The Journal of Antibiotics (2009) 62, 335–338

www.nature.com/ja

NOTE

Macrolepiotin, a new indole alkaloid from *Macrolepiota neomastoidea*

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The Journal of Antibiotics (2009) **62,** 335–338; doi:10.1038/ja.2009.30; published online 17 April 2009

Keywords: Agaricaceae; cytotoxicity; indole alkaloid; *Macrolepiota neomastoidea*; macrolepiotin

The fungi of the genus, *Macrolepiota*, are grouped under the family *Agaricaceae* (division *Basidiomycota*) and comprise ~20 species. Various biological activities of the genus, *Macrolepiota*, have been reported, including anti-microbial, antioxidant and enzyme (trypsin, monophenolase) activities.[1–5] However, few species have been studied with regard to their secondary metabolites. Only several free amino acids, fatty acids and sterols have been reported from *Macrolepiota excoriata*, *Macrolepiota procera* and *Macrolepiota thacodes*.[6] Therefore, as part of a systematic study of Korean mushrooms,[7] we investigated the constituents of the fruiting bodies of the mushroom *Macrolepiota neomastoidea*, widely distributed throughout Korea and other East Asian countries. This is a poisonous mushroom known to cause severe gastrointestinal symptoms, including intestinal irritation, vomiting and profuse diarrhea.[8] Thus far, little work has been done on the chemical constituents of *M. neomastoidea*, except for the isolation of two compounds, lepiotins A and B.[9] Recently, we reported the isolation of lepiotin C and (*R*)-5-hydroxypyrrolidin-2-one, as well as lepiotins A and B.[10] As part of a continuing study, we have further isolated a new indole alkaloid named macrolepiotin (**1**), together with four known ergosterols, (22*E*,24*R*)-5α,8α-epidioxyergosta-6,9,22-triene-3β-ol (**2**),[11] (22*E*,24*R*)-5α,8α-epidioxyergosta-6,22-dien-3β-ol (**3**),[12] (24*S*)-ergost-7-en-3β-ol (**4**)[13] and (22*E*,24*R*)-5α,6α-epoxyergosta-9(14),22-diene-3β,7α-diol (**5**).[14] In this study, we describe the isolation and structural elucidation of **1** and the cytotoxic activities of compounds **1–5**.

MATERIALS AND METHODS

General

Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA) in MeOH. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). UV spectra were recorded using a Schimadzu UV-1601 UV–visible spectrophotometer (Schimadzu, Kyoto, Japan). FAB and HR-FAB mass spectra were obtained on a JEOL JMS700 mass spectrometer (JEOL, Peabody, MA, USA). NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer (Varian, Palo Alto, CA, USA) operating at 500 (^{1}H) and 125 MHz (^{13}C), respectively. Preparative HPLC was carried out using a Gilson 306 pump (Gilson, Middleton, WI, USA) with Shodex refractive index detector (Shodex, New York, NY, USA). Low-pressure liquid chromatography was carried out over a Merck Lichroprep Lobar-A Si 60 (Merck, Darmstadt, Germany) (240×10 mm) or a Lichroprep Lobar-A RP-18 (Merck) (240×10 mm) column using a FMI QSY-0 pump (Teledyne Isco, Lincoln, NE, USA). Silica gel 60 (Merck, 70–230 and 230–400 mesh) and RP-C_{18} silica gel (Merck, 230–400 mesh) were used for column chromatography. Spots were detected on a TLC under UV light or by heating after spraying with 10% H_2SO_4 in C_2H_5OH (v/v).

Mushroom material

The fresh fruiting bodies of *M. neomastoidea* were collected in November 2005 at Mt Jiri, Namwon of Jeonbuk Province, Korea. A voucher specimen (SKKU-2005-11) of the mushroom was deposited at the College of Pharmacy in Sungkyunkwan University, Korea.

Extraction and isolation

The air-dried and powdered fruiting bodies of *M. neomastoidea* (132 g) were extracted with 80% MeOH at room temperature to afford a methanolic extract (21.4 g). This extract was suspended in H_2O and partitioned with *n*-hexane, $CHCl_3$ and *n*-BuOH successively, and the solvent was removed thereafter to yield *n*-hexane (3.3 g), $CHCl_3$ (283 mg) and *n*-BuOH fractions (10.4 g). The *n*-hexane soluble fraction (3.3 g) was subjected to silica gel column chromatography with *n*-hexane-EtOAc (1:1) as the eluent to give seven fractions (H1–H7). Fraction H4 (75 mg) was further purified by RP-C_{18} preparative HPLC (Econosil RP-18 10 μ column, 250×22 mm; 100% MeOH) to give pure compounds **2** (6 mg) and **3** (35 mg). The $CHCl_3$ soluble fraction (283 mg) was subjected to a silica Lobar A-column with $CHCl_3$–MeOH (10:1) as the eluent to give seven fractions (C1–C7). Fraction C4 (55 mg) was further purified using a silica gel Waters Sep-Pak Vac 6cc ($CHCl_3$–MeOH, 22:1; Waters, Milford, MA, USA) to afford pure compounds **4** (4 mg) and **5** (3 mg). The *n*-BuOH soluble fraction (10.4 g) was subjected to a RP-C_{18} silica gel column chromatography with a gradient solvent system of MeOH–H_2O (0:1→1:1) as the eluent to give nine fractions (B1–B9). Fraction B8 (80 mg) was subjected to a RP-C_{18} silica Lobar A-column with 50% MeOH as the eluent to give two sub-fractions (B81–B82). Sub-fraction B81 (30 mg) was further

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Received 6 February 2009; revised 19 March 2009; accepted 24 March 2009; published online 17 April 2009

purified by RP-C_{18} preparative HPLC, as described above to give the pure compound **1** (7 mg).

Physico-chemical properties

Macrolepiotin (**1**). Yellowish gum, $[\alpha]_D^{25}$: −6.5 (*c* 0.25, MeOH), UV λ_{max} (MeOH) nm (log ϵ) 231 (3.66), 280 (5.51), IR (KBr) 3443, 2253, 1662, 1028, 824, 761 cm^{-1}. ^1H- and ^{13}C-NMR spectral data are shown in Table 1. FAB-MS *m/z* 580 $[M+H]^+$. HR-FAB-MS (positive-ion mode) *m/z* 580.2665 ($[M+H]^+$, $C_{31}H_{38}N_3O_8$, calcd. for 580.2659).

Test for cytotoxicity *in vitro*

A sulforhodamin B bioassay (SRB) was used to determine the cytotoxicity of each compound against four cultured human cancer cell lines.[15] The assays were performed at the Korea Research Institute of Chemical Technology. The cell lines used were A549 (non-small-cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma) and HCT (colon adenocarcinoma). Doxorubicin was used as the positive control. The cytotoxicities of doxorubicin against A549, SK-OV-3, SK-MEL-2 and HCT cell lines were IC_{50} 0.16, 0.38, 0.04 and 0.82 μM, respectively.

Compound **1** was obtained as a yellowish gum, and was found to be positive for Dragendorff's reagent. Its molecular formula was determined to be $C_{31}H_{37}N_3O_8$ from the $[M+H]^+$ peak at *m/z* 580.2665 (calcd. for $C_{31}H_{38}N_3O_8$ 580.2659) in the positive-ion high-resolution (HR)-FAB-MS spectrum. The IR spectrum indicated that **1** possessed hydroxyl (3443 cm^{-1}) and carbonyl (1662 cm^{-1}) groups. Its UV spectrum revealed absorptions at 231 and 280 nm, suggesting chromophores of amide functional group and benzene rings in the molecule. The physico-chemical properties of **1** are summarized in the Materials and methods section. The ^1H- and ^{13}C-NMR spectral data of **1** are shown in Table 1.

Table 1 ^1H- and ^{13}C-NMR data of **1**

Position	δ_C	δ_H
1		10.93 (1H, s)
2	128.6	
3	107.5	
3a	126.1	
4	117.7	7.45 (1H, d, 7.5)
5	118.7	7.00 (1H, t, 7.5)
6	121.1	7.08 (1H, t, 7.5)
7	111.1	7.34 (1H, d, 7.5)
7a	136.2	
8	22.8	2.93 (1H, m), 3.18 (1H, m)
9	56.4	3.96 (1H, m)
10	169.4	
11	39.1	4.21 (1H, q, 7.5)
12	19.0	1.41 (3H, d, 7.5)
13	180.4	
1′		
2′	173.4	
3′	29.2	2.35 (1H, m), 2.60 (1H, m)
4′	23.5	2.02 (1H, m), 2.27 (1H, m)
5′	91.4	5.33 (1H, dd, 5.5, 1.0)
6′	129.3	
7′,11′	124.9	7.24 (2H, dd, 8.0, 2.0)
8′,10′	115.1	6.76 (2H, dd, 8.0, 2.0)
9′	155.2	
5′-OMe	52.6	3.16 (3H, s)
1″	64.3	3.76 (dd, 9.0, 6.5)
2″	37.2	2.05 (1H, m)
3″	28.5	1.42 (1H, m), 1.65 (1H, m)
4″	11.7	0.96 (3H, t, 7.5)
5″	15.0	1.01 (3H, d, 7.0)
6″	174.2	

NMR data were obtained in 500 MHz for ^1H and 125 MHz for ^{13}C in DMSO, and values in parentheses are coupling constants in Hz.

The ^1H-NMR spectrum (Table 1) of **1** showed signals for the presence of three methyl groups at δ_H 0.96 (t, H-4″), 1.01 (d, H-5″) and 1.41 (d, H-12), four methylene groups at δ_H 1.42, 1.65 (m, H-3″), 2.02, 2.27 (m, H-4′), 2.35, 2.60 (m, H-3′) and 2.93, 3.18 (m, H-8), five methine groups at δ_H 2.05 (m, H-2″), 3.76 (dd, H-1″), 3.96 (m, H-9), 4.21 (q, H-11) and 5.33 (dd, H-5′) and one methoxyl group at δ_H 3.16 (s, 5′-OMe). The 1,4-disubstituted aromatic protons were observed at δ_H 6.76 (dd, H-8′, 10′), 7.24 (dd, H-7′, 11′) and four 1,2-disubstituted aromatic protons were shown at δ_H 7.00 (t, H-5), 7.08 (t, H-6), 7.34 (d, H-7) and 7.45 (d, H-4). A signal of downfield resonance at δ_H 10.93 (s, H-1) with no heteronuclear multiple quantum coherence (HMQC) correlations with any carbon signal was assignable to the amide proton. An analysis of ^1H- and ^{13}C-NMR spectra together with HMQC indicated that 31 carbon signals of **1** were composed of 4 carbonyl carbons, 14 olefinic carbons (including 12 aromatic carbons, 2 quaternary carbons), 5 methine carbons (including 1 oxygenated methine carbon), 4 methylene carbons, 3 methyl carbons and 1 methoxyl carbon. Analysis of ^1H,^1H-COSY data, HMQC and heteronuclear multiple bond correlation (HMBC) experiments established the presence of three partial structures, namely lepiotin B (partial unit **A**), isoleucine (partial unit **B**) and indole derivative (partial unit **C**) (Figure 1).

The presence of lepiotin B (partial unit **A**) in **1** was apparent from the two sets of methine signals (δ_H/δ_C; 6.76/115.1, 7.24/124.9) on a 1,4-disubstituted aromatic ring, a methine signal (δ_H/δ_C; 5.33/91.4) adjacent to two hetero atoms and from a methyl signal (δ_H/δ_C; 3.16/52.6). The presence of a γ-lactam ring was confirmed from the HMBC correlations, in which correlations of H-5′ with C-2′ (δ_C 173.4), C-3′ (δ_C 29.2) and C-4′ (δ_C 23.5) were observed. This signal at H-5′ was further coupled with a methoxyl group (δ_C 52.6), which implied that the position of the methoxyl group was at C-5′. Therefore, the partial unit **A** was assigned as lepiotin B by the above evidence. The lepiotin B, having a γ-lactam and a phenol ring, was an unusual alkaloid, and the main constituent isolated from this mushroom.[9]

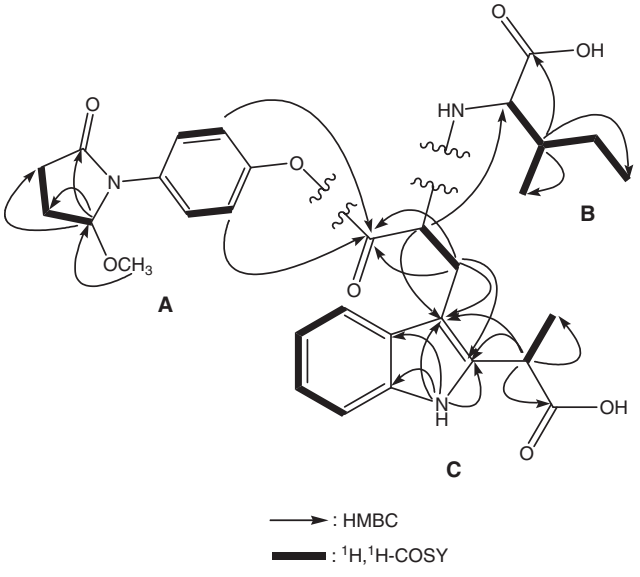

Figure 1 Key HMBC, ^1H,^1H-COSY correlations and partial units (**A**–**C**) of **1**.

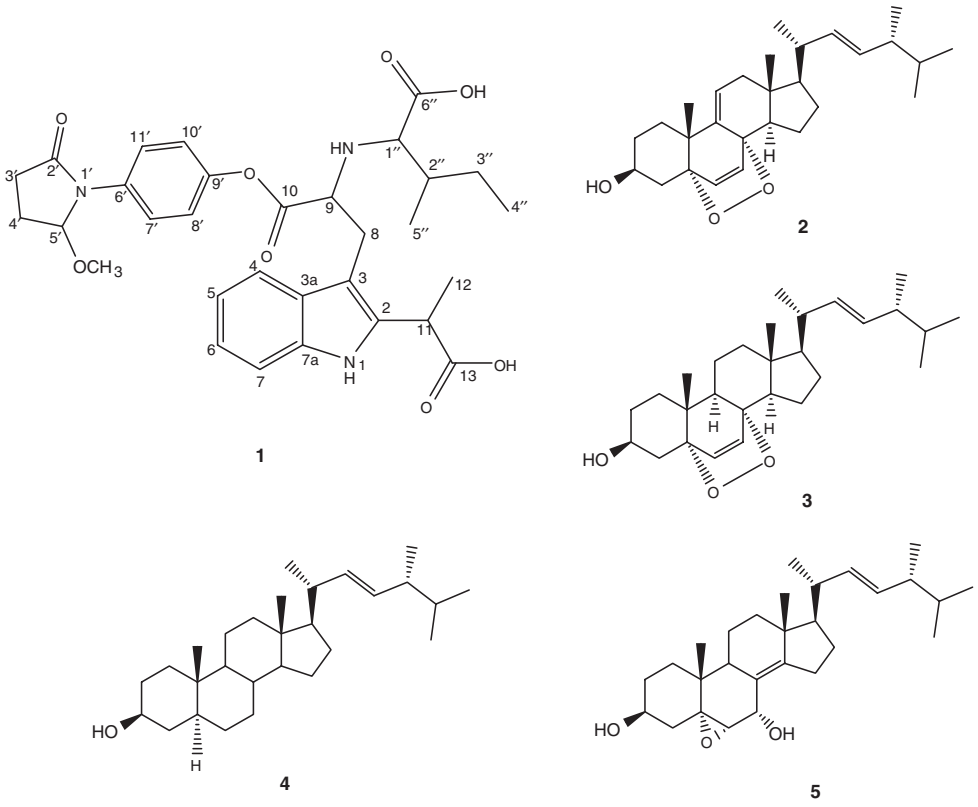

Figure 2 Structures of **1**–**5**.

Table 2 Cytotoxic activities of compounds (1–5) isolated from *Macrolepiota neomastoidea*

	IC_{50} (μM)			
Compound	*A549*	*SK-OV-3*	*SK-MEL-2*	*HCT-15*
1	>30.0	>30.0	>30.0	>30.0
2	25.6	17.5	11.8	17.1
3	14.0	17.9	12.7	10.0
4	>30.0	>30.0	>30.0	28.3
5	>30.0	16.5	>30.0	>30.0
Doxorubicin	0.16	0.38	0.04	0.82

IC_{50} value of compounds against each cancer cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*.

A second partial unit **B** was assigned as isoleucine by the following NMR data. The ^{1}H-NMR spectrum clearly indicated the appearance of two methyl groups at C-4″ (δ_H 0.96) and C-5″ (δ_H 1.01), a methylene group at C-3″ (δ_H 1.42, 1.65), as well as two methine groups at C-1″ (δ_H 3.76) and C-2″ (δ_H 2.05). The NMR resonances were similar to those of the isoleucine,[16] which showed ^{1}H,^{1}H-COSY correlations between H-1″/H-2″, H-2″/H-5″, H-2″/H-3″ and H-3″/H-4″. In addition, correlations were observed between H-2″ (δ_H 2.05) and C-4″ (δ_C 11.7), C-5″ (δ_C 15.0) and C-6″ (δ_C 174.2) in the HMBC spectrum.

The remaining fragment consisting of $C_{14}H_{13}NO_3$ was elucidated as an indole derivative (partial unit **C**) by the interpretation of the ^{1}H,^{1}H-COSY, HMQC and HMBC data. The presence of the indole skeleton was apparent from 1,2-disubstituted aromatic ring signals (δ_H; 7.00 (t, H-5), 7.08 (t, H-6), 7.34 (d, H-7), 7.45 (d, H-4), δ_C; 111.1, 117.7, 118.7, 121.1, 126.1, 136.2). The downfield signal (NH-1) of an amide proton showed HMBC correlation to four quaternary olefinic carbons (C-2, 3, 3a, 7a) (Figure 1). In addition, ^{1}H,^{1}H-COSY correlation between the methyl proton signal at δ_H 1.41 (d, H-12) and the methine proton signal at δ_H 4.21 (q, H-11) and HMBC correlations between the methine proton signal at δ_H 4.21 (q, H-11) and C-2 (δ_C 128.6), C-3 (δ_C 107.5), C-12 (δ_C 19.0) and C-13 (δ_C 180.4) were observed. This suggested that the propionic acid was located at C-2. HMBC correlations from methylene proton signals at δ_H 2.93, 3.18 (m, H-8) to C-2 (δ_C 128.6), C-3 (δ_C 107.5), C-9 (δ_C 56.4) and C-10 (δ_C 169.4) suggested a connection between the indole skeleton and 2-amino propionic acid through carbon C-3. It was assumed that this indole derivative belonged to a small group of indolyl carboxylic acids, which were isolated from solvent extracts of indole-supplemented supernatants of *Escherichia coli* and *Corynebacteria*.[17] It presented a similar structure to 2-(2-tryptophanyl) lactic acid, obtained from the condensation reaction between tryptophan and pyruvic acid.[17]

The three partial structures **A**, **B** and **C** were built into a full structure from the HMBC correlations (Figure 1). The isoleucine function was attached at C-9 of the indole derivative by the HMBC correlation between H-9 (δ_H 3.96) and C-1″ (δ_C 64.3). Biogenetically, this resembled konbamidin,[18] and may be derived from 1 mol each of isoleucine and a third partial unit, the indole derivative. The ^{13}C-NMR chemical shift of C-10 at δ_C 169.4 indicated the presence of an ester bond. This connection was confirmed from the HMBC correlation between H-8′, 10′ (δ_H 6.76) and C-10 (δ_C 169.4). Using the above data, the structure of **1** was assembled as shown in Figure 2. The stereochemistry of **1** has not yet been determined and remains to be studied in detail.

Cytotoxic activities of the isolated compounds (**1**–**5**) were evaluated against the A549, SK-OV-3, SK-MEL-2 and HCT15 human tumor cell lines *in vitro* using the SRB assay. Compounds **2** and **3** showed moderate cytotoxicity against A549, SK-OV-3, SK-MEL-2 and HCT15 cell lines as shown in Table 2. The mechanism of cytotoxic activity of the ergosterol peroxide was not well studied, but the cytotoxicity of the peroxide compounds (**2** and **3**) was probably ascribed to the presence of the peroxide functional group.[19] Compounds **4** and **5** showed moderate cytotoxicity against HCT15 and SK-OV-3 cell lines, respectively.

ACKNOWLEDGEMENTS

The authors thank Drs EJ Bang, SG Kim, and JJ Seo at the Korea Basic Science Institute for the NMR and MS spectra measurements. This work was supported by the grant 'Classification of Poisonous Mushrooms and Study of Investigating Toxic Constituents, 2006' from the Rural Development Administration in Korea.

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