that of a D,D dipeptide. This assignment was confirmed by X-ray structure determination of a de-

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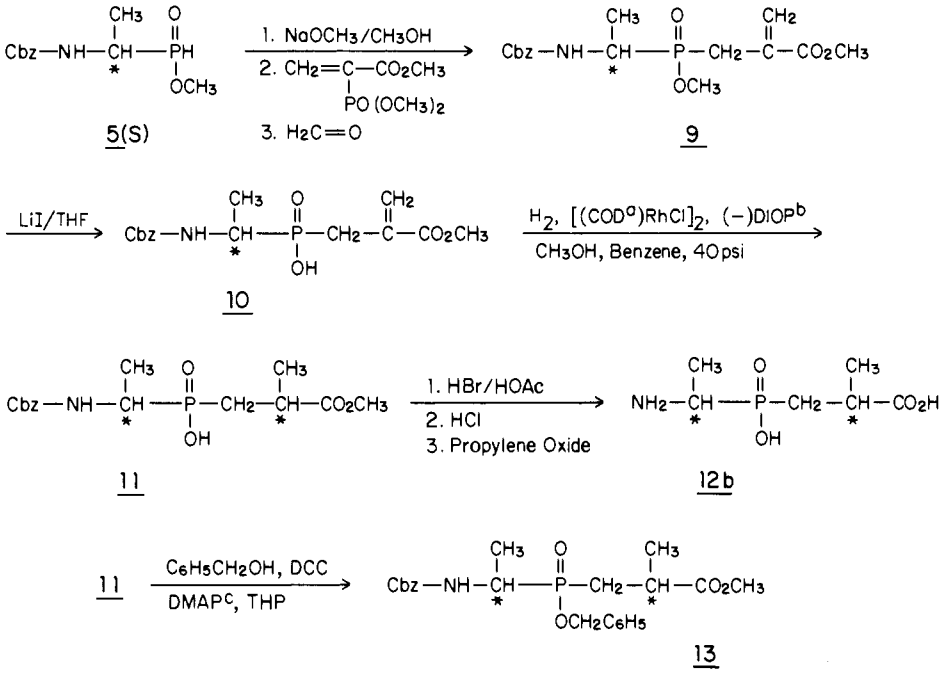
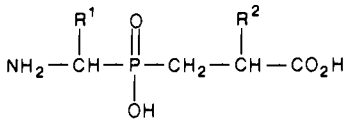
Scheme II



[a] COD = cyclooctadiene. [b] DIOP = 2,3-*O*-isopropylidene-2,3-dihydroxy-1,4-bis(diphenylphosphino)butane. [c] DMAP = 4-(dimethylamino)pyridine.

Table I. Chemical and Biological Data of Phosphinic Acid Inhibitors of D-Alanyl-D-alanine Ligase



compd	R^1	R^2	config at R^1	config at R^2	yield, %	formula	anal.	IC_{50}, μM
3a	methyl	H	*RS*	*RS*	28	$C_5H_{12}NO_4P \cdot 1.5H_2O$	C, H, N	535
3b	methyl	methyl	*RS*	*RS*	47	$C_6H_{14}NO_4P \cdot 0.75H_2O$	C, H, N	165
3c	methyl	ethyl	*RS*	*RS*	55	$C_7H_{16}NO_4P \cdot H_2O$	C, H, N	45
3d	methyl	*n*-propyl	*RS*	*RS*	45	$C_8H_{18}NO_4P \cdot H_2O$	C, H, N	20
3e	methyl	methylthio	*S*	*R*[b]	42	$C_6H_{14}NO_4PS$	C, H, N	4
3f	methyl	*n*-butyl	*RS*	*RS*	49	$C_9H_{20}NO_4P \cdot 0.25H_2O$	C, H, N	20
3g	ethyl	*n*-butyl	*RS*	*RS*	17	$C_{10}H_{22}NO_4P \cdot 0.75H_2O$	C, H, N	155
3h	methyl	isobutyl	*RS*	*RS*	26	$C_9H_{20}NO_4P \cdot H_2O$	C, H, N	30
3i	methyl	*n*-heptyl	*RS*	*RS*	37	$C_{12}H_{26}NO_4P$	C, H, N	12.5
3j	methyl	benzyl	*RS*	*RS*	64	$C_{12}H_{18}NO_4P \cdot H_2O$	C, H, N	50
3k	methyl	phenethyl	*RS*	*RS*	49	$C_{13}H_{20}NO_4P \cdot 0.25H_2O$	C, H, N	40
3l	methyl	phenylpropyl	*RS*	*RS*	28	$C_{14}H_{22}NO_4P \cdot 1.5H_2O$	C, H, N	50
3m	methyl	methyl	*R*	*RS*	55	$C_6H_{14}NO_4P \cdot H_2O$	C, H, N	22% at 1 mM
3n	methyl	methyl	*S*	*RS*	70	$C_6H_{14}NO_4P \cdot 0.25H_2O$	C, H, N	115
3o	methyl	*n*-heptyl	*R*	*RS*	51	$C_{12}H_{26}NO_4P$	C, H, N	200
3p	methyl	*n*-heptyl	*S*	*RS*	47	$C_{12}H_{26}NO_4P$	C, H, N	4
12a	methyl	methyl	*S*	*S*	18	$C_6H_{14}NO_4P \cdot H_2O$	C, H, N	215
12b	methyl	methyl	*S*	*R*	24	$C_6H_{14}NO_4P$	C, H	35
(1-aminoethyl)phosphonic acid			*RS*					930
(1-aminoethyl)phosphonous acid			*RS*					1000
D-alanyl-D-alanine			*R*	*R*				1000
D-cycloserine			*R*					300

[a] From the assay described in the Experimental Section in which product D-alanyl-D-alanine was measured after incubation of the enzymatic reaction mixture at 37 °C for 30 min with and without inhibitor. [b] This crystalline diastereomer is provisionally assigned *S,R* stereochemistry because of its high ligase inhibitory potency.

rivative of **11**. Esterification[21] of the major diastereomer of **11** with benzyl alcohol gave phosphinate ester **13** as an 80:20 mixture of diastereomers at phosphorus (Scheme II). Isolation of the major component by HPLC followed by crystallization from ether afforded a crystal suitable for X-ray analysis. Solution of the structure by standard methods[22] showed the compound to have the S,R_P,R configuration shown in Figure 2. Therefore, the configuration of the derived compound **12b** corresponds to the D,D configuration of DAla-DAla. Compound **12a** was obtained by

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(22) The following library of crystallographic programs was used: MULTAN 80, P. Main et al., University of York, York, England (1980); ORTEP-II, C. K. Johnson, Oak Ridge National Laboratory, Oak Ridge, TN (1970); SDP PLUS V1.1, Y. Okaya et al., B. A. Frenz and Associates, College Station, TX (1984).

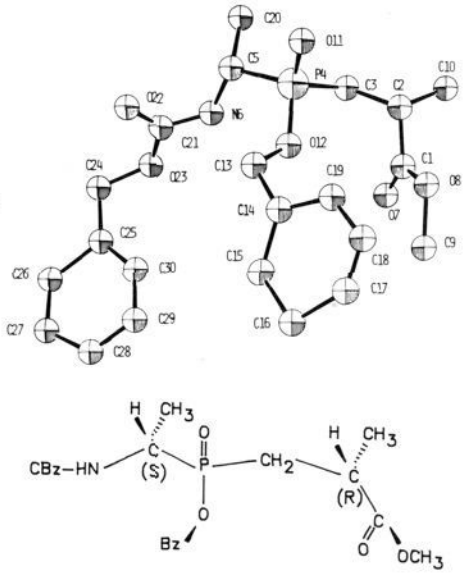


Figure 2. Molecular structure of the major diastereomer of **13** as determined by X-ray crystallography (ORTEP diagram).

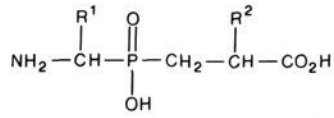
HPLC separation of the two diastereomers of compound **3n**.

Results and Discussion

Enzyme inhibition data using a crude D-alanyl-D-alanine ligase preparation from *Streptococcus faecalis* (ATCC 8043) are presented in Table I. This assay was used in initial studies to determine rank order inhibitor potencies. As expected from the enzyme's specificity,[10,11] R^1 is optimally a methyl group whose stereochemistry corresponds to that of a D-amino acid. An *RS*-ethyl group or a methyl group in an *R* configuration in that position corresponding to an L-amino acid configuration results in a major reduction in biological activity. At R^2 there is significant *improvement in enzyme inhibition obtained by replacing* hydrogen by methyl. Larger groups enhance activity even further, the most active of which are the thiomethyl analogue **3e** and the *n*-heptyl analogue **3p**. The preferred R^2 stereochemistry corresponds to that of a D-amino acid as is seen by comparing the inhibitory activities of **12a** and **12b**. Thus it would seem reasonable to conclude that residues R^1 and R^2 bind in the DAla donor and acceptor sites of the enzyme, respectively. The poor activities of (aminoethyl)phosphonic and (aminoethyl)phosphonous acids indicate that both sites must be occupied for good inhibition. The phosphinodipeptides are also more potent inhibitors than the enzyme's product DAla-DAla (IC_{50} = 1000 μM) as seen in comparison with **12b** (IC_{50} = 35 μM).

Walsh[23] has reported that the heptyl analogue **3p**, a compound made in these laboratories during the course of this study, is a high-affinity, time-dependent inhibitor of cloned *Salmonella* D-alanyl-D-alanine ligase. We also reported tight binding of this compound with our ligase preparation from the Gram-positive organism *S. faecalis*.[24] This tight-binding inhibition requires the presence of ATP in the enzyme reaction mixture. Dialysis results of ligase inactivated with **3p** and two other potent analogues are shown in Table II. A slow off-rate is evident with the heptyl inhibitor **3p**. However, those analogues more closely related to DAla-DAla (substituent R^2 = methyl or thiomethyl) appear to produce virtually irreversible inhibition, there being no evidence for recovery of any enzymatic activity after as long as 24-h dialysis.

Table II. ATP-Dependent Inhibition[a]



compd	R^1	R^2	concn, μM	% inhibn 4 h	% inhibn 24 h
(1-aminoethyl)phosphonic acid			10000	none	none
3e	*(S)-methyl*	*(R)-thiomethyl*	*10*	*96*	*96*
3p	(*S*)-methyl	(*RS*)-heptyl	10	75	53
12b	(*S*)-methyl	(*R*)-methyl	10	91	93

[a] Inhibitors were preincubated with enzyme and 0.005 M ATP at the indicated concentrations for 6 h under which conditions the enzyme is largely inactivated. Enzymatic activity was then assayed after 4- and 24-h dialysis. The data measure the degree of inhibition that persists after dialysis.

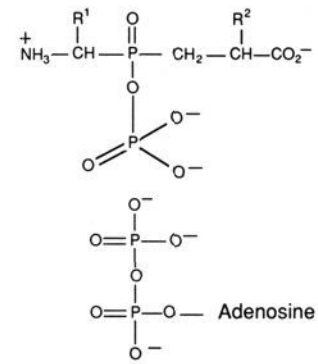


Figure 3. Proposed structure of a phosphorylated inhibitor–ADP complex bound to DAla-DAla ligase.

Precedence for the observed ATP-dependent inhibition derives from mechanisms for inhibition of glutamine synthetase by methionine sulfoximine[9] and phosphinothricin (**2**)[25] based on phosphorylation of these inhibitors by ATP within the active sites of these enzymes. By analogy, a possible structure of the enzyme–inhibitor complex formed from phosphinic acid inhibition of D-alanyl-D-alanine ligase is indicated in Figure 3.

As shown in Table III, these ligase inhibitors displayed some antibacterial activity. However, despite their exceptional enzyme inhibitory potencies, even the best of those reported here, compound **3e**, was only marginally superior to cycloserine on *S. faecalis* and *Proteus vulgaris* strains. We suspect poor transport into bacteria may contribute to this deficiency. Whereas fluoro-D-alanine and D-cycloserine are taken up by bacteria on a D-alanine/glycine transport system, D-alanyl-D-alanine appears not to penetrate bacteria as well as DAla.[26] Di- and oligopeptide derivatives have been used to facilitate the entry of antibiotic substances into bacteria,[27] but such peptides are derived from L-amino acids for good transport and/or

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Table III. Antibacterial Activities of Phosphinic Acid Inhibitors of DAla-DAla Ligase

organism[b]	in vitro antibacterial activity: MIC,[a] μg/mL						
	3b	12b	3c	3e	3p	3l	D-CS[c]
S. aureus 2865[d]	>256	>256	>256	>256	128	256	4
Strep. faecalis 2864	64	128	2	4	64	256	8
E. coli Tem 2$^+$ 4351	256	128	128	16	128	256	1
E. coli DC2 4353	256	256	8	64	32	128	4
E. coli 2891	>256	>256	256	128	128	128	1
Sal. typh. 3860	>256	>256	>256	128	128	128	8
Ent. cloacae 2647	>256	>256	256	128	256	>256	2
Klebs. pneum. 4005	256	128	256	128	64	256	1
Prot. vulg. 2829	256	64	128	4	128	>256	32
Pseud. aerug. 4279	128	128	32	64	>256	>256	16
Serratia mar. 2840	>256	>256	>256	>256	>256	>256	>256

[a] MIC = minimum inhibitory concentration in agar dilution assays with an antagonist-free medium.[31,32] [b] Full names for the organisms are given in Table IV. [c] D-Cycloserine. [d] Merck culture collection number.

Table IV. Antibacterial Activities of Inhibitor 12b (R^1, R^2 = D-CH_3) Combined with Fluoro-D-alanine

test culture	MB[b]	in vitro antibacterial activity: MIC,[a] μg/mL				
					level that no longer inhibits	
		F-D-Ala	12b	1:1 combination	F-D-Ala	1:1 combination
Staphylococcus aureus	2865	2.0	>256.0	2.0 + 2.0	8	32
Streptococcus faecalis	2864	8.0	8.0	8.0 + 8.0	>256	>256
Escherichia coli Tem 2$^+$	4351	4.0	128.0	4.0 + 4.0	128	>256
Escherichia coli DC2	4353	8.0	256.0	4.0 + 4.0	32	>256
Escherichia coli	2891	4.0	>256.0	8.0 + 8.0	64	>256
Salmonella typhimurium	3860	8.0	>256.0	8.0 + 8.0	32	>256
Enterobacter cloacae	2647	2.0	>256.0	4.0 + 4.0	16	128
Klebsiella pneumoniae	4005	1.0	128.0	1.0 + 1.0	32	256
Proteus vulgaris	2829	16.0	64.0	4.0 + 4.0	128	>256
Pseudomonas aeruginosa	4279	>256.0	128.0	4.0 + 4.0		>256
Serratia marcescens	2840	8.0	>256.0	8.0 + 8.0	32	>256

[a] MIC's were determined in agar dilution assays with an antagonist-free medium.[31,32] [b] Merck culture number.

subsequent proteolytic hydrolysis. Thus, these phosphinic acids whose preferred stereochemistry for a good ligase inhibition is that of DAla-DAla appear to be poor candidates for facilitated peptide transport into bacteria.

As shown in Table IV, potent, broad-spectrum antibacterial activity is obtained with a 1:1 combination of the alanine racemase inhibitor fluoro-D-alanine and compound **12b**. Of particular interest is the enhancement that compound **12b** provides to fluoro-D-alanine's activity toward the *Pseudomonas* and *Proteus* strains used in this panel of test organisms. Furthermore, as we also reported earlier,[24] this combination inhibits the self-reversal of antibacterial activity that is typically shown by fluoro-D-alanine at concentrations several fold higher than its MIC's. The latter phenomena has been shown to result when fluoro-D-alanine, after blocking alanine racemase, itself acts as a substrate of DAla-DAla ligase and is incorporated into cell wall peptidoglycan in place of D-alanine.[28] Inhibition of this self-reversal can be achieved with D-cycloserine,[28] and the similar effect of **12b** is in accord with the latter's activity as a DAla-DAla ligase inhibitor. Since this inhibition of F-DAla self-reversal is observed with most of the bacterial strains shown in Table IV, it would appear that DAla-DAla ligases from many species of bacteria are susceptible to inhibition by compound **12b**.

Experimental Section

D-Alanyl-D-alanine Ligase. *S. faecalis* ATCC 8043 was grown as described by Neuhaus.[10] Cell-free extracts were prepared by sonication followed by centrifugation at 27000*g* for 30 min. Protein was precipitated with 50% ammonium sulfate. 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 unit/mg of protein. No loss of activity was observed after 1 year.

Enzyme Assay. The assay mixture contained 0.05 M Tris buffer adjusted to pH 7.90 with HCl, 0.01 M KCl, 0.008 M $MgCl_2$, 0.005 M ATP, 0.005 M D-[1-^{14}C]alanine (specific activity 0.2 Ci/mol), and 625 μg of enzyme protein/mL in a volume of 50 μL. Inhibitors were preincubated with enzyme for 40 min before addition of ATP and D-alanine, and the assay was conducted at 37 °C for 30 min and then quenched by freezing in dry ice. Reaction rates were constant up to 15% conversion to product.

Product D-alanyl-D-alanine was separated from D-alanine by thin-layer chromatography on high-resolution, prechanneled silica gel plates (Altech "Adsorbosil"). Plates were developed in ethanol–ammonium hydroxide–water (11:1:8) for 3 h, and the radioactive zones were located and integrated with a Berthold linear analyzer to determine IC_{50}'s.

To study the ATP-dependent component of inhibition, enzyme and inhibitor were preincubated for 6 h in the above assay mixture in the absence of D-alanine. Samples (100 μL) were then dialyzed at 4 °C in a Bethesda Research Laboratories microdialysis apparatus against the reaction buffer and assayed for free enzyme by addition of the two substrates. Controls conducted in tandem with each inhibitor in the absence of ATP gave no evidence of irreversible inhibition but did indicate 14–24% incomplete dialysis at the 4-h time point. This produces an uncertainty of ±4% in assigning the 4-h values entirely to slowly reversible inhibition.

Antibacterial Assays. Antibacterial data were obtained with use of a synthetic agar medium describe by Atherton et al.[29] and Allen et al.[30] The minimum inhibitory concentrations (MIC) were determined by an agar plate dilution method. The investigational compounds were dissolved in water at a concentration of 256 μg/mL from which several twofold dilutions were made in water. One-milliliter aliquots of the diluted compounds were added to

(28) Kahan, F. M.; Kropp, H. *Abstracts, 15th Interscience Conference on Antimicrobial Agents and Chemotherapy*; Washington, DC, Sept 1975; No. 100.

(29) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Lambert, R. W.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 677.

(30) Allen, J. G.; Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Holmes, S. W.; Lambert, R. W.; Nisbet, L. J.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 684.

9 mL of molten agar medium resulting in final concentrations in the agar ranging from 256 to 1 μg/mL or lower. In most of the experiments, the microorganisms were subcultured from agar to trypticase soy broth and incubated at 35 °C for 20 h. The *Pseudomonas* strain was incubated on a rotary shaker at 250 rpm. MIC's were recorded as the lowest concentration of the antibacterial agent that showed no visible growth or less than five discrete colonies per inoculation spot.

Chemistry. General Methods. ^{1}H NMR spectra were recorded on a Varian XL300 pulsed Fourier transform instrument or a Varian T-60 instrument. Mass spectra were recorded on a Finnigan-MAT 731 mass spectrometer.

Elemental analyses were within ±0.4% of the theoretical values. Thin-layer chromatography was carried out on silica gel MK6L (Whatman, 0.2 mm) glass-backed plates with the following general solvent systems: 9:1 EtOAc–CH_3CN (system 1) and 1:1:1:1 *n*-BuOH–H_2O–HOAc–EtOAc (system 2). Preparative medium-pressure chromatography (MPLC) was carried out with Lobar LiChroprep Si60 (E. Merck, 40–63 μm) prepacked columns.

(±)-, (+)-, (−)-[1-[(Benzyloxycarbonyl)amino]ethyl]phosphonous acids were prepared according to the method of Baylis.[14] Methyl acrylate and methyl methacrylate were purchased from Aldrich Chemical Co. Methyl 2-ethylacrylate, methyl 2-isobutylacrylate, methyl 2-*n*-propylacrylate, methyl 2-*n*-butylacrylate, methyl 2-isobutylacrylate, methyl 2-*n*-heptylacrylate, methyl 2-benzylacrylate, methyl 2-phenethylacrylate, and methyl 2-(phenyl-*n*-propyl)acrylate, were prepared by the method of Atta-Ur-Rahman.[15] Methyl 2-(methylthio)acrylate was prepared by the procedure of Gundermann and Schultze.[17] Synthesized compounds unless otherwise specified are racemic.

Method A. (1-Aminoethyl)(2-carboxy-1-ethyl)phosphinic Acid (3a). To a stirred solution of methyl [1-[(benzyloxycarbonyl)amino]ethyl]phosphinate (0.36 g, 0.0014 mol) in 3 mL of MeOH at 0 °C was added a solution of sodium methoxide in MeOH (0.77 mL of a 2 N solution) dropwise over 10 min, following which was added dropwise methyl acrylate (0.126 mL, 0.0014 mol). The reaction mixture was then stirred for 30 min at 0 °C and for 4 h at room temperature. The reaction was quenched with 1 N HCl and extracted twice with EtOAc. The combined organic fractions were dried over Na_2SO_4, filtered through a thin pad of $MgSO_4$, and evaporated in vacuo. The product was chromatographed on silica gel (MPLC) with EtOAc–CH_3CN (9:1) as eluant to give methyl [1-[(benzyloxycarbonyl)amino]ethyl](2-carbomethoxy-1-ethyl)phosphinate **7a** (0.34 g) as a clear oil: TLC (system 1) R_f 0.47; ^{1}H NMR ($CDCl_3$) δ 1.4 (2 d, 3 H), 2.00–2.20 (m, 2 H), 2.5–2.7 (m, 2 H), 3.7 (s, 3 H), 3.7–3.9 (overlapping d, 3 H), 4.05–4.2 (q, 1 H), 5.1 (s, 2 H), 5.2 (d, $^1/_2$ H), 5.55 (d, $^1/_2$ H), 7.4 (br s, 5 H); MS (FAB), *m/e* 343 (M + 1).

This intermediate (0.3 g) was stirred for 12 h in 5 mL of 30% HBr in acetic acid. The reaction mixture was then evaporated in vacuo. The product was dissolved in H_2O (5 mL), washed twice with diethyl ether, and evaporated, again, in vacuo. The intermediate was redissolved in concentrated HCl (5 mL), stirred for 3 days at 50 °C, and subsequently evaporated. The hydrochloride salt was dissolved in MeOH (1 mL) and diluted with propylene oxide (5 mL). A solid precipitated out and was isolated by filtration and washed with diethyl ether to give the title compound as a very hygroscopic glass (28% overall yield): TLC (system 2) R_f 0.28; ^{1}H NMR (D_2O) δ 1.35–1.5 (2 overlapping d, 3 H), 1.85–2.00 (m, 2 H), 2.55–2.65 (AB q, 2 H), 3.25–3.35 (m, 1 H); MS (FAB), *m/e* 182 (M + 1).

In like manner as the above sequence and with the appropriate acrylate and phosphinate, compounds **3b–d,f–p** were prepared.

(1(*S*)-Aminoethyl)[2-carboxy-2(*R*)-(methylthio)-1-ethyl]phosphinic Acid (3e). To a stirred solution at 0 °C of methyl [1-(*S*)-[(benzyloxycarbonyl)amino]ethyl]phosphinate **5(S)** (245 mg, 0.95 mmol) in 2 mL of dry MeOH was added by syringe 0.52 mL of 2.0 N methanolic sodium methoxide (1.04 mmol). After 5 min methyl-2-(methylthio)acrylate (185 mg; 1.4 mmol) was added dropwise by syringe at 0 °C. After 10 min at 0 °C and 90 min at room temperature EtOAc and saturated aqueous NaH_2PO_4 were added, and the mixture was extracted 5 times with EtOAc. The latter extract was washed with saturated aqueous NaCl, dried over $MgSO_4$, and concentrated to dryness to give the crude Michael adduct (312 mg). The product was purified by preparative TLC on silica gel plates (solvent system; acetone–$CHCl_3$, 1:1) to give pure (*S,RS*)-methyl [1(*S*)-[(benzyloxycarbonyl)amino]ethyl][2-carbomethoxy-2(*RS*)-(methylthio)-1-ethyl]phosphinate (**7e**) (260 mg, 71%): ^{1}H NMR ($CDCl_3$) δ 1.40 (dd, 3 H), 2.16 (s, 3 H) 2.2–2.8 (m, 2 H), 3.60–3.84 (m, 7 H), 4.20 (t, 1 H), 5.10 (s, 1 H), 5.62 (dd, 1 H) 7.36 (s, 5 H); MS (FAB), *m/e* 390 (M + 1); TLC (1:1 acetone–$CHCl_3$) R_f 0.45.

The Michael adduct (250 mg) was deprotected in 10 mL of 6 N HCl at 100 °C for 75 min. The mixture was then cooled and extracted with chloroform, and the colorless aqueous layer was concentrated to dryness under water pump vacuum to give the hydrochloride of **3e** as a colorless foam (170 mg). To the latter in MeOH (3 mL) was added propylene oxide (2 mL). The precipitated **3e** was collected after 1 h: 45 mg; mp 163–165 °C; TLC (system 2) R_f 0.40 (spot visualization by ninhydrin and also iodine vapor); ^{1}H NMR (D_2O) δ 1.38 (dd, 3 H), 2.00 (m, 1 H) 2.16 (br s, 3 H), 2.30 (m, 1 H), 3.34 (m, 1 H), 3.59 (m, 1 H); MS (TMS), *m/e* 371 (M + 144), 299 (M + 72).

Concentration of the filtrate and freeze-drying of the residue gave 107 mg of amorphous solid with identical R_f. The crystalline material is provisionally assigned *S,R* stereochemistry because of its high ligase inhibitory potency.

Methyl [1(*S*)-[(Benzyloxycarbonyl)amino]ethyl](2-carbomethoxy-2-propenyl)phosphinate (9). A solution of methyl [1(S)-[(benzyloxycarbonyl)amino]ethyl]phosphinate **5(*S*)** (1.0406 g, 4.05 mmol) in 2.5 mL of distilled methanol at 0 °C was treated dropwise over 10 min with 2.2 mL of 2.0 N methanolic sodium methoxide (4.4 mmol, 1.1 equiv). When the addition of base was complete, the mixture was stirred an additional 5 min at 0 °C before a solution of 2-(trimethylphosphono)acrylate (Fluka) (0.94 mL, 6.1 mmol) in 1 mL of distilled methanol was added dropwise over 5 min. After 1 h at 0 °C, 37% aqueous formaldehyde (Formalin, 0.93 mL, 12 mmol) was added dropwise over 5 min.

The mixture was warmed to room temperature and stirred at room temperature for 3 h. The reaction was quenched at 0 °C by addition of 20 mL of EtOAc and 5 mL of 1 N HCl. The organic layer was removed, and the aqueous was layer reextracted with 5 mL of EtOAc. The combined organic extracts were washed with 2 × 5 mL of brine and then filtered through a plug of anhydrous sodium sulfate onto anhydrous magnesium sulfate. The solution was filtered, and all volatiles were removed under vacuum to afford a pale yellow oil, which was purified by medium-pressure liquid chromatography on silica, eluting with 5% MeOH–EtOAc. Purification in this manner afforded 1.091 g (3.07 mmol; 76%) of the title compound as a viscous oil: TLC (system 2) R_f 0.85; ^{1}H NMR ($CDCl_3$) δ 1.4 (dd, 3 H), 3.0 (dd, 2 H), 3.75 (m, 6 H), 4.2 (m, 1 H), 5.1 (br s, 1 H), 5.2 (s, 2 H), 5.8 (d, 1 H), 6.4 (d, 1 H), 7.3–7.4 (m, 5 H); MS, *m/e* 355 (M^+).

[1(*S*)-[(Benzyloxycarbonyl)amino]ethyl](2-carbomethoxy-2-propenyl)phosphinic Acid (10). A solution of methyl [1(S)-[(benzyloxycarbonyl)amino]ethyl](2-carbomethoxy-2-propenyl)phosphinate (**9**) (2.26 g, 6.34 mmol) in 10 mL of dry THF at room temperature was treated with anhydrous lithium iodide (0.94 g, 7.0 mmol, 1.1 equiv). The mixture was stirred at room temperature for 72 h, at which time TLC (system 2) indicated complete absence of starting material and appearance of the product with an R_f of 0.70.

All volatiles were removed under vacuum, and the residue was redissolved in 30 mL of 5% aqueous sodium bicarbonate and washed with EtOAc (5 × 10 mL). The aqueous layer was cooled to 0 °C and made acidic (pH 1) by the slow addition of 6 N HCl. The acidified mixture was extracted with EtOAc (6 × 10 mL); the combined organic extracts were washed once with brine and filtered through a plug of anhydrous sodium sulfate onto anhydrous $MgSO_4$. Filtration and removal of solvent in vacuo afforded the title compound (1.779 g, 5.22 mmol, 82%) as a pale yellow solid: TLC (system 2) R_f 0.70; ^{1}H NMR (CD_3OD) δ 1.3 (dd, 3 H), 3.0 (d, 2 H), 3.7 (s, 3 H), 4.0 (m, 1 H), 5.1 (br s, 2 H), 5.8 (d, 1 H), 6.3 (d, 1 H) 7.3–7.4 (m, 6 H); MS (FAB), *m/e* 342 (M + 1).

Asymmetric Hydrogenation of [1(*S*)-[(Benzyloxycarbonyl)amino]ethyl][2-(carboxymethyl)-2-propenyl]phosphinic Acid (10). Preparation of [1(*S*)-[(Benzyloxycarbonyl)amino]ethyl](2(*R*)-carbomethoxy-1-propyl)phosphinic Acid (11). A solution of compound **10** (0.172 g, 0.504 mmol) in 3 mL of dry MeOH and 0.5 mL of dry benzene was

hydrogenated at 40 psi in the presence of 25 mg of 1,5-cyclooctadienerhodium(I) chloride dimer and 70 mg of (–)-2,3-*O*-isopropylidene-2,3-dihydroxy-1,4-bis(diphenylphosphino)butane ("(–)-DIOP", Alfa). Hydrogen uptake was complete after 3 h. The mixture was diluted with 10 mL of ether, cooled to 0 °C, and filtered to remove the product. The crude product was recrystallized three times from aqueous acetic acid to give a single, pure diastereomer, identified as the D,D isomer [1(*S*)-[(benzyloxycarbonyl)amino]ethyl](2(*R*)-carbomethoxy-1-*n*-propyl)phosphinic acid (**11**): TLC (system 2) R_f 0.70; ^{1}H NMR (CD_3OD) δ 1.2 (d, 3 H), 1.3 (dd, 3 H), 1.8 (m, 1 H), 2.2 (m, 1 H), 2.9 (m, 1 H), 3.7 (s, 3 H), 4.0 (m, 1 H), 5.1 (dd, 1 H), 7.3–7.5 (m, 6 H); MS (NIFAB), *m/e* 342 (M – H); $[\alpha]_D$ +30.1° (CH_3OH, c 0.3).

(1(*S*)-Aminoethyl)(2(*R*)-carboxy-1-*n*-propyl)phosphinic Acid (12b). [1(*S*)-[(Benzyloxycarbonyl)amino]ethyl](2(*R*)-carbomethoxy-1-*n*-propyl)phosphinic acid (**11**) was converted according to method A to give the title compound **12b** as a single diastereomer in 60% yield: TLC (system 2) R_f 0.35; ^{1}H NMR (D_2O) δ 1.27 (d, 3 H), 1.38 (dd, 3 H), 1.68 (m, 1 H), 2.10 (m, 1 H), 2.81 (m, 1 H), 3.25 (m, 1 H); MS (NIFAB), *m/e* 194 (M – H).

(1(*S*)-Aminoethyl)(2(*S*)-carboxy-1-*n*-propyl)phosphinic Acid (12a). This chirally pure diastereomer was isolated by HPLC separation of methyl[1(*S*)-[(benzyloxycarbonyl)amino]-ethyl](2(*RS*)-carbomethoxy-1-propyl)phosphinate (**7n**) on a RP-18 (E. Merck) semipreparative column, eluting with H_2O (73%) and acetonitrile (27%) and giving diastereomers A and B. Each diastereomer was deprotected according to method A to give two pure diastereomers as determined by HPLC. Diastereomer A (t_R 29.5 min) gave on deprotection compound **12b** (NMR, MS, HPLC) and diastereomer B gave the *S,S* compound **12a** having TLC, NMR, and MS data identical with that of **12b**.

Benzyl [1(*S*)-[(Benzyloxycarbonyl)amino]ethyl](2(*R*)-carbomethoxy-1-propyl)phosphinate (13). A solution of compound **11** (0.342 g, 1 mmol) in THF (2 mL) at room temperature was treated with benzyl alcohol (0.134 mL 1.3 mmol), followed by dicyclohexylcarbodiimide (0.22 g, 1.1 mmol) and 4-(dimethylamino)pyridine. The mixture was stirred at room temperature for 6 h; then, an additional 0.22 g of DCC was added, and the reaction stirred overnight at room temperature. The mixture was diluted with ether (20 mL) and filtered, and the filtrate was washed with HCl (0.1 N), aqueous sodium bicarbonate (1%), and brine. The organic layer was dried over $MgSO_4$, filtered, and evaporated in vacuo. The residue was purified by chromatography (silica, 9:1 EtOAc–CH_3CN) to give the title compound (0.338 g, 78%) as an 8:1 mixture of diastereomers at phosphorus. The major component was isolated by preparative HPLC (silica, EtOAc) to give **13** (0.25 g) as a single diastereomer.

Crystals suitable for X-ray were obtained by slow, room temperature evaporation of a 10-mg sample of **13** in 250 μL of ether: TLC (silica; $CHCl_3$–MeOH–H_2O–HOAc, 100:20:3:0.5) R_f 0.87; ^{1}H NMR ($CDCl_3$) δ 1.20 (d, 3 H), 1.32 (q, 3 H), 1.83 (m, 1 H), 2.33 (m, 1 H), 2.82 (m, 1 H), 3.55 (s, 3 H), 4.10 (m, 1 H), 5.1 (m, 4 H), 5.62 (d, 1 H), 7.3 (br s, 10 H); MS (FAB), *m/e* 434 (M + 1).

X-ray Crystal Structure Analysis of 13. Suitable crystals of **13** ($C_{22}H_{28}NO_6P$) for X-ray diffraction studies formed from ether with space group symmetry of $P\bar{1}$ and cell constants of a = 10.482 (2) Å, b = 11.254 (3) Å, c = 5.479 (4) Å, α = 91.58 (3)°, β = 92.63 (3)°, and γ = 117.68 (2)° for Z = 1 and a calculated density of 1.261 g/cm^3. Of the 1524 reflections measured with an automatic four-circle diffractometer equipped with Cu radiation, 1433 reflections were observed [$I > 3\sigma(I)$]. The structure was solved with a multisolution tangent formula approach and difference Fourier analysis and refined by using full-matrix least-squares techniques.[22] Both phenyl groups were found to be disordered. This disordering was modeled by two separate positions for each phenyl group with occupancies of 0.5. The function $\sum\omega(|F_o| - |F_c|)^2$ with $\omega = 1/(\sigma F_o)^2$ was minimized to give an unweighted residual of 0.064. Tables V–VII containing the final fractional coordinates, temperature parameters, bond distances, and bond angles are available as supplementary material. Tables VI and VII only contain bond distances and angles for one set of phenyl positions. Figure 2 is a computer generated perspective drawing of **13** from the final X-ray coordinates showing the absolute stereochemistry.

Registry No. **3a**, 115047-74-0; **3b**, 113592-15-7; **3c**, 113592-18-0; **3d**, 113592-20-4; **3e**, 115047-75-1; **3f**, 113592-22-6; **3g**, 115047-76-2; **3h**, 113592-24-8; **3i**, 113592-29-3; **3j**, 113592-31-7; **3k**, 113592-33-9; **3l**, 113592-36-2; (I)-4, 76437-21-3; (*R*)-**4**, 115115-40-7; (*S*)-**4**, 115115-41-8; (±)-**5**, 113592-12-4; (*R*)-**5**, 113592-76-0; (*S*)-**5**, 113592-51-1; **7a**, 113592-14-6; **7b**, 113592-16-8; **7c**, 113592-19-1; **7d**, 113592-21-5; **7e**, 115047-81-9; **7f**, 113592-23-7; **7g**, 115047-82-0; **7h**, 113592-25-9; **7i**, 113592-30-6; **7j**, 113592-32-8; **7k**, 113592-35-1; **7l**, 113592-38-4; **9**, 115047-83-1; **10**, 115047-84-2; **11**, 115047-85-3; **13**, 115047-86-4; methyl acrylate, 96-33-3; methyl methacrylate, 80-62-6; methyl 2-ethylacrylate, 2177-67-5; methyl 2-propylacrylate, 3070-66-4; methyl 2-(methylthio)acrylate, 43228-10-0; methyl 2-butylacrylate, 3070-68-6; methyl 2-isobutylacrylate, 3070-69-7; methyl 2-heptylacrylate, 91213-29-5; methyl 2-benzylacrylate, 3070-71-1; methyl 2-(2-phenylethyl)acrylate, 113592-34-0; methyl 2-(3-phenylpropyl)acrylate, 88465-93-4; L-alanyl-L-alanine ligase, 9023-63-6; 2-trimethylphosphonoacrylate, 55168-74-6; fluoro-D-alanine, 35455-20-0; 1-aminoethylphosphonic acid, 16606-65-8; 1-aminoethylphosphonous acid, 65576-94-5; D-alanyl-D-alanine, 923-16-0; D-cycloserine, 68-41-7; 1-(carbobenzoxyamino)propanephosphinic acid, 115047-79-5; 1-(carbobenzoxyamino)propanephosphinic acid methyl ester, 115075-80-4.

Supplementary Material Available: Tables of the atomic positional and thermal parameters, bond distances, and bond angles for **13** (3 pages). Ordering information is given on any current masthead page.

Synthesis of (Aryloxy)alkylamines. 1. Novel Antisecretory Agents with H^+K^+-ATPase Inhibitory Activity

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A series of heterocyclic (aryloxy)alkylamines of structures II and III were prepared and found to possess gastric antisecretory activity. Of the variety of substituted thiazoles, benzoxazoles, and benzothiazoles prepared, thiazole **18**, benzoxazole **32**, and benzothiazole **47** exhibited gastric antisecretory potency comparable to that of ranitidine in vivo in the pylorous ligated rat model. In an isolated rabbit parietal system, the series of thiazoles, benzoxazoles, and benzothiazoles also demonstrated similar potency to that of ranitidine toward the inhibition of both histamine-stimulated and dcAMP-stimulated uptake of amino[^{14}C]pyrine. These compounds inhibited the H^+K^+-sensitive ATPase enzyme in isolated gastric microsomes. A direct correlation existed between inhibition of ^{14}C uptake, in vivo antisecretory activity, and inhibition of the H^+K^+-ATPase enzyme. The more potent antisecretory compounds **18**, **32**, and **47** were also the more potent enzyme inhibitors. These data suggest that the mechanism responsible for the observed in vitro and in vivo gastric antisecretory activity, in these series of compounds, is a consequence of the inhibition of the H^+K^+-sensitive ATPase enzyme.

Peptic ulcer disease results from the failure of tissues to resist the corrosive effects of gastric acid and pepsin.[1] This imbalance in the hemostatic mechanism often can be restored by reducing the exposure of these tissues to gastric

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