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 (4*E*,2*S*,3*R*)-2-*N*-octadecanoyl-4-tetradecasphingenine (**1**), (4*E*,6*E*,2*S*,3*R*)-2-*N*-eicosanoyl-4,6-tetradecasphingadienine (**2**), (4*E*,2*S*,3*R*)-2-*N*-eicosanoyl-4-tetradecasphingenine (**3**), and (4*E*,6*E*,2*S*,3*R*)-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 homogeneous fungi 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 administrated 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 C_{14} 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**, (4*E*,2*S*,3*R*)-2-*N*-octadecanoyl-4-tetradecasphingenine, was previously reported,[9] no ^{1}H and ^{13}C NMR data were available. The acidic methanolysis of **1** yielded an octadecanoic 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 $C_{34}H_{65}NO_3$ 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 ^{1}H NMR spectrum and at δ 55.2, 63.2, and 75.3 in the ^{13}C NMR spectrum,[10] respectively. In addition, the ^{1}H 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, $J \approx$ 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 ^{13}C 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 ^{1}H−^{1}H 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 ^{1}H−^{1}H COSY analysis and coupling constant data. The λ_{max} (233 nm) in the UV spectrum indicated the presence of conjugated double bonds,[11] and the ^{1}H−^{1}H COSY spectrum showed the C_3−C_4−C_5−C_6−C_7 connectivity. The $J_{4,5}$ (15.0 Hz) and $J_{6,7}$ (15.0 Hz) values indicating the *trans* geometry of the double

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bonds. Therefore, **2** was believed to be a 4*E*,6*E*-sphingadiene type ceramide, which has not been reported from natural products. Methanolysis with HCl in MeOH yielded an eicosanoic acid methyl ester, as identified by GC−MS analysis.[12] The major fragment ion at *m*/*z* 318 (296 + Na − H) in the CID (collision-induced dissociation) spectrum of the $[M + Na]^+$ ion in the FABMS of **2** indicated the presence of a C_{14} amino alcohol.[13] These results suggested that **2** was an *N*-acyl eicosanoic acid derivative of C_{14} 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 (2*S*,3*R*)-2-octanoylamidooctadeca-(4*E*,6*E*)-diene-1,3-diol, which was synthesized recently.[14] Theis evidence indicated the absolute configurations of C-2 and C-3 to be 2*S* and 3*R*, respectively. Accordingly, the structure of **2** was determined to be (4*E*,6*E*,2*S*,3*R*)-2-*N*-eicosanoyl-4,6-tetradecasphingadienine.

Compound **3** was obtained as an amorphous powder. The molecular formula of **3** was assigned as $C_{34}H_{67}NO_3$ by HRFABMS (*m*/*z* 538.5195). The ^1H and ^{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**. $^1H-^1H$ 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 $^1H-^1H$ 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_{14} amino alcohol.[13] 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 (4*E*,2*S*,3*R*)-4-sphingenine derivatives.[15] In addition, $J_{1,2}$ (ca. 3.5 Hz) and $J_{2,3}$ (ca. 3.5 Hz) values were also in good agreement with reported data on (2*S*,3*R*)-sphingosines.[15] Therefore, the structure of **3** was determined to be (4*E*,2*S*,3*R*)-2-*N*-eicosanoyl-4-tetradecasphingenine.

Compound **4** was obtained as an amorphous powder. The molecular formula of **4** was assigned as $C_{36}H_{70}NO_3$ by HRFABMS (*m*/*z* 564.5344). The IR spectrum displayed absorption bands at 3340 and 1648 cm^{-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 FABCIDMS spectrum of **4** indicated a major fragment ion at *m*/*z* 318 (296 + Na − H). On this basis, the structure of **4** was determined to be (4*E*,6*E*,2*S*,3*R*)-2-*N*-docosanoyl-4,6-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 PC12 cells. Both **2** and **4** at 10 *μ*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 PC12 cells.

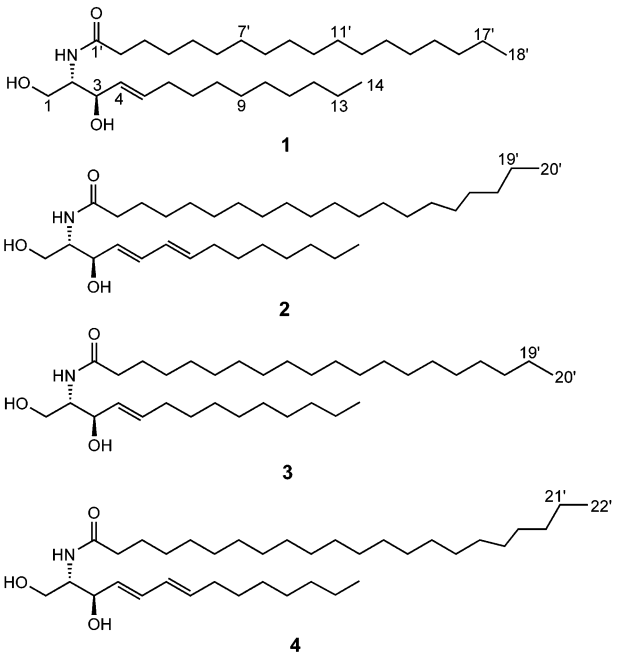

Figure 1. Structures of compounds **1**−**4**.

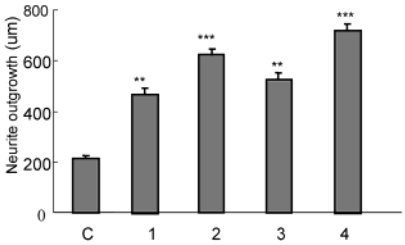

Figure 2. NGF-potentiating activities of sphingolipids from Bombycis Corpus 101A. Neurite lengths of PC12 cells cultured in the presence of compounds **1**−**4**. Compounds were added dissolved in DMSO (0.01%). PC12 cells were found to express neurites under different medium conditions. A minimum of 100 cells were examined for each data point. C, control, refers to the vehicle-treated group; lane 1−lane 4, to compounds **1**−**4** (10 *μ*M). These results show that the four compounds have NGF potentiating activities versus the control and that compound **4** is the most effective. Each value shown represents the mean ± SD ($n = 3$). **Significantly different from the control value at the $p < 0.01$ level. ***Significantly different from the control value at the $p < 0.001$ level.

Experimental Section

General Experimental Procedures. Melting points were determined on 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 $CDCl_3$. 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 QSY-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 (55 g), chloroform (6 g), and *n*-butanol (30 g) fractions. The hexane extract (55 g) was subjected to silica gel column chromatography and eluted with a solvent mixture of *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 (3:1) as an eluent to afford three subfractions (H11−H13). The H13 fraction (4.6 g) was further subjected to Sephadex LH-20

column (CH_2Cl_2−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).

(4*E*,2*S*,3*R*)-2-*N*-Octadecanoyl-4-tetradecasphingenine (1): white powder; $[\alpha]^{20}_D$ −3.5° (*c* 0.012, $CHCl_3$); IR (neat) ν_{max} 3330, 2920, 2850, 1650, 1617, 1550, 1465, 1050 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.88 (6H, t, J = 7.0 Hz, H-14 and H-18′), 1.20−1.39 (42H, m, H-7−H-13, H-4′−H-17′), 1.62 (2H, br sep, $J \approx$ 7.5 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, $J \approx$ 11.5 Hz, H-1), 3.91 (1H, ddd, J = 11.5, 4.0, 3.0 Hz, H-1), 3.96 (1H, d br q, J = 7.2, 3.5 Hz, H-2), 4.32 (1H, br dd, $J \approx$ 6.5, 3.5 Hz, H-3), 5.53 (1H, dd, J = 15.7, 6.5 Hz, H-4), 5.79 (1H, dt, J = 15.7, 6.5 Hz, H-5), 6.26 (1H, br d, J = 7.2 Hz, NH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.8 (C-14, C-18′), 23.4 (C-13, C-17′), 26.5 (C-3′), 29.8, 30.1, 30.4, 30.4 (C-7−C-11, C-4′−C-15′), 32.6 (C-12, C-16′), 33.0 (C-6), 37.6 (C-2′), 55.2 (C-2), 63.2 (C-1), 75.3 (C-3), 129.5 (C-4), 135.1 (C-5), 174.7 (C-1′); EIMS 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).

(4*E*,6*E*,2*S*,3*R*)-2-*N*-Eicosanoyl-4,6-tetradecasphingadienine (2): white powder; mp 73.6 °C; $[\alpha]^{20}_D$ −3.2° (*c* 0.05, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 233 (4.23) nm; IR (neat) ν_{max} 3340, 2930, 2850, 1648, 1625, 1535, 1470, 1070, 1050 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.88 (6H, t, J = 7.0 Hz, H-14 and H-20′), 1.20−1.39 (42H, m, H-9−H-13, H-4′−H-19′), 1.61 (2H, br sep, $J \approx$ 7.5 Hz, H-3′), 2.06 (2H, m, H-8), 2.22 (2H, t, J = 7.5 Hz, H-2′), 2.74 (1H, br s, OH), 2.88 (1H, br s, OH), 3.70 (1H, br d, J = 10.5 Hz, H-1), 3.89−3.96 (2H, m, H-1 and H-2), 4.39 (1H, br m, H-3), 5.61 (1H, dd, J = 15.0, 6.0 Hz, H-4), 5.73 (1H, dt, J = 15.0, 7.5 Hz, H-7), 6.03 (1H, dd, J = 15.0, 10.5 Hz, H-6), 6.26 (1H, br d, J = 7.0 Hz, NH), 6.29 (1H, dd, J = 15.0, 10.5 Hz, H-5); ^{13}C NMR ($CDCl_3$,125 MHz) δ 14.8 (C-14, C-20′), 23.4 (C-13, C-19′), 26.5 (C-3′), 29.9, 30.0, 30.1, 30.2, 30.3, 30.4 (C-9−C-13, C-4′−C-17′), 32.5, 32.6 (C-12, C-18′), 33.4 (C-8), 37.6 (C-2′), 55.2 (C-2), 63.2 (C-1), 75.3 (C-3), 129.6 (C-5, C-6), 133.5 (C-4), 137.5 (C-7), 174.6 (C-1′); EIMS m/z 535 $[M]^+$ (1), 517 (10), 487 (19), 388 (15), 354 (17), 337 (90), 312 (24), 306 (20), 224 (38), 206 (22), 193 (21), 57 (100); FABCIDMS m/z 558 (100), 537 (1.9), 528 (0.9), 514 (0.7), 500 (0.7), 486 (1.0), 472 (0.9), 458 (1.1), 444 (0.8), 430 (1.0), 416 (0.8), 402 (0.7), 388 (0.8), 374 (1.0), 358 (1.2), 332 (1.0), 318 (2.0), 206 (0.6); HRFABMS m/z 558.4874 (calcd for $C_{34}H_{65}NO_3$-Na, 558.4862).

(4*E*,2*S*,3*R*)-2-*N*-Eicosanoyl-4-tetradecasphingenine (3): white powder; mp 81.7 °C; $[\alpha]^{20}_D$ −4.0° (*c* 0.082, $CHCl_3$); IR (neat) ν_{max} 3332, 2920, 2851, 1650, 1615, 1550, 1465, 1045 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.88 (6H, t, J = 7.0 Hz, H-14 and H-20′), 1.20−1.39 (46H, m, H-7−H-13, H-4′−H-19′), 1.64 (2H, br sep, $J \approx$ 7.5 Hz, H-3′), 2.05 (2H, br q, J = 7.5 Hz H-6), 2.22 (2H, t, J = 7.5 Hz, H-2′), 2.84 (1H, br s, OH), 2.85 (1H, br s, OH), 3.70 (1H, m, H-1), 3.90 (1H, ddd, J = 11.3, 4.0, 3.5 Hz, H-1), 3.94 (1H, d br q, J = 7.2, 3.5 Hz, H-2), 4.31 (1H, br dd, $J \approx$ 6.3, 3.5 Hz, H-3), 5.52 (1H, ddt, J = 15.5, 6.3, 1.2 Hz, H-4), 5.79 (1H, dtd, J = 15.5, 6.3, 1.2 Hz, H-5), 6.26 (1H, br d, J = 7.2 Hz, NH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.8 (C-14, C-20′), 23.40 (C-13, C-19′), 26.5 (C-3′), 29.8, 29.9, 30.0, 30.1, 30.2, 30.3, 30.4 (C-7−C-11, C-4′−C-17′), 32.6 (C-16, C-18′), 33.0 (C-6), 37.6 (C-2′), 55.2 (C-2), 63.2 (C-1), 75.3 (C-3), 129.5 (C-4), 135.0 (C-5), 174.7 (C-1′); EIMS m/z 537 $[M]^+$ (0.6), 519 (10), 489 (12), 388 (5), 354 (18), 337 (100), 306 (22), 280 (10), 195 (15), 60 (66); FABCIDMS m/z 560 (100), 539 (1.9), 530 (0.9), 516 (0.7), 502 (0.7), 488 (1.0), 474 (0.9), 460 (1.1), 446 (0.8), 432 (1.0), 418 (0.8), 404 (0.7), 390 (0.8), 376 (1.0), 360 (1.2), 334 (1.0), 320 (2.0), 208 (0.6); HRFABMS m/z 538.5195 (calcd for $C_{34}H_{68}NO_3$, 558.5121).

(4*E*,6*E*,2*S*,3*R*)-2-*N*-Docosanoyl-4,6-tetradecasphingadienine (4): white powder; mp 71.8 °C; $[\alpha]^{20}_D$ −3.6° (*c* 0.176, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 233 (4.10) nm; IR ν_{max}^{neat} 3340, 2930, 2850, 1648, 1623, 1535, 1470, 1050 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.88 (6H, t, J = 7.0 Hz, H-14 and H-22′), 1.20−1.39 (46H, m, H-9−H-13, H-4′−H-21′), 1.63 (2H, br sep, $J \approx$ 7.5 Hz, H-3′), 2.06 (2H, m, H-8), 2.22 (2H, t, J = 7.5 Hz, H-2′), 2.85 (1H, br s, OH), 2.99 (1H, br s, OH), 3.70 (1H, br d, J = 10.5 Hz, H-1), 3.89−3.96 (2H, m, H-1 and H-2), 4.38 (1H, br m, H-3), 5.60 (1H, dd, J = 15.0, 6.0 Hz, H-4), 5.73 (1H, dt, J = 15.0, 7.8 Hz, H-7), 6.03 (1H, dd, J = 15.0, 10.5 Hz, H-6), 6.28 (1H, br d, J = 7.2 Hz, NH), 6.29 (1H, dd, J = 15.0, 10.5 Hz, H-5); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.8 (C-14, C-22′), 23.4 (C-13, C-21′), 26.5 (C-3′), 29.9, 30.0, 30.1, 30,2, 30.3, 30.4 (C-9−C-11, C-4′−C-19′), 32.5, 32.6 (C-12, C-20′), 33.4 (C-8), 37.5 (C-2′), 55.2 (C-2), 63.2 (C-1), 75.2 (C-3), 129.6, 129.7 (C-5, C-6), 133.5 (C-4), 137.4 (C-7), 174.7 (C-1′); EIMS m/z 563 $[M]^+$ (2), 545 (12), 515 (17), 416 (12), 382 (27), 365 (97), 340 (35), 224 (32), 206 (20), 194 (15), 60 (100); FABCIDMS m/z 586 (100), 565 (1.9), 556 (0.9), 542 (0.7), 528 (0.7), 514 (0.7), 500 (0.7), 486 (1.0), 472 (0.9), 458 (1.1), 444 (0.8), 430 (1.0), 416 (0.8), 402 (0.7), 388 (0.8), 374 (1.0), 358 (1.2), 332 (1.0), 318 (2.0), 206 (0.6); HRFABMS m/z 564.5344 (calcd for $C_{36}H_{70}NO_3$, 564.5277).

Methanolysis of 1−4. Compound **1** (1 mg) was heated with 5% HCl in MeOH for 24 h at 74 °C. After adding H_2O, the reaction mixture was extracted with hexane and dried with anhydrous magnesium sulfate. The resulting hexane-soluble fraction was concentrated to yield a fatty acid methyl ester **1b**, which was analyzed by GC−MS. Compounds **2**, **3**, and **4** were cleaved by the same method to afford **2b**, **3b**, and **4b**, respectively.

Octadecanoic acid methyl ester (1b): EIMS m/z (rel int) 298 (18), 267 (8), 255 (17), 241 (4), 213 (5), 199 (15), 185 (5), 171 (2), 157 (5), 143 (28), 129 (10), 97 (14), 87 (74), 74 (100), 55 (22), 43 (28).

Eicosanoic acid methyl ester (2b and 3b): EIMS m/z 326 (30), 295 (10), 283 (20), 269 (4), 255 (5), 241 (7), 227 (8), 213 (2), 199 (8), 185 (6), 171 (4), 157 (4), 143 (30), 129 (10), 97 (10), 87 (75), 74 (100), 55 (24), 43 (30).

Docosanoic acid methyl ester (4b): EIMS m/z 354 (40), 323 (8), 311 (18), 297 (4), 283 (2), 269 (5), 255 (10), 241 (4), 227 (1), 213 (2), 199 (10), 185 (6), 171 (2), 157 (3), 143 (30), 129 (12), 115 (4), 101 (8), 87 (80), 74 (100), 55 (30), 43 (45).

Measurement of Neurite Outgrowth. Measurement of NGF-potentiating activity and morphological observations of PC12 cells were performed as previously reported.[16] PC12 cells were maintained in a humidified atmosphere of 5% CO and 95% air in RPMI1640 supplemented with 1% fetal bovine serum, 2% horse serum, and 2 mM glutamine. Cells were seeded in six-well culture plates at 3×10^4 cells/well in poly-L-lysine-coated wells. PC12 cells in six-well plates were treated with each sphingolipid (**1**−**4**) (10 μM) and NGF (50 ng/mL). The culture medium was changed every day. Neurite outgrowth was measured under a microscope after 48 h of treatment,[17] when each culture was fixed with 2% glutaraldehyde in PBS and stored in PBS solution. Randomly selected fields were photographed using a camera attached to the light microscope (model CK-2; ×100 magnification). Neurite extension was evaluated in terms of lengths equivalent to one diameter of the cell body. All data are expressed as means ± SD. 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.

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