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Molecular species of ceramides from the ascomycete truffle *Tuber indicum*

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Abstract

The ceramide fractions were isolated from the chloroform/methanolic extractable of the fruiting bodies of *Tuber indicum* and separated into three kinds of molecular species TI-1, TI-2, and TI-3 by normal and reverse phase silica gel-column chromatography. By means of ^{1}H NMR and ^{13}C NMR spectroscopy, fast atom bombardment mass spectrometry (FAB–MS), and chemical degradation experiment, their component sphingoid base for TI-1 and TI-2 was uniformly (2*S*,3*S*,4*R*)-2-amino-1,3,4-octadecantriol, while the sphingoid of TI-3 was D-*erythro*-sphingosine, and their structures have been determined unequivocally to be (2*S*,2′*R*,3*S*,4*R*)-2-(2′-D-hydroxyalkanoylamino) octadecane-1,3,4-triol, the fatty acid composition of which consists of 2-hydroxydocosanoic, 2-hydroxytetracosanoic, and 2-hydroxytricosanoic acids (from major to minor); (2*S*,3*S*,4*R*)-2-(alkanoylamino)octadecane-1,3,4-triol, the fatty acid composition of which is unusual and consists of docosanoic, hexadecanoic, tricosanoic, octadecanoic and nonadecanoic acids (from major to minor); and (2*S*,3*R*,4*E*)-2-(alkanoylamino)-4-octadecene-1,3-diol, the component fatty acids of which were hexadecanoic (predominant) and octadecanoic acids, respectively.

Keywords: Higher fungus; Sphingolipid; Ceramide; Ascomycete; *Tuber indicum*; Truffle

1. Introduction

Truffles, also known as "black diamonds", are hypogeous fungi, belonging to the family Tuberaceae, the order Tuberales, the phylum Ascomycotina, which grow in symbiosis with certain trees. There are more than 60 different kinds of truffles around the world (Hawksworth et al., 1995), most of which grow in various parts of Europe, particularly in France. They are thought to be a "miracle of nature" and have been since ancient times the ultimate in gastronomy because of their highly nutritional attributes. Recent studies have proven that some truffles contain ergosteroids, and ergosterol, the most widespread fungal sterol, and brassicasterol (Gao et al., 2001a; Harki et al., 1996) as well as volatile organic compounds in truffle aroma which is characteristically sulfurous (Zeppa et al., 2004; Menotta et al., 2004; Diaz et al., 2002, 2003). More interestingly, the ability of pigs to

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detect truffles underground has been associated with the existence of trace amounts of aromatic steroidal pheromone in both black and white truffles (Claus et al., 1981).

About 25 species of the genus *Tuber* are known in China. The edible Chinese truffle, *T. indicum* Cooke et Massee., is distributed mainly in the provinces of Yunnan and Sichuan of China (Mao, 1998). This truffle looks a lot like the black winter truffle *T. melanosporum* in Europe. As part of our search for naturally occurring bioactive metabolites of higher fungi in China, the lipoid constituents of *T. indicum* were investigated. In the previous papers, we reported a polyhydroxylated ergosterol glycoside (Gao et al., 2001a), and the dominant unsaturated fatty acids as well as a minor polyhydroxylated C_{18} fatty acid from the fruiting bodies of this fungus (Gao et al., 2001b).

Although there is detailed evidence on the sterols and volatile compounds of a number of *Tuber* species, very little is known about complicated lipids of representatives of this genus. In our continuous research into chemical constituents of *T. indicum*, much attention was paid to the fact that sphingolipids, including cerebrosides and ceramides, displayed structural features uncommon for the fungal sphingosine derivatives. Since sphingolipids represent not only structural components of the cell membrane, but burden also other vitally important functions in the fungal cell (Gao et al., 2003c; Batrakov et al., 2002). Recently, some have also been reported to show antinociceptive (Koyama et al., 2002), neurotrophic (Kwon et al., 2003), antitumor and immunostimulatory (Natori et al., 1994), anti-phospholipase A_2 (Gao et al., 2003d), cholesteryl ester transfer protein inhibitory (Venkateswarlu et al., 1998) activity. Because of their important biological functions, they have given rise to considerable research interest. The present communiction deals with the isolation and structural elucidation of the ceramide molecular species present in the fungus.

2. Materials and methods

2.1. General

The dried fruiting bodies of *T. indicum* were purchased from a market of Yunnan Province in April 2000 and identified by Prof. P.G. Liu, Ms. X.H. Wang, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, People's Republic of China. A voucher specimen has been deposited at the Herbarium of Kunming Institute of Botany. All solvents used were distilled prior to experiments.

Column chromatography was performed on silica gel (200–300 mesh.) and LiChroprep reversed-phase (RP) C-8 (40–63 μm) columm (Merck, Germany). Analytical TLC was carried out with silica gel 60 F_{254} precoated plates (Qingdao Marine Chemical Ltd., People's Republic of China) using the following solvent systems for development: I, $CHCl_3$/MeOH (10:1–8:2, v/v); and II, $CHCl_3$/MeOH/H_2O (8:2:0.2, v/v). Reversed-phase TLC was performed on plates precoated with modified silica gel, RF-18F_{254}S (Merck), in 9:1–6:4 MeOH/H_2O as a developing solvent system. Fractionations were monitored by TLC. Ceramides were located by spraying the plates with 1% H_2SO_4 in anhydrous EtOH reagent and stained by spraying the plates with 10% $CuSO_4$ in H_3PO_4 reagent, followed by heating at 100–110 °C for 5–10 min, respectively.

In order to determine the alkyl chain length in fatty acid and sphingosine components, the acid hydrolysis of sphingolipids was carried out. 2-hydroxy fatty acid methyl esters (HFAMEs) or fatty acid methyl esters (FAMEs) were determined after methanolysis of 3–30 mg of the respective sphingolipid empolying 1–3 mL 0.9 mol/L hydrochloric acid in MeOH/H_2O (82:18, v/v) at 80 °C for 18 h. The reaction mixture was extracted for three times with *n*-hexane and the *n*-hexane layer was concentrated and chromatographed over a column of silica gel using *n*-hexane/EtOAc (15:1–7:3, v/v) to separate the corresponding methyl esters of fatty acids, which were analysed by GC/MS on a Finnigan 4510 GC-MS spectrometer (America) employing the EI mode (ionizing potential 70 eV) and an i.d. 3000 mm × 0.25 mm capillary column packed with 5% phenyl/95% methylsilicone on 5% phenyl-dimethylsilicone (HP-5) (Hewlett-Packard, Palo Alto, CA). Helium served as a carrier gas; column temperature was programmed from 160 to 240 °C at a rate of 5 °C/min.

For the determination of all sphingoid bases, i.e. long-chain bases, the aqueous methanol phase was neutralized with saturated Na_2CO_3 and concentrated to dryness, and then heated with 1:1 Ac_2O/Py for

1.5 h at 70 °C. The reaction mixture was diluted with 1–3 mL of H_2O and extracted with EtOAc (3 mL × 2 mL). The solvents were evaporated and the obtained residue was further purified by a column of silica gel using *n*-hexane/EtOAc (8:2, 3:2, v/v) as eluent to furnish peracetates of the sphingoid bases. Further characterization of the sphingoid acetate was performed by electronic ionization mass spectrometry (EI–MS), ^{1}H NMR and optical rotation.

The stereochemistry of ceramides was determined by comparison of the chemical shifts and coupling constants of the related protons, and/or optical rotations of the corresponding compounds.

Electronic ionization mass spectrometry at 70 eV and fast atom bombardment mass spectrometry (FAB–MS) in the positive-ion mode, using meta-nitrobenzyl alcohol as a matrix, spectra were measured with a VG Autospec-3000 mass spectrometer (VG, England). IR spectra were obtained in KBr pellets on a Bio-Rad FTS-135 IR spectrophotometer (Bio-Rad, America). Optical rotations were measured on a Horiba SEPA-300 polarimeter (Horiba, Janpan).

400 MHz ^{1}H NMR and 100 MHz ^{13}C NMR and two-dimensional NMR spectra were taken on a Bruker AM-400 and a DRX-500 spectrometers (Karlsruhe, Germany), respectively, in C_5D_5N or $CDCl_3$ with tetramethylsilane as an internal chemical-shift reference. Assignment of the signals observed was done using ^{1}H–^{1}H COSY at 125.8 MHz experiment.

2.2. Extraction, isolation, and initial fractionation

The powdered fruiting bodies (4.7 kg) were extracted successively three times with $CHCl_3$ and four times with 1:1 $CHCl_3$/MeOH at room temperature, which were concentrated to dryness in vacuo, respectively, to give both $CHCl_3$ extract (154 g) and $CHCl_3$/MeOH extract (122 g). The fractionation of the $CHCl_3$ extract led to lipids consisting mainly of sterols and fatty acids (Gao et al., 2001a, 2001b). The $CHCl_3$/MeOH (1:1) extract was chromatographed over silica gel using $CHCl_3$ and increasing concentrations of MeOH in $CHCl_3$ as eluents. The fractions eluted with $CHCl_3$/MeOH (19:1) were subjected to silica gel-column by elution of $CHCl_3$/MeOH (25:1) to give 40 mg of TI-3. The separation of the fractions eluted with $CHCl_3$/MeOH (9:1) by purification of RP-8 column using MeOH resulted in 90 mg of TI-2. The ceramide fractions eluted with $CHCl_3$/MeOH (8:2) were subjected to RP-8 column using MeOH to afford 105 mg of TI-1. The fractionation process was monitored by TLC in solvent systeme: I and II.

3. Results and discussion

From the chloroform/methanolic extractable of the fruiting bodies of *T. indicum*, three types of the ceramide molecular species TI-1, TI-2, and TI-3, have been isolated by normal-phase followed by reversed-phase silica gel-column chromatography, which appear as a single spot on normal-phase TLC.

The IR spectrum of TI-1 revealed a broad absorption band of N−H and hydroxylic O−H bonds at 3340/cm, absorption bands at 1620 and 1544/cm (secondary amide), and 2919, 2850, 723/cm (long aliphatic chains). In the positive ion FAB mass spectrum, three pseudomolecular ion peaks $[M + Na]^+$ were observed at *m/z* 678, 692, and 706.

Methanolysis of TI-1 with methanolic hydrochloric acid gave rise to a mixture of HFAMEs, and a long-chain base. Analysis of the HFAME fraction by GC/MS showed it to contain the methyl esters of the following 2-D-hydroxy fatty acids: 22:0 (57.2% of the total), 23:0 (16.5%), and 24:0 (26.3%). The long-chain base, C_{18}-phytosphingosine, was further identified after acetylation of methanolysis product of TI-1, followed by purification on silica gel-column chromatography affording its tetraacetate TI-1A, 2-acetoamino-1,3,4-triacetoxyoctadecane, and its EI mass spectrum displayed only one molecular ion $[M]^+$ at *m/z* 485. The ^{1}H NMR spectrum and the optical rotation ($[\alpha]_D$ + 10.6°) of the tetraacetate (Table 4.) was found to be identical to that of the known compound (Venkannababu et al., 1997).

The findings described indicated that TI-1 was isolated as a mixture of three homologous molecular species varying only in fatty-acyl chain length and having the same C_{18} long-chain base. The NMR signals for the three compounds in the mixture were completely identical. The ^{13}C NMR spectral data of TI-1 exhibited the characteristic signals of phytosphingosine-type ceramides containing 2-hydroxy fatty acids (Table 1). Therefore, TI-1 should be a molecular species of a phytosphingosine-type ceramide possessing 2-hydroxy fatty acids (Inagaki

et al., 1998; Gao et al., 2001e). Moreover, TI-1 was considered to possess normal types of side chains since the carbon atom signals due to terminal methyl groups were observed at δ14.3 ppm (normal form) in the ^{13}C NMR spectrum of TI-1 (Table 1).

The combined evidence presented above established unambiguously that the ceramide fraction TI-1 isolated from *T. indicum* consisted of (2*S*,3*S*,4*R*,2′*R*)-2-(2′-D-hydroxy-alkanoylamino) octadecane-1,3,4-triol, the amide-bound 2-D-hydroxy fatty acids of which were 22:0 (the predominant molecular species), 23:0, and 24:0. The relative stereochemistry at C-2, C-3, C-4, and C-2′ was proposed as 2*S*,3*S*,4*R*,2′*R*, since the chemical-shifts and coupling constants of 1-H, 2-H, 3-H, 4-H, and 2′-H (Table 1) in TI-1 were in good agreement with those of the synthetic ceramide **3**, (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino) hexadecane-1,3,4-triol (Sugiyama et al., 1991). Furthermore, the comparison of the optical rotations of TI-1 with the synthetic ceramide ($[\alpha]_D$ + 9.12°) (Sugiyama et al., 1991) suggested that TI-1 has the same absolute configuration as that of the synthetic one for chiral centres 2, 3, 4, 2′, as shown in Scheme 1.

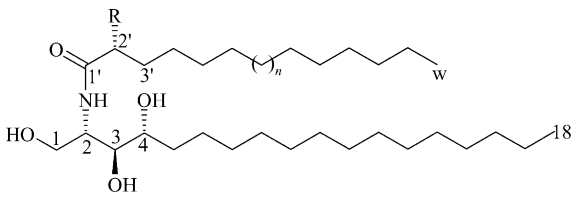
Table 1
^{1}H NMR and ^{13}C NMR spectral data for ceramides TI-1 from *T. indicum* (in C_5D_5N)

Position	δ ^{1}H ppm	Multiplicity (J Hz)	δ ^{13}C ppm
Long-chain base moiety			
1	4.52	dd, 10.6, 4.5	62.1
	4.43	dd, 10.6, 5.2	
2	5.12	m	53.1
3	4.35	dd, 6.5, 4.0	76.9
4	4.28	m	73.1
5	2.26, 1.93	m	34.2
6	1.70	m	26.7
7–16	1.25–1.41	m	29.6–30.4
17	1.25–1.41	m	22.9
18	0.86	t; 6.7	14.3
NH	8.57	d, 8.8	
2-Hydroxy fatty acid moiety			
1′			175.4
2′	4.62	dd, 7.6, 4.0	72.6
3′	2.24, 2.04	m	35.8
4′	1.76	m	25.9
5′–11′	1.25–1.41	m	29.6–30.4
ω–1	1.25–1.41	m	22.9
ω	0.86	t; 6.7	14.3

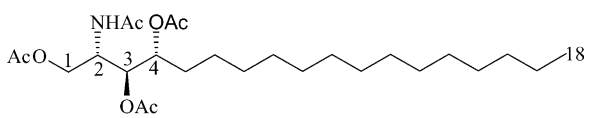
The IR spectrum of TI-2 revealed a broad absorption band of N−H and hydroxylic O−H bonds at 3340/cm, absorption bands at 1620 and 1544/cm (secondary amide), and 2919, 2850, 723/cm (long aliphatic chains). In the positive ion FAB mass spectrum, five pseudomolecular ion peaks $[M + Na]^+$ were observed at *m/z* 578, 606, 620, 662, and 676.

Drastic methanolysis of TI-2 furnished a mixture of FAMEs and a long-chain base. Analysis of the FAME fraction by GC/MS showed it to be composed of the methyl esters of the following non-hydroxy fatty acids: 16:0 (11.4%), 18:0 (6.5%), 19:0 (2.1%), 22:0 (59.1%), 23:0 (6.6%). In the same method as TI-1, the long-chain base of the ceramides TI-2 was further identified as C_{18}-phytosphingosine.

The results described above indicated that TI-2 was isolated as a mixture of five homologous molecular species varying only in fatty-acyl chain length and having the same C_{18} long-chain base. The NMR signals for the five components in the mixture were completely identical. The ^{13}C NMR spectral data of TI-2 manifested the characteristic signals of phytosphingosine-type ceramides containing non-hydroxy fatty acids (Table 2). TI-2 should be, therefore, a molecular species of a phytosphingosine-type ceramide possessing fatty acids (Inagaki et al., 1998; Gao et al., 2001f). Furthermore, the ^{1}H and ^{13}C NMR, and IR spectra of TI-2 are superimposable on those of TI-1, with only a minor difference in the fatty acyl moiety, which



TI-1: R = OH (*n* = 10, 11, 12)
TI-2: R = H (*n* = 4, 6, 7, 10, 11)



TI-1A

Scheme 1. Ceramides from *T. indicum* (TI-1 and TI-2) and phytosphingosine tetracetate (TI-1A).

was that a signal of a methylene linked to the amide carbonyl of TI-2 appeared at the chemical-shift δ 2.43 and δ 36.9 ppm instead of a methine signal at δ 4.62 and 72.6 ppm. In other words, TI-2 lacked α-hydroxy in the fatty acyl moiety.

The all above evidence obtained led to the unambiguous establishment of the ceramide fraction TI-2 isolated from *T. indicum* consisted of (2*S*,3*S*,4*R*)-2-(alkanoylamino) octadecane-1,3,4-triol, the amide-bound fatty acids of which were 16:0, 18:0, 19:0, 22:0 (the predominant molecular species), and 23:0. The relative stereochemistry at C-2, C-3, and C-4, was proposed as 2*S*,3*S*,4*R*, since the chemical-shifts and coupling constants of 1-H, 2-H, 3-H, and 4-H (Table 2) in TI-2 were in good agreement with those of the synthetic ceramide **4**, (2*S*,3*S*,4*R*)-2-(palmitoylamino) heptadecane-1,3,4-triacetate (Katiyar et al., 1998). Furthermore, the comparison of the optical rotations ($[\alpha]_D$ + 13.6°) of TI-2 with the synthetic ceramide ($[\alpha]_D$ + 12.5°) (Katiyar et al., 1998) (Table 4) suggested that TI-2 has the same absolute configuration as that of the synthetic one for the asymmetrical positions 2, 3, 4, as shown in Scheme 1.

The IR spectrum of TI-3 revealed a broad absorption band of N−H and hydroxylic O−H bonds at 3346/cm, absorption bands at 1630 and 1542/cm (secondary amide), 960/cm (*trans*-CH=CH). Two molecular ion peaks $[M + H]^+$ were observed at *m/z* 538 and 566 in the positive ion FAB mass spectrum. The characteristic signals of 2-amino-1,3-diol in hydrocarbon chain were observed at δ 4.30, 4.47, 4.75 and 4.85 ppm in the ^{1}H-NMR spectrum and at δ 56.9, 62.2, and 73.4 ppm in the ^{13}C-NMR spectrum, respectively. Furthermore, the ceramide molecular species TI-3 showed the presence of one double bond due to two olefinic carbons at δ 132.4, and 132.3 ppm in the ^{13}C NMR spectrum and two olefinic protons at δ 5.95 and 6.04 ppm in the ^{1}H NMR spectrum (Table 3). As followed from the ^{1}H–^{1}H COSY spectrum data, one of the vinyl protons (δ 6.04 ppm) was coupled, apart from another one (δ 5.95 ppm), with a proton bound to a hydroxylated carbon (C-3) adjacent to a carbon (C-2) linked to an acylamino group. In other words, the long-chain base of TI-3 had the typical *trans*-double bond at C-4, its geometry was obvious from the vinylic proton coupling constant of 15.3 Hz. Consequently, the molecular species TI-3 was indicated to be 4*E*-sphingenine type ceramide. Treatment of TI-3 with HCl in MeOH

Table 2
^{1}H NMR and ^{13}C NMR spectral data for TI-2 from *T. indicum* (in C_5D_5N)

Position	δ ^{1}H ppm	Multiplicity (J Hz)	δ ^{13}C ppm
Long-chain base moiety			
1	4.45	dd, 10.8, 4.7	62. 3
	4.40	dd, 10.8, 4.9	
2	4.99	m	53.9
3	4.31	dd, 4.8, 6.6	76.9
4	4.23	m	73.2
5	1.91	m	34.1
6	2.20	m	26.7
7–16	1.24–1.40	m	29.6–30.3
17			22.9
18	0.90	t; 6.5	14.3
NH	8.13	d, 8.3	
Fatty acid moiety			
1′			173.4
2′	2.43	t; 7.5	36.9
3′	1.82	m	26.4
4′–11′	1.24–1.40	m	29.6–30.3
ω–1	1.24–1.40	m	22.9
ω	0.90	t; 6.5	14.3

Table 3
^{1}H NMR and ^{13}C NMR spectral data for TI-3 from *T. indicum* (in C_5D_5N)

Position	δ ^{1}H ppm	Multiplicity (J Hz)	δ ^{13}C ppm
Long-chain base moiety			
1	4.47	dd, 10.9, 5.2	62.2
	4.30	dd, 10.9, 4.3	
2	4.75	dd, 5.7, 8.4	56.9
3	4.85	dd, 6.4, 12.4	73.4
4	6.04	dd, 6.6, 15.4	132.4
5	5.95	dt, 6.2, 15.4	132.3
6	2.20, 2.07	m	32.8
7–17	1.26–1.39	m	29.6–30.4
18	0.86	t, 6.7	14.3
NH	8.37	d, 8.5	
Fatty acid moiety			
1′			173.6
2′	2.46	t, 7.5	36.9
3′	1.82	m	
4′	1.91	m	
5′–15′	1.26–1.39	m	29.6–30.4
ω–1	1.26–1.39	m	22.8
ω	0.86	t, 6.7	14.3

Table 4
^{1}H NMR spectrum data and optical rotations of peracetyled sphingosines TI-1A, TI-3B, and ceramides **3**, **4** and **5** (J in Hz)

Position	TI-1A (in $CDCl_3$)	TI-3B (in $CDCl_3$)	**3** (in C_5D_5N)	**4** (in $CDCl_3$)	**5** (in $CDCl_3$)
1-Ha	4.29 (dd, 11.6, 4.3)	4.31 (dd, 12.2, 6.0)	4.52 (dd, 10.7, 4.5)	4.30 (dd, 10. 0, 5. 0)	3.95 (dd, 11.2, 3.9)
1-Hb	4.00 (dd, 11.6, 3.1),	4.05 (dd, 12.2, 4.2)	4.43 (dd, 10.6, 5.0)	4.00 (dd, 12. 0, 3. 0)	3.70 (dd, 11.2, 3.2)
2-H	4.47 (m),	4.42 (m)	5.12 (m)	4.50 (m)	3.90 (m)
3-H	5.10 (dd, 8.5, 3.1)	5.28 (dd, 6.2, 6.1)	4.36 (dd, 6.6, 6.0)	5.12 (dd, 7. 0, 4. 0)	4.31 (dd, 6.8, 6.0)
4-H	4.93 (m)	5.36 (dd, 6.0, 15.2)	4.29 (m)	4.92 (dt, 7. 0, 3. 0)	
5-H		5.80 (dt, 7.2, 15.2)			
2′-H			4.62 (dd, 7.6, 4.0)		
$[\alpha]_D$	+10.6 ($CHCl_3$)	−5.6°($CHCl_3$)	+9.12° (C_5H_5N)	+12.5° ($CHCl_3$)	−5.8° ($CHCl_3$)

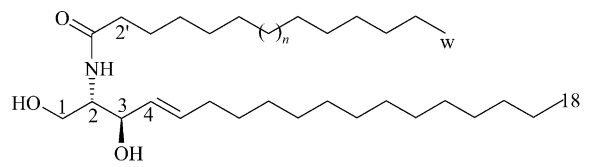
Note: Compound **3**: (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino) hexadecane-1, 3, 4-triol (Sugiyama et al., 1991); **4**: (2*S*,3*S*,4*R*)-2-(palmitoylamino) heptadecane-1,3,4-triacetate (Katiyar et al., 1998); **5**: (2*S*,3*R*,4*E*)-2-(palmitoylamino) octadecane-1,3-diol (Shin and Seo, 1995).

furnished a mixture of FAMEs and a long-chain base. Analysis of the FAME fraction by GC/MS showed it to consist of the methyl esters of the following fatty acids: 16:0 (94.9%), and 18:0 (5.1%).

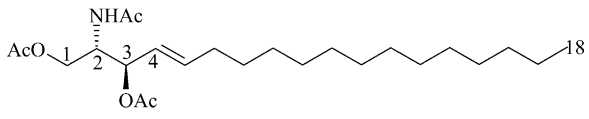
On the other hand, the long-chain base is acetylated to give its peracetate TI-3B. The ^{1}H NMR spectrum (Table 4) of TI-3B is in good agreement with that of H-1, H-2, and H-3 in triacetyl-(2*S*,3*R*,4*E*)-sphingosine (Higuchi et al., 1994). The optical rotation ([α]−4.2°) and the chemical shifts of H-1, H-2, and H-3 of TI-3 were in accordance with those of the synthetic ([α]−4.6°) and natural ([α]−5.8°) compound, (2*S*,3*R*,4*E*)-2-(palmitoylamino) octadecane-1,3-diol 5 isolated from the Gorgonian *Acabaria undulata* (Shin and Seo, 1995). Accordingly, the stereoconfiguration of the ceramide molecular species TI-3 is 2*S*,3*R*,4*E*. On the basis of the above-mentioned evidence presented, it was established that the ceramide fraction TI-3 consisted of (2*S*,3*R*,4*E*)-2-(alkanoylamino)-4-octadecene-1,3-diol, the amide-bound fatty acids of which were 16:0 (the predominant molecular species) and 18:0 (Scheme 2).

The ceramides, cleavage products of sphingolipids, including gangliosides and cerebrosides, are involved in various signal transduction pathways (Brodesser et al., 2003). Many extracellular stresses, such as tumor necrosis factors-α and human immunodeficiency virus have been shown to activate sphingomyelinases that release ceramides which inhibit cell growth and induce apoptosis (Kolter and Sandhoff, 1999; Van Veldhoven et al., 1992). Owing to the importance of ceramides, the chemistry and biology of ceramides have become a pivotal subject of the latest research of lipids in recent years (Hannun, 1994; Hannum, 1996; Jayadev et al., 1995; Okazaki et al., 1998).

The result from the present study has demonstrated the presence in ascomycete fungus *T. indicum* fruit body lipids of three ceramides consisting of C_{18}-phytosphingosine or 4-sphingenine in amide linkage with hydroxy and/or non-hydroxy fatty acids for the first time. Both fractions TI-1 and TI-2 have the same long chain base, (2*S*,3*S*,4*R*)-2-amino-1,3,4-octadecadanetriol, but differ in the structure of the fatty acids. The occurrence of the ceramides containing a phytosphingosine and an α-hydroxy fatty acid is rather common in the bonding form in higher mushrooms. More recently, a homologous series of the glycoinositol-phosphoceramides (basidiolipids) possessing this type of ceramide has been reported from some mushroom species, i.e. the basidiomycetes *Agaricus bisporus* and *Agaricus campestris* (Jennemann et al., 1999), *Amanita*



TI-3: $n = 4, 6$



TI-3B

Scheme 2. Ceramides (TI-3) from *T. indicum* and sphingosine triacetate (TI-3B).

virosa, *Calvatia exipuliformis*, *Cantharellus cibarius*, *Leccinum scabrum*, *Lentinus edodes*, and *Pleurotus ostreatus* (Jennemann et al., 2001). However, except for the fact this type of ceramide (phytosphingosine/α-fatty acid) itself is a normal constituent of the glycosylinositol-phosphoceramides in plants, fungi, yeast, protozoans and worms (Lester and Dickson, 1993; Yamada-hada et al., 2004; Aoki et al., 2004) in general, it has been reported previously to occur in the free state in fungi, such as two ceramides being composed of phytosphingosine and α-hydroxy C29:0 and C30:0 from a white-rot fungus *Phellinus pini* (Lourenco et al., 1996) and four ceramides isolated from the fruit bodies of the edible mushroom *Grifola frondosa* contain C22:0, C23:0, C25:0, and C26:0 (Yaoita et al., 2000), and one ceramide from several higher fungi contain C24:0 (Gao et al., 2003d, 2001e, 2002g, 2002h, 2002i; Liu et al., 2003; Wang et al., 2002; Koyama et al., 2002). One functional aspect of the hydroxyl group cluster of those ceramides, especially in the neighborhood of a phosphoinositol, may indeed be to strengthen the structures where it occurs. Furthermore, three molecular species of ceramides TI-1 isolated in this study were the ceramide residues of the glycospingolipids, regulosides A-C, respectively, which were recently isolated from the starfish *Pentaceraster regulus* (Venkannababu et al., 1997). One molecular species with 23:0 hydroxy fatty acid in ceramides TI-2 was the ceramide moiety of the glycospingolipids, cornuta-glycolipid A, which were isolated previously from the herbal medicine *Ilex cornuta* (Qin and Wu, 1988). These probably represent a precursor of these glycolipids. In addition, D-*erythro*-sphingosine is a structural sphingoid base unit common to almost all sphingolipids in eukaryotic cells. This type of the ceramide with 16:0 fatty acid as the major component in TI-3 obtained from this fungus was obtained previously from the Gorgonian *A. undulata* and beef spleen (Shin and Seo, 1995).

It is well-known that sphingolipids are ubiquitous membrane components of all eukaryotic cells and are abundantly located in all plasma membranes as well as in some intracellular organelles (Kolter and Sandhoff, 1999). Ceramides possessing C_{18}-sphingosine base moiety, are not only in many mammalian tissues but also in fungal and plant kingdoms. Ceramides rank among the most abundant sphingolipids of fungi (Weiss and Stiller, 1972; Lösel, 1988), the ceramides containing C_{18}-phytosphingosine being revealed in a variety of these organisms (Weiss and Stiller, 1972; Gao et al., 2003d, 2001e, 2004f, 2002g, 2002h, 2002i, Lourenco et al., 1996; Liu et al., 2003; Wang et al., 2002; Koyama et al. 2002). For the most part the fungal ceramides contain 15:0, 16:0, 18:0, 20:0 even-numbered non-hydroxylated and 2-hydroxy fatty acids as N-acyl moieties, while fatty acids with 19:0 and 23:0 odd-numbered chains are less common to these ceramides in higher fungi (Weiss and Stiller, 1972). A high level of N-acyl moieties of such kind in both ceramides TI-1 and TI-2 described here is probably related with a specific biologic function of these ceramides in the ascomycete fungus *T. indicum* and is undoubtedly of special interest. Moreover, a very high concentration of the ceramide molecular species TI-3 containing D-*erythro*-sphingosine obtained from this fungus is also of great importance to assistance in understanding the biological effects of the fungus.

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