Constituents of Leguminous Plants, XIII. New Triterpenoid Saponins from Wistaria brachybotrys

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CONSTITUENTS OF LEGUMINOUS PLANTS, XIII.[1] NEW TRITERPENOID SAPONINS FROM *WISTARIA BRACHYBOTRYS*

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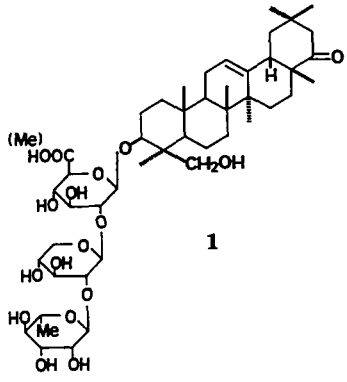
ABSTRACT.—Two new triterpenoid saponins, wistariasaponins D [**1**] and G [**2**], and the known saponin dehydrosoyasaponin I [**3**] were isolated from the knots of *Wistaria brachybotrys*. The structures of **1** and **2** were determined from their chemical and physicochemical evidence. The inhibitory effects of these saponins on the activation of Epstein-Barr virus early antigen that was induced by a tumor promoter were also tested for the primary screening of antitumor-promoting activities.

In a previous paper (1), we reported the isolation and structure determination of four major saponins, wistariasaponins A, B_1, B_2, and C, from the knots of *Wistaria brachybotrys* Sieb. et Zucc. Knots are hard swellings or masses formed in the wood. As a continuation of our chemical studies on the constituents of leguminous plants (1,2) and biological studies on the potential antitumor-promoting activities of crude drugs (3–7), we have now isolated two new triterpenoid saponins, wistariasaponins D [**1**] and G



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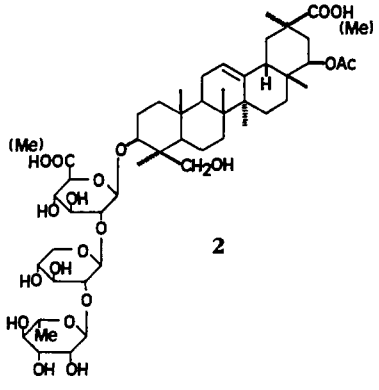

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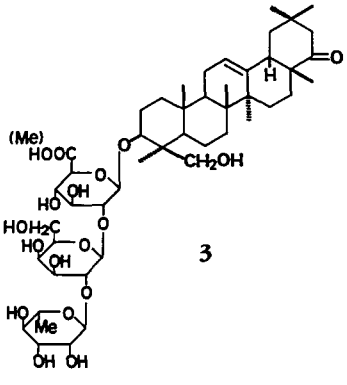

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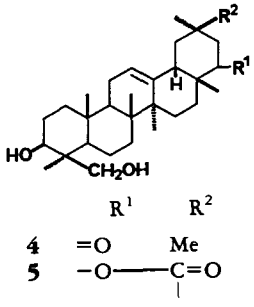

	R^1	R^2
4	=O	Me
5	–O–C=O	

[1]For Part XII, see Konoshima *et al.* (1).

[**2**], together with a known saponin, dehydrosoyasaponin I [**3**] (8) from the knots of *W. brachybotrys*. In this paper, we describe the structure elucidation and the inhibitory effects of these saponins on Epstein-Barr virus early antigen (EBV-EA) activation induced by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Biological assays were carried out according to the synergistic method described in the literature (3,4).

The crude saponin fraction was fractionated by cc on Si gel to give fractions I, II, and III, and each fraction was methylated with CH_2N_2 to afford I-Me, II-Me, and III-Me, respectively. Each methylated fraction was purified by preparative hplc repeatedly to give **1** and **2** together with **3** as methyl esters.

The methyl ester of wistariasaponin D [**1**], $C_{48}H_{76}O_{17}$, showed ir absorptions (1730 and 1700 cm^{-1}) due to an ester and a ketone. The ^{13}C- and ^{1}H-nmr spectra of **1** showed the presence of three anomeric carbons (at δ 105.52, 102.60, and 102.40) and three anomeric protons [at δ 6.37 (broad s), 5.70 (d, $J = 7.6$ Hz), and 4.96 (d, $J = 7.8$ Hz), respectively]. On acid hydrolysis of **1**, soyasapogenol E [**4**], D-glucuronic acid, D-xylose, and L-rhamnose were obtained. These observations suggested the presence of β linkages of D-xylose and D-glucuronic acid in **1**. The ^{13}C-nmr spectrum of **1** showed that the signal of C-3 was shifted to lower field (±11 ppm) by comparison with that of **4**. From this glycosylation shift (9), **1** was deduced to be a 3-*O*-monodesmoside of soyasapogenol E. As shown in Table 1, the ^{13}C chemical shift values of the oligosaccharide moiety of **1** were superimposable with those of wistariasaponin A within ±0.2 ppm. Therefore, wistariasaponin D [**1**] was characterized as 3-*O*-[α-L-rhamnopyranosyl (1↦2)-β-D-xylopyranosyl(1↦2)-β-D-glucuronopyranosyl]soyasapogenol E.

The methyl ester of wistariasaponin G [**2**], $C_{51}H_{80}O_{20}$, showed strong ir absorption bands (1730 and 1720 cm^{-1}) due to esters. The ^{13}C-nmr spectrum of **2** showed the presence of three carbonyl carbons (at δ 177.12, 170.15, and 169.65), one methyl of an acetyl group (at δ 20.87), two methyls of methoxy carbonyl (at δ 52.03 and 51.69), and three anomeric carbons (at δ 105.46, 102.44, and 102.18). The ^{1}H-nmr spectrum of **2** showed signals for one acetyl methyl proton (at δ 2.04), two methoxy protons (at δ 3.74 and 3.71), six methyl protons (at δ 1.53, 1.27, 1.17, 0.92, 0.91, and 0.76), and three anomeric protons [at δ 6.35 (s), 5.68 (d, $J = 7.5$ Hz), and 4.97 (d, $J = 7.8$ Hz)].

On acid hydrolysis of **2**, sapogenin **5**, D-glucuronic acid, D-xylose, and L-rhamnose were obtained. Sapogenin **5**, $C_{30}H_{46}O_4$, showed an ir absorption band (1680 cm^{-1}) due to a γ-lactone ring. The ^{13}C-nmr spectrum of **5** showed the presence of one carbonyl (at δ 180.00) and three oxygenated carbons (at δ 84.37, 80.00, and 64.50). The acetylation of **5** with Ac_2O and pyridine afforded a diacetate. In the eims spectra of **5** and its diacetate, retro-Diels-Alder type fragmentation peaks (10) (from the A,B rings at *m/z* 224 and 308, and from the D,E rings at *m/z* 246 and 246, respectively) were observed, suggesting the presence of two hydroxy groups on the A,B rings and a γ-lactone ring on the D,E rings. From a comparison of the ^{13}C-nmr spectrum of **5** and the ^{1}H-nmr spectrum of its diacetate with those of soyasapogenol B and wistariasapogenol B (1), it was deduced that the structure of **5** was characterized as 3β,24-dihydroxyolean-12-ene-22,30-lactone. All ^{13}C-nmr signals of **5** were reasonably assigned as listed in Table 1 by the DEPT experiment and the ^{1}H-^{13}C COSY spectrum. Further, the connectivities of the partial structure and the stereochemistry of **5** were confirmed by ^{1}H-^{13}C long range COSY (Figure 1) and difference nOe experiments (Figure 2). As shown in Figure 1, the olefinic proton at δ 5.17 (H-12) is correlated with the carbons at δ 47.90 (C-9), 45.11 (C-18), and 42.62 (C-14), the methyl proton at δ 1.00 (H_3-28) is correlated with the carbons at δ 84.37 (C-22), 45.11 (C-18), and 26.46 (C-16), and the carbonyl carbon at δ 180.00 (C-30) is correlated with the proton signals at δ 2.24 (one of H_2-21), 1.16 (one of H_2-19) and 1.21 (H_3-29). Some other significant ^{1}H-^{13}C long

TABLE 1. ^{13}C-nmr Shifts[a] of Compounds **1–5**.

Carbon	Sapogenol moiety					Carbon	Sugar moiety		
	1	**2**	**3**	**4**	**5**		**1**	**2**	**3**
C-1	38.82	38.77	38.57	38.93	38.83		glu	glu	glu
C-2	26.68	26.64	26.67	28.46	28.41	C-1′	105.52	105.33	105.52
C-3	91.13	91.02	91.29	80.14	80.00	C-2′	78.62	78.45	78.31
C-4	44.37	44.31	43.92	43.25	43.17	C-3′	76.86	76.72	76.52
C-5	56.37	56.28	56.05	56.37	56.30	C-4′	73.66	73.52	72.78
C-6	18.63	18.62	18.50	19.14	19.13	C-5′	77.59	77.58	76.59
C-7	33.09	32.82	33.03	33.35	33.55	C-6′	170.47	170.15	170.42
C-8	39.84	40.91	39.81	39.96	39.71	(OMe)	(52.13)	(52.03)	(52.17)
C-9	47.64	47.61	47.69	48.02	47.90		xyl	xyl	gal
C-10	36.58	36.49	36.47	37.08	37.03	C-1″	102.60	102.44	101.77
C-11	24.01	24.01	24.04	24.11	24.02	C-2″	79.50	79.38	77.75
C-12	123.98	123.63	124.21	124.00	124.97	C-3″	78.24	78.16	73.16
C-13	141.85	143.61	141.87	141.90	140.58	C-4″	70.90	70.79	71.20
C-14	42.07	41.82	42.02	42.06	42.62	C-5″	66.90	66.89	76.97
C-15	25.38	26.22	25.29	25.44	25.15	C-6″			61.62
C-16	27.37	26.22	27.37	27.40	26.46		rha	rha	rha
C-17	47.82	36.16	47.82	47.85	36.02	C-1‴	102.40	102.18	102.51
C-18	47.93	44.18	47.88	47.96	45.11	C-2‴	72.41	72.26	72.42
C-19	46.66	41.28	46.67	46.76	42.76	C-3‴	72.80	72.70	72.84
C-20	34.14	40.09	34.16	34.18	42.24	C-4‴	74.36	74.22	74.37
C-21	50.94	35.17	50.95	50.96	38.38	C-5‴	69.51	69.42	69.46
C-22	215.12	77.49	215.18	214.87	84.37	C-6‴	18.98	18.94	18.95
C-23	23.05	23.06	23.04	23.64	23.60				
C-24	62.86	62.74	63.57	64.61	64.50				
C-25	15.55	15.49	15.78	16.24	16.21				
C-26	16.79	16.77	16.74	16.88	16.85			169.65 (Ac)	
C-27	25.46	26.55	25.46	25.53	24.93			20.87 (Ac)	
C-28	25.27	21.24	25.29	25.31	23.59				
C-29	31.87	29.45	31.89	31.88	20.58			51.69 (COOMe)	
C-30	20.99	177.12	20.98	21.05	180.00				

[a]Recorded in pyridine-d_5. Chemical shifts are in δ (ppm). Saponins **1**, **2**, and **3** were measured as methyl esters. Chemical shift values of the sugar moiety of wistariasaponin A were: glu 105.47, 78.63, 76.81, 73.61, 77.55, 170.44; xyl 102.55, 79.41, 78.06, 70.84, 66.80; rha 102.31, 72.34, 72.67, 74.30, 69.38, 18.91 (1).

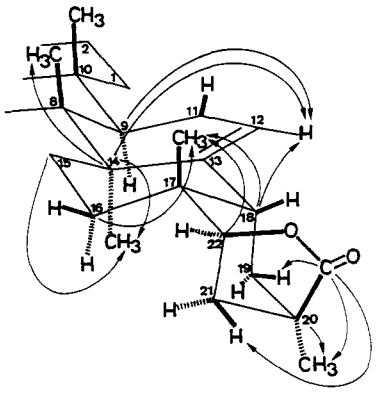

FIGURE 1. Correlation in ^{1}H-^{13}C long range COSY spectrum of **5**.

range correlations are indicated by arrows in Figure 1. Furthermore, significant nOe enhancements observed are shown by arrows in Figure 2. From these results, **5** was characterized as 3β,24-dihydroxyolean-12-ene-22β,30-lactone.

However, the spectroscopic data of the methyl ester of wistariasaponin G [**2**] showed the presence of two methyl ester groups and one acetyl group. Therefore, it was deduced that the lactone ring of the sapogenol **5** was artificially formed from the 22-*O*-acetyl-30-carboxysapogenol by acid hydrolysis of the methyl ester of wistariasaponin G [**2**].

All ^{13}C-nmr signals of **2** were reasonably assigned as listed in Table 1 by the DEPT experiment and 2D nmr spectra (^{1}H-^{1}H, ^{1}H-^{13}C, and ^{1}H-^{13}C long range COSY). Furthermore, the stereochemistry of **2** was confirmed by difference nOe experiments, as in the case of **5**. Irradiation at H-18 (δ 2.66) enhanced the signal intensities of the olefinic proton (δ 5.46), *O*-methyl protons (δ 3.71), one of the C-19 methylene protons (δ 2.17), and C-28 methyl protons (δ 0.91). Some other significant nOe results are indicated by arrows in Figure 3. The ^{13}C chemical shift values of the oligosaccharide moiety of **2** were also superimposable with those of wistariasaponin A and **1** within ±0.2 ppm. Therefore, wistariasaponin G [**2**] was characterized as 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-xylopyranosyl(1→2)-β-D-glucuronopyranosyl]-22β-*O*-acetyl-24-hydroxy-30-carboxyolean-12-ene.

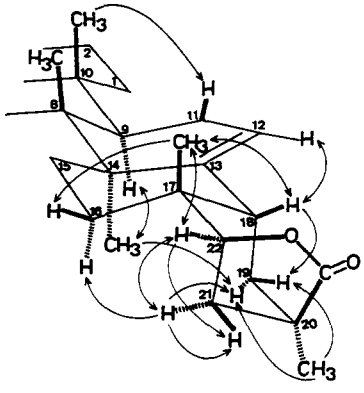

FIGURE 2. Significant enhancement of signal intensity by difference nOe experiments of **5**.

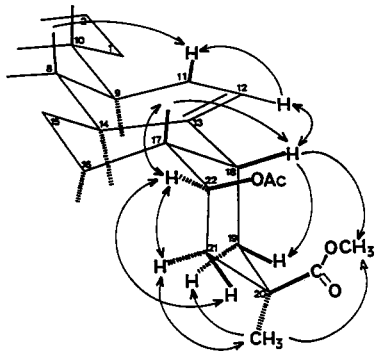

FIGURE 3. Significant enhancement of signal intensity by difference nOe experiments of **2**.

The inhibitory effects of triterpenoids **1–4** on EBV-EA activation induced by TPA are shown in Table 2. Dose concentrations are represented in mol ratio to TPA (32 pmol). Soyasapogenol E [**4**] exhibited remarkable inhibitory effects at 5×10^2 mol ratio (100% inhibition) and preserved high viability of Raji cells even at high dose (1×10^3 mol ratio). On the other hand, **1** and **2** exhibited high cytotoxicity on Raji cells at 1×10^3 mol ratio. From our experiments, it was deduced that the potency of the inhibitory activity of **4** was more than 10 times higher than that of oleanolic acid, a known inhibitor of EBV-EA activation (3,11).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURE.—Mp's were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Ir spectra were measured on a Shimadzu IR-408 spectrometer. Unless otherwise stated, ^{1}H- and ^{13}C-nmr spectra were recorded on a JEOL JNM GX-400 spectrometer in pyridine-d_5 using TMS as an internal standard. Eims was determined on a Hitachi M-80 mass spectrometer. Optical rotations were measured on a JASCO DIP-181 digital polarimeter. Preparative hplc was carried out on a Nihon Bunseki Kogyo LC-09 using a GPC column (300 mm × 2) with ri detector. Pre-coated Si gel plates (Kieselgel 60 F254, 0.25 mm, Merck) were used for analytical tlc, and triterpenoids were detected by spraying with 10% H_2SO_4 solution containing 1% $Ce(SO_4)_2$ followed by heating.

PLANT MATERIAL.—The knots of *W. brachybotrys* were collected at Shikoku, Japan, in 1986. Herbarium specimens have been deposited in the herbarium of Kyoto Pharmaceutical University.

EXTRACTION AND ISOLATION OF COMPOUNDS **1–3**.—The crude saponin fraction (42 g) was obtained from the knots of *W. brachybotrys* (2.5 kg); fractions I-Me, II-Me, and III-Me were obtained by cc on Si gel followed by methylation with CH_2N_2, and the purification of **1**, **2**, and **3** was carried out on preparative hplc as described in a previous paper (1).

METHYL ESTER OF WISTARIASAPONIN D [**1**].—Hygroscopic white powder (60 mg): $[\alpha]_D$ −11.5° (c = 0.67, MeOH); ir (KBr) 3600, 1730, 1700 cm^{-1}; ^{1}H nmr δ 6.37 (1H, br s, anomeric H), 5.70 (1H, d, J = 7.6 Hz, anomeric H), 5.25 (1H, t-like, H-12), 4.96 (1H, d, J = 7.8 Hz, anomeric H), 3.76 (3H, s, OMe), 1.54, 1.30, 1.17, 0.97, 0.89, 0.86, 0.77 (each 3H, s); ^{13}C nmr see Table 1. *Anal.* found C 59.70%, H 8.59%; calcd for $C_{48}H_{76}O_{17}\cdot 2H_2O$, C 59.98%, H 8.39%.

METHYL ESTER OF WISTARIASAPONIN G [**2**].—Hygroscopic white powder (35 mg): $[\alpha]_D$ −35.6° (c = 0.54, MeOH); ir (KBr) 3600, 1730, 1720, 1700 cm^{-1}; ^{1}H-nmr δ 6.35 (1H, br s, anomeric H), 5.68 (1H, d, J = 7.5 Hz, anomeric H), 5.46 (1H, t-like, H-12), 4.97 (1H, d, J = 7.8 Hz, anomeric H), 3.74, 3.71 (each 3H, s, OMe), 2.04 (3H, s, OAc), 1.79 (3H, d, J = 6.3 Hz, rhamnose H_3-6), 1.53, 1.27, 1.17, 0.92, 0.91, 0.76 (each 3H, s); ^{13}C nmr see Table 1. *Anal.* found C 57.13%, H 8.36%; calcd for $C_{51}H_{80}O_{20}\cdot 3H_2O$, C 57.39%, H 8.12%.

METHYL ESTER OF DEHYDROSOYASAPONIN I [**3**].—Hygroscopic white power (40 mg). Isolation of this compound from North American alfalfa (*Medicago sativa*) has been reported by Kitagawa *et al.* (8).

TABLE 2. Relative Ratio of EBV-EA Activation with Respect to Positive Control (100%) in Presence of Triterpenoids **1–4**.[a]

Sample	Concentration[b]			
	1×10^3	5×10^2	1×10^2	1×10
Wistariasaponin D [**1**]	0.0 ± 0 (10)	51.0 ± 3.8 (>80)	86.1 ± 1.8 (>80)	92.8 ± 0.7 (>80)
Wistariasaponin G [**2**]	—[c] (0)	15.4 ± 4.2 (10)	62.6 ± 2.8 (>80)	79.7 ± 2.5 (>80)
Dehydrosoyasaponin I [**3**] . . .	50.3 ± 3.6 (60)	67.8 ± 3.1 (>80)	88.5 ± 2.0 (>80)	100.0 ± 0 (>80)
Soyasapogenol E [**4**]	0.0 ± 0 (>80)	0.0 ± 0 (>80)	43.9 ± 3.3 (>80)	89.5 ± 2.3 (>80)

[a]Values represent relative percentages to the positive control value (100%), and averages of three determinations ±SD. Values in parentheses are viability percentage of Raji cells.

[b]Mol ratio/TPA (20 ng = 32 pmol).

[c]not detected.

GENERAL PROCEDURE FOR METHANOLYSIS.—A solution of the pure saponin in 1 N HCl/dry MeOH was refluxed for 2 h. The reaction mixture was neutralized with Ag_2CO_3, and the inorganic precipitate was removed by filtration. The filtrate was concentrated to half the initial volume in vacuo. The precipitate was filtered to afford sapogenols, and the MeOH filtrate was evaporated to dryness in vacuo. The residue was dissolved in dry pyridine and treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide for 1.5 h. The product was subjected to glc to identify the trimethyl derivative of the methyl monosaccharides. Conditions of glc for the identification of sugar were previously reported (1). From compound **1**, soyasapogenol E [**4**], D-glucuronic acid, D-xylose, and L-rhamnose were obtained and identified by glc with authentic samples. From compound **3**, soyasapogenol E, D-glucuronic acid, D-galactose, and L-rhamnose were obtained and identified with authentic samples. From compound **2**, sapogenol **5** was obtained together with D-glucuronic acid, D-xylose, and L-rhamnose, identified with authentic monosaccharides.

SAPOGENOL **5**.—Amorphous powder (10 mg) was obtained from wistariasaponin G [**2**] (35 mg): $[\alpha]_D$ −12.5° (*c* = 0.30, MeOH); ir (KBr) 3550, 1680 cm^{-1}; ^{1}H-nmr δ 5.17 (1H, t-like, H-12), 4.52, 3.70 (2H, ABd, *J* = 11.0 Hz, H_2-24), 4.18 (1H, d, *J* = 5.8 Hz, H-22), 3.65 (1H, dd, *J* = 11.4, 4.6 Hz, H-3), 1.57, 1.21, 1.18, 1.00, 0.93, 0.88 (each 3H, s); ^{13}C nmr see Table 1; eims *m/z* $[M]^+$ 470 (10%), 246 (base peak), 224 (12%). *Anal.* found C 76.42%, H 9.90%; calcd for $C_{30}H_{46}O_4$, C 76.55%, H 9.85%. Compound **5** was acetylated by the usual manner with Ac_2O/pyridine to afford a diacetate: ir ($CHCl_3$) 1720, 1680 cm^{-1}; ^{1}H-nmr ($CDCl_3$) δ 5.31 (1H, t, *J* = 3.4 Hz, H-12), 4.59 (1H, dd, *J* = 10.5, 5.4 Hz, H-3), 4.36, 4.15 (2H, ABd, *J* = 11.8 Hz, H_2-24), 2.06, 2.04 (each 3H, s); eims *m/z* $[M]^+$ 554 (20%), 308 (10%), 246 (base peak).

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