ELSEVIER

Fitoterapia 72 (2001) 858–864

FITOTERAPIA

www.elsevier.com/locate/fitote

Armillaramide, a new sphingolipid from the fungus *Armillaria mellea*

Jin-Ming Gao[a,b], Xue Yang[a], Chen-Ying Wang[a], Ji-Kai Liu[a,*]

[a] *Laboratory of Phytochemisty, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, PR China*

[b] *College of Life Sciences, Northwest Science & Technology University of Agriculture and Forestry, Yangling, Shaanxi 712100, PR China*

Received 19 April 2001; accepted in revised form 23 May 2001

Abstract

A new C_{18}-phytosphingosine ceramide containing non-hydroxy fatty acid, armillaramide (**1**), has been isolated together with ergosterol peroxide from the fruiting bodies of the basidiomycete *Armillaria mellea*. Its structure was established as (2*S*,3*S*,4*R*)-2-hexadecanoylamino-octadecane-1,3,4-triol by spectroscopic and chemical methods. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Armillaria mellea*; Armillaramide; (2*S*,3*S*,4*R*)-2-hexadecanoylamino-octadecane-1,3,4-triol; Ceramides; Sphingolipids; Sterols

1. Introduction

Armillaria mellea (Vahl. Ex Fr) Quél. (Tricholomataceae) is a fungus symbiotic with the Chinese medicinal herb *Gastrodia elata* Blume (Orchidaceae). A range of biologically active sesquiterpenoid aryl esters with the protoilludane skeleton have been isolated from the artificially-cultured mycelium of different strains of this basidiomycete [1]. The crude drug containing extract of artificially cultured mycelium of *A. mellea* in Chinese market is used for the treatment of geriatric patients with palsy, dizziness, headache, neurasthenia, insomnia, numbness in

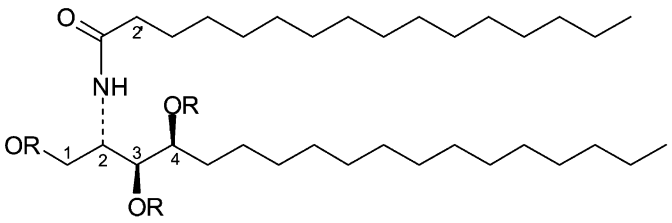
* Corresponding author. Tel.: +86-871-5216327; fax: +86-871-5150227.
E-mail address: jkliu@mail.kib.ac.cn (J.K. Liu).

0367-326X/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0367-326X(01)00319-7

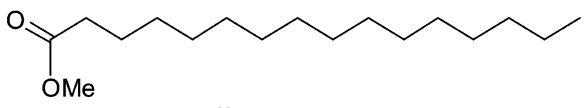
limbs, and infantile convulsion [2]. This arouses our interest to know what kind of secondary metabolites occurs in the fruiting bodies of this fungus.

Sphingolipids are found to be important building blocks of the plasma membrane of eukaryotic cells. Their function is to anchor lipid-bound carbohydrates to cell surfaces and to create an epidermal water permeability barrier, as well as to participate in antigen–antibody reactions and transmission of biological information [3]. Recently, some sphingolipids have been reported to exhibit antihepatotoxic [4], antitumor and immunostimulatory activities [5]. Because of their promising biological activities, they have aroused much increasing research interest.

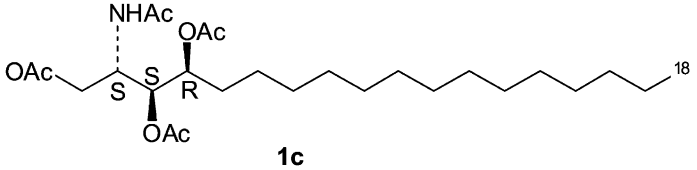
In the course of our investigation on the sphingolipid composition of higher fungi collected from Yunnan Province of China, two antifungal cerebrosides from *Russula ochroleuca* [6], a cerebroside from *Polyporus ellisii* [7], and a ceramide from *R. cyanoxantha* [8] have recently been reported. In continuation of our preceding studies on the basidiomycete-derived bioactive secondary metabolites, the chemical constituents of *A. mellea* were investigated. A new ceramide **1** was obtained by fractionation of the ethyl acetate extract of the fruiting bodies of this fungus. In this paper, the isolation and structure elucidation of the new compound are described.



1 R=H
1a R=Ac



1b



1c

Table 1
NMR data for **1** in pyridine-d_5 (500 MHz for ^{1}H-NMR, 125 MHz for ^{13}C-NMR)

Position	^{13}C (DEPT)	^{1}H (*J* in Hz)	^{1}H-^{1}H COSY
1	62.28 (CH_2)	4.57 (*dd*, *J* 12.4, 3.8)	H-2
		4.40 (*dd*, *J* 12.4, 4.0)	
2	53.79 (CH)	4.98 (*m*)	H-1, H-3, NH
3	76.86 (CH)	4.31 (*dd*, *J* 4.8, 3.9)	H-2, H-4
4	73.18 (CH)	4.23 (*m*)	H-3, H-5
5	34.09 (CH_2)	2.20 (*m*)	H-4
6	26.68 (CH_2)	1.93 (*m*)	
7–17	22.98–32.19 (11 CH_2)	1.30 (*br s*)	
18	14.29 (CH_3)	0.90 (*t*, *J* 5.6)	
NHCO		8.13 (*d*, *J* 6.7)	H-2
1′	173.44 (C)		
2′	36.91 (CH_2)	2.43 (*t*, *J* 6.0)	H-3′
3′	26.44 (CH_2)	1.83 (*m*)	H-2′
4′–15′	22.98–32.19 (11 CH_2)	1.30 (*br s*)	
16′	14.29 (CH_3)	0.90 (*t*, *J* 5.6)	

2. Results and discussion

The molecular formula of compound **1** was determined as $C_{34}H_{69}NO_4$ by high resolution EI-MS. The IR spectrum exhibited the absorption bands of hydroxyls, amide NH, and long aliphatic chains. The triacetyl derivative **1a**, obtained from **1** by reaction with pyridine-Ac_2O, still showed a sharp IR absorption band at 3331 cm^{-1}, further indicating the presence of amide NH. The ^{1}H-NMR spectrum of **1** (Table 1) showed the presence of two terminal methyls at δ 0.90, methylenes at δ 1.30 (approx. 36H, *br s*), an amide proton signal at δ 8.13 (*d*, $J = 6.7$ Hz). The ^{13}C-NMR spectrum of **1** (Table 1) showed one quaternary carbon at δ 173.44 (CONH), three methines at δ 53.79 (CHNH), 76.86 (CHOH) and 73.18 (CHOH), and one methylene at δ 62.28 (CH_2OH). All of the above spectral data revealed that **1** was a phytosphingosine-type sphingolipid [5,8].

The presence of a triplet signal at δ 2.43 (2H, $J = 6.0$ Hz) due to the methylene protons connected to amide carbonyl indicated that the *N*-acyl moiety in **1** was a non-hydroxy fatty acid. Methanolysis [8] of **1** yielded methyl palmitate (**1b**) detected by GC/MS. The existence of the palmitoyl moiety was also confirmed by high resolution EI-MS at *m/z* 239.2372 (calcd. 239.2374 for $C_{16}H_{31}O$) as well as significant fragment ions at *m/z* 239 $[CH_3(CH_2)_{14}CO]^+$, 256 $[CH_3(CH_2)_{14}CONH_2 + H]^+$, and 298 $[M\text{-}H_2O\text{-}CH_3(CH_2)_{14}CO]^+$ in the EI-MS. Moreover, the positive mode FAB-MS of the peracetate **1a** showed the molecular ion at *m/z* 681 (M^+) in addition to the ion at *m/z* 682 $(M + H)^+$. The positive ion FAB-MS also displayed the intense peak at *m/z* 622 $(M + H\text{-}AcOH)^+$ in addition to the fragment ions at *m/z* 562 $(M + H\text{-}2 \times AcOH)^+$, 502 $(M + H\text{-}3 \times AcOH)^+$. The prominent ion peak at *m/z* 264 $(M\text{-}CO(CH_2)_{14}CH_3\text{-}3 \times AcOH + H)^+$ formed by elimination of a fatty acyl group from the long-chain base moiety (681–417), established that the molecular weight of the fatty acid is 256 and that the base is a

C_{18}-phytosphingosine containing three hydroxyls and an amino group. This conclusion was in turn supported by the presence of the prominent ion peak at *m/z* 318 in the positive FAB-MS of **1** and confirmed by peracetylation of the methanolysis product of **1** to afford a tetra-acetylphytosphingosine, i.e. 2-acetoamino-1,3,4-triacetoxyoctadecane (**1c**), the ^{1}H-NMR and EI-MS spectra of **1c** was found to be identical to that of the compound reported in literature [8,9].

The proton assignments were further supported by analysis of ^{1}H-^{1}H COSY. The relative stereochemistry of **1** at C-2, C-3 and C-4 was proposed as 2*S*, 3*S* and 4*R*, since ^{1}H-NMR spectral data of **1** [1-Ha at δ 4.57 (*dd*, *J* = 12.4, 3.8 Hz), 1-Hb at δ 4.40 (*dd*, *J* = 12.4, 4.0 Hz), 2-H at δ 4.98 (*m*), 3-H at δ 4.31 (*dd*, *J* = 4.8, 3.9 Hz), and 4-H at δ 4.23 (*m*)] were in good agreement with those of natural and synthetic ceramides, (2*S*,3*S*,4*R*)-2-(2′-hydroxytetracosanoylamino)hexadecane-1,3,4-triol isolated from starfish *Acanthaster planci* [1-Ha at δ 4.52 (*dd*, *J* = 10.7, 4.5 Hz), 1-Hb at δ 4.43 (*dd*, *J* = 10.6, 5.0 Hz), 2-H at δ 5.12 (*m*), 3-H at δ 4.36 (*dd*, *J* = 4.6, 6.6 Hz), and 4-H at δ 4.29 (*m*)] [10,11]. Furthermore, the chemical shifts and coupling constants of 1-H, 2-H, 3-H and 4-H for **1a** [1-Ha at δ 4.29 (*dd*, *J* = 11.6, 4.6 Hz), 1-Hb at δ 4.01 (*dd*, *J* = 11.6, 3.1 Hz), 2-H at δ 4.47 (*m*), 3-H at δ 5.12 (*dd*, *J* = 8.7, 3.1 Hz) and 4-H at δ 4.93 (*dt*, *J* = 9.8, 3.1 Hz)] were in full agreement with those of (2*S*, 3*S*, 4*R*)-2-acetoamino-1, 3,4-triacetoxyheptadecane [1-Ha at δ 4.29 (*dd*, *J* = 11.6, 4.3 Hz), 1-Hb at δ 4.00 (*dd*, *J* = 11.6, 3.1 Hz), 2-H at δ 4.47 (*m*), 3-H at δ 5.10 (*dd*, *J* = 8.5, 3.1 Hz) and 4-H at δ 4.93 (*dt*, *J* = 9.8, 3.1 Hz)] [5]. In addition, the optical rotations of **1** ($[\alpha]_D$ + 14.4) and **1c** ($[\alpha]_D$ + 10.9) were also in accordance with those reported for the known ceramide (2*S*,3*S*,4*R*)-2-(2′-hydroxytetracosanoylamino)hexadecane-1,3,4-triol (natural $[\alpha]_D$ + 11.5; synthetic $[\alpha]_D$ + 9.1) [10,11]. These data suggested that **1** has the same absolute configuration at asymmetric centers 2, 3 and 4. Accordingly, the above evidence resulted in the establishment of the structure of **1** as (2*S*,3*S*,4*R*)-2-hexadecanoylamino-octadecane-1,3,4-triol.

Comparison of the physicochemical properties with the reported data allowed to identify the other new-isolated compound as ergosterol peroxide [5α,8α-epidioxy-(22*E*,24*R*)-ergosta-6,22-dien-3β-ol], previously isolated from *R. ochroleuca* [6] and *R. cyanoxantha* [8] and reported to possess anticomplementary [6], antitumor [12], and antimycobacterial [13] activities.

To the best of our knowledge, this is the first isolation of this type of ceramide from higher fungi and a new natural product with the configuration of 2*S*,3*S*,4*R*. The compound with the same molecular formula ($C_{34}H_{69}NO_4$) has been previously obtained by synthesis, but no data of its stereochemistry have been reported [14].

3. Experimental

3.1. General experimental procedures

Melting points were obtained on an XRC-1 apparatus and uncorrected. The optical rotations were measured with a Horiba SEPA-300 polarimeter. NMR spectra (^{1}H-, ^{13}C-NMR and ^{1}H-^{1}H COSY) were recorded on Bruker AM-400 and

DRX-500 NMR instrument with TMS as an internal standard. MS spectra were carried out with a VG Autospec-3000 mass spectrometer. IR spectra were obtained in KBr pellets on a Bio-Rad FTS-135 infrared spectrophotometer. GC-MS was performed on a Finnigan 4510 GC-MS spectrometer employing the electron impact (EI) mode (ionizing potential 70 eV) and a capillary column (30 m × 0.25 mm) packed with 5% phenyl and 95% methylsilicone on HP-5. Helium was used as carrier gas, column temperature 160–240°C (rate of temperature: 5°C/min).

TLC was carried out on plates precoated with silica gel F_{254} (Qingdao Marine Chemical Ltd., PR China) and detection was achieved by spraying with 10% copper sulfate in orthophosphoric acid solution followed by heating for 10–15 min.

3.2. Fungal material

The fresh fruiting bodies of *A. mellea* were collected from ZhongDian of Yunnan Province, in August 1998 and identified by Profs P.G. Liu and X.H. Wang, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, PR China. A voucher specimen has been deposited at the Herbarium of Kunming Institute of Botany.

3.3. Extraction and isolation

The fresh fruiting bodies (dry wt. 712 g) of *A. mellea* were extracted four times with EtOAc (4 × 1.5 l) at room temperature. The combined EtOAc layer was concentrated to dryness in vacuo to give a residue (30 g) which was subjected to Si-gel CC with a gradient elution of *n*-hexane-Et_2O.

The fraction (4.61 g) eluted with *n*-hexane–Et_2O 8:2, was passed through vacuum liquid chromatography (VLC) using petroleum ether–acetone 20:1–9:1, and the fraction eluted with 15:1 gave ergosterol peroxide (125 mg) as colorless needles (*n*-hexane–AcOEt), mp 182–184°C, $[\alpha]_D^{23}$ −34 (c 0.6, $CHCl_3$); IR, EI-MS, ^1H and ^{13}C-NMR spectra were in full agreement with those of an authentic sample [6,7].

The eluate (1.27 g) obtained from *n*-hexane–Et_2O 6:4 was passed through VLC with $CHCl_3$–MeOH 15:1–2:1 as eluents to provide eight fractions. Fraction 3 was repeatedly crystallized from *n*-hexane–Et_2O to afford compound **1** (26.8 mg).

Armillaramide (**1**). White amorphous powder, mp 113–117°C; $[\alpha]_D^{26}$ +14.4 (c 0.68, pyridine); IR bands (KBr): 3376, 3220, 2919, 2851, 2487, 1615, 1557, 1469, 1380, 1078, 1050, 720, 639, 530 cm^{-1}; ^1H- and ^{13}C-NMR: see Table 1; EI-MS (70 eV) *m/z*: 555 $[M]^+$ (3), 553 $[M\text{-}2H]^+$ (1) 537 (4.5), 328 (74), 311 (35), 299 (19), 298 (60), 281 (65), 280 (26), 256 (80), 257 (29), 239 (13), 211 (2.5), 111 (12), 97 (31), 83 (42), 69 (49), 60 (100); positive FAB-MS *m/z*: 556 $[M + 1]^+$ (100), 538 $[M + 1\text{-}H_2O]^+$ (5), 318 (2), 282 (2.5), 256 (4); HREIMS *m/z*; 555.5187 $[M]^+$ (calcd. for $C_{34}H_{69}NO_4$, 555.5226).

3.4. Acetylation of *1*

A solution of **1** (3.9 mg) in Ac_2O–pyridine 1:1 (1 ml) was left at room

temperature overnight, then diluted with 2 ml of water and extracted with EtOAc (3 × 5 ml). The extract was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue obtained was Si-gel CC (petroleum ether–EtOAc 8:2) to give 4.5 mg of **1a**.

1,3,4-Triacetoxy-(2S,3S,4R)-2-hexadecanoylamino-octadecane (**1a**). White solid; IR bands (KBr): 3331, 2922, 2846, 1737, 1679, 1529, 1468, 1440, 1372, 1272, 1246, 1224, 1103, 1048, 720, 660 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.96 (1H, *d*, *J* 9.2 Hz, NH), 5.10 (1H, *dd*, *J* 8.7, 3.1 Hz, 3-H), 4.93 (1H, *dt*, *J* 9.8, 3.1 Hz, 4-H), 4.47 (*m*, 2-H), 4.29 (1H, *dd*, *J* 11.6, 4.6 Hz, 1-Ha), 4.01 (1H, *dd*, *J* 11.6, 3.1 Hz, 1-Hb), 2.22 (2H, *t*, *J* 7.4 Hz, 2′-H_2), 2.10 (3H, *s*, OAc), 2.06 (6H, *s*, 2 × OAc), 1.66 (4H, *m*, 5-H_2 and 6-H_2), 1.12–1.27 (*n* × CH_2, *br s*), 0.90 (6H, *t*, 6.6 Hz, 2 × CH_3); positive FAB-MS *m/z*: 682 $[M + 1]^+$ (100), 622 (47), 562 (3), 502 (0.2), 264 (18).

*3.5. Methanolysis of **1** and acetylation of the long chain base*

Compound **1** (10 mg) was refluxed with 1.5 ml of 0.9 N HCl in 82% aq. MeOH for 16 h. The reaction mixture was extracted with *n*-hexane. The *n*-hexane layer was concentrated and Si-gel CC (*n*-hexane/EtOAc 9:1–7:3) to give a fatty acid methyl ester **1b**, which was subjected to GC-MS. The analysis showed only a single peak assigned to methyl hexadecanoate, major ion peaks at *m/z* 270 $[M]^+$ and 211 $[M\text{-}COOMe]^+$. The aqueous layer was neutralized with sat. Na_2CO_3, concentrated to dryness, and then heated with Ac_2O–pyridine 1:1 for 1.5 h at 70°C. The reaction mixture was diluted with water and extracted with EtOAc. The residue of the extract was Si-gel CC (*n*-hexane/EtOAc 8:2) to furnish **1c**.

2-Acetoamino-1,3,4-triacetoxyoctadecane (**1c**). White solid, $[\alpha]_D^{27}$ +10.9 (c 0.67, $CHCl_3$); EI-MS (70 eV) *m/z*: 486 $[M + 1]^+$ (1), 426 (2), 366 (9), 305 (24.5), 245 (0.5); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.97 (1H, *d*, *J* 9.2 Hz, NH), 5.10 (1H, *dd*, *J* 8.5, 3.1 Hz, 3-H), 4.93 (1H, *dt*, *J* 9.8, 3.1 Hz, 4-H), 4.47 (1H, *m*, 2-H), 4.29 (1H, *dd*, *J* 11.6, 4.3 Hz, 1-Ha), 4.00 (1H, *dd*, *J* 11.6, 3.1 Hz, 1-Hb), 2.08 (3H, *s*, 3-OAc), 2.05 (6H, *s*, 1-OAc, 4-OAc), 2.03 (3H, *s*, HNAc), 1.12–1.70 (26H, *m*), 0.88 (3H, *t*, *J* 6.1 Hz, CH_3).

Acknowledgements

We wish to acknowledge financial support from the Natural Science Foundation of Yunnan Province (2000B0066M). We are grateful to Ms X.H. Wang, Profs P.G. Liu and D.G. Ji for their help in this project.

References

[1] Donnellly DMX, Konishi T, Dunne O, Cremin P. Phytochemistry 1997;44:1473.
[2] Yang JS, Chen YW, Feng XZ et al. Planta Med 1989;55:564.
[3] Kolter T, Sandhoff K. Angew Chem Int Ed Engl 1999;38:1532.
[4] Kim SY, Choi YH, Huh H, Kim JW, Kim YC, Lee HS. J Nat Prod 1997;60:274.

[5] Natori T, Morita M, Akimoto K, Koezuka Y. Tetrahedron 1994;50:2771.
[6] Gao JM, Dong ZJ, Yang X, Liu JK. Acta Bot Yunnan 2001;23:86.
[7] Gao JM, Hu L, Dong ZJ, Liu JK. Lipids 2001;36: (in press).
[8] Gao JM, Dong ZJ, Liu JK. Lipids 2001;36:175.
[9] Lourenco A, Lobo AM, Rodriguez B, Jimeno ML. Phytochemistry 1996;43:617.
[10] Inagaki M, Isobe R, Kawano Y, Miyamoto T, Komori T, Higuchi R. Eur J Org Chem 1998:129.
[11] Sugiyama S, Honda M, Higuchi R, Komori T. Liebigs Ann Chem 1991: 349.
[12] Kahlos K, Kangas L, Hiltunen R. Planta Med 1989;55:389.
[13] Cantrell C, Rajab MS, Franzblau SG, Fronczek FR, Fischer NH. Planta Med 1999;65:732.
[14] Michel P, Bernadette L, Didier S. Eur Pat Appl EP 736 522 (Chem Abstr) 1996;125:308691.