Biosynthesis of Berninamycin: Incorporation of ^{13}C-Labeled Amino Acids[1]

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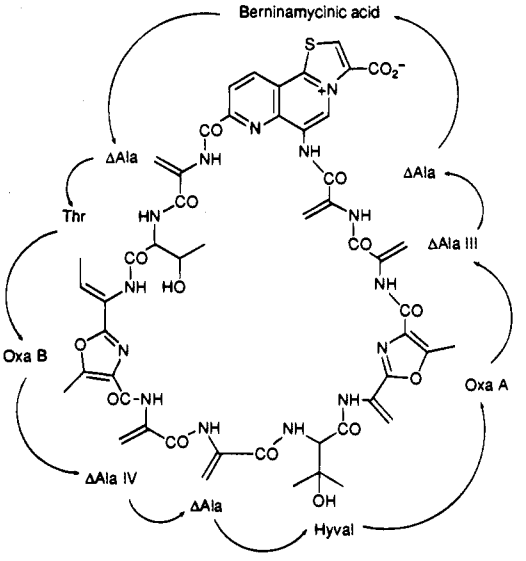
Abstract: Two-dimensional NMR and FABMS/MS studies agree with the structure for berninamycin A proposed by Abe et al. (*Tetrahedron Lett.* **1988**, *29*, 1401–1404). Biosynthetic studies with ^{13}C-enriched amino acids confirm our earlier results that the dehydroalanine units are formed by dehydration of serine. The oxazoles are formed by condensing a threonine unit with a serine or another threonine with dehydration, and the thiazole is formed by combining a cysteine with a serine with dehydration. The biosynthesis of the pyridine ring is similar to piperidine/hydroxypyridine ring formation in thiostrepton and nosiheptide. The biogenesis of berninamycin A is also discussed.

Berninamycin A belongs to the family of thiopeptide antibiotics[2] whose mode of action is the inhibition of protein synthesis in Gram-positive bacteria.[3–5] The structure of berninamycin A (**1**) was first studied by Liesch and Rinehart.[6–8] More recently, Abe et al. proposed a revised structure of berninamycin A (**2**) based on degradation findings obtained during structural studies on sulfomycin I and through COSY and NOESY studies on berninamycin A.[9,10] In the present paper we have conducted FABMS/MS and 2-D NMR studies on berninamycin A, the results of which agree with the structure proposed by Abe et al.

Pearce and Rinehart studied the biosynthesis of berninamycin A with ^{14}C-labeled amino acids.[11] Biosyntheses of nosiheptide and thiostrepton, two other members of the thiopeptide antibiotics, were recently reported by Floss, Beale, et al.[12,13] Feeding experiments with ^{13}C-enriched amino acids in the present study confirm our earlier results with ^{14}C-labeled compounds and show that the biosynthetic pattern of berninamycin A is similar to those of nosiheptide and thiostrepton.

Results and Discussion

A. Structure Studies. The structure of berninamycin A was reinvestigated in the present work since the original paper of Abe et al. provided little information on their structural studies



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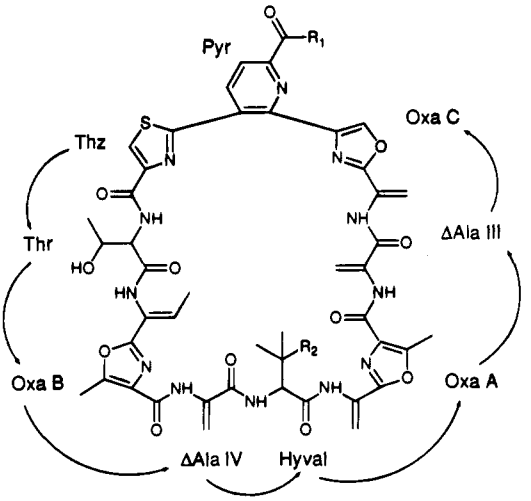

2: Berninamycin A: R_1 = ΔAla(II)-ΔAla(I)-NH_2, R_2 = OH
3: Berninamycin B: R_1 = ΔAla(II)-ΔAla(I)-NH_2, R_2 = H
4: Berninamycin C: R_1 = ΔAla(II)-NH_2, R_2 = OH
5: Berninamycin D: R_1 = NH_2, R_2 = OH

of berninamycin A.[10] Berninamycin A was purified through repeated SiO_2 chromatography. LRFABMS showed the molecular ion at *m/z* 1146 (M + H), and HRFABMS data agreed

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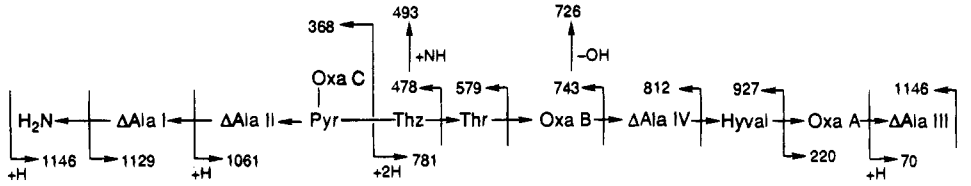
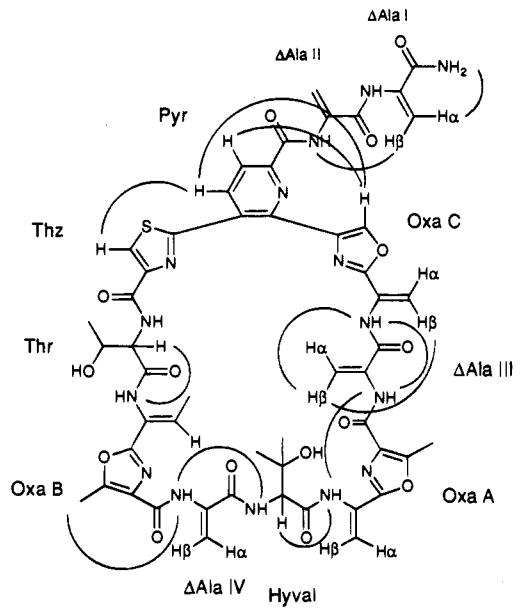
Scheme 1



Scheme 2



with the molecular formula $C_{51}H_{51}N_{15}O_{15}S$ (Δ −1.7 mmu), which contains one nitrogen and one hydrogen more and one oxygen less than our original proposal (**1**)[7,8] but agrees with the formula of Abe et al. (**2**).[10] FABMS/MS studies on the *m/z* 1146 ion suggested that the cyclic peptide undergoes a selective bond cleavage between oxazole C and ΔAla III, and the resulting linear peptide fragments to give different daughter peaks as shown in Scheme 1. This fragmentation scheme indicates the partial amino acid sequence Thz-Thr-OxaB-ΔAla IV-Hyval-Oxa A-ΔAla III in the cyclic peptide loop of berninamycin A. This partial structure is found in **2** but would not be observed in **1**.

Relay, 2Relay, and 2-D NOE studies were conducted, and the results are summarized in Scheme 2. The presence of a dipeptide side chain terminated with a primary carboxamide group was observed, and the complete amino acid sequence of the cyclic peptide loop was assigned, which agrees with the structure **2**. When the same results are applied to **1**, it is clear that the structure of berninamycin A proposed by Liesch and Rinehart cannot account for a number of the correlations observed, e.g., the primary carboxamide functional group and its correlation with the H-3α proton of ΔAla I. In summary, results of the 2-D NMR, HRFABMS, and FABMS/MS studies argue for structure **2** proposed by Abe et al.[10] as the correct structure of berninamycin A.

B. Biosynthesis. Pearce and Rinehart fed ^{14}C-labeled amino acids to *Streptomyces bernensis* and found good incorporation of L-[U-^{14}C]serine, D,L-[1-^{14}C]cysteine, L-[U-^{14}C]cysteine, L-[U-^{14}C]lysine, L-[U-^{14}C]threonine, and D,L-[1-^{14}C]valine but not of D,L-[1-^{14}C]alanine, D,L-[4-^{14}C]aspartic acid, D,L-[1-^{14}C]glutamic acid, D,L-[1-^{14}C]butyric acid, and L-[*methyl*-^{14}C]methionine. Low rates of incorporation were obtained with D,L-[6-^{14}C]-α-aminoadipic acid and D-[U-^{14}C]glucose.[11]

They concluded that (a) the dehydroalanine units are formed from the dehydration of serine, (b) cysteine is the precursor of the thiazole ring, (c) threonine provides the carbon skeleton for major portions of the oxazole rings, (d) valine gives rise to the β-hydroxyvaline moiety, and (e) lysine is the primary precursor of the pyridine ring, probably through the ε-semialdehyde of α-aminoadipic acid.

Table 1. ^{13}C Enrichments in Berninamycin A Derived from ^{13}C-Labeled Amino Acids[a]

		enrichment from precursors (times natural abundance)[c]				
enriched carbon	δ[b]	D,L-[3-^{13}C]-Ser	D,L-[1-^{13}C]-Ser	D,L-[3-^{13}C]-Cys	L-[1-^{13}C]-Val	L-[1-^{13}C]-Thr
ΔAla I, C-3	102.5	4.4				
ΔAla I, CO	161.2		3.0			
ΔAla II, C-3	101.7	5.4				
ΔAla II, CO	163.8		3.1			
ΔAla III, C-3	103.6	3.6				
ΔAla III, CO	165.0		2.9			
ΔAla IV, C-3	103.7	4.4				
ΔAla IV, CO	162.5		3.8			
Oxa A, C-2	156.8		1.6			
Oxa A, $=CH_2$	106.0	5.1				
Oxa A, CO	159.7					5.8
Oxa B, C-2	155.9					6.1
Oxa B, CO	159.4					6.4
Oxa C, C-5	140.1	3.4				
Oxa C, $=CH_2$	103.5	4.1				
Oxa C, C-2	154.4		2.9			
Pyr, C-3	120.3	2.2				
Pyr, C-4	140.7	2.6				
Pyr, C-6	146.3		2.2			
Pyr, CO	161.2		2.8			
Thz, C-2	160.2		1.7			
Thz, C-5	125.3	1.5		4.8		
Thz, CO	163.1		6.6			
Thr, CO	168.0					5.2
Hyval, CO	169.2				10.1	

[a] ΔAla, dehydroalanine; Oxa, oxazole; Pyr, pyridine; Thz, thiazole; Thr, threonine; Hyval, β-hydroxyvaline; Ser, serine; Cys, cysteine; Val, valine; Lys, lysine. [b] $CDCl_3$. [c] D,L-[1-^{13}C]Lys gave no enrichment.

The present study repeated a number of these feedings but employing ^{13}C instead of ^{14}C to locate the carbons labeled. Each sample of berninamycin A produced was isolated, purified, and subjected to ^{13}C NMR analyses. The results are summarized in Table 1 and Scheme 3.

1. Valine, Hydroxyvaline. L-[1-^{13}C]Valine labeled specifically, and with very high enrichment, the carboxyl carbon of β-hydroxyvaline, indicating that valine was hydroxylated. To determine whether hydroxylation occurs before or after incorporation of the valine unit into the peptide, D,L-[3-^{14}C]-β-hydroxyvaline was synthesized according to the procedure of Scott and Wilkinson[14] and administered to growing *S. bernensis*. The berninamycin A produced had no significant radioactivity (Table 2), arguing that the hydroxylation of valine takes place after it is assembled into the peptide. This hypothesis was further substantiated by the isolation of a minor metabolite

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Table 2. Berninamycin A Biosynthetic Studies with ^{14}C-Labeled Amino Acids

		amino acid added		berninamycin A			
medium used	amt of whole cells added (g)	compound	amt (μCi)	activity (μCi)	amt (mg)	RCP %	% incorporation
D	20	D,L-[3-^{14}C]serine	5.73	0.0203	17.8	85	0.35
C	20	D,L-[3-^{14}C]serine	5.73	0.0702	31.7	82	1.23
C	20	D,L-[3-^{14}C]-β-hydroxyvaline	6.80				
C	20	D,L-[6-^{14}C]lysine	10.0				
C	20	L-[U-^{14}C]lysine	10.8	0.0380	18.8	87	0.35

(berninamycin B) from *S. bernensis* with a valine residue replacing the β-hydroxyvaline[15] unit.

2. Cysteine. D,L-[3-^{13}C]Cysteine enriched only the C-5 carbon of the thiazole unit, with high incorporation (4.8 × natural abundance). This argued that cysteine is the true precursor of C-4, C-5, and the carbonyl carbon of the thiazole unit rather than serine (1.5 × natural abundance at Thz C-5; see Table 1), noting that serine is the biosynthetic precursor of cysteine.[16] Formation of the thiazole ring presumably involves condensing cysteine with the carboxyl of the preceding amino acid (serine/ΔAla) followed by dehydration.

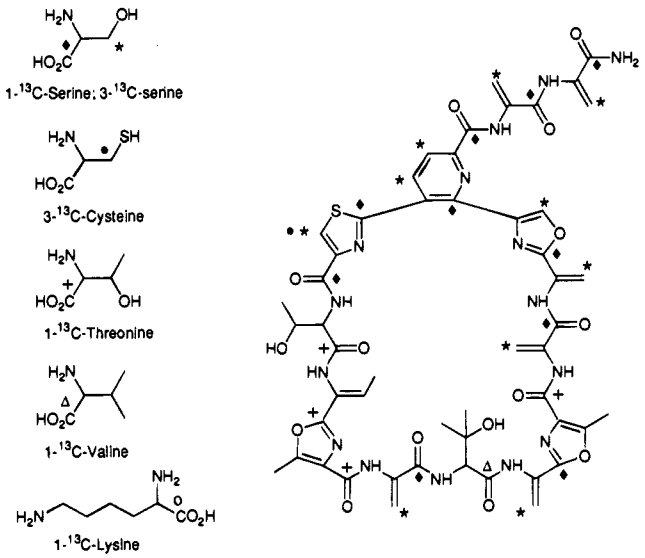
3. Threonine. L-[1-^{13}C]Threonine enriched specifically three carboxyl carbons of berninamycin A (threonine, oxazoles A and B) and the C-2 carbon of oxazole B. These enrichment patterns argue threonine condenses with serine/ΔAla to form oxazole A and with another threonine or dehydrobutyrine (ΔAba) to form oxazole B, both condensations being followed by dehydrations.

4. Serine. D,L-[3-^{13}C]Serine and D,L-[1-^{13}C]serine were fed separately. D,L-[3-^{13}C]Serine enriched the β-methylene carbons (C-3) of all four ΔAla units, the β-methylene carbons of oxazoles A and C, the C-5 carbon of oxazole C, the C-3 and C-4 carbons of the pyridine ring, and, marginally (1.5 times natural abundance), the C-5 carbon of the thiazole unit. D,L-[1-^{13}C]Serine, on the other hand, enriched the carboxyl carbons of all four ΔAla's and those of the pyridine and the thiazole units and the C-2 carbons of oxazoles A and C and the thiazole unit as well as C-6 of the pyridine ring. These enrichment patterns, shown in Scheme 3, clearly demonstrate that the ΔAla units are derived from the dehydration of serine, confirming the observation made by Pearce and Rinehart.[11]

The labeling pattern of oxazole C by D,L-[1- and 3-^{13}C]serine indicated that this unit is formed from coupling of two serine molecules, while the thiazole unit is formed from one serine and one cysteine (see above) and oxazole A is formed from one serine and one threonine, in each case followed by dehydration.

The enrichment pattern in the pyridine ring seems at first curious, with two Ser C-3 derived carbons linked to one another. More accurately, they appear to be part of two serine-derived subunits (pyridine carboxyl, C-2 and C-3; thiazole C-2, pyridine C-4 and C-5) joined tail-to-tail. This arrangement was first predicted in 1978 by Bycroft and Gowland for the pyridine ring of the micrococcin P antibiotics and the Δ^1-piperidine ring of the antibiotic thiostrepton, and an explanation was advanced.[17] Later the predicted labeling pattern (both C-3 and C-4 labeled by C-3 of serine) was substantiated by Floss, Beale, et al. for

Scheme 3



the Δ^1-piperidine ring of thiostrepton[12,13a] and for the hydroxypyridine ring of nosiheptide.[12,13b] The Floss papers also employed [1,2-$^{13}C_2$]- and [2,3-$^{13}C_2$]serine to demonstrate that serine is incorporated intact into the piperidine/hydroxypyridine rings. Since berninamycin, nosiheptide, thiostrepton, and micrococcin all belong to the same thiopeptide antibiotic family, the pyridine ring in berninamycin should be formed by a similar biosynthetic mechanism, as will be discussed below.

5. Lysine. Pearce and Rinehart earlier reported 0.24% incorporation of L-[U-^{14}C]lysine,[11] and this was repeated (0.35% incorporation, Table 2) in the present study. However, neither D,L-[6-^{14}C]Lys (Table 2) nor D,L-[1-^{13}C]Lys (Table 1) labeled berninamycin, suggesting incorporation of the uniformly labeled compound following extensive degradation, perhaps via acetoacetyl CoA, oxaloacetate, 3-phosphoglycerate, and serine.[18]

C. Biogenesis of Berninamycin A. The labeled amino acid feeding studies described above have determined the biosynthetic origins of all the modified amino acids of berninamycin A. Its biogenesis can be proposed along the following lines. A linear peptide containing all of the precursor amino acids is assembled enzymatically (nonribosomally), like most polypeptide antibiotics.[19,20] The C terminus is then constructed as a primary amide by oxidative cleavage of an adjacent amino acid, probably a Gly[21,22] or a Ser unit.[13b]

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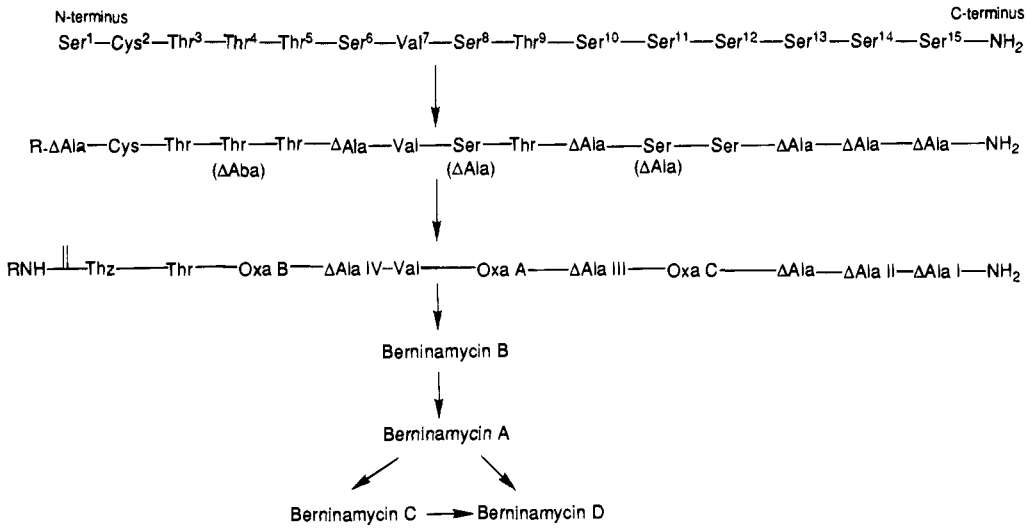
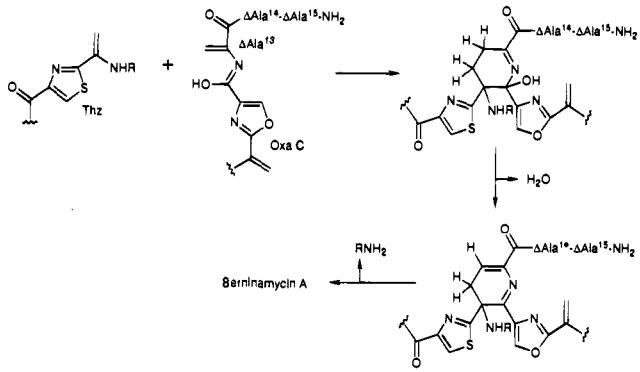
(21) Eipper, B. A.; Stoffers, D. A.; Mains, R. E. *Annu. Rev. Neurosci.* **1992**, *15*, 57–85.

Scheme 4



Scheme 5



Some insight is provided by the isolation of minor metabolites. Berninamycins C (**4**) and D (**5**) differ from berninamycin A by one or two ΔAla unit(s), respectively, at the side chain attached to the carboxyl of the picolinic acid unit.[15] Both end in primary carboxamides like berninamycin A, suggesting cleavage of a Ser unit (or perhaps hydrolysis of a ΔAla unit) to give a shorter primary amide.

The linear peptide can undergo dehydration at serines 6, 10, 13, 14, and 15 to form ΔAla's IV, III, II, and I and (perhaps) at Ser 8 and 11 to give ΔAla's that react with Thr-9 and Ser-12 to give oxazoles A and C (Scheme 4). Oxazole B and the thiazole unit can be formed similarly, while the pyridine ring would be constructed (in the penultimate step) from the ΔAla remnants of Ser^1 and Ser^{13} by a route (Scheme 5) like that proposed for micrococcin,[17] thiostrepton, and nosiheptide.[12,13] The suggested N-terminal group R, which protects the enamine from hydrolysis, is unknown. An alternative would be for the N-terminal Ser to remain until a late-stage dehydration. Perhaps the conversion of Val to Hyval is the last step in forming berninamycin A, with berninamycins C and D being formed by cleavage of berninamycin A.

Experimental Section

A. General Methods. ^{13}C, ^1H, and 2-D NMR spectra were recorded on a GN-500 spectrometer. FAB mass spectra were obtained on ZAB-SE and 70-SE-4F mass spectrometers. Microanalytical results were obtained from the School of Chemical Sciences Microanalytical Laboratory. Melting points were measured on a capillary melting point apparatus and are uncorrected; pH values were measured with a digital pH meter, Model 350. Visualization of berninamycin and other related metabolites on TLC was by exposure to UV light. Visualization of amino acids was by ninhydrin spray reagent. All media used for biosynthetic studies were sterilized at 121 °C. Incubation of media containing *S. bernensis* was conducted in a rotary shaker at 29−30 °C and 230 rpm for the time period indicated. Biological detection of antimicrobial metabolites was by the paper disk assay method, according to Loo et al., on *Bacillus subtilis*.[23]

B. Culture Conditions. *S. bernensis* was obtained as a soil stock from The Upjohn Co. and stored at 4 °C until used. A few grains of these soil stocks were added to 100 mL of sterilized seed medium and incubated at 30 °C for 3 days. An inoculum (0.1 mL) of this active seed medium was spread onto an agar plate and incubated at 30 °C for at least 4 days until a thick sporulating lawn of *S. bernensis* colonies was formed. The agar plate was then stored at 4 °C until used.

1. Medium A (Seed Medium). Medium A consisted of 2.5% Pharmamedia and 2.5% glucose. One hundred milliliters of medium A in tap water was transferred to a 500-mL Erlenmeyer flask and sterilized.

2. Medium B (Complex Production Medium). Medium B consisted of 1% dextrin, 0.1% Difco beef extract, 0.1% Difco yeast extract, 0.2% Wilson's peptone, and 0.002% cobalt chloride. The pH was adjusted to 7.2 with 1 M sodium hydroxide. One hundred milliliters of this medium was transferred to a 500-mL Erlenmeyer flask and sterilized.

3. Medium C (Synthetic Production Medium). Medium C consisted of 0.15% potassium dihydrogen phosphate, 0.05% magnesium sulfate heptahydrate, 0.004% calcium chloride dihydrate, 0.005% ferrous sulfate heptahydrate, 0.0005% zinc sulfate heptahydrate, 0.1% glucose, 0.1% L-glutamic acid, 0.05% sodium nitrate, and 10% (v/v) corn oil. The pH was adjusted to 7.2 with potassium hydroxide. One hundred milliliters of this medium was transferred to a 500-mL Erlenmeyer flask and sterilized.

4. Basal Salt Solution. The basal salt solution consisted of the same ingredients as medium C with the exclusion of glucose, L-glutamic acid, sodium nitrate, and corn oil.

C. Metabolite Production. A sterilized inoculating loop was used to scrape the surface of the sporulating lawn of *Streptomyces* colonies onto an agar plate. The inoculating loop was then dipped into 100 mL of sterilized medium A. Medium A was incubated for 72 h, and 5 mL of this seed medium was transferred to 100 mL of medium B. Medium B was incubated for 4−5 days, and the thick mycelia in 500 mL of medium B were harvested by centrifugation in a Sorvall centrifuge at 5000 rpm for 15 min. The mycelial pellet was washed with the basal salt solution and centrifuged again. This process was repeated three times, and the mycelial pellet was again suspended in

(22) Grimmelikhuijzen, C. J. P.; Darmer, D.; Schmutzler, C.; Carstensen, K.; Moosler, A.; Nothacker, H.-P.; Reinscheid, R. K.; Vollert, H.; Rinehart, K. L.; McFarlane, I. D. *Prog. Brain Res.* **1992**, *92*, 137−148.

(23) Loo, Y. H.; Skell, P. S.; Thornberry, H. H.; Ehrlich, J.; McGuire, J. M.; Savage, G. M.; Sylvester, J. C. *J. Bacteriol.* **1945**, *50*, 701−709.

20 mL of sterilized basal salt solution. This mycelial suspension was then transferred with a sterilized pipet to 100 mL of sterilized medium C and incubated for 6 days. The medium was then harvested.

D. Isolation of Antibiotic. Medium C was initially extracted twice with hexane to remove any remaining corn oil left in the medium and then extracted thrice with ethyl acetate and thrice with 1-butanol. It was sometimes necessary to use centrifugation (5000 rpm, 15 min) to disperse the thick emulsions formed. The organic extracts were combined, dried over anhydrous Na_2SO_4, and evaporated to dryness to give a brown residue. The residue was dissolved in methanol, the insoluble particulates were removed by filtration, and the methanol solution was again evaporated to dryness to give a yellow solid. This solid contained a 1:1 mixture (TLC, MeOH/CH_2Cl_2, 5:95) of berninamycin and a less polar, UV-active compound.

E. Purification of Antibiotic. Crude berninamycin (1 g) was dissolved in CH_2Cl_2 and applied to a column of SiO_2 (10 g, 100 cm × 1 cm). The column was initially washed with EtOAc/hexane (1:1) and eluted with MeOH/CH_2Cl_2 (4:96). Fractions of 7 mL were collected with a microfractionator, and berninamycin A and other metabolites were detected with TLC and UV light. Berninamycin A was eluted in fractions 21−40 and was about 85% pure. This berninamycin A was further purified by dissolving it in methanol and methylene chloride and adding 3 g of SiO_2; the mixture was then evaporated to dryness and dried under vacuum overnight. This dried berninamycin−SiO_2 mixture was placed on an SiO_2 column (10 g, 100 cm × 1 cm). The column was then eluted with acetone/toluene (3:7). Fractions of 7 mL were collected with a microfractionator, and berninamycin A and other metabolites were detected with TLC and UV light. Fractions 15−30 were combined and evaporated to dryness in a water bath at less than 50 °C. The resulting white solid showed a single spot on TLC and was identical to an authentic sample of berninamycin A: LRFABMS, *m/z* 1146 (M + H); 1300 [M + H + MB (matrix)], 1455 (M + H + 2MB).

Anal. Calcd. for $C_{51}H_{52}N_{15}O_{15}S$ (M + H): M_r 1146.3522. Found: M_r 1146.3505 (Δ −1.7 mmu, HRFABMS).

F. Feeding of Labeled Amino Acids. 1. General. D,L-[3-^{14}C]-Serine (22.92 μCi; sp act. 0.125 mCi/mmol) was purchased from Amersham; L-[U-^{14}C]lysine (10.8 μCi; sp act. 300 mCi/mmol), D,L-[6-^{14}C]lysine (10 μCi; sp act. 45 mCi/mmol) were from Research Products International Corp. D,L-[3-^{13}C]Serine (100 mg; 99.9% ^{13}C enriched) was purchased from MSD Isotopes and L-[1-^{13}C]threonine (100 mg; 99.9% ^{13}C enriched) from ICON Services Inc. D,L-[3-^{13}C]-Cysteine (100 mg; 99.9% ^{13}C enriched), D,L-[1-^{13}C]lysine (100 mg; 99.9% ^{13}C enriched), D,L-[1-^{13}C]serine (100 mg; 99.9% ^{13}C enriched), and L-[1-^{13}C]valine (100 mg; 99.9% ^{13}C enriched) were from Cambridge Isotope Laboratories. One hundred milligrams of ^{13}C labeled amino acid was dissolved in 10 mL of deionized water. The solution was neutralized with 1 M NaOH solution if necessary and added through a sterilization filter to 5 × 100 mL of sterilized medium C. Approximately 1 kg (wet weight) of *S. bernensis* whole cells, harvested from 40 × 100 mL of 4−5-day-old medium B, was added to the 5 × 100 mL of medium C, and the flasks were incubated at 30 °C for 6 days. The 6-day-old media were combined and extracted. The berninamycin A isolated was purified with SiO_2 column chromatography until chromtographically pure and subjected to ^{13}C NMR studies. Berninamycin A produced in the presence of ^{14}C-labeled amino acid was purified repeatedly with SiO_2 column chromatography until radio-TLC scanning showed constant radiochemical purity (RCP). The typical yield of purified berninamycin A in 500 mL of medium C, following feeding of ^{13}C-labeled amino acids, was between 25 and 35 mg (50−70 μg/mL).

2. Feeding of D,L-[3-^{14}C]-β-Hydroxyvaline (5). D,L-[3-^{14}C]-β-Hyval (6.8 μCi, sp act. 8.86 mCi/mmol) was dissolved in 10 mL of deionized water, adjusted to pH 7.2 with 1 M NaOH, and added to 5 × 100 mL of medium C. Berninamycin A produced was isolated and purified, and the radioactivity and radiochemical purity were determined. The result is shown in Table 2. No degradation study was conducted.

G. Preparation of Labeled Precursor. 1. *N,N*-Dibenzylglycine Ethyl Ester. Dibenzylamine (78.8 g, 0.40 mol) was added dropwise under nitrogen to a stirred solution of ethyl bromoacetate (33.4 g, 0.20 mol) in 250 mL of acetonitrile at 0 °C. The resulting white mixture was stirred at room temperature for 24 h. The white precipitate was filtered and washed with CH_2Cl_2, and the washing was combined with the filtrate and evaporated to dryness to give an oily residue. The residue was then dissolved in Et_2O, washed thrice with a 1:1 mixture of 5% $NaHCO_3$/brine and twice with deionized water, dried over anhydrous Na_2SO_4, and evaporated to dryness to give a yellow oil. The yellow oil was dissolved in absolute ethanol and allowed to crystallize at 20 °C. White needle-shaped crystals formed and were filtered, and the filtrate was concentrated and cooled to give a second crop of off-white crystals. The total yield was 4.6 g (82%): mp 55−56 °C (lit.[15] 55−56 °C).

2. [3-^{14}C]-*N,N*-Dibenzyl-β-hydroxyvaline Ethyl Ester. Lithium diisopropylamide (1.17 g, 11.0 mmol) and anhydrous $MgBr_2$ (1.01 g, 11.1 mmol) were suspended under argon in 10 mL of anhydrous THF at −78 °C, and dried *N,N*-dibenzylglycine ethyl ester (1.42 g, 5 mmol) in 10 mL of anhydrous THF was added dropwise to the stirred suspension. The mixture was stirred under argon at −78 °C for 30 min, and then [*carbonyl*-^{14}C]acetone (50 mCi, sp act. 100 mCi/mmol) diluted with 1 mL of acetone (freshly distilled over 4 Å molecular sieves) and 20 mL of anhydrous THF was added dropwise; the mixture was stirred for another 30 min. Ammonium chloride solution (15 g in 5 mL of deionized water) was added, and the yellowish mixture was allowed to stir while warming slowly to room temperature. The organic solvent was removed under vacuum, and the resulting yellow oily residue was partitioned between Et_2O and deionized water. The organic layer was separated and washed (three times) with a 1:1 mixture of 5% $NaHCO_3$ and brine and twice with deionized water, dried over anhydrous Na_2SO_4, and evaporated to dryness to give a yellowish solid. The solid was dissolved in a small amount of EtOH and allowed to crystallize at −20 °C. Recrystallization was repeated twice in EtOH to give radiochemically pure product (1.5 g, RCP = 98%, sp act. 8.63 mCi/mmol): mp 76 °C (lit.[14] 76−77 °C).

[3-^{14}C]-β-Hydroxyvaline. To a solution of [3-^{14}C]-*N,N*-dibenzyl-β-hydroxyvaline ethyl ester (1.5 g, sp act. 8.63 mCi/mmol) in 100 mL of MeOH was added 10 mL of concentrated H_2SO_4. The solution was flushed with nitrogen, and ca. 1 g of 5% Pd/C was added to the solution. A slow stream of hydrogen gas was bubbled through the stirred mixture at room temperature, and the hydrogenation was allowed to proceed overnight. The catalyst was removed by filtration and the yellow solution evaporated to dryness to give a yellow oil. The oil was dissolved in 100 mL of 1 M HCl solution, which refluxed overnight. The brown solution was concentrated to a small volume and applied to a column of Dowex 50WX8 (H^+, 100 cm × 1 cm), which was flushed with deionized water. Gradient elution with deionized water in the mixing chamber and 1 M NH_4OH in the adding chamber resulted in elution of the desired product in fractions (7 mL) 36−45. The fractions were combined and evaporated to dryness to give a yellow oil. The oil migrated as a single spot on TLC when cospotted with an authentic sample of β-hydroxyvaline (510 mg, RCP = 99%, sp act. 8.86 mCi/mmol). Precipitation with ethanol/water gave a white solid: mp 219 °C (lit.[14] 218 °C).

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