Chloroalanyl Antibiotic Peptides: Antagonism of Their Antimicrobial Effects by L-Alanine and L-Alanyl Peptides in Gram-Negative Bacteria

Kam Sing Cheung,[†,§] William Boisvert,[‡] Stephen A. Lerner,*[,‡] and Michael Johnston*[,†]

Deparments of Chemistry and of Biochemistry and Molecular Biology, Searle Chemistry Laboratory, and Departments of Medicine and Microbiology, The University of Chicago, Chicago, Illinois 60637. *Received December 27, 1985*

A large number of structurally diverse di- and tripeptides containing the alanine racemase inactivator β-chloro-L-alanine (β-Cl-LAla)[1] have been synthesized, and their antibacterial properties in vitro have been evaluated. The dipeptides **1**, **3–6**, and **8–17** and the tripeptide **20** are all broad-spectrum antibacterial agents with considerable potency against both Gram-positive and Gram-negative species, but none of these peptides improves dramatically on the antibiotic efficacy of the previously described β-Cl-LAla-β-Cl-LAla, **9** (Cheung, K. S.; Wasserman, S. A.; Dudek, E.; Lerner, S. A.; Johnston, M. *J. Med. Chem.* **1983**, *26*, 1733). Gram-negative microorganisms, such as *Escherichia coli*, *Hemophilus influenzae*, *Shigella flexneri*, and *Enterobacter* species are consistently resistant to any haloalanyl peptide containing an alanyl residue, such as the dipeptide LAla-β-Cl-LAla (**2**) and the tripeptides LMet-LAla-β-Cl-LAla (**7**), LAla-LAla-β-Cl-LAla (**18**), and LVal-LAla-β-Cl-LAla (**19**). Correspondingly, these same organisms are protected from the bactericidal effects of **9** by supplementation of the growth medium with LAla or LAla-LAla. *Escherichia coli* JSR-O exposed to **9**, but protected from lysis by sucrose stabilization, has only about 10% the normal level of intracellular alanine racemase activity. But when these cells are cultured in the presence of **9** with LAla supplementation, or in the presence of **2** with no supplementation, the alanine racemase levels are only about 20–30% below control values. These findings suggest that the resistance of Gram-negative species to chloroalanyl peptides containing alanyl units arises from the ability of LAla to protect the targeted racemase from inactivation by β-Cl-LAla in vivo, an event which otherwise leads to cell death and lysis. Inactivation of alanine racemase in Gram-positive organisms appears not to be the cellular event that confers sensitivity of these species to a haloalanyl peptide.

A number of research groups have attempted in recent years to conscript microbial peptide transport systems for intracellular delivery of peptides that contain potentially bactericidal amino acid residues. Peptide transport in bacteria displays only modest selectivity for the side-chain structures of component amino acids,[2] and this finding suggests that the potency of an impermeable amino acid antibiotic can be enhanced by its incorporation into a readily transportable peptide.[3] The prediction has been clearly anticipated in nature. There are at least a dozen naturally occurring antibiotic peptides that appear to use the microbial peptide transport systems for smuggling an animo acid analogue through the envelope of bacterial membranes.[4] Among these are bacilysin,[5] a dipeptide which contains an unusual amino acid with a cyclohexanone epoxide side chain, and tabtoxin,[6] a dipeptide with a β-lactam ring in the side-chain structure of one of its two amino acids. Only a small number of synthetic antibiotic peptides have been reported. Among these are alafosfalin and its oligopeptide derivatives,[7] peptides containing *m*-fluorophenylalanine,[8] and a set of dipeptides that incorporate into their structures the mechanism-based enzyme inactivators β-chloroalanine and propargylglycine.[9] In all of these cases, expression of the antibacterial effects of the peptide appears to require transport and subsequent intracellular hydrolysis to the component amino acids.

We have recently offered a detailed analysis of the mechanism of action of the dipeptide β-Cl-LAla-β-Cl-LAla (β-Cl-LAla = β-chloro-L-alanine)—representative of the class of haloalanyl-containing antibiotic peptides. In *Escherichia coli* strain JSR-O, the dipeptide is clearly transported across the inner membrane by a dipeptidyl permease and, once internalized, leads to inactivation of both alanine racemase and transaminase B in vivo.[10] Enzyme inactivation is almost assuredly preceded by hydrolysis of β-Cl-LAla-β-Cl-LAla; although the dipeptide alone has no inhibiting effect on the purified *E. coli* racemase, peptidase-dependent inactivation of the enzyme was demonstrated in vitro.

Insofar as both peptide transport and intracellular cleavage are key steps in this particular strategy for drug delivery, it seems obvious that both the spectrum of activity and the biological potency of a peptide will be determined by the efficiency with which it is accumulated, by its ability to be cleaved by bacterial peptidases, or by

[†] Departments of Chemistry and of Biochemistry and Molecular Biology.

[‡] Departments of Medicine and Microbiology.

[§] Present address: Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143.

(1) Other abbreviations used: LAbu, L-α-aminobutyryl; AcOH, acetic acid; LAla(P), L-(aminoethyl)phosphonate; BOC, *tert*-butoxycarbonyl; *t*-Bu, *tert*-butyl; BuOH, 1-butanol; cfu, colony-forming units; CHES, α-(*N*-cyclohexylamino)ethanesulfonic acid; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DIEA, *N,N*-diisopropylethylamine; EDTA, ethylenediaminetetraacetate; EtOAc, ethyl acetate; MIC, minimum inhibitory concentration; LNle, L-norleucyl; LNva, L-norvalyl; TFA, trifluoroacetic acid or trifluoroacetate.

(2) Payne, J. W. In *Peptide Transport in Protein Nutrition*; Matthews, D. M., Payne, J. W., Eds.; North Holland: Amsterdam, 1975; pp 283–364. Matthews, D. M.; Payne, J. W. *Curr. Top. Membr. Transp.* **1980**, *14*, 331.

(3) Payne, J. W. *Adv. Microb. Physiol.* **1976**, *13*, 55. Diddens, H.; Zahner, H.; Krauss, E.; Gohring, W.; Jung, G. *Eur. J. Biochem.* **1976**, *66*, 11. Lichliter, D. W.; Naider, F.; Backer, J. M. *Antimicrob. Agents Chemother.* **1976**, *10*, 483.

(4) Diddens, H.; Dorgerloh, M.; Zähner, H. *J. Antibiot.* **1979**, *32*, 87.

(5) Walker, J. E.; Abraham, E. P. *Biochem. J.* **1970**, *118*, 563. Neuss, N.; Molloy, B. B.; Shah, R.; DeLahiguera, N. *Biochem. J.* **1970**, *118*, 571.

(6) Stewart, W. W. *Nature (London)* **1971**, *229*, 174. Taylor, P. A.; Schnoes, H. K.; Durbin, R. D. *Biochim. Biophys. Acta* **1972**, *286*, 107.

(7) (a) Allen, J. G.; Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Holmes, S. W.; Lambert, R. W.; Nisbet, L. J.; Ringrose, P. S. *Nature (London)* **1978**, *272*, 56. Allen, 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. (b) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Holmes, S. W.; Lambert, R. W.; Lloyd, W. J.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1980**, *18*, 897.

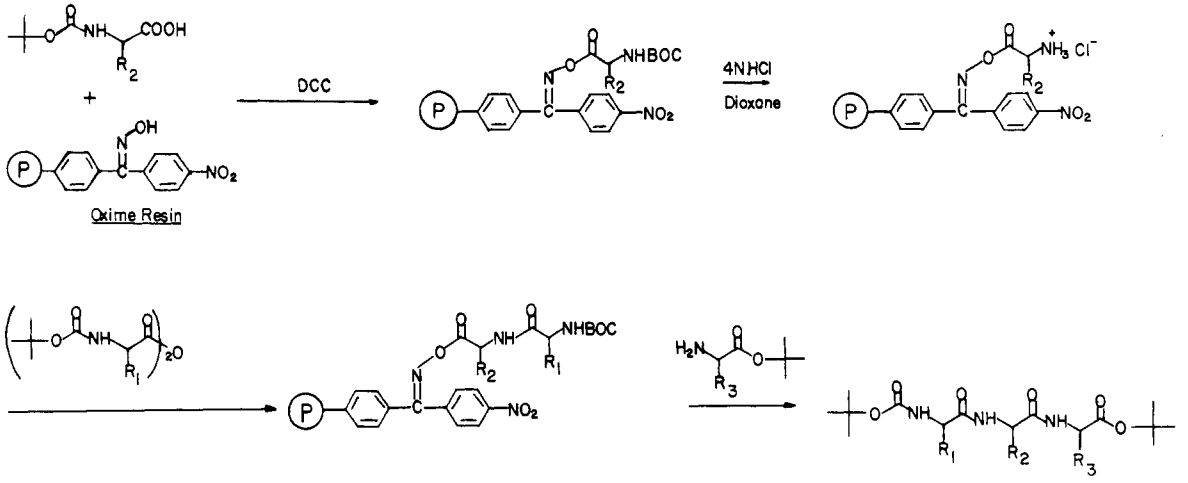
(8) Kingsbury, W. D.; Boehm, J. C.; Mehta, R. J.; Grappel, S. F. *J. Med. Chem.* **1983**, *26*, 1725.

(9) Cheung, K. S.; Wasserman, S. A.; Dudek, E.; Lerner, S. A.; Johnston, M. *J. Med. Chem.* **1983**, *26*, 1733.

(10) Boisvert, W.; Cheung, K. S.; Lerner, S. A.; Johnston, M. *J. Biol. Chem.* **1986**, *261*, 7871.

0022-2623/86/1829-2060$01.50/0

Scheme I



both. For example, if a tripeptide of β-chloroalanine were transported more rapidly by a species of bacterium than were its dipeptide cognate, then the tripeptide is likely to be the more potent antibacterial agent against that organism. Similarly, the spectrum of activity of, for example, the hypothetical LTrp-β-Cl-LAla will likely be restricted to those organisms that contain "tryptophanyl dipeptidase" activities, even though the dipeptide might be readily transported into a large number of different species.

In an initial attempt to explore the limits of the use of haloalanyl peptides as an approach to the design of antibacterial delivery systems, we have prepared a large number of chloroalanyl-containing di- and tripeptides of considerable structural diversity. When these were subjected to routine susceptibility testing, the interesting observation was made that every compound containing an alanyl residue—independent of the other structural features of the peptide—consistently lacked activity against Gram-negative species. In this paper, we report on studies which demonstrate that this finding correlates with the ability of LAla to protect in vivo the target enzyme alanine racemase from inactivation by β-Cl-LAla, which otherwise leads to cell lysis in Gram-negative organisms.

Chemistry. We have continued to use the solid-phase method developed by DeGrado and Kaiser[11] for the synthesis of chloroalanyl peptides. For dipeptide synthesis, this procedure involves the DCC coupling of an *N*-BOC-protected amino acid to a polystyrene-bound *p*-nitrobenzophenone oxime. The protected amino acid is subsequently removed from the support by aminolysis with the *tert*-butyl ester of a second amino acid, a step which forms the peptide bond and regenerates the oxime resin.

Tripeptide synthesis was accomplished by a slight modification of the DeGrado–Kaiser method (Scheme I). A resin-bound amino acid oxime—bearing the second residue of the nascent tripeptide—is first prepared by conventional DCC coupling of an *N*-BOC amino acid to the oxime group of the support. This adduct is then treated with 4 N HCl in dioxane to remove the BOC group.[12] The resulting amine is coupled to the symmetric anhydride of the *N*-BOC-amino acid that will become the N-terminal residue of the peptide. Finally, the protected tripeptide is formed by reaction of the dipeptidyl oxime with the *tert*-butyl ester of the third, and carboxy-terminal, amino acid.

N-Butoxycarbonyl and *tert*-butyl protecting groups were used throughout because of the relative ease of deprotection; both are cleaved in a single step with TFA in anisole, giving deblocked peptides as their trifluoroacetate salts. The structures of synthetic peptides are given in Table VII.

Antibacterial Activity of Chloroalanyl Dipeptides Containing an N-Terminal Residue with an *n*-Alkyl Side Chain. During the course of their extensive studies on the antibacterial peptides containing aminoethylphosphonate (Ala(P)), the Roche group described a set of dipeptides of the general structure X-Ala(P).[7b] In this series, the N-terminal residues (X) were amino acids with *n*-alkyl side chains of systematically varying carbon number. Antibacterial potency of any given peptide was found to correlate with the efficiency of its transport into bacteria, and the rate of transport, in turn, related directly to the length of the *n*-alkyl group. These observations prompted our syntheses of the dipeptides **1–5**. the minimum inhibitory concentrations (MICs) for these compounds are presented in Table I. Examination of the data reveals clearly that there is no systematic variation, by contrast to what was observed for Ala(P)-containing peptides,[7b] in the biological activities of **1–5**. The MICs of Table I are generally very similar to one another and seem not to correlate with the length of the side-chain structure of the N-terminal residue.

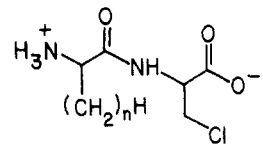
The unexpected finding of Table I is the consistent lack of susceptibility to LAla-β-Cl-LAla (**2**) among Gram-negative species, even those which are sensitive to some or all of the other four peptides (**1**, **3–5**). The two most striking examples of this trend are seen with *Hemophilus influenzae* and *E. coli*. Both of these organisms are quite susceptible to **1**, **3**, and **4**, but the MICs for **2** are >100 μg/mL in each case.

The staphylococci and *Streptococcus agalactiae* of Table I are, by contrast to the Gram-negative organisms, generally susceptible to all of the peptides, including **2**. *Streptococcus pyogenes* and *Strep. faecalis* behave, unaccountably but consistently, more like Gram-negative than like Gram-positive organisms, with reference to their sensitivity to chloroalanyl peptides. The pattern of Gram-negative resistance and Gram-positive susceptibility to haloalanyl peptides containing alanyl residues became a recurring theme in our studies (vide infra).

Antibacterial Activity of N-Terminal Methionyl Peptides. The biosynthesis of a large number of bacterial proteins appears to be initiated by *N*-formyl-L-methionyl

(11) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1980**, *45*, 1295. DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1982**, *47*, 3258.
(12) Burton, J.; Topper, R.; Ehrich, P. In *Peptides: Structure and Biological Function*; Gross, E., Meinhofer, J., Eds.; Pierce Chemical Co.: Rockford, IL, 1979; pp 605–608.

Table I. Minimum Inhibitory Concentrations (μg/mL) for Chloroalanyl Dipeptides That Contain an N-Terminal Residue Bearing an *n*-Alkyl Side Chain



bacterial species	compd 1 n = 0 (Gly)	2 n = 1 (LAla)	3 n = 2 (LAbu)	4 n = 3 (LNva)	5 n = 4 (LNle)
Gram-positive					
Staphylococcus aureus	0.025	0.0125	0.025	0.25	0.0125
Staphylococcus epidermidis	0.05	0.0125	0.025	0.025	0.0125
Streptococcus pyogenes	0.05	>100	12.5	>100	25
Streptococcus agalactiae	0.39	0.20	<0.1	0.20	<0.1
Streptococcus faecalis	>100	>100	6.25	0.39	3.12
Gram-negative					
Hemophilus influenzae	0.20	>100	<0.1	0.78	25
Escherichia coli	3.12	>100	1.56	1.56	3.12
Shigella flexneri	1.56	>100	1.56	>100	3.12
Proteus mirabilis	>100	>100	>100	12.5	>100
Morganella morganii	>100	>100	50	50	>100
Enterobacter cloacae	1.56	>100	25	6.25	6.25

tRNA;[13] newly elaborated proteins contain *N*-formyl-L-methionyl amino-terminal residues at some stage in their synthesis. In fact, LMet-LAla-LSer has been suggested as an N-terminal sequence common to prokaryotic proteins.[14] However, a majority of completed *E. coli* proteins not only lack the formyl-LMet or LMet residues, but many contain N-termini other than LAla and LSer. Thus, it has been suggested that posttranslational modification of a formyl-LMet-LAla-LSer leader sequence may be operative in protein synthesis in prokaryotes. In accord with these conclusions, an N-terminal deformylating enzyme has been identified,[15] and at least two "methionyl aminopeptidase" activities from *E. coli* have been implicated in posttranslational modification. These latter are the crystallizable dipeptidase M[16] and aminopeptidase I.[17]

It occurred to us that if methionyl aminopeptidases are, indeed, widely distributed within microbial systems, these enzymes might be coopted for delivery of β-Cl-LAla in vivo. Specifically, we imagined that chloroalanyl di- and tripeptides with methionyl N-terminal residues might have particularly broad-spectrum antibacterial activities. This reasoning led to the preparation of peptides **6**–**8**. The data for microbial susceptibility to these compounds are given in Table II.

Table II shows that LMet-β-Cl-LAla (**6**) and LMet-β-Cl-LAla-β-Cl-LAla (**8**) are, indeed, broad-spectrum antibacterial agents. However, these peptides do not appear to be particularly remarkable—with regard to either their antibacterial potency or their spectra of activity—in comparison to β-Cl-LAla-β-Cl-LAla (**9**). We have reported previously on the activity of **9**, which is the most effective antibacterial dipeptide of our design.[9,10] It would appear from the data of Table II that placement of a methionyl residue in the N-terminal position of an otherwise antibacterial chloroalanyl peptide offers no particular advantage in terms of microbial susceptibility.

Table II. Minimum Inhibitory Concentrations (μg/mL) for *N*-Methionyl Peptides of the Structure LMet-X (**6**–**8**) and for β-Cl-LAla-β-Cl-LAla (**9**)

bacterial species[a]	peptide (X value) 6 (β-Cl-LAla)	7 (LAla-β-Cl-LAla)	8 (β-Cl-LAla)$_2$	9
Gram-positive				
S. aureus	0.025	0.05	0.025	0.05
S. epidermidis	0.025	0.025	0.0125	0.05
Strep. pyogenes	1.56	25	6.25	1.56
Strep. agalactiae	0.20	<0.1	<0.1	0.39
Strep. faecalis	0.39	6.25	0.05	3.12
Gram-negative				
H. influenzae	0.78	>100	0.39	1.56
E. coli	1.56	>100	1.56	1.56
Sh. flexneri	>100	>100	12.5	12.5
M. morganii	50	>100	>100	50
Ent. aerogenes	6.25	>100	50	25
Ent. cloacae	6.25	>100	>100	12.5

[a] Full names are given in Table I.

The remarkable finding of Table II is the uniformity of resistance to LMet-LAla-β-Cl-LAla (**7**) among Gram-negative species that are otherwise susceptible to peptides **6**, **8**, and **9**. In agreement with the data of Table I, the single peptide containing an alanyl residue (**7**) is quite active against Gram-positive organisms.

Antibacterial Activity of Chloroalanyl Di- and Tripeptides. Tables III and IV report the microbial susceptibility data for a variety of di- and tripeptides, respectively, which contain a carboxy-terminal chloroalanyl residue. In Table III, the MICs for the dipeptides **10**–**17** may be compared readily with those determined for **2**, LAla-β-Cl-LAla. Note that the Gram-positive organisms are, in most cases, exquisitely sensitive to all of the dipeptides examined, including **2**. Even compounds **16** and **17**, which bear the unusual N-terminal residues β-alanine and *N*-methylalanine, respectively, are reasonably active against Gram-positive species.

As was revealed in Table I, LAla-β-Cl-LAla (**2**) lacks activity against all of the Gram-negative species tested, and yet other simple dipeptide cognates of **2** seem to be consistently, if variously, active. Even the *N*-methyl analogue (**17**) of LAla-β-Cl-LAla is demonstrably active against *E. coli* and *Shigella flexneri*, while these two organisms are

(13) Lengyel, P. In *Molecular Genetics, Part II*; Taylor, J. H., ed.; Academic Press: New York, 1967; pp 193–212. Gold, L.; Pribnow, D.; Schneider, T.; Shinedling, S.; Singer, B. S.; Stormo, G. *Ann. Rev. Microbiol.* **1981**, *35*, 365. Maitra, U.; Stringer, E. A.; Chaudhuri, A. *Ann. Rev. Biochem.* **1982**, *51*, 869.
(14) Waller, J. P. *J. Mol. Biol.* **1963**, *7*, 483.
(15) Adams, J. M. *J. Mol. Biol.* **1968**, *33*, 571.
(16) Brown, J. L. *J. Biol. Chem.* **1973**, *248*, 409.
(17) Vogt, V. M. *J. Biol. Chem.* **1970**, *245*, 4760.

Table III. Minimum Inhibitory Concentrations (μg/mL) for Dipeptides of the Structure X-β-Cl-LAla

bacterial species[a]	peptide (X value) 2 (LAla)	10 (LVal)	11 (LLeu)	12 (LIle)	13 (LPhe)	14 (LPro)	15 (LGln)	16 (β-Ala)	17 (*N*-CH_3-LAla)
Gram-positive									
S. aureus	0.0125	0.025	0.025	0.025	0.05	0.39	0.05	0.78	0.39
S. epidermidis	0.0125	<0.0125	0.0125	0.05	0.05	1.56	0.05	6.25	3.12
Strep. agalactiae	0.20	<0.1	<0.1	<0.1	<0.1	6.25	<0.1	ND[b]	ND
Strep. faecalis	>100	1.56	1.56	3.12	6.25	12.5	0.78	>100	6.25
Gram-negative									
H. influenzae	>100	<0.1	<0.1	<0.1	0.39	<0.1	0.78	>100	100
E. coli	>100	12.5	6.25	6.25	12.5	25	>100	>100	12.5
Sh. flexneri	>100	1.56	3.12	3.12	25	6.25	1.56	>100	6.25
Ent. aerogenes	>100	>100	50	25	100	>100	>100	>100	100
Ent. cloacae	>100	100	25	12.5	100	25	>100	>100	>100

[a] Full names are given in Table I. [b] ND, not determined.

Table IV. Minimum Inhibitory Concentrations (μg/mL) for Tripeptides Containing β-Cl-LAla

bacterial species[a]	tripeptides 18 ($(LAla)_2$-β-Cl-LAla	19 LVal-LAla-β-Cl-LAla	20 ($(\beta$-Cl-$LAla)_3$
Gram-positive			
S. aureus	0.025	0.05	0.20
S. epidermidis	0.025	0.025	0.10
Strep. agalactiae	0.20	0.10	0.39
Strep. faecalis	12.5	3.12	0.78
Gram-negative			
H. influenzae	>100	>100	<0.10
E. coli	>100	>100	6.25
Sh. flexneri	>100	>100	25
Ent. cloacae	>100	>100	6.25

[a] Full names are given in Table I.

completely resistant to **2**. Among the several peptides of Table III, only **16**, like **2**, is totally ineffective against Gram-negative organisms (vide infra).

Similar observations are made when one examines the data for the tripeptides of Table IV. Note that **18–20** are effective antibiotic agents against the four species of Gram-positive organisms, including, in this case, *Strep. faecalis*. On the other hand, the Gram-negative organisms are sensitive only to β-Cl-LAla-β-Cl-LAla-β-Cl-LAla (**20**). The two peptides containing LAla residues (**18**, **19**) display no activity against any Gram-negative species tested.

Physiologic Rescue Experiments. The haloalanyl antibacterial peptides described here were designed specifically to effect intracellular inactivation of alanine racemase, an event which is expected to deprive the cell of the DAla units necessary for cell wall biosynthesis. In accord with this prediction, we found that addition of DAla to the culture medium protected Gram-negative species from the bactericidal effects of β-Cl-LAla-β-Cl-LAla (**9**).[10] This finding was the first indication that alanine racemase is, in fact, a target enzyme in organisms susceptible to the antibiotic action of chloroalanyl peptides. Peptide-sensitive Gram-positive species, by contrast, were found not to be protected by DAla supplementation. Although exposure to **9** gives substantial reduction of alanine racemase activities in *Staphylococcus aureus* in vivo,[18] racemase inactivation does not appear to be the physiological event that leads to cell lysis in Gram-positive bacteria.

In Gram-negative species—where alanine racemase inactivation results in bactericidality—DAla supplementation circumvents the metabolic block and restores normal growth in otherwise peptide-sensitive cells. Correspondingly, it seems reasonable that LAla could have a similar rescuing effect. The substrate LAla may protect the racemase—by competitive binding—from inactivation by β-Cl-LAla in vivo, as it does in vitro. These considerations suggest that the uniform resistance of Gram-negative microorganisms to any chloroalanyl peptide containing an alanyl residue (**2**, **7**, **18**, **19**) may result from the antagonistic effects of simultaneous accumulation within the cell of both a racemase-inactivating (β-Cl-LAla) and a racemase-protecting (LAla) amino acid. Given these considerations, we determined whether LAla and LAla-LAla could protect cells from the action of β-Cl-LAla-β-Cl-LAla (**9**). The relevant susceptibility data are shown in Table V.

It is clear from the results of Table V that LAla and the alanyl dipeptide are able to attenuate the antibacterial effects of **9** against Gram-negative microorganisms, just as **2**, **7**, **18**, and **19** have no activity against these species. Note that LAla is able to protect the cells completely from **9** only at a very high concentration (500 μg/mL) in the growth medium, but LAla-LAla is quite effective at a much lower concentration (25 μg/mL). Perhaps the comparatively limited rescue by LAla reflects the less facile transport of the amino acid than of the dipeptide. Correspondingly, LAla-LAla may effectively compete with **9** for accumulation by the same dipeptide permease.

Note also from Table V that *S. aureus* and *S. epidermidis*, which are quite susceptible to peptides containing alanine (Tables I, II, and IV), are not at all protected from the effects of **9** by LAla. *Streptococcus faecalis* is, by contrast to the staphylococcal species, rescued by LAla, and this finding is in accord with the fact that *Strep. faecalis* is not susceptible to **2** (Table I). On the other hand, *Strep. faecalis* is sensitive to **18** and **19**, although less so than to **20** (Table IV).

It is curious that LAla-LAla does exhibit some protection of Gram-positive species from the bacteriotoxicity of **9**. Given the exquisite sensitivity of the two staphylococci to β-Cl-LAla-β-Cl-LAla, an increase in the MICs for this peptide from 0.05 to 25 μg/mL for each species would seem to demonstrate dramatic rescue by the alanyl dipeptide.

(18) Cheung, K. S. Ph.D. Thesis, University of Chicago, Chicago, IL, 1985.

(19) Moroder, L.; Hallett, A.; Wunsch, E.; Keller, O.; Wersin, G. *Hoppe-Seyler's Z. Physiol. Chem.* **1976**, *357*, 1651.

(20) The Greek letter subscripts (e.g., α_1-H) denote the amino acid units of a peptide, beginning with the N-terminal residue. Thus, α_1-H_2 designates the two methylene hydrogens of the Gly-β-Cl-LAla. Similar notations are used throughout in reporting NMR data for synthetic peptides.

(21) Each of the two prochiral β-methylene resonances of β-chloroalanyl peptides resolves into a doublet of doublets (ABX system, X = α-H) in spectra at 500 MHz. We have arbitrarily assigned the upfield resonance H_A and the downfield signal H_B.

Table V. Minimum Inhibitory Concentrations (MICs) for β-Cl-LAla-β-Cl-LAla (**9**) and for **9** in the Presence of LAla and LAla-LAla

	MICs, μg/mL								
		9 + LAla concn, μg/mL				9 + LAla-LAla concn, μg/mL			
bacterial species[a]	9	25	50	100	500	25	50	100	500
Gram-positive									
S. aureus	0.05	0.05	0.05	0.05	0.10	0.39	6.25	25	25
S. epidermidis	0.05	0.05	0.05	0.05	0.10	0.39	6.25	25	25
Strep. faecalis	3.12	>100	>100	>100	>100	>100	>100	>100	>100
Gram-negative									
E. coli ATCC	1.56	1.56	3.12	3.12	>100	>100	>100	>100	>100
E. coli JSR-O	3.12	3.12	6.25	50	>100	>100	>100	>100	>100
Sh. flexneri	1.56	1.56	3.12	6.25	>100	>100	>100	>100	>100
Ent. cloacae	12.5	12.5	12.5	12.5	>100	>100	>100	>100	>100
Ent. aerogenes	6.25	6.25	12.5	12.5	>100	>100	>100	>100	>100

[a] Full names are given in Table I.

Table VI. Enzyme Activities in Homogenates of *E. coli* JSR-O Exposed to Chloroalanyl Antibiotics and to Chloramphenicol[a]

	sp activity, μmol/min (mg protein), × 10^2	
antibacterial agent	alanine racemase	transaminase B
none[b]	1.47 (100)[c]	4.27 (100)[c]
β-Cl-LAla-β-Cl-LAla (**9**, 100 μg/mL)[d]	0.19 (12.9)	0.13 (3)
β-Cl-LAla-β-Cl-LAla (**9**, 100 μg/mL) + LAla (500 μg/mL)	0.85 (61)	0.11 (2.6)
LAla-β-Cl-LAla (**2**, 200 μg/mL)	1.17 (79.6)	0[e] (0)
chloramphenicol (200 μg/mL)	1.76 (126)	5.41 (120)

[a] Cells were harvested for enzyme assays 30 min after addition of an antibiotic to a growing culture; cultivation on Davis–Mingioli medium supplemented with LMet and LPro (50 μg/mL each) and with LLeu, LIle, and LVal (100 μg/mL each). [b] Control culture, no antibiotic added. [c] Numbers in parentheses denote percent activity, relative to the control culture lacking antibiotic addition. [d] Culture was additionally supplemented with 5% sucrose. [e] No detectable activity. Other details are given in the text.

In sharp contrast to this finding is the observation that neither DAla nor DAla-DAla rescues Gram-positive organisms from the antibacterial effects of **9**.[10] Perhaps a pathway leading toward alanine formation is the principal site of action of haloalanyl peptides in these species; we have yet to investigate this possibility.

Following on the observations of Table V, we examined more thoroughly the LAla rescue phenomenon in *E. coli* strain JSR-O. This species was chosen for further study, since it is representative of the class of organisms resistant to **2** but sensitive to most of the other haloalanyl peptides. Moreover, it is now known that the antibacterial effects of β-Cl-LAla-β-Cl-LAla (**9**) against *E. coli* JSR-O result from inactivation of alanine racemase in vivo.[10]

Microbial Susceptibility in Liquid Culture. The ability of LAla and LAla-LAla to protect *E. coli* JSR-O from the effects of **9** (Table V) are illustrated further in Figure 1. When **9** is added to a culture that lacks either LAla or LAla-LAla supplementation, an arrest in cell growth is almost immediately observed. The loss of turbidity between 180 and 270 min results from cell lysis, as was evidenced by the determination of viable cell counts during this period (data not shown). However, both LAla and the alanyl dipeptide allow for normal growth when the cells are exposed to **9** at a concentration in the medium[22] of 100 μg/mL. Note finally from Figure 1 that LAla-β-Cl-LAla (**2**) has neither bacteriostatic nor bactericidal activity against *E. coli* JSR-O cultivated under the same conditions that lead to lysis of cells exposed to **9**.

When cells are grown in the presence of 5% sucrose, which provides osmotic stabilization, the lytic effects of **9** can be circumvented. Under these conditions, we found that cells growing in the presence of **9** had dramatically

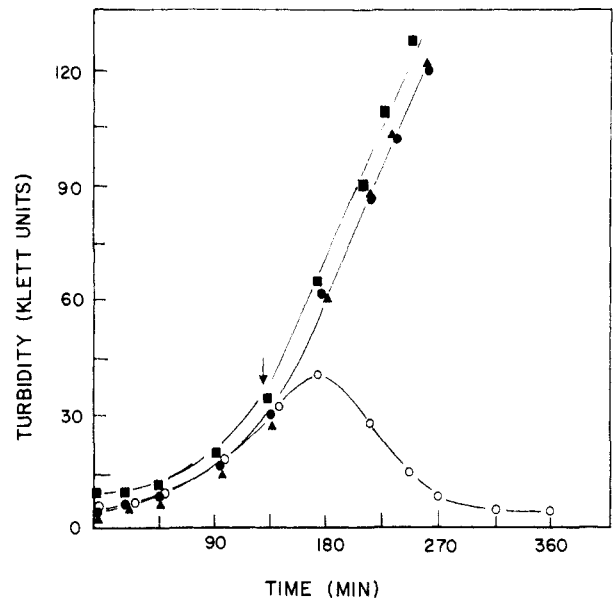


Figure 1. Antibacterial effects of peptides **2** and **9** on the growth of *E. coli* JSR-O. Cells were grown on Davis–Mingioli medium supplemented with 50 μg/mL each of LPro and LMet and 100 μg/mL each of LLeu, LIle, and LVal (see Experimental Section for details). The cultures additionally contained 100 μg/mL peptide **9** without further supplementation (○); 100 μg/mL **9** plus 200 μg/mL LAla (●); 100 μg/mL **9** plus 200 μg/mL LAla-LAla (■); and 200 μg/mL peptide **2** without further supplementation (▲). Peptides **2** and **9** were added, as indicated by the arrow in the figure, when the cultures had reached an optical density of ~30 Klett units. Control cultures, lacking the addition of an antibacterial peptide, gave growth curves identical to those obtained for cultures containing **2** and **9** with LAla or LAla-LAla supplementation (data not shown).

reduced levels of alanine racemase and transaminase B in vivo.[10] This finding, together with the results of Figure 1, prompted an analysis of the racemase and transaminase activities in cells exposed to the antibacterial peptides **2** and **9**.

(22) This is Davis–Mingioli minimal medium with the following supplementations: (1) LPro and LMet (50 μg/mL each), to satisfy the auxotrophic growth requirements of *E. coli* JSR-O, and (2) LLeu, LIle, and LVal (each at 100 μg/mL), to protect the cells from the cidal effects of haloalanyl peptides resulting from inactivation of transaminase B. See Experimental Section for details.

Enzymatic Activities in Cells Exposed to Chloroalanyl Peptides. Table VI records the activities of alanine racemase and transaminase B in *E. coli* JSR-O cells exposed to peptides **2** and **9** and to **9** in the presence of L-Ala supplementation. These results clearly show that the levels of both enzymes are dramatically reduced in cultures treated with β-Cl-LAla-β-Cl-LAla (**9**) but protected from lysis with sucrose. Note, however, that the racemase activity in the culture containing 100 μg/mL **9** and supplemented with 500 μg/mL LAla is approximately 60% of that in the control culture. This finding indicates that the ability of LAla to rescue Gram-negative organisms from the bactericidal effects of **9**, as documented in Table V and Figure 1, is due to the protection of alanine racemase in vivo. Similarly, the racemase levels in cells exposed to LAla-β-Cl-LAla (**2**) alone are only modestly depressed (to ~80%) below those in the control. Clearly, these slight reductions in the activities of the essential alanine racemase do not affect cell viability, as evidenced by the results of Figure 1.

By contrast to the results shown in Table VI for alanine racemase, transaminase B activities are dramatically reduced in cells exposed to **9**, with and without LAla supplementation, and in cells exposed to **2**. These findings are not surprising. Alanine is not a substrate for the transaminase, and it should not be expected to attenuate the inactivation of this enzyme, which is efficiently achieved by β-haloalanines in vitro[23] and by β-chloroalanyl peptides in vivo.[10] In the experiments described in Figure 1 and Table VI, the inactivation of transaminase B—which would otherwise lead to inhibition of growth—was circumvented by addition of the branched-chain amino acids to the media.

Finally, it should be noted from Table VI that cells exposed to chloramphenicol have normal control levels of alanine racemase and of transaminase B. This finding rules out the possibility that β-haloalanyl peptides alter protein biosynthesis and that the loss of enzyme activities during exposure to **2** and **9** arises from normal rapid turnover of the racemase and the transaminase without compensatory biosynthetic replacement of these enzymes.

Summary and Conclusions

β-Chloroalanyl di- and tripeptides are potent, broad-spectrum antibacterial agents with activity against both Gram-positive and Gram-negative microorganisms. Against Gram-negative species, haloalanyl peptides (e.g., **2**, **7**, **18**, **19**) containing an LAla unit are conspicuously without antibiotic efficacy. This lack of activity appears to correlate with the ability of LAla to protect the essential intracellular alanine racemase from the β-Cl-LAla residue provided to the cell by an antibacterial peptide, such as β-Cl-LAla-β-Cl-LAla (**9**). In *E. coli* JSR-O exposed to **9**, the intracellular racemase levels are dramatically reduced. But in cells exposed to LAla-β-Cl-LAla (**2**), which is *not* bacteriotoxic against *E. coli* and other Gram-negative organisms, the racemase levels are largely unaffected and are similar to those for cells grown in the presence of **9** and the rescuing LAla. It would thus appear that the lack of antibacterial activity—at least in Gram-negative species—for a peptide such as LAla-β-Cl-LAla (**2**, and its relatives **7**, **18**, and **19**), arises from the simultaneous delivery in a single peptide structure of a cytotoxic amino acid residue (β-Cl-LAla) and a residue (LAla) that antagonizes the effects of the toxophore.

It is curious that β-Ala-β-Cl-LAla (**16**), like **2**, lacks antimicrobial activity against Gram-negative organisms; β-Ala would not be expected to protect the racemase from inactivation by β-Cl-LAla. Perhaps this peptide is not transported, or, if **16** *is* accumulated, it may not be cleaved in vivo. We have yet to investigate these possibilities.

In Gram-positive organisms, compounds such as **2** are as effective as haloalanyl peptides that lack an alanyl residue, and, correspondingly, LAla and LAla-LAla are generally ineffective in reversing the antibiotic effects of peptide **9**. These facts appear to be in keeping with our conclusion that inactivation of alanine racemase in Gram-positive bacteria is *not* the physiologically significant event that confers bactericidality to a haloalanyl peptide. The exception to this generalization is *Strep. faecalis*, which behaves more like a Gram-negative than a Gram-positive organism—both in terms of its resistance to chloroalanyl peptides containing alanine and its ability to be protected by alanyl units. An understanding of the mechanisms of action of these interesting antibiotic peptides in Gram-positive species and in *Strep. faecalis* awaits further investigation.

Experimental Section

Synthesis. β-Chloro-L-alanine, L-α-aminobutyrate, *tert*-butyl L-alaninate hydrochloride, *N*-methyl-L-alanine, β-alanine, and *N*-*tert*-butoxycarbonyl derivatives of L-alanine, β-alanine, glycine, L-glutamine, L-isoleucine, L-leucine, L-methionine, L-norleucine, L-norvaline, L-phenylalanine, L-proline, and L-valine were purchased from Vega Biochemicals. The *N*-butoxycarbonyl and *tert*-butyl derivatives of β-Cl-LAla were prepared as described previously.[9] Di-*tert*-butyl dicarbonate and 4 N HCl in dioxane were obtained from Pierce Chemical Company. All other reagents were of the best grade commercially available.

Infrared spectra were recorded on a Perkin-Elmer 283 spectrometer using KBr pellets. Thin-layer chromatograms were made by using Silica Gel HLF from Analtech. Eluting solvent systems were 1-butanol/acetic acid/water, 4:1:1 (solvent A) and chloroform/methanol/acetic acid, 85:10:5 (solvent B); visualization was with ninhydrin or iodine vapor. Proton NMR spectra were recorded with a DS-1000 500-MHz instrument with internal reference of 3-(trimethylsilyl)propionic acid, sodium salt (Aldrich Chemical Co.), in D_2O, or of tetramethylsilane in $CDCl_3$. Proton magnetic resonance data for each new compound are given in the text below. Physical data for peptide products are given in Table VII. Peptide trifluoroacetate salts are hygroscopic and, consequently, give unreliable melting points. Thus, we obtained melting point data only for crystalline amino acid derivatives and protected peptides. Overall yields reported in Table VII are calculated from protected amino acids before coupling.

***p*-Nitrobenzophenone Oxime Resin.** The oxime resine was prepared essentially as described by DeGrado and Kaiser.[11] Biobeads S-XI (35 g, Biorad) were reacted with *p*-nitrobenzoyl chloride (4.0 g, 21 mmol) in 200 mL of 1,2-dichloroethane with $AlCl_3$ (4.0 g, 30 mmol) as catalyst. This afforded 36 g of *p*-nitrobenzoyl polystyrene resin. The IR spectrum gave characteristic bands at 1665, 1525, and 1310 cm^{-1}. The nitrobenzoylated beads were in turn reacted with excess hydroxylamine hydrochloride (30 g, 0.43 mol) in 200 mL of absolute ethanol and 50 mL of pyridine, which gave 36 g of the oxime resin. Strong absorbances at 3530 (oxime hydroxyl), 1525, and 1310 cm^{-1} were observed in the IR spectrum; carbonyl stretching at 1665 cm^{-1} (diagnostic of the unreacted nitrobenzoylated resin) was absent in the oxime product. The oxime substitution level was determined by elemental analysis, which gave 1.17% nitrogen, corresponding to 0.43 mmol/g of resin.

***N*-(*tert*-Butoxycarbonyl)-L-α-aminobutyrate.** *N*-*tert*-Butoxycarbonyl-protected amino acids were prepared by the di-*tert*-butyl dicarbonate method of Moroder et al.[19] The preparation of *N*-BOC-L-α-aminobutyrate is illustrative. L-α-Aminobutyrate (1.0 g, 9.7 mmol) was dissolved in 30 mL of dioxane/water (2:1), the solution was chilled to 0 °C, and the pH was adjusted to ~8.0 by addition of 1 N NaOH. Di-*tert*-butyldicarbonate (2.5 mL, 8.9 mmol) was added dropwise, and the reaction was stirred first at 0 °C for 20 min and then at room temperature for an additional 4–5 h (until the solution cleared).

(23) Arfin, S. M.; Koziell, D. A. *J. Bacteriol.* **1971**, *105*, 519.

Table VII. Physical Data for Synthetic Peptides

no.	peptide, TFA salt	formula	anal.	overall yield, %	R_f (solvent A)[a]
1	Gly-β-Cl-LAla	$C_7H_{10}N_2O_5F_3Cl$	C, H, N	99.0	0.21
2	LAla-β-Cl-LAla	$C_8H_{12}N_2O_5F_3Cl$	C, H, N, O, Cl	69.1	0.27
3	LAbu-β-Cl-LAla	$C_9H_{14}N_2O_5F_3Cl$	C, H, N	76.8	0.33
4	LNva-β-Cl-LAla	$C_{10}H_{16}N_2O_5F_3Cl$	C, H, N, O, Cl	67.4	0.41
5	LNle-β-Cl-LAla	$C_{11}H_{18}N_2O_5F_3Cl$	C, H, N	70.3	0.47
6	LMet-β-Cl-LAla	$C_{10}H_{16}N_2O_5F_3ClS$	C, H, N, O, Cl	59.5	0.41
7	LMet-LAla-β-Cl-LAla	$C_{13}H_{21}N_3O_6F_3ClS$	C, H, N, Cl	9.4	0.32
8	LMet-β-Cl-LAla-β-Cl-LAla	$C_{13}H_{20}N_3O_6F_3Cl_2S$	C, H, N, Cl	12.5	0.41
9	β-Cl-LAla-β-Cl-LAla	$C_8H_{11}N_2O_5F_3Cl_2$	C, H, N, O, Cl	78.0	0.36
10	LVal-β-Cl-LAla	$C_{10}H_{16}N_2O_5F_3Cl$	C, H, N	15.5	0.37
11	LLeu-β-Cl-LAla	$C_{11}H_{18}N_2O_5F_3Cl$	C, H, N	52.1	0.46
12	LIle-β-Cl-LAla	$C_{11}H_{18}N_2O_5F_3Cl$	C, H, N	14.7	0.43
13	LPhe-β-Cl-LAla	$C_{14}H_{16}N_2O_5F_3Cl$	C, H, N	68.1	0.48
14	LPro-β-Cl-LAla	$C_{10}H_{14}N_2O_5F_3Cl$	C, H, N	56.8	0.18
15	LGln-β-Cl-LAla	$C_{10}H_{15}N_3O_6F_3Cl$	C, H, N	75.6	0.19
16	β-Ala-β-Cl-LAla	$C_8H_{12}N_2O_5F_3Cl$	C, H, N	90.1	0.19
17	*N*-Me-LAla-β-Cl-LAla	$C_9H_{14}N_2O_5F_3Cl$	C, H, N	25.0	0.21
18	LAla-LAla-β-Cl-LAla	$C_{11}H_{17}N_3O_6F_3Cl$	C, H, N, O, Cl	35.3	0.23
19	LVal-LAla-β-Cl-LAla	$C_{13}H_{21}N_3O_6F_3Cl$	C, H, N, Cl	46.4	0.29
20	β-Cl-LAla-β-Cl-LAla-β-Cl-LAla	$C_{11}H_{15}N_3O_6F_3Cl_3$	C, H, N, Cl	44.6	0.38

[a] Conditions for TLC are given in the text.

Dioxane was then removed in vacuo; the oily residue was cooled to 0 °C, and 50 mL of EtOAc was added. This solution was acidified to pH 2–3 with $KHSO_4$ and extracted 3 times with EtOAc. The organic phases were combined, washed with H_2O, and dried over $MgSO_4$. The solvent was stripped, and the product crystallized from EtOAc/hexane: yield, 0.90 g (91%). Crystalline *N*-BOC-LAbu oils at room temperature upon removal of the solvent of crystallization; TLC of oil, R_f = 0.68 (solvent B); ^{1}H NMR ($CDCl_3$) δ 1.00 (t, 3 H, γ-H_3, J = 7.4 Hz), 1.46 (s, 9 H, *tert*-butyl), 1.75 (dq, 2 H, β-H_2, J = 7.4 Hz), 4.28 (br s, 1 H, α-H), 5.04 (br s, 1 H, NH).

***N*-(*tert*-Butoxycarbonyl)-*N*-methyl-L-alanine.** This compound was prepared as described above for *N*-BOC-LAbu. From 0.80 g (7.8 mmol) of *N*-methyl-LAla and 2.0 mL (7.1 mmol) of di-*tert*-butyldicarbonate was obtained 0.66 g (42.3%) of *N*-BOC-*N*-methyl-LAla. The product is an oil at room temperature: TLC of oil, R_f = 0.64 (solvent B); ^{1}H NMR ($CDCl_3$) δ 1.43 (d, 3 H, α-CH_3, J = 7.3 Hz), 1.46 (s, 9 H, *tert*-butyl), 2.84 (br s, 3 H, NCH_3), 3.95 (br s, 1 H, α-H).

Dipeptide Synthesis. The new dipeptides reported here (**1**, **3**, **5**, and **10–17**) were prepared according to the methodology outlined previously for the synthesis of **2**, **4**, **6**, and **9**.[9] The preparation of Gly-β-Cl-LAla (**1**) is illustrative of the synthesis of all dipeptides containing a carboxy-terminal β-Cl-LAla residue.

Glycyl-β-chloro-L-alanine Trifluoroacetate (1). The oxime resine (5.0 g) was swelled in 100 mL of CH_2Cl_2; *N*-BOC-glycine (0.44 g, 2.5 mmol) and DCC (0.62 g, 3.0 mmol) were added, and the mixture was shaken in a plastic screw-cap bottle for 48 h at room temperature. The resine was then filtered, washed with CH_2Cl_2 (3×) and CH_3OH (3×), and then air-dried: IR 1720, 1775 cm^{-1}, corresponding to the carbonyl groups of *N*-BOC-Gly-oxime-resin.

The *N*-BOC-Gly resin (4.8 g, 2.24 mmol equiv of *N*-BOC-Gly), β-Cl-LAla-O*t*-Bu·HCl (0.45 g, 2.5 mmol), DIEA (0.26 g, 2.75 mmole), and AcOH (0.08 mL, 2.75 mmol) were mixed in 100 mL of CH_2Cl_2 and shaken at room temperature for 48 h. The resin was then filtered and washed (vide supra), and the filtrates were combined and evaporated to dryness. The residue was dissolved in EtOAc and washed with 1 N HCl, 5% $NaHCO_3$, and twice with H_2O. The organic phase was recovered, dried over $MgSO_4$, and evaporated to dryness. Recrystallization from EtOAc/*n*-hexane gave *N*-BOC-Gly-β-Cl-LAla-O*t*-Bu as a white crystal, which oiled at room temperature: yield, 0.51 g (66%); R_f = 0.26 ($CHCl_3$).

The oily *N*-BOC-Gly-β-Cl-LAla-O*t*-Bu (0.51 g, 1.6 mmol) was dissolved in 2 mL of anisole, and 10 mL of cold TFA was then added. The mixture was stirred at room temperature for 2 h, and then the TFA/anisole solution was evaporated to dryness. The resulting solid residue was dissolved in 30 mL of CH_2Cl_2/H_2O (1:1), and the aqueous phase was extracted with CH_2Cl_2 (3×) to remove trace amounts of anisole and *tert*-butyl anisole. Lyophilization of the aqueous phase affoded 0.42 g of Gly-β-Cl-LAla TFA (**1**): ^{1}H NMR (D_2O) δ 3.90 (3s, 2 H, α$_1$-H_2,[20] distorted AB system), 3.93 (dd, 1 H, β$_2$-H_A,[21] J = 3.6, 11.8 Hz), 4.04 (dd, 1 H, β$_2$-H_B, J = 5.0, 11.8 Hz), 4.90 (dd, 1 H, α$_2$-H, J = 3.60, 5.0 Hz).

L-α-Aminobutyryl-β-chloro-L-alanine Trifluoroacetate (3). An *N*-BOC-L-α-aminobutyryl-oxime resin was prepared, as outlined above for the glycyl-oxime, and was reacted with β-Cl-LAla-O*t*-Bu·HCl (1.5 M excess over substituted resin). The product *N*-BOC-LAbu-β-Cl-LAla-O*t*-Bu was deprotected by use of using 5:1 TFA/anisole (vide supra), to afford 0.64 g of LAbu-β-Cl-LAla TFA (**3**): ^{1}H NMR (D_2O) δ 1.02 (t, 3 H, γ$_1$-H, J = 7.6 Hz), 1.97 (m, 2 H, β$_1$-H), 3.96 (dd, 1 H, β$_2$-H_A, J = 3.7, 11.8 Hz), 4.04 (dd, 1 H, β$_2$-H_B, J = 5.4, 11.8 Hz), 4.05 (t, 1 H, α$_1$-H, J = 6.4 Hz), 4.92 (dd, 1 H, α$_2$-H, J = 3.7, 5.4 Hz).

L-Norleucyl-β-chloro-L-alanine Trifluoroacetate (5). An *N*-BOC-LNle-oxime resin was reacted in the usual way with β-Cl-LAla-O*t*-Bu·HCl. Deprotection of the resulting *N*-BOC-LNle-β-Cl-LAla-O*t*-Bu by the TFA/anisole method gave 0.3 g of the desired LNle-β-Cl-LAla TFA (**5**): ^{1}H NMR (D_2O) δ 0.88 (t, 3 H, δ$_1$-CH_3, J = 7.0 Hz), 1.37 (m, 4 H, γ$_1$-H_2 and δ$_1$-H_2), 1.92 (m, 2 H, β$_1$-H_2), 3.95 (dd, 1 H, β$_2$-H_A, J = 3.7, 11.8 Hz), 4.03 (dd, 1 H, β$_2$-H_B, J = 5.4, 11.8 Hz), 4.08 (t, 1 H, α$_1$-H, J = 6.5 Hz), 4.84 (dd, 1 H, α$_2$-H, J = 3.7, 5.4 Hz).

L-Valyl-β-chloro-L-alanine Trifluoroacetate (10). An *N*-BOC-LVal-oxime resin was reacted in the usual way with β-Cl-LAla-O*t*-Bu·HCl. Deprotection of the resulting *N*-BOC-LVal-β-Cl-LAla-O*t*-Bu by the TFA/anisole method gave 0.09 g of the desired LVal-β-Cl-LAla TFA (**10**): ^{1}H NMR (D_2O) δ 1.09, 1.11 (2d, 6 H, β$_1$-$(CH_3)_2$, J = 7.0 Hz), 2.32 (m, 1 H, β$_1$-H), 3.93 (dd, 1 H, β$_2$-H_A, J = 3.4, 11.8 Hz), 4.08 (dd, 1 H, β$_2$-H_B, J = 4.5, 11.8 Hz), 4.29 (d, 1 H, α$_1$-H, J = 5.7 Hz), 5.07 (t, 1 H, α$_2$-H, J = 3.9 Hz).

L-Leucyl-β-chloro-L-alanine Trifluoroacetate (11). An *N*-BOC-LLeu-oxime resin was reacted in the usual way with β-Cl-LAla-O*t*-Bu·HCl. Deprotection of the resulting *N*-BOC-LLeu-β-Cl-LAla-O*t*-Bu by the TFA/anisole method gave 0.42 g of the desired LLeu-β-Cl-LAla TFA (**11**): ^{1}H NMR (D_2O) δ 0.96, 0.97 (2d, 6 H, γ$_1$-$(CH_3)_2$, J = 6.1 Hz), 1.76 (m, 3 H, β$_1$-H_2 and γ$_1$-H), 3.95 (dd, 1 H, β$_2$-H_A, J = 3.8, 11.7 Hz), 4.00 (dd, 1 H, β$_2$-H_B, J = 5.4, 11.7 Hz), 4.00 (dd, 1 H, β$_2$-H_B, J = 5.4, 11.7 Hz), 4.09 (t, 1 H, α$_1$-H, J = 6.8 Hz), 4.71 (dd, 1 H, α$_2$-H, J = 3.8, 5.4 Hz).

L-Isoleucyl-β-chloro-L-alanine Trifluoroacetate (12). An *N*-BOC-LIle-oxime resin was reacted in the usual way with β-Cl-LAla-O*t*-Bu·HCl. Deprotection of the resulting *N*-BOC-LIle-β-Cl-LAla-O*t*-Bu by the TFA/anisole method gave 0.06 g of the desired LIle-β-Cl-LAla TFA (**12**): ^{1}H NMR (D_2O) δ 0.94 (t, 3 H, δ$_1$-H_3, J = 7.4 Hz), 1.04 (d, 3 H, β$_1$-CH_3, J = 6.9 Hz), 1.27 (m, 1 H, γ$_1$-H_A), 1.55 (m, 1 H, γ$_1$-H_B), 2.02 (m, 1 H, β$_1$-H), 3.96 (dd, 1 H, β$_2$-H_A, J = 3.7, 11.7 Hz), 3.97 (d, 1 H, α$_1$-H, J = 5.5 Hz), 4.02 (dd, 1 H, β$_2$-H_B, J = 5.5, 11.8 Hz), 4.81 (dd, 1 H, α$_2$-H, J = 3.7, 5.5 Hz).

L-Phenylalanyl-β-chloro-L-alanine Trifluoroacetate (13). An *N*-BOC-LPhe-oxime resin was reacted in the usual way with β-Cl-LAla-O*t*-Bu·HCl. Deprotection of the resulting *N*-BOC-

LPhe-β-Cl-LAla-Ot-Bu by the TFA/anisole method gave 0.06 g of the desired LPhe-β-Cl-LAla TFA (**13**): ^{1}H NMR (D_2O) δ 3.21 (dd, 1 H, β_1-H_A, J = 7.3, 14.2 Hz), 3.29 (dd, 1 H, β_1-H_B, J = 7.2, 14.2 Hz), 3.91 (dd, 1 H, β_2-H_A, J = 3.9, 11.7 Hz), 3.97 (dd, 1 H, β_2-H_B, J = 5.3, 11.7 Hz), 4.34 (t, 1 H, α_1-H, J = 7.1 Hz), 4.81 (dd, 1 H, α_2-H, J = 4.0, 5.3 Hz), 7.28–7.42 (m, 5 H, phenyl).

L-Prolyl-β-chloro-L-alanine Trifluoroacetate (14). An *N*-BOC-LPro-oxime resin was reacted in the usual way with β-Cl-LAla-Ot-Bu·HCl. Deprotection of the resulting *N*-BOC-LPro-β-Cl-LAla-Ot-Bu by the TFA/anisole method gave 0.29 g of the desired LPro-β-Cl-LAla TFA (**14**): ^{1}H NMR (D_2O) δ 2.07 (m, 2 H, γ_1-H_2), 2.15 (m, 1 H, β_1-H_A), 2.48 (m, 1 H, β_1-H_B), 3.39 (m, 1 H, δ_1-H_A), 3.45 (m, 1 H, δ_1-H_B), 3.96 (dd, 1 H, β_2-H_A, J = 3.5, 11.8 Hz), 4.05 (dd, 1 H, β_2-H_B, J = 5.2, 11.8 Hz), 4.47 (dd, 1 H, α_1-H, J = 7.0, 8.1 Hz), 4.89 (dd, 1 H, α_2-H, J = 3.5, 5.2 Hz).

L-Glutamyl-β-chloro-L-alanine Trifluoroacetate (15). An *N*-BOC-LGln-oxime resin was reacted in the usual way with β-Cl-LAla-Ot-Bu·HCl. Deprotection of the resulting *N*-BOC-LGln-β-Cl-LAla-Ot-Bu by the TFA/anisole method gave 0.13 g of the desired LGln-β-Cl-LAla TFA (**15**): ^{1}H NMR (D_2O) δ 2.20 (q, 2 H, β_1-H_2, J = 7.4 Hz), 2.50, 2.51 (2t, 2 H, γ_1-H_2, J = 7.4 Hz), 3.96 (dd, 1 H, β_2-H_A, J = 3.5, 11.9 Hz), 4.05 (dd, 1 H, β_2-H_B, J = 5.3, 11.9 Hz), 4.15 (t, 1 H, α_1-H, J = 6.6 Hz), 4.88 (dd, 1 H, α_2-H, J = 2.8, 4.8 Hz).

β-Alanyl-β-chloro-L-alanine Trifluoroacetate (16). An *N*-BOC-β-Ala-oxime resin was reacted in the usual way with β-Cl-LAla-Ot-Bu·HCl. Deprotection of the resulting *N*-BOC-β-Ala-β-Cl-LAla-Ot-Bu by the TFA/anisole method gave 0.32 g of the desired β-Ala-β-Cl-LAla TFA (**16**): ^{1}H NMR (D_2O) δ 2.78 (t, 2 H, α_1-H_2, J = 6.6 Hz), 3.28 (t, 2 H, β_1-H_2, J = 6.6 Hz), 3.92 (dd, 1 H, β_2-H_A, J = 3.7, 11.7 Hz), 4.00 (dd, 1 H, β_2-H_B, J = 5.2, 11.7 Hz), 4.80 (dd, 1 H, α_2-H, J = 3.7, 5.2 Hz).

***N*-Methyl-L-alanyl-β-chloro-L-alanine Trifluoroacetate (17).** An *N*-BOC-*N*-methyl-LAla-oxime resin was reacted in the usual way with β-Cl-LAla-Ot-Bu·HCl. Deprotection of the resulting *N*-BOC-*N*-methyl-LAla-β-Cl-LAla-Ot-Bu by the TFA/anisole method gave 0.21 g of the desired *N*-methyl-LAla-β-Cl-LAla TFA (**17**): ^{1}H NMR (D_2O) δ 1.57 (d, 3 H, α_1-CH_3, J = 7.0 Hz), 2.71 (s, 3 H, NCH_3), 3.95 (dd, 1 H, β_2-H_A, J = 3.8, 11.7 Hz), 4.01 (dd, 1 H, β_2-H_B, J = 5.4, 11.7 Hz), 4.02 (q, 1 H, α_1-H, J = 7.0 Hz), 4.80 (dd, 1 H, α_2-H, J = 3.8, 5.4 Hz).

Tripeptide Synthesis. The new tripeptides reported here (**7**, **8**, **18**, and **19**) were prepared according to the methodology outlined previously[10] for the synthesis of **20**. The preparation of LMet-β-Cl-LAla-β-Cl-LAla (**8**) is illustrative.

L-Methionyl-β-chloro-L-alanyl-β-chloro-L-alanine Trifluoroacetate (8). The oxime resin (5.0 g, 2.1 mmol) was swelled in 100 mL of CH_2Cl_2, and a 1.5-fold excess of *N*-BOC-β-Cl-LAla (0.70 g, 3.1 mmol) was added. After the addition of DCC (0.47 g, 2.3 mmol), the reaction mixture was shaken at room temperature for 2 days. The resin (5.3 g) was recovered by filtration, and side products, such as DCU, were removed by washing the resin with CH_2Cl_2 and CH_3OH: IR 1720, 1775 cm^{-1}, corresponding to the oxime ester and *t*-BOC carbonyls, respectively, of *N*-BOC-β-Cl-LAla-oxime resin. The BOC group of the substituted resin was then removed by shaking the resin with 70 mL of 4 N HCl in dioxane for 1 hour. The resin was recovered and washed as described above: IR 1720 cm^{-1}; loss of the 1775 cm^{-1} stretch is diagnostic of removal of the *t*-BOC group.

N-BOC-L-Methionine (1.74 g, 7.0 mmol) was dissolved in 100 mL of dry CH_2Cl_2 and cooled to −20 °C. To this was added DCC (0.72 g, 3.5 mmol), and the reaction mixture was stirred at −20 °C for 15 min and then at 4 °C for 20 min. Dicyclohexylurea was removed by filtration, and the filtrate, containing the symmetrical anhydride of *N*-BOC-LMet, was mixed with the amino acid-oxime resin that had been deprotected as described above. To this was added 1.0 equiv of DIEA (0.61 mL, 3.5 mmol), and the mixture was shaken at room temperature for 1 h. The *N*-BOC-LMet-β-Cl-LAla-oxime resin was then collected and washed to remove side products and any unreacted starting materials: IR br 1720, 1770 cm^{-1} (oxime ester and amide carbonyls, respectively).

The dipeptidyl resin was then reacted with β-Cl-LAla-Ot-Bu·HCl (0.65 g, 3.0 mmol) in CH_2Cl_2 for 24 h, which gave the protected tripeptide, *N*-BOC-LMet-β-Cl-LAla-β-Cl-LAla-Ot-Bu. The resin-cleavage reaction and the product workup were carried out as described above for the preparation of **1**: yield, 0.21 g; ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9 H, *tert*-butyl), 1.48 (s, 9 H, *tert*-butyl), 1.98 (m, 1 H, β_1-H_A), 2.10 (s, 3 H, SCH_3), 2.17 (m, 1 H, β_1-H_B), 2.59 (t, 2 H, γ_1-H_2, J = 7.2 Hz), 3.76 (dd, 1 H, β_2-H_A, J = 4.9, 11.6 Hz), 3.91 (m, 2 H, β_3-H_2), 3.99 (dd, 1 H, β_2-H_B, J = 4.5, 11.2 Hz), 4.33 (m, 1 H, α_1-H), 4.80 (m, 1 H, α_3-H), 4.91 (m, 1 H, α_2-H), 5.34 (br d, 1 H, N_1-H) 7.17, 7.30 (2 br s, 2 H, N_2-H, and N_3-H).

The protected tripeptide was treated with TFA and anisole, as described above for deprotection of dipeptides, to afford 0.11 g of the desired LMet-β-Cl-LAla-β-Cl-LAla TFA (**8**): ^{1}H NMR (D_2O) δ 2.11 (s, 3 H, SCH_3), 2.21 (m, 2 H, β_1-H_2), 2.63 (m, 2 H, γ_1-H_2), 3.91 (dd, 1 H, β_2-H_A, J = 6.2, 11.8 Hz), 3.95 (dd, 1 H, β_3-H_A, J = 3.6, 11.8 Hz), 3.96 (dd, 1 H, β_2-H_B, J = 4.8, 11.8 Hz), 4.02 (dd, 1 H, β_3-H_B, J = 5.4, 11.8 Hz), 4.21 (t, 1 H, α_1-H, J = 6.6 Hz), 4.87 (dd, 1 H, α_3-H, J = 3.6, 5.4 Hz), 4.88 (dd, 1 H, α_2-H, J = 4.8, 6.2 Hz).

L-Methionyl-L-alanyl-β-chloro-L-alanine Trifluoroacetate (7). This peptide was prepared in a manner identical to that described for **8**, except that the synthesis began with an *N*-BOC-LAla-oxime resin: yield for LMet-LAla-β-Cl-LAla TFA (**7**), 0.21 g; ^{1}H NMR (D_2O) δ 1.43 (d, 3 H, α_2-CH_3, J = 7.2 Hz), 2.12 (s, 3 H, SCH_3), 2.19 (m, 2 H, β_1-H_2), 2.64 (m, 2 H, γ_1-H_2), 3.91 (dd, 1 H, β_3-H_A, J = 3.6, 11.5 Hz), 3.98 (dd, 1 H, β_3-H_B, J = 5.0, 11.6 Hz), 4.13 (t, 1 H, α_1-H, J = 6.5 Hz), 4.47 (q, 1 H, α_2-H, J = 7.1 Hz), 4.67 (dd, 1 H, α_3-H, J = 3.6, 5.0 Hz).

L-Alanyl-L-alanyl-β-chloro-L-alanine Trifluoroacetate (18). This peptide was prepared as described for **8**, wherein an LAla-oxime resin was reacted with the symmetric anhydride of *N*-BOC-LAla to afford an *N*-BOC-LAla-LAla-oxime. The dipeptidyl resin was then treated with β-Cl-LAla-Ot-Bu·HCl, and, after deprotection of the resulting tripeptide, 0.16 g of the desired LAla-LAla-β-Cl-LAla TFA (**18**) was obtained: ^{1}H NMR (D_2O) δ 1.43 (d, 3 H, α_2-CH_3, J = 7.2 Hz), 1.56 (d, 3 H, α_1-CH_3, J = 7.1 Hz), 3.91 (dd, 1 H, β_3-H_A, J = 3.7, 11.5 Hz), 3.96 (dd, 1 H, β_3-H_B, J = 4.7, 11.5 Hz), 4.10 (q, 1 H, α_1-H, J = 7.1 Hz), 4.42 (q, 1 H, α_2-H, J = 7.2 Hz), 4.58 (dd, 1 H, α_3-H, J = 3.9, 4.7 Hz).

L-Valyl-L-alanyl-β-chloro-L-alanine Trifluoroacetate (19). This peptide was prepared as described for **8**, wherein an L-Ala-oxime resin was reacted with the symmetric anhydride of *N*-BOC-L-Val to afford an *N*-BOC-LVal-LAla-oxime. The dipeptidyl resin was then treated with β-Cl-LAla-Ot-Bu·HCl, and, after deprotection of the resulting tripeptide, 0.35 g of the desired LVal-LAla-β-Cl-LAla TFA (**19**) was obtained: ^{1}H NMR (D_2O) δ 1.02, 1.04 (2d, 6 H, β_1-$(CH_3)_2$, J = 7.3 Hz), 1.43 (d, 3 H, α_2-CH_3, J = 7.2 Hz), 2.23 (m, 1 H, β_1-H), 3.81 (d, 1 H, α_1-H, J = 5.8 Hz), 3.92 (dd, 1 H, β_3-H_A, J = 3.5, 11.7 Hz), 4.01 (dd, 1 H, β_3-H_B, J = 5.1, 11.7 Hz), 4.47 (q, 1 H, α_2-H, J = 7.2 Hz), 4.70 (dd, 1 H, α_3-H, J = 3.5, 5.1 Hz).

Microbiology. Organisms were obtained either from the American Type Culture Collection (ATCC) or as fresh clinical isolates from the Clinical Microbiology Laboratory of the University of Chicago Medical Center. *Escherichia coli* JSR-O is a proline- and methionine-requiring strain of K-12, obtained from Prof. James Shapiro, Department of Microbiology, University of Chicago. All strains were subcultured on commercially prepared blood agar or chocolate agar from Baltimore Biological Laboratories (BBL). L-Alanine and L-alanyl-L-alanine were obtained from Sigma.

The minimum inhibitory concentration (MIC) of each peptide for each strain was determined on a defined peptide susceptibility agar medium.[24] Hemin (25 μg/mL, BBL), nicotinamide adenine dinucleotide (25 μg/mL, Calbiochem), and Isovitalex (1%, BBL) were added to the medium to support growth of *H. influenzae*, *Strep. agalactiae*, and *Strep. pyogenes*. Inocula of these three species were prepared by picking colonies of each after overnight growth on a chocolate or blood agar plate and resuspending the cells in the liquid peptide susceptibility medium to a concentration of 10^8 colony forming units (cfu)/mL. Inocula of the other species were prepared by growing the test organisms overnight in the liquid peptide susceptibility medium and diluting the cultures to approximately 1×10^7 cfu/mL. The inocula were applied with a Steers replicator[25] to plates containing peptides in serial 2-fold

(24) Atherton, F. R.; Hall, M. J.; Hassall, C. H.; Lambert, R. W.; Ringrose, P. S. *Antimicrob. Agents Chemother.* **1979**, *15*, 677.
(25) Steers, E.; Foltz, E. L.; Graves, B. S. *Antibiot. Chemother.* **1959**, *9*, 307.

dilutions, and the plates were incubated overnight at 37 °C. Plates inoculated with *H. influenzae*, *Strep. agalactiae*, and *Strep. pyogenes* were incubated in the presence of 7% CO_2. The MIC was defined as the lowest concentration of a peptide that allowed growth of fewer than 10 colonies after 16–18 h of incubation. For examination of alanyl rescue from inhibition by the toxophoric chloroalanyl peptide **9** (Table V), the media were prepared containing LAla or LAla-LAla at 25, 50, 100, or 500 μg/mL.

Growth in Liquid Culture. The antibacterial effects of **2** and **9** on the growth of *E. coli* JSR-O in Davis–Mingioli minimal medium[26] were examined (Figure 1). Since the test organism is a methionine/proline auxotroph, the minimal medium was supplemented with 50 μg/mL of LMet and LPro. For the experiments shown in Figure 1, the cultures were further supplemented with LAla (200 μg/mL) or LAla-LAla (200 μg/mL), and every culture contained 100 μg/mL each of LLeu, LIle, and LVal.

Cells were cultivated to early logarithmic phase (30 Klett units, $\sim 2 \times 10^8$ cfu/mL), at which time either peptide **2** (200 μg/mL) or **9** (100 μg/mL) was added to a culture. Cell growth was monitored by measurement of turbidity using a Klett meter and by determination of viable cell counts (cfu/mL). All cultures were grown at 37 °C.

Enzyme Assays in Cell Homogenates. L-Alanine dehydrogenase, NAD^+, pyridoxal-5′-phosphate, and the keto acids used in enzyme assays were Sigma products; NAD^+ was frozen and lyophilized before use, in order to remove traces of water, ethanol, and acetone. Alanine dehydrogenase was subjected to Sephadex G-200 (Sigma) gel filtration chromatography prior to use; this removes the small amounts of alanine racemase that often contaminate commercial preparations of the dehydrogenase. All other reagents were of the best grade commercially available and were used without further purification.

Escherichia coli JSR-O was cultivated at 37 °C in Davis–Mingioli medium supplemented with LMet (50 μg/mL), LPro (50 μg/mL), LLeu (100 μg/mL), LIle (100 μg/mL), and LVal (100 μg/mL). For the experiments described in Table VI, the cultures were variously supplemented further with 5% sucrose or with 500 μg/mL LAla. When the optical density of a culture reached approximately 100 Klett units, a test antibiotic was added (**9**, 100 μg/mL; **2**, 200 μg/mL; or chloramphenicol, 200 μg/mL). Following addition of the antibacterial agent, the culture was incubated with shaking at 37 °C for an additional 30 min, and the cells were then rapidly harvested by centrifugation at 4 °C. The resulting pellet was suspended and washed twice at 4 °C in 50 mM sodium phosphate buffer containing 0.5 mM EDTA, 1.4 mM 2-mercaptoethanol, and 10^{-5} M pyridoxal-5′-phosphate, pH 8.0. Following centrifugation, the cells in the pellet were again resuspended in 4 mL of the same buffer and were subjected to ultrasonic disruption with eight 15-s bursts separated by 45 s (on ice). The sonic extract was then centrifuged to remove cellular debris, and the supernatant fluid was retained on ice for assay of alanine racemase and transaminase B activities.

Alanine racemase activity was measured in the D to L direction using the L-alanine dehydrogenase-coupled, spectrophotometric assay of Wang and Walsh.[27] The reaction was followed by monitoring production of NADH (340 nm, $\epsilon = 6200\ M^{-1}\ cm^{-1}$) as the product L-alanine was converted to pyruvate and ammonia by saturating amounts of the dehydrogenase. A typical 1.0-mL assay mixture contained 100 μL of *E. coli* homogenate, 0.45 units of L-alanine dehydrogenase, 10 mM D-alanine, and 5 mM NAD^+ in 100 mM CHES buffer, pH 9.0, 37 °C.

The specific activity of transaminase B was determined by use of the discontinuous spectrophotometric method developed by Taylor and Jenkins[28] for assay of leucine aminotransferase. For the experiments reported here, transamination of L-valine to α-ketoisovalerate was monitored. A typical assay was carried out as follows. One milliliter of cell homogenate, prepared as described above, was mixed with 0.8 mL of 1 M Tris·HCl buffer, pH 7.8, 0.4 mL of pyridoxal-5′-phosphate (200 μg/mL), 0.8 mL of α-ketoglutarate (125 mM), and 0.2 mL of water. This mixture was incubated at 37 °C, and the reaction was initiated by addition of 0.8 mL of L-valine (250 mM). Aliquots (0.5 mL) were withdrawn at timed intervals and added to 2 mL of toluene; then 1.5 mL of 2,4-dinitrophenylhydrazine (3% in 2 N HCl) was added, and the mixture was vortexed for 2 min. This mixture was centrifuged for 2 min to disperse the emulsion. A 1.0-mL aliquot of the resulting toluene layer was added to 5.0 mL of 10% Na_2CO_3; the mixture was again vortexed and then centrifuged. A 3.0-mL portion of the resulting aqueous layer was mixed with 3.0 mL of 1.5 N NaOH, and the absorbance at 430 nm of the 2,4-dinitrophenylhydrazone of α-ketoisovalerate was measured. A standard curve of A_{430} vs. [dinitrophenylhydrazone] was constructed for use in calibrating unknowns. The specific activity of the transaminase was determined from plots of A_{430} vs. time. Protein concentrations were determined by using the method of Lowry et al.[29] Ultraviolet and visible spectrophotometric measurements were made on a Perkin-Elmer 559 spectrometer.

Acknowledgment. This work was funded by awards from the National Institutes of Health (USPHS GM 29660), the Dow Chemical Company (to M.J.), and the Infectious Disease Research Fund of the University of Chicago (to S.A.L.). We also acknowledge the NSF (CHE 8206978), the NIH (CA 14599), and the Louis Block Fund for grants allowing the purchase of NMR equipment used in this research.

Registry No. **1**, 103711-47-3; **2**, 87155-82-6; **3**, 103774-07-8; **4**, 87155-89-3; **5**, 103711-49-5; **6**, 87155-87-1; **7**, 103711-51-9; **8**, 103711-53-1; **9**, 87205-46-7; **10**, 103711-55-3; **11**, 103711-57-5; **12**, 103711-59-7; **13**, 103711-61-1; **14**, 103711-63-3; **15**, 103711-65-5; **16**, 103711-67-7; **17**, 103711-69-9; **18**, 103711-71-3; **19**, 103711-73-5; **20**, 103711-75-7; *N*-BOC-LAbu, 34306-42-8; *N*-BOC-N-methyl-LAla, 16948-16-6; *N*-methyl-L-Ala, 3913-67-5; β-Cl-LAla-O*t*-Bu·HCl, 87156-13-6; *N*-BOC-Gly-β-Cl-LAla-O*t*-Bu, 103711-76-8; *N*-BOC-LAla-β-Cl-LAla-O*t*-Bu, 103711-77-9; β-Cl-LAla-O*t*-Bu, 103775-96-8; *N*-BOC-LNle-β-Cl-LAla-O*t*-Bu, 103711-78-0; *N*-BOC-LVal-β-Cl-LAla-O*t*-Bu, 103711-79-1; *N*-BOC-L-Leu-β-Cl-LAla-O*t*-Bu, 103711-80-4; *N*-BOC-LIle-β-Cl-LAla-O*t*-Bu, 103711-81-5; *N*-BOC-LPhe-β-Cl-LAla-O*t*-Bu, 103711-82-6; *N*-BOC-LPro-β-Cl-LAla-O*t*-Bu, 103711-83-7; *N*-BOC-LGln-β-Cl-LAla-O*t*-Bu, 103711-84-8; *N*-BOC-β-Ala-β-Cl-LAla-O*t*-Bu, 103711-85-9; *N*-BOC-*N*-methyl-LAla-β-Cl-LAla-O*t*-Bu, 103730-92-3; *N*-BOC-LMet, 2488-15-5; *N*-BOC-LMet-β-Cl-LAla-β-Cl-LAla-O*t*-Bu, 103711-87-1; *N*-BOC-LVal (symmetric anhydride), 33294-55-2; *N*-BOC-LAla (symmetric anhydride), 33294-53-0; *p*-$NO_2C_6H_4COCl$, 122-04-3; L-$NH_2CH(Et)CO_2H$, 1492-24-6.

(26) Davis, B. D.; Mingioli, E. S. *J. Bacteriol.* **1950**, *60*, 17.
(27) Wang, E.; Walsh, C. *Biochemistry* **1978**, *17*, 1313.
(28) Taylor, R. T.; Jenkins, W. T. *J. Biol. Chem.* **1966**, *241*, 4391.
(29) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.