Cyclooxygenase-2 Inhibitory Cerebrosides from Phytolaccae Radix

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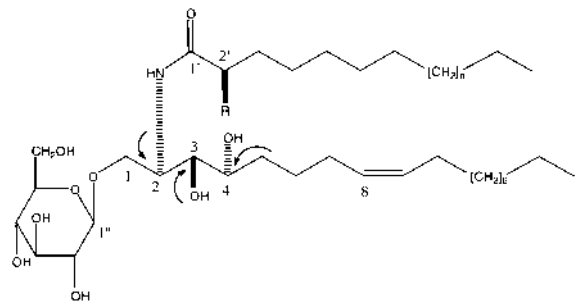
A mixture of cerebrosides, called poke-weed cerebrosides, was purified from Phytolaccae Radix (Phytolaccaceae) and characterized as 1-*O*-β-D-glucopyranosides of phytosphingosine type ceramides comprised of a common long chain base (2*S*,3*S*,4*R*,8*Z*)-2-amino-8-octadecene-1,3,4-triol and fatty acids. The fatty acyl chain of ceramide moieties was determined as (2*R*)-2-hydroxypentacosanoic acid, (2*R*)-2-hydroxylignoceric acid, (2*R*)-2-hydroxytricosanoic acid, (2*R*)-2-hydroxybehenic acid, (2*R*)-2-hydroxypalmitic acid, and palmitic acid. The poke-weed cerebroside inhibited the cyclooxygenase-2 dependent phase of prostaglandin D_2 generation in bone marrow-derived mast cells in a concentration dependent manner with an IC_{50} of 6.2 μg/ml.

Key words Phytolaccae Radix; Phytolaccaceae; cerebroside; cyclooxygenase-2 inhibitor

Plants of the *Phytolacca* family are perennial shrubs, the roots of which have been used as an indigenous medicine against edema and rheumatism in Far East Asia including China, Japan, and Korea, since ancient times. Several old dispensatories have described these plants as a diuretic agent.[1)] Plants of this family are known to contain mainly saponins,[2)] triterpenoids,[3)] and lignans.[4)] Saponins from this family exhibit diverse biological activities, including molluscicidal, fungistatic, anti-inflammatory, analgesic, spermicidal, antiduretic, and weak sedative activities.[5,6)] As part of ongoing collaborative research on anti-inflammatory activity in Korean medicinal plants,[7)] we have found considerable inhibitory activity of cyclooxygenase-2 (COX-2) in a hexane extract of Phytolaccae Radix.[8)] This plant has not been studied previously for this activity and therefore was subjected to detailed laboratory investigation, including bioassay-guided chromatographic fractionation. Herein we report novel cerebrosides as a COX-2 inhibitor of the hexane extract from Phytolaccae Radix, which were obtained as a mixture. The structural characterization of the cerebrosides using two dimensional (2D)-NMR techniques and the COX-2 inhibitory evaluation of these constituents are presented in this paper. This is the first report of COX-2 inhibitory activity by cerebrosides.

The dried Phytolaccae Radix was extracted with MeOH. The residue after evaporation of MeOH was dissolved in H_2O and partitioned with *n*-hexane. The *n*-hexane extract was subjected to silica gel column chromatography to give 14 subfractions, among which subfraction No. 14 showed the most potent inhibitory activity. Further chromatographic purification of this subfraction afforded **1**, mp 188—190 °C, as an active principle. In the IR and FAB-MS spectra of **1**, strong absorption bands typical for hydroxyl, amide, glycosidic C–O, and $(CH_2)_n$ functionalities and a series of $[M+Na]^+$ ion peaks were observed. The NMR data of **1** indicated the presence of a sugar (δ_H 4.93, 1H, d, J=7.8 Hz, anomeric H; δ_C 105.5), an amide linkage (δ_H 8.51, 1H, d, J= 9.1 Hz, N-H; δ_C 175.6) and two long chain aliphatic moieties which were essentially identical to those of aralia cerebroside,[9)] suggesting a glycosphingolipid nature.[9—15)] Methanolysis[12,16)] of **1** yielded methyl glucoside, a mixture of fatty acid methyl esters and a long chain base. Therefore, **1** must be a mixture of sphingosine-type cerebrosides composed of fatty acid and β-glucopyranose moieties; it is called poke-weed cerebroside. The fatty acid methyl esters were identified as methyl palmitate (1.4%), methyl 2-hydroxypalmitate (32.8%), methyl 2-hydroxybehenate (11.5%), methyl 2-hydroxytricosanoate (7.6%), methyl 2-hydroxylignocerate (30.6%) and methyl 2-hydroxypentacosanoate (5.8%) and other minor esters by GC/MS analysis, and the absolute configuration at C-2 of 2-hydroxy fatty acids was determined to be *R* from the specific rotation (−1.2°)[12,13,17)] as well as from the ^{1}H-NMR chemical shift for the methoxy group at δ3.649 in the *R*-(+)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) ester[18,19)] of the mixture. The positive FAB-MS spectrum of **1** showed a series of molecular ion $[M+Na]^+$ peaks at *m/z* 880, 866, 864, 852, 838, 836, 754, 738 and 732 and fragment ion at *m/z* 500 [glucosyl-long chain base+ Na].$^+$ The presence of a fragment ion at *m/z* 500 in the FAB-MS spectrum and the molecular ion peak for the long chain base at *m/z* 315 along with the identification of at least six more fatty acids as mentioned above indicated that **1** is comprised of a common long-chain base linked to varying chain lengths of fatty acid residue.[9—11)] The presence of a 1,3,4-trihydroxy unsaturated C_{18} long chain base was deduced from the ^{1}H–^{1}H correlation spectroscopy (COSY) and MS data. The signal at δ 8.51 gave a cross peak with the signal at δ 5.24 (H-2) in the ^{1}H–^{1}H COSY spectrum of **1**, which in turn showed cross peaks with methylene protons (H-1) at δ 4.49



Poke-weed cerebroside R = OH (n = 7 and 13-16)

R = H (n = 7)

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and 4.68 and δ 4.26 (H-3). The latter correlated with the signal at δ 4.17 (H-4). The chemical shift of the H-2 signal and the ^{13}C chemical shifts of C-1—C-4, C-1′ and C-2′ of glucosphingolipids are especially suitable for determination of the absolute stereochemistry of the phytosphingosine moiety.[20,21] The chemical shift of H-2 (δ 5.24) and the carbon chemical shifts at δ 70.4 (C-1), 51.7 (C-2), 75.9 (C-3), 72.4 (C-4), 175.6 (C-1′) and δ 72.5 (C-2′) in **1** were essentially identical with those of the aralia cerebroside[9] and the data reported for other (2*S*,3*S*,4*R*)-phytosphingosine moieties.[20,21] These results clearly indicate that the 1,3,4-trihydroxy phytosphingosine moiety in **1** possesses the 2*S*,3*S*,4*R* configuration. The position of the double bond in the long chain base was determined at C-8 by electron impact (EI)-MS analysis as indicated in aralia cerebroside. The *cis* (*Z*) configuration of the double bond was evidenced by the chemical shifts of the carbons next to the double bond at δ 27.5 (C-7) and δ 27.9 (C-10) in **1**. The geometry is also verified from the fact that the olefinic protons appeared at δ 5.43—5.53 as a triplet-like signal in **1**.[15,22] In light of the above evidence, the structures of poke-weed cerebrosides were deduced to be 1-*O*-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-[(2*R*)-2-hydroxypentacosanoylamino]-8-octadecene-1,3,4-triol, 1-*O*-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-[(2*R*)-2-hydroxyligneroylamino]-8-octadecene-1,3,4-triol, 1-*O*-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-[(2*R*)-2-hydroxytricosanoylamino]-8-octadecene-1,3,4-triol, 1-*O*-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-[(2*R*)-2-hydroxybehenoylamino]-8-octadecene-1,3,4-triol, 1-*O*-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-[(2*R*)-2-hydroxypalmitoylamino]-8-octadecene-1,3,4-triol, and 1-*O*-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-[palmitoylamino]-8-octadecene-1,3,4-triol. One of the major cerebrosides, 1-*O*-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-[(2*R*)-2-hydroxypalmitoylamino]-8-octadecene-1,3,4-triol, is a *Z* isomer of aralia cerebroside.[9] Taking the molecular masses of **1** in the FAB-MS and the fatty acid components into account, minor cerebrosides having 2-hydroxy normal C_{22} and C_{24} fatty acids carrying one double bond also exist in the cerebroside mixture.

Poke-weed cerebroside was found to be the only active principle (IC_{50}=6.2 μg/ml) of the hexane extract of Phytolaccae Radix. Although a limited number of natural products are reported to have been investigated for their COX-2 inhibitory effects, this seems to be the first example of cerebrosides known to have COX-2 inhibitory activity.[7,23—27]

Experimental

IR spectra were obtained on a JASCO FT/IR-5300 spectrometer. Optical rotation was determined on a Rudolph Autopol III automatic polarimeter. EI-MS were obtained on a Hewlett-Packard 5989B mass spectrometer. The FAB-MS were obtained in a 3-nitrobenzyl alcohol matrix in a positive ion mode on a VG-VSEQ spectrometer. NMR spectra were measured on either a Gemini 2000 (300 MHz) or a Bruker AMX-500 (500 MHz) instrument, and chemical shifts were referenced to tetramethylsilane (TMS). GC analysis was performed with a Hewlett Packard 5890 Series II gas chromatograph equipped with an H_2 flame ionization detector. Conditions: HP-5 capillary column (30 m×0.32 mm×0.25 μm), column temperature, 150 °C for TMS ethers of methyl glycopyranosides; DB-1 capillary column (30 m×0.25 mm), column temperature, 160→280 °C, rate of temperature increase, 5 °C /min, for fatty acid methyl esters; injector and detector temperature, 280 °C; He flow rate, 20 ml/min; HP-5 capillary column (30 m×0.32 mm×0.25 μm), column temperature, 200 °C; injector and detector temperature, 290 °C; He flow rate, 30 ml/min for the determination of absolute configuration of glucose. TLC was performed on Silica gel 60F_{254} (Merck) and cellulose plates (Art No. 5716, Merck).

Extraction and Purification Phytolaccae Radix was purchased at a crude drug store in Andong city, Kyungsangbuk-do Province, Korea, in May 1996, and was identified as the root of *Phytolacca acinosa* by Prof. Sun Q.-S. of Shenyang Pharmaceutical University, China. The powdered Phytolaccae Radix (1.8 kg) was extracted with 80% MeOH for 3 h four times. The solvent was evaporated to dryness, and the dry residue was partitioned between H_2O and *n*-hexane affording, on evaporation of solvent, 11.8 g of the hexane extract. The hexane fraction was subjected to silica gel column chromatography. Gradient elution with CH_2Cl_2–MeOH gave 14 subfractions. Subfraction 14 was further chromatographed over silica gel using EtOAc saturated with H_2O with an increasing amount of MeOH as an eluent to yield **1** (50 mg).

Poke-weed Cerebroside (1) Amorphous white powder, mp 188—190 °C. Positive FAB-MS, *m/z*: 880 $[M+Na]^+$, 866.6725 (Calcd for $C_{48}H_{93}NO_{10}$ +Na: 866.6697), 864.6572 (Calcd for $C_{48}H_{91}NO_{10}$+Na: 864.6540), 852 $[M+Na]^+$, 838.6392 (Calcd for $C_{46}H_{89}NO_{10}$+Na: 838.6384), 836.6296 (Calcd for $C_{46}H_{87}NO_{10}$+Na: 836.6227), 754.5445 (Calcd for $C_{40}H_{77}NO_{10}$+Na: 754.5446), 738 $[M+Na]^+$, 500 [glucosyl-long chain base]$^+$. IR (KBr) cm^{-1}: 3414 (OH), 2922, 2853, 1638, 1543 (amide), 1466, 1078 (glycosidic C-O), 720 $(CH_2)_n$. ^1H-NMR (500 MHz, pyridine-d_5) δ: 0.83 (6H, t-like, *J*= 6.8 Hz, 2×CH_3), 1.24 [s, $(CH_2)_n$], 2.06 (2H, m, H-10), 2.23 (2H, m, H-7), 3.85 (1H, m, H-5″), 3.97 (1H, t, *J*=8.0 Hz, H-2″), 4.13—4.18 (3H, m, H-4, 3″, 4″), 4.26 (1H, dd, *J*=5.1, 10.4 Hz, H-3), 4.30 (1H, dd, *J*=5.0, 11.6 Hz, H-6″), 4.46 (1H, br d, *J*=11.6 Hz, H-6″), 4.49 (1H, dd, *J*=4.5, 10.7 Hz, H-1), 4.54 (1H, m, H-2′), 4.68 (1H, dd, *J*=6.6, 10.7 Hz, H-1), 4.93 (1H, d, *J*=7.8 Hz, H-1″), 5.24 (1H, m, H-2), 5.43—5.53 (2.5H, olefinic H), 8.51 (1H, d, *J*=9.1 Hz, N-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ: 14.2 (CH_3), 22.9, 25.8, 26.6, 26.7, 27.5 (C-7), 27.9 (C-10), 29.5, 29.6, 29.7, 29.8, 29.9, 30.0, 30.1, 32.1, 32.9, 33.3, 33.8, 33.9, 35.5 (C-3′), 51.7 (C-2), 62.6 (C-6″), 70.4 (C-1), 71.5 (C-4″), 72.4 (C-4), 72.5 (C-2′), 75.1 (C-2″), 75.9 (C-3), 78.4 (C-5″), 78.5 (C-3″), 105.5 (C-1″), 130.1, 130.2, 130.4, 30.6, 130.8, 175.6 (C-1′).

Acid Hydrolysis of 1 Poke-weed cerebroside **1** (23 mg) was refluxed with 0.9 N HCl in 82% aqueous MeOH (12 ml) for 18 h.[16] The resulting solution was extracted with *n*-hexane, and the combined organic phase was dried over Na_2SO_4. Evaporation of the hexane yielded a fatty acid methyl ester. The H_2O layer was neutralized with conc.-NH_4OH and extracted with ether. The ether layer was dried over Na_2SO_4, filtered and then concentrated to yield a long chain base. The H_2O layer was evaporated under a N_2 stream. The residue was dissolved in pyridine (0.05 ml), then the solution was trimethylsilylated with TMS–HT (0.1 ml) at 60 °C for 30 min. After the addition of *n*-hexane and water, the *n*-hexane layer was removed and analyzed by GC. The retention times (t_R) of the peaks were 26.7 and 29.4 min for methyl glucopyranoside. The methyl glucoside was refluxed with 5% HCl in H_2O for 2 h. The reaction solution was evaporated under reduced pressure and the absolute configuration of the glucose from the hydrolysate was determined according to the method reported by Hara *et al.*[28] using GC. The t_R was 22.5 min for D-glucose. The fatty acid methyl ester was recrystallized from MeOH to give an amorphous white powder {11 mg, $[\alpha]_D^{20}$ −1.2° (*c*= 0.6, $CHCl_3$)} and then analyzed by GC/MS. Peak 1 (t_R 10.59 min, palmitic acid methyl ester), EI-MS *m/z*: 270 $[M]^+$, 239, 227 $[M-CH_3CO]^+$, 213, 199, 185, 171, 157, 143, 129, 97, 87, 74 $[CH_3OC(OH)=CH_2]^+$, 55. Peak 2 (t_R 11.44 min, 2-hydroxypalmitic acid), EI-MS *m/z*: 286 $[M]^+$, 254 $[M-CH_3OH]^+$, 227 $[M-CH_3COO]^+$, 208, 159, 145, 127 $[C_9H_{19}]^+$, 111 $[C_8H_{15}]^+$, 97 $[C_7H_{13}]^+$, 90 $[CH_3OC(OH)=CHOH]^+$, 83, 69 $[C_5H_9]^+$, 57. Peak 3 (t_R 15.07 min, 2-hydroxybehenic acid methyl ester), EI-MS *m/z*: 370 $[M]^+$, 352 $[M-H_2O]^+$, 338 $[M-CH_3OH]^+$, 311 $[M-CH_3COO]^+$, 292, 266, 227, 197, 111, 97, 90 $[CH_3OC(OH)=CHOH]^+$, 83, 57. Peak 4 (t_R 15.58 min, 2-hydroxytricosanoic acid methyl ester), EI-MS *m/z*: 384 $[M]^+$, 341 $[M-CH_3CO]^+$, 325 $[M-CH_3COO]^+$, 281, 253, 207, 145, 127, 111, 97, 90 $[CH_3OC(OH)=CHOH]^+$, 83, 57. Peak 5 (t_R 16.18 min, 2-hydroxylignoceric acid methyl ester), EI-MS *m/z*: 398 $[M]^+$, 366 $[M-CH_3OH]^+$, 339 $[M-CH_3COO]^+$, 320, 227, 139, 125, 111, 97, 90 $[CH_3OC(OH)=CHOH]^+$, 83, 57. Peak 6 (t_R 16.79 min, 2-hydroxypentacosanoic acid methyl ester), EI-MS *m/z*: 412 $[M]^+$, 353 $[M-CH_3COO]^+$, 281, 207, 125, 111, 97, 90 $[CH_3OC(OH)=CHOH]^+$, 83, 69, 57. In the total ion chromatogram, the presence of the minor 2-hydroxy unsaturated fatty acid methyl esters was also identified, among which the peak at 15.58 min showed the molecular ion at *m/z* 396 less than 2 amu to that of 2-hydroxylignoceric acid methyl ester. Other 2-hydroxy unsaturated fatty acid methyl esters were eluted slightly before corresponding saturated analogues. The position of the double bond was not determined due to a shortage of samples. The long chain base was analyzed by EI-MS (30 eV, rel. int., %) *m/z*: 315 $[M]^+$ (0.1), 279 $[M-2H_2O]^+$ (19.0), 261 $[M-3H_2O]^+$ (0.4), 167 $[C_{12}H_{23}]^+$ (40.9), 149 (100), 113 (13.8), 71 (25.0), 57 (31.0). The long chain base was identified as

2-amino-1,3,4-trihydroxy-8-octadecene by comparison with literature data.[9,10]

MTPA Esterification of 2-Hydroxy Fatty Acid Methyl Ester To a solution of fatty acid methyl ester (1 mg) in pyridine (40 μl) was added (+)-MTPA chloride (4.6 μl), and the solution was allowed to stand at 25 °C overnight. 3-[(Dimethylamino)propyl]amine (2.8 μl) was added, and after 10 min of standing, the solvent was evaporated.[29] The residue was dissolved in CCl_4 and used for ^{1}H-NMR measurement. The signals in the aliphatic region appeared at δ: 0.886 (3H, t, J=6.3 Hz, CH_3), 1.256 [s, $(CH_2)_n$], 3.649 (3H, s, OCH_3), 3.789 (3H, s, $COOCH_3$), 5.119 (2H, t, J=6.6 Hz, CH_2O).

COX-2 Inhibition Test The inhibitory activity on COX-2 was measured using aspirin-treated mouse bone marrow derived from mast cells (BMMC) as described previously.[7d,8] In brief, BMMC from male BALB/cJ mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing antibiotics, 2 mM L-glutamine, 0.1 mM nonessential amino acids and 10% fetal bovine serum) and 50% WEHI-3 cell conditioned medium as a source of interleukin (IL)-3. After 3 weeks, BMMC were suspended in enriched medium and preincubated with 10 μg/ml aspirin for 2 h in order to inactivate preexisting COX-1. The cells were activated with c-*kit* ligand (100 ng/ml), IL-10 (100 U/ml) and lipopolysaccharide (100 μg/ml) in the presence/absence of plant extract or the isolated compound for 8 h. Media were collected and prostaglandin D_2 concentrations were measured using a PGD_2 assay kit (Amersham, Buckinghamshire, U.K.). Data were the arithmetic mean of triplicate determinations.

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