# Laspartomycin, an Acidic Lipopeptide Antibiotic with a Unique Peptide Core[#]

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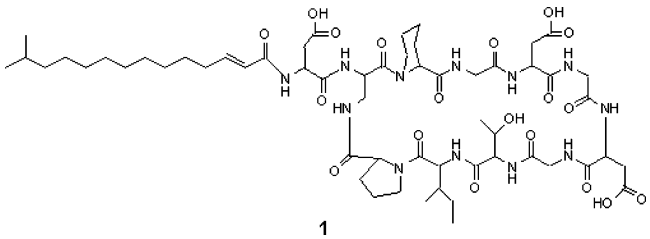
Laspartomycin was originally isolated and characterized in 1968 as a lipopeptide antibiotic related to amphomycin. The molecular weight and structure remained unknown until now. In the present study, laspartomycin was purified by a novel calcium chelate procedure, and the structure of the major component (**1**) was determined. The structure of laspartomycin C (**1**) differs from that of amphomycin and all related antibiotics as a result of its peptide region being acidic rather than amphoteric and the amino acid branching into the side chain being diaminopropionic rather than diaminobutyric. In addition, the fatty acid side chain is 2,3-unsaturated compared to 3,4-unsaturated for amphomycin and other related antibiotics. Calcium ion addition to stabilize a particular conformer was found to be important for an enzymatic deacylation of the antibiotic. A peptide resulting from the deacylation was critical for chemical structure determination by NMR studies, which also involved addition of calcium ions to stabilize a conformer.

Laspartomycin was originally reported by Umezawa et al. in 1968 as a lipopeptide antibiotic related to amphomycin with a 2,3-unsaturated $C_{15}$-fatty acid side chain.[1] At that time, full chemical and spectroscopic characterization were very difficult, and the structure and true molecular weight have remained unknown until the present. Laspartomycin was initially reported to be active against *Staphylococcus aureus* in vitro and in vivo, and current studies have shown that it is active against vancomycin-resistant *S. aureus* and methicillin-resistant strains of *S. aureus* with reduced susceptibility to vancomycin. Laspartomycin used in our studies was produced by *Streptomyces viridochromogenes* ssp. *komabensis,* ATCC-29814. The antibiotic that we obtained was compared to an authentic sample of laspartomycin obtained from the Institute of Microbial Chemistry (Japan) that was produced by the same strain of this organism. HPLC/UV and HPLC/MS analyses now have shown that the two antibiotic complexes have the same major component, laspartomycin C (**1**), but with a different number of minor components and different component ratios. More recently, the glycinocins have been reported as an antibiotic complex produced by a terrestrial Actinomycetes species. Laspartomycin C has the same molecular formula and general structure[2] as glycinocin A,[3] and these two antibiotics may be identical or may differ in the absolute configurations of one or more of the amino acid residues. A direct comparison of these antibiotics will be made in the future to determine if they are identical or diastereoisomers. The current studies have resulted in a new rapid isolation procedure for laspartomycin-type antibiotics, defined the molecular formula and structure of laspartomycin C (**1**), and shown the identity or relationship to the glycinocins.

The major component of laspartomycin, designated as laspartomycin C, has the molecular formula $C_{57}H_{90}N_{12}O_{19}$ and structure **1**.[2] All laspartomycin components have the same 11 amino acid peptide and differ in their acyl side chains. The molecular formulas of laspartomycin C and the two peptides, Peptide 1 and Peptide 2, derived from the laspartomycin complex by enzymatic deacylation (Figure 1) were determined by HRFABMS. When the deacylation was conducted in the presence of calcium ion to stabilize a particular conformation, only Peptide 2 was obtained. Amino acid analyses of the hydrolysates of laspartomycin and Peptide 2 indicated that the amino acid composition of the peptide core is three aspartic acids, three glycines, one diaminopropionic acid (Dap), one pipecolic acid (Pip), one *allo*-threonine, one isoleucine, and one proline. Amino acid analysis of the hydrolysate of Peptide 1 gave the same results as for the laspartomycin and Peptide 2 except that there were only two aspartic acid residues instead of three. The molecular formulas and amino acid composition indicated that at least a portion of Peptide 1 and Peptide 2 is cyclic rather than linear.



**1**

A substantial portion of the sequence of amino acids in laspartomycin C (**1**) was determined by ESIMS/MS, which also indicated an acyl-Asp side chain branching from a cyclic peptide derived from the 10 amino acid residues (Figure S1, Supporting Information). Amino acid sequence determination of cyclic peptides is generally not practical by MS/MS. However, in the case of laspartomycin C (**1**), the peptide linkage for the Ile-Pro unit readily fragments, and subsequent fragmentations allow sequence determination for a substantial portion of the peptide core (Figure S1, Supporting Information). Therefore, Peptide 1 and Peptide 2 contain the same 10 amino acid residues in their cyclic portions, and Peptide 2 has an additional aspartic acid residue branching from the cyclic region. The structure of Peptide 1 was established unambiguously by 2D-NMR studies in the presence calcium ions to stabilize a conformation (Figure S2, Supporting Information). Indeed, in the absence of $Ca^{2+}$, an equilibrium between several conformers gave rise to significant line broadening. The conformational changes in Peptide 1 were also indicated by broadened HPLC peaks for this compound. The characteristic broadening of chromatographic peaks due to conformational interconversion was not observed with laspartomycin C. Evidently, the long side chain resulted in mainly

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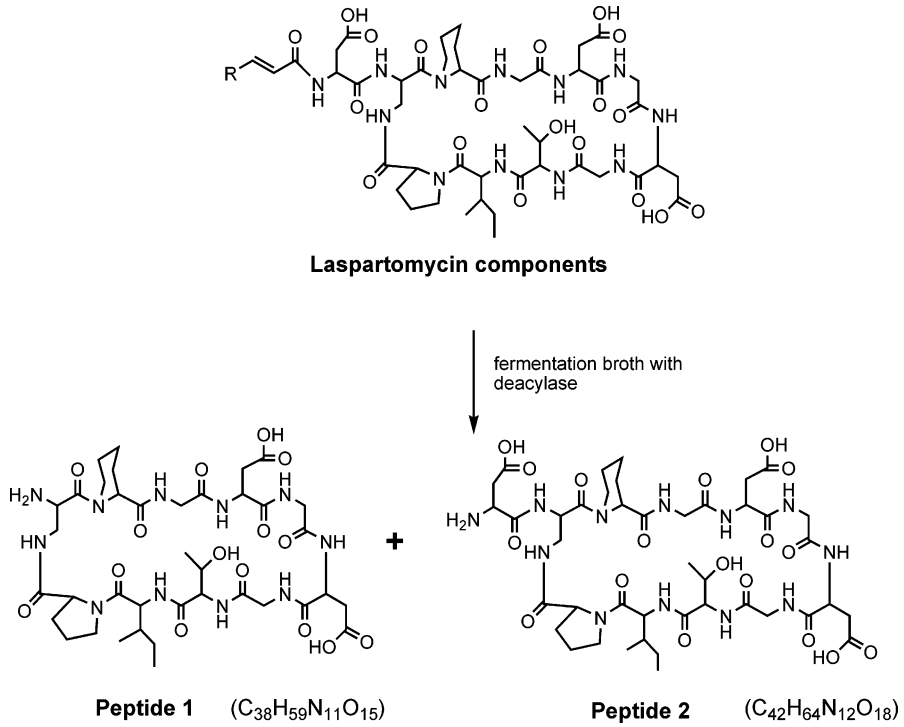


**Figure 1.** Conversion of laspartomycin to Peptides 1 and 2 with deacylase from *Actinoplanes utahensis*.

one stabilized conformation of the peptide portion of the molecule. The $C_{15}$ acyl side chain assignment for laspartomycin by Umezawa et al. was confirmed in the present studies by the mass spectrometry, UV, and $^1$HNMR spectra as **1**.

Laspartomycin is related to other cyclic lipopeptides such as amphomycin, aspartocin, and friulimicins[4] but differs by containing a unique cyclic peptide and 2,3-unsaturated acyl side chains instead of 3,4-unsaturated acyl side chains. Similar to these other lipopeptides, laspartomycin components contain a cyclic peptide with 10 amino acids. Unlike these antibiotics, the laspartomycins contain diaminopropionic acid instead of diaminobutryic acid as the amino acid for side chain attachment through aspartic acid or asparagine and have other differences in amino acid composition. The laspartomycins also differ by not having another diaminobutryic acid residue with an unsubstituted nitrogen within the ring to give some amphoteric character to the antibiotics. As a result, the ionic character of laspartomycin components depends only on the acidic groups from the three aspartic acid residues. The minor components differ from laspartomycin C (**1**) by the acyl side chains.

The use of the chelate process to purify the laspartomycin complex and other lipopeptide antibiotics from fermentation broths has generally resulted in lipopeptide complexes with 80%−90% purity.[5] The purity has been estimated by HPLC at 215 nm and also in the case of the A21978 complex by UV absorption measurements. All of the purified complexes were identified by FABMS, which readily gave $(M + H)^+$ and $(M + Na)^+$ ions for the major components. Most of the purifications by this process were performed with large excesses of calcium chloride. However, the minimum amount of calcium chloride required for maximum extraction of some of the complexes range from 2 to 6 moles per mole of antibiotic. In the case of laspartomycin, this was a 2:1 ratio. The solvent extract of the chelate of the complex allowed an aqueous extraction with base to remove impurites. Without calcium ion in this step, all of the laspartomycin would be in the aqueous phase along with the impurities. This step was followed by an acidic aqueous extraction to remove additional impurities, decompose the chelate, and remove calcium ions. A subsequent pH 7.5 wash removed other impurities and extracted the laspartomycin into the aqueous phase (Figure S3, Supporting Information).[5]

The $^1$H NMR spectrum of the free Peptide 1 recorded in water showed the presence of several conformers. At pH 6.3, three spectra corresponding approximately to 70/15/15 were identified. The line broadening of some signals was removed by a decrease of the pH from 6.3 to 3.1. At this latter pH value, all signals were sharp, permitting recording experiments based on scalar coupling (COSY, TOCSY, HSQC) essential to identify various spin systems and to determine the chemical structure. From these spectra, in spite of the presence of several conformers, most of the expected spin systems were identified for each of them (data not shown).

Given that Peptide 1 is a 10-residue cyclic peptide containing two aspartic acids groups, $Ca^{2+}$ ions were expected to interact with carboxylate and carbonyl groups, thereby favoring one macrocyclic conformer. A set of spectra at pH 6.6 (pH value above the p$K_a$ of aspartic acid) with progressive amounts of $Ca^{2+}$ ions were recorded and showed several alterations (Figure S2, Supporting Information). In the initial spectrum, most of amide signals were broadened and located in the 7.8 to 8.7 ppm area. Unexpectedly, a very broad signal was observed around 10.5 ppm. The progressive addition of the $Ca^{2+}$ ions altered the amide area in such a way that seven main amide signals are now clearly observed in the 7.6−9.0 ppm area. Moreover, although still broad, the amide signal at 10.5 ppm was observed clearly. The $\beta$ protons of Asp[2], which were broad in the initial spectrum, progressively became sharp and more inequivalent upon the addition of the $Ca^{2+}$ ions (0.59 ppm instead of 0.47 ppm in the reference). Concomitantly, the intensity of the signals belonging to the minor conformers decreased. Although still observed, they did not preclude the identification of the chemical structure of the laspartomycin core. In these conditions (pH 6.6 in the presence of $Ca^{2+}$ ions) homonuclear (TOCSY, COSY, NOESY) and heteronuclear (HSQC, HSQC-TOCSY) experiments were recorded at 280 K, and only the correlations involving the signals of the main conformer (about 90%) were considered. This low temperature was used, first to favor the most stable conformer and second to favor the observation of NOEs for this small molecule. All expected spin systems were identified from the TOCSY experiment, and their assignments were obtained by using the sequential procedure described by Wüthrich.[6] Moreover, the HSQC and the HSQC-TOCSY experiments allowed us to assign the

**Table 1.** NMR Chemical Shifts of the Laspartomycin Peptide 1 Measured in the Presence of $CaCl_2$ (water, pH 6.6, 280 K, $Ca^{2+}$/peptide ratio = 2.27)

| residue | atoms | $^1$H (ppm) | $^{13}$C (ppm) |
|---|---|---|---|
| Gly[1] | HN | 8.68 | |
| | CαH2 | 4.23, 3.87 | 45.5 |
| Asp[2] | HN | 7.72 | |
| | CαH | 4.73 | 51.9 |
| | Cβ H2 | 3.06, 2.45 | 40.3 |
| Gly[3] | HN | 8.31 | |
| | CαH2 | 4.22, 3.81 | 44.2 |
| Asp[4] | HN | 9.05 | |
| | CαH | 4.55 | 55.8 |
| | Cβ H2 | 2.72, 2.62 | 40.4 |
| Gly[5] | HN | 8.48 | |
| | CαH2 | 4.10, 4.01 | 43.8 |
| Thr[6] | HN | 8.80 | |
| | CαH | 4.26 | 62.6 |
| | Cβ H | 4.05 | 68.6 |
| | Cγ H3 | 1.32 | 22.2 |
| Ile[7] | HN | 10.70 | |
| | CαH | 4.34 | 60.0 |
| | Cβ H | 1.88 | 38.1 |
| | Cγ H2 | 1.57, 1.29 | 27.1 |
| | Cγ H3 | 1.09 | 17.5 |
| | Cδ H3 | 0.89 | 13.0 |
| Pro[8] | CαH | 4.34 | 64.6 |
| | Cβ H2 | 2.32, 1.83 | 31.7 |
| | Cγ H2 | 2.14, 1.98 | 27.9 |
| | Cδ H2 | 4.01, 3.63 | 51.0 |
| Dap[9] | CαH | 4.65 | 54.5 |
| | Cβ H2 | 4.27, 3.33 | 43.2 |
| | HNγ | 8.35 | |
| Pip[10] | CαH | 4.79 | 58.2 |
| | Cβ H2 | 2.28, 1.91 | 26.9 |
| | Cγ H2 | 1.80, 1.37 | 22.0 |
| | Cδ H2 | 1.83, 1.79 | 25.8 |
| | Cε H2 | 4.05, 3.33 | 46.3 |

$^{13}$CNMR resonances and to confirm each spin system from both their proton−proton and proton−carbon correlations. As a result, the spin systems of the three Gly, the two Asp, the Ile, the Thr, and the Pro residues and of the Dap and Pip non-natural residues were identified and assigned from the $d_{N\alpha}$ ($i$,$i$+1) NOEs. Therefore, the chemical structure of the laspartomycin core was confirmed, and the proton and carbon chemical shifts are displayed in Table 1.

The presence of several conformations could at least in part be explained either from the cis/trans conformation of the Ile[7]-Pro[8] amide bond or by the inversion of the piperidine ring as observed for cyclohexane.[7] The HαIle[7]-δδ′H Pro[8] NOE clearly characterized the trans conformation of the Ile[7]-Pro[8] amide bond, therefore suggesting that various conformers could be due to the ring inversion of the piperidine ring. Such a piperidine ring inversion that gives rise to two conformers was previously observed for a linear peptide.[8]

This NMR study has revealed the conformational mobility of the macrocycle of the laspartomycin core Peptide 1 and its capability to complex $Ca^{2+}$ ions to give rise to a major conformer from which the chemical structure was unambiguously established.

In summary, the structure of laspartomycin C (**1**) was determined mainly by amino acid analyses, HRFABMS and ESIMS/MS, and 2D-NMR studies. The analytical data for laspartomycin C, Peptide 1, and Peptide 2 along with the structure for the fatty acid side chain that was obtained by Umezawa et al.[1] provided sufficient information for a final structure proposal for laspartomycin C (**1**).

## Experimental Section

**General Experimental Procedures.** NMR spectra of Peptide 1 were recorded in water on a Bruker AMX spectrometer, operating at 600 MHz for the $^1$H nucleus and 150 MHz for the $^{13}$C nucleus. Spectra were referenced to TSP-$d_4$ as external reference set at 0 ppm. The pH was adjusted with aliquots of a 1 N NaOH or 1 N HCl solutions, and pH values were measured at room temperature with a 3 mm electrode. The freeze-dried peptide (4 mg) was dissolved in 0.5 mL of water ($H_2O$/$^2H_2O$, 95/5) and the pH of the solution adjusted to pH 6.6. A set of 1D-NMR spectra with progressive amounts of $Ca^{2+}$ ($CaCl_2$ solution in water) up to a $Ca^{2+}$/peptide ratio 2.27 was recorded at 300 K. At the end of the $Ca^{2+}$ ion addition, a set of 2D spectra was recorded at 280 K, to perform the assignment. The DQF-COSY spectrum[9] was collected into a 800 × 1024 data matrix, and the TOCSY[10] spectrum was collected with a mixing time of 80 ms. The NOESY[11]spectrum was acquired in the phase-sensitive mode using time-proportional phase incrementation with a mixing time of 250 ms. In all experiments, the carrier frequency was set at the center of the spectrum and the water resonance was suppressed using a low-power irradiation.

The ($^1$H−$^{13}$C)-HSQC[12] experiment was recorded with a delay of 3.5 ms ($^1J_{CH}$ = 143 Hz) and the ($^1$H−$^{13}$C)-HSQC-TOCSY experiment with a mixing time for proton−proton transfer of 80 ms to identify the network of one-bond and several proton−carbon connectivities, respectively.

All data were processed with the XWINNMR software, and one zero filling and a $\pi/3$ phase-shifted sine bell window function were applied in both dimensions before Fourier transform.

High-resolution mass spectra were obtained on a VG model ZAB-2SE mass spectrometer. The MS/MS data was obtained with a MicroMass model Bio-Q (Quattro-II upgrade) mass spectrometer and a PE Sciex Q-star/Pulsar ESIMS/MS mass spectrometer.

**Fermentation and Purification.** The laspartomycin complex used in these studies was produced by *Streptomyces viridochromogenes* ssp. *komabensis*, ATCC-29814, obtained from the ATCC collection. Fermentations to produce the complex were conducted successfully in flasks and bioreactors under conditions that were essentially the same as those originally reported by Umezawa et al..[1a] Purification of the complex was accomplished by the calcium chelate procedure, and HPLC analyses of the complex indicated that it was usually ∼90% pure with approximately 80% of the complex being the major component, laspartomycin C (**1**) (Figure S3, Supporting Information). The chelate extraction procedure is very selective and allows removal of impurities under basic, acidic, and neutral liquid−liquid extraction conditions, as outlined for laspartomycin in Figure S3, Supporting Information. Generally, a large excess of calcium chloride has been used for the chelate extraction procedure; however, this is not necessary. Optimal extraction and purification of the laspartomycin complex could be conducted at a 2:1 molar ratio of calcium chloride to antibiotic. A higher ratio had no detrimental effects.

**Deacylation to Obtain Peptide 1 and Peptide 2.** The deacylase was produced in a fermentation broth by culturing *Actinoplanes utahensis* NRRL 12052 with the protocol described by Boeck et al.[13] The laspartomycin complex was then treated with the deacylase broth in two separate batches in order to optimize the production of each peptide, and the batches were combined for chromatography studies. About 1.0 g of the laspartomycin complex was run at 2 mg/mL and 3.7 h to produce a Peptide 2-enriched sample (about 80−85% conversion of laspartomycin), and 1.5 g was run at 5 mg/mL for 20 h to obtain a Peptide 1-enriched sample. Both sample broths were pooled and separated from solids by centrifuge, and the product peptides were adsorbed in batch mode onto EnviChrom P resin from the broth decant. The peptides were desorbed in batch mode, desolventized, and separated on a column of EnviChromP resin to give about 600 mg of Peptide 1, HRFABMS *m*/*z* found 910.4251 $(M + H)^+$, calcd 910.4270 for $C_{38}H_{59}N_{11}O_{15}$ + H, and 100 mg of Peptide 2, HRFABMS *m*/*z* found 1025.4547 $(M + H)^+$, calcd 1025.4540 for $C_{42}H_{64}N_{12}O_{18}$ + H. When the deacylation was conducted in the presence of calcium chloride, 4−8 mg/mL of broth containing 4 mg of laspartomycin, only Peptide 2 was formed.

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**Supporting Information Available:** Figures of the ESIMS/MS of laspartomycin C (**1**), calcium effects on the NMR of Peptide 1, and the isolation procedure for laspartomycin C (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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