

BERNINAMYCINS B, C, AND D, MINOR METABOLITES FROM *Streptomyces bernensis*

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Berninamycins B, C, and D were isolated from fermentation of *Streptomyces bernensis* and their structures were studied with ¹³C NMR and FAB mass spectrometry. Berninamycin B has a valine unit in its cyclic peptide loop instead of the β-hydroxyvaline unit found in berninamycin A. Berninamycin D has two fewer dehydroalanine units attached to the carboxyl carbon of the pyridine ring. Based on FAB-MS results, berninamycin C is postulated to have only one dehydroalanine unit attached to the carboxyl carbon of pyridine. The biogenesis of berninamycins B, C, and D is discussed.

Berninamycin A belongs to the thiopeptide antibiotic family^{1~7}), a class of sulfur-containing, highly modified cyclic peptides which share some common structural characteristics—first, a heterocyclic centerpiece consisting of a pyridine (picolinic acid) or reduced pyridine ring connected to two or more thiazole or oxazole rings; second, a cyclic peptide comprised of highly modified amino acids attached to the heterocyclic centerpiece; third, a peptide side chain of dehydroalanines (ΔAla's), common in many members of the family. The mode of action of this family of antibiotics is inhibition of protein biosynthesis in Gram-positive bacteria through binding with their ribosomal subunits^{8~10}.

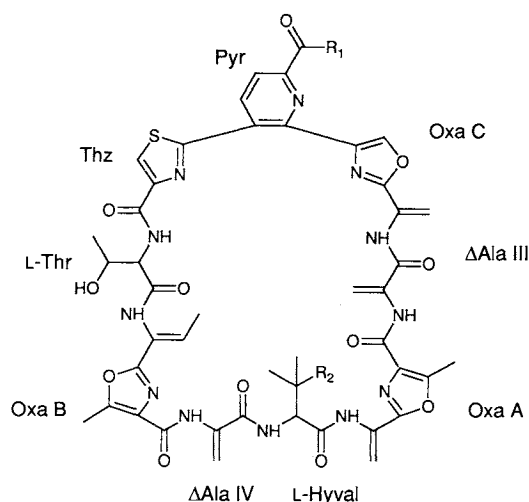
The structure of berninamycin A was first assigned by LIESCH and RINEHART^{11~13}), but a revised structure was postulated by ABE, *et al.*¹⁴) and was recently confirmed by us¹⁵). The revised structure of berninamycin A is shown in Scheme 1. LIESCH, *et al.* reported isolation of a minor metabolite, berninamycin B, from the fermentation extract of *S. bernensis*¹²). Elemental analyses suggested that the molecular formula of berninamycin B was C₅₉H₇₄N₁₄O₂₂S, which corresponded to a molecular weight of 1362, but no further structural study was reported.

In the present study five distinct peaks were observed when berninamycin was subjected to reversed-phase HPLC analysis. The compounds in these peaks were isolated and three were found to have structures related to berninamycin A. Their structural studies are reported below.

Results and Discussion

Berninamycins were isolated as a mixture of five components from the fermentation extract of

Scheme 1.



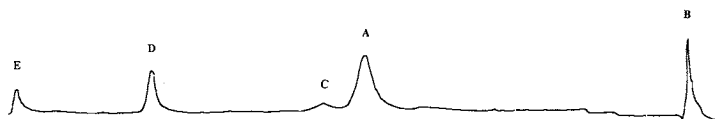
- Berninamycin A: $R_1 = \Delta\text{Ala II}-\Delta\text{Ala I}-\text{NH}_2$, $R_2 = \text{OH}$
 Berninamycin B: $R_1 = \Delta\text{Ala II}-\Delta\text{Ala I}-\text{NH}_2$, $R_2 = \text{H}$
 Berninamycin C: $R_1 = \Delta\text{Ala II}-\text{NH}_2$, $R_2 = \text{OH}$
 Berninamycin D: $R_1 = \text{NH}_2$, $R_2 = \text{OH}$

S. bernensis, with berninamycin A as the major component (45%). Individual berninamycins can be separated by reversed-phase HPLC; the chromatogram is shown in Fig. 1. The purified peaks (peaks A~E) were subjected to FAB-MS and NMR analyses. FAB-MS showed that peak E had the molecular weight 388 (LRFAB-MS, M+H, m/z 389) and is presumably structurally unrelated to berninamycin; no further study of it was conducted. LRFAB-MS and HRFAB-MS of peak A showed that its molecular weight and molecular formula, 1145 (M+H, m/z 1146) and $C_{51}H_{51}N_{15}O_{15}S$ ($\Delta 1.5$ mmu), respectively, corresponded to those of berninamycin A. The proton NMR spectrum of peak A also matches closely the proton NMR spectrum of berninamycin A.

Berninamycin B: Peak B was found to be berninamycin B. Peak B was compared to the berninamycin B sample obtained by LIESCH. Both samples had the same retention time on RP-C18 HPLC and when they were co-injected into the column, they co-eluted.

The molecular weight of berninamycin B was 1129 (LRFAB-MS, M+H, m/z 1130) and HRFAB-MS revealed the molecular formula was $C_{51}H_{51}N_{15}O_{14}S$ ($\Delta 1.2$ mmu), differing from that of berninamycin A by one oxygen atom. Thus berninamycin B can also be called deoxyberninamycin A.

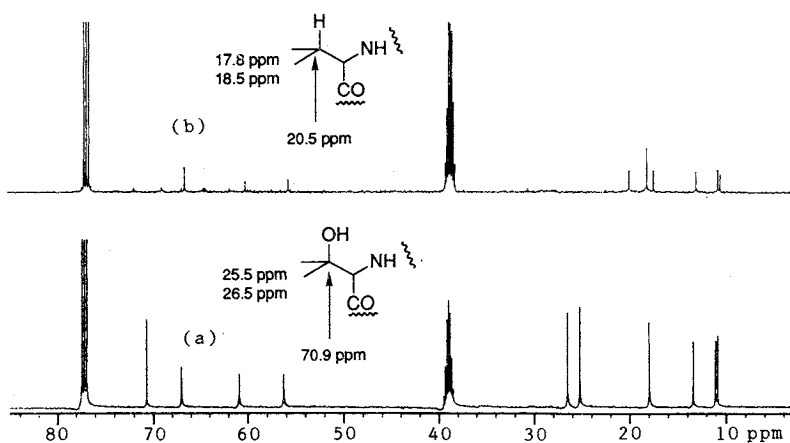
Fig. 1. Semi-preparative RPHPLC chromatogram of berninamycins.

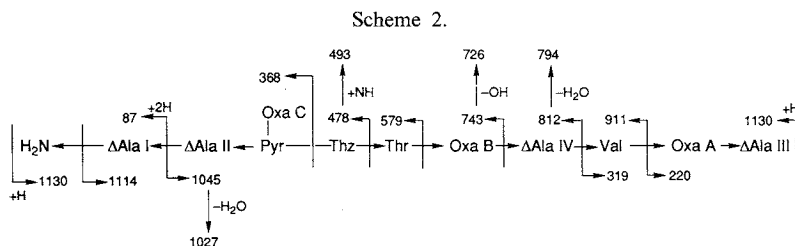


Peak	Solvent (Dioxane - Water)	Retention time (minutes)	Relative percent (%)
E	45:55	8	7.4
D	45:55	24	21.3
C	45:55	44	5.8
A	45:55	50	47.1
B	60:40	14 (64)*	18.4

* The solvent was changed after Peak A.

Fig. 2. ^{13}C NMR spectra of berninamycin A (a) and berninamycin B (b) in D_2O ; expanded region from 0 ppm to 85 ppm.





The ^{13}C NMR spectrum of berninamycin B showed that there are only minor differences between the structures of berninamycins A and B. Fig. 2 compares the ^{13}C NMR spectra of berninamycin A and berninamycin B in the 0 to 90 ppm region. In the ^{13}C NMR spectrum of berninamycin B, the signal at 70.9 ppm corresponding to the quaternary β -carbon (C-3) of β -hydroxyvaline was not found, but an extra carbon signal appeared at 20.5 ppm. The two methyl signals of β -hydroxyvaline in berninamycin A were also shifted upfield in the spectrum of berninamycin B (to 17.8 and 18.5 ppm), and the lower field signal (18.5 ppm) overlapped with the C-4 methyl carbon signal of threonine. These data suggested that berninamycin B has a valine unit in its cyclic peptide loop instead of the β -hydroxyvaline unit found in berninamycin A.

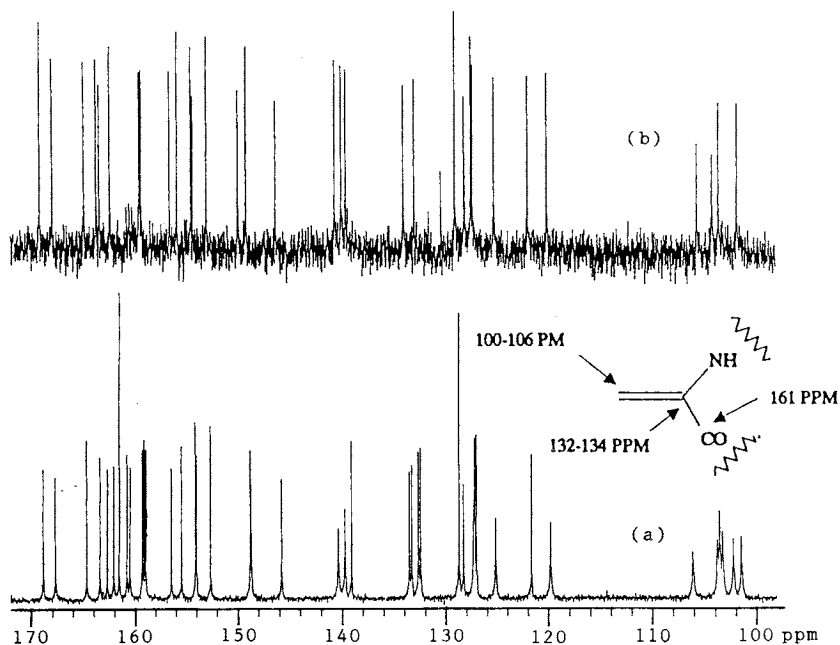
FAB-MS/MS study also provided further evidence for the structure of berninamycin B. A FAB-MS/MS spectrum of the $M+H$ (m/z 1130) ion of berninamycin B showed daughter ions (Scheme 2) indicating that berninamycin B underwent a specific bond cleavage, similar to that observed in berninamycin A FAB-MS/MS studies¹⁵⁾, between oxazole C and Δ Ala III to give a linear peptide which then fragmented to give all the daughter peaks observed in the FAB-MS/MS study. In particular, the presence of m/z 911 and 812 daughter peaks indicated that the amino acid sequence Val-Oxa A- Δ Ala III is present in the linear cleavage peptide, and confirmed the presence of a valine unit in berninamycin B in place of β -hydroxyvaline.

Berninamycin D: The compound in peak D was found to have the molecular weight 1007 (LRFAB-MS, $M+H$, m/z 1008) and molecular formula $\text{C}_{45}\text{H}_{45}\text{N}_{13}\text{O}_{14}\text{S}$ (HRFAB-MS, $\Delta 0.5$ mmu). This molecular formula differs from that of berninamycin A by 2 Δ Ala units and the compound in peak D has been named berninamycin D.

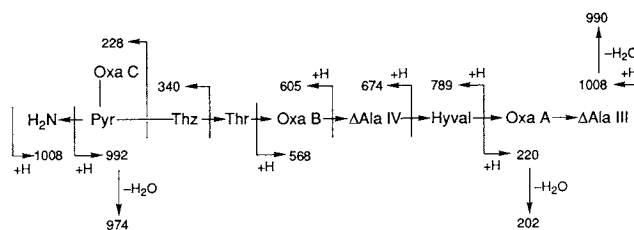
In comparing the ^{13}C NMR spectrum of berninamycin A¹⁵⁾ to that of berninamycin D it is clear that two Δ Ala units are the only structural differences. Fig. 3 compares the ^{13}C NMR spectra of berninamycin A and berninamycin D in the 100 to 170 ppm region. The two-carbonyl carbon signal at 162 ppm, corresponding to the carbonyl carbons of two Δ Ala units in the ^{13}C NMR spectrum of berninamycin A, was not found in the spectrum of berninamycin D. Four carbon signals at 132 to 134 ppm, corresponding to the α -methylene carbons of four Δ Ala units, were observed in the ^{13}C NMR spectrum of berninamycin A. Two signals were observed in the same region in the spectrum of berninamycin D. Similarly, between 100 and 106 ppm, six signals corresponding to the α -methylene carbons of oxazole A, oxazole C, and four Δ Ala units were observed in the ^{13}C NMR spectrum of berninamycin A, but in the ^{13}C NMR spectrum of berninamycin D only 4 signals were observed in the same region. These findings further confirmed that berninamycin D differs from berninamycin A by two Δ Ala units.

Additional evidence for the structure of berninamycin D was obtained from FAB-MS/MS study. Analogous to the previous MS/MS studies on berninamycins A and B, the FAB-MS/MS spectrum of berninamycin D showed fragmentation peaks at m/z 990, 974, 789, and 674. These daughter peaks were

Fig. 3. ^{13}C NMR spectra of berninamycin A (a) and berninamycin D (b) in D_2O ; expanded region from 100 ppm to 170 ppm.



Scheme 3.

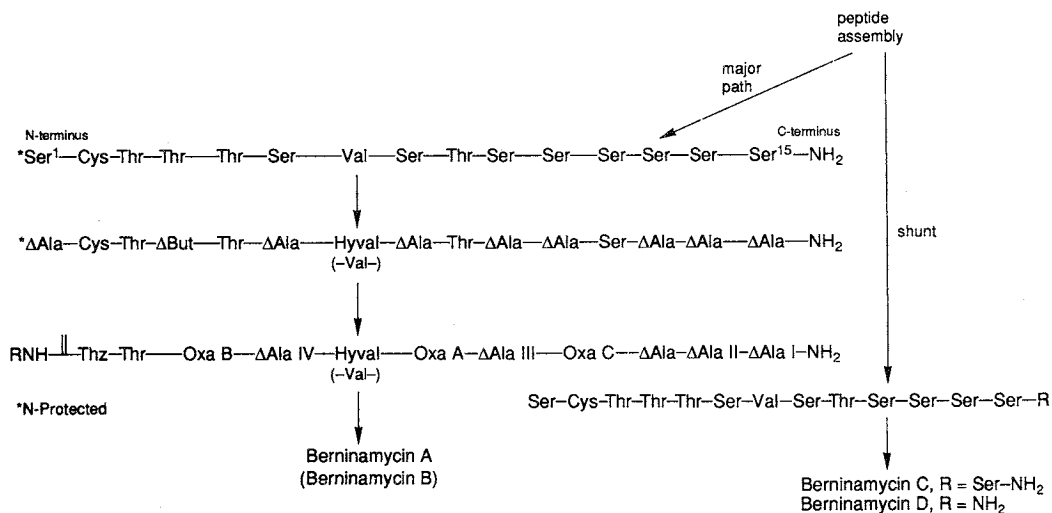


formed by fragmentation of the linear peptide resulting from the same specific bond cleavage between oxazole C and $\Delta\text{Ala III}$. The fragmentation scheme is shown in Scheme 3. The m/z 974 daughter ion fragment agrees with the postulate that berninamycin D differs from berninamycin A only in the dipeptide side chain: berninamycin D has the $-\text{NH}_2$ group of the primary carboxamide attached directly to the picolinic acid carbonyl.

Berninamycin C: Peak C contained the least abundant of all the metabolites isolated, berninamycin C. The molecular weight of the compound in peak C was 1076 (LRFAB-MS, $\text{M}+\text{H}$, m/z 1077) and the molecular formula was $\text{C}_{48}\text{H}_{48}\text{N}_{14}\text{O}_{14}\text{S}$ (HRFAB-MS, $\Delta 1.1$ mmu). This molecular formula differs from that of berninamycin A by one ΔAla unit. No further structural study was conducted since only about 1 mg of berninamycin C was isolated from about 1 g of the berninamycin mixture. We propose that berninamycin C differs from berninamycin A in their peptide side chains. Berninamycin C has a single ΔAla unit, present as a primary carboxamide, attached to the carbonyl group of the pyridine ring. The proposed structures of berninamycins A, B, C, and D are shown in Scheme 1.

Biogenesis: Berninamycins B, C and D are probably shunt metabolites of the biosynthetic pathway

Scheme 4.



that produces berninamycin A. Premature addition of the primary amide to the carboxyl terminus of the growing linear peptide may result in early termination of the linear peptide biosynthesis. The trideca- or tetradecapeptide can then undergo the same modification and cyclization processes as the linear pentadecapeptide precursor of berninamycin A to give berninamycins C and D. Hydroxylation of valine to β -hydroxyvaline may not occur with some of the linear pentadecapeptide precursor and thus result in berninamycin B. The proposed biogenesis of berninamycins B, C, and D is shown in Scheme 4.

Experimental

General Methods

¹³C, ¹H, and 2D NMR spectra were recorded on a General Electric GN-500 instrument. Mass spectra were obtained from the School of Chemical Sciences Mass Spectrometry Laboratory: LRFAB-MS on Fisons ZAB SE, HRFAB-MS and FAB-MS/MS on Fisons 70SE4F spectrometers, pH values were measured with a Beckmann 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 New Brunswick Scientific rotary shaker at 29~30°C at 230 rpm/minute for the time period indicated. Biological detection of antimicrobial metabolites was by the paper disc assay method¹⁶⁾, on *Bacillus subtilis*.

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 soil stock was added to 100 ml of sterilized seed medium and incubated at 30°C for 3 days. An 0.1-ml inoculum of this active seed medium was spread onto an agar plate and the agar plate was incubated at 30°C for at least 4 days or until a thick sporulating lawn of *Streptomyces* colonies was formed. The agar plate was then stored at 4°C until used.

Medium A (Seed Medium)

Medium A consisted of Pharmamedia 2.5%, glucose 2.5%. One hundred ml of medium A in tap water was transferred to a 500-ml Erlenmeyer flask and sterilized.

Medium B (Complex Production Medium)

Medium B consisted of Dextrin 1%, Difco beef extract 0.1%, Wilson's peptone 0.2%, cobalt chloride 0.002%. The pH was adjusted to 7.2+0.1 with 1 M sodium hydroxide. One hundred ml of this medium was transferred to a 500-ml Erlenmeyer flask and sterilized.

Medium C (Synthetic Production Medium) and Basal Salt Solution

Medium C consisted of potassium phosphate, dibasic 0.15%, magnesium sulfate heptahydrate 0.05%, calcium chloride dihydrate 0.004%, ferrous sulfate heptahydrate 0.005%, zinc sulfate heptahydrate 0.0005%, glucose 0.1%, L-glutamic acid 0.1%, sodium nitrate 0.05%, corn oil 10% (v/v). The pH was adjusted to 7.2 with potassium hydroxide. One hundred ml of medium C was transferred to a 500-ml Erlenmeyer flask and sterilized. Basal salt solution consisted of the same ingredients as medium C with the exclusion of glucose, glutamic acid, sodium nitrate and corn oil.

Metabolite Production

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

Isolation of Antibiotic

Medium C was initially extracted twice with hexane, which extracted any remaining corn oil left in the medium. The medium was then extracted thrice with ethyl acetate, and thrice with 1-butanol. It was sometimes necessary to separate by centrifugation (5000 rpm, 15 minutes) a thick emulsion formed. The organic extracts were combined, dried over anhydrous magnesium sulfate and evaporated to dryness under vacuum. The brown residue which resulted was redissolved in methanol, filtered and evaporated to give crude berninamycin.

Purification of Antibiotic

Crude berninamycin was dissolved in CH_2Cl_2 and applied to a column of SiO_2 (10 g, 100 cm \times 1 cm). The column was initially washed with EtOAc-hexane 1:1, and eluted with MeOH- CH_2Cl_2 4:96. The berninamycin eluted was 85% pure and was further purified by preparative HPLC.

HPLC Separation of Berninamycins A, B, C, and D

The berninamycin mixture previously purified by SiO_2 column chromatography was dissolved in a 1:1 mixture of CH_2Cl_2 -MeOH and passed through an RP-C18 Sep Pak twice, and the Sep Pak was washed with MeOH. The effluents were combined, concentrated, and filtered through a 2- μm membrane filter. Ten μl of the filtered solution was injected into an Alltech RP-C18 steel column (10 μm semi-preparative) and eluted with dioxane-water (45:55) at a rate of 2 ml/minute. Peaks A to E were eluted in the order EDCAB (Fig. 1). The relative amounts of the peaks and their retention times are shown in the Table in Fig. 1. The solvent ratio was changed to 60:40 (dioxane-water) after peak A was eluted to facilitate the elution of peak B.

Five hundred μl of the berninamycin solution was injected into a Rainin preparative RP-C18 steel column and eluted with dioxane-water (45:55) at a rate of 4.0 ml/minute. Each of the five peaks was collected and reinjected into an RP-C18 semi-preparative steel column to check their purities. The peaks were collected again if necessary and dried to give off-white powders.

FAB-MS: Peak E, m/z 389 (M+H); Peak D, m/z 1008 (M+H). Anal Calcd for $\text{C}_{45}\text{H}_{46}\text{N}_{13}\text{O}_{13}\text{S}$ 1008.3059, found 1008.3074 (Δ 1.5 mmu). Peak C, m/z 1077 (M+H). Anal Calcd for $\text{C}_{48}\text{H}_{49}\text{N}_{14}\text{O}_{14}\text{S}$ 1077.3273, found 1077.3262 ($-\Delta$ 1.1 mmu). Peak A, m/z 1146 (M+H). Anal Calcd for $\text{C}_{51}\text{H}_{52}\text{N}_{15}\text{O}_{15}\text{S}$

1146.3488, found 1146.3503 ($\Delta 1.5$ mmu). Peak B, m/z 1130 (M+H). Anal Calcd for $C_{51}H_{52}N_{15}O_{14}S$ 1130.3539, found 1130.3527 ($-\Delta 1.2$ mmu).

Acknowledgments

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