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Isolation and identification of berninamycin A from *Streptomyces atroolivaceus*

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ABSTRACT

Berninamycins A, a potent antibacterial agent which was originally isolated from *Streptomyces bernensis*. In this report, berninamycin A was isolated from acetone extract of *S. atroolivaceus* NBRC 12741 (type strain) and identified using NMR and MS spectra. The identification of berninamycin A was accomplished by analysis of NMR experiments including COSY, HMQC, HMBC, ROESY spectra. Since the absolute stereochemistry of berninamycin A was not determined before, in this report the absolute stereochemistries of Thr and hydroxyl Val in berninamycin A were determined to be L-form by chemical analysis of modified Marfey's Method. Taken together with analysis ROESY spectrum, the whole absolute chemistry of berninamycin A was determined. The production of berninamycin A by *S. atroolivaceus* was estimated as high as 19 μg/mg (quantity of berninamycin A /wet weight of whole cells and agar) by HPLC analysis, which indicated that *S. atroolivaceus* was efficient producer of berninamycin A.

KEYWORDS

Berninamycin;
Streptomyces atroolivaceus;
NMR spectrum;
Antibiotic;
Stereochemistry.

INTRODUCTION

The thiopeptide antibiotics are a class of sulfur containing highly modified cyclic peptides with interesting biological properties[1]. So far, structurally wide varieties of thiopeptide antibiotics including important substance such as thiostrepton have been isolated mainly from actinomycetes[2-5]. The structure of antibiotics in this class mostly contains heterocyclic rings such as thiazole, oxazole and pyridine, and dehydrated amino acids including dehydroalanine and dehydrobutyric acid[6]. The mode of action of this antibiotic family is potent inhibition of protein biosynthesis in broad range of Gram-positive bacteria by binding to their ribosomal subunits[7,8]. On the other hand, several thiopeptides were reported to induce the tipA gene, which produces the proteins TipAL and TipsAS that belong to the MerR family of transcription regulators[9]. This class of thiopeptides has very importance not only as antibacterial agents but also as biochemical reagents.

Berninamycins A-D were isolated from *S. bernensis*, however there have not been any reports of isolation of berninamycins from other streptomycetes expect for the original strain *S. bernensis*[10-13]. In the course of screening for potent antibacterial agents, we isolated and identified berninamycin A from *Strepto-*

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myces atroolivaceus. In addition, the absolute stereochemistry of berninamycin A was determined by using NOE experiments and modified Marfey's Method as shown in Figure 1. Here we describe isolation and identification of berninamycins from *S. atroolivaceus*.

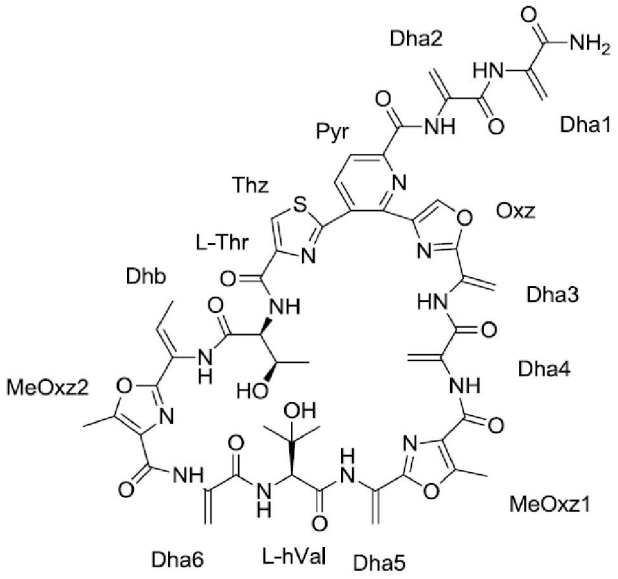

Figure 1 : Chemical structure of berninamycin A

MATERIALS AND METHODS

General methods

^{1}H and ^{13}C NMR spectra were obtained with a JOEL ECA-600 in DMSO-d_6 at 27.0 °C. The resonances of residual DMSO-d_6 at δ_H 2.49 and δ_C 39.5 were used as internal references for ^{1}H and ^{13}C NMR spectra, respectively. ESI-MS spectra were recorded by a JOEL JMS-T100LP mass spectrometer.

Bacterial strain

S. atroolivaceus (type strain, NBRC12741) was obtained from the NBRC culture collection (NITE Biological Resource Center, Japan). Cultivation was performed using ISP2 agar medium[14] with incubation at 30 °C for 6 days.

Isolation of berninamycin A

ISP2 agar medium (500 mL) which *S. atroolivaceus* grew on was extracted with 500 mL of acetone. The acetone extract were concentrated to an aqueous suspension and was subjected to open column chromatography (CHP20P, 5 × 10 cm) eluted with 20%MeOH, 60%MeOH, and MeOH. The MeOH fraction was subjected to reversed-phase HPLC using ODS column (Nacalai Tesque, Cosmosil MSII 4.6 × 250 mm). Sequential two step elution was performed for HPLC separation; step A: isocratic elution for 5 min with the solvent system consisted of MeCN/H_2O/TFA (30:70:0.1) with 1 mL/min flow rate; Step B: gradient elution for 25 min from MeCN/H_2O/TFA (30:70:0.1) to MeCN/H_2O/TFA (70:30:0.1) with 1 ml/min flow rate. The UV detector of HPLC was set at the absorbance of 220 nm to yield berninamycin A (**1**) as a colorless amorphous powder: HRFABMS *m/z* 1168.3299 $[M+Na]^+$ calcd for $C_{51}H_{51}N_{15}O_{15}SNa$ (Δ -0.78 mmu).

Marfey analysis of amino acids

Thr and hydroxyl Val was treated with 10 % Me_2CO solution of 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) or 1-fluoro-2,4-dinitrophenyl-5-D-leucinamide (D-FDLA, modified Marfey's reagent) in 1 M $NaHCO_3$ at 80-90 °C for 3 min followed by neutralization with 50 µL of 2N HCl. The reaction mixture was dissolved in 50% MeCN and subjected to reversed-phase HPLC: column, Cosmosil MS (4.6 × 250 mm), gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (60:40:0.1) in 60 min, flow rate 1ml/min, UV detector (340 nm). Retention times (min) of derivatized amino acids were found as follows:L-Thr-L-FDLA (30.0), L-Thr-D-FDLA (35.4), L-allo-Thr-L-FDLA (33.5), L-allo-Thr-D-FDLA (35.4), L-hVal-L-FDLA (30.7), L-hVal-D-FDLA (35.0).

RESULTS AND DISCUSSION

The culture of *S. atroolivaceus*[15] using ISP2 agar medium was extracted by acetone and the acetone extract was filtered and concentrated to aqueous residue by the rotary evaporator. The acetone extract was subjected to open column chromatography using hydrophobic resin eluted with 20% MeOH, 60% MeOH, and MeOH. Berninamycin A was isolated by repeatedly subjecting MeOH fraction to preparative HPLC (Figure 2).

The identification of berninamycin A was accomplished by the combination of MS and NMR spectrum data. The High Resolution ESI-MS spectrum data determined the molecular fomula as $C_{51}H_{51}N_{15}O_{15}S$. The ^{1}H and ^{13}C NMR spectral data was summarized in

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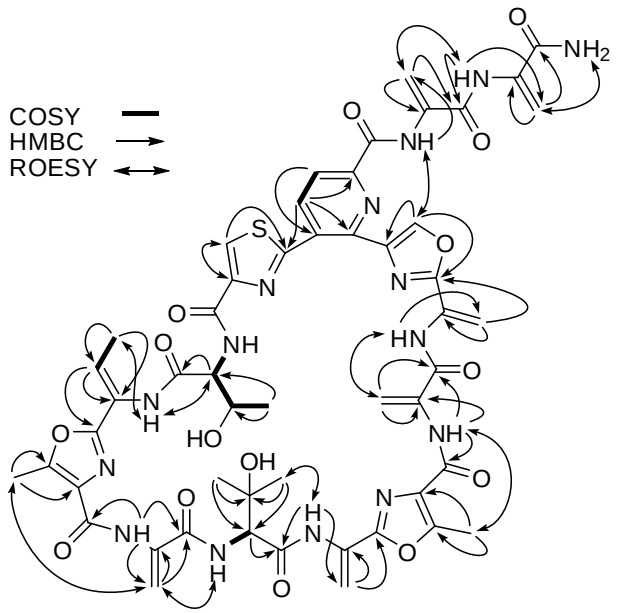

Figure 2: Selected 2D NMR correlations of berninamycin A

TABLE 1. As shown in figure 2, 6 mole of dehydroalanine (Dha) were assigned by the analysis of HMBC correlations (half end arrow) from olefinic methylene protons at β-position to α-carbon and from amide protons to β-carbons. The presence of 2,3,6-trisubstituted pyridine residue was indicated by HMBC correlations (H4/C2, H4/C6, H5/C3) and the large vicinal-coupling constant (8.3 Hz) between two aromatic doublet protons at 8.20 and 8.49 ppm. The unit of dehydrobutyric acid (Dhb) was assigned by HMBC correlations from γ-methyl protons to α- and β-carbons, and ROESY correlations between amide proton and γ-methyl protons. The heterocyclic rings including 2 mole of methyl oxazole and 1 mol each of oxazole and thiazole rings were assigned by HMBC correlations and comparison with literature data of related compounds[16,17]. The sequence of amino acids was determined using HMBC and ROESY correlations as shown in figure 2. Although 2 carbonyl carbons were not determined by NMR spectral data (TABLE 1), the identification of berninamycin A was accomplished by comparison with previously reported chemical shift values[13].

TABLE 1 : NMR spectral data for berninamycin A in DMSO-d_6

	Position	δH (ppm)		δC (ppm)		Position	δH (ppm)		δC (ppm)
Dha1	NH_2	7.45	s		Dha5	NH	9.64	s	
		7.91	s			CO			156.0
	CO			166.1		α			129.4
	NH	9.44	s			β	5.62	s	106.3
	α			136.1			6.07	s	
	β	5.67	s	106.9	hVal	NH	8.22	d, 11.0 Hz	
		5.99	s			CO			170.5
Dha2	CO			163.0		OH	5.28	br	
	NH	10.5	s			α	4.61	d, 8.2 Hz	62.8
	α			135.8		β			71.9
	β	5.81	s	107.2		γ	1.18	s	27.0
		6.39	s				1.19	s	28.6
Pyr	2			148.0	Dha6	NH	9.34	s	
	3			131.2		CO			164.9
	4	8.49	d,8.3 Hz	141.9		α			134.4
	5	8.20	d,8.3 Hz	122.3		β	5.84	s	103.9
	6			150.3			6.43	s	
	CO			ND	MeOxz2	2			156.0
Oxa	2			159.2		4			130.2
	4			140.1		5			154.6
	5	8.66	s	141.4		Me	2.56	s	12.5

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	Position	δH (ppm)		δC (ppm)		Position		δH (ppm)		δC (ppm)
Dha3	NH	9.87	s		Dhb	NH	9.58	s		
	CO			159.2		CO				157.3
	α			130.3		α				124.0
	β	5.68	s	112.1		β	6.45	d,6.9 Hz		129.8
		5.69	s			γ	1.69	d,6.9 Hz		12.9
Dha4	NH	9.38	s		Thr	NH	7.98	d, 8.9 Hz		
	CO			163.5		CO				169.9
	α			134.8		α	4.57	d, 8.9, 2.8 Hz		58.9
	β	5.78	s	107.0		β	4.25	m		68.2
		6.32	s			γ	1.10	d, 6.2 Hz		27.1
MeOxz1	2			157.3	Thz	CO				ND
	4			130.3		2				164.1
	5			155.4		4				150.1
	Me	2.58	s	12.5		5	8.47	s		127.9

ND: Not determined

To elucidate the absolute stereochemistries of Thr and hydroxy Val (hVal) in the molecule, the hydrolysate of berninamycin A was derivatized with Nα-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA), and the derivative was subjected to HPLC analysis to compare with the standard amino acid derivatives with L-FDLA or D-FDLA[18,19]. As a result, 1 mol each of L-Thr and L-hVal was detected, which determined that the confirmations of Thr and hVal in berninamycin A was all L-form. Regarding the stereochemistry of Dhb, methyl residue was determined to connect to double bond as Z-form, since ROESY correlation was observed between methyl protons and amide proton. Above all, absolute stereochemistries of berninamycin A were determined as shown in Figure 1.

The structure of berninamycin A was originally determined by Liesch and Rinehart with the combination of NMR spectral data and chemical degradation, and the structure was revised by ABE et al[20]. However the absolute stereochemistry of berninamycin A was not determined yet. In the present study, the streochemistries of Thr and hVal in bernamymycin A were determined as all L-form. Since berninamycin A is utilized as biochemical tool, the structural information including stereochemistry was important. The production of berninamycin A in *S. atroolivaceus* was estimated as high as 19 μg/mg (quantity of berninamycin A /wet weight of whole cells and agar) by HPLC analysis.

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