Solid-Phase Total Synthesis of Bacitracin A

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Received March 28, 1996[⊗]

An efficient solid-phase method for the total synthesis of bacitracin A is reported. This work was undertaken in order to provide a general means of probing the intriguing mode of action of the bacitracins and exploring their potential for use against emerging drug-resistant pathogens. The synthetic approach to bacitracin A involves three key features: (1) linkage to the solid support through the side chain of the L-asparaginyl residue at position 12 (L-Asn[12]), (2) cyclization through amide bond formation between the α-carboxyl of L-Asn[12] and the side chain amino group of L-Lys[8], and (3) postcyclization addition of the N-terminal thiazoline dipeptide as a single unit. To initiate the synthesis, Fmoc L-Asp(OH)-OAllyl was attached to a PAL resin. The chain of bacitracin A was elaborated in the C-to-N direction by sequential piperidine deprotection/HBTU-mediated coupling cycles with Fmoc D-Asp(O*t*Bu)-OH, Fmoc L-His(Trt)-OH, Fmoc D-Phe-OH, Fmoc L-Ile-OH, Fmoc D-Orn(Boc)-OH, Fmoc L-Lys(Aloc)-OH, Fmoc L-Ile-OH, Fmoc D-Glu(O*t*Bu)-OH, and Fmoc L-Leu-OH. The allyl ester and allyl carbamate protecting groups of L-Asn[12] and L-Lys[8], respectively, were simultaneously and selectively removed by treating the peptide-resin with palladium tetrakis-(triphenylphosphine), acetic acid, and triethylamine. Cyclization was effected by PyBOP/HOBT under the pseudo high-dilution conditions afforded by attachment to the solid support. After removal of the N-terminal Fmoc group, the cyclized peptide was coupled with 2-[1′(*S*)-(*tert*-butyloxycarbonylamino)-2′(*R*)-methylbutyl]-4(*R*)-carboxy-Δ^2-thiazoline (**1**). The synthetic peptide was deprotected and cleaved from the solid support under acidic conditions and then purified by reverse-phase HPLC. The synthetic material exhibited an ion in the FAB-MS at *m/z* 1422.7, consistent with the molecular weight calculated for the parent ion of bacitracin A ($MH^+ = C_{73}H_{84}N_{10}O_{23}Cl_2$, 1422.7 g/mol). It was also indistinguishable from authentic bacitracin A by high-field ^1H NMR and displayed antibacterial activity equal to that of the natural product, thus confirming its identity as bacitracin A. The overall yield for the solid-phase synthesis was 24%.

Introduction

Bacitracin A prototypifies the family of dodecapeptide lariat antibiotics produced nonribosomally by *Bacillus subtilis* and *licheniformis*.[1] These agents exhibit a novel, receptorlike mode of action—in conjunction with a divalent metal ion, bacitracins bind to and sequester bactoprenyl pyrophosphate, the lipid carrier of intermediates involved in cell wall biosynthesis.[2] Bacitracin is widely used as a component of topical antibacterial ointments and an additive in animal feeds, and it has recently been found that bacitracin eradicates intestinal colonization by vancomycin-resistant *Enterococcus faecium*.[3] Unfractionated bacitracin is not suitable for systemic use, however, because some elements of the complex, difficult to separate naturally occurring mixture are nephrotoxic.[4] Solution-phase synthetic studies directed toward the bacitracins were carried out some time ago,[5] and a combination solid-phase/solution-phase total synthesis of the inactive, nephrotoxic component bacitracin F has been reported.[6] However, no total synthesis of a biologically active bacitracin has been described. In order to probe the intriguing mode of action of the bacitracins and to further explore their potential for use against emerging drug-resistant pathogens, we undertook the development of synthetic methods for the preparation of biologically active bacitracins. In this communication, we report an efficient solid-phase method for the total synthesis of bacitracin A.

Results and Discussion

Advances with orthogonal protecting-group strategies, solid supports, and coupling reagents have led to the

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1996.

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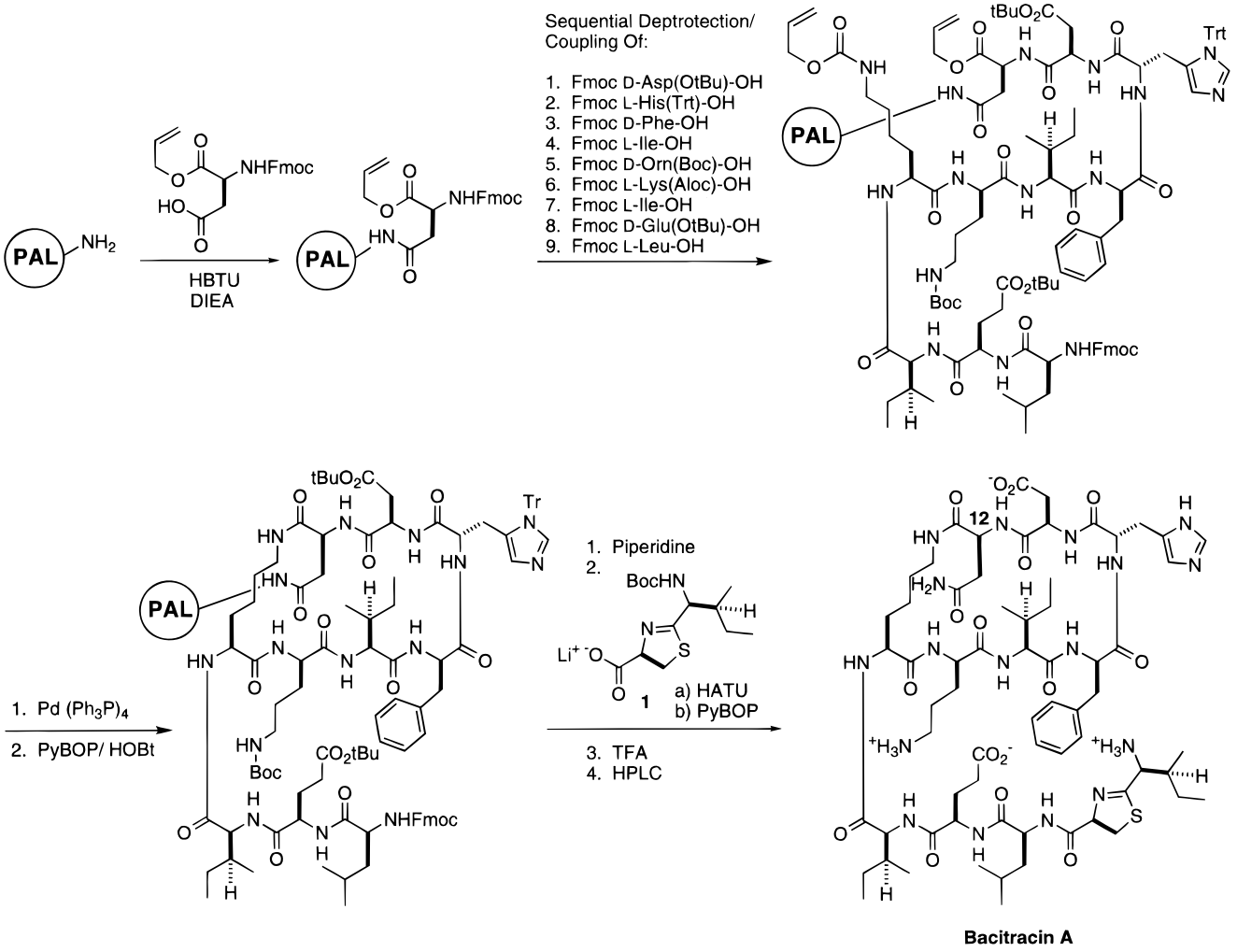
S0022-3263(96)00580-4 CCC: $12.00

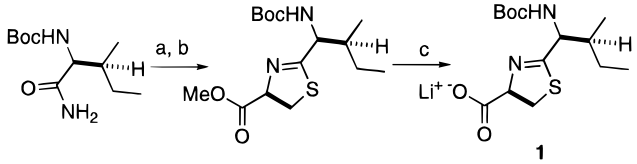
Scheme 1. Solid-Phase Total Synthesis of Bacitracin A



recent elaboration of efficient methods for the solid-phase synthesis of cyclic peptides.[7] Our synthetic approach to the lariat structure of bacitracin A involved three key features: (1) linkage to the solid support through the side chain of the L-asparaginyl residue at position 12 (L-Asn^{12}), (2) on-resin cyclization through amide bond formation between the α-carboxyl of L-Asn^{12} and the side chain amino group of L-Lys^{8}, and (3) postcyclization addition of the N-terminal thiazoline dipeptide as a single unit. This strategy allowed us to assemble bacitracin A in a simple, linear fashion (Scheme 1).

To initiate the synthesis, Fmoc L-Asp(OH)(-OAllyl[7] was linked to a PAL resin.[8] The chain of bacitracin A was then elaborated in the C-to-N direction by iterative deprotection with piperidine followed by HBTU-mediated coupling cycles with Fmoc D-Asp(O*t*Bu)-OH, Fmoc L-His(Trt)-OH, Fmoc D-Phe-OH, Fmoc L-Ile-OH, Fmoc D-Orn(Boc)-OH, Fmoc L-Lys(Aloc)-OH, Fmoc L-Ile-OH, Fmoc D-Glu(O*t*Bu)-OH, and Fmoc L-Leu-OH.[9,10] At this point, the allyl ester and allyl carbamate protecting groups used for L-Asn^{12} and L-Lys^{8}, respectively, were simultaneously and selectively removed by treating the peptide-resin with palladium tetrakis(triphenylphosphine), acetic acid, and *N*-methylmorpholine.[11] Cyclization was then effected by PyBOP and HOBT[12] under the pseudo high-dilution conditions afforded by attachment of the substrate to the solid support. After removal of the N-terminal Fmoc group, the cyclized decapeptide was coupled with Boc-protected Δ^2 thiazoline (**1**), which had been prepared by condensation of L-cysteine methyl ester and the imino ether derived from Boc L-isoleucine (Scheme 2).[13] Double-

Scheme 2[a]



[a] (a) Et_3O^+ PF_6^-, (b) L-cysteine methyl ester, (c) LiOH.

(8) PAL resin = 5-(4-((9-Fluorenylmethoxycarbonyl)aminomethyl)-3,5-dimethoxyphenoxy)valeric acid-MBHA resin. See: Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R. I.; Hudson, D.; Barany, G. *J. Org. Chem.* **1990**, *55*, 3730−3743.

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(10) Abbreviations used: HBTU, 2-(1-hydroxybenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOBt, 1-hydroxybenzotriazole; PyBOP, (benzotriazol-1-yloxy)tripyrrolidophosphonium hexafluorophosphate; Fmoc, *N*-fluorenylmethyloxycarbonyl; Aloc, allyloxycarbonyl; Asp, aspartic acid; His, histidine; Phe, phenylalanine; Ile, isoleucine; Orn, ornithine; Lys, lysine; Glu, glutamic acid; Leu, leucine; OtBu, *t*-butyl ester; Trt, trityl; Boc, *t*-butyloxycarbonyl.

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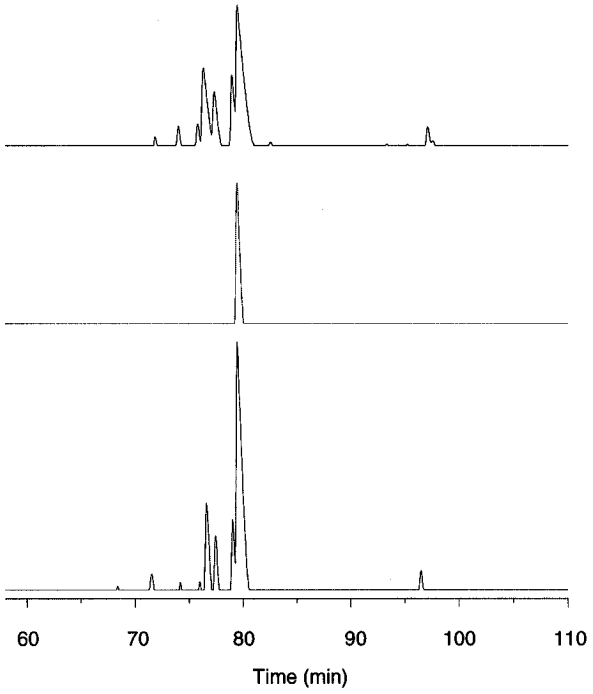

Figure 1. Reverse-phase HPLC chromatograms of commercial bacitracin (top), synthetic bacitracin A (middle), and a coinjection of synthetic and commercial materials (bottom).

coupling of **1** to the resin-bound decapeptide (mediated by HATU[14] and then by PyBOP) was carried out after the cyclization procedure in order to avoid the possibility that the sulfur-containing thiazoline moiety would inhibit the palladium-catalyzed removal of the allyl-based protecting groups.

The synthetic peptide was deprotected and cleaved from the solid support with a mixture of trifluoroacetic acid (TFA), phenol, and triisopropylsilane.[15] The crude product was fractionated by reverse-phase HPLC using a gradient of acetonitrile (+0.1% TFA) in water (+0.1% TFA). Fractions containing homogeneous material which coeluted with the bacitracin A component of a commercial sample (Figure 1) were collected and lyopholized. The resulting white powder exhibited an ion in the FAB-MS at *m/z* 1422.7, consistent with the molecular weight calculated for the parent ion of bacitracin A (M^+ = $C_{66}H_{103}N_{17}O_{16}S$, 1422.7 g/mol). The synthetic material was also indistinguishable from authentic bacitracin A by high-field ^{1}H NMR (Figure 2), and displayed antibacterial activity equal to that of the natural product (Table 1), thus confirming its identity as bacitracin A. The overall yield for the solid-phase synthesis was 24%.

In summary, we have developed an efficient, linear solid-phase method for the total synthesis of bacitracin A which should be generally applicable to the preparation of defined, homogeneous natural and non-natural bacitracins. This sets the stage for a detailed structure/function analysis of this family of antibacterial agents and efforts to elaborate bacitracin A into a reagent which not only binds to pyrophosphate-containing substrates, but also catalyzes their chemical transformation.[16]

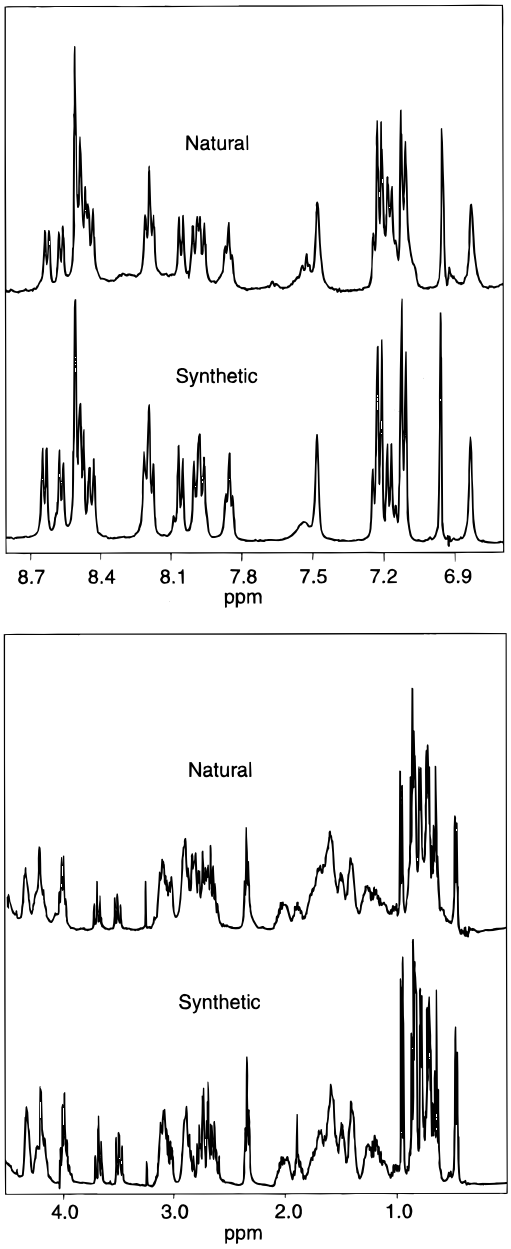

Figure 2. Overlaid portions of the 400 MHz ^{1}H NMR spectra of bacitracin A purified from the naturally occurring mixture (top spectrum of each set) and synthetic bacitracin A (bottom spectrum of each set). Spectra were recorded in 9:1 H_2O:D_2O at room temperature.

Experimental Section

General. A sample of bacitracin was generously provided by The Upjohn Co. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl. Triethylamine (Et_3N) and dichloromethane (CH_2Cl_2) were freshly distilled from CaH_2. *N*,*N*-Dimethylformamide (DMF) was dried over 4 Å molecular sieves. Other commercial reagents and solvents were used as received. Sources of reagents for peptide synthesis were as follows: Fmoc-protected amino acids, Bachem; HATU, Perseptive Biosystems; HOBt and PyBOP, Nova Biochem; All other reagents, Applied Biosystems. Reverse-phase HPLC was carried out with a Beckman 338/167 gradient chromatography system with Rainin Dynamax C18 columns using linear gradients of acetonitrile (CH_3CN)/0.1% TFA in water/0.1% TFA. ^{1}H and ^{13}C NMR spectra were obtained on a Varian XL-

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Table 1. *In Vitro* Antibacterial Activity

agent	MIC (μg/mL)[a]		
	S. aureus[b]	*S. aureus*[c]	*M. luteus*[d]
bacitracin[e]	32	16	0.5
zinc bacitracin[e]	16	16	0.5
bacitracin A[e]	16	16	1.0
bacitracin A[f]	16	16	0.5

[a] Minimal concentrations of agents required to inhibit bacterial cell growth in a broth microdilution assay. Assays were carried out in cation-adjusted Mueller−Hinton broth. [b] ATCC strain 29213, methicillin-susceptible. [c] Strain ST447, methicillin-resistant. [d] Strain X186. [e] Natural. [f] Synthetic.

400 spectrometer. Mass spectra were acquired by the Mass Spectrometry Facility at the University of California, San Francisco.

2-[1′(*S*)-(*tert*-Butyloxycarbonylamino)-2′(*R*)-methylbutyl]-4(*R*)-carboxy-Δ^2-thiazoline (1). *N*-Boc L-isoleucinyl amide (3.67 g, 1.59×10^{-2} mol) was dissolved in 40 mL of CH_2Cl_2 and treated with triethyloxonium hexafluorophosphate (4.73 g, 1.91×10^{-2} mol) to afford **2(*S*)-(*tert*-Butyloxycarbonylamino)-3(*R*)-methylpentanimino ethyl ether** (3.32 g, 81%).[13] To a solution of this compound (3.32 g, 1.29×10^{-2} mol) in 20 mL of chloroform ($CHCl_3$) was added L-cysteine methyl ester (3.30 g, 1.88×10^{-2} mol) in 15 mL of methanol at 0 °C. The mixture was stirred under Ar at 0 °C for 2 h and then at room temperature (rt) for 24 h. After the solvents had been removed under reduced pressure, 50 mL of $CHCl_3$ was added and the organic layer was washed with saturated NaCl solution. Purification of the product by flash chromatography using a 2:3 mixture of hexane:diethyl ether as eluent gave **2-[1′(*S*)-(*tert*-Butyloxycarbonylamino)-2′(*R*)-methylbutyl]-4(*R*)-(methoxycarbonyl)-Δ^2-thiazoline** (2.75 g, 65%) as a pale yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.75 (t, 3H), 0.81 (d, 3H), 0.96−1.01 (m, 1H), 1.28 (s, 9H), 1.32−1.38 (m, 1H), 1.74−1.76 (m, 1H), 3.35−3.46 (m, 2H), 3.63 (s, 3H), 4.35 (m, 1H), 4.99 (t, 1H), 5.15 (d, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.4, 15.2, 23.9, 28.0, 35.2, 38.8, 52.2, 57.3, 79.2, 155.0, 170.7, 175.4; HR-MS, calcd for $C_{15}H_{26}N_2O_4S$ (M^+): 330.1613; found: 330.1611. This ester (1.05 g, 3.18×10^{-3} mol) was dissolved in 16 mL of THF and treated with 3.18 mL of aqueous LiOH (7.6×10^{-2} g, 3.18×10^{-3} mol) at 0 °C under N_2 atmosphere. After stirring for 1 h, the THF was removed under reduced pressure while the temperature was kept below 5 °C. The aqueous layer was washed with diethyl ether and then lyopholized to afford **1** as the lithium salt, which was used directly for coupling to the resin-bound cyclized decapeptide. ^{1}H NMR (400 MHz, 60:40 D_2O:CD_3CN) δ 1.18−1.25 (m, 6H), 1.42−1.52 (m, 1H), 1.75 (s, 9H), 1.80−1.90 (m, 1H), 2.10−2.18 (m, 1H), 3.68 (dd, 1H), 3.88 (dd, 1H), 4.59 (d, 1H), 5.22 (dd, 1H).

Bacitracin A. The resin-bound linear decapeptide PAL-L-Asp(OAllyl)-D-Asp(O*t*Bu)-L-His(Trt)-D-Phe-L-Ile-D-Orn(Boc)-L-Lys(Aloc)-L-Ile-D-Glu(O*t*Bu)-L-Leu-Fmoc was assembled with an Applied Biosystems Model 431A peptide synthesizer. The synthesis was carried out on a 2.5×10^{-4} mol scale starting with 0.39 g PAL resin. Coupling reactions were mediated by a combination of HBTU and diisopropylethylamine (DIEA) in *N*-methylpyrrolidone (NMP) solvent; single-coupling cycles using a four-fold excess of Fmoc amino acid derivatives to resin-bound peptide were employed. Deprotection of terminal Fmoc protecting groups was effected by two treatments with 20% piperidine in NMP (5 and 15 min). The resin-bound linear decapeptide was dried completely *in vacuo* and then swollen with $CHCl_3$ under at atmosphere of Ar. To this was added one-half of a mixture of $Pd(PPh_3)_4$ (1.73 g, 1.49×10^{-3} mol), acetic acid (0.8 mL), and *N*-methylmorpholine (1.6 mL) in 30 mL of $CHCl_3$. After stirring at rt for 4 h, the Pd solution was drained, and the resin was treated with the second half of the Pd mixture and allowed to stir at rt for 12 h. At this point, the ^{1}H NMR spectrum of a small aliquot of peptide cleaved from the resin with TFA showed no evidence of remaining allyl groups. The deprotected resin-bound linear decapeptide was washed with DMF and NMP and then treated with PyBOP (0.26 g, 0.5×10^{-3} mol), HOBt (0.077 g, 0.5×10^{-3} mol), and DIEA (0.26 mL, 1.5×10^{-3} mol) in 10 mL of NMP for 1 day to effect cyclization. After removal of the terminal Fmoc group, the resin-bound cyclic decapeptide was treated with **1** (8.1×10^{-2} g, 2.5×10^{-4} mol) and HATU (9.5×10^{-2} g, 2.5×10^{-4} mol) in 6 mL of NMP. After addition of DIEA (8.7×10^{-2} mL, 5.0×10^{-4} mol), the reaction mixture was stirred for 1 h. The resin was washed with NMP, and a second coupling of **1** (8.1×10^{-2} g, 2.5×10^{-4} mol) was carried out using PyBOP/HOBt/DIEA (1.3×10^{-1} g, 2.5×10^{-4} mol/3.8×10^{-2} g, 2.5×10^{-4} mol/0.13 mL, 7.5×10^{-4} mol). The fully elaborated, cyclized product was deprotected and cleaved from the resin using a mixture of TFA:phenol:triisopropylsilane (93:5:2)[15] at rt for 1 h. The resulting product mixture was fractionated by reverse-phase HPLC (100 min gradient of 0−25% CH_3CN). Fractions containing homogeneous bacitracin A were combined and lyopholized to afford 8.5×10^{-2} g, 24% of the product. FaB-MS, calcd for $C_{66}H_{103}N_{17}O_{16}S$ (MH^+): 1422.7; found: 1422.7.

Biological Activity. Serial 2-fold dilutions of antibiotics were made in 96-well microtitre trays. Each well contained 100 μL of cation-adjusted Mueller-Hinton II broth. Bacteria were added to a final concentration of 10^5 colony forming units (CFU) per mL. Plates were incubated at 37 °C for 18 h and then read for visible turbidity.

Acknowledgment. This work was supported by the National Science Foundation (CHE-9457703) and the National Institutes of Health (1R01GM51122). Mass spectra were acquired by the UCSF Mass Spectrometry Facility (A. L. Burlingame, Director), supported by the Biomedical Research Technology Program of the NIH National Center for Research Resources.

JO960580B