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Phosphlipids from *Bombycis corpus* and Their Neurotrophic Effects

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Objectives

This study was carried out to investigate active constituents of *Bombysis corpus* on the neurite outgrowth from PC12 cells led to isolate 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 M). The NGF content in the culture medium was significantly increased by compound **5**, compared with the control value. These results suggest that suggest that three phospholipid compounds isolated from the methanol extract of *Bombycis corpus* may exert neurotrophic effects by stimulation of NGF synthesis in astrocytes.

Materials and Methods

1. General procedures

Mps: uncorr. NMR: in CDCl3, 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 30m 0.25mm)/Hewlett-Packard 5973 MSD system. Column Chromatography : Silica gel (Merck, 70230, 230400 mesh) and Sephadex LH-20 (Pharmacia). TLC: Merck precoated Si gel F254 plates and RP-18 F254s plates. LPLC: Merck Lichroprep Lobar -A Si 60 & Lobar -A RP-18 (240×10 mm).

2. 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.

3. 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 ℃. 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 (B1B6). 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 (B11B14). 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 (4.3g) 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 (3.3g) 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); 1H-NMR (500 MHz, CD3OD) : 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); 13C-NMR (125 MHz, CD3OD) : 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, 1H-NMR (500 MHz, CD3OD) : 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); 13C-NMR (125 MHz, CD3OD) : 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, 1H-NMR (500 MHz, DMSO-d6) : 8.47 (1H, s, H-2), 8.86 (1H, br.s, H-6)

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

Colorless oil, [α]D +11.1。 (c 0.48, CH3OH) ; FAB-MS m/z (rel. int.) : 785 ([M+H]+, 100); 1H-NMR (500MHz, CD3OD) : 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''); 13C-NMR (125MHz, CD3OD) : 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, [α]D +64.5。 (c 0.08, CH3OH) ; FAB-MS m/z (rel. int.) : 757 ([M+Na]+, 100) ; 1H-NMR (500MHz, CD3OD) : 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); 13C-NMR (125MHz, CD3OD) : 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-Di-*O*-9*Z*-octadecenoyl-*sn*-glycero-3-phosphorylcholine (6)

Colorless oil, [α]D +11.5。 (c 0.72, CH3OH) ; FAB-MS m/z (rel. int.) : 809 ([M+Na]+, 100); 1H-NMR (500MHz, CD3OD) : δ 0.91 (6H, m, H-18', H-18"), 1.25-1.38 (40H, m, H-4'H-7', H-12'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), 5.295.34 (4H, m, H-9',10',9",10"); 13C-NMR (125MHz, CD3OD) : 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 ℃ for 2hrs. After addition of H2O, 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 9*Z*-Octadecenoic acid methyl ester, that is, it was same with **4b**.

. 8*Z*,11*Z*-Octadecadienoic acid methyl ester(4a)

Rt 18.012 min; EI-MS m/z (rel. int.) : 294 (20), 263 (18), 220 (4), 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)

4. Measurement of neurite outgrowth

PC12 cells in 6 well plates were treated with compounds (10μM) and NGF (50ng/ml), and neurite outgrowth was measured under a microscope at 48 hours post treatment15). 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.

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]+) by FAB-MS. The characteristic signals of diacyl-glycerol were observed at δ4.01 (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 1H-NMR spectrum and at δ 64.20, 66.77 and 71.11 in the 13C-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 1H-NMR and at δ 53.99, 59.75 and 62.98 in the 13C-NMR spectrum. The 1H-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 13C-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 13C-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 9*Z*-octadecenoic acid methyl ester and 8*Z*,11*Z*-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 1H-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*-(9*Z*-octadecenoyl)-2-*O*-(8*Z*,11*Z*-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. 1H- and 13C-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. 1H- and 13C-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 9*Z*-octadecenoic acid methyl ester and glycerophosphorylcholine. On this basis, the structure of **6** was assigned as 1,2-di-O-9*Z*-octadecenoyloxy-*sn*-glycero-3- phosphorylcholine (Ishihara and Sano, 1996; Solodin *et al.* 1996).

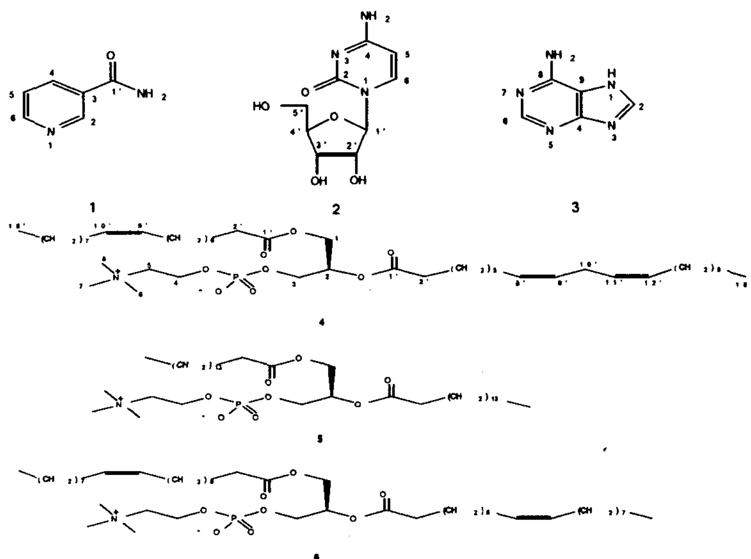

Fig. 1. The structures of compounds **1**- **6** isolated from *Bombycis corpus*

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 10M. 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 6hrs, and NGF content was measured directly in culture medium.

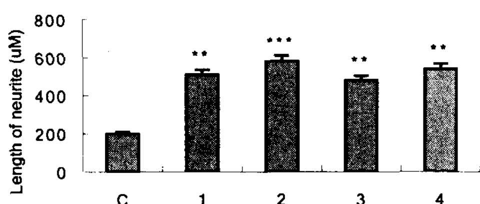

Fig. 2. The effects of phospholipid compounds (10M) and NGF (50ng/ml) on neurite outgrowth. PC12 cells in 6-well plates were treated with compounds and NGF. And neurite outgrowth was measured under a microscope at 6 days. Randomly selected field was photographed with camera attached with light microscope. Each value represents the mean∂S.E.M. (n=30) C represents 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 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.

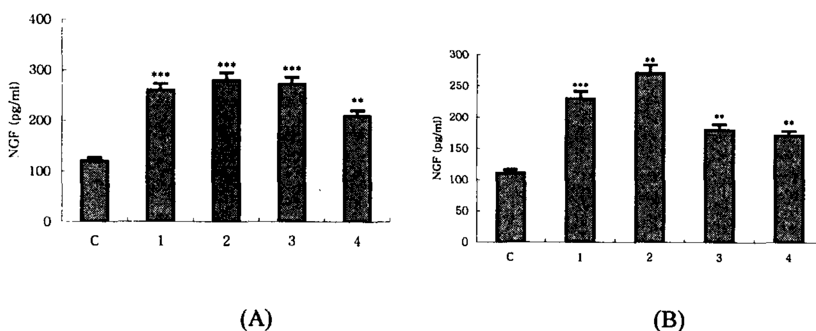

Fig. 3. The Effects of compounds on NGF protein expression in C6 glioma cell line (A) and Primary rat astrocytes (B). After incubation with each compounds for 6h, NGF released from each cells were measured using NGF protein ELISA kit. C represents control; lane 1 represents compound 4 ; lane 2 represents 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.

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 6hr 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 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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