Structures of 1,4-Benzodioxane Derivatives from the Seeds of *Phytolacca americana* and Their Neuritogenic Activity in Primary Cultured Rat Cortical Neurons

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The methanol extract of the seeds of *Phytolacca americana* was reinvestigated to yield three new 1,4-benzodioxane-type compounds, americanoic acid methyl ester (1), isoamericanoic acid A methyl ester (2), and 9′-*O*-methylamericanol A (3) along with the previously isolated neolignans 6—9. The structures of 1—3 were characterized by 2D NMR and long-range selective proton-decoupling (LSPD) techniques. The neuritogenic effects of compounds 1—3, and dicarboxilic acids 4 and 5, which had been previously synthesized with horseradish peroxidase-catalyzed oxidative coupling of caffeic acid, were examined in primary cultured rat cortical neurons. Americanoic acid A methyl ester (1) exhibited neurite outgrowth-promoting activity at concentration of 0.01—1.0 μM, whereas dicarboxilic acids 4 and 5 were found to induce neuritogenesis dose dependently at the concentrations from 0.1 μM to 10 μM.

Key words neolignan; 1,4-benzodioxane; neuritogenic activity; antioxidant; *Phytolacca americana*; Phytolaccaceae

Phytolacca americana L. (Phytolaccaceae) is a plant originating in North America, and its dried roots have been used as a diuretic drug in place of the Chinese medicinal plant *Phytolacca esuclenta*. A number of triterpenes, their glycosides, and neolignans have been isolated from the roots of *P. americana*.[1—7] Previously, we reported the structures of americanol A (**6**) and isoamericanol A (**7**) isolated from seeds of *P. americana*,[8,9] as well as those of triterpene glycosides produced from the cultures of *P. americana*.[10] Americanol A (**6**) and isoamericanol A (**7**) in particular showed interesting neurite outgrowth-promoting activity in primary cultured rat cortical neurons.[9] Their significant biological properties stimulated our interest in developing the preparative synthesis of **6** and **7**, resulting in the successful one-step syntheses of **6** and **7** through horseradish peroxidase (HRP)-catalyzed oxidative coupling of 3,4-dihydroxycinnamyl alchohol.[11,12] The methanol extract of the seeds of *P. americana* showed antioxidant activities, *i.e.*, free radical-scavenging activity of the α,α-diphenyl-β-picrylhydrazyl radical (DPPH)[13] and superoxide anion-scavenging activity (O_2^-). This result encouraged us to reinvestigate the chemical constituents in *P. americana*.

In this paper, we describe the structures of three new 1,4-benzodioxane compounds **1**—**3** named americanoic acid A methyl ester, isoamericanoic acid A methyl ester, and 9′-*O*-methylamericanol A, and their neuritogenic activity together with neuritogenesis of dicarboxilic acids **6** and **7**[11,12] in primary cultured rat cortical neurons.

Results and Discussion

The methanol extract of the seeds of *P. americana* was subjected to silica gel chromatography and then purified by reverse-phase HPLC to yield compounds **1**—**3** along with the previously isolated neolignans, isoamericanol A (**7**), americanin A (**8**), and isoamericanin A (**9**) (Fig. 1), and the triterpenes glycoside esculentoside B and esculentside S.[10]

Compounds **1** and **2** not only had the same molecular formula $C_{17}H_{16}O_7$ determined by high-resolution (HR) electron impact (EI)-MS, but also similar ^{1}H- and ^{13}C-NMR spectral data. Their IR spectra showed the presence of hydroxyl

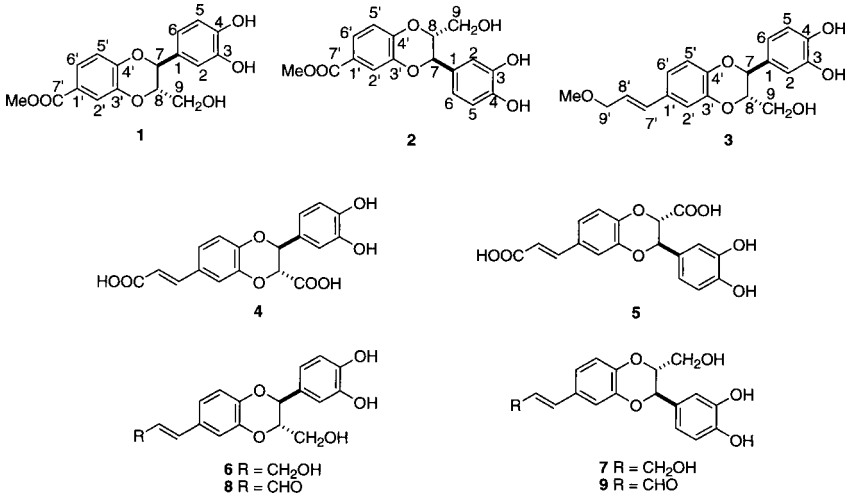

Fig. 1. Structures of **1**—**9**

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Table 1. ^{1}H-NMR Spectral Data of **1**—**3**

H	**1**[a)]	**2**[a)]	**3**[a)]	**3**[b)]
2	6.84 d (1.9)	6.86 d (1.5)	6.84 d (1.8)	6.77 d (1.7)
5	6.80 d (8.2)	6.78 d (8.1)	6.80 d (8.1)	6.75 d (8.0)
6	6.80 dd (8.2, 1.9)	6.76 dd (8.1, 1.5)	6.78 dd (8.1 ,1.8)	6.67 dd (8.0, 1.7)
7	4.89 d (8.2)	4.83 d (7.3)	4.80 d (7.7)	4.81 d (7.6)
8	4.02 ddd (8.2, 4.6, 2.2)	4.07 ddd (7.3, 4.4, 2.2)	3.98 ddd (7.7, 4.8, 2.6)	4.02 ddd (7.6, 4.6, 2.9)
9	3.49 dd (12.5, 4.6)	3.49 dd (12.4, 4.4)	3.47 dd (12.4, 4.8)	3.65 dd (14.9, 3.9)
	3.70 dd (12.5, 2.2)	3.70 dd (12.4, 2.2)	3.66 dd (12.4, 2.6)	3.63 (m)
2′	7.60 d (1.9)	7.53 d (2.0)	7.03 d (1.8)	7.00 d (1.9)
5′	6.97 d (8.5)	7.01 d (8.4)	6.83 d (8.4)	6.82 d (8.2)
6′	7.54 dd (8.4, 1.9)	7.56 dd (8.4, 2.0)	6.91 dd (8.4, 1.8)	6.92 dd (8.2, 1.9)
7′			6.52 d (16.1)	6.48 d (16.0)
8′			6.15 dt (16.1, 6.6)	6.19 dt (16.0, 6.0)
9′			4.05 dd (6.6, 1.5)	3.98 dd (6.0, 1.5)
OMe	3.86 s	3.85 s	3.36 s	3.25 s

a) Recorded in CD_3OD at 400 MHz. *b*) Recorded in DMSO-d_6 at 400 MHz.

Table 2. ^{13}C-NMR Spectral Data of **1**—**3** and **6**—**7**

C	**1**[a)]	**2**[a)]	**3**[b)]	**6**[b),9)]	**7**[b),9)]
1	128.74	129.08	127.51	127.54	127.60
2	115.36	115.57	115.07	114.91	114.94
3	147.07	146.70	145.37	145.24	145.24
4	146.46	147.29	145.95	145.78	145.78
5	116.21	116.41	115.62	115.46	115.49
6	120.26	120.45	118.82	118.80	118.83
7	78.01	77.50	75.73	75.66	75.63
8	79.86	80.50	78.20	78.21	78.30
9	61.92	61.91	60.18	60.14	60.17
1′	124.06	124.27	130.03	130.48	130.30
2′	119.30	119.47	114.39	114.12	114.18
3′	144.43	145.08	143.28	142.99	142.66
4′	149.58	149.44	143.36	143.23	143.60
5′	117.78	117.93	116.87	116.69	116.73
6′	124.18	124.36	119.41	119.27	119.37
7′	167.95	168.26	124.49	128.15	128.15
8′			131.31	128.81	128.81
9′			72.32	61.54	61.57
OMe	52.47	52.45	57.25		

a) Recorded in CD_3OD at 100 MHz. *b*) Recorded in DMSO-d_6 at100 MHz.

groups (**1**: 3367 cm^{-1}; **2**: 3380 cm^{-1}), a carbonyl group (**1**: 1694 cm^{-1}; **2**: 1698 cm^{-1}), and an aromatic moiety (**1**: 1505 cm^{-1}; **2**: 1505 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectral data (Tables 1, 2) of **1** and **2** resembled those of americanol A (**6**) and isoamericanol A (**7**) coexisting in *P. americana*, except for missing the vinyl alcohol part (C-7′—C-9′) existing in **6** and **7** and the new appearance of a methyl ester (**1**: δ_H 3.85, δ_C 52.45, 167.95; **2**: δ_H 3.86, δ_C 52.47, 168.26). These spectral data indicate that the C_3 vinyl alcohol unit in **6** and **7** is degraded, presumably by oxidation into a C_1 methyl ester in the case of **1** and **2**. The heteronuclear multiple-bond connectivity (HMBC) experiments substantiated the methyl ester bonded to C-1′ by the observation of correlating H-2′ (**1**: δ_H 7.60, **2**: δ_H 7.53) and H-6′ (**1**: δ_H 7.53, **2**: δ_H 7.56) to the ester carbonyl resonance and other correlations, as shown in Fig. 2. However, the HMBC was not useful in determining which substituents are located at the C-7 and C-8 positions on the 1,4-benzodioxane ring since there was no HMBC correlation between H-7 or H-8 and C-3′ or C-4′. This drawback of HMBC was also observed in **6** and **7**.[8)] Therefore long-range selective proton-decoupling (LSPD)[14)] method

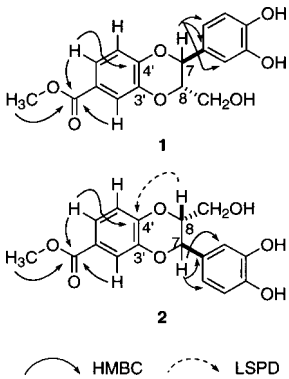

Fig. 2. Representative HMBC and LDPS Correlations of **1** and **2**

was applied to **1** and **2**. Selective irradiation at H-8 in **2** collapsed the carbon signals for C-4′, which became significantly sharper, thereby indicating that compound **2** has a hydroxymethyl group and a chatechol unit attaching to C-8 and C-7, respectively. Thus, although compound **1** showed an indistinct difference in the carbon signals for C-3′ and 4′ with selective irradiation at H-7 and H-8, the structure of **1** should be of the americanol type. A H-7/H-8 *trans* relationship on the 1,4-benzodioxane ring was elucidated by a large coupling constant between H-7 and H-8 (**1**: 8.2 Hz; **2**: 7.3 Hz). Compounds **1** and **2** had no optical rotation, which was analyzed by HPLC using a chiral column,[15)] indicating that they are racemic with regard to C-7 and C-8 like compounds **1** and **2**. Accordingly, the structure of americanoic acid A methyl ester is formulated as **1**, whereas that of isoamericanoic acid A methyl ester is represented as **2**.

Compound **3** had the molecular formula $C_{19}H_{20}O_6$ as determined by HR-EI-MS. The ^{1}H- and ^{13}C-NMR spectra were very similar to those of **6** and **7**, except for the presence of additional signals at δ_H 3.25 and δ_C 57.3 due to a methoxy group. These data suggest that **3** is a 9-*O*-methyl derivative of **6** or **7**. The methoxy signal showed an HMBC correlation to the C-9′ resonance at δ_C 72.32. It remains problem whether **3** belongs to the americanol or isoamericanol type. HMBC and LSPD were not useful for **3**. Waibel *et al.*[16)] reported a comparatively large difference of 0.2 ppm between H-2′ and H-5′ in americanol A (**6**), whereas the difference between the two protons in isoamericanol A (**7**) is as small as

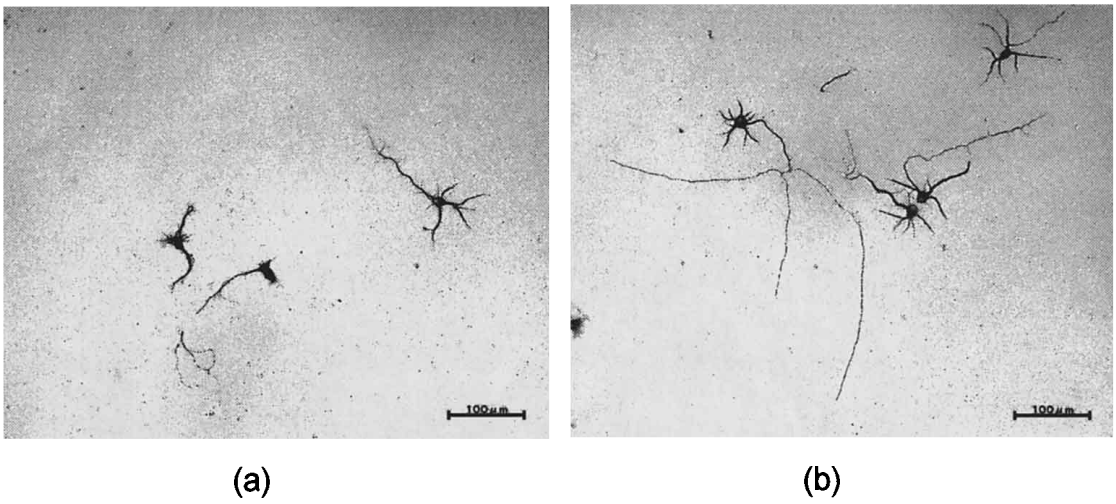

Fig. 3. Neurotrophic Effect of **1** in Primary Cultured Rat Cortical Neurons
(a) Control culture treated with 0.5% EtOH; (b) culture treated with **1** (0.1 μM).

Table 3. Antioxidant Activity of **1**—**9**

	IC_{50} (μM)[a]	
	DPPH radical[b]	O_2^{-} [c]
1	16	68
2	38	64
3	9	8
4	11	9
5	39	29
6	5	24
7	16	23
8	10	9
9	16	58
Catechin	7	4

a) Inhibitory activity was expressed as the mean of 50% inhibitory concentration of triplicate determination, obtained by interpolation of concentration–-inhibition curves. *b*) Chemically stable radical scavenging activity (α,α-diphenyl-β-picrylhydrazyl (DPPH) radical). *c*) Superoxide anion scavenging activity.

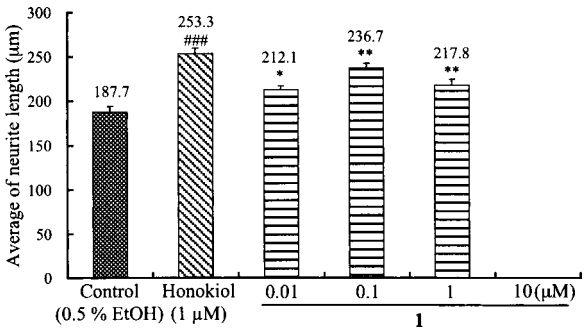

Fig. 4. Morphometric Analysis of the Neurons Affected by **1**
After the neuronal cells (9000 cell cm^{-2}) cultured for 6 d in the presence of 0.5% EtOH, honokiol and **1** were fixed with 4% paraformaldehyde, morphometric analysis was carried out on these neurons according to the criteria described. The data are expressed±S.E. (n=80); Student's t-test *vs.* control, ### $p<0.001$; Dunnett's t-test *vs.* control, * $p<0.05$, ** $p<0.01$.

0.07 ppm. The ^{1}H-NMR spectral data of **3** showed a large difference of 0.2 ppm between H-2′ (δ 6.83) and H-5′ (δ 7.03). Additionally, the ^{13}C-NMR data of **7**, as summarized in Table 3, showed a large difference of 0.94 ppm between C-3′ and C-4′, whereas a difference between the carbon signals of both in **6** is as small as 0.2 ppm. The ^{13}C-NMR data of **3** showed a difference of 0.08 ppm between C-3′ (δ_C 143.28) and C-4′ (δ_C 143.36). These difference values for **3** are consistent with those of the americanol A type. Thus the structure of **3** was determined to be 9′-*O*-methylamericanol A.

We previously reported that americanol A (**6**) and isoamericanol A (**7**) exhibited neurotrophic properties, *i.e.*, the promotion of neurite outgrowth and the enhancement of choline acetyltransferase activity in primary cultured fetal rat cortical neurons.[8,9] Based on those results, we achieved the efficient biomimetic syntheses of **6** and **7** by HRP-catalyzed oxidative phenol coupling of caffeic acid, and dicarboxylic acids **4** and **5**, which are 1,4-benzodioxane-type compounds, were obtained as the main coupling products. Some neurotrophic compounds such as mastigophorene A and B,[17] and honokiol[18] showed antioxidant activity. Therefore, prior to evaluation of neurotrophic activity, compounds **1**—**9** were all tested for antioxidant properties (DPPH and O_2^{-}). The results are summarized in Table 3. Most showed antioxidant activity at an IC_{50} value less than 70 μM. These antioxidant properties are generally due to the cathechol moiety.

Next, we examined the neurotrophic activity of **1**—**5** in primary cultured rat cortical neurons. Americanoic acid A methyl ester (**1**) was found to exhibit potent neurite outgrowth activity at 0.1 μM, as shown in Figs. 3 and 4. The morphometric analyses of the neurons affected by the other compounds were comparable with that in control cultures. Although compounds **4** and **5** did not promote neurite outgrowth, it should be noted that they induced significant neuritogenesis such as increasing the number of neurite branches at a concentration range of from 0.1 μM to 10 μM in a manner similar to basic fibroblast growth factor (bFGF). In particular, americanin-type **4** induced more potent neuritogenesis than isoamericanin-type **5**. These results are shown in Figs. 5 and 6.

Although the relationship between neurotrophic activity and structure must wait for more examples of active compounds, 1,4-benzodioxane-type neolignans and their related compounds may have potential as candidates for nonpeptide neurotrophic agents.

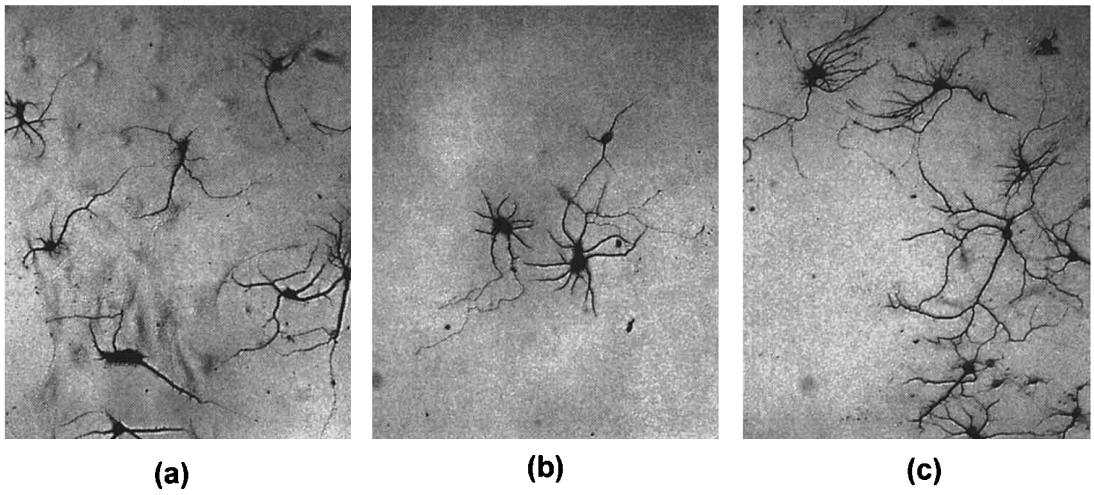

Fig. 5. Neuritogenesis of **4** and **5** in Primary Cultured Rat Cortical Neurons

(a) Control culture treated with 0.5% EtOH; (b) and (c) culture treated with **4** (0.1 μM) and **5** (0.1 μM), respectively.

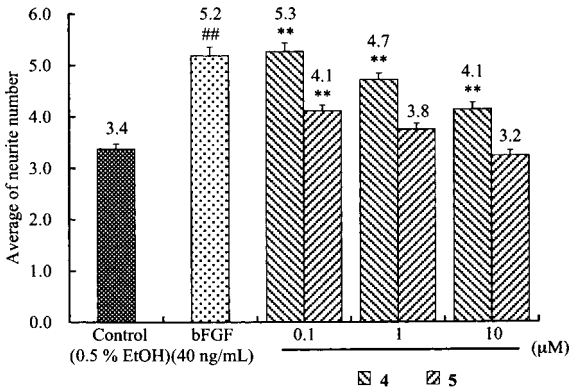

Fig. 6. Morphometric Analysis of the Neurons Affected by **4** and **5**

After the neuronal cells (9000 cell cm^{-2}) cultured for 6 d in the presence of 0.5% EtOH, bFGF, **4**, and **5** were fixed with 4% paraformaldehyde, morphometric analysis was carried out on these neurons according to the criteria described. The data are expressed±S.E. (n=80); Student's t-test *vs.* control, $^{\#\#}p<0.01$; Dunnett's t-test *vs.* control, $^{**}p<0.01$.

Experimental

General Optical rotations were measured with a Jasco DIP-1000 digital polarimeter. IR spectra were recorded on a Jasco FT-IR 5300 IR spectrophotometer. NMR spectra were recorded on a JEOL ECP-400 instrument. MS were recorded on a JEOL AX-500 instrument. Column chromatography was carried out on WAKOGEL C-300 and Sephadex LH-20.

Plant Material The seeds of *P. americana* were collected in Tokushima, Japan, and a voucher specimen is deposited at the Institute of Pharmacognosy, Tokushima Bunri University.

Extraction and Purification Dried seeds (2.6 kg) of *P. americana* collected in Tokushima were extracted three times with MeOH at room temperature for 1 month. The combined MeOH extracts were evaporated *in vacuo* to leave a crude extract (278 g). The extract (57.3 g) was mixed with celite and eluted with *n*-hexane, CH_2Cl_2, EtOAc, EtOAc–MeOH (9 : 1) and MeOH to give five fractions. The third fraction was subjected to column chromatography on silica gel to give fractions 6—16. Fraction 9 was further separated by Sephadex LH-20 and HPLC [Cosmosil 5C_{18}-ARII ϕ 10×250 mm, MeOH–CH_3CN–H_2O (1 : 1 : 2), 2.0 ml min^{-1}] to give **1** (3 mg), **2** (6 mg), **3** (10 mg), and **9** (32 mg). Fraction 4 was separated by Sephadex LH-20 and finally by HPLC [Cosmosil 5C_{18}-ARII ϕ 10×250 mm, MeOH–H_2O (11 : 9), 2.0 ml min^{-1}] to give **7** (9 mg), **8** (1 mg), **9** (12 mg), esculentoside B (1 mg), and esculentside S (23 mg).

Americanoic Acid A Methyl Ester (**1**): $[\alpha]_D^{20}$ ±0° (c=0.31, MeOH); UV λ_{max} (EtOH) nm (ε): 290 (3000), 261 (4500), 206 (30200); EI-MS *m*/*z*: 332 $[M]^+$ (100), 314 (33), 179 (24), 166 (23), 123 (37); HR-EI-MS *m*/*z*: 332.0885 (Calcd for $C_{17}H_{16}O_7$, 332.0896); IR (FT film) cm^{-1}: 3367 (OH), 1694 (CO), 1505 (aroma.); ^{1}H- and ^{13}C-NMR: see Tables 1 and 2.

Isoamericanoic Acid A Methyl Ester (**2**): $[\alpha]_D^{20}$ ±0° (c=0.71, MeOH); UV λ_{max} (EtOH) nm (ε): 288 (5000), 261 (6900), 204 (40000); EI-MS *m*/*z*: 332 $[M]^+$ (100), 314 (31), 179 (28), 166 (26), 123 (35); HR-EI-MS *m*/*z*: 332.0900 (Calcd for $C_{17}H_{16}O_7$, 332.0896); IR (FT film) cm^{-1}: 3380 (OH) , 1698 (CO) , 1505 (aroma.); ^{1}H- and ^{13}C-NMR: see Tables 1 and 2.

9′-*O*-Methylamericanol A (**3**): $[\alpha]_D^{20}$ ±0° (c=0.12, MeOH); UV λ_{max} (EtOH) (ε): 263 (16700), 203 (64300); EI-MS *m*/*z*: 344 $[M]^+$ (100), 166 (58), 148 (67), 123 (61), 110 (37); HR-EI-MS *m*/*z*: 344.1265 (Calcd for $C_{19}H_{20}O_6$, 344.1260); IR (FT film) cm^{-1}: 3262 (OH), 1505 (aroma.); ^{1}H- and ^{13}C-NMR: see Tables 1 and 2.

Neurotrophic Bioassay Neuronal cells were separated from the cerebral hemispheres of a fetal 18-day-old Sprague–Dawley rats (Japan SLC, Inc.) and suspended in 10% FAB/MEM, then seeded at 12000 cells/cm^2 into poly-L-lysine-coated 24-well culture plates.[19] After 48 h, the medium was changed to a serum-free medium, neurobasal medium, supplemented with B27, in the presence or absence of the compounds at 0.1, 1, and 10 μM. After incubation for 5 d, the cells were fixed with 4% paraformaldehyde/PBS for anti-MAP-2 immunohistochemical staining. The neurite outgrowths affected by samples were analyzed under a microscope and photographs taken at magnification of 200.

Superoxide Anion-Scavenging Assay Superoxide anion-scavenging activity was determined using the xanthine/xanthine-oxidase reduced WST-1 system with a SOD assay kit (Dojindo Co. Ltd.)[20,21] according to the manufacturer's protocol.

Radical-Scavenging Activity A solution of 180 μl of DPPH (0.1 mM) in EtOH was added to 20 μl of a solution of the test compounds in EtOH. After 20 min, the absorbance at 517 nm was measured. The scavenging activity of the tested compounds was measured as the decrease in absorbance of DPPH expressed as a percentage of the absorbance of a control DPPH solution without test compounds.

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