Fitoterapia 81 (2010) 838–843

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Cerebroside and ceramide from the pollen of *Brassica napus* L.

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ARTICLE INFO

Article history:
Received 21 February 2010
Accepted in revised form 10 May 2010
Available online 17 May 2010

Keywords:
Pollen of *Brassica napus* L.
Cerebroside
Ceramide
Cytotoxic activity

ABSTRACT

The new cerebroside 1-O-(β-D-glucopyranosyl)-(2 S, 3 S, 4R, 8E)-2-[(2′R)-2′-hydroxytetracosenoilamino]-8-octadecene-1,3,4-triol (1) and ceramide (2 S, 3 S, 4R, 8E)-2-[(2′R)-2′-hydroxytetracosenoilamino]-8-octadecene-1,3,4-triol (2) were isolated from the ethyl acetate extract of the pollen of *Brassica napus* L. The structures of 1 and 2 were elucidated on the basis of chemical and spectroscopic method. Two new compounds were evaluated for activity in vitro assays for the cytotoxic activities against human tongue squamous carcinoma cell line (Tca8113).

1. Introduction

The pollen of *Brassica napus* L. has been used in China to treat benign prostatic hyperplasia (BPH) for over decades. In previous studies, the ethyl acetate extract of the pollen of *Brassica napus* L. showed strong activity on decreasing the secretion of prostate specific antigen (PSA) in LNCaP cells [1]. Sterols, terpenoids, flavones, long chain hydrocarbons, and brassinolide have been reported from the EtOAc-soluble fraction [1–3]. This study deals with the isolation and characterization of a new cerebroside and ceramide from the EtOAc-soluble fraction of EtOH extract of this pollen. The cytotoxic activity of compounds 1 and 2 was tested in vitro against human tongue squamous carcinoma cell line Tca8113. Compound 2 was shown to possess significant cytotoxic activity.

Sphingolipids, e.g. ceramides, cerebrosides, are important constituents of cellular membranes and are emerging as important second messengers for various cellular processes such as cell cycle arrest, differentiation, senescence, apoptosis [4–6]. Furthermore, some sphingolipids have been reported to exhibit anti-ulcerogenic [7], antihepatotoxic [8], antitumor and immunostimulatory activities [9].

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doi:10.1016/j.fitote.2010.05.006

2. Experimental

2.1. General experimental procedures

Optical rotations were measured using a Rudolph-Research Autopol-III automatic polarimeter. UV spectra were measured on a T6 UV–vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd, Beijing, China). IR spectra were obtained on a Nicolet-170SX FT-IR spectrometer (Madison, WI, USA) using KBr pellets in cm^{-1}. NMR spectra were recorded on a Varian Inova-400 FT-NMR spectrometer (Palo Alto, CA, USA) with TMS as an internal standard, δ in ppm, J in Hz. HR-ESI-MS were measured on Bruker APEXII mass spectrometer in m/z. EI-MS were measured on a VG ZABHS mass spectrometer at 70 eV. Silica gel (200–300 mesh, Qingdao Mar. Chem. Ind. Co. Ltd.).

2.2. Plant material

The pollen of *Brassica napus L.* was collected in September 2007 in Gansu, China. The material was identified by Prof. Huan-Yang Qi of Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, China. A voucher specimen (ZY200701) has been deposited at the Key Laboratory of Chemistry of Northwestern Plant Resources,

Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, China.

2.3. Extraction and isolation

The air-dried pollen of *B. napus L.* (5000 g) was extracted with 70% EtOH (3×20 L, 7 days each) at room temperature and the EtOH was removed under reduced pressure to give a residue (1750 g), which was suspended in distilled water and extracted with n-hexane and EtOAc, respectively. The EtOAc extract (80 g) was subjected to column chromatography over silica gel (200–300 mesh, 2500 g) and eluted with $CHCl_3$, $CHCl_3$–MeOH (95/5), (90/10), (85/15), (80/20), (70/30), (50/50), (30/70), (10/90) and MeOH to yield 10 fractions (Fr.1–Fr.10) . Fr.2 was subjected to a silica gel column eluting with $CHCl_3$–MeOH (19/1) to give compound 2 (55 mg). Fr.3 was subjected to a silica gel column eluting with $CHCl_3$–MeOH (10/1 and 8/2) to give 10 subfractions. Subfraction 4 was recrystallized from MeOH to give compound 1 (125 mg).

Compound 1 : colorless solid; m.p. 104–106 °C; $[\alpha]^{20}_D$ + 10.2 (c = 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 224.0 (0.49) nm; IR (KBr) ν_{max} cm^{-1}: 3387 (hydroxyl), 1632, 1541 (amide); ^1H and ^{13}C NMR data: see Table 1; HR-ESI-MS: m/z 864.6529 $[M+Na]^+$ (calcd for $C_{48}H_{91}NO_{10}Na$: 864.6535). The structure of 1 was determined to be 1-O-(β-D-glucopyranosyl)-(2 S,3 S,4R,8E)-2-[(2′R)-2′-hydroxytetracosenoilamino]-8-octadecene-1,3,4-triol (Fig. 1).

Compound 2 : white amorphous powder; m.p. 138–139 °C; $[\alpha]^{20}_D$ + 11.0 (c = 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 212.0 (0.24) nm; IR (KBr) ν_{max} cm^{-1}: 3332 (hydroxyl), 1624, 1545 (amide); ^1H and ^{13}C NMR data: see Table 2; HR–ESI–MS: m/z 680.6173 $[M+H]^+$ (calcd for $C_{42}H_{82}NO_5$: 680.6188). The structure of 2 was determined to be (2 S, 3 S, 4R, 8E)-2-[(2′R)-2′-hydroxytetracosenoilamino]-8-octadecene-1,3,4-triol (Fig. 1).

Table 1
^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data of 1 in DMSO-d_6 (TMS, δ in ppm, J in Hz).

No.	^1H (m, J, Hz)	^{13}C	^1H–^1H COSY	HMBC (H–C)
1a	3.61(m)	68.9	1b, 2	1″, 2
1b	3.78(m)		1a, 2	
2	4.08(m)	49.9	3, 1a, 1b	1, 3, 1′
3	3.39(m)	74.1	2, 4	2, 4
4	3.36(m)	70.5	5a, 5b, 3	3, 5
5a	1.47(m)	31.7	5b, 4, 6	4, 6
5b	1.57(m)		5a, 4, 6	
6	1.49(m)	25.7	5a, 5b, 7	5, 7
7	1.95(m)	32.1	6, 8	6, 8
8	5.34(m)	130.2	7, 9	7, 9
9	5.34(m)	129.8	8, 10	8, 10
10	1.95(m)	32.4	9	9
11-15	1.30(m)	28.6–29.1		
16	1.22(m)	31.3		
17	1.22(m)	22.1		
18	0.83(t)	13.9		
1′		173.8		
2′	3.82(m)	70.9	3′a, 3′b	1′, 3′
3′a	1.47(m)	34.4	3′b	2′
3′b	1.57(m)		3′a	
14′,17′	1.96(m)	26.7		
15′	5.29(m)	129.5	14′,16′	14′,16′
16′	5.29(m)	129.3	15′,17′	15′,17′
CH_2	1.22-1.30(m)	22.1–31.3		
24′	0.83(t)	13.9		
1″	4.12(d,7.6)	103.5	2″	2″, 1
2″	2.92(m)	73.4	1″, 3″	1″, 3″
3″	3.15(m)	76.5	2″, 4″	2″, 4″
4″	3.04(m)	69.9	3″, 5″	3″, 5″
5″	3.10(m)	76.9	4″, 6″a, 6″b	4″, 6″
6″a	3.44(d,11.2)	61.0	6″b, 5″	5″
6″b	3.65(d,11.2)		6″a, 5″	5″

2.4. Methanolysis of compounds 1 and 2

After each compound (15 mg) was refluxed with 0.9 M HCl in 82% aq. MeOH (10 mL) for 18 h, the reaction mixture was extracted with n-hexane. The concentrated n-hexane layer was subjected to silica gel column chromatography (hexane/EtOAc, 20:1) to yield fatty acid methyl ester (FAM). The aq. MeOH layer was neutralized with $NH_3 \cdot H_2O$ and extracted with EtOAc. The combined EtOAc extract was washed with H_2O, dried over Na_2SO_4 and evaporated to give the long chain base (LCB). Methanolysis of compounds 1 and 2 afforded Methyl 2-hydroxy-tetracosenoate. $[\alpha]^{20}_D$ −2.0 (c = 0.1, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ_H: 5.33 (2H, m, H-15, H-16), 4.17 (1H, m, H-2), 3.77 (3H, s, OCH_3), 1.99 (4H, m, H-14, 17), 0.86 (3H, t, J = 7.2 Hz, H-24). EI-MS: m/z: 396 $[M]^+$, 337$[M\text{-}59]^+$. The data were identical to those published earlier [10,11].

2.5. Dimethyl disulfide derivative of FAMs from compounds 1 and 2

FAM-1 and FAM-2 (5 mg) were dissolved respectively in carbon disulfide (0.5 mL), and then dimethyl disulfide (0.5 mL) and iodine (10 mg) were added, successively. The reaction mixture was then kept at 60 °C for 48 h in a small sealed vial, which was subsequently quenched with 5% aq. $Na_2S_2O_3$. And then the mixture was extracted with n-hexane. The n-hexane layer was dried over Na_2SO_4, filtered and concentrated to give the dimethyl disulfide (DMDS) derivative of FAMs. Finally, the DMDS derivatives were analyzed by EI-MS. FAM-1 DMDS derivative, EI-MS: m/z = 317 $[M\text{-}C_{10}H_{21}S]^+$, 173 $[C_{10}H_{21}S]^+$. FAM-2 DMDS derivative, EI-MS: m/z = 317 $[M\text{-}C_{10}H_{21}S]^+$, 173 $[C_{10}H_{21}S]^+$ (Fig. 2).

2.6. MS analysis of LCB from compounds 1 and 2

The LCBs of compounds 1 and 2 were subjected to ESI-MS analysis. The results were as follows: LCB-1, ESI-MS: m/z = 316 $[M+H]^+$. LCB-2, ESI-MS: m/z = 316 $[M+H]^+$.

2.7. Dimethyl disulfide derivatives of LCB from compounds 1 and 2

LCBs DMDS derivatives from compounds 1 and 2 were synthesized according to the procedure described above. Finally, the DMDS derivatives were analyzed by EI-MS, both showed a characteristic fragment-ion peak at m/z = 187 $[C_{11}H_{23}S]^+$ (Fig. 2).

2.8. Acid hydrolysis of compound 1

Compound 1 (10 mg) was dissolved in MeOH–H_2O (1:1, 10 mL) and then 5% HCl (5 mL) solution was added. After the solution was refluxed for 8 h at 70 °C and cooled to room temperature, MeOH in the reaction mixture was evaporated

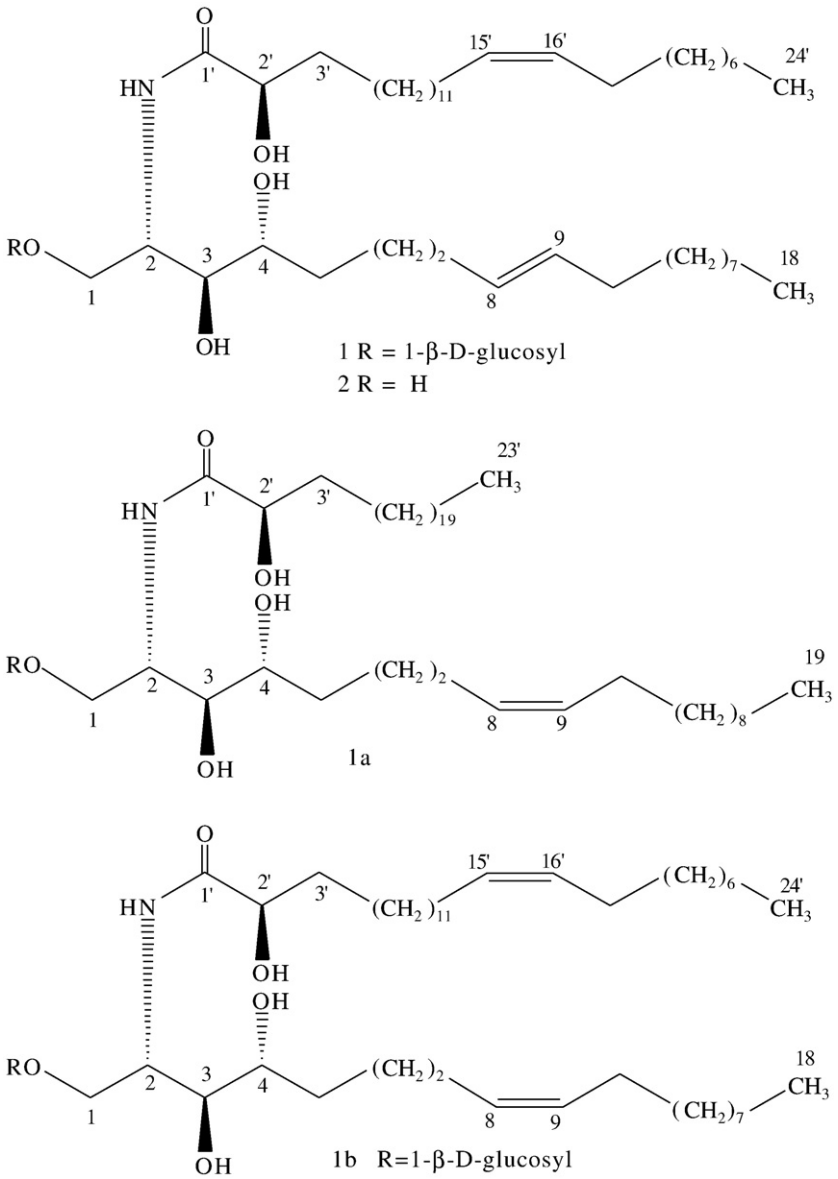

Fig. 1. Chemical structures of compounds 1, 1a, 1b and 2.

in vacuo. The residue mixture was extracted twice with $CHCl_3$. The combined extract was evaporated to yield a residue, which was compared with standard sugar units on silica gel plates [12] using n-BuOH-EtOAc-iso-PrOH-HOAc-H_2O (7:20:12:7:6) solvent system and the sugar was found to be D-glucose.

2.9. Cells and cytotoxic assays

2.9.1. Cell cultures

Human tongue squamous carcinoma cell line (Tca8113) was obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Tca8113 cells were cultured in RPMI-1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone), 100 μg/mL streptomycin, 100 IU/mL penicillin, and 0.03% L-glutamineand and maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

2.9.2. Cytotoxity assay

Tca8113 cells were cultured at 5×10^4 cells/well in 96-well plates (Corning Costar, Milano, Italy) precultured under the same conditions. Twenty-four hours later, the medium was replaced by the 10% fetal bovine serum (FBS) RPMI-1640 containing 0.1% DMSO or samples. After incubating cells with 1 and 2 (2.5, 5, 10, and 20 μg/mL) for 72 h, the cell viability was assayed by MTT method [13]. Briefly, 4 h before the end of incubation, 20 μL of MTT solutions (5.0 mg/L) was added to each well. Then, 150 μL of DMSO was added to the culture system to dissolve formazan crystal. Finally, formazan absorbance was assessed at 490 nm by an automated microplate

Table 2
^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data of 2 in DMSO-d_6 (TMS, δ in ppm, J in Hz).

No.	^{1}H (m, J, Hz)	^{13}C	^{1}H–^{1}H COSY	HMBC (H–C)
1a	3.64(dd, 10.8, 4.4)	60.4	1b, 2	2
1b	3.57(dd, 10.8, 4.4)		1a, 2	
2	3.98(m)	51.0	3, 1a, 1b	1, 3, 1′
3	3.43(m)	74.7	2, 4	2, 4
4	3.40(m)	71.1	5a, 5b, 3	3, 5
5a	1.54(m)	31.8	5b, 4, 6	4, 6
5b	1.65(m)		5a, 4, 6	
6	1.56(m)	25.6	5a, 5b, 7	5, 7
7	1.94(m)	32.1	6, 8	6, 8
8	5.35(m)	130.0	7, 9	7, 9
9	5.35(m)	129.6	8, 10	8, 10
10	1.94(m)	32.3	9	9
11–15	1.35(m)	28.6–29.1		
16	1.25(m)	31.3		
17	1.25(m)	22.1		
18	0.87(t)	13.8		
1′		173.7		
2′	3.89(m)	71.1	3′a, 3′b	1′, 3′
3′a	1.54(m)	34.3	3′b	2′
3′b	1.65(m)		3′a	
14′,17′	1.99(m)	26.6		
15′	5.30 (m)	129.3	14′,16′	14′,16′
16′	5.30 (m)	129.3	15′,17′	15′,17′
CH_2	1.25-1.35(m)	22.1-31.3		
24′	0.87(t)	13.8		

reader EL311s spectrophotometer (BIO-TEK Instruments, INC. Winooski, Vermount, USA).

3. Results and discussion

Compound 1 was obtained as a colorless solid. Its molecular formula was determined as $C_{48}H_{91}NO_{10}$ on the basis of the HR-ESI-MS $[M+Na]^+$, m/z 864.6529 (calcd for $C_{48}H_{91}NO_{10}Na$: 864.6535). The IR (KBr) spectrum of 1 showed absorption bands typical for a secondary amide (1632 cm^{-1}, 1541 cm^{-1}), and hydroxyl (3387 cm^{-1}) functionalities. The NMR data of 1 indicated the presence of a sugar (δ_H 4.12, 1H, d, $J=7.6$ Hz, anomeric H; δ_C 103.5), an amide linkage (δ_H 7.53, 1H, d, $J=9.6$ Hz, N–H; δ_C 173.8), and two long chain aliphatic moieties and olefinic functions (δ_H 0.83, 6H, t, $J=7.0$ Hz, CH_3; δ_H 1.22, brs, CH_2; δ_H 5.29 and 5.34, 4H; δ_C 130.2, 129.8, 129.5, 129.3). Compared the ^{1}H and ^{13}C NMR spectras of 1 with those of cerebrosides [14–16], the compound 1 was a cerebroside analogue, obviously. Methanolysis of 1 yielded three parts (Fig. 3): methyl glucoside, a fatty acid methyl ester (FAM-1) and a long-chain base (LCB-1).

The FAM-1 was identified as methyl 2-hydroxy-tetracosenoate through EI-MS and NMR experiments. The EI-MS of the FAM-1 exhibited a base peak at m/z 396 $[M]^+$ and an ion at m/z 337 $[M\text{-}COOCH_3]^+$, which determined the number of carbons in the fatty acid (FAM-1) to be 24 and a double bond in it. The presence of an α-hydroxy fatty acid side chain could be supported by the HMBC correlation from H-2′ to the carbonyl carbon, and the proton OH-2′ to C-2′. The absolute configuration at C-2′ of the 2′-hydroxy fatty acid was presumed to be R from the specific rotation value, $[\alpha]^{20}_D$ -2.0° ($c=0.1$, $CHCl_3$) [17]. The position of the double bond at C-15 in the FAM-1 was determined by the characteristic fragment ions (m/z 317 and 173) due to cleavage between the carbons bearing the methylthio group in the EI-MS analysis of the corresponding dimethyl disulfide derivative. The cis (Z) configuration of the double bond was evidenced by the chemical shifts of the carbons next to the double bond at δ 26.7 (C-14′ and C-17′) in 1 [8,17]. The FAM-1 part was determined to be methyl 2-hydroxy-tetracosenoate, which was also proved by the NMR data.

The presence of a 1,3,4-trihydroxy unsaturated C_{18} long chain base (LCB-1) was deduced from the gCOSY, NMR and MS experiments. The location of three hydroxy groups at C-1,

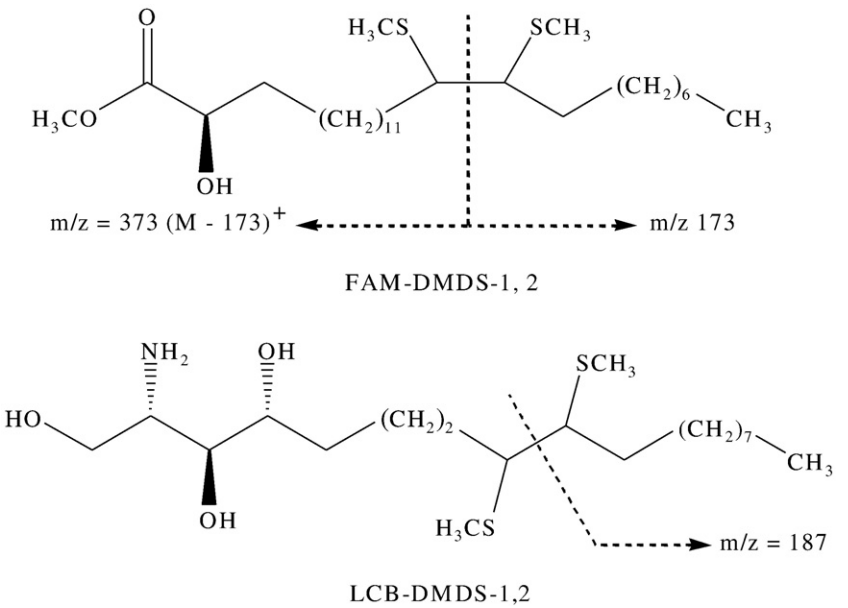

Fig. 2. FAM-DMDS derivatives and LCB-DMDS derivatives of 1 and 2.

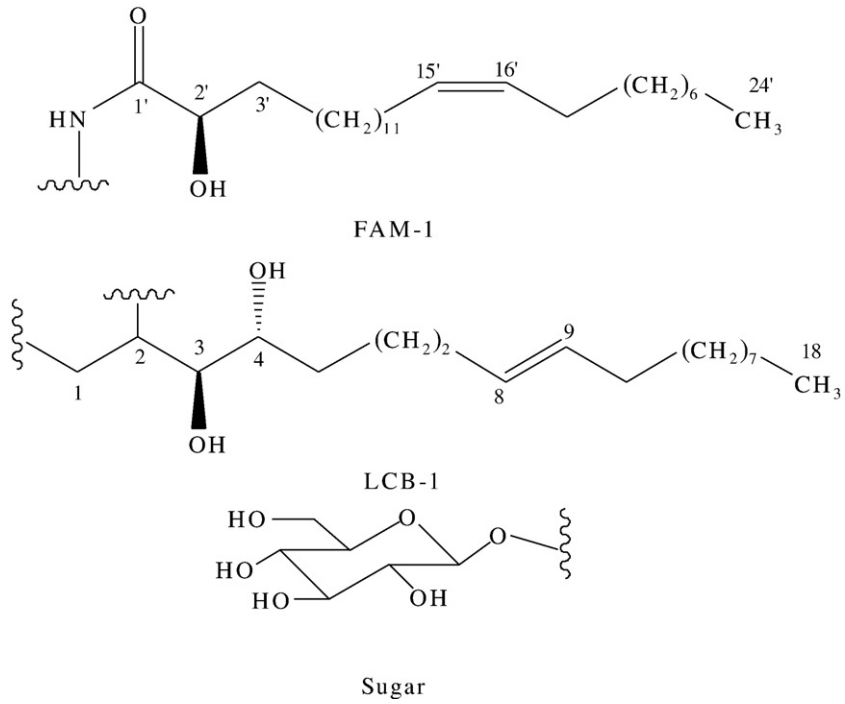

Fig. 3. The structural parts of 1.

C-3 and C-4 could be confirmed by the gCOSY correlations between N–H (δ 7.53) and H-2, H-1, H-3 and H-2, H-3 and H-4, which could be supported by HMBC correlations (Fig. 4). Furthermore, The proton signal at δ 4.08 (H-2) and the carbon signals at δ 68.9 (C-1), 49.9 (C-2), 74.1 (C-3), 70.5 (C-4) in ^{1}H, ^{13}C NMR spectra of 1 were in good agreement with those of a reported cerebroside(1a) (Fig. 1), possessing a 1,3,4-trihydroxy unsaturated C_{18} long chain base, which had been isolated from the pollen of *Typha angustifolia* [14]. The ESI-MS of the LCB-1 exhibited a base peak at *m/z* 316 $[M+H]^+$, which determined the number of carbons in the long chain base to be 18 and a double bond in it. The position of the double bond was determined by EI-MS analysis of the corresponding dimethyl disulfide derivative of LCB-1. Characteristic fragment ion at *m/z* 187, from cleavage between the carbons bearing the methylthio group, indicated that the double bond was at C-8. The cis (E) configuration of the double bond which is different from the known compound (1b, $\delta_{C\text{-}7/C\text{-}10} = 27.8$) (Fig. 1) was confirmed by the chemical shifts at δ 32.1 (C-7/C-10) and δ 32.4 (C-7/C-10), the special carbons next to the double bond group [8,16,17].

Additionally, the anomeric proton at δ 4.12 (1H, H-1″) and the *J* value (7.6 Hz) suggested the sugar group was a β-D-glucospyranoyl moiety which also supported by the acidic hydrolysis experiment. The linkage of three parts of 1 was determined by HMBC experiment (Fig. 4). The HMBC correlation from H-2 to C-1′ determined the linkage of FAM-1 part with C-2 of LCB-1; Furthermore, the HMBC correlation from the anomeric proton to C-1 (δ 68.9) of the long chain base determined the linkage of sugar part with LCB-1.

The chemical shifts of the carbon signals of C_2–C_4 of glucosphingolipids are especially suitable for determination of the absolute stereochemistry of the phytosphingosine moiety [14,18]. Based on the literature and the ^{13}C NMR spectral data, the relative stereochemistries of C-2 (δ 49.9),

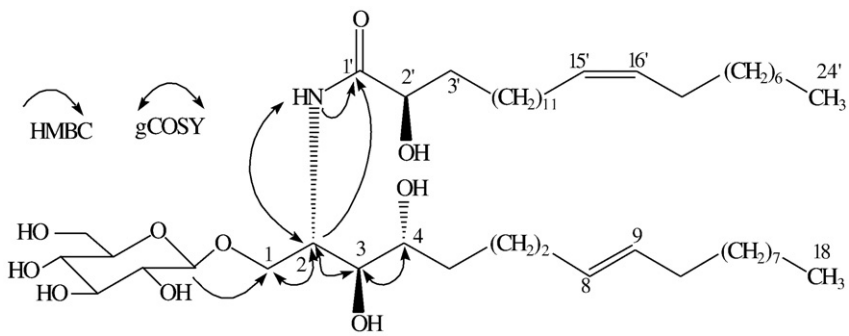

Fig. 4. Key correlations of 1.

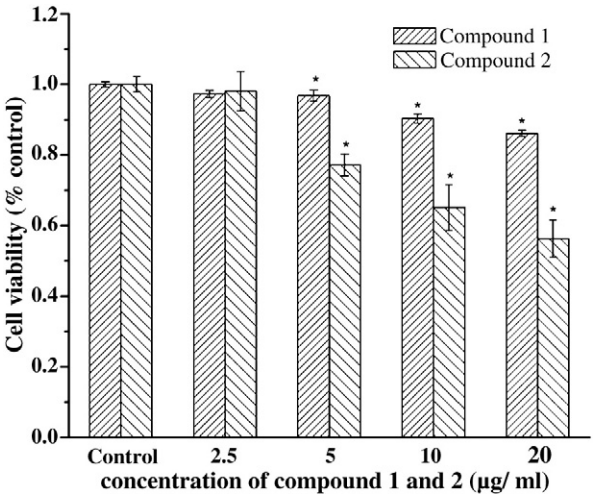

Fig. 5. Cytotoxicity of compounds 1 and 2 against Tca8113 cells by MTT analysis. Tca8113 cells were treated with compound 1 and 2 (2.5, 5, 10, and 20 μg/mL) for 72 h, and cell growth inhibition rate was measured by MTT assay. The data points are presented as mean ± S.E.M. of four experiments. $^{*}p<0.05$, significance from control (the cells with 10% FBS RPMI-1640 containing 0.1% DMSO).

C-3 (δ 74.1) and C-4 (δ 70.5) were determined as 2S, 3S, and 4R. Thus, the structure of 1 was concluded as 1-O-(β-D-glucopyranosyl)-(2S, 3S, 4R, 8E)-2-[(2′R)-2′-hydroxytetracosenoilamino]-8-octadecene-1,3,4-triol.

Compound 2 was obtained as an amorphous white powder. Its molecular formula was determined as $C_{42}H_{81}NO_5$ by HR-ESI-MS $[M+H]^+$, m/z 680.6173 (calcd for $C_{42}H_{82}NO_5$: 680.6188). Compared the IR, 1D, 2D NMR and the HR-ESI-MS data of 2 with compound 1, it was found that both structures were almost identical except for the absent glucose of 2 (Table 2). The similar chemical degradation of 2 further confirmed that the length of the chain and the location of the double bond were the same as in 1. Thus, the structure of 2 was determined to be (2S, 3S, 4R, 8E)-2-[(2′R)-2′-hydroxytetracosenoilamino]-8-octadecene-1,3,4-triol.

The cytotoxic activity of compounds 1 and 2 was tested in vitro against human tumor cell line Tca8113. Fig. 5 showed compound 2 (2.5, 5, 10, and 20 μg/mL) significantly inhibited Tca8113 cells viability in a dose-dependent manner compared with the control cell treat with FBS only. However compound 1 did not show significant anti-proliferation effect on Tca8113 cells. Compound 2 shows higher cytotoxic activity than 1, which also demonstrated that the polarity of the molecule plays an important role in the neuritogenic activity of sphingolipids [19].

Acknowledgements

This work was supported by the “Hundred Talents Program” of Chinese Academy of Sciences and the National Natural Sciences Foundation of China (NSFC No. 20775083) as well as the Science and Technology Key Project of Gansu Province (2GS64-A43-019-06).

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