therapy was used as an indication of drug toxicity. Determination of the sensitivity of ascitic neoplasms to these agents was based on the prolongation of survival time afforded by the drug treatments. Each experiment was repeated at least one time.

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**Registry No.** 1, 51-21-8; 2, 17902-23-7; 3, 98652-97-2; 4, 98652-98-3; 5, 98652-99-4; 6, 98653-00-0; 7, 98653-01-1; 8, 98653-02-2; 9, 98653-03-3; 10, 98653-04-4; 11, 98653-05-5; 12, 98653-06-6; 13, 98653-07-7; 14, 98653-08-8; 15, 98653-09-9; 16, 98653-10-2; 17, 98653-11-3; 18, 98653-12-4; 19, 98653-13-5; 20, 98653-14-6; 21, 98653-15-7; 22, 98653-16-8; 23, 98653-17-9; 24, 98653-18-0; 2′-deoxy-5-fluorouridine, 50-91-9.

# Peptides of 2-Aminopimelic Acid: Antibacterial Agents That Inhibit Diaminopimelic Acid Biosynthesis

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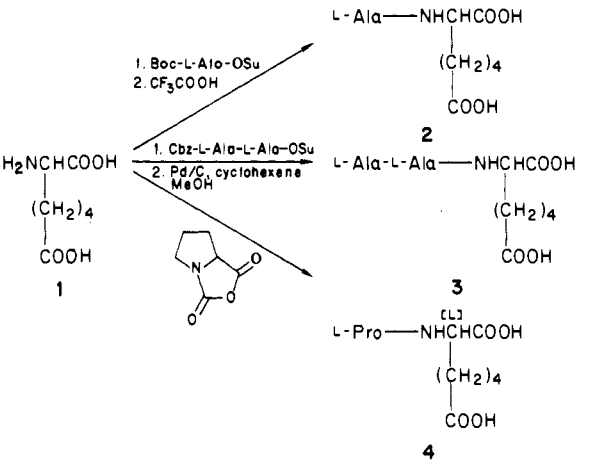
Succinyl-CoA:tetrahydrodipicolinate-*N*-succinyltransferase is a key enzyme in the biosynthesis of diaminopimelic acid (DAP), a component of the cell wall peptidoglycan of nearly all bacteria. This enzyme converts the cyclic precursor tetrahydrodipicolinic acid (THDPA) to a succinylated acyclic product. L-2-Aminopimelic acid (L-**1**), an acyclic analogue of THDPA, was found to be a good substrate for this enzyme and was shown to cause a buildup of THDPA in a cell-free enzyme system but was devoid of antibacterial activity. Incorporation of **1** into a di- or tripeptide yielded derivatives that exhibited antibacterial activity against a range of Gram-negative organisms. Of the five peptide derivatives tested, (L-2-aminopimelyl)-L-alanine (**6**) was the most potent. These peptides were shown to inhibit DAP production in intact resting cells. High levels (30 mM) of 2-aminopimelic acid were achieved in the cytoplasm of bacteria as a result of efficient uptake of the peptide derivatives through specific peptide transport systems followed, presumably, by cleavage by intracellular peptidases. Finally, the antibacterial activity of these peptides could be reversed by DAP or a DAP-containing peptide. These results demonstrate that the peptides containing L-2-aminopimelic acid exert their antibacterial action by inhibition of diaminopimelic acid biosynthesis.

Ever since its discovery in *Corynebacterium diphtheriae* by Work,[1,2] diaminopimelic acid (DAP) has been recognized as a prime site for the design of rationally based antibiotics. This conviction derives from the finding that the compound is widely distributed in prokaryotes[3] where it serves as a component of the cell wall peptidoglycan of nearly all bacteria (Gram-positive cocci excepted)[4,5] and as a precursor of lysine.[6,7] Additionally, since DAP is not found as a constituent of animal tissues and its biosynthetic pathway is absent in animals, there is a reasonable expectation that an antibiotic acting on DAP biosynthesis is unlikely to be toxic to humans. Furthermore, interference with the DAP pathway has been shown to be a lethal event; growth studies on a strain of *Escherichia coli* auxotropic for DAP showed that the organism lysed when exogenously supplied DAP had been depleted.[8]

Given the above biological rationale, it is surprising that the literature records few attempts at interdicting the biosynthesis of diaminopimelate[9] or interfering with its incorporation into peptidoglycan precursors. This is all the more remarkable since all of the intermediates in the biosynthetic pathway are known and the steps leading to DAP and lysine in bacteria have been fully elucidated (Figure 1).[10] Of the eight enzymes necessary for the conversion of pyruvate and aspartate to *meso*-DAP, the isomer usually present in the peptidoglycan of Gram-negative bacteria, four have been purified to homogeneity:

**Scheme I**




the aldolase (dihydrodipicolinate synthase, EC 4.2.1.52),[11] the reductase (dihydrodipicolinate reductase, EC

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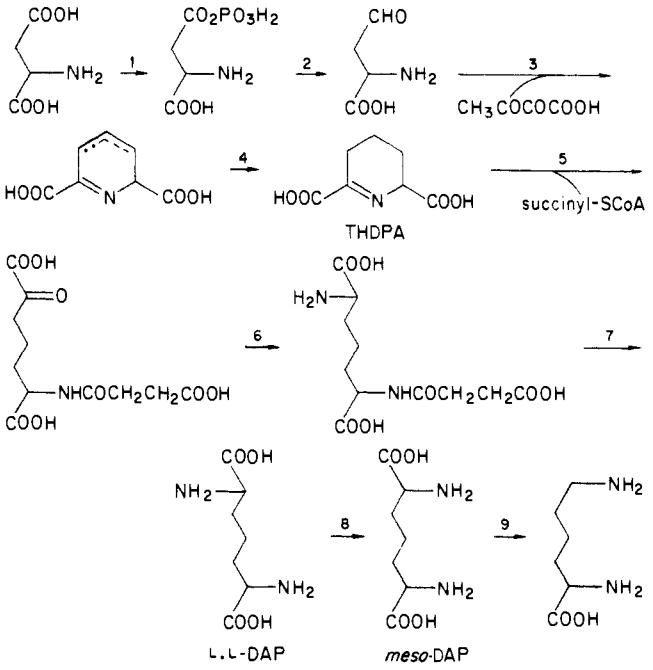



**Figure 1.** Biosynthetic pathway to diaminopimelic acid and lysine in bacteria. The pathway enzymes are as follows: 1, kinase; 2, dehydrogenase; 3, aldolase; 4, reductase; 5, succinylase; 6, transaminase; 7, deacylase; 8, epimerase; 9, decarboxylase.

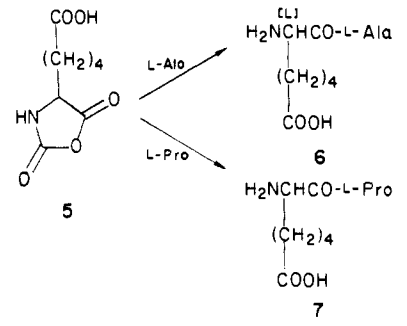
1.3.1.26),[12] and, very recently, the succinylase (succinyl-CoA:tetrahydrodipicolinate-*N*-succinyltransferase)[13] and the epimerase (diaminopimelate-2-epimerase, EC 5.1.1.7).[14]

As a part of efforts directed at the discovery of new antibacterial agents we have been investigating the design and syntheses of inhibitors of key enzymes in the DAP pathway. Recently, in the course of investigating the mechanism of the succinylase (Figure 1, enzyme 5) DL-2-aminopimelic acid (**1**) was found to be a substrate and an inhibitor for this enzyme.[15] Though compound **1** itself did not interfere with the growth of intact cells, we found that, when incorporated into a peptide, it was inhibitory to a range of bacteria.

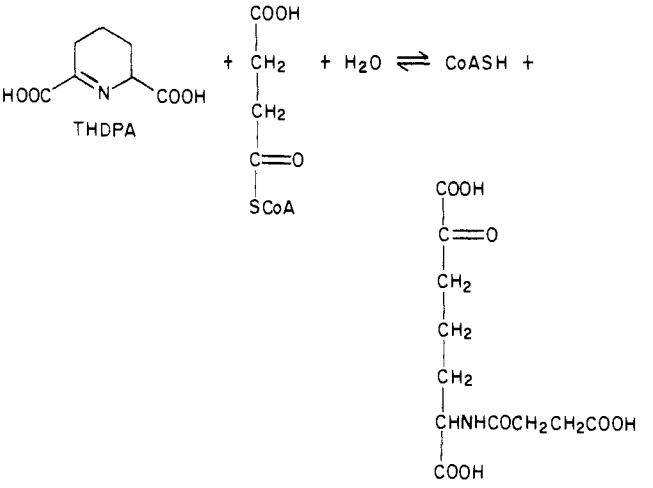
This paper reports the antibacterial properties of several peptide derivatives of 2-aminopimelic acid and presents evidence establishing the mechanism by which these peptides interfere with microbial growth.

**Chemistry.** Synthesis of the 2-aminopimelyl peptides was accomplished by the use of standard peptide procedures. For L-alanyl-DL-2-aminopimelic acid (**2**) and L-alanyl-L-alanyl-DL-2-aminopimelic acid (**3**), a benzyloxycarbonyl- or *tert*-butoxycarbonyl-protected amino acid or peptide, activated as its *N*-hydroxysuccinimide ester, was coupled with commercial DL-2-aminopimelic acid followed by hydrogenolytic or acidic deblocking. For L-prolyl-L-2-aminopimelic acid (**4**), the *N*-carboxyanhydride of L-proline

**Scheme II**




**Scheme III**




was used in the coupling reaction (Scheme I). The dipeptides (L-2-aminopimelyl)-L-alanine (**6**) and (DL-2-aminopimelyl)-L-proline (**7**), in which 2-aminopimelic acid is the N-terminal amino acid, were synthesized by reaction of the *N*-carboxyanhydride of DL-2-aminopimelic acid with an appropriate amino acid in aqueous buffer (Scheme II). The L-alanyl derivative, **8**, of LL-2,6-diaminopimelic acid (LL-DAP) was prepared by coupling of the Boc-protected amino acid *N*-hydroxysuccinimide activated ester with LL-DAP under aqueous conditions followed by deblocking with trifluoroacetic acid. The peptides were purified either by open column chromatography or preparative HPLC of the blocked intermediates or the free peptides.

Peptides containing D-amino acids are known to be very poorly transported in bacteria and are not substrates for intracellular peptidases.[16] Thus, stereochemical assignments were made on the basis of bioactivity of the isolated fractions. In two instances, compounds **4** and **6**, pure diastereoisomers were isolated when they fortuitously crystallized from solution. The stereochemistry of **6** was established as the LL diastereomer by the fact that it was hydrolyzed by aminopeptidase M (which cleaves only LL peptides). Compound **4** was assigned LL stereochemistry on the basis of cleavage by leucine aminopeptidase.

## Results and Discussion

Bacteria utilize two types of acyl transferases for DAP biosynthesis, succinylases and acetylases, with the former occurring more widely.[17] The acylase that we used for assay of potential inhibitors is obtained from an *E. coli* strain and is a succinylase.[13] This enzyme catalyzes the conversion of the cyclic pathway intermediate tetra-

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**Table I.** In Vitro Antibacterial Activities[a]

| compd | minimum inhibitory concentration, μg/mL | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *E. coli* CB64 | *E. cloacae* P99 | *S. enteritidis* E497 | *S. marcescens* ATCC13880 | *Citrobacter sp.* 2404 | *K. pneumoniae* 4200 | *P. rettgeri* AAB-137 | *P. aeruginosa* 647 |
| 1 | >1000 | >1000 | >1000 | >1000 | >1000 | 500 | NT[b] | >1000 |
| 2 | 16 | 4 | 16 | 8 | 8 | 16 | 4 | 1000 |
| 3 | 31 | 63 | 2 | 16 | 250 | 1000 | 125 | NT |
| 4 | 500 | 250 | >1000 | >1000 | 250 | 63 | 250 | NT |
| 6 | 2 | 4 | 8 | 2 | 2 | 8 | ≤1 | >1000 |
| 7 | >1000 | 8 | >1000 | >1000 | >1000 | 16 | 4 | NT |
| AF[c] | ≤0.1 | 100 | 50 | 1.6 | 25 | 1.6 | >100 | >100 |

[a] See the Experimental Section. [b] Not tested. [c] Alafosfalin.

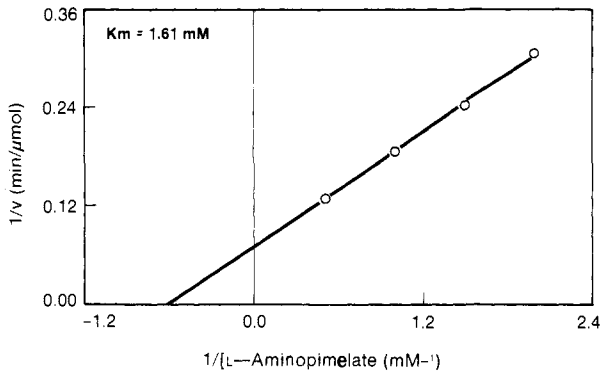


**Figure 2.** Lineweaver–Burk plot of L-2-aminopimelic acid as a substrate for the succinylase. The data were fitted by computer to the equation $v = V_mS/K_m + S$, which describes normal hyperbolic saturation by a substrate. The symbols represent the experimental data and the lines are the best fit of the experimental data to the equation.

hydrodipicolinic acid (THDPA), in the presence of succinyl-CoA, to the acyclic product 2-(succinylamino)-6-oxo-L-pimelic acid (Scheme III). L-2-Aminopimelic acid was found to be a good substrate for the *E. coli* succinylase; it has a $V_{max}$ comparable to that of the natural substrate, but it exhibits a relatively poor $K_m$ (1.61 mM) (Figure 2) compared to that of THDPA (20 μM). The D enantiomer was not a substrate for this enzyme.[18] When tested in a disk assay using an *E. coli* strain as the indicator organism, DL-2-aminopimelic acid (1) showed no inhibition of growth at concentrations up to 2 μmol/disk.

It has been well established that most bacteria can utilize peptides present in their external environment for nutritional purposes. Often these peptides are transported across the cellular membrane by specific peptide permeases and then are hydrolyzed inside the cell.[16] Small, naturally occurring antibiotics are known that use this specific transport and hydrolytic process to advantage to deliver toxic amino acids into bacterial cells.[19,20] Examples of synthetic compounds also exist that were designed to take advantage of this concept.[21] With this information in mind we synthesized several peptide derivatives of 2-aminopimelic acid with the aim of evaluating their ability to interfere with DAP biosynthesis in intact cells and to inhibit the growth of bacteria.

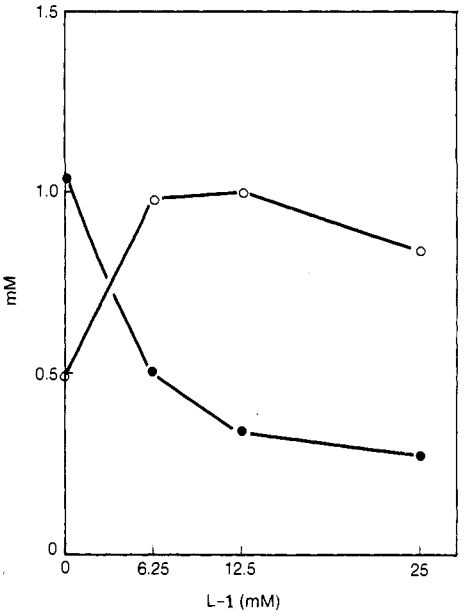


**Figure 3.** Effect of L-1 on levels of THDPA (○) and DAP (●) in a cell-free enzyme preparation. Data points represent concentrations after incubation at 37 °C. See Experimental Section for detailed description.

When assayed in a minimal medium containing lysine against a number of clinical isolates of different Gram-negative bacteria, two peptides, L-alanyl-DL-2-aminopimelic acid (2) and (L-2-aminopimelyl)-L-proline (6) showed surprisingly good antibacterial activity (Table I). The two proline-containing peptides L-prolyl-L-2-aminopimelic acid (4) and (DL-2-aminopimelyl)-L-proline (7) were significantly less active than their alanine-containing counterparts. The poorer activity of these derivatives is probably due to either less efficient transport or greater resistance to cleavage by intracellular peptidases or perhaps a combination of the two.

Are these peptides delivering 2-aminopimelic acid into the cytoplasm, and if so, is the antibacterial effect due to 2-aminopimelic acid inhibiting an enzyme in the DAP pathway? Compounds **2**, **3**, and **6** were studied further in order to determine the mechanism by which they inhibited the growth of bacteria.

**Effects in a Cell-Free Enzyme Preparation.** With use of a cell-free preparation from *E. coli* M26-26 which contains all of the enzymes necessary to synthesize DAP from aspartate, L-2-aminopimelic acid (L-1) was found to inhibit DAP production with a concomitant increase in the levels of THDPA (Figure 3). Similarly, inhibition was observed when THDPA instead of aspartate was used as the added substrate for the pathway enzymes, indicating

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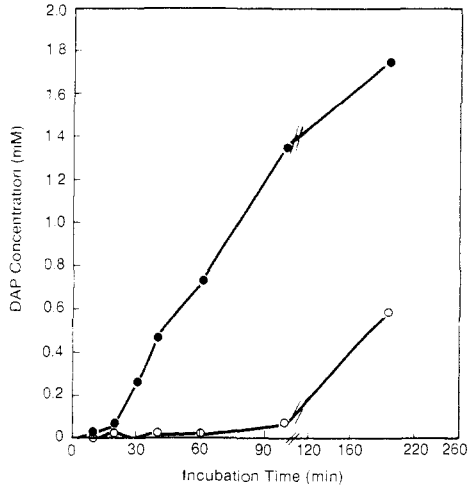


**Figure 4.** Effect of peptide **6** on accumulation of DAP in *E. coli* M26-26 resting cells. Untreated (●) and treated with **6** at a concentration of 2.4 mM (○). See Experimental Section for a detailed description of the method.

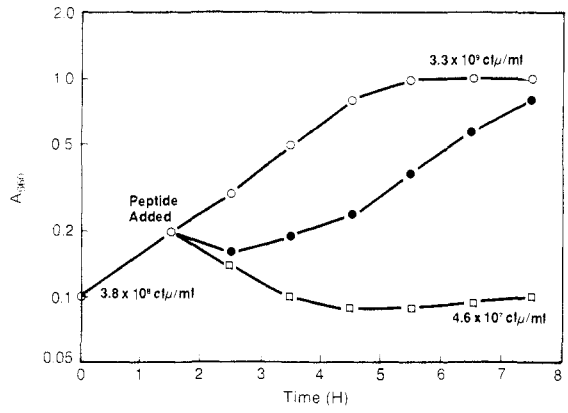


**Figure 5.** Effect of peptide **2** on the growth of *E. cloacae* P99. Untreated control (○), 100 μM **2** (●), 200 μM **2** (□). The incubation temperature was 37 °C. The organism was grown in peptide-free minimal medium.

that L-1 was acting in the second half of the pathway, consistent with the succinylase being the site of inhibition. Since L-1 is also a substrate for the succinylase,[15] its succinylation product, L-2-succinylaminopimelic acid, was assayed and shown not to inhibit the succinylase or DAP production at 3 mM in the cell-free enzyme mixture.[22]

**Effects in a Resting Cell System.** A "resting" cell system has been described[10a] that, because the organism lacks *meso*-DAP decarboxylase, excretes DAP. When these cells were incubated in the presence of peptide **6** (2.4 mM), DAP production was inhibited by up to 95%. After 3 h DAP levels recovered somewhat to reach 33% of controls (Figure 4). Since 2-aminopimelic acid is a substrate for the succinylase, recovery is presumably due to removal of L-2-aminopimelic acid by conversion to the innocuous *N*-succinyl derivative.

**Effects on Growing Cells.** The addition of dipeptide **2** (200 μM) to a logarithmically growing culture of *Enterobacter cloacae* P99 in defined medium in the presence of lysine caused a marked decline in the growth rate of this organism compared to that of an untreated control (Figure 5). The viable cell count after 7.5 h (3–4 doubling times) had decreased 8-fold ($4.6 \times 10^7$ cfu/mL) below the initial value ($3.8 \times 10^8$ cfu/mL) and 70-fold below the final value for the control ($3.3 \times 10^9$ cfu/mL). The turbidimetric data

(22) Unpublished results.

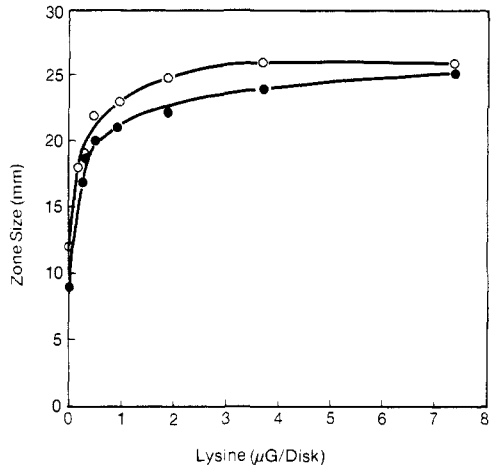


**Figure 6.** Enhancement of the in vitro antibacterial activity of peptides **2** (○) and **3** (●) by added lysine.

indicate that significant cell lysis occurred following exposure to dipeptide **2**. This was confirmed by microscopic examination of a treated sample which showed a high level of cell debris and lysed cells compared to a control sample. These observations are in accord with the expectation that inhibition of DAP biosynthesis will lead to lysis of growing cells.[8]

When treated at a lower concentration of dipeptide **2** (100 μM), however, the growth rate was slowed but within about 5 h had resumed a growth rate which closely paralleled that of the control (Figure 5).

**Intracellular Levels of 2-Aminopimelic Acid.** In view of the relatively poor $K_m$ of L-2-aminopimelic acid for the succinylase, its peptide derivatives exhibited surprisingly good antibacterial activity. This may be due in part to the fact that the succinylase has the lowest specific activity of the pathway enzymes.[10e] Moreover, determination of the intracellular concentrations of L-1 in an actively growing culture of *E. cloacae* P99 which had been exposed to compound **2** (200 μM) showed that within 5 min a high enough level (30 mM) of L-1 had been achieved in the cytoplasm to effectively inhibit the succinylase in spite of its weak affinity for the enzyme. Similarly high levels (25–30 mM) of L-1 were observed in *E. coli* M26-26.

**Effects of Lysine and DAP on in Vitro Antibacterial Activity.** The bioactivity of peptides **2** and **3** and, presumably the other L-1-containing peptides, is enhanced dramatically by adding lysine to the growth medium (Figure 6). In the absence of lysine both compounds gave zones of inhibition that were clear in the center but hazy on the outer edges. However when lysine was added, clear, sharp zones of inhibition were observed. Of the 20 naturally occurring amino acids, only lysine produced this enhancement. This observation is in agreement with the conclusion that these compounds act in the DAP pathway and may be explained as follows: the succinylase competitor L-1 shuts down DAP biosynthesis, causing a concomitant cut-off of lysine biosynthesis since *meso*-DAP is the immediate precursor to lysine in bacteria. Consequently, in the defined growth medium used, which contains only glucose and inorganic nitrogen, protein synthesis also is impaired, causing the bacteria to go into stasis. Addition of lysine to the medium has two effects. First, and most importantly, it restores protein synthesis, allowing the cells to grow, but now the cells are not producing sufficient DAP; hence the rate of new cell wall synthesis is slowed and the bacteria lyse. Second, lysine has been reported to act as a feedback inhibitor of the DAP pathway[10b] serving to further impair DAP biosynthesis.

**Table II.** Reversal of the Antibacterial Activity of Tripeptide 3 by LL-Diaminopimelic Acid (LL-DAP) and L-Alanyl-LL-diaminopimelic Acid (L-Ala-LL-DAP)

| LL-DAP,[a] μg/disk | zone size, mm | L-Ala-LL-DAP, μg/disk | zone size, mm |
|---|---|---|---|
| 0.76 | no zone | 26 | no zone |
| 0.38 | no zone | 13 | no zone |
| 0.19 | 17 (hazy) | 6.5 | 9 (hazy) |
| | 17–21 (clear) | | 9–15 (clear) |
| 0.00 | 25 (clear) | 0.0 | 25 (clear) |

[a] A standard disk diffusion assay was performed with a clinical isolate of *Enterobacter cloacae* P99 grown on minimal agar medium containing 40 μg/mL of lysine. Each disk contained LL-DAP or L-Ala-LL-DAP at the concentrations specified and L-Ala-L-Ala-DL-2-aminopimelic acid (3) at a fixed concentration of 80 μg/disk.

As proof of both peptide transport and intracellular activation, peptide 3 was tested in an agar disk diffusion assay against *E. cloacae* P99 in the presence of varying concentrations of LL-DAP or L-Ala-LL-DAP on each disk (Table II). Reversal of antibacterial activity by both compounds was clearly observable, indicating that peptide 3 was interfering with DAP biosynthesis. The antibacterial activity of peptides 2 and 6 also was reversed when tested similarly. It is noteworthy that a higher concentration of L-Ala-LL-DAP compared to LL-DAP is required to observe reversal of the antibacterial activity of tripeptide 3. This difference could be due to more efficient transport of LL-DAP relative to L-Ala-LL-DAP. Some support for this contention comes from studies carried out in *E. coli*. In this organism it was determined that the binding of DAP to its transport system[23] ($K_m = 3 \times 10^{-7}$ M) is far superior to the binding of L-Ala-LL-DAP to its transport system[24] ($K_m = 7.5 \times 10^{-3}$ M).

The peptide derivatives of 1 were also shown to be taken up by bacteria via specific peptide permeases. When assayed against *E. coli* CB64 (TOR/TOR, DPT$^-$), a strain that lacks the ability to transport both di- and tripeptides,[25] neither 2 nor 3 showed activity. However, against *E. coli* CB64 (TOR/TOR), a strain known to transport dipeptides but not tripeptides,[26] dipeptide 2 inhibited growth but tripeptide 3 did not. Furthermore, in peptide reversal studies against a strain of *E. coli*, the activity of dipeptide 2 was antagonized by di-L-Ala but not by tri-L-Ala and that of tripeptide 3 was antagonized by tri-L-Ala but not by di-L-Ala.

In summary, we have demonstrated what we believe to be the first example a compound that kills bacteria by inhibition of diaminopimelic acid biosynthesis. Di- and tripeptide derivatives of the simple amino acid 2-aminopimelic acid, a weak competitor of one of the DAP pathway enzymes, the succinylase, were shown to inhibit the growth of a number of Gram-negative bacteria. After uptake into bacteria by bacterial peptide permeases, the peptides are cleaved, presumably by intracellular peptidases, to produce high levels (30 mM) of L-2-aminopimelic acid in the cytoplasm. Strong evidence that 1 acts by interfering with DAP biosynthesis was provided by the following observations: (1) reversal of their bioactivity by exogenous DAP or a DAP peptide, (2) accumulation of THDPA, the substrate for the succinylase, when 1 is added to a cell-free extract (*E. coli*) containing all of the enzymes required for DAP synthesis, and (3) enhancement of bioactivity by the addition of lysine to the bacterial growth medium.

A logical extension of the results presented here suggests that a strong inhibitor of the succinylase, provided that it could be delivered in sufficient concentration into the cytoplasm of bacterial cells, ought to display potent antibacterial activity against a broad spectrum of bacterial genera.

## Experimental Section

Melting points were obtained on a Thomas-Hoover melting point apparatus and are uncorrected. IR spectra were obtained with a Perkin-Elmer 137 spectrophotometer. NMR spectra were obtained on either a Varian T-60 or a JEOL FX-90Q spectrometer using $(CH_3)_4Si$ or SDSS as internal standard. IR and NMR spectra were obtained on all compounds and were consistent with assigned structures. Elemental analyses were performed in the Analytical, Physical and Structural Chemistry Department of Smith Kline & French Laboratories.

TLC was performed on glass-backed silica gel GF (250 μm) or cellulose MN300F (250 μm) plates obtained from Analtech, Inc., Newark, DE, and on glass-backed reverse phase KC18F (200 μm) plates from Whatman, Inc., Clifton, NJ. Silica gel (particle size, 40 μm) and microcrystalline cellulose (particle size, 100 μm) for column and flash chromatography were obtained from J. T. Baker, Phillipsburg, NJ. Preparative HPLC was performed on a Whatman Partisil ODS-3 Magnum 20 column using a Beckman-Altex Model 332 HPLC fitted with a 254-nm UV detector.

Reactions were monitored by TLC using the following systems: (I) silica gel, $CH_2Cl_2$/EtOH/HCOOH (8:2:1, v/v/v), (II) reverse phase, MeOH/5% aqueous NaCl (1:1, v/v), (III) cellulose, MeCN/$H_2O$/HCOOH (75:25:2, v/v/v).

The following compounds were purchased from Sigma Chemical Co.: L-proline, L-alanine, *N*-(benzyloxycarbonyl)-L-alanine, and *N*-(benzyloxycarbonyl)-L-alanyl-L-alanine. Disuccinimidyl carbonate was purchased from Fluka Chemical Co., *N*-Boc-L-alanine *N*-hydroxysuccinimide ester was purchased from Chemical Dynamics Corp. and DL-2-aminopimelic acid was purchased from Vega Biochemcials Co.

All organic extractions were dried over anhydrous $MgSO_4$.

**Resolution of DL-2-Aminopimelic Acid (1).** Commercially obtained DL-2-aminopimelic acid was acetylated, and the mixture of *N*-acetyl enantiomers was resolved enzymatically with use of hog renal acylase I as described by Wade et al.[27] The products were separated by chromatography on an AG-50W × 8 ($H^+$) column. Elution with $H_2O$ gave the N-acetylated D enantiomer, which was hydrolyzed (4 N HCl, 100 °C, 90 min), and the product was recrystallized from $H_2O$/EtOH to give D-2-aminopimelic acid as a white solid; $[\alpha]^{26}_D$ −21.4° (*c* 1.0, 5 N HCl) [lit.[27] $[\alpha]^{26}_D$ −21.0° (*c* 1.0, 5 N HCl)].

Elution with 1 N $NH_4OH$ and lyophilization of the eluates yielded L-2-aminopimelic acid as its ammonium salt; $[\alpha]^{26}_D$ +20.4° (*c* 1.0, 5 N HCl) [lit.[27] $[\alpha]^{26}_D$ +21.5° (*c* 1.0, 5 N HCl)].

**L-Alanyl-DL-2-aminopimelic Acid (2).** To a cold (5 °C) solution of DL-2-aminopimelic acid (2.0 g, 0.011 mol) in a mixture of DMF (100 mL) and $H_2O$ (50 mL) was added dropwise with stirring a solution of *N*-Boc-L-alanine *N*-hydroxysuccinimide ester (3.27 g, 0.11 mol) in DMF (20 mL). The pH of the reaction mixture was maintained between 8.0 and 9.0 during the addition by adding $Et_3N$. After the addition was completed (10 min), the solution was stirred at ambient temperature for 1.5 h. The reaction mixture was evaporated to a syrup, which was dissolved in $H_2O$ (40 mL) and layered with EtOAc (100 mL), and the aqueous layer was acidified to pH 1.0 with 3 N HCl. The layers were separated, the aqueous layer was extracted again with EtOAc (100 mL), and the combined organic layers were dried and evaporated to give 5 g of crude product as a glassy solid. Flash chromatography (silica gel, $CH_2Cl_2$/EtOH/HCOOH, 95:5:1 v/v/v) gave 2.2 g of purified *N*-Boc-L-alanyl-DL-2-aminopimelic acid. This product was dissolved in a cold (5 °C) mixture of TFA (9 mL) and 1,3-dimethoxybenzene (1 mL). After stirring at 5 °C for 1 h, the solution was warmed to room temperature and added

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dropwise with stirring to $Et_2O$ (250 mL). The $Et_2O$ was decanted from the colorless, sticky precipitate and replaced with fresh $Et_2O$. After this $Et_2O$ was decanted, the sticky solid was dissolved in $H_2O$ and lyophilized to give 1.7 g (43%) of **2** as a glassy solid. Anal. ($C_{10}H_{19}N_3O_5$ $CF_3COOH$) C, H, N.

**L-Alanyl-L-alanyl-DL-2-aminopimelic Acid (3).** Disuccinimidyl carbonate (6.11 g, 0.024 mol) was added in one portion to a suspension of *N*-(benzyloxycarbonyl)-L-alanyl-L-alanine in $CH_3CN$ (200 mL). Pyridine (2.5 mL) was added, and the resulting solution was stirred overnight at room temperature. The reaction mixture was evaporated to a syrup which was dissolved in EtOAc. The solution was washed with $H_2O$ (2 × 50 mL) and brine (25 mL), dried, and evaporated to a glassy residue. TLC (system I) indicated that formation of the activated ester was incomplete. The residue was redissolved in $CH_3CN$ (200 mL) and treated again with pyridine (2 mL) and disuccinyl carbonate (5 g, 0.02 mol) for an additional 18 h at room temperature. Following workup 7.1 g (79%) of crude activated ester was obtained as a glassy solid.

A solution of the activated ester (1.13 g, 3 mmol) in $CH_3CN$ (10 mL) was added during 2 min to a solution of DL-2-aminopimelic acid (0.525 g, 3 mmol) in $H_2O$ (15 mL) containing $NaHCO_3$ (0.504 g, 6 mmol). After the solution was stirred at room temperature for 2.5 h, the solvent was evaporated, the residue was dissolved in $H_2O$ (2.5 mL), the solution was filtered, and, following purification by preparative HPLC (100% $H_2O$ to 100% MeOH gradient, 1 h), 0.21 g (16%) of blocked peptide was obtained.

The blocked peptide (370 mg, 0.8 mmol) was dissolved in MeOH (20 mL), 10% Pd/C (200 mg) and cyclohexene (4 mL) were added, and the mixture was heated at reflux for 1 h. The cooled mixture was filtered, the filter cake was washed liberally with $H_2O$, and the combined filtrate and washings were concentrated in vacuo to remove methanol. The remaining aqueous solution was lyophilized to give 233 mg (89%) of **3** as a white lyophilizate; single spot on TLC (system II). Anal. ($C_{13}H_{23}N_3O_6 \cdot 1.5H_2O$) C, N, H: calcd 7.61; found, 7.14.

**L-Prolyl-L-2-aminopimelic Acid (4).** A solution of the *N*-carboxy anhydride of L-proline[28] (6.5 g, 0.046 mol) in 1:1 THF/acetone (30 mL) was added during 15 min to a cold (0–2 °C), stirring solution of DL-2-aminopimelic acid (8.07 g, 0.046 mol) in 180 mL of buffer (1:1 1 N $NaHCO_3$/1 N $Na_2CO_3$ diluted with 9 parts $H_2O$ and adjusted to pH 10.2 with 10 N NaOH). The reaction mixture was maintained at pH 10–10.2 during the addition by simultaneous addition of the 1 N NaOH. When the addition was complete, the solvent was evaporated and the residue was dissolved in $H_2O$ and chromatographed on an AG-50 W × 8 ($H^+$) column. Sequential elution with $H_2O$, 1.7 N $NH_4OH$, and finally 4 N $NH_4OH$ gave, in the latter eluates, 1.2 g of syrup. The syrup was dissolved in $CH_3CN/H_2O$/HCOOH (40:10:1, v/v/v) in preparation for column chromatography on cellulose, but serendipitously, crystals formed prior to chromatography and were collected to give 100 mg of **4**; mp 213–215 °C. Anal. ($C_{12}H_{20}N_2O_5$) C, H, N.

The identification of **4** as the LL isomer was established by enzymatic assay using leucine aminopeptidase.

**DL-2,5-Dioxooxazolidine-4-pentanoic Acid (*N*-Carboxy Anhydride of DL-2-Aminopimelic Acid) (5).** Phosgene was bubbled during 1 h into a suspension of DL-2-aminopimelic acid (5.25 g, 0.03 mol) in dry THF (165 mL), which was immersed in an oil bath heated at 50 °C. A clear solution was obtained after 30 min of phosgene treatment. Nitrogen was bubbled through the reaction mixture for 30 min at 50 °C to remove residual phosgene, and then the mixture was filtered and evaporated to give a syrup. The syrup was dissolved in EtOAc (40 mL), and the solution was diluted with petroleum ether (40 mL), to give upon standing, 4.19 g (70%) of crystalline **5**: mp 103–104.5 °C. Anal. ($C_8H_{11}NO_5$) C, H, N.

**(L-2-Aminopimelyl)-L-alanine (6).** The *N*-carboxy anhydride **5** (402 mg, 2 mmol) was added in small portions during 10 min to a cold (1–3 °C), stirring solution of L-alanine (535 mg, 6 mmol) in 25 mL of buffer (1:1 1 N $NaHCO_3$/2 N $Na_2CO_3$ adjusted to pH 10.3 with 1 N NaOH). During the addition the reaction mixture was maintained at pH 10.2 by addition of 1 N NaOH. After stirring in the cold for 1 h followed by 0.5 h at ambient temperature, TLC (system III) indicated formation of a new product. The solution was adjusted to pH 8.5 with HCOOH, stored overnight in a freezer, and then was lyophilized. The lyophilizate was dissolved in 1:1 $CH_3CN/H_2O$ (15 mL) and purified by flash chromatography on microcrystalline cellulose ($CH_3CN/H_2O$/HCOOH, 75:25:2, v/v/v). Fractions containing the desired product (TLC) were combined, and the solvent was evaporated to give 243 mg of a gummy solid. The solid was dissolved in methanol and upon standing afforded 76 mg (31%) of LL isomer **6** as colorless crystals; single spot on TLC (system III): mp 208.5–209.5 °C. Anal. ($C_{10}H_{18}N_2O_5 \cdot 0.25\ H_2O$) C, H, N.

The identification of **6** as the LL isomer was established by enzymatic assay using aminopeptidase M.

**(DL-2-Aminopimelyl)-L-proline (7).** To a stirring, cold solution (0–3 °C) of L-proline (431 mg, 3.75 mmol) in 37 mL of buffer (1:1 1 N $NaHCO_3$/2 N $Na_2CO_3$ adjusted to pH 10.2 with 1 N NaOH) was added simultaneously a solution of **5** (500 mg, 2.49 mmol) in $CH_3CN$ (8 mL) and enough 1 N NaOH to maintain the reaction mixture at pH 10.3. After the addition was complete (5 min), 1 N HCl was added to adjust the reaction mixture to pH 7, and the solvent was evaporated. A solution of the residue in a minimum amount of $H_2O$ was adjusted to pH 2 (1 N HCl) and chromatographed on a column of AG-50 W × 8 ($H^+$) resin. After washing with $H_2O$ (to remove salts), elution with 3 N $NH_4OH$ gave, after lyophilization, a mixture (848 mg) of product and L-proline (TLC system III). Chromatography on microcrystalline cellulose using ($CH_3CN/H_2O$/HCOOH, 85:15:1, v/v/v) as eluant gave, after lyophilization, 405 mg (60%) of **7**; single spot on TLC (system III). Anal. ($C_{12}H_{20}N_2O_5$ $H_2O$) C, H, N.

**L-Alanyl-LL-DAP (8).** A suspension of *N*-Boc-L-alanine *N*-hydroxysuccinimide ester (286 mg, 1.0 mmol) in EtOH (6 mL) was added during 15 min at room temeprature to a stirring solution of LL-DAP[10h] (190 mg, 1.0 mmol) in $H_2O$ (6 mL) containing $KHCO_3$ (220 mg, 2.2 mmol). After the solution was stirred for 1 h, TLC ($CH_2Cl_2$/MeOH, 20:1 v/v) showed complete disappearance of activated ester. The reaction mixture was concentrated to 2–3 mL to remove the EtOH, and the aqueous residue was acidified to pH 2.8 (3 N HCl) and then extracted with EtOAc (2 × 5 mL). The aqueous layer was adjusted to pH 4.5 (1 N $NH_4OH$), concentrated in vacuo (1.5 mL), and chromatographed in two batches (1.3 mL and 0.5 mL) using preparative HPLC (100% $H_2O$ to 70% $H_2O$/MeOH gradient, 30 min, for the 1.3-mL fraction and 100% $H_2O$ to 40% $H_2O$/MeOH, 45 min, for the 0.5-mL fraction). Fractions (25 mL) from each run were assayed quantitatively with trinitrobenzenesulfonate (TNBS) to detect the presence of compounds containing a primary amino group. The first TNBS-positive fractions contained DAP. Fractions containing the second TNBS-positive compound to be eluted from the column were assayed further by analytical HPLC (Whatman ODS-3 column, 25 cm × 4.6 mm i.d. 20% aqueous MeOH, 1.25 mL/min, 220 nm) and TLC (95% EtOH/5% aqueous NaCl, 70:30, v/v) and were shown to be homogeneous and free of DAP. The fractions from both preparative runs were combined, concentrated in vacuo, and then lyophilized to give 36.7 mg (10%) of *N*-Boc-L-alanyl-LL-DAP as a white solid.

To the Boc-protected peptide (31.3 mg, 0.087 mmol) was added 1,3-dimethoxybenzene (0.32 mL) followed by TFA (2.9 mL). After stirring at room temperature for 1 h, the solution was evaporated in vacuo, the glassy residue was triturated with $Et_2O$, and the resulting solid was collected. This solid was dissolved in $H_2O$ (5 mL) and treated portionwise with Amberlite IR-45 ($OH^-$) until the pH rose to 6.0 (45 min). After removal of the resin, the filtrate was lyophilized to give 33 mg (100%) of **8** as a tacky solid; single ninhydrin-positive spot on TLC (95% EtOH/5% aqueous NaCl, 70:30, v/v). Anal. ($C_{10}H_{19}N_3O_5$) C, H, N.

**Microbiological Testing. (A) Disk Diffusion Assay.** Seeded plates of a suitable organism were prepared as follows. The Gram-negative organism was grown from a frozen-glycerolized culture initially made in either M9 medium[29] or in the Davis-Mingioli minimal medium,[30] supplemented, where necessary (*E.*

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*coli* CB64 variants), with methionine and using 0.2–0.5% w/v glucose as carbon source. One percent inocula were grown for 16 h at 37 °C on a New Brunswick rotary shaker set at 250 rpm. One liter of 1.5% agar in the same medium was inoculated with 1 mL of the grown cells diluted to give $1 \times 10^8$ cfu/mL. Petri dishes containing 15 mL of inoculated medium were poured and stored at 4 °C in closed bags for up to 14 days. When necessary, the inoculated agar contained suitable supplements at the levels shown in the tables.

**(B) Minimum Inhibitory Concentration (MIC).** Cells were grown either on agar slants or in the liquid media referred to above for periods of 7–16 h at 37 °C. MIC's were determined by a conventional microtiter technique, using $10^5$ cfu/mL of the cells in M-9 broth supplemented with thiamine and lysine, following 16 h of incubation at 37 °C.

**(C) Treatment of Peptides and Source of Organisms.** Peptides and/or amino acids were dissolved in potassium phosphate buffer at 50 mM, pH 7.4, adding solid $NaHCO_3$ where necessary to complete dissolution. Dilutions, serial 2-fold or 10-fold, were made in the same buffer. Suitable aliquots were placed on 6.25-mm filter paper disks and placed on top of seeded plates or sterilized by filtration through 0.45-$\mu$M filters (Millex-HA, Millipore Corp.) and used for MIC or growth tests. Organisms were either from the SK&F culture bank or were obtained from the Princeton collection (Charles Gilvarg).

**Biochemical Testing. (A) DAP Determinations.** Levels of diaminopimelic acid in cell extracts and in cell-free synthesizing systems were measured by a spectrophotometric assay based on the color produced by reaction with acidic (pH 0.3) ninhydrin followed by heating at 100 °C for 5 min[31] (max 420 nm, $\epsilon_M$ 6000). Tetrahydrodipicolinic acid (THDPA) was measured by reversal of the *Bacillus sphaericus* enzyme (*meso*-2,6-diaminopimelate-D-dehydrogenase) prepared and assayed as reported by Misono et al.,[32] monitoring the oxidation of NADPH at 30 °C and pH 7 in a Beckman DU8B spectrophotometer.

**(B) Cell-Free Production of Diaminopimelic Acid from Aspartic Acid.** A dialyzed cell-free extract of *E. coli* M26-26 (a lysine requiring auxotroph)[33] was prepared by sonic lysis of cells (grown in 100 L of M9 medium supplemented with 40 $\mu$g/mL of L-lysine), centrifugation at 30000*g* (Sorvall RC5B), and dialysis of the supernatant solution against a 50 mM solution of potassium phosphate (pH 7.4). This solution, containing 40 mg/mL of protein (Lowry et al.[34]), was stable at −70 °C for up to 2 years and contained all of the enzymes necessary to produce diaminopimelic acid. The reaction conditions were as follows: 100 mM imidazole HCl, pH 7.2, 10 mM $MgSO_4$; 25 mM DL-aspartate; 2 mM DL-glutamate; 20 mM pyruvate; 10 mM ATP; 10 $\mu$M GTP; 154 $\mu$M NAD; 150 $\mu$M NADP; 12.5 $\mu$M succinate; succinyl-CoA synthetase (Boehringer Mannheim) 0.0025 U/mL. A reaction volume of 0.5 mL and a temperature of 37 °C were used. The reaction was started by addition of a suitable volume of cell-free extract and was terminated by addition of an equal volume of glacial acetic acid. Inhibitors to be tested were added to the reaction mixture at a suitable concentration prior to adding the cell-free extract. All determination of diaminopimelic acid levels were made on duplicate samples from duplicate treatments and were reproducible (SD ± 10%).

**(C) Cell-Free Production of Diaminopimelic Acid from Tetrahydrodipicolinic Acid (THDPA).** THDPA was produced by use of *B. sphaericus meso*-2,6-diaminopimelate-D-dehydrogenase at pH 10.5 as described by Misono et al.[32] with the modifications made by Simms et al.[13] The active fractions from a Sephadex G15 column were combined and stored at 4 °C and pH 4. The same cell-free extract was used as a source of enzymes as was described above for the production of DAP from aspartic acid, but the supplements were changed to the following: 250 mM imidazole buffer, pH 7.0; 40 mM $MgSO_4$; 50 mM DL-glutamate; 250 mM succinate; 50 mM ATP; 700 mM CoA; 250 mM 2-mercaptoethanol. A reaction volume of 0.5 mL and a temperature of 37 °C were used.

**(D) Succinylase Assay.** Removal of the succinyl group from succinyl-CoA was followed by measurement of the release of the free sulfhydryl group of CoA with 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB, Ellman's reagent). The reaction conditions were as follows: 100 mM potassium phosphate, pH 7.4; 0.5 mM DTNB; 0.125–1.0 mM L-2-aminopimelic acid; 200 $\mu$M succinyl-CoA; 0.01 U/mL succinylase. A reaction volume of 1 mL and a temperature of 25 °C were used. The reaction was started by addition of succinyl-CoA. Initial velocities were determined by spectrophotometric measurement at 215 nm in a Beckman DU8B spectrophotometer using a blank lacking the enzyme. The experimental data were fitted by computer to the equation $v = V_m S/K_m + S$ using the HYPER program described by Cleland.[35]

**(E) Determination of Intracellular Levels of L-2-Aminopimelic Acid.** An actively growing culture of *E. cloacae* P99 in M-9 medium supplemented with lysine was treated at 37 °C with a 200 $\mu$M solution of L-alanyl-DL-2-aminopimelic acid (**2**). Aliquots were removed at suitable time intervals (1–15 min) and were immediately filtered through a 0.45-$\mu$m filter which had been prewashed with medium. The filter and cells were boiled in water for 3 min, the filter was removed, the suspension was centrifuged (15000*g*, 1 min), and the supernatant solution was lyophilized. The lyophilizate was taken up in buffer (pH 2.2 sodium citrate) and assayed for L-2-aminopimelic acid by use of an amino acid analyzer. The intracellular concentration was calculated on the basis of known cell densities and a volume of 1.3 fL/cell.

**(F) Resting Cells.** *E. coli* M26-26 was recovered from the growth medium by centrifugation at 10000*g* for 20 min and were washed twice with 33 mM potassium phosphate (pH 7.0). The cell density was adjusted to 63.5 mg wet weight of cells/mL, and the cells were stored at 4 °C. The incubation mixture contained 23 mM potassium phosphate in a final volume of 1310 $\mu$L; 8.4 mM $MgSO_4$ (200 $\mu$L), 44 mM potassium L-aspartate, 51 mM glucose, and 350 $\mu$L of the cell suspension. The mixture was incubated at 33 °C with shaking in the absence (150 $\mu$L of $H_2O$) and presence of 20 mM L-2-aminopimelyl-L-alanine (**6**) (150 $\mu$L). Aliquots were removed and assayed as described previously[10a] except that only 0.6 mL of 50% aqueous 2-propanol was used as diluent.

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**Registry No.** D-**1**, 32224-57-0; L-**1**, 26630-55-7; L-**1**·$NH_3$, 98839-15-7; DL-**1**, 627-76-9; L-**2**, 98839-10-2; **3**, 98839-11-3; **4**, 98839-12-4; **5**, 98839-13-5; **6**, 94696-85-2; **7**, 98839-14-6; **8**, 94731-14-3; Boc-L-Ala-OSu, 3392-05-0; Cbz-L-Ala-L-Ala-OSu, 16946-96-6; L-Ala, 56-41-7; L-Pro, 147-85-3; LL-DAP, 14289-34-0; *N*-Boc-L-alanyl-LL-DAP, 98919-97-2; *N*-Boc-L-alanyl-DL-2-aminopimelic acid, 98839-16-8; *N*-(benzyloxycarbonyl)-L-alanyl-L-alanine, 16012-70-7; *N*-(benzyloxycarbonyl)-L-alanyl-L-alanyl-DL-2-aminopimelic acid, 98839-17-9; *N*-carboxy-L-proline anhydride, 45736-33-2; diaminopimelic acid, 583-93-7.

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