Natural Product Sciences
27(3) : 161-168 (2021)
https://doi.org/10.20307/nps.2021.27.3.161

Neuroprotective Effects of *N*-Acetyldopamine Dimers from Cicadidae Periostracum

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Abstract – The chemical investigation of the 90% EtOH extract from Cicadidae Periostracum led to the isolation and identification of seven known *N*-acetyldopamine dimers (**1-7**). These compounds were identified by comparing mass spectrometry data and NMR spectroscopic data with those previously reported. In this study, complete interpretation of 1D and 2D NMR data of **1** and **2** were reported for the first time. In addition, compounds **3** and **4** were isolated from this material for the first time. All isolates were obtained as racemic mixtures, as confirmed by chiral HPLC. Furthermore, we evaluated the neuroprotective activities of compounds **1–7** and found that compounds **1**, **5**, and **6** significantly attenuated rotenone-induced death of SH-SY5Y neuroblastoma cells at a concentration of 100 μM. Parallel to this result, compounds **3** and **6** displayed antioxidant effects in the cytoplasm, as determined by CM-H2DCFDA fluorescence intensity, while compounds **1** and **5** showed antioxidant effects in the mitochondria, as assessed by MitoSox fluorescence intensity. Overall, these results suggest that some of these compounds protect neuroblastoma cells by ameliorating the release of reactive oxygen species. Further studies are warranted to elucidate the underlying mechanisms by which these compounds exhibit antioxidant and neuroprotective actions.
Keywords – Cicadidae Periostracum, *N*-acetyldopamine, neuroprotective effect, antioxidant effect

Introduction

Insects are the dominant form of life on earth, making up 80%–90% of recorded animals, and they produce unique natural products as part of their defense mechanisms.[1] Insect metabolites and their biological activities have not been well studied as compared to those of plants or microorganisms and are thus an untapped source for new drug development.[2] In this investigation, we explore insect metabolites and their physiological activities. Cicadidae Periostracum, the cast-off shell of the *Cryptotympana pustulata* Fabricius which is called 'Seontoi' in Korea, has been reported to show whitening,[3] anti-oxidative,[4] anti-inflammatory,[4] anti-convulsive,[5] sedative,[5] and hypothermic effects.[5] The shell of insects is mainly composed of chitin, proteins, and catecholamine derivatives.[3] In addition, *N*-acetyldopamine monomers, dimers, trimers, tetramers to oligomers have also been reported from previous studies.[4,6-9]

Sclerotization is a process of hardening the insect shells by enzymatically activating 1,2-dehydro-*N*-acetyldopamine from reactive intermediates and chitin-catechol adducts.[10] *O*-Benzoquinone and quinone methide are two sclerotizing agents which proceed with oxidation and coupling to generate cross-links forming benzodioxane type dimers.[11] In a similar way, the sclerotizing agent reacts with the catechol group of dopamine dimers, resulting in trimers and then tetramers to oligomers of *N*-acetyldopamine.[11,12]

In this study, fractionation and isolation of an ethyl acetate (EtOAc) extract obtained by liquid-liquid partition of a 90% ethanol (EtOH) crude extract of Cicadidae Periostracum led to the isolation of seven *N*-acetyldopamine dimeric derivatives (**1–7**) (Fig. 1), which were obtained as enantiomeric mixtures. The chemical structures of the isolated compounds were identified by spectroscopic analysis and by comparison with reported values. All compounds have been described in previous reports.[13-15] However, this is the first study to analyze and report the

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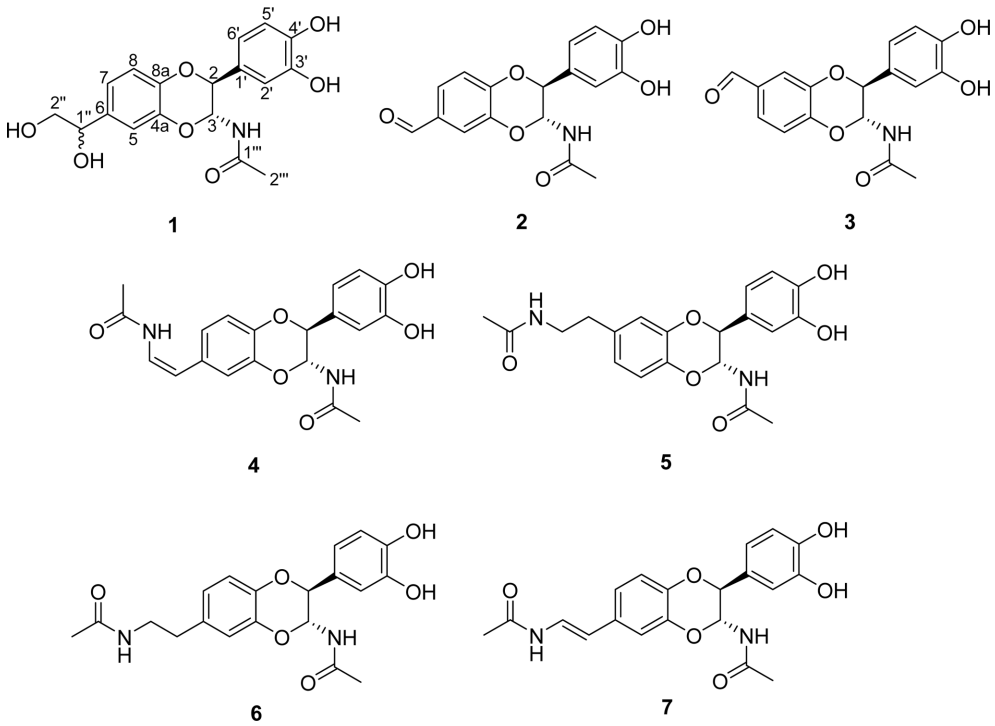


Fig. 1. The structures of **1**–**7** isolated from Cicadidae Periostracum.

NMR data of compounds **1** and **2**. We further analyzed the pharmacological activities of compounds **1**–**7** by assessing the viability of SH-SY5Y neuroblastoma cells treated with rotenone and the cytoplasmic level of reactive oxygen species (ROS). Herein, we report that the compounds found in Cicadidae Periostracum have neuroprotective properties, which might be attributable to their antioxidant effects.

Experimental

General experimental procedures – Optical rotations were measured on a JASCO DIP-1000 in methanol (Tokyo, Japan). Low resolution electrospray ionization mass spectrometry (LR-ESI-MS) was performed on an Agilent 6120 series LC-MS system (Agilent Technologies, Santa Clara, CA, USA). The 1D and 2D NMR spectra, in methanol-d_4, were acquired on a 600-MHz Varian NMR spectrometer (VNS-600, Palo Alto, CA, USA) at the Core Research Support Center for Natural Products and Medical Materials (CRCNM). NMR spectra were also acquired on a Bruker Avance DPX 250 spectrometer (Bruker, Billerica, USA) and a Bruker Biospin GmbH 400 MHz spectrometer (Bruker AG, Fällanden, Switzerland). Column chromatography was performed using silica gel (230–400 mesh; Merck, Germany) and YMC gel ODS-A (12 nm, S-150 μm; YMC, Japan). Semi-preparative HPLC was conducted on a Waters system (Waters Corporation, Milford, CT, USA) using a YMC-Pack ODS-AQ column (10 × 250 mm, 5 μm). Analytical chiral profiles were obtained using a CHIRALPAK IC (4.6 × 250 mm, 5 μm) column. Thin-layer chromatography (TLC) was carried out on silica gel 60 F_{254} (0.2 mm thickness; Merck) and RP-18 F_{254S} (Merck) plates. Spots were detected under UV light at 254 nm and by heating after spraying with 10% (v/v) H_2SO_4 in ethanol.

Insect materials – Dried Cicadidae Periostracum was purchased from Naemome Dah (Ulsan, Korea), a traditional medicine company in Korea. A voucher specimen (YNPC006) of the insect was deposited at the Natural Product Chemistry Laboratory at Yeungnam University, Gyeongsan, Korea.

Extraction and isolation – Dried and powdered Cicadidae Periostracum (3 kg) were extracted three times with 90% EtOH at room temperature, 2 days each time. The resultant extracts were evaporated under vacuum to yield 70 g of dried extract, which was suspended in water (1 L) and partitioned with hexanes, (3 × 1 L) methylene chloride (MC, 3 × 1 L), ethyl acetate (EtOAc, 3 × 1 L), and water-saturated *n*-BuOH (3 × 1 L) yielding 1.5, 5.0, 10.5, and 43.0 g, respectively. A portion of the EtOAc fraction (10 g) was purified by silica gel column chromato-

graphy, eluted with hexanes/EtOAc (95:5 to 0:100) and MC/MeOH (100:0 to 0:100) to give 27 subfractions (CP1− CP27). CP11 was purified using a reverse-phase semi-prep HPLC column (RP-C_{18}, 40% MeOH) to yield **3** (1.1 mg, t_R = 28.1 min) and **2** (1.0 mg, t_R = 33.6 min). Compounds **5** (20.6 mg, t_R = 21.5 min), **7** (8.1 mg, t_R = 40.7 min), and **4** (1.1 mg, t_R = 50.7 min) were obtained from subfraction CP17 by RP-HPLC using an isocratic solvent system of 40% MeOH in water. CP19-21 (1.2 g) was subjected to reverse-phase column chromatography, eluting with a gradient solvent system consisting of H_2O/MeOH (30:70 to 0:100), resulting in 13 subfractions (CP.RP.1-CP.RP.13). Subfraction CP.RP.2 was purified by RP-C_{18} prep HPLC (25% MeOH) to give **1** (1.2 mg, t_R = 37.1 min). Similarly, RP-HPLC was used for purification of subfraction CP.RP.3 to give compounds **5** (20.2 mg, t_R = 33.2 min) and **7** (12.5 mg, t_R = 44.92 min) using 30% MeOH. Subfraction CP.RP.4 was purified with RP-HPLC using 20% ACN to give compounds **6** (1.8 mg, t_R = 32.4 min). The racemic mixture of **5** was separated by analytical HPLC equipped with a CHIRALPAK IC column using MeOH/H_2O (55:45, 1 mL/min) to yield **5a** (2.8 mg, t_R 10 min) and **5b** (2.2 mg, t_R 25 min).

Compound (1) – white powder; $[\alpha]_D^{25}$: −3.24 (*c* 0.12, MeOH); ^{1}H, ^{13}C NMR: see Tables 1 and 2; LR-ESI-MS *m/z*: 362.1 $[M+H]^+$ (calcd for $C_{18}H_{20}NO_7^+$: 362.1).

Compound (2) – white powder; $[\alpha]_D^{25}$: +2.59 (*c* 0.10, MeOH); ^{1}H and ^{13}C NMR: see Tables 1 and 2; LR-ESI-MS *m/z*: 330.2 $[M+H]^+$ (calcd for $C_{17}H_{16}NO_6^+$: 330.1).

Parvamide B (3) – white powder; $[\alpha]_D^{25}$: +1.91 (*c* 0.10, MeOH); ^{1}H and ^{13}C NMR: see Tables 1 and 2; LR-ESI-MS *m/z*: 330.2 $[M+H]^+$ (calcd for $C_{17}H_{16}NO_6^+$: 330.1).

Aspongopisamide B (4) – white powder; $[\alpha]_D^{25}$: +0.37 (*c* 0.11, MeOH); ^{1}H and ^{13}C NMR: see Tables 1 and 2; LR-ESI-MS *m/z*: 385.1 $[M+H]^+$ (calcd. for $C_{20}H_{21}N_2O_6^+$: 385.1).

(2*R,3*S**)-2-(3',4'-Dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2''-aminoethyl)-1,4-benzodioxane (5)** – white powder, $[\alpha]_D^{25}$: +5.09 (*c* 0.18, MeOH). The optical rotation of the pure enantiomers (after chiral separation) were measured and gave the following results: white powder (**5a**), $[\alpha]_D^{25}$: 31.78 (*c* 0.13, MeOH); and white powder (**5b**), $[\alpha]_D^{25}$: −25.04 (*c* 0.14, MeOH). ^{1}H NMR (CD_3OD, 600 MHz): δ 6.84 (1H, d, *J* = 1.9 Hz, H-2'), 6.80 (1H, d, *J* = 8.0 Hz, H-5'), 6.80 (1H, d, *J* = 1.9 Hz, H-8), 6.76 (1H, d, *J* = 7.5 Hz, H-5), 6.74 (1H, dd, *J* = 1.9, 7.5 Hz, H-6'), 6.72 (1H, dd, *J* = 1.9, 7.5 Hz, H-6), 5.67 (1H, d, *J* = 7.1 Hz, H-3), 4.69 (1H, d, *J* = 7.1 Hz, H-2), 3.35 (2H, t, *J* =

Table 1. ^{1}H NMR Spectroscopic Data of Compounds **1**–**4**

positions	**1**	**2**	**3**	**4**
	δ_H Multi (*J* in Hz)	δ_H Multi (*J* in Hz)	δ_H Multi (*J* in Hz)	δ_H Multi (*J* in Hz)
2	4.70, d (7.2)	4.84, d (7.2)	4.78, d (7.2)	4.72, d (7.2)
3	5.70, d (7.2)	5.77, d (7.2)	5.83, d (7.2)	5.72, d (7.2)
4a				
5	6.92, d (1.9)	7.45, d (1.9)	7.07, d (8.3)	6.97, d (1.8)
6			7.51, dd (8.3, 1.8)	
7	6.88, dd (8.2, 1.9)	7.50, dd (8.2, 1.9)		6.88, dd (8.2, 1.8)
8	6.90, d (8.2)	7.12, d (8.2)	7.49, d (1.8)	6.91, d (8.2)
8a				
1'				
2'	6.84, d (1.8)	6.85, d (1.8)	6.86, d (1.5)	6.85, d (1.8)
3'				
4'				
5'	6.76, d (8.2)	6.78, d (8.2)	6.77, d (8.2)	6.77, d (8.1)
6'	6.74, dd (8.2, 1.8)	6.76, dd (8.2, 1.8)	6.76, dd (8.2, 1.5)	6.75, dd (8.1, 1.8)
1"	4.60, dd (7.0, 4.1)	9.81, s	9.81, s	5.66, d (9.8)
2"	3.58, m			6.71, d (9.8)
3"				
4"				2.07, s
1'"				
2'"	1.88, s	1.89, s	1.89, s	1.88, s

J values are in Hz; δ in ppm; the assignments were confirmed by ^{1}H-^{1}H COSY, HSQC, and HMBC experiments; ^{1}H NMR spectra were acquired at 600 MHz.

Table 2. ^{13}C NMR Spectroscopic Data of Compounds **1**–**4**

positions	1	2	3	4
	δ_C	δ_C	δ_C	δ_C
2	78.3	78.8	78.2	78.4
3	78.3	78.3	78.9	78.4
4a	143.5	144.3	149.7	143.6
5	116.1	119.1	118.7	117.8
6	137.3	132.6	125.7	130.9
7	120.8	125.5	132.4	123.2
8	117.7	118.7	118.9	118.1
8a	143.8	150.3	145.0	143.4
1'	128.8	127.9	127.9	128.6
2'	115.6	115.6	115.6	115.6
3'	146.5	146.6	146.6	146.6
4'	147.2	147.4	147.5	147.2
5'	116.2	116.2	116.2	116.1
6'	120.6	120.7	120.6	120.6
1"	75.4	192.6	192.7	112.4
2"	68.7			121.9
3"				171.8
4"				22.6
1'"	173.2	173.3	173.3	173.3
2'"	22.6	22.6	22.6	22.6

^{13}C NMR spectra were acquired at 62.5 MHz for **1** and 150 MHz for **2**–**4.**

7.1 Hz, H-2"), 2.69 (2H, t, *J* = 7.1 Hz, H-1"), 1.90 (3H, s, CH_3-4"), 1.87 (3H, s, CH_3-2'"). ^{13}C NMR (CD_3OD, 150 MHz): δ 173.2 (C-1'"), 173.2 (C-3"), 147.1 (C-4'), 146.4 (C-3'), 144.3 (C-8a), 142.2 (C-4a), 134.1 (C-7), 128.8 (C-1'), 123.2 (C-6), 120.5 (C-6'), 118.1 (C-8), 117.9 (C-5), 116.1 (C-5'), 115.6 (C-2'), 78.3 (C-3), 78.2 (C-2), 42.1 (C-2"), 35.8 (C-1"), 22.6 (C-4"), 22.5 (C-2'"). LR-ESI-MS *m/z* 387.1 $[M+H]^+$.

(2*R,3*S**)-2-(3',4'-Dihydroxyphenyl)-3-acetylamino-6-(*N*-acetyl-2''-aminoethyl)-1,4-benzodioxane (6)** – white powder, $[\alpha]_D^{25}$: +1.01 (*c* 0.22, MeOH). ^{1}H NMR (CD_3OD, 600 MHz): δ 6.85 (1H, d, *J* = 1.8 Hz, H-2'), 6.83 (1H, d, *J* = 8.2 Hz, H-5'), 6.76 (1H, d, *J* = 8.2 Hz, H-8), 6.70 (1H, dd, *J* = 8.2, 2.0 Hz, H-7), 6.74 (1H, dd, *J* = 8.2, 1.8 Hz, H-6'), 6.74 (1H, d, *J* = 2.0 Hz, H-5), 5.69 (1H, d, *J* = 7.2 Hz, H-3), 4.68 (1H, d, *J* = 7.2 Hz, H-2), 3.34 (2H, t, *J* = 7.2 Hz, H-2"), 2.68 (2H, t, *J* = 7.2 Hz, H-1"), 1.90 (3H, s, CH_3-4"), 1.87 (3H, s, CH_3-2'"). ^{13}C NMR (CD_3OD, 150 MHz): δ 173.2 (C-1'"), 173.2 (C-3"), 147.1 (C-4'), 146.4 (C-3'), 144.3 (C-8a), 142.9 (C-4a), 134.2 (C-6), 128.8 (C-1'), 123.0 (C-7), 120.6 (C-6'), 118.1 (C-5), 117.8 (C-8), 116.1 (C-5'), 115.6 (C-2'), 78.3 (C-3), 78.1 (C-2), 42.1 (C-2"), 35.7 (C-1"), 22.6 (C-4"), 22.5 (C-2'"). LR-ESI-MS *m/z*: 387.2 $[M+H]^+$.

(2*R,3*S**)-2-(3',4'-Dihydroxyphenyl)-3-acetylamino-6-(*N*-acetyl-2''-aminoethylene)-1,4-benzodioxane (7)** – white powder, $[\alpha]_D^{25}$: +2.48 (*c* 0.18, MeOH). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.30 (1H, dd, *J* = 14.7 Hz, H-2"), 6.87 (1H, d, *J* = 8.1 Hz, H-5'), 6.86 (1H, dd, *J* = 8.1, 1.9 Hz, H-6'), 6.84 (1H, d, *J* = 1.6 Hz, H-8), 6.85 (1H, dd, *J* = 1.9 Hz, H-7), 6.76 (1H, dd, *J* = 8.2 Hz, H-8), 6.74 (1H, d, *J* = 8.2, 1.6 Hz, H-7), 6.10 (1H, d, *J* = 14.7 Hz, H-1"), 5.69 (1H, d, *J* = 7.3 Hz, H-3), 4.69 (1H, d, *J* = 7.3 Hz, H-2), 2.04 (3H, s, CH_3-4"), 1.88 (3H, s, CH_3-2'"). ^{13}C NMR (CD_3OD, 100 MHz): δ 173.4 (C-1'"), 170.8 (C-3"), 147.5 (C-4'), 146.8 (C-3'), 143.8 (C-8a), 143.4 (C-4a), 134.0 (C-6), 128.7 (C-1'), 123.0 (C-2"), 120.2 (C-6'), 120.7 (C-7), 118.3 (C-5), 118.3(C-8), 115.6 (C-5'), 114.9 (C-2'), 114.1 (C-1"), 78.5 (C-2), 78.5 (C-3), 22.7 (C-4"), 22.7 (C-2'"). LR-ESI-MS *m/z*: 385.2 $[M+H]^+$.

SH-SY5Y cell culture, viability assay, and ROS measurement – SH-SY5Y neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (HyClone Laboratories) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 humidified chamber.

The cells were exposed to 10, 100, and 200 μM of each compound for 6 h, followed by 1 μM of rotenone for 12 h. Next, cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)–2H-tetrazolium (MTS, Thermo Fisher Scientific) as described in the manufacturer's manual.

The cytoplasmic level of ROS was determined using a CM-H2DCFDA probe (Thermo Fisher Scientific). Briefly, the media was gently aspirated, and the cells were washed with pre-warmed phosphate buffered saline (PBS). The cells were then incubated for 30 min in PBS containing the CM-H2DCFDA probe at a final concentration of 10 μM. The fluorescence intensity of oxidized 2',7'-dichlorofluorescein (DCF) was analyzed using a microplate fluorometer (Fluostar spectrofluorometer, BMG; λ_{ex} = 485 nm and λ_{em} = 520 nm).

We assessed mitochondrial ROS using MitoSOX™ Red (Thermo Fisher Scientific), an indicator which is rapidly oxidized only by mitochondria-derived superoxide. Briefly, the cells were subject to 1.0 mL of the 5 μM MitoSOX™ working solution. Then, the cells were incubated for 10 min at 37 °C avoiding light. The cells were gently washed three times with warm PBS. Fluorescence was measured with a microplate reader and fluorescence microscope using the respective excitation and emission wavelengths (λ_{ex} = 510 nm and λ_{em} = 580 nm).

Statistical analysis – All values are expressed as the mean ± SEM of multiple independent experiments. One-

way analysis of variance (ANOVA) was performed, followed by the Student–Newman–Keuls test to calculate statistical differences between various groups using GraphPad Prism V5.0 (San Diego, CA, USA). A value of $p<0.05$ was considered statistically significant.

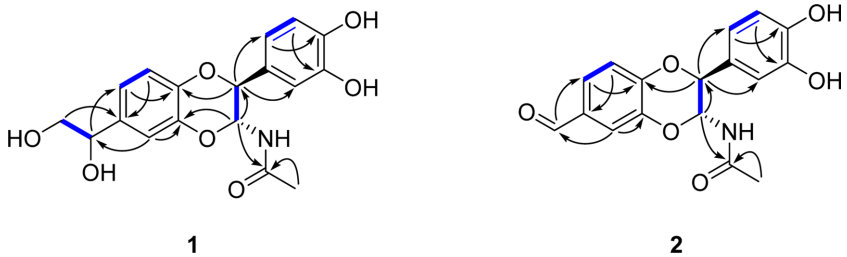
Results and Discussion

Chromatographic separation of the EtOAc fraction of Cicadidae Periostracum has led to the isolation of seven *N*-acetyl dopamine dimers (**1**–**7**) (Fig. 1). The structures of **1**–**7** were confirmed by spectroscopic data analysis, including MS and NMR, and by comparison with the reported values. The present study includes the first report of the complete sets of 1D and 2D NMR data for compounds **1** and **2**. As shown in the chiral HPLC profiles in Fig. 3, all the isolates were obtained as racemic mixtures, further evidenced by optical rotation values near zero.

Compound **1** was obtained as a white powder. The LR-ESI-MS displayed a molecular ion peak $[M+H]^+$ at *m/z* 362.1 (calcd for $C_{18}H_{20}NO_7^+$: 362.1234) with 10 degrees of unsaturation. The ^{1}H NMR spectrum (Table 1) of **1** displayed characteristic signals of ABX systems at δ_H 6.92 (1H, d, J = 1.9 Hz, H-5), 6.90 (1H, d, J = 8.2 Hz, H-8), 6.88 (1H, dd, J = 8.2, 1.9 Hz, H-7), 6.84 (1H, d, J = 1.8 Hz, H-2'), 6.76 (1H, d, J = 8.2 Hz, H-5'), and 6.74 (1H, dd, J = 8.2, 1.8 Hz, H-6') suggesting the presence of two 1,2,4-trisubstituted benzene rings. Moreover, three methines at δ_H 5.70 (1H, d, J = 7.2 Hz, H-3), 4.70 (1H, d, J = 7.2 Hz, H-2), and 4.60 (1H, dd, J = 7.0, 4.1 Hz, H-1"), one oxygenated methylene at δ_H 3.58 (2H, m, H-2") and one methyl at 1.88 (3H, s, H-2'") were also observed in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum showed 18 carbons including six unprotonated sp2 carbons at δ_C 147.2 (C-4'), 146.5 (C-3'), 143.8 (C-8a), 143.5 (C-4a), 137.3 (C-6), and 128.8 (C-1'); one carbonyl carbon at δ_C 173.2 (C-1'"); six carbons of two ABX systems attributable to benzene rings at δ_C 120.8 (C-7), 120.6 (C-6'), 117.7 (C-8), 116.2 (C-5'), 116.1 (C-5), and 115.6 (C-2'); three methine carbons at δ_C 78.3 (C-2), 78.3 (C-3), and 75.4 (C-1"); one oxygenated aliphatic methylene at δ_C 68.7 (C-2"); and one methyl carbon at δ_C 22.6 (C-2'"). These spectral data closely resembled the *N*-acetyldopamine dimer derivative, (2*R*,3*S*)-2-(3',4'-dihydroxyphenyl)-3-acetylamino-6-hydroxyethyl)-1,4-benzodioxane,[16] except for the presence of a hydroxy group at the C-1" position [δ_H 4.60 (1H, dd, J = 7.0, 4.1 Hz); δ_C 75.4]. The *N*-acetyldopamine dimer scaffold was further verified by extensive analysis of COSY, HSQC, and HMBC correlations (Fig. 2). The key HMBC crosspeaks of H-3 and H-5 to C-4a, H-2 and H-7 to C-8a, and H-3 to C-2 indicate the presence of a 1,4-benzodioxane block including one ABX spin system. The attachment of the 1",2"- dihydroxyethyl group at C-6 position was established by HMBC correlations of H-1" to C-7, H-2" and H-8 to C-6, and H-5 to C-1". In addition, the connection of an acetamide unit at C-3 was confirmed by the HMBC correlation from H-2'" and H-3 to C-1'". The position of the 3',4'-dihydroxyphenyl, which suggests another ABX spin system in the structure, was confirmed using the long-range couplings of H-2 to C-2' and C-6', H-5' to C-3' and H-6' to C-4' in the HMBC spectrum.

The relative configurations between H-2 and H-3 were determined as *trans* (J = 7.2 Hz) based on the coupling constant reported in previous studies (J = 7.3 Hz for *trans*[13,17,18]; J = 1.5 Hz[14] or br.s[6] for *cis*). To determine the absolute configurations of C-2 and C-3 in **1**, the optical rotation was measured and a low value was obtained, $[\alpha]_D^{25}$: −3.24 (*c* 0.12, MeOH), which indicates the presence of a racemic mixture. This was further supported by the chiral HPLC profile, as shown in Fig. 3. Further purification and chemical reactions for determining the absolute configuration of C-1" were not conducted owing to the limited amount of material. Based on these observations, compound **1** was identified as (2*R**,3*S**)-2-(3',4'-dihydroxyphenyl)-3-acetylamino-6-(1",2"-dihydroxyethyl)-1,4-benzodioxane.[13,16]

Compound **2** was obtained as a white powder. Its molecular formula, $C_{17}H_{15}NO_6$, was confirmed based on NMR spectral data as well as the LR-ESI-MS molecular ion peak $[M+H]^+$ at *m/z* 330.2 (calcd for $C_{17}H_{16}NO_6^+$:



1 **2**

Fig. 2. COSY (—) and key HMBC (→) correlations of **1** and **2**.

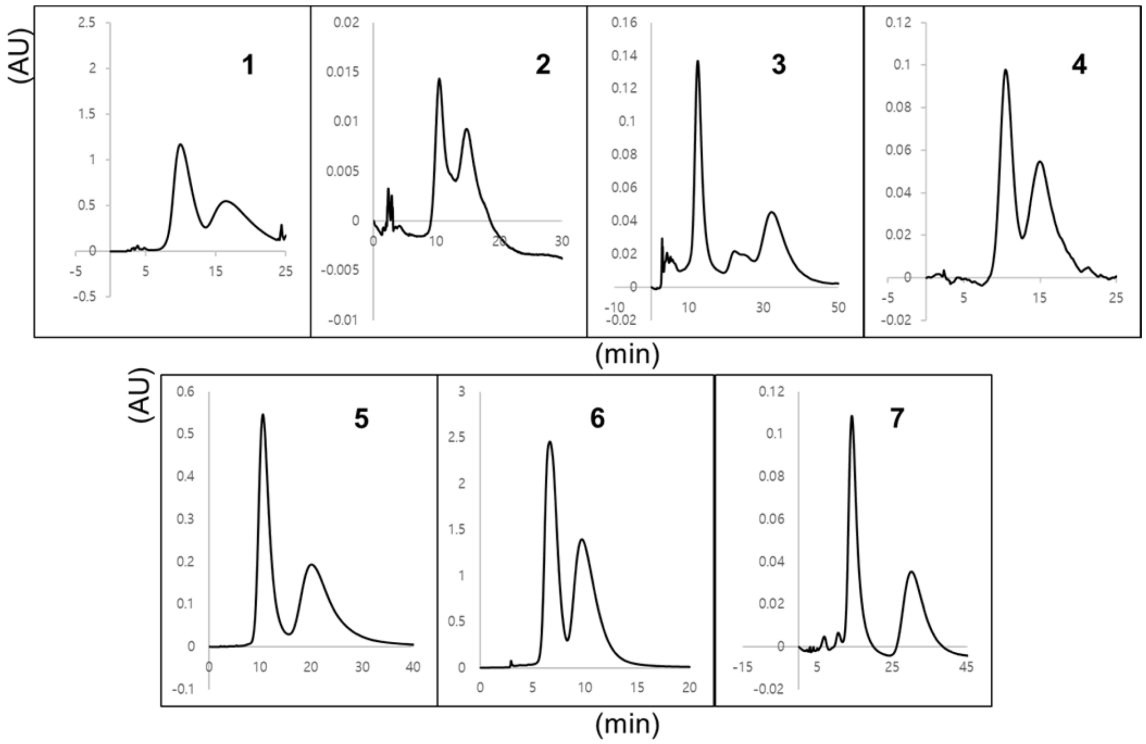


Fig. 3. HPLC chromatograms of the racemic mixtures of **1–7** [CHIRALPAK IC, 4.6×250 mm; mobile phase-MeOH:H_2O (30:70) for compound **1**, MeOH:H_2O (51:49) for compounds **2–4** and MeOH:H_2O (55:45) for compounds **5–7**.

330.0972) with 11 degrees of unsaturation. The ^{1}H and ^{13}C NMR spectra of compound **2** were similar to those of compound **1**, suggesting it was another derivative of the *N*-acetyldopamine dimer, where the 1", 2"-dihydroxy ethyl group at the C-6 position of **1** is replaced by an aldehyde group. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) of **2** consist of two ABX spin systems at δ_H 7.50 (1H, dd, J = 8.2, 1.9 Hz, H-7)/125.5 (C-7), 7.45 (1H, d, J = 1.9 Hz, H-5)/119.1 (C-5), 7.12 (1H, d, J = 8.2 Hz, H-8)/118.7 (C-8) and 6.85 (1H, dd, J = 1.8 Hz, H-2')/115.6 (C-2'), 6.78 (1H, d, J = 8.2 Hz, H-5')/116.2 (C-5') and 6.76 (1H, dd, J = 8.2, 1.8 Hz, H-6')/120.7 (C-6'), two methines δ_H 5.77 (1H, d, J = 7.2 Hz, H-3)/78.3 (C-3), 4.84 (1H, d, J = 7.2 Hz, H-2)/78.8 (C-2), one aldehyde δ_H 9.81 (1H, s, H-1")/192.6 (C-1") and one methyl δ_H 1.89 (1H, s, H-2"')/22.6 (C-2"'). After a comprehensive analysis of the COSY, HSQC, and HMBC spectra (Fig. 2), the planar structure of **2** was established. The HMBC correlations from H-1" to C-7, from H-8 to C-6, from H-5 to C-1" and C-4a, from H-7 and H-2 to C-8a established the position of the aldehyde group in the 1,4-benzodioxane framework. Additionally, attachment of an acetamide group was confirmed by the HMBC correlation from H-3 and H-2"' to C-1"' and the position of a 3',4'-dihydroxyphenyl group was supported by long-range couplings of H-2 to C-2' and C-6', H-5' to C-3' and H-6' to C-4' in the HMBC spectrum. These observations were comparable to **1**, with the exception of showing the attachment of an aldehyde group at the C-6 position instead of a 1",2"-dihydroxyethyl group. The *trans* configuration at C-2 and C-3 of **2** was established by analyzing the coupling constant between H-2 and H-3 (J = 7.2 Hz)[13] and comparing it with **1**. Optical rotation was measured to determine the absolute configuration of **2**. The result showed a low value for the optical rotation, $[\alpha]_D^{25}$: +1.91 (*c* 0.10, MeOH), indicating a racemic mixture similar to that of compound **1**. This was further confirmed by the chiral HPLC profile seen in Fig. 3. Accordingly, compound **2** was identified as (2*R**,3*S**)-2-(3',4'-dihydroxyphenyl)-3-acetylamino-6-(formyl)-1,4-benzodioxane[13, 16], thus providing the first report of the complete NMR data for this compound.

The structures of compounds **3**–**7** structures were also identified by comparing ^{1}H/^{13}C NMR and MS spectral data with those in the literatures to be parvamide B (**3**),[13] aspongopusamide B (**4**),[15] (2*R**,3*S**)-2-(3',4'-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2"-aminoethyl)-1,4-benzodioxane (**5**),[14] (2*R**,3*S**)-2-(3',4'-dihydroxyphenyl)-3-acetylamino-6-(*N*-acetyl-2"-aminoethyl)-1,4-benzodioxane (**6**),[14] and (2*R**,3*S**)-2-(3',4'-dihydroxyphenyl)-3-acetylamino-6-(*N*-acetyl-2"-aminoethylene)-1,4-benzodioxane (**7**).[15] Among

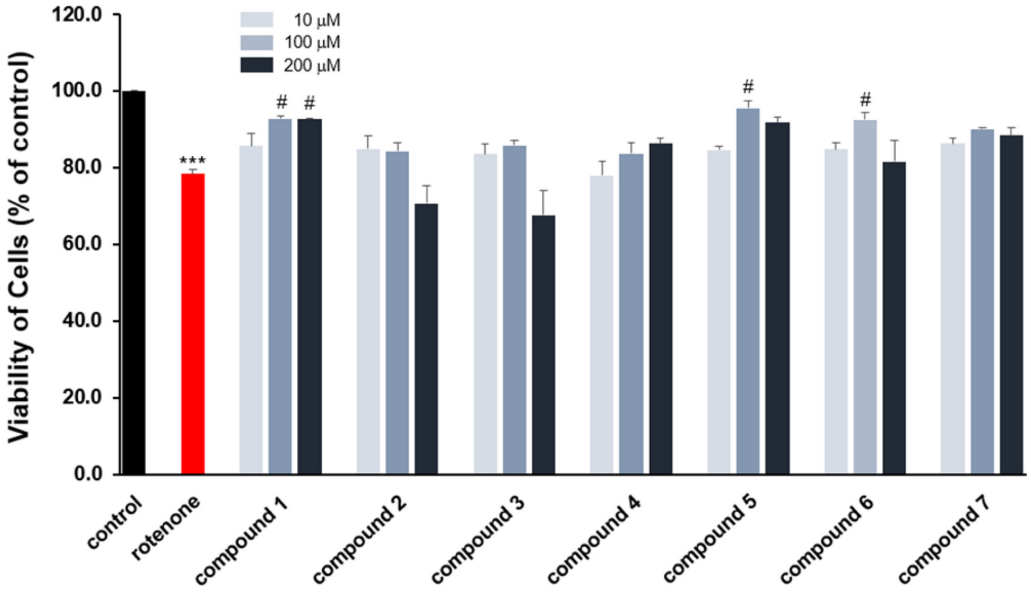


Fig. 4. Neuroprotective effects of the compounds against rotenone-induced toxicity. Results of MTS assay show that compounds **1** (100 and 200 μM), **5** (100 μM), and **6** (100 μM) significantly protect from rotenone (1 μM)-induced loss of SH-SY5Y neuroblastoma cells. ***, $p<0.001$ compared to control; #, $p<0.05$, ##, $p<0.01$ compared to rotenone group. All values represent mean ± S.E.M. from four independent experiments.

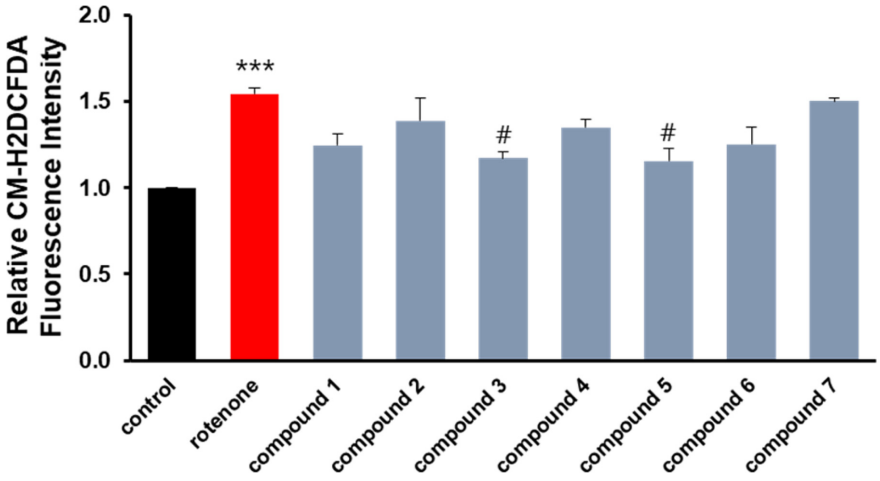


Fig. 5. Attenuation of rotenone-mediated ROS production in the cytoplasm by the compounds. Results of CM-H2DCFDA assay show that compounds **3** and **5** significantly reduce rotenone (1 μM)-induced ROS production in the SH-SY5Y neuroblastoma cells. ***, $p<0.001$ compared to control; #, $p<0.05$ compared to rotenone group. All values represent mean ± S.E.M. from four independent experiments.

them, compounds **3** and **4** were isolated from this material for the first time.

To assess the neuroprotective activities of compounds **1**–**7**, we treated SH-SY5Y cells with each compound (10, 100, and 200 μM), followed by 1 μM of rotenone. The results of the MTS assay showed that rotenone significantly decreased cell viability by 23% compared to the control (Fig. 4). Among the compounds, compound **1** (100 and 200 μM), compound **5** (100 μM), and compound **6** (100 μM) clearly reduced rotenone-induced cytotoxicity. Overall, 100 μM of the compounds appeared to be more effective than 10 or 200 μM.

We speculated that the neuroprotective effects might be due to the antioxidant effects of the specific compounds. Thus, we tested whether 100 μM of the compounds could ameliorate ROS production in cells. We observed that rotenone increased ROS release, and compounds **3** and **5** significantly reduced rotenone-mediated ROS production

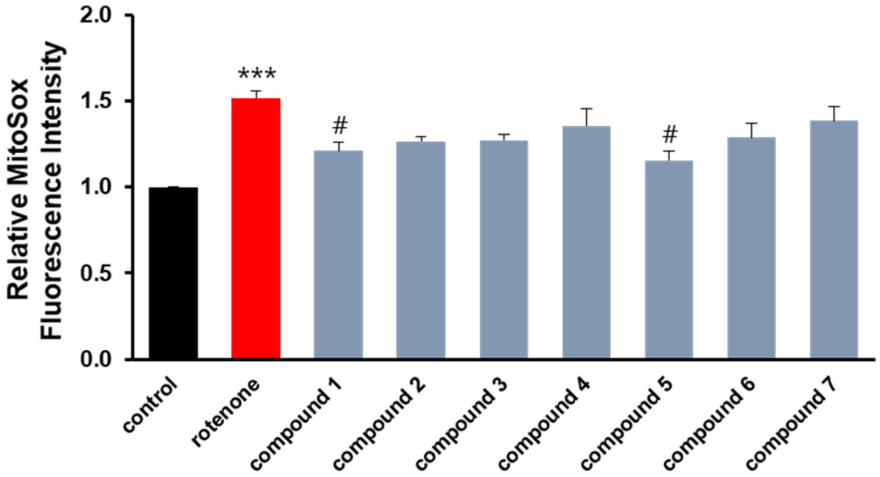


Fig. 6. Attenuation of rotenone-mediated ROS production in the mitochondria by the compounds. Results of MitoSox assay show that compounds **1** and **5** significantly reduce rotenone (1 μM)-induced mitochondrial superoxide production in the SH-SY5Y neuroblastoma cells. ***, $p<0.001$ compared to control; #, $p<0.05$ compared to rotenone group. All values represent mean ± S.E.M. from four independent experiments.

in the cells (Fig. 5). The effect of compound **1** was not statistically significant, but there was a trend toward lowering ROS levels ($p = 0.06$).

Further, we tested whether the compounds ameliorated rotenone-induced mitochondrial superoxide production. Rotenone dramatically increased mitochondrial superoxide levels, and compounds **1** and **5** significantly reduced superoxide production mediated by rotenone challenge (Fig. 6).

Overall, these results indicate that the neuroprotective activities of the *N*-acetyldopamine dimers might be related to their antioxidant properties, as compounds **1** and **5** consistently protected the SH-SY5Y neuroblastoma cells from rotenone-induced injury and alleviated the cytoplasmic and mitochondrial ROS production induced by rotenone. Further studies are necessary to elucidate the specific mechanisms by which the compounds spare neuroblastoma cells from rotenone toxicity and to evaluate their neuroprotective effects in animal models of neurodegenerative diseases.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education [NRF-2019R1C1C1009929].

References

(1) Dossey, A. T. *Nat. Prod. Rep.* **2010**, *27*, 1737-1757.
(2) Zhu, H. J.; Yan, Y. M.; Tu, Z. C.; Luo, J. F.; Liang, R.; Yang, T. H.; Cheng, Y. X.; Wang, S. M. *Fitoterapia* **2016**, *114*, 163-167.
(3) Tada, T.; Ohnishi, K.; Suzuki, K.; Tomita, H.; Okamori, M.; Katuzaki, H.; Komiya, T.; Imai, K. *J. Oleo Sci.* **2002**, *51*, 355-358.
(4) Xu, M. Z.; Lee, W. S.; Han, J. M.; Oh, H. W.; Park, D. S.; Tian, G. R.; Jeong, T. S.; Park, H. Y. *Bioorg. Med. Chem.* **2006**, *14*, 7826-7834.
(5) Hsieh, M. T.; Peng, W. H.; Yeh, F. T.; Tsai, H. Y.; Chang, Y. S. *J. Ethnopharmacol.* **1991**, *35*, 83-90.
(6) Yang, L.; Li, G. Y.; Wang, H. Y.; Zhang, K.; Zhu, Y.; Zhao, W.; Wang, H.; Wang, J. H. *Phytochem. Lett.* **2016**, *16*, 97-102.
(7) Noda, N.; Kubota, S.; Miyata, Y.; Miyahara, K. *Chem. Pharm. Bull. (Tokyo)* **2000**, *48*, 1749-1752.
(8) Yang, L.; Li, G. Y.; Li, Q. R.; Wang, J. H. *J. Asian Nat. Prod. Res.* **2012**, *14*, 204-209.
(9) Li, G.; Yang, L.; Lu, L.; Yu, F.; Wang, J. China Patent. **2011**, CN102285958.
(10) Sugumaran, M.; Semensi, V.; Kalyanaraman, B.; Bruce, J. M.; Land, E. J. *J. Biol. Chem.* **1992**, *267*, 10355-103561.
(11) Sugumaran, M. *Int. J. Mol. Sci.* **2016**, *17*, 1576.
(12) Abebe, A.; Zheng, D.; Evans, J.; Sugumaran, M. *Insect Biochem. Mol. Biol.* **2010**, *40*, 650-659.
(13) Li, J.; Li, Y. P.; Qin, F. Y.; Yan, Y. M.; Zhang, H. X.; Cheng, Y. X. *Fitoterapia* **2020**, *142*, 104534.
(14) Lee, W. H.; Lee, H. S.; Kim, M. A.; Choi, J. H.; Kim, K. M.; Hwang, J. S.; Na, M. K.; Bae, J. S. *Sci. Rep.* **2017**, *7*, 7934.
(15) Shi, Y. N.; Tu, Z. C.; Wang, X. L.; Yan, Y. M.; Fang, P.; Zuo, Z. L.; Hou, B.; Yang, T. H.; Cheng, Y. X. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 5164-5169.
(16) Yan, Y. M.; Li, L. J.; Qin, X. C.; Lu, Q.; Tu, Z. C.; Cheng, Y. X. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 2469-2472.
(17) Thapa, P.; Gu, Y.; Kil, Y. S.; Baek, S. C.; Kim, K. H.; Han, A. R.; Seo, E. K.; Choi, H.; Chang, J. H.; Nam, J. W. *Bioorg. Chem.* **2020**, *102*, 104095.
(18) Lee, S. R.; Yi, S. A.; Nam, K. H.; Park, J. G.; Hwang, J. S.; Lee, J.; Kim, K. H. *Bioorg. Chem.* **2020**, *95*, 103554.

Received July 2, 2021
Revised July 27, 2021
Accepted August 1, 2021