Structures of Americanol A and Isoamericanol A Having a Neurotrophic Property from the Seeds of *Phytolacca americana*

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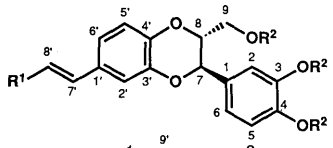
The structures of new *neo*-lignans, isoamericanol A (1) and americanol A (2) isolated from the seeds of *Phytolacca americana* have been elucidated on the basis of spectroscopic data and then confirmed by chemical correlation with the previouslty known isoamericanin A (3) and americanin A (4). Isoamericanol A, americanol A, and americanin A have been found to enhance choline acetyltransferase activity at 10^{-5} M in a cultured neuronal cell system derived from fetal rat hemisphere.

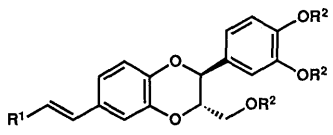
Keywords americanol A: isoamericanol A; americanin A; isoamericanin A; *neo*-lignan; 1,4-benzodioxane; *Phytolacca americana*; Phytolaccaceae; choline acetyltransferase; neurotrophic activity

Phytolacca americana L. (Phytolaccaceae), a plant originating in North America, has been used as a traditional crude drugs instead of the Chinese medicinal plant, *Phytolacca esuclenta*. Its rhizome, in particular, is known as a strong diuretic agent,[1] and the seed elaborates several kinds of antihepatotoxic *neo*-lignans.[2] As a part of our search for neurotrophic substances in natural products,[3,4] we have investigated the methanol extract of the seed of *P. americana*. In a previous paper,[5] we reported the isolation and structure of a prostaglandin I_2 inducer, isoamericanin A (**3**) and also confirmed the reversed structure[6,7] of americanin A (**4**) based on long-range selective decoupling (LSPD) technique. Our proposed structures were later substantiated by Tanaka's syntheses of both compounds.[8] A further study for biologically active substances in the seeds of *Phytolacca americana* resulted in the isolation of other new *neo*-lignans **1** and **2** named isoamericanol A and americanol A. These two new *neo*-lignans have been found to enhance choline acetyltransferase (ChAT) activity in a primary neuronal cell culture of fetal rat hemisphere.[9,10] In this paper, we deal with the isolation and structure of new compounds **1** and **2** and report their neurotrophic properties.

Isoamericanol A (**1**) and americanol A (**2**) were isolated as colorless prisms by successive chromatographies on silica gel, Sephadex LH-20, and reverse phase RP-8. Compounds **1** and **2** had not only the same molecular formula $C_{18}H_{18}O_6$ established by the high resolution electron im-



1: $R^1 = CH_2OH$, $R^2 = H$
1a: $R^1 = CH_2OAc$, $R^2 = Ac$
3: $R^1 = CHO$, $R^2 = H$
3a: $R^1 = CHO$, $R^2 = Ac$



2: $R^1 = CH_2OH$, $R^2 = H$
2a: $R^1 = CH_2OAc$, $R^2 = Ac$
4: $R^1 = CHO$, $R^2 = H$
4a: $R^1 = CHO$, $R^2 = Ac$

Chart 1

TABLE I. ^{1}H-NMR Data (400 MHz) of **1**, **1a**, and **2a**

Proton	**1**[a]	**1a**[b]	**2**[a]	**2a**[b]
2	6.80 d (2.0)[c]	7.29—7.24	6.80 d (2.0)	7.29—7.24
5	6.75 d (7.8)		6.75 d (7.7)	
6	6.70 dd (7.8, 2.0)		6.70 dd (7.7, 2.0)	
7	4.84 d (7.5)	4.96 d (7.8)	4.82 d (7.7)	4.96 d (7.7)
8	4.03 ddd (7.5, 5.1, 3.1)	4.21 ddd (7.8, 3.9, 3.9)	4.03 ddd (7.7, 4.3, 2.6)	4.21 ddd (7.7, 4.4, 3.7)
9	3.51 ddd (12.2, 5.1, 3.1)	4.38 dd (12.4, 3.9)	3.52 ddd (12.2, 5.0, 2.6)	4.38 dd (12.4, 3.7)
	3.32 ddd (12.2, 5.1, 5.1)	4.00 dd (12.4, 3.9)	3.32 ddd (12.2, 5.0, 4.3)	4.00 dd (12.4, 4.4)
9-OH	4.91 t (5.1)	—	4.90 t (5.0)	—
2′	6.96 d (2.0)	7.03 d (2.0)	6.97 d (2.0)	7.03 d (1.7)
5′	6.87 d (8.1)	6.91 d (8.2)	6.83 d (8.2)	6.90 d (8.4)
6′	6.92 dd (8.1, 2.0)	6.95 dd (8.2, 2.0)	6.90 dd (8.2, 2.0)	6.93 dd (8.4, 1.7)
7′	6.42 d (15.9)	6.54 dt (15.6, 1.1)	6.44 d (15.5)	6.59 dt (15.8, 1.3)
8′	6.20 dt (15.9, 5.4)	6.14 dt (15.6, 6.4)	6.21 dt (15.5, 5.2)	6.16 dt (15.8, 6.7)
9′	4.08 t (5.4)	4.70 dd (6.4, 1.1)	4.08 t (5.2)	4.70 dd (6.7, 1.3)
9′-OH	4.78 t (5.2)	—	4.79 d (5.2)	—
$COCH_3$		2.30 s, 2.29 s		2.30 s, 2.29 s
		2.09 s, 2.04 s		2.09 s, 2.04 s

a) In DMSO-d_6. *b*) In $CDCl_3$. *c*) *J*/Hz in parentheses.

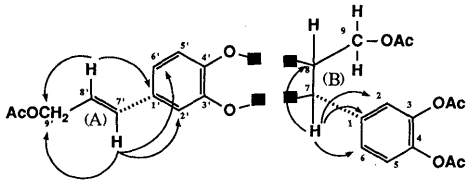


Fig. 1. Long-Range ^{13}C and ^{1}H Correlations Based on HMBC Spectra of Isoamericanol A Tetraacetate (**1a**) and Americanol A Tetraacetate (**2a**)

Bond formations are indicated by dotted lines.

TABLE II. ^{13}C-NMR Data[a] (100 MHz) of **1**, **1a**, **2**, and **2a**

Carbon	**1**[b]	**1a**[c]	**2**[b]	**2a**[c]
1	127.60	134.56	127.54	134.69
2	114.94	122.52	114.91	122.67
3	145.24	142.53	145.24	142.95
4	145.78	142.76	145.78	142.99
5	115.49	123.70	115.46	123.86
6	118.83	125.21	118.80	125.37
7	75.63	75.95	75.66	76.18
8	78.30	75.41	78.21	75.45
9	60.17	62.38	60.14	62.52
1′	130.30	130.56	130.48	130.87
2′	114.18	115.04	114.12	115.15
3′	142.66	142.83	142.99	142.72
4′	143.60	143.26	143.23	143.43
5′	116.73	117.13	116.69	117.33
6′	119.37	120.47	119.27	120.54
7′	128.15	133.36	128.15	133.60
8′	128.81	122.15	128.81	122.31
9′	61.57	64.80	61.54	64.95
$\underline{CO}CH_3$		170.39		170.63
		169.97		170.20
		167.53		167.70 (×2)
		167.50		
$CO\underline{CH_3}$		20.66		20.76
		20.30 (×2)		20.40 (×2)
		20.23		20.34

a) Assignments were based on the C/H COSY and HMBC spectra. *b*) In DMSO-d_6. *c*) In $CDCl_3$.

pact mass spectra (HREIMS) at *m/z* 330.1100 [M^+], but also almost identical physical data. Their infrared (IR) spectra displayed the presence of hydoxy (3300 cm^{-1}) and aromatic (1580 and 1510 cm^{-1}) groups, and additional styrene moieties, the presence of which was also supported by the ultraviolet (UV) spectra.[11] Moreover, the ^{1}H- and ^{13}C- nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra (Tables I and II) for **1** and **2** showed an extreme similarity to each other. Usual acetylations of **1** and **2** yielded tetraacetates **1a** and **2a**, the ^{1}H-NMR spectra of which could not again make them distinguishable. The ^{1}H-NMR spectra (Table I) of **1a** and **2a** indicated the presence of two aromatic acetyl (δ 2.29 and 2.30) and two aliphatic acetyl (δ 2.04 and 2.09) groups, a 1,3,4-trisubstituted benzene ring [δ 6.91 (d, J=8.2 Hz), 6.95 (dd, J=8.2, 2.0 Hz), and 7.03 (d, J=2.0 Hz) for **1a**: δ 6.90 (d, J=8.4 Hz), 6.93 (dd, J=8.4, 1.7 Hz), and 7.03 (d, J=1.7 Hz) for **2a**], and an additional trisubstituted benzene moiety as well as of the partial structures (A) and (B) shown in Fig. 1, which were obtained on the basis of analyses of the ^{1}H–^{1}H and ^{13}C-^{1}H correlation spectroscopies (H/H and C/H COSYs). The ^{13}C-NMR data of **1a** and **2a**, in contrast, were closely related to those of isoamericanin A tetraacetate (**3a**) and americanin A tetraacetate (**4a**)[5] except for the presence of oxygen-bearing methylene signals (δ 64.80 for **1a** and 64.95 for **2a**) instead of the aldehyde carbonyl carbon resonances existing in **3a** and **4a**. These spectral features disclosed that **1** and **2** comprised the same structure units involving a 1,4-dioxane ring as **3** and **4**, and the aldehyde groups at C-9′ existing in **3** and **4** were just reduced into the hydroxy groups in **1** and **2**. In fact, the EIMS spectra (Fig. 2) of **1** and **2** revealed the prominent fragment ions at *m/z* 166 and 148 derived from a retro-Diels Alder cleavage, in addition to the dihydroxy tropylium cation at *m/z* 123 as a base ion peak.[12] Further, the ^{1}H detected multiple bond heteronuclear multiple quantum coherence spectra (HMBC) of **1a** and **2a** summarized in Fig. 1 allowed us to connect one (A) of the partial structure units to the C-1′ (δ 130.56 for **1a**; 130.87 for **2a**) by the distinct three bond correlations of the H-7′ (δ 6.54 for **1a**, 6.59 for **2a**) to the C-1′ aromatic quaternary carbons. It also permitted linking of the partial structure (B) to the C-1 (δ 134.56 for **1a**; 134.69 for **2a**) based on the correlation of the H-7 with the corresponding carbon aromatic resonances (C-1, 2, and 6) through two or three bonds; thereby the dioxane ring with a trans relationship ($J_{7,8}$=7.5 Hz) at the C-7 and C-8 was elucidated. Thus, the structures of isoamericanol A and americanol A can be formulated as **1** or **2**, but we have met the same difficulty in distinguishing these two compounds as we did with isoamericanin A (**3**) and americanin A (**4**).[13] This ambiguity was cleared up by the high performance liquid chromatography (HPLC) comparison of reduced compounds **5** and **6** derived from isoamericanin A (**3**) and americanin A tetraacetate (**4a**) by $NaBH_4$ and $CeCl_3$[14] with **1** and **2**. In HPLC analysis, compounds **5** and **6** had retention times corresponding to those of **1** and **2**, respectively. These results thus corroborated the structures of isoamericanol A and americanol A to be **1** and **2**, respectively. Neither compound **1** nor **2** showed optical rotation or circular dichroism (CD) Cotton curve indicating racemic mixtures, and could presumably be biosynthetic precursors for optical inactive isoamericanin A (**3**) and americanin A (**4**).

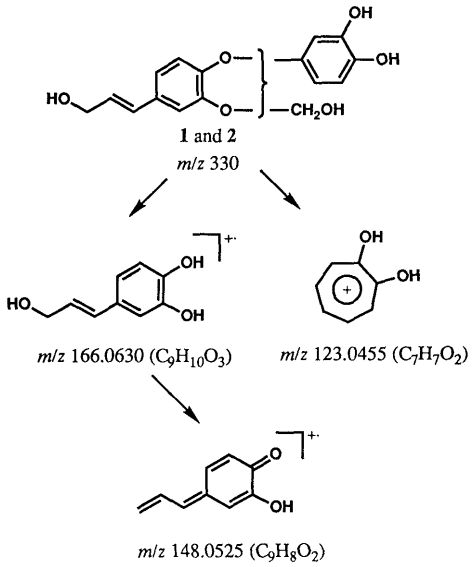
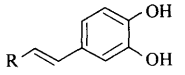


Fig. 2. Mass Spectral Fragmentations of Isoamericanol A (**1**) and Americanol A (**2**)

TABLE III. Effect of Each Compound on ChAT Activity in a Primary Culture of Fetal Rat Cerebral Hemisphere 10 d after Seeding

Compound	Conc. (M)	ChAT activity	
		pmol/min/dish	pmol/min/mg·protein
0.5% EtOH		4.15±0.26	35.5±1.6
1	10^{-5}	12.81±0.46[a]	78.1±4.1[a]
2	10^{-5}	16.68±0.01[a]	90.5±6.5[a]
3	10^{-5}	15.34±0.26[a]	98.6±3.0[b]
9	10^{-5}	1.84±0.61[b]	15.6±6.5[b]
0.5% EtOH		7.51±0.27	53.5±1.6
3	10^{-5}	23.65±1.79[c]	154.4±14.5[b]
7	10^{-5}	10.56±0.38	79.0±9.7
8	10^{-5}	4.61±0.32	56.9±3.6

The dissociated Trypan blue-negative cells derived from fetal rat (17th d of gestation) cereberal hemisphere were seeded at a density of 1.5×10^6 cells/35 mm dish containing 2.5 ml of 20% FCS-DMEM. Each compound dissolved in 0.5% EtOH was added 24 h after seeding. Each value represents the mean±S.E. ($N=3$). *a*) $p<0.001$, *b*) $p<0.01$, *c*) $p<0.05$ *vs.* 0.5% EtOH.



7: R=CH_2OH
8: R=CHO
9: R=CO_2H

In a primary culture of fetal rat cerebral hemisphere,[9] isoamericanol A (**1**), americanol A (**2**), and isoamericanin A (**3**) enhanced neurrite sprouting morphologically, and also increased ChAT activity[10] at 10^{-5} M. However, the cinnamic acid derivatives **7**, **8**, and **9** regarded as the monomeric units of the active *neo*-lignans failed to enhance ChAT activity at the same concentration. On the contrary, cinnamic aldehyde (**8**) which is one unit of isoamericanin A was found to reduce ChAT activity. Although only a limited number of compounds had been screened to date by this primary neuronal cell culture, a dimeric stucture having a 1,4-dioxane ring may be essential for an interesting neurotrophic activity.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and were uncorrected. UV spectra were recorded on a Shimadzu UV-300 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained at 400 MHz (^{1}H-NMR) and 100.16 MHz (^{13}C-NMR), using a JEOL GX-400 spectrometer. Chemical shifts were expressed in δ (ppm) downfield from tetramethylsilane as an internal standard. Abbreviations are as follows: s, singlet; d, doublet; t, triplet; m, multiplet. The EI- and HREIMS were taken on a JEOL JMS SX-102. HPLC was performed on a Jasco-PU pump using Cosmosil 5C_{18} (10 i.d. × 250 mm).

Extraction and Purification Dried seeds (2.6 kg) of *Phytolacca americana* collected in Tokushima were extracted three times with MeOH. The combined MeOH extracts were evaporated *in vacuo* to leave a crude extract (278 g), which was chromatographed over silica gel (Wakogel C-200) using a stepwise gradient of CH_2Cl_2–MeOH (9:1, 4:1, and then 7:3) to divide into the seven fractions. The sixth fraction (45 g) was rechromatographed on Sephadex LH-20 eluting MeOH to give frs. 1—20. Fraction 12 (7.8 g) was purified by silica gel (Wakogel C-300) chromatography eluting with $CHCl_3$–MeOH (95:5) to yield isoamericanin A (**3**) (0.985 g) and americanin A (**4**) (0.160 g) as a crystal, respectively. Fraction 14 (15.2 g) was subjected to a Toyopearl HW-40F chromatography with MeOH–H_2O (3:2) and then purified by Lobar RP-8 (type C) using MeOH–H_2O (1:1) to give isoamericanol A (**1**) (1.60 g) and americanol A (**2**) (0.33 g) as a crystal, respectively.

Isoamericanol A (1) Colorless prism, mp 157—159 °C (EtOAc–acetone). $[\alpha]_D^{27}$ ±0° ($c=1.05$, EtOH). HREIMS *m/z*: 330.1100 [M$^+$], Calcd 330.1104 for $C_{18}H_{18}O_6$. EIMS *m/z* (rel. int.): 330 [M$^+$] (11.4), 166 (55), 148 (59), 123 (100). UV λ_{max}^{EtOH} nm (ε): 262 (16100), 267 (16500), 300 (5800), 312 (3900). IR ν_{max}^{KBr} cm^{-1}: 3300 (OH), 1610 (C=C), 1580, 1510 (aroma.). ^{1}H- and ^{13}C-NMR: see Tables I and II.

Isoamericanol A Tetraacetate (1a) A mixture of **1** (34 mg), acetic anhydride (2 ml), and puridine (2 ml) was allowed to stand at room temperature for 3 h. Ice-water was added to the reaction mixture, and then the resultant solution was extracted with EtOAc. The organic layer was washed with 10% HCl sol. and sat. NaCl sol., and dried over $MgSO_4$. After filtration, the solvent was evaporated *in vacuo* to afford the tetraacetate **1a** (54 mg) as an oil. EIMS *m/z* (rel. int.): 498 [M$^+$] (24), 456 [M−42] (6), 396 [M−42−60] (4), 354 [M−60−42×2] (5), 294 (10), 250 (16), 208 (42), 148 (60), 43 (100). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1770 ($COCH_3$), 1740 ($COCH_3$), 1590, 1502 (aroma.), 1240. ^{1}H- and ^{13}C-NMR: see Tables I and II.

Americanol A (2) Colorless prism, mp 125—128 °C (EtOAc–acetone). $[\alpha]_D^{27}$ ±0° ($c=1.11$, EtOH), HREIMS *m/z*: 330.1100 [M$^+$], Calcd 330.1104 for $C_{18}H_{18}O_6$. EIMS *m/z* (rel. int.): 330 [M$^+$] (47), 166 (70), 148 (87), 123 (100). UV λ_{max}^{EtOH} nm (ε): 260 (18800), 268 (18800), 300 (6400), 312 (4100). IR ν_{max}^{KBr} cm^{-1}: 3300 (OH), 1610 (C=C), 1580, 1510 (aroma.). ^{1}H- and ^{13}C-NMR: see Tables I and II.

Americanol A Tetraacetate (2a) A mixture of **2** (131 mg), acetic anhydride (1.5 ml), and pyridine (1.6 ml) was allowed to stand at room temperature for 3 h. Usual work-up afforded the tetraacetate **2a** (206 mg) as an amorphous material. EIMS *m/z* (rel. int.): 498 [M$^+$] (38), 456 [M−42] (6), 396 [M−42−60] (14), 354 (9), 366 (10), 294 (30), 250 (28), 208 (62), 148 (95), 43 (100). IR ν_{max}^{KBr} cm^{-1}: 1770 ($COCH_3$), 1740 ($COCH_3$), 1590, 1510 (aroma.), 1240. ^{1}H- and ^{13}C-NMR: see Tables I and II.

Reduction of Isoamericanin A (3) To a mixture of **3** (13.7 mg, 0.042 mmol) and $CeCl_3\cdot7H_2O$ (17.9 mg, 0.04 mmol) in MeOH (0.2 ml) was added $NaBH_4$ (3.2 mg, 0.084 mmol) with vigorous stirring at room temperature. The reaction mixture was stirred until americanin A (**3**) had disappeared on thin layer chromatography (TLC). 0.1 N HCl sol. was added to the reaction mixture and then the solution was extracted with EtOAc. The organic layer was washed with sat. NaCl sol. and dried over $MgSO_4$. The removal of solvent *in vacuo* gave an oil (12.4 mg), which was purified by column chromatography on silica gel eluting with $CHCl_3$–MeOH (9:1) to afford a reduced product **5** (2.5 mg). Its spectral data and HPLC behavior [retention time, 31 min; mobile phase, MeOH–CH_3CN–H_2O (2:2.5:5.5); flow rate, 2.5 ml/min; detector, UV 254 nm] were identical with those of isoamericanol A (**1**).

Reduction of Americanin A Tetraacetate (4a) To a mixture of **4a** (21 mg, 0.047 mmol) and $CeCl_3\cdot7H_2O$ (17.6 mg, 0.047 mmol) in MeOH (1 ml) and sat. $NaHCO_3$ sol. (0.2 ml) was added $NaBH_4$ (3.4 mg, 0.09 mmol). The reaction mixture was stirred at room temperature for 3 h. The reaction was terminated by addition of 0.1 N HCl sol. and then the solution was extracted with EtOAc. The organic layer was washed with sat. NaCl sol. and dried over $MgSO_4$. After filtration, the solvent was evaporated *in vacuo* to leave the residue (25mg), which was purified by silica gel chromatography ($CHCl_3$–MeOH; 95:5) to afford an alcohol **6**. Its HPLC behavior [retention time 33 min] was in complete agreement with that of americanol A (**2**).

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