Novel Ceramides from the Fungus *Lactarium volemus*

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Two novel ceramides, lactariamides A (*N*-2′-hydroxytetracosanoyl-2-amino-3,4-epoxyoctadecan-1-ol (**1**)) and B ((4*E*,8*E*)-*N*-2′-hydroxyoctadecanoyl-2-amino-9-methyl-4,8-octadecadine-1,3-diol (**2**)), were isolated from the fungus *Lactarium volemus*, together with a known compound, cerebroside D (**3**). Their structures were determined on the basis of chemical evidence and spectral methods.

Lactarium volemus Fr. (Russullaceae) is a fungus widely distributed in the southwest of China, and its ethanol extract was reported to inhibit the growth of several tumor cell lines in vitro.[1] A number of sterols and their derivatives have been isolated from this fungus in the search for bioactive components.[2,3] In the continuation of our research on this fungus, two novel ceramides, lactariamides A (*N*-2′-hydroxy tetracosanoyl-2-amino-3,4-epoxyoctadecan-1-ol (**1**)) and B ((4*E*,8*E*)-*N*-2′-hydroxyoctadecanoyl-2-amino-9-methyl-4,8-octadecadine-1,3-diol (**2**)), were isolated. The current report describes the isolation and structural elucidation of lactariamides A (**1**) and B (**2**), along with a known compound, cerebroside D (**3**). Ceramides are key compounds in the metabolism of sphingolipids and are emerging as important second messengers for various cellular processes including cell cycle arrest, differentiation, senescence, apoptosis, and others. Because of their biological importance, a series of research efforts have been made to understand their function and metabolism.[4]

Lactariamide A (**1**) was obtained as a white amorphous powder. Its molecular formula was determined as $C_{42}H_{83}NO_4$ on the basis of HREIMS. Compound **1** exhibited a molecular ion peak at *m*/*z* 665 $[M]^+$ in the EIMS and a significant ion at *m*/*z* 670 $[M + Na - H_2O]^+$ in the positive FABMS. IR data of compound **1** indicated that it was a secondary amide (1625, 1545 cm^{-1}). The ^1H and ^{13}C NMR spectra of **1** (Table 1) were consistent with the presence of a secondary amide group (δ_H 8.65, 1H, d, J = 9.9 Hz, δ_c 53.1, δ_c 175.4).[5,6] The IR spectrum also showed strong absorption bands at 3334 and 3217 cm^{-1}, indicating the possible existence of hydroxyl groups together with the presence of a secondary amide. Proton signals at δ 4.35−4.75 in the ^1H NMR and four oxygenated carbons at δ 62.2, 72.6, 73.2, and 76.9 in the ^{13}C NMR supported the presence of hydroxyl groups or other oxygenated groups. The overlapped proton signals at δ1.25−1.45 in the ^1H NMR spectrum and the carbon signals at δ 22.9−35.7 in the ^{13}C NMR spectrum inferred the occurrence of long aliphatic chains.[7]

The structural elucidation and complete ^1H and ^{13}C signal assignments were achieved by 2D NMR techniques and chemical methods. In the $^1H-^1H$ COSY (Figure 1, Supporting Information), the H-1a and H-1b protons were taken as the starting point; both H-1a and H-1b correlated with H-2. H-2, assumed to be geminal with the nitrogen of the secondary amide group, correlated with H-3. H-3 correlated with H-4; both the protons were considered geminal to an oxygen functional group. H-4 correlated with H-5a and H-5b, and H-5a and H-5b also correlated with H-6a and H-6b. H-6a and H-6b showed cross-peaks with the multiple proton signals at δ 1.25−1.45, which showed correlation with methyl proton signals at δ 0.90. Thus, the connectivity $-OCH_2-CH(NH-)-CH(O-)-CH(O-)-CH_2-CH_2-(CH_2)_m-CH_3$ was established. Another structural fragment $(-CH(OH)-CH_2-CH_2-(CH_2)_n-CH_3)$ was also evident from the $^1H-^1H$ COSY spectrum. This was supported by the proton signals at δ_H 1.25−1.45 and two terminal methyls at δ_H 0.90 (6H, t, 7.2 Hz) in the ^1H NMR spectrum, indicating the presence of two long aliphatic chains.

There were two degrees of unsaturation in the molecule according to the molecular formula. One secondary amide group accounted for one of those. The remaining degree of unsaturation was assumed to be a cyclic ether group on the basis of the ^1H NMR and ^{13}C NMR data. An ether linkage between C-1 and C-3, or between C-1 and C-4, was ruled out since the C-1 signal (δ 62.8) was typical of a primary alcohol. If an ether linkage between C-1 and C-3 or between C-1 and C-4 was present, the C-1 signal should be shifted downfield about $\Delta\delta$ 5−10.[8] The presence of an epoxy group between C-3 and C-4 was thus indicated.

The carbonyl group was assigned to C-1′ on the basis of long-range HMBC correlations between H-2′ at δ 4.75 and C-1′ at δ 175.4, and H-2′ and C-3′ at δ 35.8. The two fragments were finally connected by the correlation between the proton on the secondary amide (NH) and the carbonyl carbon, and H-2 and the carbonyl carbon. The planar structure of compound **1** was thus elucidated. These assignments were also confirmed by the correlation of NOESY spectrum (Table 1a, Supporting Information). In the NOESY spectrum, the proton on the secondary amide (NH) correlated with H-2 and H-2′, and H-2′ correlated with the NH and H-3a′, 3b′.

Basic hydrolysis of compound **1** yielded a new amine (**1a**). Positive ESIMS exhibited an ion at *m*/*z* 304 $[M + Na - H_2O]^+$ corresponding to the molecular formula $C_{18}H_{37}NO_2$. The structure of **1a** was elucidated on the basis of ^1H NMR and positive ESIMS. The chain lengths of both the amino alcohol and acyl group in compound **1** were thus unambiguously determined. The H-2 and H-3 protons having strong correlations with H-5 in the NOESY spectrum (Table 1a, Supporting Information) indicated a *cis*-configuration of the epoxy ring. The structure of lactariamide A

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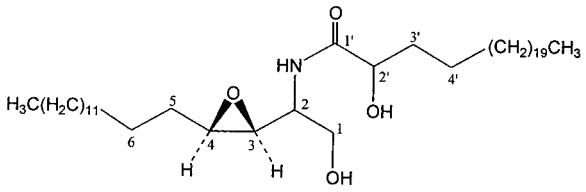
Table 1. ^{1}H NMR (400 MHz) and ^{13}CNMR (100 MHz) of **1** and **2**

compound **1**[a]			compound **2**[b]		
no.	δ_H (*J*/Hz)	δ_C (DEPT)	no.	δ_H (*J*/Hz)	δ_C (DEPT)
1	4.50 (1H, dd, 10.8, 5.0, H-1a) 4.60 (1H, dd, 10.8, 4.5, H-1b)	62.2	1	3.72 (1H, m, H-1a) 3.78 (1H, m, H-1b)	62.2
2	5.20 (1H, m)	53.1	2	4.22 (1H,m)	54.6
3	4.45(1H, dd, 6.5, 4.7)	76.9	3	4.08 (1H, 7.2, 4.4)	74.3
4	4.35 (1H, td, 7.7, 2.3)	73.2	4	5.10 (1H, m)	134.1
5	2.35, 2.08 (each 1H, m, H-5a, 5b)	34.3	5	5.78 (1H, m)	128.9
6	1.80, 1.51 (each 1H, m, H-6a, 6b)	26.8	6	2.05 (2H, m)	32.5
7−15	1.33−1.37(m)	29.7−30.6	7	2.05 (2H,m)	28.7
16	1.25−1.45 (m)	32.3	8	5.50 (1H, dd, 15.6, 6.4)	123.1
17	1.25−1.45 (m)	23.1	9		136.3
18	0.90, (6H, t, 7.2)	14.4	10	1.95 (2H, t, 6.8)	39.7
NH	8.65 (1H, d, 9.9)		11−15	1.22−1.32 (10H, m)	29.4−29.7
1′		175.4	16	1.22−1.32 (2H, m)	31.9
2′	4.75 (1H, dd, 7.7, 3.8)	72.6	17	1.22−1.32(2H, m)	22.7
3′	2.18, 2.28 (each 1H, m, H-3′a, 3′b)	35.8	18	0.90 (3H, t, 7.3)	14.1
4′	1.49, 1.90 (each 1H, m, H-4′a, 4′b)	26.0	19	1.60 (3H, s)	16.0
5′−21′	1.25−1.45 (m)	29.7−30.6	NH	7.21 (1H, d, 7.8)	
22′	1.25−1.45 (m)	32.3	1′		175.1
23′	1.25−1.45 (m)	23.1	2′	3.90 (1H, dd, 10.8, 4.0)	72.5
24′	0.90, (6H, t, 7.2)	14.4	3′	1.55, 1.75 (each 1H, m)	34.9
			4′	1.39 (2H, m)	25.1
			5′−15′	1.22−1.32 (m)	29.4−29.7
			16′	1.22−1.32 (m)	31.9
			17′	1.22−1.32 (m)	22.7
			18′	0.90 (1H, t, 7.3)	14.1

[a] The ^{1}H NMR and ^{13}C NMR of compound **1** were measured in pyridine-d_5. [b] The ^{1}H NMR and ^{13}C NMR of compound **2** were measured in CCl_3D.

was therefore assigned to be *N*-2′-hydroxytetracosanoyl-2-amino-3,4-epoxyoctadecan-1-ol (**1**).

The molecular formula $C_{37}H_{71}NO_4$ for compound **2** was deduced on the basis of ESIMS and ^{13}C NMR data. Compound **2** exhibited a protonated molecular ion at *m*/*z* 594 $[M + H]^+$ and a major fragment peak at *m*/*z* 576 $[M + H - H_2O]^+$. The IR of **2** indicated secondary amide (1653, 1620 cm^{-1}) and hydroxyl groups (3275 cm^{-1}).[5,6] The ^{1}H and ^{13}C NMR data of **2** showed the presence of an amide linkage and two long chain aliphatic moieties. The ^{1}H and ^{13}C NMR spectral data of **2** showed that one of the long chain aliphatic units had two double bonds and was branched. The proton signal at δ 1.60 (3H, s) indicated that a methyl group was attached to one of the double bonds.[7] Analyses of the ^{1}H and ^{13}C NMR, IR, and EIMS spectral data of compound **2** clearly indicated that it was a ceramide. Furthermore, the ^{1}H and ^{13}C NMR data of compound **2** were nearly identical with those of a known ceramide, (4*E*,8*E*,2*S*,3*R*,2′*R*)-*N*-2′-hydroxyhexadecanoyl-2-amino-9-methyl-4,8-octadecadine-1,3-diol,[9] the only difference being the integral intensities for the methylene proton signals, suggesting that they are analogues.



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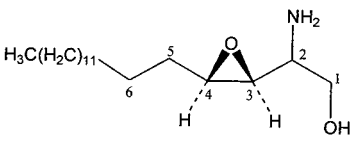
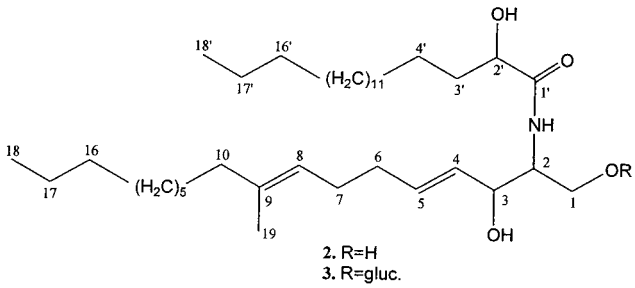


1a



2. R=H
3. R=gluc.

Direct comparison of the ^{1}H NMR, ^{13}C NMR, IR, and EIMS spectral data of compound **2** with those of the known compound cerebroside D (**3**), which was also isolated from this fungus, showed that compound **2** might be the aglycone of compound **3**. Acidic hydrolysis of **3** yielded glucose and an aglycone, which was identical with **2** judging from a comparison of its ESIMS and co-TLC (both on silica gel and on RP-18) with those of compound **2**. The structure of lactariamide B (**2**) was thus determined to be (4*E*,8*E*)-*N*-2′-hydroxyoctadecanoyl-2-amino-9-methyl-4,8-octadecadine-1,3-diol.

Compound **3** was identified as cerebroside D by comparison of its NMR, MS spectra, and optical rotation data with the reported data in the literature.[7]

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Nicolet Magna 750 spectrometer with KBr disks. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM-400 spectrometer with TMS as internal standard. ^{1}H NMR (400.13 MHz) and ^{13}C NMR (100.61 MHz, broad band and DEPT) were measured in solvents $CDCl_3$ and pyridine-d_5. EIMS (70 eV) and FABMS were carried out on a VG Auto Spec 3000 instrument. The matrix for positive FABMS was *meta*-nitrobenzyl alcohol (MNBA). HREIMS was performed on a Finnigan MAT-95 MS spectrometer. ESIMS was measured on a Finnigan LCQ DECA mass spectrometer. All solvents used were of analytical grade (Shanghai Chemical Plant). Silica gel (200−300 mesh, Qingdao Haiyang Chemical Plant) was used for column chromatography, and precoated silica gel GF254

plates (Qingdao Haiyang Chemical Plant) were used for TLC. C18 silica gel (Merck) and MCI GEL CHP20P (75−150μ) (Mitsubishi Chemical Industries LTD) were used for column chromatography.

Plant Material. *Lactarius volemus* Fr. was collected from the Lijiang district of Yunnan province of China and authenticated by Prof. Zang Mu of the Kunming Institute of Botany, where a voucher specimen (HKAS 30221) is deposited.

Extraction and Fractionation. Fresh mushroom bodies (20 kg) were collected from the mountains (over 3000 m above sea level) near the town of Qiao-Tou in Lijiang district. The fresh *L. volemus* was crushed with a blender and immediately immersed in 95% EtOH. The ethanol-immersed sample was then extracted four times with 95% EtOH at room temperature (10 h each), and the liquid was obtained by suction filtration. The residue (276 g) yielded by removal of the solvent in a vacuum was dissolved in H_2O (2 L) to form a suspension. The aqueous suspension was successively partitioned with petroleum ether (to defat) and ethyl acetate to obtain a petroleum ether-soluble fraction (89 g) and an ethyl acetate-soluble fraction (52 g), respectively. The aqueous part was condensed to give a H_2O-soluble fraction (135 g). The ethyl acetate-soluble fraction (52 g) was subjected to CC containing MCI GEL CHP20P eluting with 70% MeOH in H_2O, MeOH, and acetone to give fraction 1 (18 g), fraction 2 (27 g), and fraction 3 (5.5 g). Fraction 1 was separated by CC (packed with 240 g silica gel H) eluted with CH_2Cl_2−acetone (4:1 and acetone) to obtain six major fractions, 1a−1f. Fractions 1a and 1b contained mainly unsaturated fatty acids and sterols. Fraction 1c, containing a major blue spot on TLC (C18 silica gel precoated plate, developed by 70% MeOH in H_2O, $R_f = 0.4$, color-reacted by 20% H_2SO_4 in EtOH and heating), was subjected to C18 silica gel column chromatography eluted with 60% MeOH in H_2O to yield **1** (87 mg). Fraction 1d was extensively chromatographed on a C18 silica gel column eluted with 70% MeOH in H_2O to give **2** (57 mg). Fraction 1f was extensively subjected to a C18 silica gel column and eluted with 60% and 70% MeOH in H_2O to afford **3** (536 mg).

Lactariamide A (1): white amorphous powder; $[\alpha]_D^{20}$ +11.4° (*c* 0.51, $CHCl_3$−MeOH, 1:1); no UV absorption between 210 and 600 nm (in MeOH); IR (KBr disk) ν_{max} 3334, 3216, 2918, 1625, 1544, 1467, 723 cm^{-1}; EIMS *m/z* (%) 665 $[M]^{\bullet+}$ (12), 384 (36), 357 (10) 57 (100); ^1H and ^{13}C NMR data (Table 1); positive FABMS *m/z* (%) 670 $[M + Na - H_2O]^+$ (100), 384 (7), 286 (5); HREIMS *m/z* 665.6329 (calcd for $C_{42}H_{83}NO_4$, 665.6322).

Hydrolysis of 1. Compound **1** (18 mg) was dissolved in 4 mL of methanolic solution of 1 M NaOH and refluxed for 3 h. The pH of the reaction mixture was then adjusted to 6−7 with dilute HCl. The solvent was removed, and the residue was subjected to silica gel column chromatography, eluted with $CHCl_3$−MeOH (6:1), to give **1a** (8 mg) as a white amorphous powder.

Compound 1a: white amorphous powder; ^1H NMR (CD_3-OD, 400 MHz) δ 3.75 (1H, dd, $J = 10.7$, 4.5 Hz, H-1a), 3.56 (1H, dd, $J = 10.7$, 5.0 Hz, H-1b), 3.35 (2H, m, H-2α, H-3α), 3.27 (1H, m, H-4α), 1.60 (2H, m), 1.40 (2H, m), 1.20 (22H, m), and 0.70 (3H, t, $J = 7.2$ Hz); positive ESIMS *m/z* 304 $[M + Na - H_2O]^+$ and 286 $[M + Na - 2H_2O]^+$.

Lactariamide B (2): white amorphous powder; $[\alpha]_D^{20}$ +8.38° (*c* 0.39, MeOH); no UV absorption between 210 and 600 nm (in MeOH); IR (KBr) ν_{max} 3350, 3274, 2919, 1652, 1538, 1467, 721 cm^{-1}; EIMS *m/z* (%) 594 $[M]^+$ (3), 396 (23), 342 (45), 325 (81), 60 (100); ^1H and ^{13}C NMR data (Table 1).

Hydrolysis of 3. Compound **3** (40 mg) was dissolved in 10 mL of a methanolic solution of 1 M H_2SO_4 and refluxed for 8 h. The reaction mixture was then adjusted to pH 6−7 with a solution of NaOH. The solvent was removed in vacuo, and the residue was subjected to silica gel column chromatography, eluted with $CHCl_3$−MeOH (6:1), to yield **2** (19 mg).

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Supporting Information Available: Key correlations in the $^1H-^1H$ COSY spectrum of **1** and major 2D NOESY correlations of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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