Sphingolipids from Bombycis Corpus 101A and Their Neurotrophic Effects

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Three new (2–4) and one known (1) sphingolipid were identified in the MeOH extract of Bombycis Corpus 101A. Their structures were elucidated as (4E,2S,3R)-2-N-octadecanoyl-4-tetradecasphingenine (1), (4E,6E,2S,3R)-2-N-eicosanoyl-4,6-tetradecasphingadienine (2), (4E,2S,3R)-2-N-eicosanoyl-4-tetradecasphingenine (3), and (4E,6E,2S,3R)-2-N-docosanoyl-4,6-tetradecasphingadienine (4) on the basis of spectroscopic data. Their neurotrophic effects were evaluated by examining PC12 cell neurite outgrowth.

Bombycis Corpus is a Bombyx mori larvae (silk moth larvae, Bombycidae) killed by infecting with the fungus Beauveria bassiana and has been used in Korean traditional medicine to treat palsy, headache, convulsion, and speech problems induced by stroke and tremor.1,2 Several sterols have been isolated from Bombycis Corpus.3 Bombycis Corpus 101A was produced by inoculating Bombyx mori larvae with the homogenous fungus strain Beauveria bassiana 101A, which was fermented at the National Institute of Agricultural Science and Technology, Korea. We have previously reported the presence of two cytotoxic sterols and two cyclodepsipeptides in the methanolic extract of Bombycis Corpus 101A.4,5 In this study, three new sphingolipids (2–4) and one known sphingolipid (1) were isolated from the hexane-soluble fraction of the methanol extract, and their neurotrophic effects were evaluated by examining their ability to induce neurite outgrowths from PC12 cells. Neurotrophic factors such as NGF (nerve growth factor) are secreted peptides that act as growth factors during phenotypic development and for the maintenance of specific neuronal populations in the developing and adult vertebrate nervous system. Neurotrophic factor is necessary for the development and maintenance of the peripheral and central nervous systems, and it has been reported that NGF has a therapeutic role in the treatment of neurodegenerative diseases, including Alzheimer’s disease and cerebrovascular dementia.6 Recent research upon the efficacy and function of NGF in the basal forebrain cholinergic neuron system suggested that NGF may be used as a therapeutic agent to prevent the degeneration of cholinergic neurons in Alzheimer patients.7 However, NGF can be used for medical treatment only when directly injected into the brain, since it is a large molecular weight polypeptide, which cannot cross the blood–brain barrier and because it is easily metabolized by peptidases when administered peripherally. Recently, several synthetic compounds and natural products have been found to enhance the action of NGF in terms of neurite outgrowth from PC12 cells and to enhance neuronal cell survival. Therefore, it is of some importance that NGF-like agents be developed. The rat pheochromocytoma cell line (PC12) is a convenient cell model for sympathetic neurons and has been proven useful in the study of the downstream signaling pathways involved in neuronal survival and death. In response to NGF, PC12 cells cease division and differentiate into sympathetic neuron-like cells with extensive neuritis.8 This study describes the isolation and structural elucidation of the above sphingolipids and reports upon their neurotrophic effects.

Results and Discussion

Purification of the n-hexane fraction of Bombycis Corpus 101A by several column chromatographic methods has afforded four unusual ceramides with C14 sphingosine skeleton (1–4). The structure elucidation of these ceramides was performed by HRFABMS, FAB-CID MS, GC−MS, and 2D NMR experiments. Although the compound 1, (4E,2S,3R)-2-N-octadecanoyl-4-tetradecasphingenine, was previously reported,9 no 1H and 13C NMR data were available. The acidic methanolysis of 1 yielded an octadecanoyl acid methyl ester, which was identified by GC−MS analysis. Compound 2 was obtained as an amorphous powder. The molecular formula of 2 was assigned as C34H58NO3 by HRFABMS ([M + Na]+ m/z 558.4874). The IR spectrum displayed absorption bands at 3340 and 1648 cm−1, indicating the presence of hydroxyl and amide functionalities. The characteristic signals of 2-amino-1,3-diol of the hydrocarbon chain were observed at δ 3.70 (1H, br d, J = 10.5 Hz), 3.89–3.96 (2H, m), and 4.39 (1H, br m) in the 1H NMR spectrum and at δ 55.2, 63.2, and 75.3 in the 13C NMR spectrum, respectively. In addition, the 1H NMR spectrum showed signals corresponding to aliphatic hydrocarbons at δ 0.88 (6H, t, J = 7.0 Hz), 1.20–1.39 (42H, m), 1.61 (2H, br sep, J = 7.5 Hz), 2.06 (2H, m), 2.22 (2H, t, J = 7.5 Hz) and four olefinic protons at δ 5.61 (1H, dd, J = 15.0, 6.0 Hz), 5.73 (1H, dt, J = 15.0, 7.5 Hz), 6.03 (1H, dd, J = 15.0, 10.5 Hz), 6.29 (1H, dd, J = 15.0, 10.5 Hz). The 13C NMR spectrum showed signals due to two terminal methyl groups in aliphatic hydrocarbon chains at δ 14.8, four olefinic carbons at δ 129.6 (×2), 133.5, and 137.5, and an amide carbon at δ 174.6. Analysis of the 2−1H COSY, HMBC, and HMQC spectra led to the assignment of proton and carbon signals for 2. The position and geometry of the double bonds were confirmed by 1H−1H COSY analysis and coupling constant data. The λmax (233 nm) in the UV spectrum indicated the presence of conjugated double bonds.10 and the 1H−1H COSY spectrum showed the C3=C4−C5=C6−C7 connectivity. The J4,5 (15.0 Hz) and J6,7 (15.0 Hz) values indicating the trans geometry of the double bonds

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bonds. Therefore, 2 was believed to be a 4E,6E-sphingadiene type ceramide, which has not been reported from natural products. Methanolation with HCl in MeOH yielded an eicosanoic acid methyl ester, as identified by GC-MS analysis.\textsuperscript{12} The major fragment ion at m/z 318 (296 + Na — H) in the CID (collision-induced dissociation) spectrum of the [M + Na]\textsuperscript{+} ion in the FABMS of 2 indicated the presence of a C\textsubscript{36} amino alcohol.\textsuperscript{13} These results suggested that 2 was an N-acyl eicosanoic acid derivative of C\textsubscript{36} amino alcohols. The optical rotation (−3.2°) and the chemical shifts of C-1 (δ 63.2), C-2 (δ 55.2), C-3 (δ 75.3), and C-1’ (δ 174.6) were very similar to those of (2S,3R)-2-octanoylamidocadeca-(4E,6E)-diene-1,3-diol, which was synthesized recently.\textsuperscript{14} These results indicated that the double configurations of C-2 and C-3 to be 2S and 3R, respectively. Accordingly, the structure of 2 was determined to be (4E,6E,2S,3R)-2-N-eicosanoyl-4,6-tetradecasphingadienine.

Compound 3 was obtained as an amorphous powder. The molecular formula of 3 was assigned as C\textsubscript{36}H\textsubscript{67}NO\textsubscript{3} by HRFABMS (m/z 538.5195). The \textsuperscript{1}H and \textsuperscript{13}C NMR spectra of 3 showed that it had a structure similar to that of 2. The major difference between 3 and 2 was the presence of only one double bond in 3. \textsuperscript{1}H—\textsuperscript{1}H COSY, HMBC, and HMQC spectral analysis led to the assignment of proton and carbon signals for 3. The position and geometry of the double bond was confirmed by \textsuperscript{1}H—\textsuperscript{1}H COSY analysis and coupling constant data. In the FABCIDMS spectrum, the major fragment ion at m/z 320 (298 + Na — H) indicated the presence of a C\textsubscript{34} amino alcohol.\textsuperscript{15} Treatment of 3 with HCl—MeOH furnished an eicosanoic acid methyl ester. The optical rotation (−4.0°) and the chemical shift of C-1 (δ 63.2), C-2 (δ 55.2), C-3 (δ 75.3), and C-1’ (δ 174.6) were in good agreement with previously published values of (4E,2S,3R)-4-sphingadienine derivatives.\textsuperscript{15} In addition, J\textsubscript{1H,2H} (ca. 3.5 Hz) and J\textsubscript{2H,3H} (ca. 3.5 Hz) values were also in good agreement with reported data on (2S,3R)-sphingosines.\textsuperscript{15} Therefore, the structure of 3 was determined to be (4E,2S,3R)-2-N-eicosanoyl-4-tetradecasphingadienine.

Compound 4 was obtained as an amorphous powder. The molecular formula of 4 was assigned as C\textsubscript{38}H\textsubscript{70}NO\textsubscript{3} by HRFABMS (m/z 564.5344). The IR spectrum displayed absorption bands at 3340 and 1648 cm\textsuperscript{-1}, indicating the presence of hydroxyl and amide functionalities. The optical rotation (−3.6°) and NMR spectra of 4 were in good agreement with that of 2 except for the integral value of a signal at δ 1.20—1.39 (46H, m). Treatment of 4 with HCl—MeOH furnished a docosanoic acid methyl ester. The \textsuperscript{1}H—\textsuperscript{1}H COSY, HMBC, and HMQC spectral analysis led to the assignment of proton and carbon signals of 4. The position and geometry of the double bond was confirmed by \textsuperscript{1}H—\textsuperscript{1}H COSY analysis and coupling constant data. In the FABCIDMS spectrum, the major fragment ion at m/z 318 (296 + Na — H) indicated the presence of a C\textsubscript{36} amino alcohol.\textsuperscript{13} These results showed that it had a structure similar to that of 2. The major difference between 3 and 2 was the presence of only one double bond in 3. \textsuperscript{1}H—\textsuperscript{1}H COSY, HMBC, and HMQC spectral analysis led to the assignment of proton and carbon signals for 3. The position and geometry of the double bond was confirmed by \textsuperscript{1}H—\textsuperscript{1}H COSY analysis and coupling constant data. In the FABCIDMS spectrum, the major fragment ion at m/z 320 (298 + Na — H) indicated the presence of a C\textsubscript{34} amino alcohol.\textsuperscript{15} Treatment of 3 with HCl—MeOH furnished an eicosanoic acid methyl ester. The optical rotation (−4.0°) and the chemical shift of C-1 (δ 63.2), C-2 (δ 55.2), C-3 (δ 75.3), and C-1’ (δ 174.6) were in good agreement with previously published values of (4E,2S,3R)-4-sphingadienine derivatives.\textsuperscript{15} In addition, J\textsubscript{1H,2H} (ca. 3.5 Hz) and J\textsubscript{2H,3H} (ca. 3.5 Hz) values were also in good agreement with reported data on (2S,3R)-sphingosines.\textsuperscript{15} Therefore, the structure of 3 was determined to be (4E,2S,3R)-2-N-eicosanoyl-4-tetradecasphingadienine.

The neurite outgrowth promoting activity of compounds 1–4 was examined in PC 12 cells by measuring neurite length (Figure 2). All these sphingolipids promoted neurite outgrowth in PC 12 cells. Both 2 and 4 at 10 \textmu M exhibited a neurite outgrowth promoting activity greater than that of NGF (50 ng/mL). As shown in Figure 2, compounds possessing the conjugated double bond of an N-acyl fatty acid and a long aliphatic carbon chain were found to exhibit good neurite outgrowth activity in PC 12 cells.

**Experimental Section**

**General Experimental Procedures.** Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Optical rotations were measured on a Jasco P-1020 polarimeter. UV spectra were recorded with a Shimadzu UV 1601 spectrophotometer. IR spectra were recorded with a Nicolet model 205 instrument. NMR spectra were recorded on either a Bruker AMX or a Varian UNITY INOVA 500 NMR spectrometer in CDCl\textsubscript{3}. EIMS and HRFABMS data were obtained on a JEOL JMS700 mass spectrometer. Open column chromatography was carried out over silica gel (Merck, 70–230) or Sephadex LH-20 (Pharmacia). Low-pressure liquid chromatography was carried out over Merck Lichroprep Lobar-A Si 60 (240 × 10 mm) with a FMI OSY-0 pump (ISCO).

**Material.** Bombycis Corpus 101A was supplied by the National Institute of Agricultural Science and Technology, Suwon, Korea. A voucher specimen (SKK-118b) is deposited at the College of Pharmacy in Sung Kyun Kwan University.

**Extraction and Isolation.** The dried and ground Bombycis Corpus 101A (1.5 kg) was extracted with MeOH (4 L) five times at room temperature and then three times at 60 °C. The resulting methanol extract (120 g) was suspended in water and successively partitioned to provide n-hexane–EtOAc–MeOH with increasing methanol content, to provide four fractions (H1—H4). The H1 fraction (30 g) was then subjected to silica gel column chromatography using n-hexane–EtOAc–MeOH at the College of Pharmacy in Sung Kyun Kwan University.
column (CH2Cl2-MeOH, 1:1) and purified using a Lobar A RP-18 column (90% MeOH) to afford 1 (3 mg), 2 (5 mg), 3 (15 mg), and 4 (17 mg).

(4E,6E,2R,3R)-2'-N-Octanoyl-4-tetradecaphingine (1): white powder; [α]D20~265° (c 0.012, CHCl3); IR (neat) νmax 3340, 2930, 2850, 1648, 1625, 1535, 1470, 1070, 1050 cm⁻¹; 1H NMR (CDCl3, 500 MHz) δ 0.88 (6H, t, J = 7.0 Hz, H-14 and H-18), 1.20-1.39 (34H, m, C-13, C-17), 1.56 (1H, d, J = 15.5 Hz, H-3), 2.05 (2H, br q, J = 7.0 Hz, H-6), 2.23 (2H, t, J = 7.5 Hz, H-2), 2.65 (2H, br s, OH), 3.71 (1H, br d, J = 11.5 Hz, H-1), 3.96 (1H, d br q, J = 7.2, 3.5 Hz, H-3), 4.22 (2H, br m, H-5), 7.26 (1H, d, J = 15.7, 6.5 Hz, H-4), 5.26 (1H, dd, J = 10.5, 7.0 Hz, H-2), 3.70 (1H, br s, OH), 3.90 (1H, ddd, J = 15.0, 10.5 Hz, H-14 and H-22), 4.13 (1H, br d, J = 15.0, 10.5 Hz, H-5); 13C NMR (CDCl3, 125 MHz) δ 14.8 (C-1, C-2), 63.2 (C-1), 75.3 (C-3), 129.5 (C-4), 135.1 (C-5), 174.7 (C-13), 53.5 (C-6), 75.3 (C-7), 75.3 (C-9), 115.3 (C-11), 63.2 (C-2), 75.3 (C-3), 129.5 (C-4), 135.1 (C-5), 174.7 (C-13); EI/MS m/z 509 [M]+ (0.5), 491 (6), 461 (6), 360 (4), 326 (35), 309 (100), 278 (23), 267 (16), 226 (11), 208 (18), 60 (97).

(4E,6E,5S,6R)-2'-N-Eicosanoyl-4,6-tetradecaphingine (2): white powder; mp 73.6 °C; [α]D20~6.3° (c 0.05, CHCl3); UV (EtOH) λmax (log ε) 233 (4.43) nm; IR (neat) νmax 3083, 2920, 2850, 1648, 1535, 1470, 1070, 1050 cm⁻¹; 1H NMR (CDCl3, 500 MHz) δ 0.88 (6H, t, J = 7.0 Hz, H-14 and H-18), 1.26-1.82 (46H, m), 1.64 (2H, br sep, J = 15.5 Hz, H-3), 2.06 (2H, M) and NGF (50 ng/mL). The culture medium was changed every day. Neurite outgrowth was evaluated in terms of lengths equivalent to one microscope (model CK-2; Olympus). Fields were photographed using a camera attached to the light microscope (Eclipse E600; Nikon). Statistical significance was tested by one-way ANOVA.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes
(1) Chicago Science and Technology Publisher and Shougakukan. The Dictionary of Chinese Drugs; Shougakukan: Tokyo, 1995; pp 2238–2240.

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