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A new cerebroside from the fruiting bodies of *Hericium erinaceus* and its applicability to cancer treatment

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ABSTRACT

A new cerebroside, cerebroside E (**1**) was isolated from the fruiting bodies of *Hericium erinaceus* (Hericiaceae). The structure of **1** was elucidated by a combination of extensive spectroscopic analyses, including extensive 2D NMR, HR-MS, and chemical reactions. Compound **1** was evaluated for its applicability to medicinal use in several human diseases using cell-based assays. As a result, compound **1** attenuated cisplatin-induced nephrotoxicity in LLC-PK1 cells and exhibited a significant inhibitory effect on angiogenesis in HUVECs. These results collectively reflect the beneficial effects of compound **1** in cancer treatment.

Cerebrosides are present in almost all species of fungi, and in some plants and animals where they are crucial constituents of a wide diversity of cell membranes and tissues. Cerebrosides are glycosphingolipids consisting of a ceramide and a single sugar residue at C-1, thus are usually named monohexosylceramides or ceramide monohexosides.[1] Structurally, ceramide has a long-chain sphingosine or sphingol and an amide-linked long-chain fatty acid, with the sugar moiety being either glucose or galactose.[2] This amphipathic molecules, cerebrosides, have been reported to exhibit a variety of biological effects on ulcer gastritis and tumors, immune reinforcement, as well as anti-adipogenic effects in vitro inhibiting lipid accumulation in cells.[3–5]

Hericium erinaceus (Hericiaceae) also called Lion's Mane mushroom, is an edible and medicinal mushroom of Korea, Japan, and China. This edible mushroom has long been used in traditional Chinese medicine for treating dyspepsia, gastric ulcers, and enervation.[6] It is now widely available as a health functional food and dietary supplement, especially in East Asia and Europe. *H. erinaceus* has been reported to be a plentiful source of bioactive compounds that exhibit pharmacological effects on many illnesses.[7–11] A number of works have proven that *H. erinaceus* has beneficial activities such as antioxidant, antiproliferative, antimicrobial and anti-inflammatory effects.[12]

In our ongoing effort to characterize new natural compounds with interesting chemical structures and/or pharmaceutical activities from Korean mushrooms, we have previously investigated the active components of the MeOH extract of the fruiting bodies of *H. erinaceus*. Recently, we reported the identification of a new cytotoxic isoindolinone alkaloid and benzyl alcohol derivatives with anti-inflammatory effects.[13,14] Our continuing attempts to search for biologically active compounds from *H. erinaceus* led to the isolation and identification of a new cerebroside, named cerebroside E (**1**) (Fig. 1).[15] Here, we describe the isolation and structural elucidation of the new compound, and the evaluation of its applicability to cancer treatment.

Compound **1** was isolated as an optically active white amorphous powder with a positive optical rotation ($[\alpha]_D^{25}$ +2.9, $CHCl_3$). The molecular formula of **1** was determined to be $C_{40}H_{75}NO_9$ from its positive mode HR-ESIMS data at *m/z* 736.5346 $[M+Na]^+$ (calcd for $C_{40}H_{75}NO_9Na$, 736.5340), which was compatible with the ^{13}C NMR data. The IR spectrum of **1** displayed characteristic absorption bands of hydroxyl and amide NH (3347 cm^{-1}) and amide carbonyl

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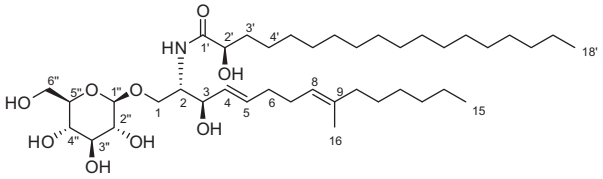
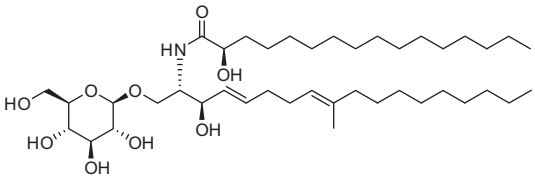
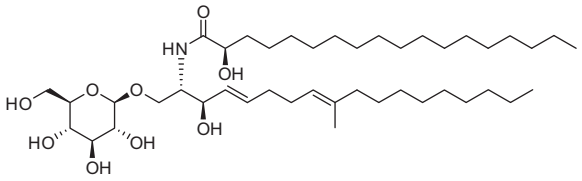


1



cerebroside B



cerebroside D

Figure 1. Chemical structures of compound **1** and cerebrosides B and D.

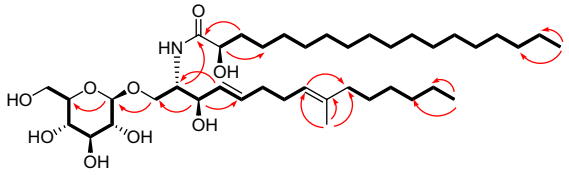


Figure 2. Key ^{1}H–^{1}H COSY (bold) and HMBCs (→) of **1**.

(1646 cm^{-1}) functionalities. The ^{1}H and ^{13}C NMR spectral data (Table 1) of **1** indicated that the spectral signals were ascribable to a monosaccharide with an anomeric proton at δ_H 4.28, an amide linkage with a nitrogenated methine proton at δ_H 4.00, and a carbonyl carbon at δ_C 177.3, together with an aliphatic long chain (terminal methyl protons at δ_H 0.92 and methylene protons at δ_H 1.31), suggesting that the compound was a glycosphingolipid.[16–18] The ^{1}H and ^{13}C NMR spectra also showed the presence of a typical disubstituted Δ^4 double bond [δ_H 5.50 (dd, J = 15.5, 7.5 Hz, H-4), δ_C 131.2 (C-4); δ_H 5.77 (dt, J = 15.5, 7.5 Hz, H-5), δ_C 134.7 (C-5)] and an additional trisubstituted double bond [δ_H 5.08 (m, H-8), δ_C 124.9 (C-8); δ_C 136.9 (C-9)] as a sphingosine part.[17,18] The large vicinal coupling constant of the olefinic protons ($J_{H\text{-}4,H\text{-}5}$ = 15.5 Hz) clearly indicated an *E* geometry for the double bond.[17,18] The *trans* geometry of the Δ^8 double bond was established by the chemical shift of methyl (δ_C 16.2) attached to C-9, since the chemical shift of a methyl group connected to a *trans*-alkene typically resonates around 15 ppm, whereas for *cis*-alkenes they typically resonate around 23 ppm.[17–19] Analyses and comparisons of spectroscopic data with those of cerebrosides B and D strongly suggested that the glycosphingolipid of **1** was a cerebroside B analogue with the molecular formula $C_{40}H_{75}NO_9$,[20] which was confirmed by the COSY and HMBC correlations (Fig. 2).

Table 1
^{1}H (700 MHz) and ^{13}C (175 MHz) NMR data of **1** in CD_3OD[a] (δ in ppm)

Position	1	
	δ_C	δ_H
1	69.8 t	4.13 m; 3.74 dd (10.0, 3.5)
2	54.7 d	4.00 m
3	73.0 d	4.16 m
4	131.2 d	5.50 dd (15.5, 7.5)
5	134.7 d	5.77 dt (15.5, 7.5)
6	33.9 t	2.08 m
7	28.8 t	2.10 m
8	124.9 d	5.16 m
9	136.9 s	
10	40.9 t	2.00 t (7.5)
11	29.2 t	1.42 m
12	31.0–30.5 t	1.31 m
13	33.2 t	1.35 m
14	23.9 t	1.30 m
15	14.6 q	0.92 t (7.0)
16	16.2 q	1.62 s
1′	177.3 s	
2′	73.2 d	4.00 m
3′	36.0 d	1.73 m; 1.58 m
4′–15′	31.0–30.5 t	1.31 m
16′	33.2 t	1.35 m
17′	23.9 t	1.30 m
18′	14.6 q	0.92 t (7.0)
1″	104.8 d	4.28 d (8.0)
2″	75.1 d	3.23 dd (10.0, 7.5)
3″	71.7 d	3.31 m
4″	78.1 d	3.30 m
5″	78.0 d	3.38 m
6″	62.8 t	3.88 dd (12.0, 2.0); 3.69 dd (12.0, 5.5)

[a] The assignments were based on ^{1}H–^{1}H COSY, HSQC, and HMBC experiments. Well-resolved couplings are expressed with coupling patterns, and coupling constants (in parentheses) are in Hz.

Methanolysis of **1** yielded a long chain sphingosine base (LCSB), a fatty acid methyl ester (FAME), and a methyl glucopyranoside.[21] The absolute configuration of the LCSB was determined by comparison to similar compounds. The LCSB was peracetylated using acetic anhydride in pyridine, giving the desired triacetyl derivative ($C_{22}H_{37}NO_5$, ESI-MS *m/z* 418 $[M+Na]^+$), establishing it as 2-acetoamino-1,3-diacetoxy-9-methyl-4,8-pentadecanediene. The triacetyl LCSB showed the negative sign of optical rotation ($[\alpha]_D^{25}$ −17.2, $CHCl_3$), which indicated the D-*erythro*-triacetylsphingosine.[22] In addition, the chemical shifts of C-2 (δ_C 54.7) and C-3 (δ_C 73.0) of **1** supported an D-*erythro*-configuration of C-2/C-3, which was in agreement with the configuration of D-glucosyl-D-*erythro*-ceramide [δ_C 53.8 (C-2); δ_C 72.6 (C-3)], while the L-*threo*-isomers of related compounds have been reported to show chemical shifts of C-2 (δ_C 53.7) and C-3 (δ_C 70.0).[23] The absolute configuration at C-2 of most natural cerebrosides was all-2*S*.[16–18,24,3] Thus, the D-*erythro*-configuration, the specific rotation of the triacetyl LCSB, and the biogenetic pathway of the natural cerebrosides suggested a 2*S*,3*R*-configuration of the sphingosine part. The FAME, one of the products of the chemical degradation of **1**, was identified as methyl 2-hydroxyoctadecanoate by the ESI-MS data at *m/z* 337 $[M+Na]^+$ The optical rotation ($[\alpha]_D^{25}$ −12.7, $CHCl_3$) identified it as the *R* isomer.[17,18,24] The specific rotation of the methyl glucopyranoside ($[\alpha]_D^{25}$ +72.4, MeOH), separated from the methanolysis reaction mixture, defined glucose as a D-configuration.[17,18,3] On the basis of the above data, the structure of cerebroside E (**1**) was elucidated as 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*,4*E*,9*E*)-2-[(2′*R*)-2′-hydroxyoctadecanoylamino]-9-methyl-pentadeca-4,8-dien-1,3-diol.[25]

As mentioned above, cerebrosides have been reported to show a wide range of pharmacological effects.[3–5] Therefore, in the present study, several cell-based and enzyme assays were conducted for the evaluation of the applicability of cerebroside E (**1**) to medicinal use. Cerebroside E (**1**) was tested for its effects on free radical generation,[26] cancer cell growth,[27] angiogenesis,[28] acetylcholine esterase (AChE) inhibition,[29] and anticancer drug induced nephrotoxicity.[30] Cerebroside E was shown to have no antioxidant, anticancer (data not shown), or acetylcholinesterase (AChE) inhibitory effects up to 200 μM (Fig. 3A). However, pre-

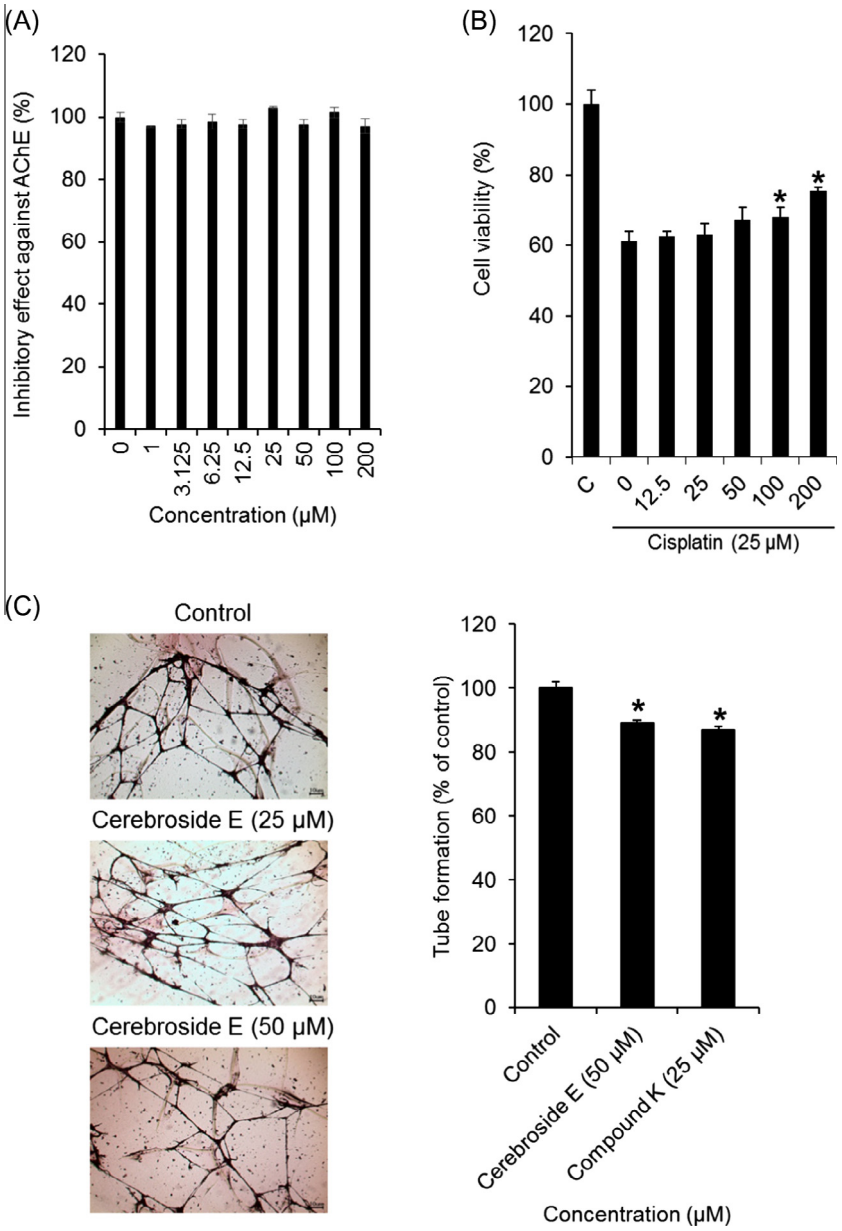


Figure 3. Biological activities of cerebroside E. (A) Effect of cerebroside E on AChE activity. (B) Effect of cerebroside E on cisplatin-induced nephrotoxicity in LLC-PK1 cells. (C) Effect of cerebroside E on tube formation in HUVECs. Compound K was used as a positive control.

treatment with cerebroside E at high concentrations of 100 and 200 μM significantly abrogated cisplatin-induced nephrotoxicity in LLC-PK1 cells (Fig. 3B). Cisplatin is an anticancer drug used for the treatment of various types of solid tumors. However, the clinical use of cisplatin has been limited due to its nephrotoxicity.[31,32] In addition, treatment with cerebroside E up to a concentration of 50 μM had no cytotoxic effects on human umbilical vein vascular endothelial cells (HUVECs), whereas it significantly inhibited vascular endothelial growth factor (VEGF)-induced tube formation that is importantly involved in the process of cancer cell metastasis (Fig. 3C).[33] The inhibitory effect of cerebroside E at 50 μM on tube formation in HUVECs was as strong as compound K at 25 μM (Fig. 3C). Therefore, cerebroside E was found to have beneficial effects in cancer treatment, not only by inhibiting angiogenesis, but also by attenuating anticancer drug-induced side effects. There has been an ever growing interest in treatment using natural compounds as an adjuvant therapy for the inhibition of cancer metastasis and the reduction of anticancer drug-induced side effects.[34]

In conclusion, we isolated and identified a new cerebroside, cerebroside E (**1**), which was isolated from the fruiting bodies of *H. erinaceus* and its various biological activities were evaluated for the identification of its medicinal applicability. Mushrooms have been known to be rich in cerebrosides, however, cerebrosides from *H. erinaceus* have rarely been reported.[35,36] This study suggests beneficial effects of the cerebroside isolated from *H. erinaceus* in cancer treatment. To the best of our knowledge, this is the first report to investigate the inhibitory effect on angiogenesis and the reduction in anticancer drug-induced nephrotoxicity of a cerebroside isolated from *H. erinaceus*.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.10.092. These data include MOL files and InChiKeys of the most important compounds described in this article.

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15. The fruiting bodies of *H. erinaceus* were purchased from Pochun Mushroom Development Co., Ltd at Pochun-gun in Gyunggi-Do province, Korea, in July 2010. A voucher specimen (SKKU-2010-07) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University. Partially dried fruiting bodies of *H. erinaceus* (1.5 kg) were extracted twice with 80% MeOH at room temperature. The resultant 80% MeOH extracts (115 g) were suspended in water (2 L) and successively solvent-partitioned with *n*-hexane (800 mL × 3), CH_2Cl_2 (800 mL × 3), EtOAc (800 mL × 3), and *n*-BuOH (800 mL × 3), yielding residues weighing 13.8 g, 1.0 g, 0.5 g, and 3.1 g, respectively. The most abundant *n*-hexane soluble fraction (13.8 g) was chromatographed on a silica gel column using a gradient solvent system of *n*-hexane–EtOAc (50:1 to 1:1, v/v) to yield five fractions (A–E). Among these, the last fraction E (1.9 g) was separated over RP-C_{18} silica gel using a gradient solvent system of MeOH–H_2O (1:9 to 1:0, v/v). According to TLC analysis, three subfractions (E1–E3) were collected. Fraction E1 (250 mg) was separated using a reverse-phase RP-C_{18} silica gel column (a gradient solvent of 40–90% MeOH) and purified further by semi-preparative reverse-phase HPLC (C18 column, Phenomenex Luna, 250 × 10.0 mm, 5 μm, flow rate: 2 mL/min) using a solvent system of 90% MeOH to yield compound **1** (12 mg).
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21. A solution of **1** (4.0 mg) in 9% HCl in MeOH (1.0 mL) was refluxed at 90 °C for 6 h (N_2 atmosphere). The reaction mixture was neutralized by passage through an Amberlite IRA-67 column. The residue, obtained by removal of the solvent, was partitioned into MeOH (10 mL) and hexane (10 mL) to give the hexane phase and the MeOH phase. The hexane layer was evaporated, and purified by a silica gel Waters Sep-Pak Vac 6cc (hexane/EtOAc, 5:1) to yield the FAME of **1** (0.5 mg): $[\alpha]_D^{25}$ −12.7 (*c* 0.03, $CHCl_3$); ESI-MS *m*/*z*: 337 $[M+Na]^+$. The MeOH layer was concentrated, and the residue was partitioned into EtOAc and H_2O. The EtOAc portion was dried, and peracetylated using acetic anhydride (0.5 ml) in pyridine (1 ml) at room temperature for 10 h (N_2 atmosphere). The reaction mixture was dried, and subjected to a silica gel Waters Sep-Pak (hexane/EtOAc, 5:1) to yield triacetyl LCSB of **1** (1.1 mg): $[\alpha]_D^{25}$ −17.2 (*c* 0.06, $CHCl_3$); ESI-MS *m*/*z*: 418 $[M+Na]^+$. The H_2O portion was concentrated, and applied to the reverse-phase C_{18} column chromatography (methanol/H_2O, 1:9) to afford methyl glucopyranoside (0.8 mg): $[\alpha]_D^{25}$ +72.4 (*c* 0.04, MeOH); ESI-MS *m*/*z*: 195 $[M+H]^+$.
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25. *Cerebroside E* (**1**): white amorphous powder; $[\alpha]_D^{25}$ +2.9 (*c* 0.07, $CHCl_3$); IR (neat) ν_{max} cm^{-1}: 3347, 2921, 2852, 1646, 1535, 1466, 1247, 1076, 760; ^1H (CD_3OD, 700 MHz) and ^{13}C (CD_3OD, 175 MHz) NMR data, see Table 1; HR-ESIMS (positive-ion mode) *m*/*z*: 736.5346 $[M+Na]^+$ (calcd for $C_{40}H_{75}NO_9Na$, 736.5340).
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