Chloroalanyl and Propargylglycyl Dipeptides. Suicide Substrate Containing Antibacterials[1]

Kam-Sing Cheung,[†] Steven A. Wasserman,[‡] Edward Dudek,[§] Stephen A. Lerner,[§] and Michael Johnston*[,†]

Departments of Chemistry and Biochemistry, Searle Chemistry Laboratory, and the Departments of Medicine and Microbiology, The University of Chicago, Chicago, Illinois 60637, and the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received March 21, 1983*

A set of dipeptides containing the amino acid residues β-chloroalanine and propargylglycine, which are mechanism-based inactivators of purified microbial enzymes (alanine racemase and cystathionine γ-synthase, respectively), have been synthesized, and their antibacterial properties in vitro have been evaluated. Dipeptides containing a single β-chloro-L-alanyl residue (e.g., **3**, **5**, **9**, and **10**) or a single L-propargylglycyl residue (e.g., **12** and **15**) are potent antibacterials. The in vitro antibiotic activity of β-chloro-L-alanine and of L-propargylglycine is increased as much as 4000-fold by incorporation of these residues into a dipeptide. Compounds that contain only a single enzyme-inactivating amino acid together with a second L-alanyl residue (**3**, **5**, **12**, and **15**) have a restricted range of activity: of the species tested, only *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* are sensitive. However, peptides that contain two suicide-substrate residues [e.g., β-Cl-LAla-β-Cl-LAla (**8**) or LppGly-LppGly (**18**)] are broad-spectrum antibacterials; as many as 12 different species of the 16 surveyed are sensitive. Dipeptides that contain an amino-terminal L-methionyl (**9**) or an L-norvalyl (**10**) residue and a carboxy-terminal β-chloro-L-alanyl unit are also effective against a large number of organisms; the spectra of activity are like those seen for **8** and **18**. A "mixed" dipeptide [β-Cl-LAla-LppGly, (**21**)] gives apparent synergism of antibiotic action of β-chloro-L-alanine and of L-propargylglycine when these two residues are incorporated into a single structure. Peptides of the D,D configuration (**4**, **6**, **13**, **16**, and **20**) and ones of L,D stereochemistry (e.g., **7**) are not antibacterials. Peptides containing one (**11** and **14**) and two (**17**) D,L-propargylglycyl residues are unresolved sets of diastereomers; the mixtures of compounds are between two- and fourfold less active than the correspondingly resolved L,L dipeptides (**12**, **15**, and **18**). These findings are consistent with a mechanism of action for these antibiotics involving stereoselective processing of the peptidyl unit in vivo.

A number of enzymes involved in peptidoglycan biosynthesis, including alanine racemase, DAla:DAla[2] ligase, and D-amino acid transaminase, are inhibited by derivatives of alanine.[3] A major effort in the design of new antibiotics has long focused, therefore, on the preparation of alanine analogues that might serve to abort the construction of an intact, functional cell wall. Renewed interest in this strategy emerged following several discoveries that confirmed that a number of the sometimes antibacterial alanine analogues, notably cycloserine,[4] *O*-carbamoylserine,[4b,5] and the β-haloalanines,[4b,6] are mechanism-based inactivators of at least the bacterial racemases—which appear to be nearly uniformly sensitive—and perhaps also of the ligases and the transaminases from certain species. The possibility for development as potential pharmaceuticals of suicide substrates specifically for the racemases is attractive. The target enzyme is both essential and restricted to microbial systems. There is, additionally, the general expectation that mechanism-based inactivators ought to have a high degree of in vivo specificity.[7]

The promise of clinical utility, however, would appear to have been compromised thus far by problems of transport and of host cytotoxicity. For example, whereas β,β,β-trifluoroalanine is a very efficient inactivator of *Escherichia coli* alanine racemase,[8] it is not an exceedingly effective antibiotic—probably because it is not well transported into bacteria. The trifluoromethyl group lowers the α-amino pK_a of β,β,β-trifluoroalanine to 5.8. As a result, at physiological pH it lacks the zwitterionic character required for transport of neutral amino acids.[9] Similarly, *O*-carbamoyl-D-serine is a poor antibacterial agent because it is not readily transported by the D-alanyl-glycine permease.[10] β-Chloro-D- and -L-alanine are, as Manning and co-workers have shown,[11] bacteriostatic against both Gram-positive and Gram-negative organisms, as well as inactivators of purified alanine racemases. The D compound, however, is also a substrate for renal D-amino acid oxidase—generating β-chloropyruvate.[12] Since

[†] The University of Chicago, Departments of Chemistry and Biochemistry.
[‡] Massachusetts Institute of Technology.
[§] The University of Chicago, Departments of Medicine and Microbiology.

(1) Dedicated to Professor Joseph S. Fruton.
(2) The omission of the hyphen in abbreviations of amino acid residues (e.g., DAla) conforms with suggestions cited in *Biochemistry*, **5**, 2485 (1966). Other abbreviations used are: Ala(P), (1-aminoethyl)phosphonic acid; Boc, *tert*-butoxycarbonyl; *t*-Bu, *tert*-butyl; BuOH, 1-butanol; DCC, dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; Gly(P), (1-aminomethyl)phosphonic acid; MIC, minimum inhibitory concentration (in micrograms per milliliter); Nva, norvaline; ppGly, propargylglycine; TFA, trifluoroacetic acid or trifluoroacetate.
(3) An excellent review of the topic is F. C. Neuhaus and W. P. Hammes, *Pharmacol. Ther.*, 14, 265 (1981).
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chloropyruvate inactivates a number of mammalian enzymes,[13] it may be expected to show toxicity in human cells.

Circumventing host toxicity is a constant concern in drug design. The problem of transport, however, may be more easily remedied. Indeed, selective drug delivery and *specific* accumulation only into targeted cells may reduce the incidence and severity of adventitious cytotoxicity. An approach of this type that has met with success is the incorporation of alanine analogues into peptides that are designed for accumulation by the several bacterial di- and oligopeptide transport systems.

Among the antibacterials reported to be transported by a dipeptide permease is D-norvalyl-D-alanine, which inhibits the growth of *E. coli* K12, acts synergistically with D-cycloserine, and appears to replace DAla-DAla as a substrate in the synthesis of the UDP *N*-acetylmuramyl pentapeptide.[3,14] The naturally occurring bacilysin, a dipeptide of L-alanine and L-anticapsin, is active against a wide range of bacteria and against the yeast *Candida albicans*. Anticapsin is a powerful inhibitor of glucosamine synthase isolated from both bacilysin-sensitive and -resistant strains of *Staphylococcus aureus*, but this amino acid alone has poor activity against whole bacterial cells.[15]

Perhaps the most successful exploitation of the peptide-transport strategy for delivery of antibacterial amino acids has been achieved by workers at Roche. They have synthesized a large number of di- and oligopeptides that contain a carboxy-terminal L-(1-aminoethyl)phosphonic acid residue.[16] (Aminoethyl)phosphonate (phosphonoalanine) is a potent inhibitor of alanine racemase in vitro[17a] and interferes with cell-wall biosynthesis in vivo.[17b] The mechanism of action of phosphonoalanyl peptides appears to involve specific transport and intracellular hydrolysis to generate (aminoethyl)phosphonate.[16b] As a consequence, the action of the racemase in both Gram-positive and Gram-negative organisms is blocked in vivo; nonetheless, the phosphonoalanine by itself has little antibacterial action.

We have similarly reasoned that antimicrobial activity can be enhanced by targeting the bacterial racemase with a well-characterized, mechanism-based inactivator that is incorporated into a peptide to facilitate its delivery. Thus, we have prepared a number of β-chloroalanyl dipeptides and report here on their antibacterial properties. We have prepared also a series of dipeptides containing propargylglycine, designed for inactivation in vivo of bacterial cystathionine γ-synthase, an essential enzyme in microbial methionine biosynthesis. The acetylenic amino acid propargylglycine (2-amino-4-pentynoate) is a natural product elaborated by *Streptomyces*;[18] the synthetic compound is bacteriostatic against some strains of *E. coli*.[19] The detailed mechanism of propargylglycine-induced inactivation of two pyridoxal phosphate enzymes has now been elucidated.[20] Although propargylglycine has effective antibacterial activity, it is also a suicide substrate for rat liver γ-cystathionase.[21] Therefore, clinical utility of this acetylene will probably require selective delivery.

Chemistry. Conventional methods for solution-phase peptide synthesis have been used in the preparation of compounds containing propargylglycyl residues as the single suicide substrate. For example, *N*-Boc-LAla-LppGly-O-*t*-Bu was readily produced by dicyclohexylcarbodiimide coupling in tetrahydrofuran. *N*-Butoxycarbonyl and *tert*-butyl protecting groups were chosen for ease of deprotection; both were cleaved in a single step with TFA in anisole, giving the deblocked peptide as its trifluoroacetate salt.

We have found the solid-phase method recently developed by DeGrado and Kaiser[22] to be especially well suited for the synthesis of chloroalanyl-containing peptides. 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 that forms the peptide bond and regenerates the oxime resin. This method has allowed the use of an *unprotected* β-chloroalanine for cleavage of a resin-bound *N*-Boc-protected amino acid, thus affording the introduction of a carboxy-terminal chloroalanyl residue without first having to prepare the *tert*-butyl ester of that compound (see Synthesis under Experimental Section). In all cases, the oxime resin method gives haloalanyl-containing peptides of exceedingly high purity and in very good yield. The structures of the synthetic peptides are given in Table IV.

NMR of Peptides. Four of the propargylglycyl-containing peptides (**11**, **14**, **17**, and **19**) were synthesized with racemic DL-propargylglycine, a preparation that obviously yields a mixture of diastereomers. In the 500-MHz proton spectra of these compounds, pairs of signals are resolved that are consistent with diastereotopic resonances for a single proton (or set of protons). In the case, for example, of DLppGly-LAla (**14**), the alanyl methyl resonance appears as a pair of doublets (δ 1.45, 1.46), which together integrate to three protons. Similarly, the acetylenic proton is a pair of triplets (δ 2.58, 2.60), the α-hydrogen of propargylglycine is a pair of triplets (δ 4.22, 4.23), and the α-hydrogen of

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alanine is a pair of quartets (δ 4.43, 4.45).[23] Usually, the chemical-shift differences in these signals are great enough that integration is possible, and this allows an estimate of the ratio of diastereomers. Thus, analysis of the data for **14** suggests that DppGly-LAla and LppGly-LAla are present in equal concentrations. For the pair LAla-DLppGly, the ratio of diastereomers is 6:4.

By contrast to the diastereomeric mixtures, peptides prepared from resolved enantiomers of propargylglycine (**12**, **13**, **15**, **16**, **18**, **20**, and **21**) give single signals, as is expected for a single, pure diastereomer. Thus, for LppGly-LAla (**15**) the alanyl methyl resonance is a doublet (δ 1.44), the acetylenic hydrogen is a triplet (δ 2.58), the propargylglycyl α-hydrogen is a triplet (δ 4.21), and the α-hydrogen of alanine is a quartet (δ 4.42). Peptides composed of D,D residues give, obviously, the same set of NMR signals as their L,L cognates (see Synthesis under Experimental Section).

Antibacterial Activity. Dipeptide analogues of LAla-LAla, which contain a single β-chloro-L-alanyl substitution in either the amino-terminal (**3**) or the carboxy-terminal (**5**) position, show substantial antibacterial activity against three Gram-positive cocci: *Streptococcus agalactiae*, *S. aureus* and *Staphylococcus epidermidis* (Table I). In the very best of these cases (i.e., **5** against *S. aureus* and *S. epidermidis*), the minimum inhibitory concentrations are an exceedingly low 0.0125 μg/mL, corresponding to 0.06 μM peptide. LAla-β-Cl-LAla (**5**) is an analogue of alafosfalin, wherein the racemase-inhibiting (aminoethyl)phosphonate is also (and necessarily) carboxy terminal. Against *S. aureus*, alafosfalin has an MIC that corresponds to 41 μM peptide.[16a]

The two control alanylalanine peptides (**1** and **2**) show no antibacterial effects up to 100 μg/mL. Four organisms are inhibited by β-chloro-L-alanine under our experimental conditions. The MICs determined, however, are substantially greater for the free amino acid than for either peptide containing a single β-chloro-L-alanyl residue. Attention is drawn, for example, to the action of **5** on *S. epidermidis*, in which case the antibacterial action of β-chloro-L-alanine is potentiated by a factor of 2×10^3 when the haloalanine is incorporated into a peptide. In the "worst" case, the enhancement of activity is only eightfold, as observed for the action of **3** against *Strep. agalactiae*. The dipeptides **3** and **5** have, on an equivalent-weight basis, only one-half as much "active drug" as does free chloroalanine. Thus, **3** is actually 16-fold more active than β-Cl-LAla against *Strep. agalactiae*; and **5** effectively enhances the activity of chloroalanine against *Strep. epidermidis* by a factor of 4000.

β-Chloro-D-alanine, by contrast to the L enantiomer, is a broad-spectrum, if weak, antibacterial against the organisms screened in this study. The two chloroalanyl peptides of the D,D configuration (**4** and **6**), however, have no detectable biological activity. Indeed, none of the D,D dipeptides reported here is antibacterial (vide infra). Similarly, peptides of mixed stereochemistry, such as **7** (LAla-β-Cl-DAla) and **19** (β-Cl-DAla-DLppGly, which is a pair of D,D and D,L diastereomers) show no antibacterial action. Peptide **7** has been synthesized previously and is described in the patent literature.[24] This compound and

Table I. Minimum Inhibitory Concentration[a] for β-Chloroalanyl Peptides and β-Chloroalanine Controls

bacterial species[b]	peptides										amino acids	
	1	2	3	4	5	6	7	8	9	10	β-Cl-L-Ala	β-Cl-D-Ala
S. aureus	>100	>100	0.05	>100	0.0125	>100	>100	0.05	0.025	0.025	3.12	12.5
S. epidermidis	>100	>100	0.025	>100	0.0125	>100	>100	0.05	0.025	0.025	25	25
Strep. pyogenes	>100	>100	>100	>100	>100	>100	>100	1.56	>100	>100	3.12	12.5
Strep. agalactiae	>100	>100	0.39	>100	0.20	>100	>100	0.39	0.20	0.20	3.12	12.5
Strep. faecalis	>100	>100	>100	>100	>100	>100	>100	3.12	0.39	0.39	>100	50
H. influenzae	>100	>100	>100	>100	>100	>100	>100	1.56	0.78	0.78	>100	6.25
E. coli	>100	>100	>100	>100	>100	>100	>100	1.56	1.56	1.56	>100	6.25
Sal. typhimurium	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	100
Sh. flexneri	>100	>100	>100	>100	>100	>100	>100	12.5	>100	>100	>100	50
P. mirabilis	>100	>100	>100	>100	>100	>100	>100	50	100	12.5	>100	>100
K. pneumoniae	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	100
M. morganii	>100	>100	>100	>100	>100	>100	>100	50	50	50	>100	>100
Ser. marcescens	>100	>100	>100	>100	>100	>100	>100	>100	50	>100	>100	25
Ent. aerogenes	>100	>100	>100	>100	>100	>100	>100	25	6.25	>100	>100	12.5
Ent. cloacae	>100	>100	>100	>100	>100	>100	>100	12.5	6.25	6.25	>100	100
Ps. aeruginosa	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

[a] In micrograms per milliliter. [b] For full names, see Table III.

(23) Occasionally, the chemical-shift differences for two diastereotopic resonances are not great enough to be resolved even in the 500-MHz spectrum; these are reported as multiplets. An especially complicated set of signals is given, for example, by the two nonequivalent, prochiral β-methylene hydrogens of propargylglycyl residues in diastereomeric mixtures (vide infra, especially ref 33).

Table II. Minimum Inhibitory Concentration[a] (μg/mL) for Propargylglycyl Peptides and Propargylglycine Controls

	peptides								amino acids	
bacterial species[b]	11	12	13	14	15	16	17	18	LppGly	DppGly
S. aureus	>100	>100	>100	50	>100	>100	12.5	3.12	>100	50
S. epidermidis	3.12	1.56	>100	3.12	1.56	>100	3.12	0.78	>100	500
Strep. pyogenes	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Strep. agalactiae	50	100	>100	>100	>100	>100	6.25	3.12	>100	>100
Strep. faecalis	>100	100	>100	>100	>100	>100	>100	>100	>100	>100
H. influenzae	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
E. coli	>100	>100	>100	>100	>100	>100	>100	25	>100	>100
Sal. typhimurium	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Sh. flexneri	>100	>100	>100	>100	>100	>100	>100	12.5	>100	>100
P. mirabilis	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
K. pneumoniae	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
M. morganii	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Ser. marcescens	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Ent. aerogenes	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Ent. cloacae	>100	>100	>100	>100	>100	>100	>100	50	>100	>100
Ps. aeruginosa	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

[a] In micrograms per milliliter. [b] For full names, see Table III.

its β-fluoro-D-alanyl cognate are exceptionally potent antibiotics in synergism with cycloserine and with β-lactams. Neither peptide, however, has appreciable antibacterial activity alone, an observation underscored by our results for **7** shown in Table I.

Peptides composed of D-amino acid residues are known to be transported by bacteria,[14,25] but intracellular peptidase activity may be largely restricted to cleavage of the peptide bond formed by L-amino acids.[26] For example, a highly purified dipeptidase from *E. coli* B has broad specificity for peptides composed of L residues; compounds such as DLeu-Gly or Gly-DLeu are competitive inhibitors.[27] It seems reasonable that β-chloro-D-alanine will *not* be generated from peptides such as **4**, **6**, and **7** even if these are concentrated in situ, which may very well occur. Thus, we suspect that the mechanism of action of all the active β-chloroalanyl peptides described here involves transport—perhaps nonselective with regard to stereochemistry—and intracellular hydrolysis with stereospecificity for the peptide bond between two amino acid residues of the L configuration.

Twelve of sixteen organisms surveyed are inhibited by β-Cl-LAla-β-Cl-LAla (**8**) at concentrations less than 100 μg/mL, and seven are inhibited at ≤ 3.12 μg/mL. *Strep. agalactiae*, *S. aureus*, and *S. epidermidis* are particularly susceptible to the antibacterial action of this chloroalanyl peptide. Indeed, no other organism is as exquisitely sensitive to **8** as are these three. On the other hand, *Streptococcus pyogenes*, *Streptococcus faecalis*, *E. coli*, and *Hemophilus influenzae* are quite susceptible to **8** and yet not inhibited by the peptides containing only a single haloalanyl unit (**3** and **5**).

It is not clear why a second β-chloro-L-alanyl residue should broaden the spectrum of antibiotic action of a dipeptide. It seems unlikely that this effect relates to increased efficiency of any enzymatic inactivation in vivo. The group at Roche has shown that LNva-LAla(P) exhibits a significantly higher level of antibacterial action than does alafosfalin and does so against a larger number of organisms. Similarly, alaphosphono dipeptides with varying amino-terminal substitutions (including arginyl, leucyl, norleucyl, and methionyl) are as much as 30-fold more active against *E. coli* than is LAla-LAla(P).[16c] These differences probably reflect in large measure differing rates of peptide transport. Very recently Atherton and coworkers have shown that LNva-Gly(P) is transported into *E. coli* at a substantially higher rate than is LAla-Gly(P).[28]

These observations prompted our syntheses of the N-terminal methionyl- and norvalyl-β-chloroalanyl dipeptides (**9** and **10**). Table I shows that these two compounds are largely equivalent to **8** with regard to their spectra of activity. In most cases, peptides **9** and **10** are slightly more active than **8**. The remarkable exception to this trend is seen for *Strep. pyogenes*, which is sensitive to β-Cl-LAla-β-Cl-LAla and to no other peptide.

We suspect, based on these results, that β-chloroalanyl peptides of varying structure are transported with differing facility by the dipeptide permeases of different species. We are currently investigating the transport properties of several of the organisms of Table I for ability to accumulate chloroalanyl peptides. It may emerge, however, that the special properties of **8**–**10** relate not merely to discrimination in transport but also to the characteristic specificities of bacterial peptidases. In this regard, we note with curiosity that *E. coli* is quite sensitive to at least **8**–**10**, while *Salmonella* is not susceptible to any of the chloroalanyl peptides, despite the fact that both dipeptides and oligopeptides are actively transported by both these organisms.[3,26a]

The antibacterial activities of propargylglycyl peptides are given in Table II. Compounds containing a single propargylglycyl residue (**11**, **12**, **14**, and **15**) are active only against *S. epidermidis*. Introduction of a second DL-propargylglycyl residue (**17**) improves the biological activity somewhat; *S. aureus* and *Strep. agalactiae*, as well as *S. epidermidis*, are sensitive to **17**. However, only **18**, which is a single diastereomer (LppGly-LppGly), has demonstrable activity against many Gram-negative organisms.

It is of some interest to note the apparent correlation between the observed MIC and the diastereomeric "purity" of a propargylglycyl peptide. Compounds **11** and **14**, both of which are a pair of diastereomers in approximately equal

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Table III. Minimum Inhibitory Concentration[a] for Peptides Containing β-Chloroalanyl and Propargylglycyl Residues[b]

bacterial species	19	20	21
Staphylococcus aureus	>100	>100	0.10
Staphylococcus epidermidis	>100	>100	0.05
Streptococcus pyogenes	>100	>100	25
Streptococcus agalactiae	>100	>100	0.39
Streptococcus faecalis	>100	>100	3.12
Hemophilus influenzae	>100	>100	1.56
Escherichia coli	>100	>100	3.12
Salmonella typhimurium	>100	>100	>100
Shigella flexneri	>100	>100	3.12
Proteus mirabilis	>100	>100	>100
Klebsiella pneumoniae	>100	>100	>100
Morganella morganii	>100	>100	>100
Serratia marcescens	>100	>100	>100
Enterobacter aerogenes	>100	>100	25
Enterobacter cloacae	>100	>100	25
Pseudomonas aeruginosa	>100	>100	>100

[a] In micrograms per milliliter. [b] Values for amino acid controls are given in Tables I and II.

concentration (vide supra), have MICs that are twice those for the corresponding L,L peptides (**12** and **15**). Similarly, the diastereomerically pure **18** (LppGly-LppGly) has an MIC against *S. epidermidis* fourfold lower than that of **17**—which consists of *two* pairs of diastereomers. This enhancement of antimicrobial action corresponds, obviously, to an effective fourfold increase in the concentration of the L,L diastereomer in **18** vs. **17**. An identical pair of results obtains for the action of **17** vs. **18** on *S. aureus*. These findings draw particular attention to the relationship between biological activity of a peptide and the stereochemical configuration of its component amino acids. Moreover, it would seem that only peptides of the L,L configuration are effective antibacterials, a conclusion supported by the observation that neither of the D,D compounds of Table II (**13** and **16**) has antibiotic activity.

Our data do not rule out the possibility that D,D and D,L peptides are both transported and hydrolyzed intracellularly, although we suspect that this is unlikely. The reduced antibacterial activity for compounds such as **11**, **14**, and **17** vs. **12**, **15**, and **18** may relate simply to the fact that the putative target, cystathionine γ-synthase (at least the purified one from *S. typhimurium*), does not process D-propargylglycine.[20] Thus, cells exposed to diastereomers containing D- and L-propargylglycyl residues may concentrate both the "right" and the "wrong" enantiomers of the inactivating amino acid.

Compounds **19–21** (Table III), which contain chloroalanyl and propargylglycyl residues, were designed specifically with the hope of targeting both the racemase and the synthase in vivo. Given the results presented above, it is not surprising that **19** (which consists of a pair of D,D and D,L diastereomers) and **20** (the single D,D diastereomer) lack demonstrable antibiotic effect. By contrast to these, however, **21** (β-Cl-LAla-LppGly) is antibacterial against 10 of 16 organisms screened, and it is antibacterial against seven species at ≤3.12 μg/mL. The spectrum and levels of activity for this compound are similar to those observed for **8**.

The enhanced biological activity of **21** may reflect only the inherent susceptibility of a large number of organisms to β-haloalanines. Nonetheless, distinct patterns of sensitivity are discerned from the data, which suggest multiple sites of action for **21** in vivo. For example, **21** inhibits the growth of a number of organisms that are resistant to peptides (**3** and **5**) containing only a single chloroalanyl residue. *Shigella*, moreover, is apparently fourfold more sensitive to **21** than to both **8** and **18**, the peptides containing two units each of haloalanine and propargylglycine, respectively. If, in fact, **21** is actually cleaved to its component amino acids in situ, it would appear that β-chloroalanine and propargylglycine act synergistically.

We have been guided in the design of all our peptides by the expectation that their modes of action would involve intracellular inactivation of alanine racemase and of cystathionine synthase. The results reported here do not, of course, verify that these two enzymes are the actual targets in vivo, and we are aware that the peptides may undergo a variety of transformations in the cell that render them cytotoxic. β-Chloroalanine, for example, inhibits threonine deaminase and transminase B from *Sal. typhimurium*. Thus, Arfin and Koziell[29] suggest that the bacteriostatic effect of haloalanines is linked to inhibition of branched-chain amino acid biosynthesis.

Moreover, there is no reason to expect that a toxophoric amino acid need interact identically with the same target enzyme in different species of microorganisms. For example, both β-chloro-L- and -D-alanines are suicide substrates for the purified *E. coli* racemase (although the kinetic constants for the two enentiomers differ).[4b] By contrast β-chloro-D-alanine is a strong (K_I = 0.005 mM) but only competitive inhibitor of the enzyme from *B. subtilis*; the L enantiomer binds relatively weakly (K_I = 1.71 mM) and inhibits noncompetitively.[30]

We have undertaken a number of projects—including transport experiments with radiolabeled compounds, competition experiments, and susceptibility studies with racemase-minus mutants—with a view toward identifying the intracellular targets of our peptides and of describing more fully their reactions with susceptible enzymes. We hope to be able to present a detailed analysis of the mechanisms of action of these interesting antibacterials in a subsequent report.

Experimental Section

Synthesis. β-Chloro-L-alanine, β-chloro-D-alanine, L- and D-alanine derivatives, *N*-(*tert*-butoxycarbonyl)-L-methionine and *N*-(*tert*-butoxycarbonyl)-L-norvaline were purchased from Vega Biochemicals. DL-Propargylglycine and hog kidney acylase were from Sigma Chemical Co. Di-*tert*-butyldicarbonate was obtained from Pierce Chemical Co. All other reagents were of the best commercial grade available.

Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. Peptide trifluoroacetate salts are hygroscopic and give, consequently, unreliable melting points. We report here, therefore, melting point data only for crystalline amino acid derivatives and protected peptides. Infrared spectra were recorded on a Perkin Elmer 283 spectrometer with KBr pellets. 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 Me_4Si in $CDCl_3$. Thin-layer chromatograms were made 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 with iodine vapor. Physical data for the antibacterial peptides are given in Table IV. Proton magnetic resonance data are given for each compound in the text below. Overall yields for peptide trifluoroacetate salts are calculated from starting amino acid derivatives before coupling.

***p*-Nitrobenzophenone Oxime Resin.** The oxime resin was prepared essentially as described by DeGrado and Kaiser.[22] Biobeads S-XI (10 g, Bio-Rad Laboratories) were reacted with nitrobenzoyl chloride (1.2 g, 5 mmol) in 1,2-dichloroethane with $AlCl_3$ as catalyst. This afforded 10.4 g of *p*-nitrobenzoyl poly-

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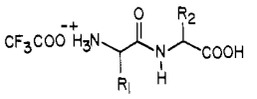
Table IV. Physical Data for Synthetic Peptides



no.	R_1	R_2	name	formula	anal.	overall yield, %	R_f (solvent A)[a]
1	CH_3	CH_3	L Ala-L Ala	$C_8H_{13}N_2O_5F_3$	C, H, N, O	56.0	0.22
2	CH_3	CH_3	D Ala-D Ala	$C_8H_{13}N_2O_5F_3$	C, H, N, O	56.0	0.23
3	CH_2Cl	CH_3	β-Cl-L Ala-L Ala	$C_8H_{12}N_2O_5F_3Cl$	C, H, N, O, Cl	91.4	0.29
4	CH_2Cl	CH_3	β-Cl-D Ala-D Ala	$C_8H_{12}N_2O_5F_3Cl$	C, H, N, O, Cl	55.3	0.29
5	CH_3	CH_2Cl	L-Ala-β-Cl-L Ala	$C_8H_{12}N_2O_5F_3Cl$	C, H, N, O, Cl	31.9	0.26
6	CH_3	CH_2Cl	D Ala-β-Cl-D Ala	$C_8H_{12}N_2O_5F_3Cl$	C, H, N, O, Cl	12.6	0.26
7	CH_3	CH_2Cl	L Ala-β-Cl-D Ala	$C_8H_{12}N_2O_5F_3Cl$	C, H, N, O, Cl	60.4	0.26
8	CH_2Cl	CH_2Cl	β-Cl-L Ala-β-Cl-L Ala	$C_8H_{11}N_2O_5F_3Cl_2$	C, H, N, O, Cl	42.8	0.36
9	$(CH_2)_2SCH_3$	CH_2Cl	L Met-β-Cl-L Ala	$C_{10}H_{16}N_2O_5F_3ClS$	C, H, N, O, Cl	59.5	0.34
10	$(CH_2)_2CH_3$	CH_2Cl	L Nva-β-Cl-L Ala	$C_{10}H_{16}N_2O_5F_3Cl$	C, H, N, O, Cl	67.4	0.34
11	CH_3	$CH_2C{\equiv}CH$	L Ala-DL ppGly	$C_{10}H_{13}N_2O_5F_3$	C, H, N, O	51.4	0.22
12	CH_3	$CH_2C{\equiv}CH$	L Ala-L ppGly	$C_{10}H_{13}N_2O_5F_3$	C, H, N, O	64.4	0.22
13	CH_3	$CH_2C{\equiv}CH$	D Ala-D ppGly	$C_{10}H_{13}N_2O_5F_3$	C, H, N, O	79.3	0.22
14	$CH_2C{\equiv}CH$	CH_3	DL ppGly-L Ala	$C_{10}H_{13}N_2O_5F_3$	C, H, N, O	21.0	0.30
15	$CH_2C{\equiv}CH$	CH_3	L ppGly-L Ala	$C_{10}H_{13}N_2O_5F_3$	C, H, N, O	39.1	0.25
16	$CH_2C{\equiv}CH$	CH_3	D ppGly-D Ala	$C_{10}H_{13}N_2O_5F_3$	C, H, N, O	8.4	0.25
17	$CH_2C{\equiv}CH$	$CH_2C{\equiv}CH$	DL ppGly-DL ppGly	$C_{12}H_{13}N_2O_5F_3$	C, H, N, O	42.9	0.35
18	$CH_2C{\equiv}CH$	$CH_2C{\equiv}CH$	L ppGly-L ppGly	$C_{12}H_{13}N_2O_5F_3$	C, H, N, O	59.2	0.35
19	CH_2Cl	$CH_2C{\equiv}CH$	β-Cl-D Ala-DL ppGly	$C_{10}H_{12}N_2O_5F_3Cl$	C, H, N, O, Cl	30.6	0.32
20	CH_2Cl	$CH_2C{\equiv}CH$	β-Cl-D Ala-D ppGly	$C_{10}H_{12}N_2O_5F_3Cl$	C, H, N, O, Cl	64.2	0.32
21	CH_2Cl	$CH_2C{\equiv}CH$	β-Cl-L Ala-L ppGly	$C_{10}H_{12}N_2O_5F_3Cl$	C, H, N, O, Cl	33.2	0.32

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

styrene resin. The IR spectrum gave characteristic bands at 1665, 1525, and 1310 cm^{-1}. The nitrobenzoylated beads (4.4 g) were in turn reacted with excess hydroxylamine hydrochloride (4.4 g, 63.3 mmol) in 50 mL of absolute ethanol and 6 mL of pyridine, which gave 4.5 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) is absent in the oxime product. The oxime substitution level was determined by titration with *N*-(*tert*-butoxycarbonyl)-L-alanine on the resin and gave a value of 0.44 mmol/g of resin. Elemental analysis gave 1.46% nitrogen, which corresponds to 0.52 mmol/g of resin.

Enzymatic Resolution of DL-Propargylglycine. DL-Propargylglycine was resolved according to the procedures of Scannell and co-workers.[18] This method involves reaction of the racemic amino acid (5.0 g, 44.2 mmol) with acetic anhydride to afford *N*-acetyl-DL-propargylglycine: yield 5.71 g (83.3%); mp 137–139 °C. The *N*-acetyl product (7.6 g, 49.0 mmol) was treated with hog kidney acylase (30 mg, 37 °C, 21 h), followed by ion-exchange chromatography (Amberlite IR-120), which separates unreacted *N*-Ac-DppGly from LppGly.

D-Propargylglycine Hydrochloride. The Amberlite column above was washed with 2 L of distilled H_2O, and the eluent was evaporated to dryness. The residue was refluxed with 2 N HCl (40 mL) for 3 h and afforded 2.35 g of DppGly hydrochloride (64.1%): mp 212–214 °C dec; R_f 0.23 (solvent A); $[\alpha]^{25}_D$ +8.1° [lit.[18] $[\alpha]^{25}_D$ +6.0° (5 N HCl)]; ^1H NMR (D_2O) δ 2.57 (t, 1 H, C≡CH, J = 2.6 Hz), 2.93 (m, 2 H, β-H_2), 4.18 (t, 1 H, α-H, J = 5.5 Hz).

L-Propargylglycine. The ion-exchange column, after elution of *N*-Ac-DppGly, was then eluted with 10% aqueous pyridine. Fractions containing significant amounts of ninhydrin-positive material were combined, concentrated, and recrystallized from water/ethanol to give LppGly: yield 2.46 g (88.8%); mp 243–245 °C dec; R_f 0.19 (solvent A); $[\alpha]^{25}_D$ −31.56° (lit.[18] $[\alpha]^{25}_D$ − 32.6°); ^1H NMR (D_2O) δ 2.53 (t, 1 H, C≡CH, J = 2.6 Hz), 2.86 (m, 2 H, β-H_2), 3.91 (t, 1 H, α-H, J = 5.5 Hz).

***N*-(*tert*-Butoxycarbonyl)-DL-propargylglycine.** *N*-*tert*-Butoxycarbonyl-protected amino acids were prepared by the di-*tert*-butyl dicarbonate method of Moroder et al.[31] The preparation of *N*-(*tert*-butoxycarbonyl)propargylglycine is illustrative. DL-Propargylglycine (0.3 g, 2.6 mmol) was dissolved in 9 mL of dioxane/water (2:1), and 2.6 mL of 1 N NaOH was added at 0 °C. Di-*tert*-butyl dicarbonate (0.62 g, 2.86 mmol) was added dropwise. The reaction mixture was stirred for 15 min at 0 °C, brought to room temperature and stirred for an additional 2 h. Dioxane was then removed in vacuo, cooled to 0 °C, and 10 mL of ethyl acetate was added. The mixture was acidified to pH 2–3 with $KHSO_4$ and extracted three times with ethyl acetate. The organic phases were combined, washed with water, and dried over $MgSO_4$. The solvent was stripped, and the product was crystallized from ethyl acetate/hexane: yield 0.42 g (74.3%); mp 95–97 °C; R_f 0.64 (solvent B); ^1H NMR ($CDCl_3$) δ 1.46 (s, 9 H, *tert*-butyl), 2.08 (t, 1 H, C≡CH, J = 2.5 Hz), 2.77 (m, 2 H, β-H_2), 4.52 (br s, 1 H, α-H), 5.33 (br s, 1 H, α-NH).

N-(*tert*-Butoxycarbonyl)-L-propargylglycine was prepared in an identical way: yield 0.42 g (74.3%); mp 95–97 °C; R_f 0.64 (solvent B); ^1H NMR ($CDCl_3$) same as for the DL compound (vide supra).

N-(*tert*-Butoxycarbonyl)-D-propargylglycine was synthesized as described above: 0.50 g of DppGly hydrochloride afforded 0.60 g of *N*-Boc-DppGly (84.2%): mp 95–97 °C; R_f 0.64 (solvent B); ^1H NMR ($CDCl_3$) same as for the DL compound (vide supra).

***tert*-Butyl DL-Propargylglycinate Hydrochloride.** The *tert*-butyl propargylglycinate was prepared by the isobutylene method of Roeske.[32] DL-Propargylglycine (1.0 g, 8.8 mmol) was added to 50 mL of *p*-dioxane and cooled to −78 °C in a 125-mL pressure bottle. Concentrated sulfuric acid (5 mL) and 50 mL of liquid isobutylene were added. The mixture was shaken at room temperature for 12 h and then poured into a 400-mL solution of ether and saturated Na_2CO_3 previously chilled in an ice bath. This mixture was then extracted several times with ether; the organic phases were combined, dried over $MgSO_4$, and evaporated to dryness. The resulting yellow oil was dissolved in 200 mL of dry ethyl acetate, and a white crystalline product was obtained by passing dry HCl gas through the solution: yield 1.04 g (57.2%); mp 195–197 °C dec; R_f 0.47 (solvent A); ^1H NMR (D_2O) δ 1.52 (s, 9 H, *tert*-butyl), 2.60 (t, 1 H, C≡CH, J = 2.6 Hz), 2.89 (ddd, 1 H, β-H_A, J_{AB} = 18.0 Hz, J_{AC} = 4.6 Hz, J_{AD} = 2.7 Hz),[33] 3.00 (ddd,

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1 H, β-H_B, J_{BA} = 17.9 Hz, J_{BC} = 5.7 Hz, J_{BD} = 2.7 Hz), 4.22 (t, 1 H, α-H, J = 5.1 Hz).

tert-Butyl L-propargylglycinate hydrochloride was prepared in the same way: yield 0.54 g (59.4%); mp 195–197 °C dec; R_f 0.47 (solvent A); ^{1}H NMR (D_2O) same as for the DL compound (vide supra). For *tert*-butyl D-propargylglycinate hydrochloride: yield 1.2 g (87.3%); mp 195–197 °C dec; R_f 0.47 (solvent A); ^{1}H NMR (D_2O) same as for the DL compound (vide supra).

N-(*tert*-Butoxycarbonyl)-β-chloro-L- and -D-alanine. The *N*-Boc derivatives of β-chloro-L- and -D-alanine were prepared as described for propargylglycine (vide supra): yield for *N*-Boc-β-Cl-LAla is 2.34 g (83.8%); mp 127–129 °C; R_f 0.63 (solvent B); ^{1}H NMR ($CDCl_3$) δ 1.47 (s, 9 H, *tert*-butyl), 3.87 (dd, 1 H, β-H_A, J = 2.3 and 11.0 Hz),[34] 4.02 (dd, 1 H, β-H_B, J = 1.9 and 11.0 Hz), 4.76 (br s, 1 H, α-H), 5.42 (br s, 1 H, α-NH).

For *N*-Boc-β-Cl-DAla the yield is 0.43 g (77.0%); mp 127–129 °C; R_f 0.63 (solvent B); ^{1}H NMR ($CDCl_3$) same as for the L compound (vide supra).

***tert*-Butyl β-Chloro-L- and -D-alaninate Hydrochloride.** *tert*-Butyl β-chloro-L- and -D-alaninate hydrochlorides were prepared in the same way as described for the *tert*-butyl DL-propargylglycinate. β-Cl-LAla-O-*t*-Bu·HCl: yield 0.33 g (48.9%); mp 142–145 °C dec; R_f 0.54 (solvent A); ^{1}H NMR (D_2O) δ 1.53 (s, 9 H, *tert*-butyl), 4.03 (dd, 1 H, β-H_A, J = 3.0 and 12.7 Hz), 4.21 (dd, 1 H, β-H_B, J = 4.2 and 12.7 Hz), 4.50 (t, 1 H, α-H, J = 3.2 Hz). For β-Cl-DAla-O-*t*-Bu·HCl: yield 0.28 g (41.5%); mp 175–180 °C dec; R_f 0.54 (solvent A); ^{1}H NMR (D_2O) same as for the L compound above.

L-Alanyl-L-alanine Trifluoroacetate (1). To a mixture of *tert*-butyl L-alaninate hydrochloride (0.6 g, 3.3 mmol) and Et_3N (0.49 mL, 3.5 mmol) in 15 mL of THF was added *N*-(*tert*-butoxycarbonyl)-L-alanine (0.62 g, 3.3 mmol) in 10 mL of THF at 0 °C with stirring. *N,N'*-Dicyclohexylcarbodiimide (0.76 g, 3.6 mmol) was added, and the reaction stirred at 4 °C for 25 h. Dicyclohexylurea and Et_3N·HCl were removed by filtration, and the filtrate was evaporated to dryness. The oily residue was dissolved in methylene chloride and washed with 1 N HCl, 5% $NaHCO_3$, and water, dried over $MgSO_4$, and evaporated to dryness.

The crude oily product was dissolved in 4 mL of cold anisole, to which 20 mL of TFA was added dropwise. The mixture was then stirred for 3 h at room temperature. The TFA/anisole mixture was pumped to dryness. The solid residue was dissolved in 20 mL of CH_2Cl_2/H_2O (1:1), and the aqueous phase was extracted with CH_2Cl_2 to remove trace amounts of anisole. Lyophilization of the aqueous phase afforded 0.5 g of LAla-LAla·TFA: ^{1}H NMR (D_2O): δ 1.38 (d, 3 H, α_2-CH_3, J = 6.9 Hz), 1.56 (d, 3 H, α_1-CH_3, J = 7.0 Hz), 4.14 (q, 1 H, α_1-H, J = 6.9 Hz), 4.27 (q, 1 H, α_2-H, J = 7.0 Hz).[35]

D-Alanyl-D-alanine Trifluoroacetate (2). Peptide **2** was prepared as described above for LAla-LAla (1): yield 0.5 g; ^{1}H NMR (D_2O) δ 1.42 (d, 3 H, α_2-CH_3, J = 7.3 Hz), 1.54 (d, 3 H, α_1-CH_3, J = 7.1 Hz), 4.08 (q, 1 H, α_1-H, J = 7.1 Hz), 4.36 (q, 1 H, α_2-H, J = 7.3 Hz).

β-Chloro-L-alanyl-L-alanine Trifluoroacetate (3). The synthesis of peptide **3** is illustrative for the preparation of all the β-chloroalanyl-containing peptides (**3–10** and **19–21**). The oxime resin (6.2 g) was swelled in 100 mL of CH_2Cl_2. *N*-(*tert*-butoxycarbonyl)-β-chloro-L-alanine (0.76 g, 3.4 mmol) and DCC (0.81 g, 3.9 mmol) were added, and the mixture was shaken in a plastic screw-cap bottle for 20 h at room temperature. The resin was then washed with CH_2Cl_2 (4 times) and CH_3OH (4 times) and dried: IR 1720, 1775 cm^{-1}, corresponding to the carbonyl groups of *N*-Boc-β-Cl-LAla-resin.

In 100 mL of CH_2Cl_2 were mixed *N*-Boc-β-Cl-LAla-resin (7.03 g, 2.91 mmol equiv amino acid), LAla-O-*t*-Bu·HCl (0.61 g, 3.4 mmol), DIEA (0.60 mL, 3.4 mmol), and CH_3COOH (0.20 mL, 3.4 mmol). The reaction was allowed to shake for 24 h at room temperature, after which the resin was washed with CH_2Cl_2 (3 times) and CH_3OH (3 times). The filtrates were combined and evaporated to dryness. The residue was dissolved in ethyl acetate and washed with 5% citric acid (3 times), 5% $NaHCO_3$, and water (3 times). The organic phase was recovered, dried over $MgSO_4$, and evaporated to dryness to yield 1.0 g (oil) of *N*-Boc-β-Cl-LAla-LAla-O-*t*-Bu: R_f 0.24 (single iodine spot in $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.40 (d, 3 H, α_2-CH_3, J = 7.0 Hz), 1.47 (s, 9 H, *tert*-butyl), 1.48 (s, 9 H, *tert*-butyl), 3.72 (dd, 1 H, β_1-H_A, J = 5.0 and 11.2 Hz), 4.03 (dd, 1 H, β_1-H_B, J = 4.1 and 11.2 Hz), 4.45 (m, 1 H, α_2-H), 4.46 (br s, 1 H, α_1-NH), 5.32 (br s, 1 H, α_2-NH), 6.88 (m, 1 H, α_1-H).

The oily product was dissolved in 8 mL of anisole and 40 mL of TFA was added dropwise at 0 °C. The reaction was then stirred for 3.5 h at room temperature. The TFA/anisole mixture was pumped off, and the residue was dissolved in 25 mL of CH_2Cl_2/H_2O (1:1). The aqueous phase was washed with CH_2Cl_2, recovered, and lyophilized to afford a white solid of β-Cl-LAla-LAla·TFA: yield 0.82 g (92.1%); ^{1}H NMR (D_2O) δ 1.41 (d, 3 H, α_2-CH_3, J = 7.5 Hz), 4.07 (dd, 1 H, β_1-H_A, J = 6.0 and 12.0 Hz), 4.14 (dd, 1 H, β_1-H_B, J = 4.0 and 12.0 Hz), 4.30 (q, 1 H, α_2-H, J = 7.5 Hz), 4.45 (t, 1 H, α_1-H, J = 4.2 Hz).

β-Chloro-D-alanyl-D-alanine Trifluoroacetate (4). This peptide was prepared as described above for **3**: yield 0.3 g; ^{1}H NMR (D_2O) δ 1.43 (d, 3 H, α_2-CH_3, J = 7.3 Hz), 4.05 (dd, 1 H, β_1-H_A, J = 5.8 and 11.8 Hz), 4.10 (dd, 1 H, β_1-H_B, J = 3.7 and 11.8 Hz), 4.36 (q, 1 H, α_2-H, J = 7.3 Hz), 4.45 (t, 1 H, α_1-H, J = 4.5 Hz).

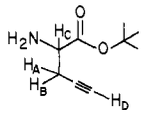
L-Alanyl-β-chloro-L-alanine Trifluoroacetate (5). *N*-(*tert*-Butoxycarbonyl)-L-alanyl-resin was prepared as outlined above for *N*-(*tert*-butoxycarbonyl)-β-chloro-L-alanyl-resin. The resin cleavage reaction (peptide bond formation) was carried out in the way usual for solid-phase peptide synthesis, except that unprotected β-chloro-L-alanine hydrochloride (0.64 g, 4 mmol), rather than its *tert*-butyl ester, was used. Deprotection was again accomplished with TFA in anisole to give **5**: yield 0.20 g; ^{1}H NMR (D_2O) δ 1.57 (d, 3 H, α_1-CH_3, J = 7.0 Hz), 3.95 (dd, 1 H, β_2-H_A, J = 3.6 and 11.8 Hz), 4.03 (dd, 1 H, β_2-H_B, J = 5.3 and 11.8 Hz), 4.17 (q, 1 H, α_1-H, J = 7.2 Hz), 4.84 (t, 1 H, α_2-H, J = 4.3 Hz).

D-Alanyl-β-chloro-D-alanine Trifluoroacetate (6). β-Cl-DAla-O-*t*-Bu·HCl was reacted with an *N*-(*tert*-butoxycarbonyl)-D-alanyl-resin prepared in the ordinary way. After deprotection of the resultant *N*-Boc-DAla-β-Cl-DAla-O-*t*-Bu in TFA/anisole, **6** was afforded from lyophilization: yield 0.11 g; ^{1}H NMR (D_2O) δ 1.57 (d, 3 H, α_1-CH_3, J = 7.1 Hz), 3.94 (dd, 1 H, β_2-H_A, J = 3.6 and 11.7 Hz), 4.03 (dd, 1 H, β_2-H_B, J = 5.3 and 11.7 Hz), 4.16 (q, 1 H, α_1-H, J = 7.1 Hz), 4.82 (t, 1 H, α_2-H, J = 3.9 Hz).

L-Alanyl-β-chloro-D-alanine Trifluoroacetate (7). This peptide was synthesized as outlined for **6**: yield 0.16 g; ^{1}H NMR (D_2O) δ 1.56 (d, 3 H, α_1-CH_3, J = 7.1 Hz), 3.95 (dd, 1 H, β_2-H_A, J = 3.6 and 11.8 Hz), 4.02 (dd, 1 H, β_2-H_B, J = 5.5 and 11.8 Hz), 4.17 (q, 1 H, α_1-H, J = 7.1 Hz), 4.88 (dd, 1 H, α_2-H, J = 3.6 and 5.5 Hz).

β-Chloro-L-alanyl-β-chloro-L-alanine Trifluoroacetate (8). An *N*-(*tert*-butoxycarbonyl)-β-chloro-L-alanyl-resin was reacted in the usual way with β-Cl-LAla-O-*t*-Bu·HCl. After isolation and deprotection, **8** was obtained by lyophilization: yield 0.33 g; ^{1}H

(33) For the *tert*-butyl esters of propargylglycine, we have obtained spectra wherein the prochiral β-methylene protons display exceedingly well-resolved geminal (J_{AB}, J_{BA}), vicinyl (J_{AC}, J_{BC}) and long-range (J_{AD}, J_{BD}) couplings. Each methylene hydrogen appears as a doublet of doublets of doublets. In most other spectra (e.g., diastereomeric mixtures of propargylglycyl peptides), these resonances are unresolved and are reported as multiplets, two protons.



(34) Each of the two prochiral, diastereotopic β-methylene hydrogens of β-chloroalanines and derivatives and β-chloroalanyl peptides resolves into a doublet of doublets (ABX system, X = α-H) in the 500-MHz spectrum. We have arbitrarily assigned the upfield signal H_A and the downfield resonance H_B.

(35) The subscripts 1 and 2 (e.g., α_1-H) refer to the carbons of the amino- and carboxy-terminal residues, respectively (see structure in Table IV).

NMR (D_2O) δ 3.97 (dd, 1 H, β_2-H_A, J = 3.5 and 11.8 Hz), 4.05 (dd, 1 H, β_2-H_B, J = 5.1 and 11.8 Hz), 4.09 (dd, 1 H, β_1-H_A, J = 5.7 and 12.5 Hz), 4.13 (dd, 1 H, β_1-H_B, J = 3.9 and 12.5 Hz), 4.55 (t, 1 H, α_1-H, J = 5.3 Hz), 4.91 (t, 1 H, α_2-H, J = 4.2 Hz).

L-Methionyl-β-chloro-L-alanine Trifluoroacetate (9). *N*-(*tert*-Butoxycarbonyl)-L-methionine was coupled to the oxime resin in the usual way and then cleaved by β-Cl-LAla-O-*t*-Bu·HCl to yield the protected peptide. Deprotection afforded 0.19 g of **9**: ^{1}H NMR (D_2O) δ 2.12 (s, 3 H, SCH_3), 2.22 (q, 2 H, β_1-H_2, J = 7 Hz), 2.66 (m, 2 H, γ_1-H_2), 3.96 (dd, 1 H, β_2-H_A, J = 3.6 and 11.8 Hz), 4.05 (dd, 1 H, β_2-H_B, J = 5.5 and 11.8 Hz), 4.22 (t, 1 H, α_1-H, J = 6.6 Hz), 4.86 (dd, 1 H, α_2-H, J = 3.6 and 5.5 Hz).

L-Norvalyl-β-chloro-L-alanine Trifluoroacetate (10). This peptide was synthesized as described for **9**: yield 0.17 g; ^{1}H NMR (D_2O) δ 0.95 (t, 3 H, δ_1-H_3, J = 7.3 Hz), 1.43 (q, 2 H, β_1-H_2, J = 6.6 Hz), 1.89 (m, 2 H, γ_1-H_2), 3.95 (dd, 1 H, β_2-H_A, J = 3.7 and 11.8 Hz), 4.02 (dd, 1 H, β_2-H_B, J = 5.4 and 11.8 Hz), 4.08 (t, 1 H, α_1-H, J = 6.6 Hz), 4.86 (dd, 1 H, α_2-H, J = 3.7 and 5.4 Hz).

L-Alanyl-DL-propargylglycine Trifluoroacetate (11). *tert*-Butyl DL-propargylglycinate hydrochloride (0.5 g, 2.4 mmol) was dissolved in 20 mL of THF and chilled to 0 °C; Et_3N (0.35 mL, 2.5 mmol) was added dropwise. To this mixture was added *N*-Boc-LAla (0.46 g, 2.4 mmol) and DCC (0.56 g, 2.5 mmol), and the reaction was stirred at 4 °C for 23 h. The precipitated dicyclohexylurea and Et_3N·HCl were removed by filtration, and the filtrate was evaporated to dryness. The solid residue was then redissolved in $CHCl_3$ and washed with 1 N HCl, 5% $NaHCO_3$, and water. The chloroform was dried over $MgSO_4$ and then evaporated in vacuo. A solid residue was obtained, which crystallized from ethyl acetate/hexane, giving 0.43 g (52.0%) of *N*-Boc-LAla-DL-ppGly-O-*t*-Bu as a white powder: mp 85–89 °C; R_f 0.43 (single iodine spot in $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.38 (d, 3 H, α_1-CH_3, J = 7.4 Hz), 1.45 (s, 9 H, *tert*-butyl), 1.48 (s, 9 H, *tert*-butyl), 1.98 (t, 1 H, C≡CH, J = 2.6 Hz), 2.74 (m, 2 H, β_2-H_2), 4.20 (br s, 1 H, α_1-NH), 4.56 (m, 1 H, α_2-H), 4.96 (br s, 1 H, α_2-NH), 6.80 (m, 1 H, α_1-H).

The protected dipeptide (0.20 g) was then stirred with TFA/anisole, as described above, to give LAla-DLppGly·TFA (**11**): yield 0.17 g (95.8%); ^{1}H NMR (D_2O) δ 1.56 and 1.57 (2 d, 3 H, α_1-CH_3, J = 6.9 Hz), 2.39 (t, 1 H, C≡CH, J = 2.5 Hz), 2.72 (m, 2 H, β_2-H_2), 4.14 and 4.16 (2 q, 1 H, α_1-H, J = 6.9 Hz), 4.33 and 4.39 (2 t, 1 H, α_2-H, J = 6.0 Hz).

L-Alanyl-L-propargylglycine Trifluoroacetate (12). The synthesis was carried out as described above for **11**: yield 0.17 g; ^{1}H NMR (D_2O) δ 1.56 (d, 3 H, α_1-CH_3, J = 7.1 Hz), 2.45 (t, 1 H, C≡CH, J = 2.6 Hz), 2.81 (m, 2 H, β_2-H_2), 4.14 (q, 1 H, α_1-H, J = 7.1 Hz), 4.61 (t, 1 H, α_2-H, J = 6.0 Hz).

D-Alanyl-D-propargylglycine Trifluoroacetate (13). Two equivalents of *N*-Boc-DAla was reacted in methylene chloride at −20 °C with 1 equiv of DCC to afford the symmetrical anhydride of *N*-Boc-DAla. The DCU was removed by filtration. The anhydride was, without isolation, then reacted with DppGly-O-*t*-Bu·HCl, together with 1 equiv of DIEA to afford *N*-Boc-DAla-DppGly-O-*t*-Bu. After recrystallization, the protected peptide was treated in the usual way with TFA and anisole: yield 0.17 g; ^{1}H NMR (D_2O) same as **12**, vide supra.

DL-Propargylglycyl-L-alanine Trifluoroacetate (14). *N*-(*tert*-Butoxycarbonyl)-DL-propargylglycine (0.58 g, 2.7 mmol) was coupled to *tert*-butyl L-alaninate (0.50 g, 2.7 mmol) by the DCC coupling method described above for **11**. After deprotection, 0.2 g of DLppGly-LAla was afforded as the TFA salt: ^{1}H NMR (D_2O) δ 1.45 and 1.46 (2 d, 3 H, α_2-CH_3, J = 7.3 Hz), 2.58 and 2.60 (2 t, 1 H, C≡CH, J = 2.6 Hz), 2.91 (m, 2 H, β_1-H_2), 4.22 and 4.23 (2 t, 1 H, α_1-H, J = 6.0 Hz), 4.43 and 4.45 (2 q, 1 H, α_2-H, J = 7.4 Hz).

L-Propargylglycyl-L-alanine Trifluoroacetate (15) and D-Propargylglycyl-D-alanine Trifluoroacetate (16). Both peptides were prepared as described for **14**. For LppGly-LAla·TFA (**15**): yield 0.20 g; ^{1}H NMR (D_2O) δ 1.44 (d, 3 H, α_2-CH_3, J = 7.3 Hz), 2.58 (t, 1 H, C≡CH, J = 2.6 Hz), 2.92 (m, 2 H, β_1-H_2), 4.21 (t, 1 H, α_1-H, J = 5.9 Hz), 4.42 (q, 1 H, α_2-H, J = 7.3 Hz). For DppGly-DAla·TFA (**16**): yield 0.07 g; ^{1}H NMR (D_2O) same as for the L,L isomer **15**.

DL-Propargylglycyl-DL-propargylglycine Trifluoroacetate (17) and L-Propargylglycyl-L-propargylglycine Trifluoroacetate (18). Both peptides were prepared by DCC coupling, as outlined above, using the appropriately protected amino acids. For the mixture of diastereomers **17**, 0.26 g of the TFA salt was obtained: ^{1}H NMR (D_2O) δ 2.434 and 2.437 (2 t, 1 H, C≡CH of residue 2, J = 2.3 Hz), 2.60 and 2.61 (2 t, 1 H, C≡CH of residue 1, J = 2.5 Hz), 2.78 (m, 2 H, β_2-H_2), 2.95 (m, 2 H, β_1-H_2), 4.28 (m, 1 H, α_1-H), 4.52 and 4.56 (2 t, 1 H, α_2-H, J = 5.5 and 5.9 Hz).

For the LL diastereomer **18**, 0.3 g of the TFA salt was obtained: ^{1}H NMR (D_2O) δ 2.45 (t, 1 H, C≡CH of residue 2, J = 2.5 Hz), 2.59 (t, 1 H, C≡CH of residue 1, J = 2.5 Hz), 2.80 (m, 2 H, β_2-H_2), 2.94 (m, 2 H, β_1-H_2), 4.27 (t, 1 H, α_1-H, J = 6.0 Hz), 4.58 t (1 H, α_2-H, J = 5.9 Hz).

β-Chloro-D-alanyl-DL-propargylglycine Trifluoroacetate (19). This peptide was synthesized by the oxime resin method described above, wherein DLppGly-O-*t*-Bu·HCl (0.31 g, 1.5 mmol) was used to cleave an *N*-Boc-β-Cl-DAla-resin. After deprotection, 0.21 g of **19** was obtained: ^{1}H NMR (D_2O) δ 2.43 (t, 1 H, C≡CH, J = 2.6 Hz), 2.75 (m, 2 H, β_2-H_2), 4.09 (m, 2 H, β_1-H_2), 4.50–4.55 (m, 2 H, α_1-H and α_2-H).

β-Chloro-D-alanyl-D-propargylglycine Trifluoroacetate (20) and β-Chloro-L-alanyl-L-propargylglycine Trifluoroacetate (21). Both peptides were prepared in a fashion identical with that described for **19**. For β-Cl-DAla-DppGly·TFA (**20**): yield 0.16 g; ^{1}H NMR (D_2O) δ 2.45 (t, 1 H, C≡CH, J = 2.4 Hz), 2.81 (m, 2 H, β_2-H_2), 4.07 (dd, 1 H, β_1-H_A, J = 5.6 and 12.8 Hz), 4.12 (dd, 1 H, β_1-H_B, J = 3.7 and 12.8 Hz), 4.52 (dd, 1 H, α_1-H, J = 3.7 and 5.6 Hz), 4.60 (t, 1 H, α_2-H, J = 5.9 Hz). For β-Cl-LAla-LppGly·TFA salt (**21**): yield 0.25 g; ^{1}H NMR (D_2O) same as D,D diastereomer.

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 Hospitals and Clinics. All strains were subcultured on commercially prepared blood agar or chocolate agar from Baltimore Biological Laboratories (BBL).

The minimum inhibitory concentration (MIC) of each peptide for each strain was determined on a defined peptide susceptibility medium.[15a] 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 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 medium and diluting the cultures to approximately 1×10^7 cfu/mL. The inocula were applied with a Steers replicator[36] to plates containing peptides in serial twofold dilutions, and the plates were incubated overnight at 37 °C. The plates 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 peptide that allowed growth of fewer than ten colonies after 16–18 h of incubation.

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Registry No. 1, 87155-76-8; 2, 87155-77-9; 3, 87155-79-1; 4, 87155-80-4; 5, 87155-82-6; 6, 87155-84-8; 7, 87155-85-9; 8, 87205-46-7; 9, 87155-87-1; 10, 87155-89-3; 11, 87155-91-7; 12,

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87155-93-9; 13, 87155-95-1; 14, 87155-97-3; 15, 87155-99-5; 16, 87156-01-2; 17, 87156-03-4; 18, 87156-05-6; 19, 87156-07-8; 20, 87156-09-0; 21, 87156-11-4; DL-ppGly, 50428-03-0; D-ppGly·HCl, 87205-47-8; L-ppGly, 23235-01-0; *N*-Boc-DL-ppGly, 61172-66-5; DL-ppGly *tert*-butyl ester hydrochloride, 87246-71-7; *N*-Boc-β-Cl-L-Ala, 71404-98-3; *N*-Boc-β-Cl-D-Ala, 87156-12-5; β-Cl-L-Ala *tert*-butyl ester hydrochloride, 87156-13-6; β-Cl-D-Ala *tert*-butyl ester hydrochloride, 87156-14-7.

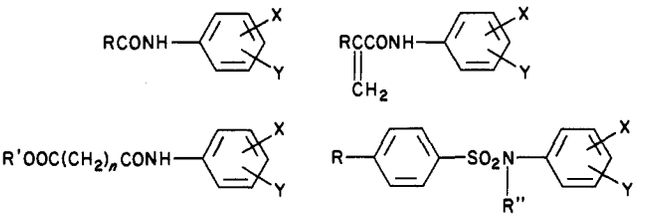
Antibacterially Active Substituted Anilides of Carboxylic and Sulfonic Acids[1]

Warner M. Linfield,* Thomas J. Micich, Thomas J. Montville, John R. Simon, Ermellina B. Murray, and Raymond G. Bistline, Jr.

Eastern Regional Research Center,[2] Philadelphia, Pennsylvania 19118. *Received October 21, 1982*

Anilides of carboxylic and sulfonic acids were prepared and tested for antimicrobial activity. While these anilides were ineffective against Gram-negative organisms, there was a good correlation between chemical structure and biological activity against Gram-positive species. Both the nature and position of the benzene ring substituents and the length of the carbon side chain affected the activity and specificity of the compounds. The highest activity was observed when the acyl or sulfuryl moiety had a C_7–C_9 side chain attached. The CONH and SO_2NH bridging groups were equally effective. The attachment of COOH or $COOCH_3$ groups in the ω-position did not effect activity, but the substitution of the acidic proton of the sulfonamide group by an alkyl group rendered the compound inactive. Six compounds, which were substituted anilides of sulfonic acids, fatty acids, or the analagous α-methylene-substituted acids, were bacteriostatic at 10 ppm against *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus faecalis*, and *Lactobacillus plantarum*. One of these compounds, 2-hydroxy-5-nitroanilide of α-methylenedecanoic acid, was bactericidal at 1 ppm.

The need for effective antibacterial agents, nontoxic to mammals and useful in the disinfection of skin and hard surfaces, has posed a continuing research problem, particularly in view of the banning of hexachlorophene and tribromosalicylanilide. This study was carried out to gain greater insight into the chemical structure–biological activity relationship of substituted aniline derivatives. Comprehensive studies by Beaver et al.[3,4] on diarylureas pinpointed the high antibacterial properties of 3,4,4′- and 3,3′,4-trichlorocarbanilides, while Hoffman et al.[5] investigated *N*-hydroxycarbanilides. Substituted alkylureas also possess bacteriostatic properties,[6] as do some substituted acylureas.[7] The antibacterial activity of anilides of carboxylic and sulfonic acids have received little attention. Baker et al.[8] found some nitrohaloanilides to be bacteriostatic, and Chase and Weller[9] described several active carbanilides and sulfonanilides not covered by previous investigators. A recent study from our laboratory reported the correlation between chemical structure and bacteriostatic properties of substituted anilides of fatty acids. In spite of some inconsistencies, all of the above-mentioned studies reveal parallels in the structure–activity correlations of these compounds. This study extends the work of Bistline et al.[10] in an effort to broaden our knowledge of biologically active anilides. The following structural types were examined:



X and Y are substituents such as Cl, NO_2, or OH; R is an alkyl, alkylaryl, or arylalkyl group; R′ is H or CH_3; R″ is H, CH_3, or p-$ClC_6H_4CH_2$; and n is an integer from 2 to 8.

While the synthesis of the above compounds is not new, their bacteriostatic properties have not been reported. This study focuses on the effect of side-chain length and structure, unsaturation of the side chain, introduction of a carboxyl or carboxymethyl group into the side chain, the nature of the bridging group CONH vs. SO_2NH, and the nature and position of substituents on the aromatic ring upon antibacterial activity.

Results and Discussion

The initial screening of the compounds showed that while some were active against *S. aureus*, all were ineffective against Gram-negative organisms. Considering the highly lipophilic nature of the compounds, the lack of activity against Gram-negative bacteria is not surprising. The structure of the Gram-negative cell wall is complex, as well as higher in lipids, than the simpler Gram-positive cell wall. Compounds that are most effective against Gram-negative bacteria are considerably less lipophilic

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