New Antihepatotoxic Cerebroside from *Lycium chinense* Fruits

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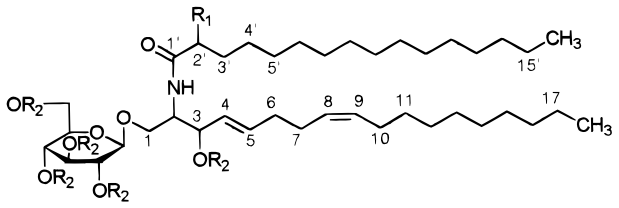
Received October 1, 1996[⊗]

Two cerebrosides isolated from *Lycium chinense* fruits have been characterized as 1-*O*-*β*-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-palmitoyloctadecasphinga-4,8-dienine (**1**) and 1-*O*-*β*-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-(2′-hydroxypalmitoyl)octadecasphinga-4,8-dienine (**2**). While **2** is already known, the structure of **1** was determined by spectral and chemical studies. Incubation of CCl_4-intoxicated hepatocytes with **1** and **2**, respectively, significantly reduced the levels of glutamic pyruvic transaminase (GPT) and sorbitol dehydrogenase (SDH) released by injured cells.

In a continuation of our search for antihepatotoxic compounds from natural sources using primary cultured rat hepatocytes as a screening system,[1] a $CHCl_3$/MeOH extract of the fruits of *Lycium chinense* was found to significantly protect against CCl_4-induced injury in this system. The dried ripe fruits of *L. chinense* Miller (Solanaceae) have been used as a tonic in Oriental medicine. A number of neutral volatile, steroidal, and alkaloidal compounds as well as betaine are known to be constituents of the fruits of this plant.[2−5] These fruits have been reported as having antihypertensive activity, an inhibitory effect on the development of fatty liver, and the ability to reduce the content of sugar in the blood.[6] To date, however, no precise correlation has been made between a particular constituent of these fruits and any observed pharmacological activities. Subsequent activity-guided fractionation resulted in the isolation of a new cerebroside, **1**, and a known cerebroside, **2**, both of which showed antihepatotoxic activity.



	R_1	R_2
1	H	H
2	OH	H
3	H	$COCH_3$
4	$OCOCH_3$	$COCH_3$

The molecular formula of $C_{40}H_{75}NO_8$ for compound **1** was determined by FABMS and confirmed by HR-FABMS. In the positive FABMS, compound **1** exhibited significant fragment peaks at *m*/*z* 721 $[M + Na]^+$, 698 $[M + H]^+$, 680 $[M + H - H_2O]^+$, and 536 [M + H − hexose]$^+$. The acetate derivative **3** of compound **1** gave peaks at *m*/*z* 908 $[M + H]^+$ and 578 [M^+ + H − tetraacetyl hexose] in the positive FABMS. The IR data of **1** indicated it to be a secondary amide (1660, 1640 cm^{-1}), bearing hydroxyl (3310 cm^{-1}) functionality.

The ^{1}H- and ^{13}C-NMR spectral data of **1** indicated the presence of a sugar, an amide linkage, and two long-chain aliphatic moieties, strongly suggesting the glycolipid nature of the molecule (Table 1). In the ^{1}H-NMR spectrum, an anomeric signal indicative of the sugar unit was observed at *δ* 4.27, 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* 103.7, 77.9, 77.8, 74.1, 70.7, and 62.0 also suggested that the sugar in **1** was in the *β*-D-glucopyranose form.[7] The sugar part of **1** was identified as D-glucose by GC after hydrolysis of **1**.

An intense signal at *δ* 1.27 and two terminal methyls at *δ* 0.89 (t, *J* = 6.9 Hz) in the ^{1}H-NMR spectrum indicated the presence of either two long aliphatic chains or a single branched aliphatic chain in the molecule of **1**. Signals of a tertiary carbon at *δ* 53.8 and a quaternary carbon at *δ* 176.4 in the ^{13}C-NMR spectrum supported the presence of a carbon attached to a nitrogen and an amide carbonyl, respectively. Four CH carbons observed at *δ* 129.1, 131.1, 130.0, and 134.1 suggested that this compound possessed two double bonds. All of the above spectral information revealed that **1** is a cerebroside of the C_{18}-sphinga-4,8-dienine type.[8] The geometry of the C_4/C_5 alkene bond was *trans*, as evidenced by the large vicinal coupling constant (J_{4-5} = 16.0 Hz). The *trans* geometry of this double bond was also supported by the chemical shift of the C-6 carbon (*δ* 33.0). Typically, the signals of a carbon next to a *trans* double bond appear between *δ* 32−33, while those of a *cis* double bond appear between *δ* 27−28.[9] The geometry of the C_8/C_9 alkene bond was determined to be *cis*, as evidenced by the carbon signals of C-7 (*δ* 28.0 or 27.2) and C-10 (*δ* 28.3 or 27.2). In the ^{13}C-NMR spectrum of **1**, another CH_2 carbon signal appeared at *δ* 36.0 (C-2′ at the acyl moiety), and the signals observed at *δ* 25.1, 29.7, and 29.8−30.3 suggested that this acyl moiety is a linear acyl chain without any branching.[10]

The relative stereochemistry of **1** at C_2, C_3 was proposed as 2*S*,3*R*, identical to that of D-sphingosine on the basis of the ^{13}C-NMR spectral data, since the chemical shifts of C-2 (*δ* 53.8) and C-3 (*δ* 72.5) were in agreement with those of synthetic *N*-octadecanoyl-D-*erythro*-sphingosine (*δ* 54.7, 73.1)[11] and glucosyl-*erythro*-ceramide (*δ* 53.8, 72.6).[12] Methanolysis of **1** demon-

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1997.

S0163-3864(96)00670-2 CCC: $14.00

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data of Compounds **1** and **2** (solvents; $CDCl_3$:CD_3OD = 2:1)

	^{1}H NMR δ (m, J (Hz))		^{13}C MNR, δ	
position	**1**	**2**	**1**	**2**
1	4.07		68.9	69.0
2	4.02		53.8	54.6
3	4.12		72.5	72.9
4	5.48 (d, d, 16)		129.1	134.4
5	5.75 (d, t, 16)		134.1	134.4
6	2.05 (m)	2.10 (m)	33.0	33.7
7	1.98 (m)	2.01 (m)	27.8[a]	28.0[a]
8,9	5.35 (t, 8)		130.0, 131.1	131.2, 132.0
10	1.98 (m)		27.8[a]	28.2[a]
11	1.37 (m)		32.4	30.8
12−17	1.27(m)	1.30 (m)	23.2, 25.6, 27.2, 27.8	23.7, 26.2, 30.2, 30.7
1″	4.27 (d, 7.8)	4.26 (d, 8.0)	103.7	104.7
2″	3.19	3.25	74.1	75.0
3″	3.24 (t, 8.8)	3.38 (t, 9.0)	77.8[b]	77.9[b]
4″	3.28	3.35	70.7	70.1
5″	3.24 (t, 8.8)	3.30 (t, 9.0)	77.9[b]	78.0[b]
6″	3.86		62.0	62.7
1′			176.4	177.1
2′	2.15	3.99	36.0	73.0
3′	1.75 (m)		25.1	36.1
4′	1.42 (m)	1.55 (m)	29.7	33.1
5′−16′	1.27 (m)	1.30 (m)	29.8, 29.9, 30.0, 30.3	23.7, 26.2, 30.2, 30.5
CH_3	0.89 (t, 6.9)	0.90 (t, 6.9)	14.3	14.5

[a,b]Assignments with the same superscript may be reversed.

strated that palmitate was the fatty acyl moiety. Thus, the chemical structure of **1** was assigned as 1-*O*-*β*-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-palmitoyloctadecasphinga-4,8-dienine.

^{1}H and ^{13}C NMR spectra of **2** (Table 1) were also almost identical with those of **1**, with only a subtle difference in the acyl moiety. In the positive FABMS of **2**, peaks at m/z 737 $[M + Na]^+$, 714 $[M + H]^+$, 696 $[M + H - H_2O]^+$, and 552 $[M + H -$ hexose$]^+$ were observed. Methanolysis of **2** afforded 2-(hydroxymethyl)palmitate as the acyl moiety of **2**. Accordingly, the chemical structure of **2** was identified as 1-*O*-*β*-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-(2′-hydroxypalmitoyl)octadecasphinga-4,8-dienine, which was previously isolated from various plant sources.[13−16]

The antihepatotoxic activities of **1** and **2** were quantified by measuring their preventive effects on the release of GPT and SDH into culture media of primary cultures of hepatocytes injured with CCl_4. Compounds **1** and **2** markedly blocked the release of both GPT and SDH from CCl_4-intoxicated primary cultured rat hepatocytes.

The present study has shown that compounds **1** and **2** have significant antihepatotoxic effect on CCl_4-induced cytotoxicity in primary cultured rat hepatocytes. As such, **1** and **2** might hold significant therapeutic value in the prevention or treatment of liver disease.

Experimental Section

General Experimental Procedures. ^{1}H-NMR and ^{13}C-NMR spectra were run on a JEOL GSX 400 spectrometer in $CDCl_3$ and CD_3OD, at 400 MHz and 100 MHz, respectively, with TMS as an internal standard. FT-IR spectra were recorded on a Perkin-Elmer 1710 spectrophotometer. UV spectra were recorded on a Shimadzu UV-2100 spectrophotometer. HR-FABMS and FABMS spectra were obtained on a VG 70-VSEQ mass spectrometer with direct inlet system using PEG600/glycerol as a matrix. GC/MS was performed on a JMS-AX505WA spectrometer employing the EI mode and a column packed with DB-5 on Lichrosorb, carrier gas He (20 mL/min), column temperature 200−300 °C (rate of temperature increase; 5 °C/min). TLC was carried out on silica gel precoated plates (Art No., 1.05715, Merck).

Plant Material. Fruits of *L. chinense* Miller (Solanaceae) were purchased from Chungyang Agriculture Cooperative, Chungnam Province, Korea, and were identified by Dr. Dae S. Han, Professor Emeritus, College of Pharmacy, Seoul National University. Voucher specimens documenting this purchase have been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

Extraction and Isolation. Dried plant material (40 kg) was extracted three times for 6 h with $CHCl_3$−MeOH (3:1) using a reflux apparatus that yielded an extract (2.8 kg) upon removal of the solvent *in vacuo*. This extract was suspended in H_2O, and partitioned between *n*-hexane and H_2O, with the aqueous extract then extracted with ethyl acetate. The ethyl acetate extract (199.7 g), showing antihepatotoxic activity (73% protection against CCl_4 toxicity, concentration 100 μg/mL, $P < 0.01$), was chromatographed on a silica gel column (2.5 kg, 230−400 mesh, column size 12 × 130 cm) eluted with a stepwise gradient from $CHCl_3$−MeOH (30:1) to MeOH to give 150 fractions (1 L each). Antihepatotoxic activity was detected in the fractions 95 through 120, and the active fractions were combined, evaporated, and chromatographed on a silica gel column (650 g, 230−400 mesh, column size 5 × 100 cm). Fractions were eluted by a stepwise gradient using the lower phases of $CHCl_3$−MeOH−H_2O (100:4:1) to $CHCl_3$−MeOH−H_2O (6:5:1), with 65 500 mL fractions being obtained. Fractions 25−41 (1 g) showed antihepatotoxic activity (79% protection against CCl_4 toxicity, concentration 100 μg/mL, $P < 0.01$) and were combined, dried, and purified with Sephadex LH-20 (50 g, column size 2.0 × 60 cm) using CH_2Cl_2−MeOH (2:1) as the eluting solvent system. Fractions 30−120 were evaporated to dryness *in vacuo*, and the residue was recrystallized from MeOH−H_2O to give an amorphous powder (100 mg). This powder was chromatographed on a SPE with H_2O, H_2O−MeOH (5:1), and MeOH as eluents, and two

Table 2. Antihepatotoxic Activities of Compounds **1** and **2**

compd	GPT assay[c] (%)	SDH assay[c] (%)
ctrl[a]	100 ± 0.1	100 ± 0.1
ctrl[b]	0 ± 0.1	0 ± 0.1
silybin[d]	76.0 ± 0.5	58.9 ± 0.8
1	95.6 ± 0.8[e]	80.2 ± 0.9[e]
2	86.4 ± 0.6[f]	69.5 ± 0.9[f]

[a] Control is the value for hepatocytes cultures not challenged with CCl_4. [b] Control is the value for untreated hepatocytes challenged with CCl_4. [c] The percent of activity is calculated as 100(GPT or SDH activity of Ctrl[b]−GPT or SDH activity of sample)/(GPT or SDH activity of Ctrl[b]−GPT or SDH activity of Ctrl[a]). [d] Sillybin was used as a positive control. Each sample was tested at 25 μM concentration, which showed the highest activity. [e] $P < 0.01$, $P < 0.05$.

spots with R_f 0.15 and R_f 0.20 (MeOH:H_2O = 10:1) were isolated. Each compound was precipitated from MeOH−H_2O to give compounds **1** (50 mg) and **2** (15 mg).

Compound 1: white amorphous powder; $C_{40}H_{75}NO_8$ $[\alpha]^{25}_D$ +1.2° (*c* 0.1, MeOH); IR (KBr) ν 3310, 2920, 1660, 1640, 1000−1100 cm^{-1}; ^{1}H-, ^{13}C-NMR (400 MHz, 100 MHz, $CDCl_3$−CD_3OD (2:1)) see Table 1; HR-FABMS *m/z* 698.5537 $[M + H]^+$ ($C_{40}H_{75}NO_8$ requires 698.5570); FABMS 721 $[M + Na]^+$, 698 $[M + H]^+$, 680 $[M + H - H_2O]^+$, 536 $[M + H -$ hexose$]^+$; R_f 0.47, developing solvent, $CHCl_3$−MeOH−H_2O (15:4:1, lower phase), silica gel plate; R_f 0.15, developing solvent, MeOH−H_2O (10:1), RP-18 F_{254} plate.

Compound 2: white amorphous powder; $C_{40}H_{75}NO_9$; $[\alpha]^{25}_D$ +1.7° (*c* 0.1, MeOH); IR (KBr) ν 3430 (OH), 2960, 1550, 1520, 1000−1100 (CO) cm^{-1}; ^{1}H-, ^{13}C-NMR (400 MHz, 100 MHz, $CDCl_3$−CD_3OD (2:1)) see Table 2; FABMS *m/z* 737 $[M + Na]^+$, 714 $[M + H]^+$, 696 $[M + H - H_2O]^+$, 552 $[M + H -$ hexose$]^+$; R_f 0.47 developing solvent, $CHCl_3$−MeOH−H_2O (15:4:1, lower phase), silica gel plate; R_f 0.20 developing solvent, MeOH−H_2O (10:1) RP-18 F_{254} plate.

Acid Hydrolysis of 1 and 2. Compound **1** or **2** (3 mg) was dissolved in 2 mL of 2 N 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 aglycons for further analysis, the reaction mixture from **1** or **2** was evaporated to half its volume to remove MeOH and then was extracted several times with $CHCl_3$ by shaking vigorously in a test tube. In each case, the aglycon separated into the $CHCl_3$ fraction and the sugar into 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) were vortexed and reacted for 1 h at 73 °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 of 250 °C; column temperature of 240 °C; detector temperature of 250 °C; a flow rate of 0.5 mL/min using He as a carrier gas.

Methanolysis of Compounds 1 and 2. Using previously published conditions,[17] compound **1** or **2** (5 mg) was dissolved in 5% HCl−MeOH (2 mL) and the mixture refluxed for 10 h. In each case, the reaction mixture was extracted with *n*-hexane, and the *n*-hexane layer was then concentrated *in vacuo*. The residue obtained from the hexane extract was subjected to GC/MS under the conditions stated above.

Acetylation of Compounds 1 and 2. Compounds **1** (5 mg) and **2** (2 mg) were separately dissolved in pyridine (200 mL), and the mixtures were treated with acetic anhydride (200 mL) and left overnight. Each mixture was then diluted with 3 mL of H_2O and extracted with $CHCl_3$. The $CHCl_3$ residue was further purified by Sephadex LH-20 column chromatography using a 3:1 mixture of CH_2Cl_2 and MeOH until the peracetate (**3** and **4**) of compounds **1** and **2**, respectively, was obtained.

Compound 3: FABMS *m/z* 908 $[M + H]^+$, and 578 $[M^+ + H -$ tetraacetyl hexose].

Compound 4: FABMS *m/z* 966 $[M + H]^+$, 636 $[M^+ + H -$ tetraacetyl hexose].

Biological Evaluation. The isolated rat hepatocytes were plated and then cultured for 1 day. The 1-day-old cultures of hepatocytes were exposed to 10 mM CCl_4 for 1.5 h to induce cytotoxicity. The activity of GPT (glutamic pyruvic transaminase) in the culture medium was determined by the Reitman−Frankel method[18] using an assay kit. The activity of SDH (sorbitol dehydrogenase) in the cultured medium was determined by the method of Gerlach[19] with minor modifications.

Statistical Analysis. The evaluation of statistical significance was determined by the "ANOVA" test.

Acknowledgment. This work was supported in part by grants from the Research Center for New Drug Development (KOSEF-RCNDD), Seoul National University, and the Ministry of Health and Welfare (HMP-96-D-1-1005), Korea.

References and Notes

(1) Lee, M. K.; Choi, Y. J.; Sung, S. H.; Shin, D. I.; Kim, J. W.; Kim, Y. C. *Planta Med.* **1995**, *61*, 493−590.
(2) Sannai, A.; Fujimori, T.; Kato, K. *Agric. Biol. Chem.* **1983**, *47*, 2397−2399.
(3) Nishiyama, R. *Nippon Shokuhin Kogyo Gakkaishi* **1963**, *17*, 14−18; *Chem. Abstr.* **1965**, *63*, 4660*h*.
(4) Itoh, T.; Ishii, T.; Tamura, T. *Phytochemistry* **1978**, *17*, 971−977.
(5) Noguchi, M.; Mochida, K.; Shingu, T.; Kozuka, M.; Fujitani, K. *Chem. Pharm. Bull.* **1984**, *32*, 3584−3587.
(6) Kurokawa, X. *Shikoku Igkuzasshi* **1962**, *18*, 127−143; *Chem. Abstr.* **1962**, *57*, 11822e.
(7) Falk, K. E.; Karlsson, K. A.; Samuelsson, B. E. *Arch. Biochem. Biophys.* **1979**, *192*, 164−176.
(8) Manas, C.; Archana, B.; Baruna, A. K. *J. Nat. Prod.* **1994**, *57*, 393−395.
(9) Stothers, J. B. *Carbon-13 NMR Spectroscopy*; Academic Press: New York, 1972.
(10) Zhao, H.; Zhao, S. *J. Nat. Prod.* **1994**, *57*, 138−141.
(11) Julina, R.; Herzig, T. *Helv. Chim. Acta* **1986**, *69*, 368.
(12) Sarimentos, F.; Schwarzmann, G.; Sandhoff, K. *Eur. J. Biochem.* **1985**, *146*, 59.
(13) Okuyama, E.; Yamazaki, M. *Chem. Pharm. Bull.* **1983,** *31*, 2209.
(14) Yoshioka, A.; Etoh, H.; Sakata, K.; Ina, K. *Agric. Biol. Chem.* **1990,** *54,* 3355.
(15) Higuchi, R.; Inagaki, M.; Togawa, K.; Miyamoto, T.; Komori, T. *Liebigs Ann. Chem.* **1994**, 653.
(16) Shibuya, H.; Kawashima, K.; Sakagami, M.; Kawanishi, H.; Shimomura, M.; Kitagawa, I. *Chem. Pharm. Bull.* **1990,** *38*, 2933.
(17) Brady, R. O.; Koval, G. J. *J. Biol. Chem.* **1958**, *233*, 26−31.
(18) Reitmann, S.; Frankel, S. *Am. J. Clin. Pathol.* **1957**, *28*, 56−61.
(19) Gerlach, U. In *Methods of Enzymatic Analysis;* Bergmeyer, H. U., Ed.; VCH Publishers: Weinheim, 1983; Vol. III, pp 112−117.

NP960670B