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Short Communication

Isolation and Sequence Analysis of New Peptaibol, Boletusin, from *Boletus* spp.

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Abstract: A new peptaibol, boletusin, was isolated from the methanol extract of the fruiting body of the mushroom, *Boletus* spp. Sequential determination by positive FAB MS/MS showed that boletusin is a peptide consisting of 19 amino acids, with one acetylated *N*-terminus residue, phenylalanine, and a *C*-terminal amino alcohol, tryptophanol. This peptide showed antimicrobial activity against several Gram-positive bacteria. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptaibol; *Boletus* spp.; boletusin; α-aminoisobutyric acid

INTRODUCTION

Peptaibols originate from fungal organisms, some of which exhibit antibiotic activity against phytopathogenic fungi and Gram-positive bacteria. These compounds are of considerable biological interest as they are known to facilitate the transport of ions across membranes *via* a mechanism involving pore formation [1–3]. Peptaibols share the characteristics of lipophilic linear peptides with α,α-dialkylated amino acids, such as α-amino isobutyric acid (Aib, U) and isovaline (Iva, J), and exhibit an *N*-terminal acylated residue and a *C*-terminal amino alcohol [4,5]. In the course of our study, searching for new antibiotic substances from various fruiting bodies of mushrooms, we isolated one new peptaibol class antibiotic from *Boletus* spp. (Boletaceae), boletusin, which is composed of 19 residue amino acids, together with four known peptaibols. The four known peptaibols, compounds **2**, **3**, **4** and **5**, are identical with chrysospermins previously reported from *Apiocrea chrysosperma* [6] and the new peptaibol, boletusin, is also structurally related to chrysospermins.

In the present paper, we report on the sequential determination of boletusin **1** by fast-atom bombard-

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Boletusin **1**	AcPh	Aib	**Ala**	Aib	**Iva**	Leu	Gln	Gly	Aib	Aib	Ala	Ala	Aib	Pro	**Aib**	Aib	Aib	Gln	Trpol
Compound **2**	AcPh	Aib	**Ser**	Aib	**Aib**	Leu	Gln	Gly	Aib	Aib	Ala	Ala	Aib	Pro	**Aib**	Aib	Aib	Gln	Trpol
Compound **3**	AcPh	Aib	**Ser**	Aib	**Aib**	Leu	Gln	Gly	Aib	Aib	Ala	Ala	Aib	Pro	**Iva**	Aib	Aib	Gln	Trpol
Compound **4**	AcPh	Aib	**Ser**	Aib	**Iva**	Leu	Gln	Gly	Aib	Aib	Ala	Ala	Aib	Pro	**Aib**	Aib	Aib	Gln	Trpol
Compound **5**	AcPh	Aib	**Ser**	Aib	**Iva**	Leu	Gln	Gly	Aib	Aib	Ala	Ala	Aib	Pro	**Iva**	Aib	Aib	Gln	Trpol

Bold-faced letters indicate amino acids which differ in sequence: Aib, U: α-aminoisobutyric acid; Iva, J: isovaline; Trpol, Tol: tryptophanol.

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ment mass spectrometry/mass spectrometry (FAB MS/MS). In addition, the isolation of boletusin and its biological activities are described.

Boletusin **1** was isolated from the methanol extracts of fresh, fruiting bodies of *Boletus* spp. The body of mushroom (1 kg) was extracted twice with 5 l of MeOH. Combined aqueous methanol extracts were evaporated *in vacuo* to an aqueous layer and successively partitioned with hexane (1 l × 3), chloroform (1 l × 3), and ethyl acetate (1 l × 3). The chloroform layer was evaporated *in vacuo* to give a gummy residue (2.5 g). The obtained residue was subjected to silica gel chromatography (Silica Gel 60, 0.063 ~ 0.1 mm, E. Merck) and eluted with a $CHCl_3$/MeOH mixture, with the amount of MeOH gradually increasing from 20:1 to 3:1. The residue eluted with 4:1 $CHCl_3$/MeOH was purified by a semi-preparative HPLC [MAXSIL 5 μ C_{18}, Phenomenex, 10 × 250 ml, 80:20 MeOH/H_2O] to yield compounds **1** ($t_R = 45$ min, 4 mg), mixtures **2**/**3** ($t_R = 23$ min, 7 mg), and **4**/**5** ($t_R = 30$ min, 15 mg). The structural elucidation of peptaibols **2**, **3**, **4**, and **5** was performed by using the FAB MS/MS technique and by direct comparison with previously published data [6].

Amino acid analysis of the total acidic hydrolysates of boletusin **1** provided the following amino acid composition: Aib (8), Ala (3), Gln (2), Gly (1), Iva (1), Leu (1), Pro (1), and Phe (1). To determine the absolute configuration of each amino acid, GC analysis of the total hydrolysates with derivatization was employed. The GC analysis results indicated that the chirality of all the amino acids and the amino alcohol was in L form, except for isovaline which was in D form.

The amino acid sequence was determined by FAB MS. In the FAB MS spectrum of boletusin **1**, a protonated molecular ion with an m/z of 1897 and a $[M + Na]^+$ ion with m/z of 1919 were observed, and the molecular formula of the compound was determined to be $C_{91}H_{142}N_{22}O_{23}$. Analysis of the continuous series of b_n acylium ions generated from peptide fragmentation led to a sequence determination. However, difficulty arose from the presence of an Aib-Pro tertiary amide link frequently observed in this peptide. This amide bond generally undergoes a preferential cleavage generating a b_n type *N*-terminal acylium ion N^+ and a y_n type *C*-terminal ammonium ion $(CH, H)^+$ according to the nomenclature of Roepstorff *et al.* [7]. The abundant fragmentation ion at m/z 1226 later turned out to correspond to the *N*-terminal part of the boletusin, providing sequence-specific ions at m/z 1140, 1069, 998, 913, 828, 644, 530, 431, 346, and 275. The fragmentation ion at m/z 671 corresponded to the *C*-terminal part of boletusin. However, sequence information of the *C*-terminal peptide could not be obtained from

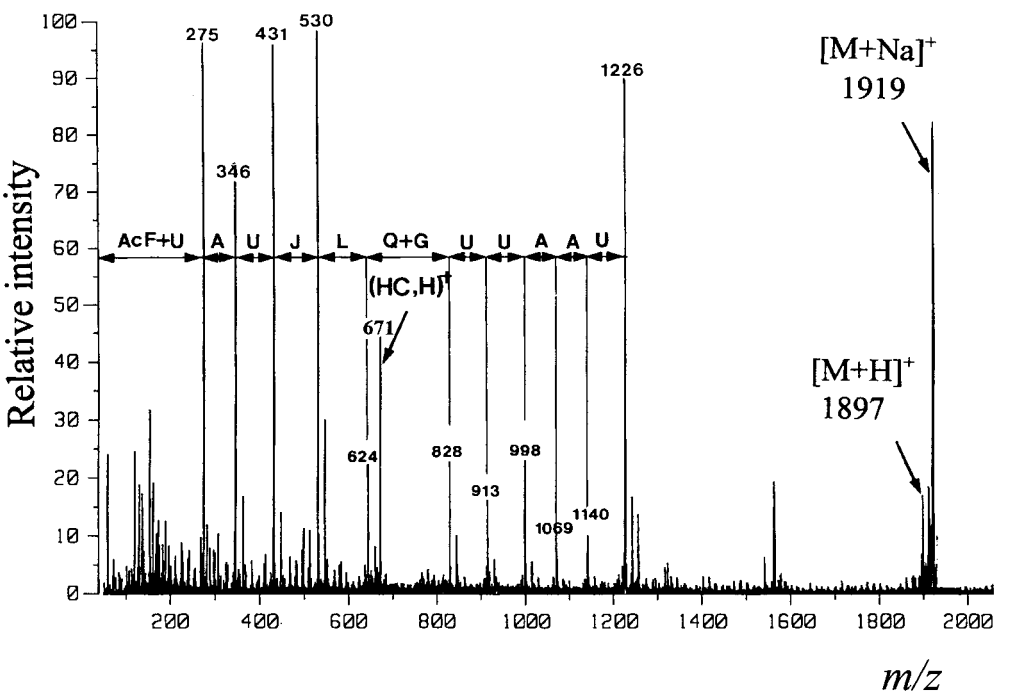

Figure 1 Positive ion FAB mass spectrum of boletusin (U = Aib, J = Iva).

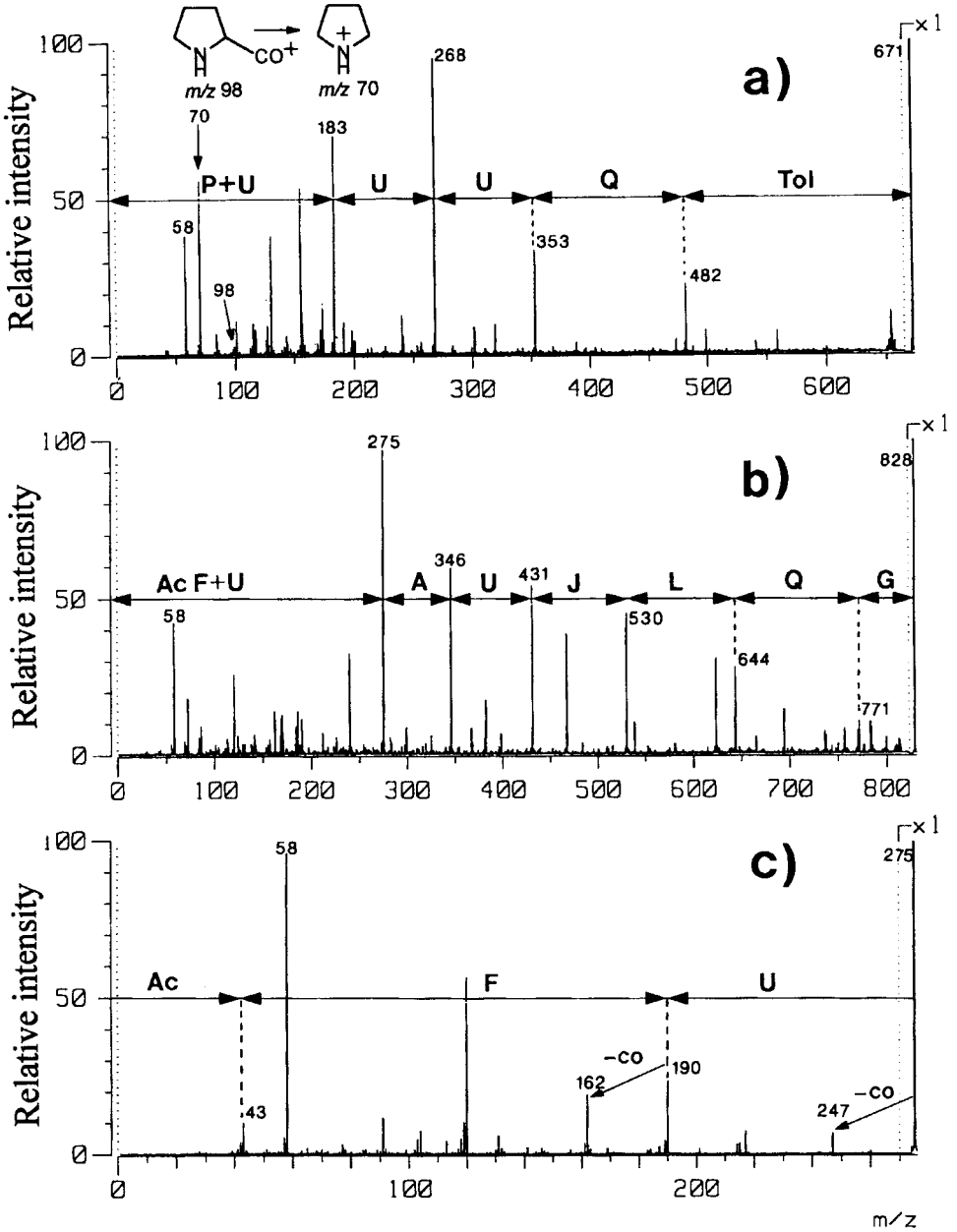

Figure 2 CID spectra of the m/z 671 (a); 828 (b); and 275 (c). (U = Aib, J = Iva, Tol = Trpol).

this spectrum due to the absence of sequence-specific ions. Also, complete sequence information of the *N*-terminal peptide was not shown in this spectrum. To clarify these points, FAB MS/MS technique was employed in our experiment.

A collision-induced dissociation (CID) spectrum of the $(M+H)^+$ ion and *N*-terminal and *C*-terminal fragment ions were recorded in the normal FAB MS spectrum. Figure 1 shows a part of the CID spectrum of the peak at m/z 1226. The mass differences of the sequence-specific daughter ions observed at m/z 828, 913, 998, 1069, and 1140 are attributable to Aib, Aib, Ala, Ala, and Aib residues for the sequence 9–13, respectively, and m/z 275, 346, 431, 530, and 644 are attributable to AcPh + Aib, Ala, Aib, Iva, and Leu residues for the sequence 1–6, respectively. The sequence 7–8 could not be determined because sequence-specific ions were not found between m/z 644 and 828 in the spectrum. However, as shown in Figure 2(b), the fragment ions observed in the CID

spectrum of the *m*/*z* 828 indicated Gln-Gly for the sequence 7–8. Sequence 1–2 was also determined by the CID spectrum (Figure 2(c)). Therefore, the amino acid sequence of the *N*-terminal part of boletusin in the FAB MS spectrum (Figure 1) was determined as: Ac-Phe-Aib-Ala-Aib-Iva-Leu-Gln-Gly-Aib-Aib-Ala-Ala-Aib.

The CID spectrum of the peak at *m*/*z* 671 afforded the sequence of *C*-terminal peptide shown in Figure 2(a). Continuous fragment ion series arising from such ions were clearly observed. The sequence-specific acylium ion series lost Trpol, Gln, Aib, Aib, and Pro + Aib, successively, for the sequence 14–19. Sequence-specific ions for the sequence 14–15 were not observed in this CID spectrum and the location of Pro could not be determined, but the spectrum showed the significantly abundant B-type ion *m*/*z* 70 which was deduced to originate from the loss of CO for Pro. Thus, the amino acid sequence of the *C*-terminal peptide of boletusin was determined as: Pro-Aib-Aib-Aib-Gln-Trpol.

The antimicrobial activity of boletusin **1**, compound **3** and compound **5** was examined against phytopathogenic fungi and Gram-positive and Gram-negative bacteria. As shown in Table 1, boletusin and other tested peptaibols were active against the Gram-positive bacteria, *Bacillus subtillis*, *Staphylococcus aureus* R-109, *S. aureus* IFO-12732, and *Corynebacterium lilium*, and were slightly active against the fungus *Mucor ramannianus* and the yeast *Saccharomyces cerevisiae*. However, they were completely inactive against several Gram-negative bacteria.

EXPERIMENTAL

FAB mass spectra were taken with the first (MS-1) of the two mass spectrometers of a JMS-HX110A/110A tandem mass spectrometer (JEOL, Tokyo, Japan) using a JMS-DA9000 data system. The ion source was operated at 10 kV accelerating voltage in the positive-ion mode with a mass resolution of 1000 (10% valley). Ions were produced by fast-atom bombardment using a cesium ion gun operated at 22 kV. Approximately 10 μg of the sample dissolved in methanol was mixed with 1 μg of *m*-nitrobenzyl alcohol (Sigma) in the positive-ion mode. MS/MS was

Table 1 Antimicrobial Activity of Boletusin **1** and Compounds **3** and **5**

Test organism[a]		Diameter of inhibition zone[b] (mm)		
		1	**3**	**5**
1	*Bacillus subtilis* IAM 1609	16	15	15
2	*Staphylococcus aureus* R-209	13	14	13
3	*Staphylococcus aureus* IFO-12732	15	16	15
4	*Corynebacterium lilium* (wild-type)	23	25	24
5	*Streptococcus* sp. (wild-type)	–[c]	9	–
6	*Salmonella typhimurium* KCTC 1926	–	–	–
7	*Escherichia coli* AB 1157	–	–	–
8	*Pasteurella haemolytica* (wild-type)	–	–	–
9	*Pasteurella multocida* (wild-type)	–	–	–
10	*Candida albcans* IAM 4905	–	–	–
11	*Saccharomyces cerevisiae* KCTC7039	10	11	11
12	*Mucor ramannianus* IAM 6218	10	12	11
13	*Aspergillus niger* ATCC 9642	–	–	–
14	*Magnaporthe gricea* IFO 5994	–	–	–
15	*Colletotrichum lagenarium* IFO 7513	–	–	–
16	*Fusarium solani* (wild-type)	–	–	–
17	*Alternaria mali* IFO 8594	–	–	–
18	*Penicillium notatum* (wild-type)	–	–	–

[a] Bacteria was tested on LB agar, fungi on PDA agar, and yeast on SAB agar medium.
[b] Determined by the agar diffusion test with 50 μg of peptides on an 8 mm paper disc.
[c] No activity.

carried out using a four-sector instrument with the $E_1B_1E_2B_2$ configuration. CID of the precursor ions selected with MS-1 (E_1B_1) occurred in the collision cell located between B_1 and E_2 and floated at 3.0 kV. Both MS-1 and MS-2 were operated as double-focusing instruments. The collision gas, helium, was introduced into the collision chamber at a pressure sufficient to reduce the precursor ion signal by 70%.

Absolute configurations of component amino acids were determined by GC analysis after hydrolysis of boletusin **1**. Boletusin (1 mg) was hydrolyzed by 1 ml of 6 N HCl for 24 h at 120°C. Derivatization of the given amino acids and the amino alcohol was conducted as previously described [8]. Retention times of the *N*-trifluoroacetyl isopropyl ester derivatives were compared with those of the standards. The GC analyses were performed with a Hewlett Packard series II 5890 gas chromatograph on a chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) quartz capillary column (Chrompack, 25 m length, 0.2 mm i.d.). The analysis conditions were the same as those previously described in reference [9].

The antimicrobial activity was determined against several phytopathogenic fungi and Gram-positive and Gram-negative bacteria by the agar diffusion method using a paper disc with an 8 mm diameter. The peptide samples were dissolved in MeOH at a concentration of 1 mg/ml. Other concentrations were obtained by dilution. Each 50 μg of the peptides was deposited into the paper disc. Inhibition zones were measured after 24 h of incubation at 37°C for bacteria and 27°C for yeast, and after 48 h of incubation at 27°C for fungi.

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