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Lignans from the Fruits of *Schizandra chinensis* and Their Inhibitory Effects on Dopamine Content in PC12 Cells

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Abstract – Five lignans including gomisin N (**1**), wuweizisu C (**2**), gomisin L1 (**3**), (+)-deoxyschizandrin (**4**), and gomisin J (**5**) have been isolated from the fruits of *Schizandra chinensis*. The structures of the isolated compounds were elucidated by analyzing MS and NMR spectra. Effects of the compounds isolated in this study on the dopamine content in PC12 cells were investigated to evaluate their inhibitory effectiveness. The gomisin N, wuweizisu C, and gomisin J showed 25.4%, 39.8%, and 35.1%, respectively, inhibition effect on dopamine content in PC12 cells at the concentration of 50 μg/ml.
Keywords – *Schizandra chinensis*, Schizandraceae, lignans, dopamine content

Introduction

The fruits of *Schizandra chinensis* (Schizandracea) have been used for treating anti-tussive, astringent and tonics in oriental medicine (Lea *et al.*, 1997). They also have anti-oxidative and anti-inflammatory efficacy (Liu *et al.*, 1982). Biologically effective components of the drugs were believed to be the presence of lignans. Several dibenzocyclooctadiene lignans from the fruits of *S. chinensis* have recently been found to have some beneficial effects due to antioxidant activity (Yao *et al.*, 1997), and expected to be used for the regulation of neural system (Lee *et al.*, 1986; Baek *et al.*, 2000).

Lignans isolated from the fruits of *S. chinensis* can be classified into the following five groups : 1) *R*-biphenyl configuration (+)-deoxyschizandrin group, 2) *S*-biphenyl configuraton-gomisin N group (gomisin J, gomisin k, dimethyl gomisin J), 3) *R*-biphenyl configuraton and a hydroxy group at C-7-gomisin A, schizandrin, gomisin H, 4) *S*-biphenyl configuation and a hydroxyl group at C-6-gomisin O, epigomisin O, and 5) *S*-biphenyl configuration and two hydroxy group at C-6 and C-7-gomisin B, deangeloylogomisins B and F, gomisin P (Yukinobu *et al.*, 1980).

PC12 cells have been characterized from a chromaffin tumor of rat adrenal pheochromocytoma and have various functions like the synthesis, storage and secretion of catecholamines (Greene and Tischler, 1982). They also express the catecholamine biosynthetic enzymes, a dopamine β-hydroxylase (Tischler *et al.*, 1983).

The purpose of this study was to investigate an inhibitory effectiveness of the compounds isolated from the fruits of *S. chinensis* on the dopamine content in PC12 cells to ascertain their physiological functions.

Experimental

General – Melting points were determined on an Electrothermal 9200 apparatus and reported uncorrected. Optical rotations were obtained using a JASCO P-1020 polarimter. ^{1}H and ^{13}C-NMR, ^{1}H-^{1}H correlation spectroscopy (COSY), NOE spectroscopy (NOESY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC) NMR spectra were recorded on a varian UI 500 spectrometer at the operating frequency of 500 MHz (^{1}H) and 125 MHz (^{13}C) at the Korea Basic Science Institute in Seoul, korea. EI-MS data were also obtained using a JEOL JMS-600W. Open column chromatography was performed with silica gel (40-100

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μm mesh) and the Sephadex LH-20 (Amersham Biosciences Co. Ltd.). TLC was carried out on precoated silica gel 60 F_{254} plates (merck) and spots were detected using 50% H_2SO_4 reagent. Solvents were used without further purification.

Plant material – The fruit of *S. chinensis* was purchased from Muju Province in October 2001. It was air-dried and ground before its extraction. The voucher specimens are deposited at the Korea Forest Research Institute, Seoul, Korea.

Extraction and isolation – The dried and ground *S. chinensis* fruit (15 kg) was extracted with EtOH for 72h with three times at room temperature. The crude extracts were successively partitioned with organic solvents, such as petroleum ether, diethyl ether (Et_2O), and ethyl acetate (EtOAc). The ether fraction (195 g) afforded 6 fractions (SCE 1~6) on silica gel column chromatography with a benzene-EtOAc (20:1) solvent system. The SCE-3 (356 mg) was chromatographed on silica gel with *n*-hexane-Et_2O (4:1) to give compound 1 (270 mg). The SEC-2 (654 mg) was chromatographed on silica gel with benzene-Et_2O (40:1) to obtain four fractions (SCE2-1~4). The SCE2-3 (220 mg) was purified by preparative TLC with *n*-hexane-Et_2O (3:1) and benzene-EtOAc (30:1) to give compound 2 (20 mg). The SCE-5 (20.8 g) was chromatographed on silica gel with benzene-EtOAc (10:1) to give four fractions (SCE5-1~4). The SCE5-1 (296 mg) was chromatographed on centrifugal partition chromatography (CPC) with *n*-hexane-CH_3CN-EtOAc-H_2O (8:7:5:1) to give three fractions. The SCE5-1-1 (51 mg) was purified to produce three fractions with *n*-hexane-MeOH-EtOH-H_2O (10:5:5:1) to give compound 3 (3 mg). The fraction 5-3 (2.4 g) was chromatographed Sephadex LH-20 with MeOH-EtOH (3:7) to obtain three fractions (SCE-5-3-1~3). The fraction SCE-5-3-1 (1.3 g) was chromatographed using Sephadex LH-20 with MeOH-H_2O (7:3) and MeOH-H_2O (3:7) to get compound 4 (3 mg). The SCE-5-3-2 (650 mg) was chromatographed using Sephadex LH-20 with MeOH-H_2O (1:1) to give three fractions (SCE-5-3-2-13). The SCE-5-3-2-2 was purified using the Sephadex LH 20 with MeOH-H_2O (1:1) to give compound 5 (103 mg).

Compound 1 – Pale yellow oil. $[\alpha]^{22}_D$-80.1 (*c*=0.64, CH_2Cl_2). EI-MS *m/z* : 400 (M^+, base ion), 344 (M^+-56), 312, 235 (M^+-165), 219 (M^+-181), 181, 165, 56. ^{1}H-NMR (500 MHz, CD_3OD) : δ 0.73 (3H, d, *J* = 7.5 Hz, CH_3-18), 0.97 (3H, d, *J* = 7.5 Hz, CH_3-17), 1.76 (1H, m, H-8), 1.89 (1H, m, H-7), 2.05 (1H, m, H-9a), 2.22 (1H, dd, *J* = 9.0, 13.5 Hz, H-9b), 2.44 (1H, dd, *J* = 2.5, 13.5 Hz, H-6a), 2.60 (1H, dd, *J* = 8.5, 13.5 Hz, H-6b), 3.48 (3H, s, OMe-3), 3.76 (3H, s, OMe-14), 3.80 (3H, s, OMe-2), 3.86 (3H, s, OMe-1), 5.96 (2H, s, OCH_2O), 6.53 (1H, s, H-11), 6.68 (1H, s, H-4). ^{13}C-NMR (125 MHz, CD_3OD) : δ 12.37 (q, CH_3-18), 21.10 (q, CH_3-17), 33.83 (d, C-7), 35.36 (t, C-9), 38.94 (t, C-6), 41.11 (d, C-8), 55.59 (q, OMe-1), 58.88 (q, OMe-14), 59.96 (q, OMe-3), 60.23 (q, OMe-2), 101.03 (t, OCH_2O), 102.84 (d, C-11), 111.13 (d, C-4), 123.59 (s, C-16), 31.88 (s, C-15), 34.04 (s, C-5), 134.89 (s, C-13), 137.91 (s, C-10), 140.59 (s, C-2), 141.47 (s, C-14), 149.02 (s, C-12), 152.02 (s, C-3), 152.21 (s, C-1).

Compound 2 – Colorless solid, mp 122-123°C. $[\alpha]^{22}_D$ -115.6 (*c*=0.53 CH_2Cl_2). EI-MS *m/z* : 384 (M^+, base ion), 328 (M-56), 219 (M-165), 165, 56. ^{1}H-NMR (500 MHz, CD_3OD) : δ 0.73 (3H, d, *J* = 7.0, Hz, CH_3-18), 0.97 (3H, d, *J* = 7.0 Hz, CH_3-17), 1.75 (1H, m, H-8), 1.87 (1H, m, H-7), 2.01 (1H, d, *J* = 13.5 Hz, H-9a), 2.21 (1H, d, *J* = 9.5, 13.5 Hz, H-9b), 2.44 (1H, dd, *J* = 2.0, 13.5 Hz, H-6a), 2.60 (1H, dd, *J* = 7.5, 13.5 Hz, H-6b), 3.76 (3H, s, OMe-14), 3.78 (3H, s, OMe-1), 5.96 (4H, s, OCH_2O), 6.53 (1H, s, H-4), 6.52 (1H, s, H-11) ^{13}C-NMR (125 MHz, CD_3OD) : δ 12.14 (q, CH_3-18), 21.19 (q, CH_3-17), 33.86 (d, C-7), 35.17 (t, C-9), 38.70 (t, C-6), 41.07 (d, C-8), 55.93 (q, OMe-1), 58.94 (q, OMe-14), 101.01 (t, OCH_2O), 102.85 (d, C-11), 105.88 (d, C-4), 121.52 (s, C-15), 122.66 (s, C-16), 132.74 (s, C-5), 134.69 (s, C-13), 135.03 (s, C-2), 138.22 (s, C-10), 141.44 (s, C-14), 141.69 (s, C-1), 147.99 (s, C-3), 149.05 (s, C-12).

Compound 3 – Colorless solid. mp 194-196°C. $[\alpha]^{22}_D$ -80.8 (*c*=0.48, CH_2Cl_2). EI-MS *m/z* : 386 (M^+, base ion). ^{1}H-NMR (500 MHz, $CDCl_3$) : δ 0.73 (3H, d, *J* = 7.0 Hz, CH_3-18), 1.01 (3H, d, *J* = 7.0, Hz, CH_3-17), 1.80 (1H, m, H-8), 1.90 (1H, m, H-7), 2.05 (1H, d, *J* = 13.5 Hz, H-9a), 2.32 (1H, dd, *J* = 10.0, 13.5 Hz, H-9b), 2.45 (1H, dd, *J* = 2.0, 13.5 Hz, H-6a), 2.57 (1H, dd, *J* = 7.5, 13.5 Hz, H-6b), 3.89 (3H, s, OMe-1), 3.91 (3H, s, OMe-12), 3.92 (3H, s, OMe-13), 5.96 (2H, s, *J* = 12.0 Hz, OCH_2O) , 6.38 (1H, s, H-11), 6.53 (1H, s, H-4). ^{13}C-NMR (125 MHz, $CDCl_3$) : δ 13.16 (q, CH_3-18), 22.19 (q, CH_3-17), 34.08 (d, C-7), 35.63 (t, C-9), 39.17 (t, C-6), 41.03 (d, C-8), 55.92 (q, OMe-12), 60.00 (q, OMe-1), 61.24 (q, OMe-13), 101.04 (t, OCH_2O), 104.13 (d, C-11), 106.72 (d, C-4), 115.95 (s, C-15), 121.57 (s, C-16), 133.34 (s, C-13), 134.76 (s, C-5), 135.20 (s, C-2), 140.15 (s, C-10), 141.41 (s, C-1), 146.95 (s, C-14), 148.07 (s, C-3), 151.91 (s, C-12).

Compound 4 – Colorless solid. mp 114-116°C. $[\alpha]^{23}_D$ -48.3 (*c*=0.67, CH_2Cl_2) EI-MS *m/z* : 416 (M^+, base ion). ^{1}H-NMR (500 MHz, $CDCl_3$) : δ 0.73 (3H, d, *J* = 7.0 Hz, CH_3-18), 1.01 (3H, d, *J* = 7.0 Hz, CH_3-17), 1.80 (1H, m, H-8), 1.90 (1H, m, H-7), 2.05 (1H, d, *J* = 13.5 Hz, H-9a) 2.32 (1H, dd, *J* = 10.0, 13.5 Hz, H-9b), 2.45 (1H, dd, *J* = 2.0, 13.5 Hz, H-6a), 2.57 (1H, dd, *J* = 7.5, 13.5 Hz, H-6b)

3.89 (3H, s, OMe-1), 3.91 (3H, s, OMe-12), 3.92 (3H, s, OMe-13), 5.96 (3H, s, J = 12.0 Hz, OCH_2O), 6.38 (1H, s, H-11), 6.53 (1H, s, H-4). ^{13}C-NMR (125 MHz, $CDCl_3$) : δ 12.88 (q, CH_3-17), 21.99 (q, CH_3-18), 33.98 (d, C-8), 35.82 (t, C-6), 39.37 (t, C-9), 41.00 (d, C-7), 56.12 (q, OMe-3), 56.12 (q, OMe-12), 60.74 (q, OMe-14), 60.74 (q, OMe-1), 61.17 (q, OMe-2), 61.17 (q, OMe-13), 107.43 (d, C-4), 110.74 (d, C-11), 122.57 (s, C-16), 123.62 (s, C-15), 134.17 (s, C-10), 139.40 (s C-5), 139.97 (s, C-13), 140.32 (s, C-2), 151.62 (s, C-14), 151.75 (s, C-1), 153.09 (s, C-3), 153.09 (s, C-12).

Compound 5 – Pale yellow oil. $[\alpha]^{25}_D$-20.3 (c=1, CH_2Cl_2). EI-MS m/z : 388(M^+, base ion). ^{1}H-NMR (500 MHz, $CDCl_3$) : δ 0.74 (3H, d, J = 7.5 Hz, CH_3-18), 0.98 (3H, d, J = 7.5 Hz, CH_3-17), 1.80 (1H, m, H-8), 1.90 (1H, m, H-7), 2.03 (1H, d, J = 13.5 Hz, H-9a), 2.03 (1H, d, J = 13.5 Hz, H-9a), 2.26 (1H, dd, J = 9.5, 13.5 Hz, H-9b), 2.47 (1H, dd, J = 2.0, 13.5 Hz, H-6a), 2.55 (1H, dd, J = 7.5, 13.5 Hz, H-6b), 3.53 (3H, s, OMe-14), 3.54 (3H, s, OMe-1), 3.94 (3H, s, OMe-13), 3.95 (3H, s, OMe-2), 6.64 (2H, s, H-4, 11). ^{13}C-NMR (125 MHz, $CDCl_3$) : 12.77 (q, CH_3-18), 21.93 (q, CH_3-17), 31.13 (d, C-7), 33.98 (t, C-9), 39.08 (t, C-6), 31.13 (d, C-7), 41.18 (d, C-8), 60.28 (s, OMe-1, 14), 61.23 (d, OMe-2, 13), 110.34 (d, C-11), 113.41 (d, C-4), 121.66 (s, C-15), 122.69 (s, C-16), 135.11 (t, C-5), 137.64 (s, C-13), 137.93 (s, C-2), 140.43 (s, C-10), 147.77 (s, C-3), 148.96 (s, C-12), 150.48 (s, C-14), 150.60 (s, C-1).

PC12 cell incubation – PC cells were routinely maintained in RPMI 1640 medium supplemented with 10% HS (heat-inactivated horse serum) and 5% FCS (fetal calf serum) plus penicillin/streptomycin at 37°C (Greene and Tischler, 1982). PC12 cells (cell density 2-5×10^4 cells/cm^2) were incubated for 36(-48) hours. These cells (1×10^5 cells/cm^2) were used for sample treatments, and then incubated for 24 (or 48) hours. The cells were centrifuged. The cell pellets were used for the measurement of dopamine content.

Measurement of dopamine content – The dopamine content was determined using the method described by Mitsui *et al.* (1984) and a modified method (Lee *et al.*, 1986). To the pellet extract, 1*M* perchloric acid 300 μl and 0.2 nmol/ml isoproterenol (internal standard) 100 μl were added and then centrifuged. After the pre-treatment with Toyopak IC-SP M cartridge (Na^+ form, Tosoh, Tokyo, Japan), the eluate was derivatized with 1,2-diphenylethylenediamine. The final reaction mixture (100 μl) was injected into a high performance liquid chromatography (HPLC) system (Tosoh). The conditions of HPLC analysis were the same as described by Lee *et al.* (1986).

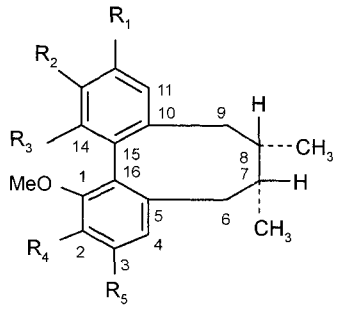
Results and Discussion

The isolated compound 1 was pale yellow oil. In the ^{13}C- and ^{1}H-NMR spectra, the signals at δ 152.21, 140.59, 152.01, and 111.13 (C-1, 2, 3, 4), together with the HMBC spectrum, which showed coupling between an olefinic proton (H-4, δ 6.68) and three olefine carbons at C-5, C-3, and C-2 (δ 134.04, 152.01, 140.59, respectively). The other aromatic signals at δ 137.91, 102.84, 149.02, 134.89, and 141.47 were assigned to C-10, C-11, C-12, C-13, and C-14, respectively, on the basis of long-range correlations. In the HMBC spectrum of 1, the cross peaks between C-12, C-13 and a dioxymethylene (δ 5.96) signal indicated the presence of an oxygenated benzene moiety. The proton signals at δ 6.68 assigned to H-4 was correlated with C-2, C-16, and C-6. and the δ 6.53 assigned to H-11 was correlated with C-13, C-15, and C-9. The four methoxyl groups (δ 3.86, 3.80, 3.48, 3.76) at C-1, C-2, C-3, and C-14 could be predicted from the HMBC spectrum. The equatorial methyl (CH_3-17) and axial methyl (CH_3-18) proton signal at δ 0.97 (3H, d, J = 7.5 Hz, CH_3-17) and δ 0.73 (3H, d, J = 7.5 Hz, CH_3-18) were conjugated C-7 and C-8. These results indicated that the compound 1 was identified to be gomisin N (Yukinobu *et al.*, 1979).

The isolated compound 2 was a colorless solid. The two methoxyl signals at δ 55.93 and δ 58.94 suggested that two methoxyls are located at the C-1 and C-14 positions. Two dioxymethylene moietis must be at the C-(2 and 3) and C-(12 and 13) positions. The doublet aromatic carbon signals at δ 105.88 and δ 102.85 can be assigned to C-4 and C-11, respectively. The ^{1}H-NMR spectrum of the compound 2 shows the presence of two dioxymethylene moieties, two methoxyls on the aromatic rings, two secondary methyls, and two benzylic methylene in the cyclooctadiene ring. On the basis of the above physical constants and spectral data (NMR), the compound 2 was identified to wuweizisu C (Yukinobu *et al.*, 1982).

The isolated compound 3 was colorless solid. The three methoxyl signals at δ 60.00, δ 55.92, and δ 61.24 suggested that these are located at the C-1, C-12, and C-13 positions and one dioxymethylene moietis must be at the C-2 and 3 positions. Three methoxyl signals at δ 3.89, δ 3.91, and δ 3.92 are located at the C-1, C-12, and C-13. The ^{13}C-NMR spectrum of the compound 3 showed the presence of one dioxymethylene moietis and three methoxyls on the aromatic rings. Therefore, the compound 3 was identified to gomisin L1 (Yukinobu *et al.*, 1982).

The isolated compound 4 was as colorless solid. In the ^{13}C NMR spectra, the signals at δ 35.82, δ 41.00, δ 33.98, and 39.37 were assigned to C-6, C-7, C-8, and C-9,



gomisin N (1) : R_1=R_2=-O-CH_2-O-, R_3=R_4=R_5=OMe
wuweizisu C (2) : R_1=R_2=R_4=R_5=-O-CH_2-O-, R_3=OMe
gomisin L1 (3) : R_1=R_2=OMe, R_3=OH, R_4=R_5=-O-CH_2-O-
(+)-deoxyschizandrin (4) : R_1=R_2=R_3=R_4=R_5=OMe
gomisin J (5) : R_1=R_5=OH, R_2=R_3=R_4=OMe

Fig. 1. Compounds isolated from the fruits of *S. chinensis*.

respectively. The axial methyl (CH_3-17) and equatorial methyl (CH_3-18) proton signal at 1.01 (3H, d, J = 7.0 Hz, CH_3-17), and 0.73 (3H, d, J = 7.0 Hz, CH_3-18) was conjugated C-7 and C-8. The six methoxyl signals at δ 60.74, 61.17, 56.12, 56.12, 61.17, and δ 60.74 suggested that six methoxyls are located at the C-1, C-2, C-3, C-12, C-13, and C-14 position, respectively. Therefore, the isolated compound 4 was identified to be (+)-deoxyschizandrin (Yukinobu *et al.*, 1979).

The isolated compound 5 was pale yellow oil. The four methoxy signals at δ 60.28, δ 61.23, δ 60.28, and δ 61.23 suggested that four mehtoxyls were located at the C-1, 2, 14, and 13. The equatorial methyl (CH_3-17) and axial methyl (CH_3-18) proton signal at 0.98 (3H, d, J = 7.5 Hz, CH_3-17), and 0.74 (3H, d, J = 7.5 Hz, CH_3-18) was conjugated C-7 and C-8. Thus, the compound 5 was identified to be gomisin J (Yukinobu *et al.*, 1980).

Among these five compounds isolated from the fruits of *S. chinensis*, gomisin N, wuweizisu C, and gomisin J were used to evaluate their inhibitory effectiveness on the dopamine content in PC12 cells. The inhibitory effectiveness of gomisin N, wuweizisu C, and gomisin J on the dopamine content in PC12 cells were investigated, and the results were shown in Table 1.

Three isolated compounds, i.e., gomisin N (1), wuweizisu C (2), and gomisin J (5) showed, 25.4%, 39.8%, and 35.1% of inhibition effect on the dopamine content at a concentration of 50 μg/ml. The treatment of the wuweizisu C significantly decreased the dopamine content dose in PC12 cell as shown Table 1. The decreased dopamine level was maintained for almost 24 hours. The cell viability was examined by lactate dehydrogenase activity. The wuweizisu C at the concentrations up to 50 μM was not toxic toward PC12 cell. The

Table 1. Inhibitory effectiveness of gomisin N, wuweizisu C, and gomisin J on the dopamine content in PC12 cells

Compounds	Dopamine content (nmol/mg protein)
Control	3.54 ± 0.35 (100)
gomisin N (1)	
10 μg/ml	3.94 ± 0.23 (111.3)
25 μg/ml	3.63 ± 0.21 (102.5)
50 μg/ml	2.64 ± 0.25 (74.6)
wuweizisu C (2)	
10 μg/ml	3.39 ± 0.28 (95.8)
25 μg/ml	3.16 ± 0.24 (89.3)
50 μg/ml	2.13 ± 0.16 (60.2)*
gomisin J (5)	
10 μg/ml	3.75 ± 0.23 (109.3)
25 μg/ml	3.05 ± 0.19 (86.0)
50 μg/ml	2.30 ± 0.14 (65.3)*

Values in parentheses are the percentages of the control. PC12 cells were treated with sample compounds, and then incubated at 37°C for 24 hr. The cells were harvested and the dopamine content was measured by an HPLC method. Results represent means±SEM of 5-7 dishes. *Statistically significant at a p value of 0.05 (Student's t-test).

wuweizisu C did not alter the secretion of dopamine in to the medium. Therefore, the total dopamine content in PC12 cells was reduced for the wuweizisu C. The mechanisms of decreased dopamine content with the use of the wuweizisu C in PC12 cells are under investigation.

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