methyl ester, 99-75-2; *p*-toluic acid ethyl ester, 94-08-6; *p*-toluic acid propyl ester, 6976-69-8; *p*-toluic acid *tert*-butyl ester, 13756-42-8; coumalic acid ethyl ester, 5942-96-1; *N*-phenyl-*p*-toluamide, 620-84-8; *N*-cyclohexyl-*p*-toluamide, 10386-93-3; *N*-benzyl-*p*-toluamide, 5405-15-2; *N*-methyl-*N*-benzyl-*p*-toluamide, 69267-39-6; *N,N*-diphenyl-*p*-toluamide, 4316-53-4; cyclohexylamine, 108-91-8; benzylamine, 100-46-9; *N*-methylbenzylamine, 103-67-3; 2,1,3-benzothiadiazole-2-5^{IV}, 118514-40-2; 1,2-phenyldiamine, 95-54-5; *N*-phenyl-1,2-phenyldiamine, 534-85-0; 2,3-diaminobenzoic acid ethyl ester, 37466-88-9; 1,8-diaminonaphthalene, 479-27-6; *o*-aminophenol, 95-55-6; *o*-aminothiophenol, 137-07-5; *N*,2-diphenylbenzimidazole, 2622-67-5; 2-(2-methylphenyl)benzimidazole, 2963-64-6; *N*-phenyl-2-(2-methylphenyl)benzimidazole, 109744-85-6; 2-(4-methylphenyl)-4-benzimidazolecarboxylic acid ethyl ester, 109744-86-7; 2-(4-(2,13-benzothiadiazolyl))-4-benzimidazolecarboxylic acid ethyl ester, 109744-87-8; 2-*p*-tolylbenzoxazole, 835-71-2; 2-*p*-tolylbenzothiazole, 16112-21-3; 2-phenyl-1*H*-perimidine, 15666-84-9; 3-phenylpropanoic acid, 501-52-0; 3-phenylbutanoic acid, 4593-90-2; 3,3-diphenylpropanoic acid, 606-83-7; 3,3,3-triphenylpropanoic acid, 900-91-4; *o*-phenylbenzoic acid, 947-84-2; 5-phenylpentanoic acid, 2270-20-4; *N*-phenylglycine, 103-01-5; indanone, 83-33-0; 3-methylindanone, 6072-57-7; 3-phenylindanone, 16618-72-7; 3,3-diphenylindanone, 55010-17-8; fluorenone, 486-25-9; 1-tetralone, 529-34-0; 1-benzosuberone, 826-73-3; indoxyl, 480-93-3; 5-phenylpentanoic anhydride, 118514-42-4.

Structure Determination of Lysobactin, a Macrocyclic Peptide Lactone Antibiotic[1]

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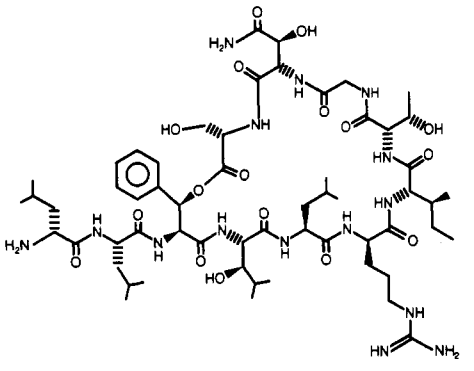
A new antibiotic, lysobactin (**1**), was isolated from fermentations of *Lysobacter* sp. ATCC 53042. Lysobactin was shown to be a potent agent against Gram-positive aerobic and anaerobic bacteria in vitro, and its efficacy in vivo was found to compare favorably with the clinically useful antibiotic vancomycin. Physicochemical characterization identified the antibiotic as a dibasic peptide of nominal mass 1275 Da. The structure of lysobactin, including stereochemical details, was determined by a combination of chemical and enzymatic degradations that were analyzed primarily by mass spectrometry. On the basis of synthetic modifications, the macrocyclic lactone and N-terminal D-amino acid of lysobactin are important structural elements contributing to the antibacterial activity.

Introduction

Inhibition of bacterial cell wall synthesis has proven to be a desirable mode of action for antimicrobial chemotherapeutic agents.[2] Notable examples of antibiotics having this mode of action are the penicillins, cephalosporins, bacitracin, and vancomycin. Microbial resistance to β-lactam antibiotics, however, is a widely occurring phenomenon. Recently, a clinically important increase in the incidence of methicillin-resistance Gram-positive infections has been noted.[3] The drug of choice for such infections,[4] vancomycin, is a natural product isolated from streptomycete fermentations. In contrast to the enzyme inhibitory activity of the β-lactams, vancomycin and related glycopeptide antibiotics inhibit bacterial cell wall biosynthesis by specific binding to D-alanyl-D-alanine cell wall precursors.[5] This mode of antibacterial action is consistent with the observed selectivity and potency of the vancomycin-type antibiotics.

Strains of vancomycin-resistant bacteria, although rare, have now been isolated and have been shown to cause infections.[6] Consequently, an alternative antibacterial agent with the same or similar cell wall precursor binding ability could be medically useful. In order to find such an agent, microbial fermentations were screened for antibiotics with biological activity antagonized by a *Staphylococcus aureus* cell wall preparation. Using this approach, *Lysobacter* sp. SC13,067 (ATCC 53042) was identified as a producer of a novel antibiotic having preferential activity against Gram-positive bacteria.[7] The structure determination of lysobactin (**1**) and structural requirements for its biological activity are the subjects of this paper. An independent structure elucidation of lysobactin, illustrating the utility of 2D NMR techniques, will be presented in a separate report.[8]



1

(1) This paper is dedicated to Professor Kenneth L. Rinehart Jr. on the occasion of his 60th birthday. Portions of this work were presented previously: (a) Tymiak, A. A.; McCormick, T. J.; Unger, S. E. 193rd National Meeting of the American Chemical Society, Denver 4/87, ORGN-56. (b) Tymiak, A. A.; Kirsch, D. R.; O'Sullivan, J.; McCullough, J. E. U.S. Patent 4,754,018; June 28, 1988.

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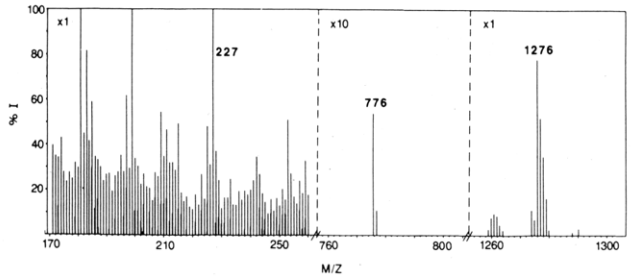
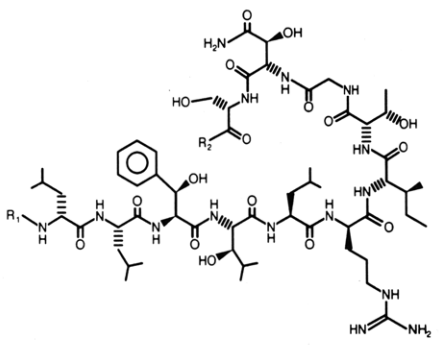


Figure 1. FAB mass spectrum (+ ion mode) of lysobactin (1).

Results

The antibiotic from the *Lysobacter* sp. cell mass was isolated by extraction and reversed-phase chromatography by using aqueous acetonitrile containing trifluoroacetic acid as the eluting solvent.[7] Preliminary stability studies indicated that lysobactin is extremely base labile and also suffers some decomposition during prolonged exposure to acid. Upon concentration of the bioactive fractions from reversed-phase columns, lysobactin precipitated as its bis(trifluoroacetate) salt (lysobactin·2TFA), thus facilitating isolation and minimizing decomposition. Although lysobactin·2TFA was clearly crystalline, the microscopic plates formed from acetonitrile–water mixtures were not suitable for X-ray studies. Attempts to obtain adequate crystals of the TFA salt and also of the HBr and HCl salts were unsuccessful. Chemical and spectroscopic methods were therefore employed for the structure elucidation.

Physicochemical measurements were obtained exclusively on lysobactin·2TFA. In addition to end absorption, weak UV absorbance bands at 250, 257, 263, and 269 nm indicated the presence of a phenyl group in the antibiotic. A weak shoulder at 1740 cm^{-1} on a strong amide absorption in the IR spectrum suggested a peptide containing an ester function. Fast atom bombardment (FAB) mass spectrometry (MS) in the positive and negative ion modes established the nominal mass of lysobactin (free base) as 1275 Da, although the negative ion spectra were typically weak. In the positive ion mode (Figure 1), only two major fragment ions, m/z 227 and 776, were observed. MS/MS analysis of the parent ion at m/z 1276 verified 776^+ as a daughter ion but gave little other significant information. Hydrolysis of **1** with dilute NH_4OH resulted in product **2** in which the antibiotic activity was destroyed. The IR shoulder at 1740 cm^{-1} disappeared and the molecular ion shifted 18 amu higher in the FAB mass spectrum, providing the first evidence that lysobactin contains a lactone. It is noteworthy that MS/MS spectra of **2** were also devoid of significant fragment ions useful for sequencing the peptide.



	R_1	R_2
2	H	OH
3	H	OCH_3
4	H	$NHCH_2Ph$
5	PhNHCS	$NHCH_2Ph$

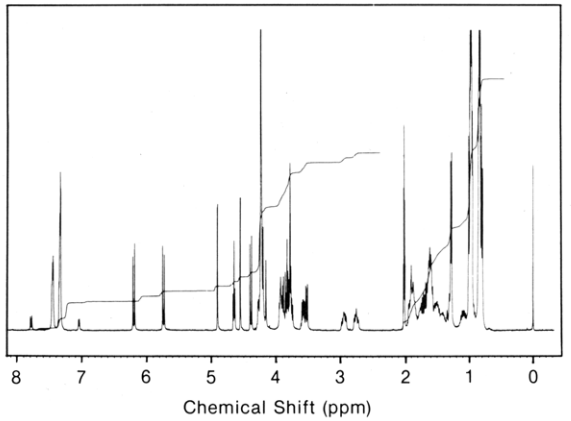
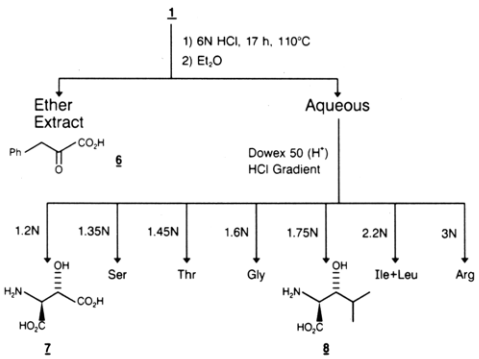


Figure 2. 400-MHz ^1H NMR spectrum of **1** in CD_3CN–D_2O–TFA (500:500:1) after three lyophilizations from the same solvent mixture. Signals at ca. 7 and 7.8 ppm are due to slowly exchanging amide protons.

Scheme I



High-resolution FABMS measurements yielded a probable formula of $C_{58}H_{98}N_{15}O_{17}$ for protonated lysobactin free base (1276.7264 calcd, 1276.7252 measured). This was consistent with elemental analyses of various lysobactin salts. A carbon count from the ^{13}C NMR spectrum of **1** further supported the proposed formula. Although the ^1H NMR spectrum of lysobactin (Figure 2) was also consistent, a full proton count for the antibiotic was impractical. Lysobactin·2TFA displayed poor solution dynamics in DMSO, leading to very broad ^1H NMR spectra even at 60 °C and in the presence of additional TFA. It is also unstable in methanol and insoluble in water and most neat organic solvents. A few well-resolved signals in the ^1H NMR spectrum measured in CD_3CN–D_2O–TFA (Figure 2) provided evidence for some unusual hydroxylated residues in lysobactin. Very slow exchange of amide NH protons at 7 and 7.8 ppm and wide dispersion of the phenyl and certain methine and geminal protons were observed for lysobactin but not for its hydrolysis product **2**, suggesting a fairly rigid solution conformation for the lactone. Due to the complexities in the NMR spectra, especially with the limited dispersion achieved at 400 MHz, a degradative approach to the structure elucidation commenced. A detailed analysis of this ^1H NMR spectrum (Figure 2) is presented elsewhere.[8]

Conventional amino acid analysis of a lysobactin hydrolysate detected ammonia, threonine, serine, glycine, arginine, isoleucine, and leucine in a 1:1:1:1:1:1:3 molar ratio. Preparative separation of the amino acids from a large-scale hydrolysis (Scheme I) allowed identification of two unusual amino acids, β-hydroxyleucine (**8**) and β-hydroxyaspartic acid (**7**). Both were assigned the threo configuration on the basis of ^1H NMR comparisons to authentic standards (Table I). The final fragment of the

Table I. Comparison of Lysobactin-Derived Amino Acids with Authentic β-Hydroxyamino Acid Diastereomers

compound	^{1}H NMR (D_2O) α-CH δ	α-CH J, Hz	β-CH δ	β-CH J, Hz	TLC,[a] R_f
threo-β-OH-Leu·HCl[b,c]	4.17	3.2	3.86	3.4, 9.0	
erythro-β-OH-Leu·HCl[b,c]	4.28	2.6	3.56	3.0, 9.5	
lysobactin β-OH-Leu·HCl[c] (8)	4.13	3.1	3.83	3.3, 8.8	
threo-β-OH-Asp·HCl[d,e]	4.42	3.2	4.88	2.7	0.13
erythro-β-OH-Asp·HCl[e,f]	4.58	2.6	4.72g	g	0.07
lysobactin β-OH-Asp·HCl[e] (7)	4.50	2.5	4.92	3.0	0.12

[a] Merck silica gel; *n*-PrOH–NH_4OH (7:3). [b] Mixture of diastereomers from US Biochemical Corp. Data was interpreted according to ref 9. [c] Internal *t*-BuOH reference for ^{1}H NMR data (1.25 ppm). [d] Commercial sample from Calbiochem-Behring. [e] Internal dioxane reference for ^{1}H NMR data (3.75 ppm). [f] Synthetically prepared sample. [g] Peak overlapped with HOD signal.

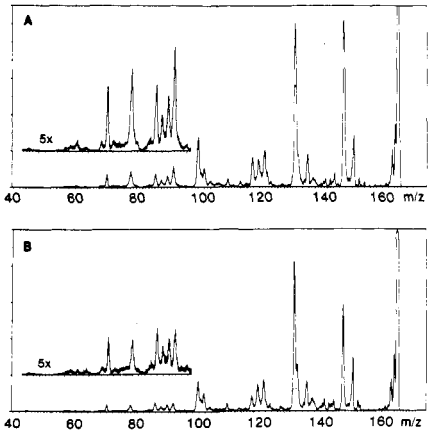


Figure 3. MS/MS fingerprints for lysobactin-derived **6** (A) and authentic phenylpyruvate (B).

antibiotic structure was deduced from the ether-extractable portion of the acid hydrolysate. The main component in the ether extract was identified as phenylpyruvic acid (**6**) from its mass spectrum and particularly by comparison with MS/MS spectra of the protonated molecular ion with that from authentic phenylpyruvic acid (Figure 3).

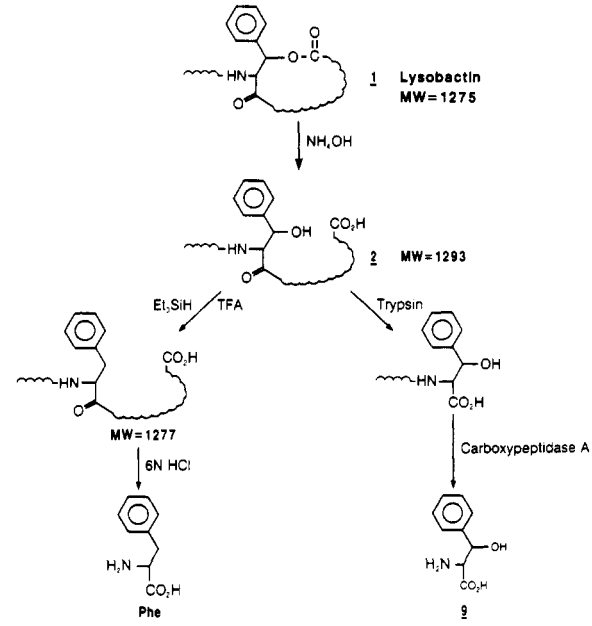
These 11 fragments account for all the carbon atoms required by the proposed formula. However, the keto function of phenylpyruvic acid is absent in the intact antibiotic and is an artifact of acid hydrolysis. Evidence for a β-hydroxyphenylalanine residue as the source of phenylpyruvic acid (**9**) was obtained by inspection of the ^{1}H NMR spectra of lysobactin base hydrolysis and methanolysis products (Table II). This data also implicates β-hydroxyphenylalanine as the alcohol contributor to the lactone. Direct evidence for β-hydroxyphenylalanine was obtained by two routes (Scheme II). First, a reduction product, MW = 1277, of lysobactin was generated from the reaction of **2** with triethylsilane/TFA.[10] Acid hydrolysis and gas chromatography (GC)/MS analysis showed the presence of phenylalanine. Secondly, the base-hydrolysis product (**2**) of lysobactin was treated with trypsin followed by carboxypeptidase A to liberate intact β-hydroxyphenylalanine, which was identified by HPLC and ^{1}H NMR comparison with authentic material. The number of rings and double bonds prescribed by the formula (18) could now be accounted for with the 11 amino acid residues and one lactone ring. Since lysobactin does

Table II. Spectroscopic Evidence for an Esterified β-Hydroxyphenylalanine Residue in Lysobactin

sample	FABMS (M + H$^+$), *m/z*	^{1}H NMR[a] H_β δ	H_β J	H_α δ	H_α J
lysobactin (**1**)	1276	6.19	10.2	5.73	10.3
hydrolysis product (**2**)	1294	5.28	4.8	4.75	5.5
methanolysis product (**3**)	1308	5.30	5.1	4.77	5.1

[a] 400-MHz spectral data collected in CD_3CN–D_2O–TFA (500:500:1) with internal TMS standard; chemical shifts (δ) in ppm; coupling constants (*J*) in hertz.

Scheme II



not react with diazomethane, no free carboxylic acid groups are present. A continuous peptide chain was established by hydrolysis to a single product (**2**). Therefore, two potentially acidic sites, one from the C-terminus and one from β-hydroxyaspartic acid, must be masked in lysobactin. The lactone, of course, accounts for one of these functional groups. A primary carboxamide was a likely candidate for the other site since it would also satisfy the nitrogen balance required by the proposed formula. Hofmann reaction[11] of **1** and subsequent amino acid analysis of the degraded peptide verified that β-hydroxyaspartic acid was the only residue altered and, therefore, that it exists as β-hydroxyasparagine in the original peptide. Similar degradation of hydrolysis product **2** led to a loss of serine and β-hydroxyaspartic acid in the subsequent amino acid analysis of the major peptide fragment, demonstrating a C-terminal serine adjacent to β-hydroxyasparagine. This experiment also allowed a second conclusion to be drawn, that the macrocyclic lactone involves the C-terminal serine.

The regiochemistry of the β-hydroxyasparagine linkage, however, was not resolved unambiguously by carboxamide degradation. Although the observed parent ions from the Hofmann reaction ($1236^+/1234^-$) were suggestive of an isoasparagine linkage (Scheme III), the actual product structures were not defined (HR-FABMS mass measurements were inconclusive). Furthermore, complications from a skeletal rearrangement during the Hofmann reaction could not be ruled out. Studies have shown that the

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Scheme III

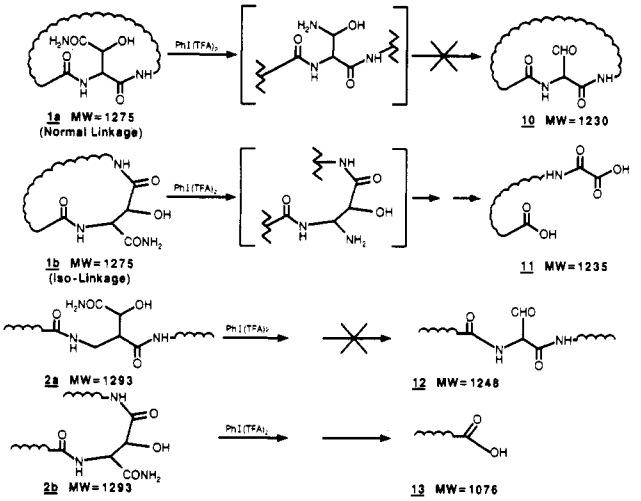


Table III. Summary of Edman Degradation of 1

step	assigned residues	PTH Amino Acid[a] TLC	CIMS	LCMS	residual peptide,[b] FABMS $(M + H)^+$
0					1276
1	Leu	+	+		1163, 1181[c]
2	Leu	+	+		1050d, 1068[c]
3	hydroxy-Phe	+	+		905
4	hydroxy-Leu	+	+		776
5	Leu	+	+		663
6	Arg	+	+		507
7	Ile	+	+		*e*
8	Thr	−	−	+	*e*
9	Gly	−	−	+	*e*
10		−	−	−	*e*

[a] PTH amino acids were isolated and compared with authentic standards: TLC (Merck silica, $CHCl_3$–EtOAc, 9:1); CIMS (direct probe, see Experimental Section); LCMS (see Experimental Section). A positive identification is indicated by +. [b] Residual peptides were analyzed directly by FABMS (+ ion mode). [c] Some lactone hydrolysis was observed. [d] The ion at m/z 1050 is attributed to the protonated molecule of macrocyclic lactam **14**, presumably formed by transacylation of the lactone. This component was resistant to Edman degradation and carried through the remaining steps. [e] Not detected by FABMS or LCMS.

α-amide bond in aspartyl and asparaginyl peptides can be readily converted to the β-aspartyl linkage via an imide intermediate.[12] There are several examples of isoglutamine linkages in nature,[13] but the isoasparagine linkage is notably rare. It was, in fact, initially misassigned in the case of vancomycin.[14] Several chemical and enzymatic strategies designed to distinguish between the two possible skeletal arrangements in lysobactin afforded mixtures of hydroxyasparagine (or hydroxyaspartate) regiomers. (A recently reported mass spectrometric method for distinguishing aspartate linkages[15] circumvents chemical or enzymatic skeletal rearrangements but unfortunately does not apply to lysobactin due to the β-hydroxy substituent, which would affect the fragmentation.) The most convincing evidence for the β-hydroxyasparagine linkage in the natural product ultimately came from ^1H NMR and $^1H/^{13}C$ HETCOR experiments.[8] On this basis, an α-linked β-hydroxyasparagine residue is assigned to lysobactin.

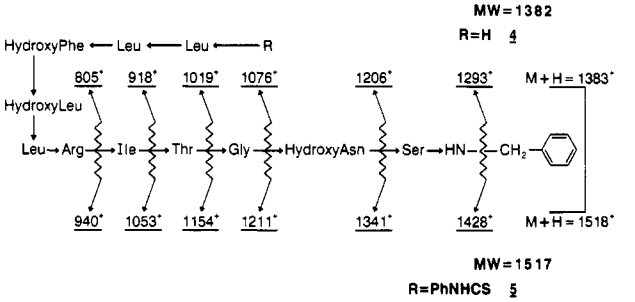
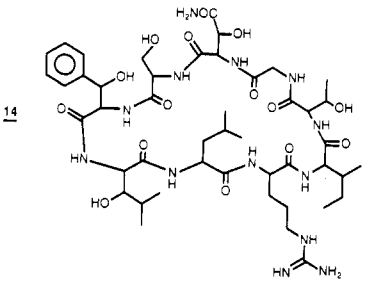


Figure 4. FAB mass spectral fragmentation pattern observed (+ ion mode) for benzylamide derivatives of lysobactin.

Direct mass spectrometric sequencing of the peptide was not possible since lysobactin and the open chain derivative **2** did not yield high mass fragment ions by FABMS or MS/MS. Sequencing of lysobactin was accomplished primarily by Edman degradation[16] of a sample of the lactone. After each cycle, the cleaved PTH amino acid and the residual peptide mixture were analyzed separately (Table III). During the third cycle of the degradation, only a portion of the didesleucyl peptide reacted with phenylisothiocyanate. After cleavage, the third residue from the N-terminus was identified as β-hydroxyphenylalanine. Since the lactone ring closure occurs at the β-hydroxyphenylalanine residue, it is possible that the lactonized peptide undergoes partial transacylation during Edman degradation to form the macrocyclic lactam **14**. This



product, of course, would no longer be susceptible to Edman degradation and would remain unaltered during the remaining sequencing. Thus, the N-terminal Leu-Leu sequence occurs as an exocyclic tail on the macrocyclic lactone of lysobactin. It follows that a major fragment ion in FAB mass spectra of **1** can be ascribed to the leucylleucine side chain cleavage at m/z 227 (227.1759 measured, 227.1759 calcd for $C_{12}H_{23}N_2O_2$). Subsequent Edman degradation was conducted on the open chain peptide present in the mixture with the presumed macrocyclic lactam (Table III). Nine residues from the N-terminus were sequenced by this method. The residual peptide was detectable through the sixth step after which only the PTH amino acids could be identified by thermospray liquid chromatography (LC)/MS. Failure of the degradation at the tenth cycle is consistent with an iso-linked hydroxyasparagine residue, but this is presumed to be the result of a skeletal rearrangement. It should be noted that MS/MS analyses were attempted at each step on the residual peptides with very little, if any, fragmentation re-

(12) (a) Kovacs, J.; Kovacs, H. N.; Konyves, I.; Csaszar, J.; Vajda, T.; Mix, H. *J. Org. Chem.* **1961**, *26*, 1084–1091. (b) Barany, G.; Merrifield, R. B. In *The Peptides*; Gross, E., Meinhofer, J., Eds.; Academic Press: New York, 1979; Vol. 2, pp 192–208. (c) Capecchi, J. T.; Miller, M. J.; Loudon, G. M. *J. Org. Chem.* **1983**, *48*, 2014–2021.

(13) (a) Nitecki, D. E.; Goodman, J. W. In *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*; Weinstein, B., Ed.; Marcel Dekker: New York, 1971; Vol. 1, Chapter 4. (b) Jeschkeit, H.; Losse, G. *Z. Chem.* **1965**, *3*, 81–96.

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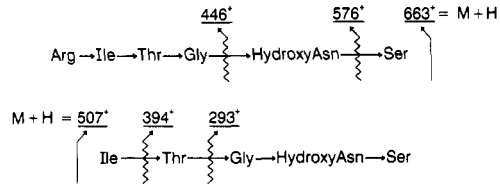
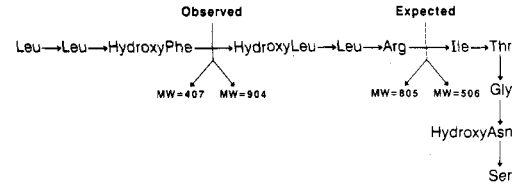
(15) Lloyd, J. R.; Cotter, M. L.; Ohori, D.; Rittgarn, P.; Doyle, D. *Spectra* **1987**, *11*, 26–30.

(16) Unger, S. E.; McCormick, T. J. *Trends Anal. Chem.* **1985**, *4*, 178–183.

Scheme IV



Scheme V



flecting the remaining sequence.

The Hofmann reaction revealed the C-terminal dipeptide as β-hydroxyasparagylserine, but the order of the preceding two residues (threonine and glycine) remained uncertain with only PTH amino acid evidence from Edman degradation. The C-terminal sequence of lysobactin and its hydrolysis product **2** were remarkably resistant to mass spectral fragmentation. On close inspection, the methanolysis product of lysobactin (**3**) gave some weak high mass ions in its FAB mass spectrum that were suggestive of the C-terminal sequence. In contrast, the benzylamide of lysobactin (**5**) provided a series of strong fragment ions using FABMS that could be assigned to the C-terminal sequence with confidence (Figure 4). These assignments could be verified further by observing mass shifts in the fragmentation pattern of the phenylthiocarbamyl derivative of benzylamide **4**. Thus, the covalent skeleton of lysobactin is unambiguously defined as shown in Figure 4.

With the full amino acid sequence determined for lysobactin, some previous observations could be interpreted. In the FAB mass spectrum of **1**, the major high mass fragment ion at m/z 776$^+$ is clearly due to β-elimination at the site of lactonization (leading to the open chain peptide) and cleavage of the linear peptide with charge retention by the C-terminal heptapeptide containing arginine. This fragment ion is prevalent in the mass spectra of the first three residual peptides from Edman degradation. The preferred cleavage site is one residue removed from the arginine peptide bond itself. After five cycles of Edman degradation, the N-terminal arginine hexapeptide gave MS/MS fragments attributable to the two residues lost from the C-terminus (Scheme IV). With the cleavage of arginine at the sixth cycle of Edman degradation, the resulting pentapeptide provided, instead, fragment ions reflecting losses from its N-terminus. Apparently the preformed cation of the arginine guanidino function mediates the fragmentation pathways for lysobactin and its derivatives in FAB mass spectra. This phenomenon has been described previously for electron impact mass spectra of peptides.[17]

Susceptibility of lysobactin and its derivatives to enzymatic digestions was studied throughout the structure determination. Although total hydrolysis was not achieved with papain, it did appear to liberate free β-hydroxyasparagine. (At this stage there was evidence for a mixture of regiomers from ^{1}H NMR analysis of the crude papain digest, which could have been a result of either chemical or enzymatic rearrangement.) When trypsin and thermolysin digestions of **2** were attempted, the same unexpected profile was observed (Scheme V). In both cases, the site of hydrolysis was reminiscent of chymotrypsin specificity. This was used, as described previously (Scheme II), to generate free β-hydroxyphenylalanine. In theory, the aminodipeptidase activity of cathepsin C would be a shortcut to N-terminal sequencing of lysobactin. In practice, neither **2** nor its des-D-leucyl-shortened peptide showed any susceptibility to cathepsin C digestion. The C-terminal heptapeptide (m/z 776$^+$) obtained during Edman degradation was also treated with cathepsin C, and in this case, a loss of the dipeptide Leu-Arg (leaving a signal for the residual pentapeptide at m/z 507$^+$) was observed. Perhaps these results reflect a narrow tolerance of cathepsin C for unusual (D and hydroxylated) amino acid residues. Carboxypeptidase A digestions proved to be useful for confirming the C-terminal sequence. When Hofmann reaction product **13** was treated with carboxypeptidase A, a sequential loss of glycine (m/z 1020$^+$/1018$^-$ for the residual peptide) followed by threonine (m/z 919$^+$/917$^-$ for the residual peptide) was evident. Further incubation did not reveal more of the sequence, perhaps due to reduced ionization efficiency of the shortened peptides in the digestion mixture.

The stability of lysobactin in basic media was also studied on account of the extraordinary lability of the biologically crucial lactone ring. The half-life of the lactone in 50 mM phosphate buffer, pH 7, was approximately 4 h at room temperature on the basis of biological activity and TLC densitometry. Under more severe conditions (pH 7 buffer, 100 °C, overnight or 5 N NaOH, room temperature, 1 h), the base hydrolysis product **2** was further degraded to shorter peptides (mainly due to cleavage of two or three C-terminal residues). None of the high mass base decomposition products gave sequence ions in MS/MS spectra.

The amino acid chiralities were determined by a variety of methods (Table IV). Since β-hydroxyphenylalanine did not survive hydrolysis, it was analyzed by an indirect approach. Phenylalanine produced by reduction of **1** (Scheme II) had the L configuration. Enzymatically liberated β-hydroxyphenylalanine (Scheme II) was assigned the L-threo configuration by a combination of enzymatic and chromatographic comparisons to authentic material. The locations of the isomers D-Leu, L-Leu, and L-Ile were determined by chiral GC/MS analyses (using both electron impact and negative chemical ionization) of hydrolysates of partially degraded peptide intermediates from Edman sequencing. Although arginine was initially misassigned, apparently due to a coeluting contaminant, unambiguous identification of D-Arg was performed on a reisolated sample. The presence of D-Arg may explain the failure of trypsin cleavage at the arginine residue (Scheme V). Finally, the residue assigned as threonine by conventional amino acid analysis of lysobactin was identified as L-allothreonine by chiral GC/MS comparison with standards. This completed the skeletal and stereochemical structure elucidation of lysobactin,[18] as shown in **1**.

(17) Shemyakin, M. M.; Ovchinnikov, Y. A.; Vinogradova, E. I.; Feigina, M. Y.; Kiryushkin, A. A.; Aldonova, N. A.; Alakhov, Y. B.; Lipkin, V. M.; Rosinov, B. V. *Experientia* **1967**, *23*, 428–430.

(18) During final preparation of this manuscript, a report on katanosins A and B came to our attention (see ref 19). Katanosin B was assigned the same structure as **1** with the exception of D-allothreonine in place of the L-allothreonine found in lysobactin. We have now confirmed our assignment of L-allothreonine in lysobactin with the L-leucylated dipeptide method utilized in the katanosin B structure elucidation. Conformational allowances for this and other stereochemical changes in the macrocycle will be considered once the solution conformation of **1** is defined.

Table IV. Amino Acid Chirality Determinations

residue	assignment	method[a] chiral GC/MS	GITC LC	amino acid oxidase	TLC[b]	NMR[b]
Leu_1[c]	D	+				
Leu_2[c]	L	+				
Leu_3[c]	L	+				
β-hydroxy-Phe	L-threo	+[d]	+	+		
β-hydroxy-Leu[e]	L-threo	+				+
β-hydroxy-Asn[ef]	L-threo	+			+	+
Ile	L	+				
Ser	L	+				
Thr	L-allo	+	+	+		
Arg	D		+	+		

[a] Details and references are presented in the Experimental Section. [b] Used only for distinction of erythro and threo forms. [c] These amino acids were isolated from the total acid hydrolysate of 1 as a mixture of D-Leu/L-Leu/L-Ile (1:2:1). Subscripts indicate the order of appearance from the N-terminus as determined by analysis of hydrolsates of partially degraded intermediates from Edman sequencing. [d] The L configuration was determined on phenylalanine produced by reduction of 1 (see Scheme II). [e] Optical rotations are given in the Experimental Section. [f] Isolated and analyzed as β-hydroxy-Asp from total acid hydrolysates.

Table V. Antibacterial Activity of Lysobactin and Selected Derivatives

organism	SC no.[a]	MIC, μg/mL 1	2	17	18	19
Staphylococcus aureus	1276	0.2	>100	50	3.1	0.4
Staphylococcus aureus	2399	0.4	>100	100	6.3	0.4
Staphylococcus aureus	2400	0.4	>100	50	6.3	0.4
Staphylococcus aureus	10165	0.8	>100	100	12.5	0.8
Streptococcus faecalis	9011	0.8	>100	50	6.3	1.6
Streptococcus agalactiae	9287	0.4	>100	50	3.1	0.2
Micrococcus luteus	2495	0.8	>100	50	1.6	0.1
Escherichia coli	8294	25	>100	>100	>100	50
Escherichia coli	10857	12.5	>500	>100	100	12.5
Escherichia coli	10896	6.3	>100	>100	25	6.3
Escherichia coli	10909	6.3	>100	>100	12.5	3.1
Klebsiella aerogenes	10440	50	>100	>100	>100	100
Klebsiella pneumoniae	9527	50	>100	>100	>100	100
Proteus mirabilis	3855	100	>100	>100	>100	>100
Proteus rettgeri	8479	100	>100	>100	>100	100
Proteus vulgaris	9416	25	>100	>100	100	50
Salmonella typhosa	1195	50	>100	>100	>100	50
Shigella sonnei	8449	25	>100	>100	100	25
Enterobacter cloacae	8236	50	>100	>100	>100	100
Enterobacter aerogenes	10078	50	>100	>100	>100	100
Citrobacter freundii	9518	50	>100	>100	>100	100
Seratia marcescens	9783	50	>100	>100	>100	>100
Pseudomonas aeruginosa	9545	25	>100	>100	>100	>100
Pseudomonas aeruginosa	8329	25	>100	>100	>100	>100
Acinetobacter calcoaceticus	8333	12.5	>100	>100	>100	50

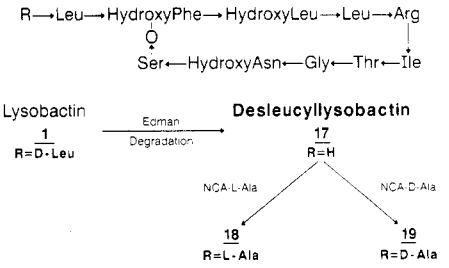
[a] Squibb culture access number.

Discussion and Biological Activity

Lysobactin is equal to or greater in potency than vancomycin against Gram-negative aerobes both in vitro and in vivo in a mouse infection and against anaerobes. However, 1 was more toxic than vancomycin.[20] In an effort to find a semisynthetic peptide with an improved therapeutic index, some lysobactin derivatives were prepared for biological testing (Scheme VI). Hydrolysis of the lactone, phenylthiocarbamylation, and degradation to desleucyllysobactin all resulted in loss of antibacterial activity. When desleucyllysobactin was reacylated with D- or L-alanine, the D-analogue was 10-fold greater in antibacterial potency (Table V). Thus, the biological data indicate that the rigid solution conformation (enforced by the lactone linkage) and N-terminal D-amino acid of lysobactin are important factors in the antimicrobial activity.

Unlike vancomycin and closely related glycopeptides, lysobactin antibiosis is not antagonized by D-Ala-D-Ala-containing peptides. Other recently reported peptide antibiotics, specifically a cyclic biphenyl-containing tripeptide[21] and a family of acidic lipopeptolides,[22] display a similar biological spectrum to 1 and do not appear to

Scheme VI



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bind to D-Ala-D-alanyl cell wall precursors. Cell wall biosynthesis is clearly a complicated, multistep process involving unusual metabolites. It is therefore plausible that other steps or intermediates in the biosynthetic pathway are good targets for selective anti-Gram-positive antibacterial agents. The specific mode of action of lysobactin, implied by the narrow structural requirements for antimicrobial activity, is an intriguing problem. Biochemical studies on this subject have been reported elsewhere.[20] We hope that the solution conformation of 1, currently under study, will shed light on the mechanism of action of this novel anti-Gram-positive agent.

Experimental Section

General Procedures. Melting points were determined on a microscope hot stage apparatus and were not corrected. TLC analyses were performed on Merck silica gel 60 layers (0.25 mm) with and without (for mass spectrometry) fluorescent indicator. NMR spectra were obtained with a JEOL GX 400 (400 MHz) spectrometer; all chemical shifts (δ) are reported downfield from internal tetramethylsilane (0 ppm), *p*-dioxane (3.75 ppm in D_2O), or *tert*-butyl alcohol (1.25 ppm in D_2O) standard. A Finnigan TSQ-4600 triple quadrupole instrument was used to obtain CI, thermospray mass, and MS/MS spectra while FAB mass and MS/MS spectra were measured on a VG Analytical Model ZAB-2F mass spectrometer. For FAB analyses, samples were dissolved in either a thioglycerol or a dithiothreitol–dithioerythritol mixture and sputtered using 8 keV Xe atoms. High-resolution analyses were performed at 1:8000 resolution by peak matching; low-resolution spectra were recorded at 1:1500 using 20-s scans. For CI analyses, a Finnigan direct exposure probe was used to desorb the analyte. Two-second scans were used to record positive and negative spectra in a methane–nitrous oxide mixture or ammonia as reagent gas. For thermospray, a Vestec thermospray source was fitted onto the Finnigan TSQ-4600 mass spectrometer. The analyte was injected onto a Whatman C_{18} column with various proportions of 0.1 M ammonium acetate–acetonitrile as mobile phase. Both UV and MS detection were employed in series. MS/MS spectra were obtained under 30 eV/1 mTorr Ar conditions on the TSQ-4600 MS and at 8 keV/50% beam reduction on the VG-ZAB-2F MS. UV spectra were recorded on a Shimadzu UV-260 instrument and IR spectra were measured with a Mattson Sirius 100 FTIR spectrometer. Amino acid analyses were performed by Sequemat Inc., Watertown, MA. Low-voltage paper electrophoresis was performed at 12 V/cm for 1 h; mobility was scored relative to vitamin B_{12} (0) and *p*-nitrobenzenesulfonate anion (+1.00). Antibiotic assays were carried out by conventional paper disks agar diffusion assay on *Bacillus subtilis* Br151V seeded agar plates. Antibiotic zones were measurable after 6 h of incubation at 37 °C.

Isolation of Lysobactin (1). Fermentation broths were centrifuged at 62500*g* to sediment bacterial cells. As described elsewhere,[6] a typical extraction of the cell mass (33 g) from a 5-L fermentation yields 73 mg of crystalline lysobactin bis(trifluoroacetate) salt: mp 224–228 °C; $[\alpha]^{25}_D$ –68.3° (*c* 2.0, MeOH); UV (MeOH) λ_{max} ($E^{1\%}$) 250 (sh, 1.4), 257 (1.5), 262 (1.4), 268 nm (0.9); IR (KBr) 3345 (br), 2965, 2937, 2878, 1745 (sh), 1666 (s), 1530 (s), 1203, 1138, 838, 800, 722, and 702 cm^{-1}; ^1H NMR (CD_3CN–D_2O–CF_3COOH, 500:500:1) see Figure 3; ^{13}C NMR (CD_3CN–D_2O–CF_3COOH, 500:500:1) δ 176.33, 175.38, 174.31, 173.96, 173.85, 172.90, 172.56, 172.17, 171.71, 170.56, 169.99, 168.46, 156.58, 135.61, 130.64, 129.75 (2 C), 128.47 (2 C), 76.21, 75.68, 71.55, 70.85, 62.37, 61.54, 60.89, 60.20, 58.47, 56.88, 56.60, 56.35, 55.85, 53.42, 52.75, 44.01, 41.84, 41.28 (2 C), 40.00, 36.76, 31.35, 28.78, 26.75, 26.41, 25.18 (2 C), 25.02, 23.98, 23.79, 22.42, 22.17, 20.89, 20.61, 20.50, 19.49, 19.41, 15.92, and 11.01 ppm; FABMS (+ ion) see Figure 1; FABMS (– ion) *m/z* 1274. Anal. Calcd for $C_{58}H_{97}N_{15}O_{17}\cdot C_4H_2O_4F_6$: C, 49.47; H, 6.64; N, 13.97; F, 7.58. Found (after drying in vacuo at 60 °C for 3 h):[23] C, 50.01; H, 6.92; N, 13.98; F, 7.4.

Hydrolysis of 1. Lysobactin (246.6 mg) was dissolved in 12 mL of CH_3CN and 12 mL of sodium 50 mM phosphate buffer (pH 7.0). The solution was heated in a boiling water bath for 2 h and then allowed to cool at room temperature. The reaction mixture was diluted with 10 mL of CH_3CN and was kept at 4 °C over night. The white solid was collected by vacuum filtration and was dried in vacuo to give **2** (212.9 mg) as its amorphous monophosphate salt. A second precipitation from 5 mL of CH_3CN–H_2O (1:1) at 4 °C overnight provided a homogeneous sample of **2** (96.5 mg) for analysis: mp 192–204 °C; IR (KBr) 3358 (br), 2962, 2876, 1660 (s), 1536 (s), 1468, 1387, 1370, 1270, 1142, 1064, 946, and 704 cm^{-1}; ^1H NMR (CD_3CN–D_2O–CF_3COOH, 500:500:1) see Table II; FABMS (+ ion) *m/z* 1294; FABMS (– ion) *m/z* 1292. Anal. Calcd for $C_{58}H_{99}N_{15}O_{18}\cdot H_3PO_4\cdot 5H_2O$: C, 46.97; H, 7.62; N, 14.18; P, 2.09. Found: C, 47.13; H, 7.58; N, 14.0; P, 1.94.

Alternately, the TFA salt of **2** was obtained by treating **1** with CH_3CN–H_2O–concentrated NH_4OH (49:49:2) for 2 h at room temperature and then removing the solvents and volatile salts by evaporation and drying in vacuo.

Methanolysis of 1. Lysobactin (5 mg) was dissolved in 0.5 mL of CH_3OH, and 5 μL of 57% NH_4OH was added. After being allowed to stand at room temperature for 3 h, the solution was diluted with CH_3CN and evaporated under a stream of nitrogen to provide 4.5 mg of amorphous **3**: mp 159–161 °C; IR (KBr) 3343 (br), 2966, 2879, 1665 (s), 1529 (s), 1469, 1391, 1371, 1203, 1139, 1087, 1062, 837, 801, 722, and 703 cm^{-1}; ^1H NMR (CD_3CN–D_2O–CF_3COOH, 50:50:0.1) see Table II; FABMS (+ ion) *m/z* 1308.

Formation of Benzylamide 5. Lysobactin (1, 102.4 mg) was dissolved in benzylamine (2 mL) and allowed to stand at room temperature for 1 h. Diethyl ether was added (20 mL) to precipitate the product. The supernatant was removed after centrifugation. The residudal solid was dissolved in methanol, and the product was reprecipitated with ether. Acetonitrile trituration of the white solid afforded **5** as an amorphous white solid: mp 152–155 °C; IR (KBr) 3330 (br), 2962, 2876, 1662 (s), 1530 (s), 1468, 1386, 1369, 1255, 1203, 1137, 1062, 835, 801, 722, and 699 cm^{-1}; ^1H NMR (see ref 7); FABMS (+ ion) *m/z* 1383. Anal. Calcd for $C_{65}H_{106}N_{16}O_{17}\cdot 5H_2O\cdot C_2HO_2F_3$: C, 50.68; H, 7.43; N, 14.11; F, 3.6. Found for **5**: C, 52.73; H, 7.23; N, 14.72; F, 2.4.

Reaction of 5 with Phenyl Isothiocyanate. Benzylamide **5** (5.0 mg) was dissolved in 0.5 mL of pyridine–water (1:1), and 100 μL of PhNCS was added. The reaction was allowed to stand at 37 °C for 1 h under a nitrogen atmosphere, and then the biphasic mixture was extracted with toluene (4 × 1 mL) to remove excess reagent. The residual aqueous layer was evaporated under a stream of nitrogen and triturated with acetonitrile to yield **6** (4.7 mg) as an off-white amorphous solid: FABMS (+ ion) *m/z* 1518.

Total Acid Hydrolysis of Lysobactin (Scheme I). Lysobactin bis(trifluoroacetate) salt (145.5 mg) was dissolved in 5 mL of constant boiling HCl and was heated at 110 °C for 17 h. The hydrolysate was cooled to room temperature and extracted with diethyl ether (3 × 2 mL). The ether extract was vacuum dried to provide an oily mixture (22.5 mg) containing phenylpyruvic acid (**6**). The aqueous layer was filtered through cotton and vacuum dried to yield an amino acid mixture. The mixture was chromatographed on a 35 × 1.1 cm column of Dowex 50-X2 (200–400 mesh, hydrogen form) resin by eluting with a linear gradient between 0.01 N HCl (150 mL) and 3 N HCl (150 mL). Fractions were pooled on the basis of R_f on silica gel TLC (*n*-BuOH–HOAc–H_2O, 4:1:1, ninhydrin detection) and vacuum dried to give the following amino acids, in order of elution, as their hydrochloride salts: β-hydroxyaspartic acid (**7**, 20.6 mg), serine (5.0 mg), serine + threonine (6.6 mg), threonine (5.5 mg), threonine + glycine (3.6 mg), glycine (12.0 mg), β-hydroxyleucine (**8**, 14.3 mg), leucine + isoleucine (137.0 mg), and arginine (21.4 mg, impure). For **7**: $[\alpha]^{25}_D$ +2.9° (*c* 2.0, 5 N HCl) [lit.[24] $[\alpha]^{25}_D$ +6.4°]; ^1H NMR (see Table I); HRFABMS (+ ion) *m/z* 150.0413 ($C_4H_8NO_5$ requires 150.0402). For **8**: $[\alpha]^{25}_D$ +11.3° (*c* 1.3, 5 N HCl) [lit.[25] $[\alpha]^{25}_D$ + 15°]; ^1H NMR (see Table I); HRFABMS (+

(23) Crystalline lysobactin·TFA typically analyzed for 5 mol of water prior to drying.

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ion) m/z 148.0973 ($C_6H_{14}NO_3$ requires 148.0974).

Reduction of Hydrolysis Product 2 To Form Phenylalanine (Scheme II). Hydrolysis product **2** (11.6 mg) was dissolved in 300 μL of TFA at 0 °C, and 30 μL of Et_3SiH was added slowly. The mixture was stirred at 0 °C for 1 h. Addition of 1 mL of Et_2O–CH_3CN (1:1) precipitated a white solid that was separated by centrifugation and dried in vacuo to give a mixture of peptides (9.4 mg): FABMS (+ ion) m/z 1278 and 1294. This mixture (MW = 1277 and 1293) was hydrolyzed in 0.5 mL of 6 N HCl (as described above for the total hydrolysis of **1**), and the resulting amino acid mixture was analyzed for the presence of phenylalanine by TLC, GITC/HPLC, and chiral GC/MS methods (see details given below).

Enzymatic Digestions of Hydrolysis Product 2 (Scheme VI). A. Trypsin.[26] Hydrolysis product **2** (1.0 mg) was dissolved in 1 mL of 50 mM Tris buffer (pH 8.5), and 100 μL of CH_3CN was added. To this, 4.8 mg of trypsin (Sigma) was added, and the clear solution was incubated at 37 °C for 17 h. The digest was then evaporated with a stream of nitrogen, and the residue was chromatographed on a C_{18} Sep Pak column, eluting with a step gradient of CH_3CN in water. The fractions eluting with 50% aqueous CH_3CN were combined to give a white solid: FABMS (+ ion) m/z 905 and 407; FABMS (– ion) m/z 903 and 406.

B. Thermolysin. Hydrolysis product **2** (1.0 mg) was dissolved in 1 mL of 50 mM HEPES buffer (pH 7.0) and 100 μL of CH_3CN. Thermolysin (3.8 mg, Sigma) was added, and the suspension was incubated at 37 °C for 48 h. The digest was then evaporated and chromatographed as described for the trypsin digest. All buffer-free fractions were combined to give a white solid: FABMS (+ ion) m/z 905 and 408; FABMS (– ion) m/z 903 and 406.

Formation of Free β-Hydroxyphenylalanine (Scheme II). A solution was prepared by combining the white solid obtained from trypsin digestion (described above), 900 μL of 1 M NH_4Cl (adjusted to pH 8), 100 μL of CH_3CN, and 40 μL of carboxypeptidase A suspension (40 μL, Sigma). After incubation at 37 °C for 17 h, the mixture was evaporated with a stream of nitrogen and chromatographed on a 3-mL column of MCI Gel CHP20P with use of a gradient from 0.1 N HCl to CH_3CN. Fractions containing β-hydroxyphenylalanine (identified in the 0.1 N HCl eluate by TLC) were combined, dried and analyzed: ^1H NMR (D_2O) 7.46 (5 H), 5.30 (1 H, d, J = 4.4 Hz), and 3.92 (1 H, d, J = 4.4 Hz) ppm.

Hofmann Reaction of Lysobactin. [*I,I*-Bis(trifluoroacetoxy)iodo]benzene (BTI, 35.2 mg) was dissolved in 3.5 mL of CH_3CN–H_2O (1:1), and 3.0 mL of this solution was added to 30.5 mg of lysobactin·2TFA. The reaction mixture was held at 60 °C for 4 h under a nitrogen atmosphere. After the mixture was cooled to room temperature, 30 μL of TFA was added, and the solution was evaporated until a solid precipitated. The resulting suspension was extracted with 3 × 1 mL of Et_2O, and the aqueous residue was dried under nitrogen to yield a white solid (30.9 mg): FABMS (+ ion) m/z 1236; FABMS (– ion) m/z 1234; IR (KBr) 3352 (br), 2969, 2880, 1750 (sh), 1664 (s), 1532 (s), 1468, 1433, 1392, 1372, 1204, 1139, 1072, 839, 801, 723, and 702 cm^{-1}; ^1H NMR (CD_3CN–D_2O–TFA 50:50:0.1) 7.35 (5 H, m), 6.15 (1 H, d), and 5.02 ppm (1 H, d), indicative of an intact lactone at β-hydroxyphenylalanine. Amino acid analysis was performed on the product: threonine, serine, glycine, arginine, β-hydroxyleucine, isoleucine, and leucine in a 1:1:1:1:1:1:3 molar ratio.

Hofmann Reaction of Hydrolysis Product 2. Hydrolysis product **2** (9.2 mg) was treated with 1 mL of a 10 mg/mL solution of BTI as described above. The aqueous residue provided 7.5 mg of a white solid containing two ninhydrin positive components. The solid was chromatographed on a 3-mL column of MCI Gel CHP20P with use of a step gradient from 0.1% aqueous TFA to 0.1% TFA in CH_3CN. Fractions were pooled conservatively to give a pure sample of the guanidine-containing (Sakaguchi-positive) peptide as a white solid (2.8 mg) after vacuum drying: FABMS (+ ion) m/z 1077; FABMS (– ion) m/z 1075. Amino acid analysis was performed on the peptide: threonine, glycine, arginine, β-hydroxyleucine, isoleucine, and leucine in a 1:1:1:1:1:3 molar ratio.

Enzymatic Digestions for Mass Spectral Studies. A. Papain. A solution of the peptide (ca. 10 mg), papain (Sigma, 7 mg), and thioglycerol (2 μL) in 2 mL of sodium 50 mM phosphate buffer, pH 7, and 1 mL of CH_3CN was prepared. The mixture was incubated at 37 °C for 4 h (adequate cleavage was detected by TLC monitoring). The sample was concentrated to dryness and chromatographed on a column of Sephadex G-25M with C_5H_5N–H_2O (1:19) elution to remove the enzyme. Fractions containing peptide fragments and amino acids were combined and vacuum dried prior to analysis by MS.

B. Cathepsin C. The enzyme solution was prepared by dissolving cathepsin C (Sigma, 0.3 mg) in 50 mL of 10 mM NH_4Cl adjusted to pH 5.5 with 0.1 M HCl and adding 1 μL of thioglycerol. The peptide (ca. 0.5 mg) was suspended in 0.5 mL of the enzyme-free NH_4Cl/thioglycerol solution, and then the enzyme solution (30 μL) was added. The solution was incubated at 37 °C, and subsamples were monitored directly by FABMS.

C. Carboxypeptidase A. The peptide to be digested (ca. 1 mg) was dissolved in 1 mL of 40 mM NH_4Cl adjusted to pH 8 with 1 M NH_4OH, and 10 μL of carboxypeptidase A solution (Sigma) was added. The mixture was incubated at 37 °C for 1 day, and then another 10 μL of enzyme suspension was added. Subsamples were analyzed directly by FABMS.

Amino Acid Chirality Determinations. A. GC/MS. Amino acid unknowns, amino acid standards and peptide hydrolysates were derivatized for analysis. Typically, an 0.5-mg sample was treated with 0.5 mL of 1.75 N HCl in nPrOH for 30 min at 80 °C in a sealed vial. The reagents were evaporated with a stream of nitrogen, and the residual solid was treated with 0.5 mL of pentafluorobutyric anhydride–CH_2Cl_2 (1:3) for 10 min at 50 °C in a sealed vial. The reagents were evaporated with a stream on nitrogen, and the resulting *N,O*-pentafluorobutyrate *n*-propyl esters were analyzed by GC/MS. In the case of hydroxyamino acids, the *N*-TFA-*O*-acetyl isopropyl ester derivatives were also prepared as described elsewhere.[27] Analyses were performed on a Finnigan MAT Model 9611 gas chromatograph interfaced with the Finnigan TSQ-4600 mass spectrometer using a Chirasil-Val III column (Applied Science, 25 m × 0.31 mm) programmed to run isothermally at 80 °C for 3 min followed by a 4 °C/min gradient to 220 °C. Detection was by positive or negative EIMS or CIMS with use of a CH_4/N_2O mixture as reagent gas.

B. GITC/LC. Amino acid unknowns and standards were derivatized with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC, Polyscience) by modifying a previously reported protocol.[28] Each amino acid (1 mg) was dissolved in 1 mL of H_2O and 6 μL of Et_3N. The GITC reagent was prepared in CH_3CN (2 mg/mL). A portion of the amino acid solution (50 μL) was combined with 220 μL of GITC solution and was left to stand at room temperature for 30 min. Water (230 μL) was added to quench the reaction, and 10 μL subsamples were immediately analyzed by HPLC with use of an IBM C_{18} (5 μm) column (4.5 mm × 25 cm) in a Varian 5000 liquid chromatograph. The derivatives were eluted with MeOH–CH_3CN–0.1% aqueous H_3PO_4 (10:35:55) at a 1.3 mL/min flow rate and detected by their UV absorption at 254 nm.

C. Amino Acid Oxidase Transaminations. Amino acids were treated with D- and L-amino acid oxidases (Sigma) and then analyzed by LVE with ninhydrin visualization of the unreacted amino acid as described previously.[29]

Edman Sequencing. General Protocol. The peptide (5.4 mg of starting material **1**) was dissolved in 1 mL of C_5H_5N–H_2O (1:1), and 200 μL of phenyl isothiocyanate (PhNCS, Aldrich) was added. The reaction was allowed to stand at 37 °C for 1 h under a nitrogen atmosphere and then the biphasic mixture was extracted with toluene (4 × 1 mL) to remove excess reagent. The residual aqueous material was diluted with 0.5 mL of CH_3CN and evaporated under a stream of nitrogen. The (phenylthio)-carbamylation product was treated with neat TFA (0.2 mL) under a nitrogen atmosphere for 1 h at 37 °C. The TFA was removed by dilution with CH_3CN and evaporation under a stream of

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nitrogen. The residual solid was then dissolved in 1 mL of 0.2 N TFA in CH_3CN–H_2O (1:1) and heated for 10 min at 80 °C. The solution was concentrated to approximately half volume. The (phenylthio)hydantoin (PTH) of the N-terminal amino acid was separated by Et_2O extraction (3 × 0.5 mL) and concentrated for TLC and MS analyses. The aqueous layer containing the degraded peptide was evaporated to dryness under a stream of nitrogen and then subjected to another cycle of Edman degradation.

Scaled-Up Edman Degradation of Lysobactin To Form Desleucyllysobactin (17). PhNCS (5 mL) was added to lysobactin·2TFA (326.7 mg) dissolved in C_5H_5N–H_2O, 1:1 (30 mL). The mixture was allowed to stand at 37 °C under nitrogen for 1 h with occasional swirling. Toluene extraction (3 × 10 mL) removed the excess reagent (C_5H_5N–H_2O, 1:1, was added as necessary to maintain a biphasic extraction). The aqueous residue was concentrated and lyophilized to yield the (phenylthio)-carbamyl derivative as a white solid. TFA (3 mL) was added to the solid, and the solution was heated at 40 °C for 15 min under a nitrogen atmosphere. After being chilled to 0 °C, the reaction mixture was diluted with Et_2O (0 °C) to precipitate desleucyllysobactin. The cleaved amino acid derivative was removed by Et_2O trituration (2 × 10 mL), and the residual peptide was vacuum dried to yield **17** as a white solid: FABMS (+ ion) m/z 1163; FABMS (− ion) m/z 1161. Anal. Calcd for $C_{52}H_{86}N_{14}O_{16}$·2TFA·H_2O: C, 45.38; H, 6.67; N, 13.24; F, 7.7. Found: C, 45.73; H, 6.34; N, 13.43; F, 6.4.

Acylation of Desleucyllysobactin with *N*-Carboxy Anhydrides. General Method (Scheme VI). All *N*-carboxy amino acid anhydrides (NCA's) were prepared according to ref 30. The details given below for synthesis of **19** also apply to **18**. Desleucyllysobactin (800 mg) was dissolved in 50 mL of DMF containing triethylamine (250 μL), diluted with 150 mL of solvent mix A (THF–EtOAc–DMF, 12:4:1), and chilled to −65 °C. The NCA of D-alanine (115.7 mg) was dissolved in 50 mL of solvent mix A via sonication and was slowly added to the peptide solution under a nitrogen atmosphere at −65 °C with rapid stirring. After the solution was mixed for 10 min, 25 mL of 1 M HOAc (0 °C) was added with stirring. The reaction mixture was concentrated to an oily residue in vacuo, from which the Et_2O-insoluble material was chromatographed on a column of MCI Gel CHP20P resin (5 × 42 cm), eluting with a 4-L linear gradient from 0.1% TFA–H_2O to 0.1% TFA in CH_3CN–H_2O (4:1) at a flow rate of 4 mL/min. The bioactive fractions were pooled on the basis of TLC appearance and lyophilized to give **18** as a white solid: mp 206–212 °C; FABMS (+ ion) m/z 1235; FABMS (− ion) m/z 1223.

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A Synthesis of (−)-Talaromycin A

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The total synthesis of (−)-talaromycin A utilizing the addition of the lithium acetylide of 1-methoxy-1-buten-3-yne to 4(*R*)-ethylvalerolactone is described. The ethylvalerolactone is prepared in seven steps from allyl bromide. The required 1,3-diol of talaromycin A is introduced in a regio- and stereospecific fashion via a tin-mediated radical cyclization.

The talaromycins A and B (**1** and **2**) are toxic metabolites that were isolated by Lynn and co-workers in 1982 from the fungus *Talaromyces stipitatus*.[2] Their structures were assigned primarily through the use of two-dimensional ^1H NMR studies of a mixture of the two toxins. More recently Lynn[3] has isolated and identified the minor components that accompany talaromycins A and B, and these have been assigned the names talaromycins C, D, E,[4] and F (**3**–**6**). It is important to note that the less stable talaromycin A (**1**), which possesses an axially disposed hydroxymethyl, can be quantitatively converted to talaromycin B (**2**) (equatorial hydroxymethyl) by acid catalysis. Several reports of syntheses of talaromycin B have appeared.[5-8,13,14] Most of these approaches take advantage of a key thermodynamically controlled spiroketalization to establish all stereogenic centers on the spiroketal and thus preclude (with the exception of Schreiber's[9] approach) the preparation of talaromycin A. In contrast, the thermodynamically less stable talaromycin A has received less attention.[10-12] We describe here a highly enantioselective synthesis of talaromycin A (**1**), uncontaminated by talaromycin B, which takes advantage of the dioxaspiro-[5.5]undecene system as a template to control the stereochemistry of the 1,3 diol function.

Results and Discussion

Previously we had reported a method for the preparation of spiroketals such as **7** by the addition of the lithium acetylide of 1-methoxy-1-buten-3-yne to lactones. The acetylenic ketones that resulted were exposed to a two-step

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