Journal of Natural Products
Vol. 58, No. 9, pp. 1378-1383, September 1995

SAPONINS AND TRITERPENES FROM CALLUS TISSUES OF *AKEBIA TRIFOLIATA* AND COMPARISON WITH THE CONSTITUENTS OF OTHER LARDIZABALACEOUS CALLUS TISSUES

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ABSTRACT.—Four triterpenoid saponins [**1–4**], including three new compounds, were isolated as major saponins from callus tissues of *Akebia trifoliata*. The structures of these new glycosides, named trifosides A–C, were elucidated as 3β-hydroxyolean-12-en-28,29-dioic acid 3-*O*-β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-β-D-glucopyranoside [**2**], 3β-hydroxy-30-nor-olean-12,20(29)-dien-28-oic acid 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranoside [**3**], and 3β,23-dihydroxy-30-nor-olean-12,20(29)-dien-28-oic acid 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranoside [**4**], respectively. The known saponin was identified as mubenoside A [**1**]. Additionally, nine known triterpene aglycones were isolated and identified by comparing their spectral data and tlc R_f values with those of authentic samples. The compounds identified in the present study were compared with the terpenoid profiles of callus cultures of other plants in the Lardizabalaceae.

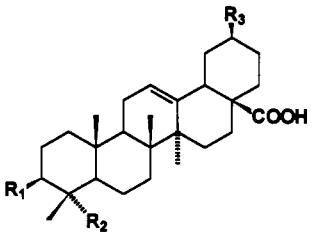
Akebia trifoliata Koidz. (Lardizabalaceae) is widely distributed in thickets in the hills and low mountains of Japan and the People's Republic of China. It is a glabrous climber growing as a woody vine or sometimes a shrub, with plants reaching up to more than 3 meters high, whose pale-purple flowers, usually unisexual, bloom in April and May (1). Its air-dried stems are employed as a crude drug to substitute for *Akebia quinata*, which is one of the most important antiphlogistic, diuretic, analgesic, and antirheumatic crude drugs in traditional Japanese medicine.

Prior to the present study, the constituents of the callus tissues of two lardizabalaceous plants (*A. quinata* and *Stauntonia hexaphylla*) have been investigated, and new 30-nor-oleanane-type triterpenes and their saponins have been reported (3–8). We report herein the isolation and the structural elucidation of three novel saponins [**2–4**] isolated from callus tissues of *A. trifoliata*. The isolation and identification of a known saponin and eight related oleanane-type triterpenes are also described, and the constituents identified are compared with those of other lardizabalaceous callus tissues from a chemotaxonomic point of view.

RESULTS AND DISCUSSION

The crude saponin mixtures obtained from MeOH extracts of callus tissues derived from the stem of *A. trifoliata* were chromatographed on Si gel and subsequently by reversed-phase cc; four major saponins [**1–4**] were obtained. The ^{1}H-nmr spectrum of saponin **1** as well as the ^{13}C-nmr spectrum of both the aglycone and sugar moieties of **1** were superimposable on those of mubenoside A reported previously from the callus tissues of *S. hexaphylla* (7). This is the second report of compound **1** from a natural source.

The fdms of saponin **2** exhibited a molecular peak at *m/z* 935 $[M+Na]^+$ indicating a mol wt of 912 daltons. Saponin **2** showed the presence of six singlet methyl groups (δ 0.80, 0.98, 1.04, 1.25, 1.30, 1.59) in the ^{1}H-nmr spectrum, one fewer methyl group than found in oleanolic acid. Further, an olefinic proton at δ 5.53 (1H, t, J=3.5 Hz) and three anomeric protons at δ 4.85 (1H, d, J=7.5 Hz), 5.24 (1H, d, J=7.5 Hz), and 5.57 (1H, d, J=7.5 Hz) were observed indicating the presence of three sugar moieties in **2**. The ^{13}C-nmr spectrum of **2** showed the presence of two carboxyl groups at δ 179.94 (C-29) and



1 R_1=Glc(2→3)Xyl, Ara; R_2=$-CH_3$, R_3=$-CH_2OH$

2 R_1=Glc(2→3)Xyl, Ara; R_2=$-CH_3$, R_3=$-COOH$

3 R_1=Ara(2→3)Xyl, Glc; R_2=$-CH_3$, R_3=$=CH_2$

4 R_1=Ara(2→3)Xyl, Glc; R_2=$-CH_2OH$, R_3=$=CH_2$

181.10 (C-28). On the basis of these data, the aglycone moiety of **2** was determined as serratagenic acid by comparison with ^{13}C-nmr data reported previously for the same isolate obtained from the callus tissues of *Stauntonia hexaphylla* (6). In addition, the ^{13}C-nmr spectrum of **2** showed signals of three anomeric carbons at δ 104.94, 104.82, and 104.18, respectively, which were identical with those of **1** (Table 1). Therefore, the structure of **2** was determined as 3β-hydroxyolean-12-en-28,29-dioic acid 3-*O*-β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-β-D-glucopyranoside [**2**]. This is a new natural product and has been designated trifoside A.

The fdms of saponin **3** exhibited a molecular peak at *m/z* 865 $[M-1]^+$ indicating a mol wt of 866 daltons, and the eims exhibited a fragment ion corresponding to the aglycone at *m/z* 440 $[M-426]^+$. In the ^{1}H-nmr spectrum, saponin **3** showed the presence of five methyl groups (δ 0.82, 0.96, 1.09, 1.25, 1.29), and characteristic signals for exomethylene protons were exhibited at δ 4.75 (1H, s) and 4.81 (1H, s). The signals of three anomeric protons of the sugar moieties were also observed at δ 4.76 (1H, d, J=7.5 Hz, overlapping the exomethylene proton signals, δ 4.76 and 4.80, 1H each, s), 5.32 (1H, s, J=8.0 Hz), and 5.42 (1H, d, J=7.5 Hz). The ^{13}C-nmr spectrum of **3** showed the presence of an olefinic bond at δ 122.92 (C-12) and δ 144.12 (C-13), and an exomethylene group at δ 149.10 (s) and 107.08 (t), in addition to all the other carbon signals of the aglycone moiety of **3**, which were identical to those reported for akebonoic acid from the callus tissues of *A. quinata* (3) (Table 1). The ^{13}C-nmr spectrum of **3** also showed the presence of three anomeric carbon signals at δ 105.11, 105.17, and 105.73, and thirteen carbon signals for three sugar moieties (Table 1). The assignments of the protons in arabinose, xylose, and glucose, and one-bond correlations between the ^{1}H and ^{13}C nuclei were established by HOHAHA (10) and HMQC (11) nmr experiments. Interglycosidic cross-peaks were also determined from NOESY nmr experiments. Therefore, the structure of saponin **3** was determined to be akebonoic acid 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranoside [**3**], a new compound that has been designated trifoside B.

The fdms of saponin **4** exhibited a molecular peak at *m/z* 905 $[M+Na]^+$ indicating

TABLE 1. ^{13}C-Nmr Spectral Data (δ values) of Compounds **1–4** in Pyridine-d_5.

Carbon	Compound				Carbon	Compound			
	1	**2**	**3**	**4**		**1**	**2**	**3**	**4**
1	38.67	38.42	38.77	38.87	glc				
2	26.55	26.31	28.28	26.18	1	105.29	104.94	105.11	104.93
3	89.55	89.31	89.23	81.77	2	79.43	79.14	75.32	75.31
4	39.71	39.47	39.74	43.82	3	87.62	87.20	78.40	78.38
5	55.85	55.58	55.94	47.30	4	72.88	72.56	71.58	71.57
6	18.45	18.22	18.48	18.23	5	79.02	78.66	78.52	78.53
7	33.18	32.89	33.16	32.88	6	62.58	62.28	62.57	62.55
8	39.61	39.33	39.80	39.80	xyl				
9	47.93	47.69	47.89	48.02	1	104.52	104.18	105.17	105.06
10	36.91	36.69	36.99	36.90	2	76.31	76.02	76.07	76.07
11	23.86	23.56	23.77	23.81	3	77.90	77.54	79.09	79.15
12	122.58	122.90	122.92	122.45	4	71.38	71.15	71.36	71.35
13	144.46	144.13	144.12	145.32	5	67.92	67.59	67.13	67.12
14	42.12	42.35	42.08	42.14	ara				
15	28.31	28.02	28.28	28.30	1	105.12	104.82	105.73	105.19
16	23.70	23.53	23.74	23.81	2	69.89	69.51	77.43	77.50
17	46.77	46.42	47.01	47.11	3	74.84	74.55	83.69	83.91
18	44.40	40.86	47.98	48.15	4	69.68	69.42	68.98	69.01
19	48.16	47.67	41.96	42.06	5	67.21	66.87	66.19	66.23
20	69.89	41.88	149.10	149.10					
21	35.18	29.04	38.35	38.49					
22	33.18	32.16	29.90	30.48					
23	27.72	27.46	27.82	63.58					
24	16.46	16.25	16.49	13.27					
25	15.43	15.20	15.48	16.07					
26	17.37	17.12	17.31	17.45					
27	26.06	25.87	26.14	26.37					
28	180.18	179.94	179.37	179.76					
29	—	181.10	107.08	106.94					
30	25.70	19.78	—	—					

a mol wt of 882 daltons. Compound **4** showed the presence of only four methyl groups (δ 0.90, 0.98, 1.04, 1.18) in the ^{1}H-nmr spectrum, one fewer than **3**, and characteristic signals of an exomethylene group at δ 4.73 (1H, s) and 4.78 (1H, s), respectively. Further, three anomeric protons appeared at δ 5.09 (1H, d, J=4.5 Hz), 5.28 (1H, d, J=8.0 Hz), and 5.41 (1H, d, J=7.6 Hz). The ^{13}C-nmr spectrum of **4** showed the presence of olefinic carbons, an exomethylene carbon, and additionally, the carbon signal of a C-23 hydroxymethyl group at δ 63.58 (t). Thus, the aglycone moiety of **4** was identified as 30-nor-hederagenin by comparison with ^{13}C-nmr chemical shifts reported previously from the callus tissues of *A. quinata* (4) and *Paeonia japonica* (9) (Table 1), with the sugar moieties of **4** being identical to those of **3**. Therefore, the structure of saponin **4** was determined to be 30-nor-hederagenin 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranoside [**4**], which is a new compound that has been designated trifoside C.

In addition, nine known triterpenes from the $CHCl_3$ extract of the callus tissue of *A. trifoliata* were identified by comparing their spectral data with those reported (3,4,6,8) and by co-tlc with authentic samples: oleanolic acid, 3-*epi*-oleanolic acid, akebonoic acid (30-nor-oleanolic acid), 3-*epi*-akebonic acid, quinatic acid, 3β-hydroxy-29-al-olean-12-en-28-oic acid, mesembryanthemoidigenic acid, serratagenic acid, and β-amyrin.

These triterpenes were compared with those obtained from the callus tissues of other lardizabalaceous plants (*A. trifoliata* and *S. hexahylla*) already reported (3,6) (Table 2). *A. trifoliata* (At) callus tissues produce 30-nor-oleanane type triterpenes [**c** and **e**] as the main products, as do *A. quinata* (Aq) and *S. hexaphylla* (Sh) callus tissues, in addition to a number of stepwise biogenetic intermediates [**a**→**g**→**h**→**c**] (Aq, At, and Sh) and [**b**→**j**→**k**→**d**] (Sh) of 30-nor-oleanolic acid, as already reported from *S. hexaphylla* callus tissues (6). These results suggest that 30-nor-oleanane-type triterpenes may be considered significant chemotaxonomic markers for lardizabalaceous plants in studies utilizing callus tissues. Interestingly, free 30-nor-oleanane-type triterpenes have not been detected from the original plant (*A. quinata*) (A. Ikuta, unpublished data). Furthermore, pairs of triterpenes having 3β- and 3α-hydroxy groups [(**a**-**b**, **c**-**d**) (Aq, At, and Sh), (**g**–**j** and **h**–**k**) (Sh)] were also produced characteristically from these three callus tissues (Table 2).

The callus tissues of certain paeoniaceous plants (*Paeonia japonica, P. sucsiflora,* and *P. lactiflora*) produced triterpene compounds of both the oleanane and lupane type, as the main skeletons in common. A number of biogenetic intermediates representing different oxidation stages for oleanane-type triterpenes were also produced in the same manner as mentioned above in the case of lardizabalaceous plant callus tissues (12,15). Similar findings have also been reported from the callus tissues of papaveraceous, ranunculaceous, berberidaceous, and menispermaceous plants (13,14). Even though the original plants showed varying alkaloid profiles, the skeletons of the alkaloids produced from callus tissues derived from sixteen species belonging to the Papaveraceae were of the benzophenanthridine, protopine, or aporphine type, and also, the skeletons of the alkaloids produced from the callus tissues derived from twelve species belonging to the Ranunculaceae, Berberidaceae, and Menispermaceae were of the protoberberine or aporphine type. In addition, alkaloids produced from callus tissues were similar to one another and displayed simpler biosynthetic pathways than those isolated from the original plants (13,14).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The nmr spectra were taken on JEOL GX-500 and Varian XL-400 spectrometers. The chemical shifts were given in δ (ppm) with pyridine-d_5 (δ 7.22 and 135.5 ppm, respectively) as internal standard; the multiplicities for the ^{13}C-nmr signals were determined by DEPT experiments at 90° and 135°. ^{1}H- and ^{13}C-nmr chemical shifts have been assigned on the basis of HOHAHA, HMQC, and HMBC experiments. Eims and fdms were taken with a Hitachi-80 mass spectrometer. The optical rotations were recorded on a Jasco DIP-4 polarimeter.

PLANT MATERIAL.—*Akebia trifoliata* Koidz. (Lardizabalaceae) was collected in October 1981 at the Medicinal Plant Garden of Tokyo College of Pharmacy, where a voucher specimen has been deposited.

TABLE 2. Triterpenes Produced by Lardizabalaceous Plant Callus Tissues.

Species	Compound[a,b]											
	a	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**	**k**	Reference
Akebia												
A. quinata	+	+	++	+	++	+	+	+	−	−	−	3
A. trifoliata ...	+	+	++	+	++	−	+	+	+	−	−	
Stauntonia												
S. hexaphylla ..	+	+	++	+	++	+	+	+	+	+	+	6

[a] ++ large amount, + present, − not detected.

[b] Key to compounds detected: **a** Oleanolic acid, **b** 3-*epi*-Oleanolic acid, **c** Akebonoic acid, **d** 3-*epi*-Akebonoic acid, **e** Quinatic acid, **f** 3β-Hydroxy-29-al-olean-12-en-28-oic acid, **g** Mesembryanthemoidigenic acid, **h** Serratagenic acid, **i** β-Amyrin, **j** 3-*epi*-Mesembryanthemoidigenic acid, and **k** 3-*epi*-Serratagenic acid.

DERIVATION AND CULTURE OF CALLUS TISSUES.—The callus tissues from the stem of *A. trifoliata* were established in Murashige and Skoog medium (minus glycine) containing 2,4-D (1 mg/liter, 3 mg/liter) and kinetin (0.1 mg/liter) as plant growth regulators. The callus tissues were subcultured every 5–6 weeks onto fresh Murashige and Skoog medium containing 2,4-D (1 mg/liter) and kinetin (0.1 mg/liter) at 25±1° in the dark.

EXTRACTION AND ISOLATION.—The callus tissues (ca. 1600 g fresh wt, 59.5 g dry wt) were extracted with cold MeOH and EtOAc in a Waring blender. The extracts were combined and concentrated under reduced pressure. The residue was partitioned between $CHCl_3$ and H_2O to obtain the organic solvent-soluble fraction. The residual H_2O solution was further partitioned with saturated *n*-BuOH with H_2O, which was concentrated under reduced pressure. The $CHCl_3$ solution was evaporated under reduced pressure and chromatographed over a Si gel column (Fuji gel BW 350), after which gradient elution with $CHCl_3$ with increasing proportions of MeOH (0–100%) afforded the crude triterpene mixtures. The mixtures were purified repeatedly by cc on a Si gel column (CIG column system, Kusano) with hexane-EtOAc-CH_3CN (5:1–5:0.5), which afforded eight known triterpenoids. These compounds were identified by spectroscopic means (^{1}H- and ^{13}C-nmr) and by Si gel tlc [Merck 5317; solvent system, hexane-EtOAc-CH_3CN (5:1:0.5) and $CHCl_3$-MeOH-H_2O (65:30:15), lower layer]. Furthermore, the BuOH solution was chromatographed over a Si gel column (Merck 9385) and eluted with $CHCl_3$ containing increasing proportions of MeOH to afford crude saponin mixtures. The mixtures were purified repeatedly by re-chromatography over RP-18 (Fuji gel 18–37) with CH_3CN-H_2O (5:5–4:6) and MeOH-H_2O (5:5–4:6), and afforded saponins **1** (5.5 mg), **2** (5.0 mg), **3** (4.0 mg), and **4** (4.5 mg).

Mubenoside A [**1**].—Colorless powder; $[\alpha]^{25}$D +4.89° (*c*=0.36, pyridine); ^{1}H nmr (pyridine-d_5) δ 0.79, 0.98, 1.05, 1.26×2, 1.58 (each 3H, each s), 2.45 (1H, t, *J*=14.0 Hz), 3.27 (1H, dd, *J*=4.5 and 11.5 Hz), 3.37 (1H, dd, *J*=4.0 and 13.5 Hz), 4.86 (1H, d, *J*=7.5 Hz), 5.23 (1H, d, *J*=7.5 Hz), 5.53 (1H, t, *J*=3.5 Hz), 5.57 (1H, d, *J*=8.0 Hz); ^{13}C-nmr data, see Table 1; fdms *m/z* 883 $[M-1]^+$.

Trifoside A [**2**].—Colorless powder; $[\alpha]^{25}$D −0.62° (*c*=0.29, pyridine); ^{1}H nmr (pyridine-d_5) δ 0.80, 0.98, 1.04, 1.25, 1.30, 1.59 (3H each, each s), 2.62 (1H, t, *J*=14.0 Hz), 3.24 (1H, dd, *J*=4.5 and 11.5 Hz), 3.43 (1H, dd, *J*=4.5 and 15.0 Hz), 4.85 (1H, d, *J*=7.5 Hz), 5.24 (1H, d, *J*=7.5 Hz), 5.53 (1H, t, *J*=3.5 Hz), 5.57 (1H, d, *J*=7.5 Hz); ^{13}C-nmr data, see Table 1; fdms *m/z* 935 $[M+Na]^+$, 911 $[M-1]^+$.

Trifoside B [**3**].—Colorless powder; $[\alpha]^{25}$D +72.8° (*c*=0.08, pyridine); ^{1}H nmr (pyridine-d_5) δ 0.82, 0.96, 1.09, 1.25, 1.29 (3H each, each s), 3.24 (1H, dd, *J*=5.5 and 14.0 Hz), 3.27 (1H, dd, *J*=4.5 and 10.0 Hz), 4.74 (1H, s), 4.76 (1H, d, *J*=7.5 Hz), 4.78 (1H, s), 5.48 (1H, t, *J*=3.5 Hz), 5.32 (1H, d, *J*=8.0 Hz), 5.42 (1H, d, *J*=7.5 Hz); ^{13}C-nmr data, see Table 1; eims *m/z* 440 $[M-426]^+$; fdms *m/z* 865 $[M-1]^+$.

Trifoside C [**4**].—Colorless powder; $[\alpha]^{25}$D +65.1° (*c*=0.11, pyridine); ^{1}H nmr (pyridine-d_5) δ 0.90, 0.98, 1.03, 1.18 (3H each, each s), 2.59 (1H, t, *J*=14.5 Hz), 3.22 (1H, dd, *J*=12.5 Hz), 4.73 (1H, s), 4.78 (1H, s), 5.09 (1H, d, *J*=4.5 Hz), 5.25 (1H, d, *J*=8.0 Hz), 5.41 (1H, d, *J*=7.6 Hz), 5.46 (1H, t); ^{13}C-nmr data, see Table 1; fdms *m/z* 905 $[M+Na]^+$.

ACKNOWLEDGMENTS

K. Tokunaga and N. Tamura, Department of Applied Biological Science, College of Agriculture and Veterinary Medicine, Nihon University, are thanked for their assistance in the experimental work. I also wish to acknowledge H. Hatano, Central Analytical Center of Kitasato University, for measurement of the HMBC and HMQC nmr spectra, and Dr. K. Arai, Research Center, Taisho Pharmaceutical Co., Ltd., for meaasurement of the HOHAHA nmr spectrum. Thanks are due to N. Sawabe and T. Hasegawa, Faculty of Pharmaceutical Science, Science University of Tokyo, for measurement of the ^{1}H-nmr, ^{13}C-nmr, and fdms data.

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Received 16 November 1994