Triterpene Glycosides from the Cultures of *Phytolacca americana*

Hironobu TAKAHASHI, Yuki NAMIKAWA, Masami TANAKA, and Yoshiyasu FUKUYAMA*

Institute of Pharmacognosy, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770–8514, Japan. Received August 21, 2000; accepted October 14, 2000

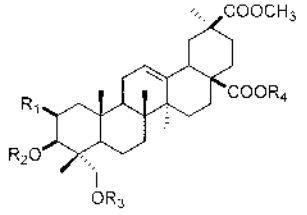
A new triterpene glycoside 1 was isolated together with the five known triterpene glycosides 2—6 from the cultures of *Phytolacca americana.* The structure of 1 was elucidated by analysis of spectroscopic data and comparison of its NMR data with those of 2—7 and chemical degradation.

Key words *Phytolacca americana*; triterpene glycoside; cell culture

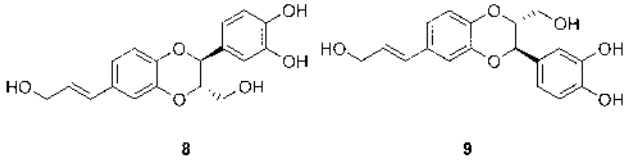
Phytolacca americana L. (Phytolaccaceae) is a perennial plant originating from North America. Its rhizome has been used as a traditional crude diuretic drug in spite of having strong toxicity. A number of triterpenes, their glycosides, and neolignanes have been isolated from the roots of *P. americana* and identified.[1—7] As a part of our search for neurotrophic substances in natural products, we reported the isolation and structure of americanol A (**8**) and isoamericanol A (**9**) from the seeds of *P. americana.*[8,9] Moreover, we accomplished the convenient syntheses of americanol A (**8**) and isoamericanol A (**9**) by horseradish peroxidase (HRP) catalyzed oxidative coupling of caffeic acid.[10] In this paper, we describe our next attempt aiming at production of neurotrophic substances **8** and **9** from the cultures of *P. americana.* First of all, we tried to establish the cell cultures of *P. americana* independently of the previously known procedure.[11]

A cell culture of *P. americana* was cultured in the Murashige & Skoog (MS) liquid medium[12] (pH 5.6) containing 10 mM 1-naphthaleneacetic acid (1-NAA) and 3% of glucose, on a rotary shaker (90 rpm) at 25 °C. The cell cultures were subcultured at intervals of 4 weeks. The filtered cells were freeze-dried and extracted with methanol. The methanol extract was subjected to silica gel, Sephadex LH-20 and HPLC (octadecyl silica gel (ODS) column) to afford a new triterpene glycoside **1** and the five known triterpene glycosides, esculentoside B (**2**),[4,13,14] phytolaccoside E (**3**),[3,4,13] esculentoside S (**4**),[13,15] esculentoside L1 (**5**),[13,15] and esculentoside G (**6**).[3,13,14] The spectral data of **2**—**6** were in full agreement with those described in the literature.

Compound **1** had the molecular formula $C_{42}H_{66}O_{15}$ determined by its high-resolution FAB-MS at *m/z* 833 $[M+Na]^+$ and showed IR absorption at 3387 and 1712 cm^{-1} attributable to a hydroxyl and a carbonyl group, respectively. The ^{1}H-NMR (CD_3OD) spectrum of **1** revealed the signals due to five tertiary methyl groups at δ_H 0.71, 0.82, 0.97, 1.12, and 1.17, one methoxyl group at δ_H 3.68 and an olefin proton at δ_H 5.28 as well as anomeric protons at δ_H 4.47 (d, J=7.1 Hz), 4.67 (d, J=7.7 Hz) indicating the presence of two sugars. Acetylation of **1** gave the heptaacetate **1a** (*m/z* 1127 $[M+Na]^+$), which was then treated with trimethylsilyldiazomethane yielding the methyl ester **1b** (*m/z* 1141 $[M+Na]^+$), thereby indicating the presence of seven hydroxyl groups and one carboxyl group. The ^{13}C-NMR (CD_3OD) (Table 1) and distortionless enhancement by polarization transfer (DEPT) spectra displayed the 42 carbons which consisted of two carbonyl groups (δ_C 184.1, 176.7), one trisubstituted double bond (δ_C 122.5, 144.5), two anomeric carbons (δ_C 105.3, 104.0), one oxygen-bearing methylene group (δ_C 50.9), five methyl groups, ten methylenes, three methines, six quaternary carbons and eleven oxygen-bearing carbons. The acid hydrolysis of **1** afforded D-xylose and D-glucose, which were identified with authentic sugars by using chiral HPLC detection. Additionally, the coupling constants [δ_H 4.47 (d, J=7.1 Hz), 4.67 (d, J=7.7 Hz)] for the anomeric protons of xylose and glucose in **1** indicated that the anomeric configurations of D-xylose and D-glucose are β. These results indicated that **1** was the triterpene glycoside linked with D-xylose and D-glucose. In the ^{13}C-NMR spectrum, the aglycone part of **1** was similar to phytolaccinic acid (**7**)[13] except for C-3 which was shifted down-field by 8.1 ppm in comparison with that of **7**, thus indicating that the sugar part was attached at the C-3 position. In order to confirm the linkages of the two sugar parts and the structure of the aglycone, heteronuclear multi-bond correlation (HMBC) experiments (Fig. 1) were carried out. Correlations were observed between the H-1 signal at δ 4.47 in xylose and the C-3 signal (δ_C 83.9) of the aglycone and between the H-1 signal at δ 4.67 in glucose and the C-2 signal (δ_C 81.2) of xy-



1 R_1 = H, R_2 = xyl(4→1)glc, R_3 = H, R_4 = H
1a: R_1 = H, R_2 = xyl(OAc)$_2$(4→1)glc(OAc)$_4$, R_3 = Ac, R_4 = H
1b: R_1 = H, R_2 = xyl(OAc)$_2$(4→1)glc(OAc)$_4$, R_3 = Ac, R_4 = CH_3
2 R_1 = OH, R_2 = glc, R_3 = H, R_4 = H
3 R_1 = OH, R_2 = xyl(4→1)glc, R_3 = H, R_4 = H
4 R_1 = OH, R_2 = xyl, R_3 = H, R_4 = glc
5 R_1 = H, R_2 = xyl(2→1)glc, R_3 = H, R_4 = glc
6 R_1 = OH, R_2 = xyl(4→1)glc, R_3 = H, R_4 = glc
7 R_1 = H, R_2 = H, R_3 = H, R_4 = H



8 **9**

Chart 1

* To whom correspondence should be addressed. e-mail: fukuyama@ph.bunri-u.ac.jp

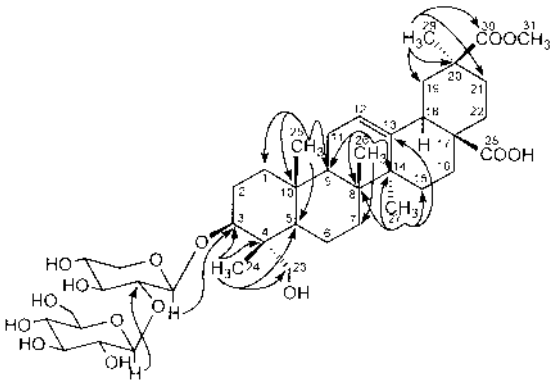


Fig. 1. HMBC Correlations of **1**

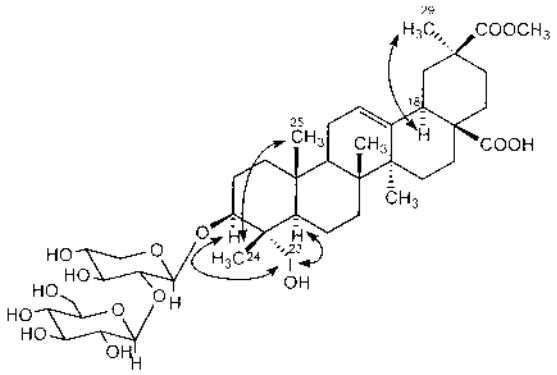


Fig. 2. ROESY Correlations of **1**

lose. In addition to these HMBC, the other methy groups at δ_H 0.71, 0.82, 0.97, 1.12, and 1.17 had correlations in the HMBC spectrum 1 as shown in Fig. 1 confirming the structure of the aglycone part of **1**. These results showed that the aglycone part was **7**, and D-xylose was attached at the C-3 position and D-glucose was attached at the C-2 of xylose. The relative stereochemistry of the aglycone was determined to be identical with that of **7** by rotating frame nuclear Overhauser and exchange spectroscopy (ROESY) experiments (Fig. 2). Thus, compound **1** was represented as 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl] phytolaccinic acid. Furthermore, we assigned the ^{13}C-NMR spectral data for **2**—**6** in CD_3OD which are summarized in Table 1.[16]

In this study, we obtained six triterpene glycosides, among which three compounds **2**, **3** and **6** were isolated from the same plant roots,[3,4] and **4** and **5** were reported from the roots of *P. acinosa*[13] and *P. dodecandra.*[15] Although, the production of betacyanin from the cell cultures of *P. americana*[11] has been reported, this is the first report on the production of the triterpene glycosides from the cultures of *P. americana.* However, we did not succeed in the production of **8** and **9**. We are continuing our efforts to find culture conditions for the production of **8** and **9**.

Table 1. ^{13}C-NMR (CD_3OD) Data for **1**—**6**

	1	**1** (py)	**2**	**2** (py)	**3**	**4**	**5**	**6**	**7** (py)
1	39.5	38.2	44.4	43.6	44.4	44.5	39.6	44.5	38.8
2	26.6	25.5	71.1	70.4	71.5	71.6	26.6	71.5	27.7
3	83.9	81.6	83.5	82.2	83.4	83.4	83.9	83.4	73.5
4	44.1	42.9	43.1	42.2	43.1	43.1	44.1	43.1	42.9
5	48.2	47.4	48.0	47.1	48.0	48.0	48.2	48.1	48.7
6	18.9	17.6	18.6	17.3	18.5	18.6	18.8	18.6	18.6
7	33.5	32.4	33.5	32.3	33.4	33.4	33.4	33.4	33.0
8	40.5	39.1	40.6	39.2	40.6	40.7	40.6	40.7	39.8
9	49.3	47.6	49.3	47.9	49.3	49.4	49.9	49.4	48.2
10	37.7	36.3	37.5	36.3	37.5	37.5	37.6	37.5	37.2
11	24.5	23.2	24.6	23.3	24.6	24.6	24.6	24.7	23.9
12	123.5	122.5	124.2	122.7	124.2	124.2	124.3	124.4	123.7
13	145.6	144.5	144.9	143.8	144.9	144.5	144.5	144.5	144.5
14	42.9	41.5	43.0	41.5	43.0	43.0	42.9	43.0	42.1
15	29.1	27.9	28.8	27.7	28.8	28.8	28.9	28.8	28.4
16	24.5	23.4	24.3	23.2	24.2	24.1	24.1	24.1	23.9
17	47.6	45.7	47.0	45.5	47.0	47.4	47.4	47.4	46.2
18	44.3	42.9	44.0	42.8	44.0	43.9	43.9	43.9	43.4
19	43.8	42.5	43.4	42.0	43.3	43.3	43.3	43.3	42.1
20	45.1	43.6	45.0	43.5	45.0	44.9	44.9	44.9	44.2
21	31.6	30.4	31.3	30.2	31.3	31.3	31.3	31.3	30.8
22	35.4	34.1	35.0	33.9	35.0	34.4	34.4	34.4	34.5
23	64.6	64.2	65.4	64.6	65.3	65.4	64.6	65.4	68.0
24	13.4	12.8	14.7	14.4	14.7	14.7	13.3	14.7	13.1
25	16.4	15.5	17.5	16.6	17.5	17.6	16.5	17.6	16.2
26	18.1	17.0	17.8	16.8	17.8	17.7	17.7	17.7	17.5
27	26.4	25.4	26.4	25.5	26.4	26.3	26.3	26.3	26.1
28	184.2		178.8		181.3	177.5	177.5	177.5	179.8
29	28.9	27.9	28.7	27.8	28.7	28.6	28.6	28.6	28.4
30	179.0	176.7	178.8	176.5	178.8	178.7	178.7	178.7	177.2
31	52.3	50.9	52.3	51.0	52.3	52.3	52.4	52.4	51.7
Xyl-1	104.5	104.0	106.4	106.2	106.3	106.4	104.5	106.3	
2	81.2	83.2	75.3	74.7	75.1	75.2	81.2	75.1	
3	78.1	77.5	78.1	77.9	76.3	78.0	78.1	76.3	
4	71.0	70.1	71.5	70.4	78.5	71.1	71.0	78.5	
5	66.6	66.1	66.9	66.6	64.6	66.9	66.6	64.6	
Glu-1	105.1	105.3			103.5		105.1	103.4	
2	76.1	76.1			74.6		76.1	74.6	
3	77.8	77.4			78.1		77.8	77.8	
4	71.7	70.9			71.6		71.7	71.6	
5	78.3	77.6			77.8		78.4	78.1	
6	63.0	62.0			62.6		63.0	62.6	
Glu-1′						95.7	95.7	95.7	
2′						73.9	73.9	73.9	
3′						78.3	78.3	78.3	
4′						71.0	71.0	71.0	
5′						78.7	78.7	78.7	
6′						62.3	62.3	62.3	

Experimental

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 Varian Unity 600 instrument. MS were recorded on a JEOL AX-500 instrument.

Plant Material Fresh stems of *P. americana* plants, grown in the botanical garden of Tokushima Bunri University, were washed in water, dipped for 30 s in 70% EtOH, sterilized in a 1% sodium hypochlorite solution for 30 s, and washed three times with sterilized H_2O. The stems were placed on MS medium containing 1 mM 1-NAA, 3% of glucose and 0.9% agar and incubated at 25 °C. The cell cultures then subcultured on the same medium several times. The cell cultures were routinely subcultured using the same medium under continuous light of 2000 lux. The suspension culture was cultivated on a rotary shaker at 90 rpm in 300 ml flask with 100 ml of same liquid medium and subcultured at intervals of 4 weeks.

Extraction and Isolation The MeOH extract (7.9 g) of the freeze-dried cell culture material (35.3 g) was chromatographed on silica gel (WACO-GEL, C-300) eluting with a $CHCl_3$–MeOH gradient and elutant were separated into 1—12 fractions. Fraction 8 was purified by Sephadex LH-20 chromatography [$CHCl_3$: MeOH (1 : 2)] and silica gel chromatography [Merck, 230—400 mesh, $CHCl_3$: MeOH (2 : 1)] to give **2** (29 mg). Fraction 10 was purified by Sephadex LH-20 [$CHCl_3$: MeOH (1 : 2)] and silica gel chromatography [Merck, $CHCl_3$: MeOH (2 : 1)] and finally by medium pressure liquid chromatography (MPLC) [Merck, Lobar RP-8; MeOH–H_2O (3 : 1)] to give **1** (23 mg). Fraction 11 was purified by silica gel chromatography [Merck, $CHCl_3$: MeOH (2 : 1)] and MPLC [Merck, Lobar RP-8; (3 : 1)] to give **3** (13 mg). Fraction 12 was purified by silica gel chromatography

[WACOGEL, $CHCl_3$: MeOH : H_2O (7 : 3 : 0.5)] and finally by HPLC [cosmosil $5C_{18}$-AR (ϕ10×250 mm); MeOH–H_2O (3 : 1; 2.0 ml min^{-1}); UV 220 nm] to give **4** (23 mg), **5** (36 mg) and **6** (26 mg).

3-*O*-[β-D-Glucopyranosyl-(1→2)-β-D-xylopyranosyl]phytolaccinic Acid (1) Amorphous powder. $[\alpha]_D^{21.8}$ +46.1° (c=0.74, MeOH). IR cm^{-1}: 3387 (OH), 1712 (C=O). HR-FAB-MS *m/z* 833.4295 (Calcd for $C_{42}H_{66}O_{15}Na$, 833.4299). ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 0.71 (3H, s), 0.82 (3H, s), 0.97 (3H, s), 1.12 (3H, s), 1.17 (3H, s), 3.20 (3H, m), 3.27 (1H, d, J=11.5 Hz), 3.34 (1H, t, J=7.8 Hz), 3.48 (1H, m), 3.50 (1H, dd, J=3.8, 9.0 Hz), 3.59 (1H, m), 3.60 (1H, dd, J=6.0, 12.0 Hz), 3.68 (3H, s), 3.71 (1H, d, J=11.5 Hz), 3.82 (1H, dd, J=2.2, 12.0 Hz), 3.83 (1H, dd, J=5.2, 10.2 Hz), 4.47 (1H, d, J=7.1 Hz), 4.67 (1H, d, J=7.7 Hz), 5.28 (1H, br s). ^{13}C-NMR: Table 1.

Acetylation of 1 Acetic anhydride (1 ml) was added to a solution of **1** (30 mg) in pyridine (1 ml), and the mixture was stored at room temperature for 16 h. The reaction mixture was poured onto water and extracted with EtOAc. After evaporation of solvent, the residue was chromatographed by silica gel (hex–EtOAc, 1 : 1) to give the acetate **1a** (28 mg) as an oil. HR-FAB-MS: *m/z* 1127.5050 (Calcd for $C_{56}H_{80}O_{22}Na$, 1127.5037). IR cm^{-1}: 3210 (OH), 1751 (C=O). ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 0.72 (3H, s), 0.74, 0.94 (3H, s), 1.11 (3H, s), 1.15 (3H, s), 2.000 (3H, s), 2.007 (3H, s), 2.009 (3H, s), 2.02 (3H, s), 2.09 (3H, s), 2.109 (3H, s), 2.112 (3H, s), 2.68 (1H, dd, J=3.8, 13.7 Hz), 3.36 (1H, dd, J=8.8, 12.1 Hz), 3.52 (1H, dd, J=4.7, 11.8 Hz), 3.68 (1H, m), 3.70 (3H, s), 3.71 (1H, dd, J=6.8, 8.8 Hz), 4.01 (1H, d, J=11.5 Hz), 4.02 (1H, dd, J=5.5, 12.1 Hz), 4.08 (1H, dd, J=2.5, 12.6 Hz), 4.16 (d, J=11.5 Hz), 4.30 (1H, dd, J=5.2, 12.3 Hz), 4.45 (1H, d, J=6.8 Hz), 4.66 (1H, d, J=8.0 Hz), 4.86 (1H, ddd, J=5.2, 8.5, 8.8 Hz), 4.92 (1H, dd, J=8.0, 9.6 Hz), 5.11 (2H, m), 5.35 (1H, dd, J=3.3, 3.6 Hz).

Methylation of 1a Trimethylsilyldiazomethane (0.5 ml, in 1.0 M hexane) was added to a solution of **1a** (28 mg) in methanol (1 ml), and the mixture was stirred at room temperature for 14 h. After evaporation of solvent, the residue was chromatographed on Sephadex LH-20 ($CHCl_3$–MeOH, 1 : 1) to give the methyl ester **1b** (21 mg) as an oil. FAB-MS: *m/z* 1141.5240 (Calcd for $C_{50}H_{86}O_{27}Na$, 1141.5252). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.70 (3H, s), 0.74 (3H, s), 0.93 (3H, s), 1.11 (3H, s), 1.15 (3H, s), 2.00 (3H, s), 2.01 (6H, s), 2.02 (3H, s), 2.08 (6H, s), 2.11 (3H, s), 2.71 (1H, br dd, J=3.4, 12.2 Hz), 3.36 (1H, dd, J=9.3, 11.7 Hz), 3.52 (1H, dd, J=4.4, 11.7 Hz), 3.58 (3H, s), 3.70 (3H, s), 4.01 (1H, dd, J=5.8, 12.7 Hz), 4.06 (1H, d, J=11.7 Hz), 4.15 (1H, d, J=11.7 Hz), 4.29 (1H, dd, J=5.4, 12.7 Hz), 4.43 (1H, d, J=6.8 Hz), 4.66 (d, J=7.8 Hz), 4.89 (2H, m), 5.12 (3H, m), 5.36 (1H, br s).

Acid Hydrolysis of Compound 1 Compound **1** (11 mg) was hydrolyzed with HCl/MeOH under reflux for 1 h. The reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The aq. layer was neutralized with Amberlite IRA-35 and evaporated *in vacuo* to dryness. The sugar was determined by using refractive index (RI) detection and chiral detection (Shodex OR-1) on HPLC (Shodex NH_2P-50G, 80% CH_3CN, 1 ml/min, 45 °C) in comparison with authentic sugars.

Acknowledgements We are grateful to Prof. Shigenobu Arihara for the analyses of sugars and Ikuko Okamoto for the mass spectroscopic measurements.

References

1) Johnson A., Shimizu Y., *Tetrahedron*, **30**, 2033—2036 (1974).
2) Woo W. S., Kang S. S., *Phytochemistry*, **15**, 1315—1317 (1976).
3) Suga Y., Maruyama Y., Kawanishi S., Shoji J., *Chem. Pharm. Bull*., **26**, 520—525 (1978).
4) Woo W. S., Kang S. S., Wagner H., Seligmann O., Chari V. M., *Planta Medica*, **34**, 87—92 (1978).
5) Woo W. S., Kang S. S., *Tetrahedron Lett*., **1978**, 3239—3242.
6) Woo W. S., Kang S. S., *Tetrahedron Lett*., **21**, 4255—4258 (1980).
7) Kang S. S., Woo W. S., *J. Nat. Prod*., **43**, 510—513 (1980).
8) Hasegawa T., Fukuyama Y., Koshino K., Nakagawa K., Asakawa Y., *Chem. Lett*., **1987**, 329—332.
9) Fukuyama Y., Hasegawa T., Toda M., Kodama M., Okazaki H., *Chem. Pharm. Bull*., **40**, 252—254 (1992).
10) Matsumoto K., Takahashi H., Miyake Y., Fukuyama Y., *Tetrahedron Lett*., **40**, 3185—3186 (1999).
11) Hirose M., Yamakawa T., Kodama T., Komamine, A., *Plant Cell Physiol*., **31**, 267—271 (1990).
12) Murashige T., Skoog F., *Physiol. Plant*., **15**, 473—497 (1962).
13) Strauss A., Spengel S. M., Schaffner W., *Phytochemistry*, **38**, 861—865 (1995).
14) Yi Y.-H., Wang C.-L., *Planta Med*., **55**, 551—552 (1989).
15) Spengel S. M., Luterbacher S., Schaffner W., *Planta Med*., **61**, 385—386 (1995).
16) The ^{13}C-NMR data of compound **3** and **5**, **6** have been described in dimethyl sulfoxide-d_6[4] and pyridine-d_5,[13,14] respectively.