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NOTE

# NMR spectroscopic and MS/MS spectrometric characterization of a new lipopeptide antibiotic bacillopeptin $B_1$ produced by a marine sediment-derived *Bacillus amyloliquefaciens* SH-B74

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*Bacillus* species have the ability to secrete lipopeptide antibiotics under both *in vivo* and *in vitro* conditions.[1–4] *Bacillus* lipopeptides are versatile antibiotics composed mainly of three families—iturins, plipastatins (or fengcyins) and surfactins. Iturins and plipastatins are fungicides and have potential utilization in the biological control of fungal plant pathogens.[5] Marine-derived microorganisms have arisen as a focus of research during recent decades, and numerous novel metabolites with excellent biological activity have been reported.[6,7] In the present work, compound **1** was isolated and purified from the fermentation broth of a marine sediment-derived *B. amyloliquefaciens* SH-B74. The structure of compound **1** was characterized by one-dimensional and two-dimensional NMR spectroscopy as well as tandem mass spectrometry (MS/MS) analysis. Finally, the antagonistic activities of compound **1** to several plant pathogens were also evaluated *in vitro* by the paper disc-agar diffusion assay.

Compound **1** was obtained as a white amorphous powder. The HRESI MS spectrum of compound **1** showed *m/z* 1057.5177 ($[M+Na]^+$) for chemical formula $C_{47}H_{74}N_{10}NaO_{16}$, which was calculated for 1057.5182. The result could be further demonstrated by the full mass spectrum of compound **1**, which showed *m/z* 1035.49 ($[M+H]^+$) and 1057.48 ($[M+Na]^+$).

The low-field region of $^1$H-NMR (600 MHz, DMSO-$d_6$) spectrum of compound **1** showed 12 N-bonded protons (–NH– or –$NH_2$, $\delta$ 8.29–6.95), a *para*-substituted benzene ring ($\delta$ 6.63, 7.00, each d, $J=8.4$ Hz, 2 H) and seven α-protons ($\delta$ 4.47–3.98) of peptide bonds, a long fatty-acid chain ($\delta$ 1.38–1.13) and a terminal methyl group ($\delta$ 0.84, t, 3H) in the highest-field part of $^1$H-NMR spectrum. The $^{13}$C-NMR (150 MHz, DMSO-$d_6$) spectrum of compound **1** showed the presence of 11 carbonyl groups ($\delta$ 174.3–169.6), a *para*-substituted benzene ring ($\delta$ 155.9, 130.1, 130.1, 128.1, 115.2, 115.2) and α-carbons or β-carbons of seven amino acids ($\delta$ 66.0–50.3). The two signals of methyl carbons, $\delta$ 46.3 and $\delta$ 40.9, were the characteristic signals of the β-amino fatty-acid residue of iturinic lipopeptides.[8] The terminal methyl group of the fatty-acid residue within the iturinic lipopeptides was very particular in one-dimensional NMR spectra and different from other methyl groups, which could be distinguished by signals from the high field of the $^1$H-NMR or $^{13}$C-NMR spectrum. More specifically, the terminal methyl carbon ($\delta$ 14.1), as well as the shape of the terminal methyl group ($\delta$ 0.84, t, 3H) of the $^1$H-NMR spectrum, indicated that the branch at the end of the fatty-acid chain residue of compound **1** was *normal*-type.[9,10] Seven amino acids, including Asn (2 ×), Ser (2 ×), Gln (1 ×), Thr (1 ×) and Tyr (1 ×), as well as a *normal*-type C15 β-amino fatty-acid residue (Figure 1a), were completely identified on the basis of two-dimensional NMR spectroscopy, such as $^1$H-$^1$H COSY, NOESY and HSQC as well as HMBC experiments (Table 1). Selected key correlations of $^1$H-$^1$H COSY, HMBC and NOESY of compound **1** are shown in Figure 1b.

The sequence of the amino-acid residues of compound **1** was confirmed by MS/MS analysis, which provided all the needed information for the sequence determination of amino acids by means of different type of fragments. More specifically, a series of b and y types of ion fragmentations was found from the MS/MS spectrum of compound **1**; the fragmentation ions are shown in Figure 1c. All these ion fragments supported the sequence of $Asn_1$, C15 β-amino fatty-acid $residue_2$, $Thr_3$, $Ser_4$, $Glu_5$, $Ser_6$, $Asn_7$ and $Tyr_8$ existing within compound **1**. The results coincided well with the collision-induced dissociation fragmentation of the mass spectrometry experiment undertaken by Ishikawa and colleagues[11] as well as NMR analysis of bacillopeptins family taken by Kajimura and colleagues.[8] The branch of the fatty-acid residue indicated that compound **1** was a new compound belonging to the bacillopeptin family and could be characterized as bacillopeptin $B_1$. More specifically, compound **1**

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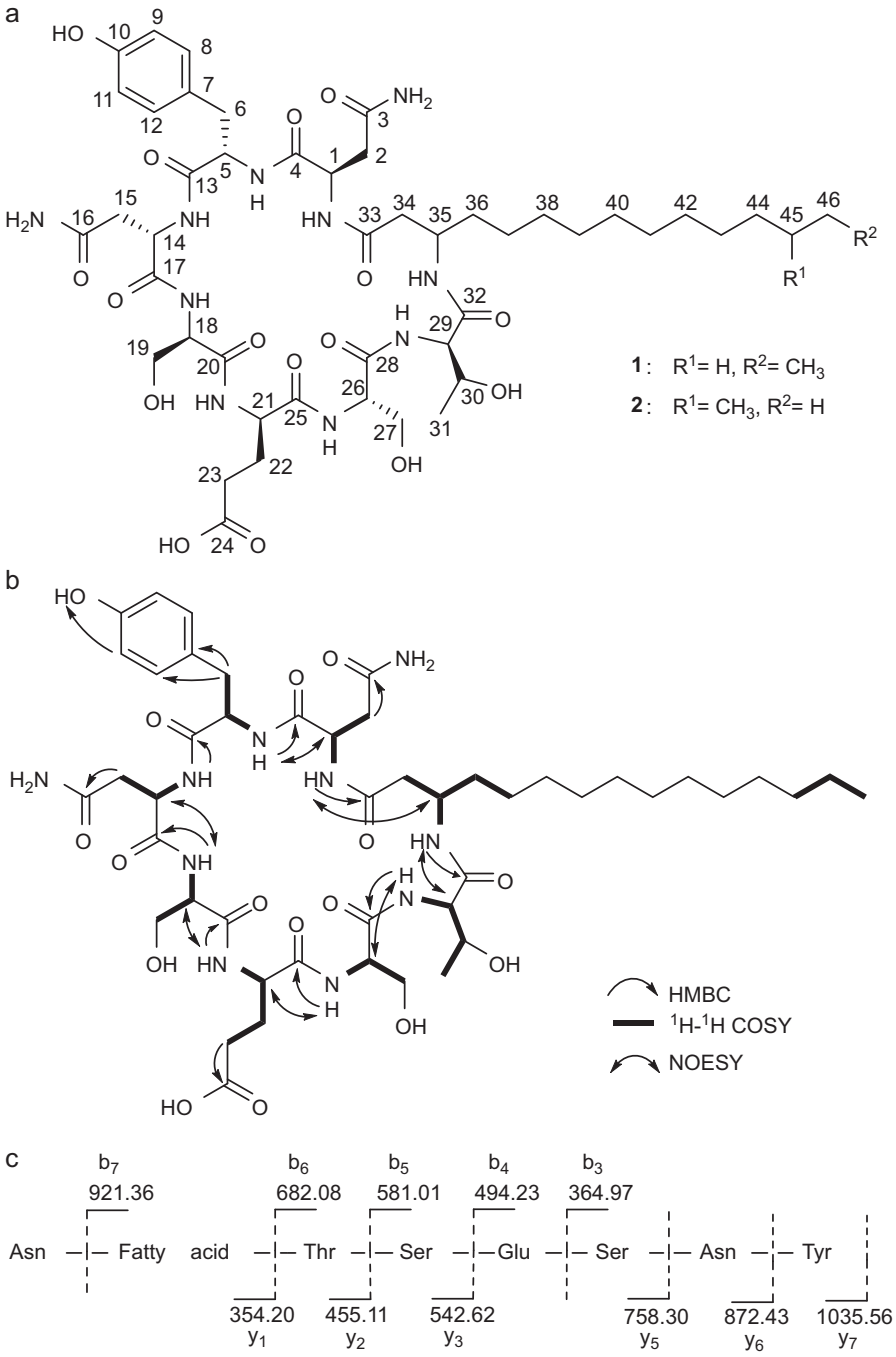


**Figure 1** Chemical structure of bacillopeptin $B_1$ (**1**) and bacillopeptin B (**2**) (**a**), selected key correlations (**b**), MS/MS fragmentations (**c**) of bacillopeptin $B_1$ (**1**) isolated and purified from the fermentation broth of *B. amyloliquefaciens* SH-B74.

had a different fatty-acid residue and the same amino-acid sequence, as well as same molecular weight compared with bacillopeptin B (**2**) (Figure 1a).[8] The configuration of each amino acid of bacillopeptins had been completely determined via TLC and chiral HPLC analysis by Kajimura and colleagues,[8] confirmed by Marfey's derivation combined with Edman-degradation and RP-HPLC analyses after partial or total hydrolysis by Eshita and colleagues[10] and also reassured mainly via chemical synthesis of analog of bacillomycin (bacillopeptin) by Volpon and colleagues.[8,10,12] The results showed that the stereochemistry of L-$Asn_1$, D-$Tyr_2$, D-$Asn_3$, L-$Ser_4$, L-$Glu_5$, D-$Ser_6$ and L-$Thr_7$ existed within bacillopeptins. Bacillopeptin $B_1$ (**1**) had antagonistic activities toward the tested plant fungal pathogens, such as *Valsa mali*, *Fusarium oxysporum* f.sp. *cucumerinum* and *Rhizoctonia solani*, when the concentration was over 2 mg ml$^{-1}$.

## METHODS

### General experimental procedure

A semi-preparative HPLC system (Dionex U3000, Sunnyvale, CA, USA) was used in this study for further purification and analysis. One-dimensional and two-dimensional NMR spectroscopy ($^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$H COSY, HSQC, HMBC and NOESY) analyses were recorded on a Bruker AV600 spectrometer (Leipzig, Germany, 600 and 150 MHz for $^1$H- and $^{13}$C-NMR),

**Table 1** $^{1}H$- and $^{13}C$-NMR data ($\delta$, p.p.m.) assignments of bacillopeptin $B_1$ (1)

| | | *1* | |
|---|---|---|---|
| *Moiety* | *Position* | $\delta_C$ | $\delta_H$ *(J in Hz)* |
| Asn1 | 1 | 50.3 | 4.39 m |
| | 2 | 36.6 | |
| | 3 | 171.6[a] | |
| | 4 | 171.9[a] | |
| | 1-NH | | 7.92 d (6.4) |
| | 3-$NH_2$ | | 6.95 s, 7.31 s |
| Tyr2 | 5 | 55.7 | 4.22 m |
| | 6 | 35.6 | 2.69 dd (10.6, 10.0)/2.94 dd (4.0, 3.9) |
| | 7 | 128.1 | |
| | 8/12 | 130.1 | 7.00 d (8.4) |
| | 9/11 | 115.2 | 6.63 d (8.4) |
| | 10 | 155.9 | |
| | 13 | 171.4[a] | |
| | 5-NH | | 8.29 d (7.1) |
| | 10-OH | | 9.20 s |
| Asn3 | 14 | 50.8 | 4.47 m |
| | 15 | 36.8 | 2.50 m/2.56 dd (5.8, 5.3) |
| | 16 | 171.5[a] | |
| | 17 | 171.3[a] | |
| | 14-NH | | 8.04 d (6.9) |
| | 16-$NH_2$ | | 6.95 s, 7.31 s |
| Ser4 | 18 | 55.2 | 4.23 m |
| | 19 | 61.2 | 3.57 m |
| | 20 | 169.6 | |
| | 18-NH | | 7.56 d (7.2) |
| Glu5 | 21 | 53.0 | 4.18 m |
| | 22 | 25.4 | 1.88 m/1.94 m |
| | 23 | 31.4 | 2.22 m |
| | 24 | 174.3 | |
| | 25 | 172.4 | |
| | 21-NH | | 8.00 d (5.9) |
| Ser6 | 26 | 55.3 | 4.33 m |
| | 27 | 61.4 | 3.60 m |
| | 28 | 170.3 | |
| | 26-NH | | 8.13 d (7.0) |
| Thr7 | 29 | 58.8 | 4.05 m |
| | 30 | 66.0 | 4.06 m |
| | 31 | 20.1 | 1.01 d (6.12) |
| | 32 | 170.7 | |
| | 29-NH | | 7.69 d (8.0) |
| Fatty acid | 33 | 171.2[a] | |
| | 34 | 40.9 | 2.28 m |
| | 35 | 46.3 | 3.98 m |
| | 36 | 34.0 | 1.34 m/1.38 m |
| | 37 | 25.4 | 1.12 m/1.21 br s |
| | 38-42 | 28.8–29.2 | 1.21 br s |
| | 43 | 28.8–29.2 | 1.21 br s |
| | 44 | 28.8–29.2 | 1.21 br s |
| | 45 | 31.4 | 1.24 m |
| | 46 | 22.2 | 1.22 m |
| | 47 | 14.1 | 0.84 t |
| | 33-NH | | 7.28 d (8.1) |

[a]The chemical shifts may be changed in the column.

using DMSO-$d_6$ ($\delta_H$ 2.49 p.p.m. and $\delta_C$ 39.6 p.p.m.) as a solvent. The HRESI MS experiments were performed on a Bruker micro Q-TOF MS (Germany), and the MS/MS experiment was conducted on a LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

### Strains and cultural conditions

SH-B74 was isolated from the deep-sea sediment (17°53′59″N, 114°34′58.6″E) collected from the South China Sea, and the strain was characterized as *Bacillus amyloliquefaciens* on the basis of the morphological, physiological and biochemical characteristics as well as the sequence analysis of 16S rRNA (GenBank accession number: KC517141). The gradient of the cultural medium (1 liter) used for the liquid fermentation procedure was as follows: 20 g sucrose, 2 g $NH_4NO_3$, 3 g $KH_2PO_4$, 10 g $Na_2HPO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g yeast extracts, 0.7 μg $CaCl_2$, 1 μg $MnSO_4 \cdot 4H_2O$ and pH 7.0–7.2. The *B. amyloliquefaciens* SH-B74 was inoculated and cultured in several 250 ml flasks containing 50 ml of medium at 28 °C for 20 h as the seed culture. The seed culture was inoculated into 3-liter flasks each containing 1-liter of medium, cultured for 48 h at a stirring rate of 180 r.p.m. at 28 °C and then harvested.

### Isolation and purification procedure

The supernatant of the fermentation broth (16 liters) of *B. amyloliquefaciens* SH-B74 was collected after centrifugation at 4000 *g* for 30 min and then acidified to pH 2.0 with 6 N hydrochloric acid and kept overnight at 4 °C. The precipitate was collected by centrifugation at 4000 *g* for 30 min, followed by extraction of the residue with methanol (400 ml) three times. Finally, the solvent was evaporated under vacuum to yield 11.3 g of the crude extract. The crude extract was eluted by dichloromethane/methanol (eight different ratios (v/v), 100/0, 98/2, 95/5, 90/10, 80/20, 70/30, 50/50 and 0/100) using vacuum flash chromatography over silica gel (600–800 mesh). The antagonistic fraction from vacuum flash chromatography eluted via 0/100 (dichloromethane/methanol, v/v) was further purified by a semi-preparative HPLC system using C18 YMC-Pack ODS-A column (5 μm, φ 10 × 250 mm) at a flow rate of 2.5 ml $min^{-1}$ with UV detection at 220 nm and eluted via 75% methanol/$H_2O$ system (v/v), afforded compound **1** (3.5 mg, 22.6 min).

### *In vitro* antifungal activity test

The direct antagonistic inhibition of purified lipopeptide was measured using the method of paper disc-agar diffusion assay.[13] Compound **1** was prepared as stock solutions for further *in vitro* testing. Selected fungal pathogens, such as *Valsa mali, Fusarium oxysporum* f. sp. *Cucumerinum* and *Rhizoctonia solani*, were incubated on the potato sucrose agar plates at 28 °C.

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