Chemistry of Natural Compounds, Vol. 46, No. 2, 2010

A NEW CERAMIDE FROM THE ROOT OF *Isatis indigotica* AND ITS CYTOTOXIC ACTIVITY

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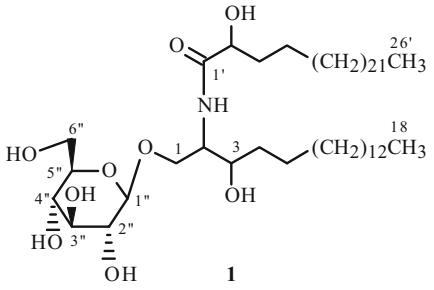
UDC 547.29+547.917

In addition to two ceramides 1-O-β-*D-glucopyranosyl-(2*S*,3*R*)-*N*-(2′-hydroxyhexadecanoyl)-octadeca-4*E*,8*E*-sphingenine (**2**) and (2*S*,3*S*,4*R*)-*N*-(2′-hydroxytetracosanoyl)-octadecasphingenine (**3**), which separated for the first time, a new ceramide 1*-O-β-*D-glucopyranosyl-(2*S*,3*R*)-*N*-(2′-hydroxyhexacosanoyl)-octadecasphingenine (**1**) was isolated from the traditional Chinese medicinal herb* Isatis indigotica*. Their cytotoxic effects were evaluated by the MTT method.*

Keywords: *Isatis indigotica*, ceramides, cytotoxic activity.

Ceramides have been isolated from many kinds of marine organisms [1–7]. Some show cytotoxic [8–10], immunostimulatory [11], antifungal [10], antimicrobial [9, 12], antiviral, or Ca^{2+} ATPase activity [13]. *Isatis indigotica* is used in traditional Chinese medicine for its antiviral properties [14]. In previous studies, we have reported on the isolation, structural elucidation, and cytotoxic activity of two ceramides from the root of *Isatis indigotica* [15]. In this investigation one additional new ceramide, together with two known ceramides, were isolated from this Chinese herb. The isolation, structural determination, and cytotoxic activities of these compounds are reported herein.

Purification of the *n*-BuOH fraction of the root of *I. indigotica* afforded two pure known ceramides 1-*O*-*β*-D-glucopyranosyl-(2*S*,3*R*)-*N*-(2′-hydroxyhexadecanoyl)-octadeca-4*E*,8*E*-sphingenine (**2**) and (2*S*,3*S*,4*R*)-*N*-(2′-hydroxytetracosanoyl)-octadecasphingenine (**3**) [16, 17] and the new ceramide **1**.



1

The molecular formula of **1** was determined to be $C_{50}H_{99}NO_9$ by HR-ESI-MS. The IR spectra displayed absorption bands at 3352 and 1648 cm^{-1}, indicating the presence of hydroxyl and amide groups. The NMR spectra of **1** showed an amide N–H signal [δ 8.59, d, J = 9.0 Hz, 1H], a carbonyl (δ 175.8), and a group of proton signals [δ 1.20–1.38 (m), 0.88 (6H, t, J = 6.9 Hz)], which indicated that **1** might belong to the sphingolipid class of compounds. The NMR data of **1** also suggested the glycolipid nature of the molecule. The HMBC spectra showed the correlation of the anomeric proton and C1.

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 0009-3130/10/4602-0180

TABLE 1. ^{1}H and ^{13}C NMR of **1** (300 and 75 MHz, DMSO-d_6, δ, ppm, J/Hz)

C atom	δ_H	δ_C	HMBC	^{1}H–^{1}H COSY
1	4.65 (2H, d, J = 4.8)	61.5	1″, 2-H	2-H
2	5.10 (1H, m)	50.1	1,3-H, 3-OH, -NH-	1,3-H, -NH-
3	4.32 (1H, m)	74.5	2,4-H, 3-OH	2,4-H, 3-OH
4	2.01 (2H, m)	33.2	3,5-H, 3-OH	3,5-H
2′-OH	4.85 (1H, s)			2′-H
3-OH	4.96 (1H, s)			3-H
5–17 (-CH_2-)	1.20–1.38	22–31		
NH	8.59 (1H, d, J = 9.0)			
1′		175.8	-NH-, 2′-H, 2′-OH	
2′	4.52 (1H, t, J = 8.0)	72.5	3′-H, 2′-OH	2′-OH, 3′-H
3′	2.04 (2H, m)	32.9	2′,4′-H	2′,4′-H
4′–25′	1.20–1.38	22–31		
18 or 26′ (-CH_3)	0.88 (6H, t, J = 6.9)	14.1	17,25′-H	17 or 25′-H
1″	4.90 (1H, d, J = 7.8)	104.5	2″-H, 2″-OH	2″-H
2″	3.96 (1H, dd, J = 6.8)	74.8	1″,3″-H, 2″,3″-OH	1″,3″-H, 2″-OH
3″	3.79 (1H, m)	76.9	2″,4″-H, 2″,3″,4″-OH	2″,4″-H, 3″-OH
4″	3.77 (1H, m)	70.1	3″,5″-H, 3″,4″-OH	3″,5″-H, 4″-OH
5″	3.38 (1H, m)	77.6	4″,6″-H, 4″-OH	4″,6″-H
6″	3.91 (2H, m, J = 5, 10)	62.1	5″-H, 6″-OH	5″-H, 6″-OH

TABLE 2. Cytotoxicity of **1**, **2** and **3**

Compound	IC_{50}, μg/mL			
	Hep G2	Hep 3B	BGC-823	A-549
1	4.32	>20	>20	>20
2	3.91	19.02	>20	>20
3	4.69	>20	>20	>20
Doxorubicin[a]	0.28	0.25	0.40	0.23

[a]Positive standard.

In the ^{1}H NMR spectra, an anomeric signal indicative of the sugar unit was observed at δ 4.90, and the coupling constant (d, J = 7.8 Hz) of this signal suggested it to be the β-anomer. The ^{13}C NMR signals at δ 104.5, 77.6, 76.9, 74.8, 70.1, and 62.1 also suggested that the sugar in **1** was of the β-D-glucopyranose form [18]. The sugar part of **1** was identified as D-glucose by GC after hydrolysis of **1**. The characteristic signals of 2-amino-3-hydroxyl-1-ol of the hydrocarbon chain were observed at δ 5.10 (1H, m, H-2), 4.65 (2H, d, J = 4.8, H-1), and 4.32 (1H, m, H-3) in the ^{1}H NMR spectra and at δ 50.1 (C-2), 61.5 (C-1), and 74.5 (C-3) in the ^{13}C NMR spectra. The characteristic signals of 1′-carbonyl-2′-ol of the hydrocarbon chain were observed at δ 4.52 (1H, t, J = 8.0 Hz, H-2′) in the ^{1}H NMR spectra and at δ 175.8 (C-1′) and 72.5 (C-2′) in the ^{13}C NMR spectra. In addition, the ^{1}H NMR spectra showed signals corresponding to aliphatic hydrocarbons at δ 0.88 (6H, t, J = 6.9 Hz), 1.20–1.38 (m), 2.01 (2H, m), and 2.04 (2H, m). The ^{13}C NMR spectra showed signals due to two terminal methyl groups in aliphatic hydrocarbon chains at δ 14.1, and an amide carbon at δ 175.8. Analysis of the 2D NMR spectra of HMBC and ^{1}H–^{1}H COSY led to the assignment of proton and carbon signals for **1**. ESI-MS fragment analysis and the major fragment ion at *m/z* $[M + Na]^+$ 418 indicated the presence of a fatty chain of 26 carbon atoms. It is reported that the absolute configurations of C-2 and C-3 in all sphingolipids isolated from natural plants were 2*S* and 3*R*, respectively [19], and the chemical shifts of C-2 (δ 50.1) and C-3 (δ 74.5) were very similar to those of ceramides, which have the same configuration [20]. Accordingly, the structure of **1** was determined to be 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*)-*N*-(2′-hydroxyhexacosanoyl)-octadecasphingenine.

The cytotoxicities of compounds **1**, **2**, and **3** were evaluated against the cell lines of human hepatocellular carcinoma Hep G2 and Hep 3B, human stomach carcinoma BGC-823, and human lung carcinoma A-549. Compounds **1**, **2**, and **3** showed selective activities against the Hep G2 cancer cell line (IC_{50} 4.32, 3.91, and 4.69 μg/mL, respectively). It exhibited no evident effect on the growth inhibition of Hep 3B, BGC-823, and A-549 cell lines (Table 2).

EXPERIMENTAL

General. Melting points: Gallenkamp apparatus. Optical rotations: Jasco P-1020. IR: Hitachi 260-30. NMR: Bruker AMX or Varian UNITY INOVA 300 NMR in DMSO-d_6. ESI-MS^n: API 3000. HR-ESI-MS: PE LC/MS spectrometer. Open column chromatography: silica gel (70–230).

Plant. *I. indigotica* root Fort. (Brassicaceae) were purchased from a traditional Chinese medicine company (An-guo Co.) in Hebei Province in 2007 and identified by Professor Jian-Wei Chen (School of Pharmacy, Nanjing University of Chinese Medicine, Jiangsu, China). The samples were authenticated and deposited in the Herbarium of Nanjing University of Chinese Medicine, Nanjing, P. R. China (SDD-BLG-003).

Extraction and Isolation. The root (30 kg) was extracted with 95% EtOH at room temperature. The extract (1300 g) was suspended in water and partitioned to provide $CHCl_3$ (180 g) and *n*-BuOH (150 g) fractions. The *n*-BuOH extract (150 g) was subjected to Si-gel CC and eluted with mixtures of $CHCl_3$–MeOH to provide five fractions (H1–H5).The H2 fraction (25 g) was then subjected to Si-gel CC using $CHCl_3$–MeOH (6:1) as an eluent to afford five other fractions (H12–H16).The H14 fraction (5 g) was further subjected to Sephadex LH-20 CC ($CHCl_3$–MeOH 7:2) and purified using a Lobar A RP-18 column (80% MeOH) to afford **1** (11 mg) and **2** (25 mg); the H13 fraction (4 g) was further subjected to Sephadex LH-20 CC ($CHCl_3$–MeOH 4:1) and purified using a Lobar A RP-18 column (85% MeOH) to afford **3** (20 mg).

The known ceramides **2** and **3** were identified by comparison of their ^1H and ^{13}C NMR spectral data with those in the literature [16, 17].

1-*O*-*β*-D-Glucopyranosyl-(2*S*,3*R*)-*N*-(2′-hydroxyhexacosanoyl)-octadecasphingenine (1). White amorphous powder: mp 225°C; $[\alpha]_D^{20}$ –4.2° (*c* 0.1, pyridine); IR (KBr, ν_{max}, cm^{-1}): 3500–3100 and 1650; ESI-MS: *m/z* 880 $[M + Na]^+$ 880, 418, 390, 306, 291, 264; HR-ESI-MS: *m/z* $[M + Na]^+$ 880.7210 (calcd for $C_{50}H_{99}NO_9Na$, 880.7218).

^1H and ^{13}C NMR data, see Table 1.

Hydrolysis of 1. Compound **1** was dissolved in 2 mL of 2N HCl–MeOH (1:1) in a 10 mL round-bottomed flask and heated at 100°C for 1 h. To isolate the sugar and the aglycon for further analysis, the hydrolysate was evaporated to half its volume to remove MeOH and then extracted several times with $CHCl_3$ by shaking vigorously in a test tube. In that case, the aglycon separated into the $CHCl_3$ fraction and the sugar into the H_2O. For the analysis of sugar by GC, equal portions of sugar and of silylating agent (Tri-Sil/BSA in DMF, Pierce Chemicals, Rockford, IL, USA) were vortexed and reacted for 1 h at 75°C. A GC 353 (GL Science, Japan) system equipped with a flame ionization detector and DB-1 capillary column (30 m × 0.25 mm, i.d., film thickness 0.25 mm) was used. The GC conditions were as follows: injector temperature 250°C; column temperature 240°C; detector temperature 250°C; flow rate 0.5 mL/min using He as a carrier gas.

Cytotoxicity Assays. Compounds **1**, **2**, and **3** were assayed for cytotoxicity against Hep G2, Hep 3B, BGC-823, and A-549 cells using the MTT method. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000–10,000 cells per well with the tested compounds added from DMSO-diluted stock. After 3 days in culture, attached cells were incubated with MTT (0.5 mg/mL, 1h) and subsequently solubilized in DMSO. The absorbance at 550 nm was then measured using a microplate reader. The IC_{50} is the concentration of agent that reduced cell growth by 50% under the experimental conditions.

ACKNOWLEDGMENT

The authors would like to thank the assistance from Prof. Han-Qing Zhang, Institute of Botany, Jiangsu Province and the Chinese Academy of Sciences.

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