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Phospholipids from *Bombycis corpus* and Their Neurotrophic Effects

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Three phospholipids (**4-6**) and three aromatic amines (**1-3**) were obtained from the methanol extract of *Bombycis corpus*. Based on spectral data, their structures have been elucidated as nicotiamide (**1**), cytidine (**2**), adenine (**3**), 1-*O*-(9*Z*-octadecenoyl)-2-*O*-(8*Z*,11*Z*-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (**4**), 1,2-di-*O*-hexadecanoyl-*sn*-glycero-3-phosphorylcholine (**5**) and 1,2-di-*O*-9*Z*-octadecenoyl-*sn*-glycero-3-phosphorylcholine (**6**). We examined the effects of compounds on synthesis of NGF in cultured astrocytes. By RT-PCR analysis, expresison of NGF mRNA in astrocytes cultured in serum-starvation increased after the addition of phospholipid (10 μM). The NGF content in the culture medium was significantly increased by compound **5**, compared with the control value. These results suggest that three phospholipid compounds isolated from the methanol extract of *Bombycis corpus* may exert neurotrophic effects by stimulation of NGF synthesis in astrocytes.

Key word : *Bombycis corpus*, Phospholipid, Diacylglycerophosphorylcholines, Neurotrophic effect

INTRODUCTION

Bombycis corpus has been used in Korean traditional medicine to treat palsy, headache, convulsion, stroke induced speech problem and tremor (Shanghai Science and Technologic Publisher *et al.*, 1985; Pemberton 1999). Several sterols were reported from *Bombycis corpus* (Cheng *et al.*, 1977). Our research on constituents promoted neurite growth related to traditional use of *Bombycis corpus* led to the isolation of three diacylglycerophosphorylcholines (**4-6**) and three aromatic amines (**1-3**) from the butanol soluble portion. Diacylglycerophosphorylcholines were major component of cell membrane and play an important role in membrane functions (Arora and Gupta, 1997). To date, they have been detected by HPLC and MS analysis as mixture from natural sources (Singleton *et al.*, 1993; Gamo *et al.*, 1999), and several synthetic phosphorylcholines have been reported (Ishihara *et al.*, 1996; Solodin *et al.*, 1996). Compounds **4**, **5** and **6** were isolated as pure compound from natural sources. It has been reported that nerve growth factor (NGF) stimulates the outgrowth of neurites in neuronal cells and plays an important role in the survival and maintenance of neuron in the central nervous system (Woodruff *et al.*, 1997). Thus, neurotrophic factors such as NGF have been considered as good candidates for the prevention and treatment of neurodegenerative diseases. Since such endogenous factors are too large in size to pass through the blood-brain barrier, exogenous low-molecular-weight compounds that mimic the activity of neurotrophic factors might be developed as promising therapeutic drugs to treat various neurodegenerative disease. We investigated the enhancement of neurites and effects of phospholipids on synthesis of NGF in cultured astrocytes. The present paper describes the isolation, structural elucidation and neurotropic effects of these phosphorylcholines **4-6**.

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MATERIALS AND METHODS

General procedures

Mps: uncorr. NMR: in $CDCl_3$, Bruker AMX 500 and Varian UNITY INOVA 500. IR: Brucker Vector 22 FT-IR spectrophotometer. UV: Shimadzu UV-1601 UV-Visible Spectrophotometer. Polarimeter : JASCO P-1020. MS: mass spectrometer. GC-MS: Hewlett-Packard 6890 GC (column : HP-5MS 30 m 0.25 mm)/Hewlett-Packard 5973 MSD system. Column Chromatography : Silica gel (Merck, 70~230, 230~400 mesh) and Sephadex LH-20 (Pharmacia). TLC: Merck precoated Si gel F_{254} plates and RP-18 F_{254s} plates. LPLC: Merck Lichroprep Lobar®-A Si 60 & Lobar®-A RP-18 (24010 mm).

Material

Bombycis corpus was purchased at Kyung-Dong Herbal Market in September, 2000, Seoul, Korea. The voucher specimen (SKK-118b) is deposited in the College of Pharmacy at SungKyunKwan University.

Extraction and purification

The dried and ground *Bombycis corpus* (2 kg) were extracted with MeOH (4L) five times at room temperature and three times at 60°C. The resultant methanol extract (180 g) was suspended in water and then successively partitioned to give hexane (65 g), chloroform (6 g) and butanol (30 g) soluble fractions. The butanol extract (30 g) was subjected to silica gel column chromatography, eluted with a solvent mixture of ethylacetate/methanol/water (9:3:1 and 9:5:2) to give six fractions (B1~B6). B1 fraction (5 g) was then applied to a silica gel column chromatography using ethylacetate/methanol/water (9:1:0.5) as eluent to afford four subfractions (B11~B14). B12 fraction was further subjected to silica Sep-Pak column (ethylacetate/methanol=20:1) to afford **1** (10 mg) and **2** (5 mg). B14 fraction was subjected to silica Sep-Pak column (ethylacetate/methanol=20:1) to afford **3** (5 mg). B5S fraction (4.3 g) which are supernant of B5 fraction in water was applied to a HP-20 column chromatography using water, methanol and aceton as eluent to afford four subfractions (B5S1-B5S4). B5S1 fraction (3.3 g) was subjected to silica gel column chromatography (ethylacetate/methanol/water=9:5:2) to give three subfractions (B5S11-B5S13). B5S12 fraction purified with RP Sep-Pak column (80% methanol) and with repeated prep. HPLC (95 % methanol/0.1 M ammonium acetate) to afford **4** (Rt. 25.0 min, 30 mg). B5S13 fraction purified with RP Sep-Pak column (80% methanol) and with repeated prep. HPLC (methanol/0.1 M ammonium acetate) to afford **5** (Rt. 39.5 min,10 mg) and **6** (Rt. 27.4 min, 40 mg).

Nicotiamide (1)

Yellowish powder, EI-MS *m/z* (rel. int.) : 122 (M^+, 100), 106 (80), 104 (20), 85 (18), 78 (74); ^{1}H-NMR (500 MHz, CD_3OD) : δ 7.52 (1H, ddd, *J* = 8.0, 4.7, 0.9 Hz, H-5), 8.27 (1H, ddd, *J* = 8.0, 2.0, 1.5 Hz, H-4), 8.67 (1H, dd, *J* = 4.7, 1.5 Hz, H-6), 9.00 (1H, d, *J* = 2.0 Hz, H-2); ^{13}C-NMR (125 MHz, CD_3OD) : δ 123.40 (C-5), 130.73 (C-3), 136.60 (C-4), 148.74 (C-6), 152.12 (C-2), 169.10 (C-1).

Cytidine (2)

White powder, ^{1}H-NMR (500 MHz, CD_3OD) : δ 3.73 (1H, dd, *J* = 12.3, 3.2 Hz, H-5), 3.83 (1H, dd, *J* = 12.3, 2.9 Hz, H-5), 4.00 (1H, ddd, *J* = 4.4, 3.2, 2.9 Hz, H-4), 4.15 (1H, dd, *J* = 5.3, 4.4 Hz, H-3), 4.17 (1H, dd, *J* = 5.3, 4.5 Hz, H-2), 5.70 (1H, d, *J* = 8.0 Hz), 5.90 (1H, d, *J* = 4.5Hz, H-1), 8.00 (1H, d, *J* = 8.0 Hz, H-6); ^{13}C-NMR (125 MHz, CD_3OD) : δ 61.56 (C-5), 70.58 (C-3), 75.01 (C-4), 85.64 (C-2), 90.01 (C-1), 101.91 (C-5), 142.00 (C-6), 151.74 (C-2), 165.47 (C-4).

Adenine (3)

Yellowish powder, ^{1}H-NMR (500 MHz, DMSO-d_6) : δ 8.47 (1H, s, H-2), 8.86 (1H, br.s, H-6).

1-*O*-(9*Z*-octadecenoyl)-2-*O*-(8*Z*,11*Z*-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (4)

Colorless oil, $[\alpha]_D$ +11.1° (c 0.48, CH_3OH); FAB-MS *m/z* (rel. int.) : 785 ($[M+H]^+$, 100); ^{1}H-NMR (500 MHz, CD_3OD) : δ 0.91 (6H, m, H-18, H-18"), 1.29-1.38 (34H, m, H-4'~H-7', H-12'~H-17', H-4"~H-6", H-14"~H-17"), 1.61 (4H, m, H-3', H-3"), 2.09 (8H, m, H-8', H-11', H-7", H-13"), 2.32 and 2.35 (each 2H, t, *J* = 7.0 Hz, H-2', H-2"), 2.82 (2H, t, *J* = 5.5 Hz, H-10"), 3.24 (9H, s, H-6,7,8), 3.65 (2H, m, H-5), 4.01 (2H, t, *J* = 6.0 Hz, H-3), 4.18 (1H, dd, *J* = 12.0, 7.0 Hz, H-1a), 4.28 (2H, br.s, H-4), 4.44 (1H, dd, *J* = 12.0, 3.0 Hz, H-1b), 5.25 (1H, m, H-2), 5.34-5.40 (6H, m, H-9', 10', 8", 9", 11", 12"); ^{13}C-NMR (125 MHz, CD_3OD) : δ 13.78 and 14.00 (C-18' and 18"), 25.74 (C-10"), 20.82, 22.95, 23.06, 25.33, 27.50, 29.48, 29.55, 29.67, 29.79, 29.97, 30.05, 30.11, 31.99 and 32.39 (C-3'~C-8', C-11'~C-17', C-3"~C-7", C-13"~C-17"), 34.21 and 34.39 (C-2' and 2"), 53.99 (C-6,7,8), 59.75 (C-4), 62.98 (C-5), 64.20 (C-3), 66.77 (C-1), 71.11 (C-2), 127.55, 128.20, 128.52, 130.14, 130.35 and 132.04 (C-9', 10', 8", 9", 11" and 12") , 173.84 and 174.14 (C-1' and 1")

1,2-Di-*O*-hexadecanoyl-*sn*-glycero-3-phosphorylcholine (5)

Colorless oil, $[\alpha]_D$ +64.5° (c 0.08, CH_3OH) ; FAB-MS *m/z* (rel. int.) : 757 ($[M+Na]^+$, 100); ^{1}H-NMR (500 MHz, CD_3OD) : δ 0.87 (6H, m, H-16', H-16"), 1.25-1.35 (48H, m, H-4'-H-15', H-4"-H-15"), 1.57 (4H, m, H-3', H-3"), 2.28 and 2.31

(each 2H, t, *J* = 7.0 Hz, H-2', H-2"), 3.19 (9H, s, H-6,7,8), 3.61 (2H, m, H-5), 3.97 (2H, t, *J* = 5.8 Hz, H-3), 4.14 (1H, dd, *J* = 12.0, 7.0 Hz, H-1), 4.24 (2H, br.s, H-4), 4.40 (1H, dd, *J* = 12.0, 3.0 Hz, H-1), 5.21 (1H, m, H-2); ^{13}C-NMR (125 MHz, CD_3OD) : δ 13.76 (C-16' and 16"), 23.05, 25.30 27.49, 29.50, 29.79, 29.93, 30.10, 32.38 (C-3'~C-15', C-3"~C-15"), 34.19 and 34.37 (C-2' and 2"), 53.98 (C-6,7,8) 59.80 (C-4), 62.92 (C-5), 64.20 (C-3), 66.76 (C-1), 71.14 (C-2), 173.87 and 174.24 (C-1' and 1").

1,2-D-*O*-9Z-octadecenoyl-*sn*-glycero-3-phosphorylcholine (6)

Colorless oil, $[\alpha]_D$ +11.5° (c 0.72, CH_3OH); FAB-MS *m/z* (rel. int.) : 809 ([M+Na]$^+$, 100); ^{1}H-NMR (500 MHz, CD_3OD) : δ 0.91 (6H, m, H-18', H-18"), 1.25-1.38 (40H, m, H-4'~H-7', H-2"~H-17', H-4"~H-7", H-12"~H-17"), 1.56 (4H, m, H-3', H-3") 2.04 (8H, m, H-8', 11', 8", 11"), 2.28 and 2.31 (each 2H, t, *J* = 7.0 Hz, H-2', H-2"), 3.19 (9H, s, H-6,7,8), 3.61 (2H, m, H-5), 3.96 (2H, t, *J* = 6.0 Hz, H-3), 4.15 (1H, dd, *J* = 12.0, 7.0 Hz, H-1), 4.24 (2H, br.s, H-4), 4.40 (1H, dd, *J* = 12.0, 3.0 Hz, H-1), 5.20 (1H, m, H-2), 5.29~5.34 (4H, m, H-9', 10', 9", 10"); ^{13}C-NMR (125 MHz, CD_3OD) : δ 13.83 and 14.00 (C-18' and 18"), 20.84, 22.98, 23.08, 25.35 27.54, 29.52, 29.55, 29.57, 29.61, 29.83, 30.01, 30.13 32.01 and 32.42 (C-3'~C-8', C-11'~C-17', C-3"-C-8", C-11'-C-17"), 34.25 and 34.40 (C-2' and 2"), 53.99 (C-6,7,8) 59.76 (C-4), 63.00 (C-5), 64.20 (C-3), 66.78 (C-1), 71.13 (C-2), 128.21, 128.54, 130.28 and 130.35 (C-9',10', 9" and 10") , 173.80 and 174.14 (C-1' and 1").

Alkaline hydrolysis of 4, 5 and 6

Compound **4** (1mg) was stirred with 2.3% NaOMe in MeOH at 74°C for 2 h. After addition of H_2O, the reaction mixture was extracted with hexane and dried by magnesium sulfate anhydrous. The resultant hexane soluble fraction was concentrated to yield fatty acid methyl ester **4a** and **4b**, which was analyzed by GC-MS. **5** and **6** were cleaved by same method to afford **5a** and **6a**, respectively. **6a** was determined as 9Z-octadecenoic acid methyl ester, that is, it was same with **4b**.

8Z,11Z-octadecadienoic acid methyl ester (4a)

Rt 18.012 min; EI-MS *m/z* (rel. int.) : 294 (20), 263 (18), 220 (41), 178 (5), 164 (15), 150 (18), 135 (16), 123 (20), 109 (40) 95 (65), 81 (95), 67(100), 55 (60).

9Z-octadecenoic acid methyl ester (4b)

Rt 18.087 min; EI-MS *m/z* (rel. int.): 296 (12), 264 (50), 222 (30), 180 (22), 152 (10), 137 (12), 123 (20), 110 (24), 97 (60), 83 (62), 69 (75), 55 (100).

Hexadecanoic acid methyl ester (5a)

Rt 15.706 min; EI-MS *m/z* (rel. int.) : 270 (18), 239 (10), 227 (20), 199 (10), 185 (12), 171 (10), 157 (6), 143 (22), 129 (10), 115 (5), 97 (8), 87 (70), 74 (100), 55 (22).

Measurement of neurite outgrowth

PC12 cells in 6 well plates were treated with compounds (10 μM) and NGF (50 ng/mL), and neurite outgrowth was measured under a microscope at 48 h post treatment[15]. Fresh medium with compounds or NGF was changed everyday. Randomly selected fields were photographed using a camera attached to light microscope (model CK-2; X100 magnification). Neurite extension was evaluated with lengths equivalent to one diameter of the cell body. All data are expressed as the mean±S.D. The evaluation of statistical significance was determined by one way ANOVA.

NGF gene expression assay

RNA extraction and cDNA synthesis

Total RNA was extracted from C6 cell line by using the method of Trizol Kit (Gibco). RNA was analyzed by gel electrophoresis and its concentration was measured by spectrophotometer reading at 260 nm and 280nm (1A260 absorbance unit equal to 40 mg/mL).

1 μg total RNA was analyzed by reverse transcription (RT) and followed polymerase chain reaction (PCR)(RT-PCR). RT was performed at a volume of 20 μL. RT solution contained : 5×PCR buffer, dNTP mix (dATP dCTP dGTP dTTP each 0.5 mM), 0.1MDTT, 40U/mL RNAase-inhibitor and 200U SuperscriptII reverse transcriptase (Gibco BRL, USA). Aliquotes of cDNA were amplified in 25 μL of PCR cocktail containing a 200 nM concentration of NGF primer and 0.125U Taq polymerase (TaKaRa, Shiga Japan) and 1.5 mM. $MgCl_2$, dNTP mix (dATP dCTP dGTP dTTP each 0.5 mM). The sets of primers were the following: NGF, 5′ TGGCCAGTGGTCGTGCAGTC-3′ 5′-AAGTCAGCCTCTTGCAGC-3′. PCR was carried out in a thermal cycler (Ommi; Gnen, Hybaid, Tedington, UK). Thirty cycles of PCR were performed (For NGF: initial template denaturation at 95°C for 30 sec; annealing with NGF primers at 60°C; primer extention at 72°C for 160 sec; for GAPDH : denaturation at 95°C for 30 sec:; annealing at 56°C for 30sec; primer extention at 72°C for 180 sec). The 30th cycle was followed by a final extention step at 72°C for 3 min.

PCR products were analyzed using 1.5% agarose gels and visualized using Ethdium bromide staining.

NGF Protein measurement

A two-enzyme immune assay for NGF was performed as recommended by the supplier of reagent (Boehringer-Mannheim). Monoclonal anti-NGF antibody (clone 27/21) specific for mouse, rat, beef and human NGF were used. The wells of microtiter plates (microplates, Falcon 96well)

were coated with buffer solution (100 μL/well) containing 50 mM $NaCO_3/NaHCO_3$ buffer, 0.05% sodium azide, pH 9.6 and 0.5 μg/mL anti-NGFβ monoclonal antibodies for 2 h at 37°C. Non specific binding sites were saturated with 150 μL/well blocking solution containing 1% FBS. The wells were washed 3 times with washing buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, 0.1% TritonX-10, 0.05% sidium azide, pH 7.0). The samples and the standard solution (0-1 ng/mL of NGF) were added and the microtiter plates were incubated overnight at 4°C. After washing 3 times, anti-NGF-β monoclonal antibodies conjugated to β-galactosidase (clone 27/21) diluted with buffer solution were added at a 50 μL/well. The microtiter plates were incubated for 5 h at 37°C and then washed 3-7 times. The well was filled with freshly substrate solution contained chlorophenol red β-galactopyranoside (2 mg/mL, 60 μL/well), diluted in substrate buffer (100 Mm Hepes, 150 Mm NaCl, 10 Mm $MgCl_2$, 1% Bovine serum albumin, 0.1% sodium azide pH7.0) and the color developed after incubation at 37°C for 4 h was measured photometically by enzyme-linked immunosorbent assay (ELISA) plate reader at 574 nm. Standard curves were prepared with 2.5S NGFβ standard purified from mouse submaxilmally glands by assaying parallel wells containing increasing amounts of NGFβ (0-1 ng/mL). The amount of NGFβ in the samples was calculated from the standard curve.

Statistical analysis

All data are expressed as the mean±S.D. The evaluation of statistical significance was determined by one way ANOVA.

RESULTS AND DISCUSSION

Three known amines, nicotiamide (**1**) (Pouchert and Behnke, 1993a), cytidine (**2**) (Pouchert and Behnke, 1993b) and adenine (**3**) (Sekiya and Suzuki, 1972), were characterized by comparing their physical and spectroscopic data with those of authentic samples.

Compound **4** was obtained as amorphous powder and its quasimolecular ion peak showed at m/z 785 ($[M+H]^+$)

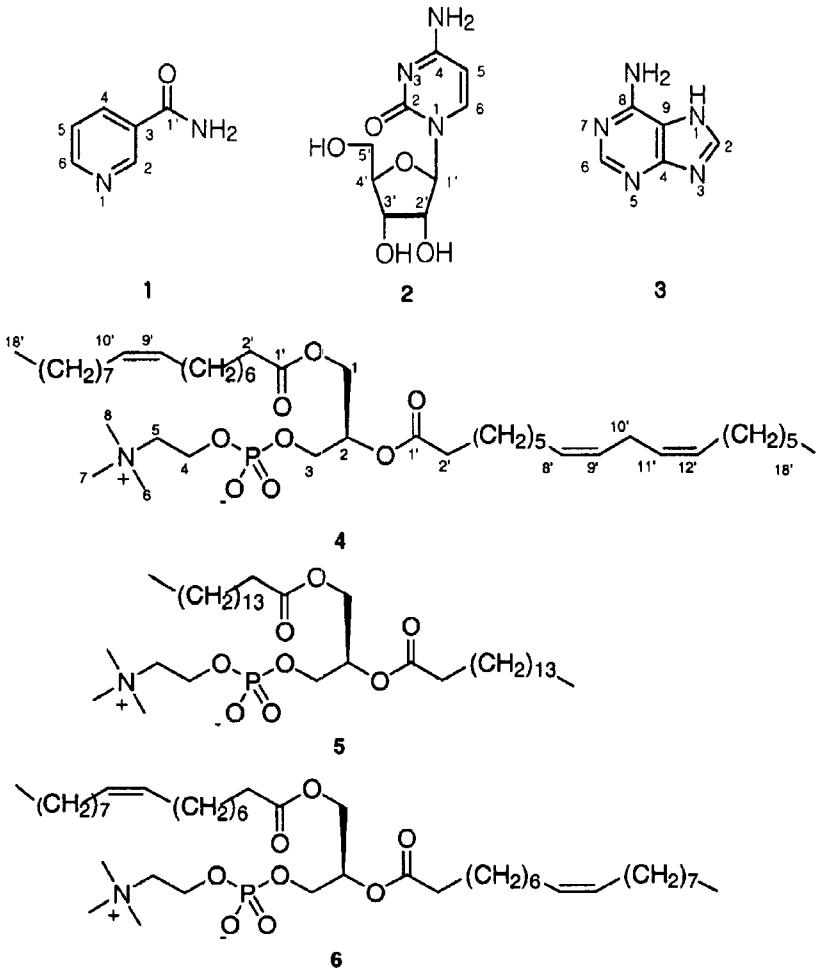

Fig. 1. The structures of compounds **1~6** isolated from *Bombycis corpus*

by FAB-MS. The characteristic signals of diacyl-glycerol were observed at δ 4.01 (2H, t, *J* = 6.0 Hz), 4.18 (1H, dd, *J* = 12.0, 7.0 Hz), 4.44 (1H, dd, *J* = 12.0, 3.0 Hz), 5.25 (1H, m) in the ^{1}H-NMR spectrum and at δ 64.20, 66.77 and 72.17 in the ^{13}C-NMR spectrum (Jung *et al.*, 1996). The signals by *N,N,N*-trimethylamino-ethanol were observed at δ 3.24 (9H, s), 3.65 (2H, m) and 4.28 (2H, br.s) in the ^{1}H-NMR and at δ 53.99, 59.75 and 62.98 in the ^{13}C-NMR spectrum. The ^{1}H-NMR spectrum showed the signals corresponding to long chain aliphatic hydrocarbons at δ 0.91 (6H, m), 1.29-1.38 (34H, m), 1.61 (4H, m), 2.09 (8H, m), 2.32 and 2.35 (each 2H, t, *J* = 7.0 Hz), 2.82 (2H, t, *J* = 5.5 Hz), 5.34-5.40 (6H, m). The ^{13}C-NMR spectrum showed the signals by two terminal methyl groups in aliphatic hydrocarbon chains at δ 13.78 and 14.00, six olefinic carbons at δ 127.55, 128.20, 128.52, 130.14, 130.35 and 132.04 and two ester carbon at δ 173.84 and 174.14. The geometry of the double bonds in the fatty acid moiety was presumed to be *cis* based on the chemical shift (29.48, 29.55 29.67 and 29.79) of the adjacent carbons in the ^{13}C-NMR data. The signals of carbons next to a double bond appear at 27-28 in a *cis*-configuration, while in a *trans*-configuration at 32-33 (Kwon *et al.*, 1998). The hexane layer of alkaline hydrolysis of **4** yielded 9Z-octadecenoic acid methyl ester and 8Z,11Z-octadecenoic acid methyl ester (Jung *et al.*, 1996). The major spot in the methanol layer of alkaline hydrolysis responded positively on TLC to the malachite green reagent and the periodate-Schiffs reagent, indicated phosphorus-containing substances and 1,2-diol groups, respectively (Batrakov *et al.*, 2001). Above mentioned data indicated **4** was 1,2-diacyl-glycero-3-phosphorylcholine derivatives. The stereochemistry of glycerol moiety is determined to *sn*-glycerol based on $J_{1a,2}$ (7.0 Hz) and $J_{1b,2}$ (3.0 Hz) values in the ^{1}H-NMR spectrum (Arora *et al.*, 1997). On basis of the comparison the FAB-MS data of **4** with those of the previous literatures (Gamo *et al.*, 1999; Limb *et al.*, 1999; Fallani *et al.*, 1976), we suggested 9Z-octadecenoyl group and 8Z, 11Z-octadecadienoyl group were bonded at C-1 and C-2, respectively. Accordingly, the structure of **4** was determined to 1-*O*-(9Z-octadecenoyl)-2-*O*-(8Z,11Z-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine.

Compound **5** was obtained as amorphous powder and its quasimolecular ion peak showed at m/z 757 ([M+Na]$^+$) by FAB-MS. ^{1}H- and ^{13}C-NMR spectra of **5** were in good agreement with those of **4** except for signals by long hydrocarbons chain. Alkaline hydrolysis of **5** yielded hexadecanoic acid methyl ester and positive to the malachite green reagent and the periodate-Schiffs reagent. Based on the spectral data, the structure of **5** was assigned as 1,2-di-O-hexadecanoyl-*sn*-glycero-3-phosphorylcholine (Ishihara and Sano, 1996; Solodin *et al.*, 1996).

Compound **6** was obtained as amorphous powder and its quasimolecular ion peak showed at *m/z* 809 ([M+Na]$^+$) by FAB-MS. ^{1}H- and ^{13}C-NMR spectra of **6** were in good agreement with those of **4** except for signals by long hydrocarbons chain. Treatment of **6** with NaOMe-MeOH furnished 9Z-octadecenoic acid methyl ester and glycerophosphorylcholine. On this basis, the structure of **6** was assigned as 1,2-di-*O*-9Z-octadecenoyloxy-*sn*-glycero-3-phosphorylcholine (Ishihara and Sano, 1996; Solodin *et al.*, 1996).

The neurite outgrowth activities of compounds **1-6** from *Bombycis corpus* were examined in PC 12 cells by measuring the length of neurites (Fig. 2). Phospholipids (**4**, **5** and **6**) promoted neurite outgrowth in PC12 cells with lengths equivalent to two diameters of the cell body at 10 μM. Interestingly, compound **5** was more effective than other compounds in promoting neurite outgrowth. We measeured NGF protein by using highly sensitive ELISA system. Rat astrocytes were incubated compounds **4**, **5**, **6** and NGF for 6 h, and NGF content was measured directly in culture medium. Fig. 3 showed NGF induction in C6 glial cell line. As like C6 glial cell line, the NGF content in the primary cultured rat astrocytes medium were increased. Compound **5** was the most potent in amount of secreted NGF protein. To rule out that increase in NGF contents might have been due to leakage from the cells, we investigated expression of NGF mRNA. Because of the low level of NGF mRNA transcripts were analyzed by RT-PCR (Fig. 4). Expressions of NGF mRNA in cultured C6 glial cells were increased in all treated group at 6 h after the addition of compounds. Compared with C6 glial cells, compound **5** reached its maximal intensity in expression of NGF mRNA in primary cultures rat astrocytes

To our knowledge, phospholipids isolated from *Bombycis*

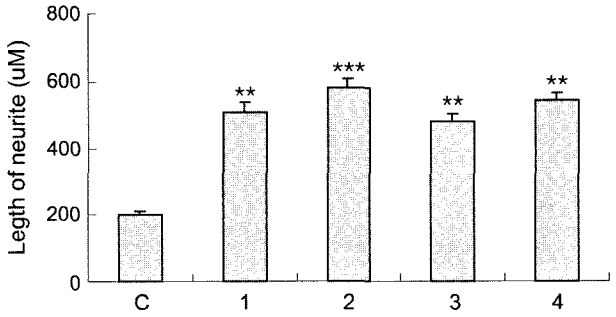

Fig 2. The effects of phsopholipid compounds (10 μM) and NGF (50 ng/mL) on neurite outgrowth. PC12 cells in 6-well plates were treated with compounds and NGF. And neurite outgrowth was measured under a microscope at 6 days. Randomly selected field was photographed with camera attached with light microscope. Each value represents the meanS.E.M. (n=30) C represent s control; lane 1 represents compound **4**; lane 2 represents compound **5**; lane 3 represents compound **6**; lane 4 represents NGF **significantly different from Control value at the level of $p<0.01$.,*** significantly different from control value at the level of $p<0.001$.

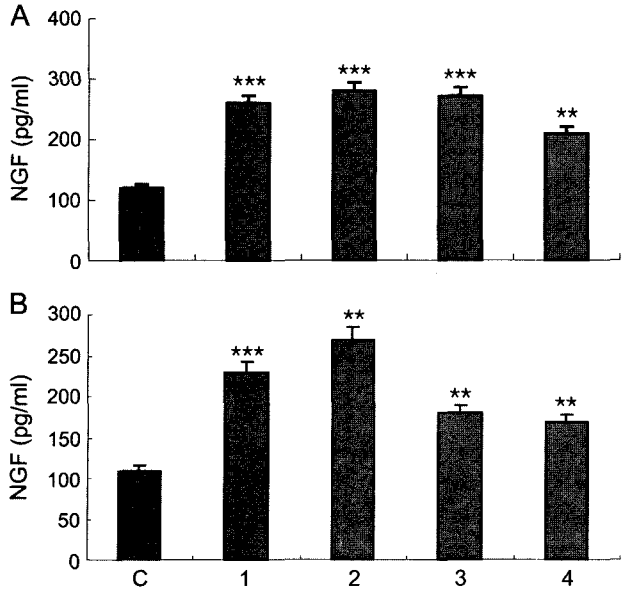

Fig. 3. The Effects of compounds on NGF protein expression in C6 glioma cell line (A) and Primary rat astrocytes (B). After incubation with each compounds for 6 h, NGF released from each cells were measured using NGF protein ELISA kit. C represents control; lane 1 represents compound **4**; lane 2 represents compound **5**; lane 3 represents compound **6**; lane 4 represents NGF. Values are the mean±S.D. of three plates. **significantly different from Control value at the level of $p<0.01$.,*** significantly different from control value at the level of $p<0.001$.

corpus increased NGF synthesis in astrocytes. The results suggest that phospholipids from *Bombycis corpus* may be useful to regulation of neurofrophic factors in neuronal degeneration.

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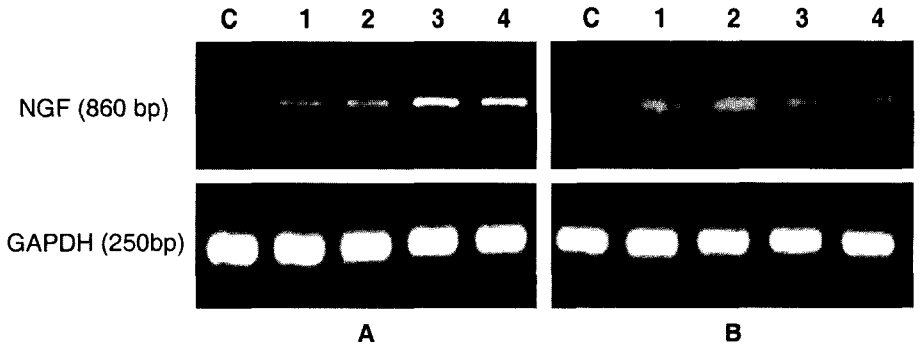

Fig. 4. Expression of NGF mRNA in C6 cell (A) cultured rat cortical astrocytes (B) after exposure to phospholipids (10 μM) for 6 h in serum-starvation medium. The RT-PCR products were electrophoresed in 1.5% agarose gel. The photograph represents NGF mRNA expression in the treated groups. C represents control; lane 1 represents compound **4**; lane 2 represents compound **5**; lane 3 represents compound **6**; lane 4 represents NGF. The results were reproduced with three different cultures.

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