A New Bidesmosidic Triterpenoidal Saponin from the Roots of *Symphytum officinale*

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Abstract

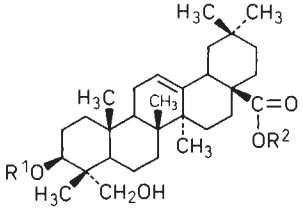
A new triterpenoidal saponin having hederagenin as the aglycone was isolated from the roots of *Symphytum officinale* L. The structure of this saponin was elucidated by FAB-MS, ^{1}H-, ^{13}C-NMR, 2D-NMR analyses and chemical studies as 3-*O*-[*β*-D-glucopyranosyl-(1→4)-*α*-L-arabinopyranosyl]-hederagenin 28-*O*-[*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl] ester.

Key words

Symphytum officinale, Boraginaceae, triterpenoid saponin, hederagenin, structural elucidation, 2D-NMR.

Introduction

Symphytum officinale L. (Boraginaceae) grows in North Asia, England, and Europe (1) and is found abundantly in Turkey. Its various parts are highly reputed for their medicinal properties in the indigenous system of medicine (2, 3). This paper describes the isolation and structural elucidation of a new triterpenoidal saponin **1**, isolated from the roots of *Symphytum officinale* L.



	R^1	R^2
1	Glc$^{1\to4}$Ara–	Glc$^{1\to6}$Glc–
2	H	H
3	Glc$^{1\to4}$Ara–	H
4	Ara–	H

Glc = *β*-D-glucopyranosyl
Ara = *α*-L-arabinopyranosyl

Materials and Methods

General analytical techniques

The melting points (m.p.) were determined in an H_2SO_4 bath (capillary) and are uncorrected. Optical rotation was measured on a Jasco DIP-360 digital polarimeter. The UV spectra were recorded with a Hitachi U-3200 spectrophotometer in MeOH and IR with a Shimadzu infrared spectrophotometer IR-460 in KBr discs. ^{1}H- and ^{13}C-NMR spectra of saponin **1** were recorded on a Bruker AM-300 spectrometer operating at 300 and 75 MHz, respectively. All NMR spectra were recorded in CD_3OD and chemical shifts values are given on a δ (ppm) scale with TMS as an internal standard. EI-mass spectra were determined on a Finnigan MAT-312 Varian MAT-112 double focusing mass spectrometer connected to a PDP 11/34 (DEC) computer system. Kieselgel 60 (70–230 mesh) was used for CC. TLC was carried out on Si gel PF-254 precoated glass plates (Merck 70–230 mesh ASTM) using the following solvent systems (A) $CHCl_3$-MeOH-H_2O (7.5 : 2.5 : 0.2) (B) *n*-BuOH-HOAc-H_2O (12 : 3 : 5). The purity of the sample was checked on RP-8, $F_{254}S$ precoated glass plates (E. Merck, Art. No. 15684, size 5 × 10 cm, layer thickness 0.25 mm). The chromatograms were sprayed with 0.1% $Ce(SO_4)_2$ in 2N H_2SO_4 followed by heating.

The 2D COSY-45° experiment was acquired at 300 MHz with a sweep width of 2016 Hz (1K data point in ω_2) and 1008 Hz (256 t_1 values 1K) in ω_1. A 1.5 sec relaxation delay was used and 16 transients were performed for each t_1 value. The heteronuclear two dimensional ^{1}H-^{13}C chemical shift correlation experiments were carried out at 300 MHz with a sweep width of 10000 Hz (2K data points in ω_2) and 1008 Hz (256 t_1 values, no zero filling 2K) in ω_1. A 1.5 sec relaxation delay was used and 32 transients were accumulated for each t_1 value.

Paper chromatography for sugar was performed on Whatman No. 1, with solvent system (C) *n*-BuOH-pyridine-H_2O (10 : 3 : 3) using the descending mode. A saturated solution of aniline phthalate in *n*-BuOH was used as a staining agent for visualization followed by heating.

Plant material

Roots of *Symphytum officinale* L. were collected from Mudyana-Bursa, Ankara, Turkey during the flowering stage in 1989. The plant specimen has been deposited at the herbarium of the Faculty of Pharmacy, Gazi University, Ankara, Turkey and was identified by Prof. Dr. Bilge Sener, Department of Pharmacognosy, Gazi University.

Extraction, isolation, and identification of the saponin

The air-dried and coarsely powdered roots (3 kg) of *S. officinale* L. were defatted with petroleum ether. The defatted mass was exhaustively extracted with EtOH and concentrated under reduced pressure. The combined extract was dissolved in a small amount of MeOH and Et_2O was added to give a yellowish brown precipitate. This procedure was repeated several times, and the yellow precipitate was collected by filtration. The crude mixture (55 g) was chromatographed on a silica gel column using a gradient of MeOH in $CHCl_3$ as eluent. The fractions eluted with $CHCl_3$: MeOH (78 : 22) were mixed on the basis of similar TLC profiles consisting of a new saponin **1** and further purified by semi-preparative HPLC on LiChrosorb RP-8 column, using 35 % MeOH to afford **1** (34 mg).

Saponin **1**: colourless crystals; m.p. 168–170 °C (dec.); $[\alpha]_D^{25}$: +18.29° (*c* 0.16, MeOH); molecular formula $C_{53}H_{86}O_{23}$; UV λ_{max} (MeOH) nm: 201.8 (end absorption); IR ν_{max}^{KBr} (KBr) cm^{-1}: 3400 (OH), 2920 (C-H), 1740 (C=O, ester), 1089 (C-O group); ^{1}H-NMR (300 MHz, CD_3OD): δ = 0.71 (3H, s, 24-CH_3), 0.79 (3H, s, 26-CH_3), 0.90 (3H, s, 29-CH_3), 0.93 (3H, s, 30-CH_3), 0.97 (3H, s, 25-CH_3), 1.16 (3H, s, 27-CH_3), 2.84 (m, H-18), 3.62 (1H, m, H-3*a*), 4.33 (1H, d, *J* = 7.3 Hz, H-1′), 4.36 (1H, d, *J* = 7.8 Hz, H-1‴), 4.49 (1H, d, *J* = 7.4 Hz, H-1″), 5.35 (1H, d, *J* = 7.9 Hz, H-1⁗), 5.24 (1H, distorted t, H-12); Negative FAB-MS: m/z = 1089 [M – H]$^-$, 927 [M – glucose – H]$^-$, 765 [M – (2 × glucose) – H]$^-$, 603 [M – (3 × glucose) – H]$^-$, 471 [M – (3 × glucose + pentose) – H]$^-$; Positive FAB-MS: *m/z* 1113 [M + Na]$^+$; ^{13}C-NMR data are given in Table **1**.

Acid hydrolysis of **1**: compound **1** (11.5 mg) was refluxed with 10 % HCl (5 ml) and MeOH (5 ml) on a boiling water bath for 3 h. The reaction mixture was concentrated under reduced pressure to remove methanol. It was then diluted with water and the hydrolysate was then extracted with chloroform. The combined $CHCl_3$ layer was evaporated to afford hederagenin **2**, which was crystallized from MeOH, m.p. 325 °C, identified by direct comparison with an authentic sample (co-TLC, m.m.p., IR, MS, ^{1}H-NMR) (4).

Identification of the sugar moieties of **1**: the aqueous layer thus separated was neutralized with Ag_2CO_3, filtered, and concentrated under reduced pressure. The residue obtained was compared with standard sugars on TLC (silica gel, H_2O : MeOH : AcOH : EtOAc, 15 : 15 : 20 : 65); spots were detected by spraying with a solution of freshly prepared aniline phthalate in BuOH (sugar reagent) followed by heating which showed that the sugars in **1** were arabinose and glucose. Moreover, the identity was further confirmed by paper chromatography (*n*-BuOH-pyridine-water, 10 : 3 : 3) along with authentic samples (glucose, galactose, arabinose, and xylose).

Basic hydrolysis of **1**: compound **1** (30 mg) was refluxed with 2 % NaOH (30 ml) in MeOH for 2 h. After cooling, the reaction mixture was slightly acidified with dilute HCl, and extracted with BuOH. The BuOH solution was washed with H_2O, evaporated under reduced pressure, and crystallized with MeOH to yield the prosapogenin **3**: colourless crystals, m.p. 238–240 °C, $[\alpha]_D^{25}$: +49.0° (*c* 0.40, C_5D_5N), IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1695 (COOH), 1075 (C-O); ^{1}H-NMR (400.13, CD_3OD): δ = 0.71 (3H, s, 24-CH_3), 0.81 (3H, s, 26-CH_3), 0.90 (3H, s, 29-CH_3), 0.94 (3H, s, 30-CH_3), 0.97 (3H, s, 25-CH_3), 1.17 (3H, s, 27-CH_3), 2.84 (1H, dd, *J* = 13.9, 3.8 Hz, H-18), 4.32 (1H, d, *J* = 6.7 Hz, H-1′), 4.48 (1H, d, *J* = 7.6 Hz, H-1″), 5.25 (1H, t, *J* = 3.4 Hz, H-12); Negative FAB-MS: *m/z* 765 [M – H]$^-$, 603 [M – glucose – H]$^-$, 471 [M – glucose – arabinose – H]$^-$; ^{13}C-NMR: see Table **1**, which was identical with hederagenin 3-*O*-*β*-D-glucopyranosyl-(1→4)-*α*-L-arabinopyranoside (leontoside B) (5, 6) (^{13}C-NMR, m.m.p., and co-TLC).

Partial acid hydrolysis of compound **3**: compound **3** (10 mg) was refluxed with 0.5 M HCl in aqueous MeOH (5 ml) for 2 h. The MeOH was evaporated under reduced pressure

Carbon	1	3	4	Carbon		1	3	4
1	39.58	39.51	39.53	Ara	1′	106.29	106.33	106.27
2	26.28	26.22	26.33		2′	73.42	73.45	72.99
3	83.81	83.65	83.43		3′	74.46	74.52	74.54
4	43.90	43.89	43.91		4′	79.84	79.87	69.74
5	48.87	49.02	49.04		5′	66.65	66.62	66.79
6	18.98	18.92	18.93	Glc	1″	106.11	106.22	
7	33.22	33.50	33.90	(terminal)	2″	75.41	75.45	
8	40.71	40.57	40.59		3″	78.13	78.00	
9	48.31	48.25	48.23		4″	71.51	71.33	
10	37.76	37.74	37.75		5″	77.93	77.89	
11	24.09	24.11	24.16		6″	62.63	62.67	
12	123.80	123.65	123.53	Glc	1‴	95.78		
13	145.02	145.31	145.33	(inner)	2‴	73.83		
14	43.06	43.01	43.04		3‴	77.93		
15	28.94	28.86	28.90		4‴	70.96		
16	24.66	24.59	24.61		5‴	77.73		
17	48.09	47.65	47.73		6‴	69.59		
18	42.57	42.75	42.81	Glc	1⁗	104.62		
19	47.30	47.28	47.34	(terminal)	2⁗	75.10		
20	31.59	31.63	31.65		3⁗	77.82		
21	34.97	34.95	34.99		4⁗	71.29		
22	33.45	33.88	33.52		5⁗	77.93		
23	65.13	65.03	64.99		6⁗	62.71		
24	13.59	13.47	13.48					
25	16.74	16.50	16.49					
26	17.97	17.83	17.89					
27	26.55	26.58	26.54					
28	178.27	181.92	182.19					
29	33.68	33.50	33.64					
30	24.20	24.05	24.07					

Table 1 ^{13}C-NMR chemical shifts of saponins **1**, **3** and **4** in CD_3OD.

Ara: *α*-L-arabinopyranosyl, glc: *β*-D-glucopyranosyl.

and the mixture was diluted with H_2O, neutralized with Ag_2CO_3, filtered, and extracted with *n*-BuOH. The *n*-BuOH extract was evaporated under reduced pressure which gave the prosapogenin **4**: m.p. 274–276 °C; $[\alpha]_D^{26}$: +40.0° (*c* 0.8, C_5D_5N), IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1695 (COOH), 1080 (C-O); ^{1}H-NMR (400.13, CD_3OD): δ 0.71 (3H, s, 24-CH_3), 0.82 (3H, s, 26-CH_3), 0.91 (3H, s, 29-CH_3), 0.94 (3H, s, 30-CH_3), 0.98 (3H, s, 25-CH_3), 1.15 (3H, s, 27-CH_3), 2.85 (1H, dd, J = 13.6, 4.12 Hz, H-18), 3.20–3.85 (sugar protons), 4.32 (1H, d, J = 6.5, H-1′), 5.23 (1H, t, J = 3.6 Hz, H-12); FAB-MS: *m/z* 603 $[M - H]^-$, 471 $[M -$ arabinose $- H]^-$; ^{13}C-NMR: see Table **1** which was identical with hederagenin 3-*O*-α-L-arabinopyranoside (leontoside A) (5, 6).

Results and Discussion

Saponin **1**, $C_{53}H_{86}O_{23}$, m.p. 168–170 °C (decomp.), $[\alpha]_D^{25}$: +18.29° (*c*, 0.164, MeOH), was obtained as colourless crystals after repeated silica gel column chromatography and by employing an RP-8 semi-preparative column. The UV spectrum displayed only an end absorption at 201.8 nm. The IR spectrum exhibited broad absorption bands at 3400 and 1080 cm^{-1} for hydroxys, indicating its glycosidic nature. It also showed the characteristic absorption band assigned to the ester group at 1740 cm^{-1}.

Saponin **1** on acid hydrolysis yielded hederagenin as the aglycone, identified by comparison of physical and spectral properties (4, 5) together with D-glucose and L-arabinose as the sugar components. The sequence of the sugars was established by negative ion FAB-MS which exhibited a strong $(M - H)^-$ ion at *m/z* 1089 indicating a molecular mass of 1090 in good agreement with the empirical formula $C_{53}H_{86}O_{23}$, indicating the degree of unsaturation as 11 double bond equivalents, and a fragment ion at *m/z* 927 $(M - 162 - H)^-$ consistent with the loss of a terminal glucose from the molecular ion, respectively. The fragment ion at *m/z* 765 was attributed to the loss of a disaccharide unit (glucose-glucose) from the molecular ion. The peaks at *m/z* 603 and 471 corresponding to the loss of a trisaccharide unit (3 glc) and a tetrasaccharide unit (3 glc + ara) from the molecular ion, respectively. The fragment ion at *m/z* 603 also indicates that the arabinose moiety is attached directly to the aglycone. The molecular ion peak was confirmed by a peak at *m/z* 1113 $(M + Na)^+$ in the positive FAB-MS.

The ^{1}H-NMR spectrum of **1** displayed the presence of six tertiary methyl singlets at δ = 0.71, 0.79, 0.90, 0.93, 0.97, and 1.16 ppm. These signals were correlated with C-24, C-25, C-26, C-27, C-29 and C-30 at δ = 13.59, 16.74, 17.97, 26.55, 33.68 and 24.20 ppm in the heteroCOSY spectrum (7). The ^{1}H-NMR spectrum also showed a one proton distorted triplet at δ = 5.24 ppm characteristic for the Δ^{12}-H in pentacyclic triterpenes. A heteroCOSY experiment also revealed interaction between this vinylic proton at δ = 5.24 with C-12 at δ = 123.80 ppm. Four anomeric proton signals were also observed in the ^{1}H-NMR spectrum of **1** at δ = 4.33 (d, J = 7.3 Hz, H-1′), 4.36 (d, J = 7.8 Hz, H-1′′′′), 4.49 (d, J = 7.4 Hz, H-1″), and 5.35 ppm (d, J = 7.9 Hz, H-1‴). In the ^{1}H-^{13}C COSY spectrum these signals were correlated with δ = 106.29, 104.62, 106.11, and 95.78 ppm peaks, respectively. Considering the coupling constants of the anomeric protons and chemical shifts of the anomeric carbons, the C-1 atoms of three glucoses were established as having the β-configuration but the C-1 atoms of arabinose as having the α-configuration and supported by their carbon signals (see Table **1**).

The broad-band ^{13}C-NMR spectrum of **1** showed 53 carbon resonances indicating the presence of a tetrasaccharide moiety (three hexoses and one pentose). The sequence and configuration of the sugar moieties were also verified by the ^{13}C-NMR spectrum which exhibited four anomeric signals at δ = 95.78, 104.62, 106.11, and 106.29 ppm indicating the presence of four sugar moieties. The DEPT spectrum (7) showed the presence of 6 × CH_3, 15 × CH_2, 24 × CH, and 8 quaternary carbon atoms.

The olefinic resonances at δ = 145.02 and 123.80 ppm, corresponding to quaternary and methine behavior, reveal the presence of unsaturation at the 12 position in an oleanane skeleton. The ^{13}C-NMR shielding data were analyzed by analogy with the reported data of oleanane triterpenoids which led to the identification of the aglycone as 3β,23-dihydroxyolean-12-ene-28-oic acid (**2**, hederagenin) (5, 8).

The appearance of an ester absorption band in its IR spectrum (1740 cm^{-1}) and a carboxyl carbon signal (δ = 178.27) and one of the anomeric carbon signals (δ = 95.78 ppm) at rather high-field in its ^{13}C-NMR spectrum strongly indicated that one of the sugars was linked to the 28-carboxylic group of the genin in the ester form (9, 10). Alkaline hydrolysis of **1** gave the prosapogenin **3**, m.p. 238–240 °C which was identified as hederagenin 3-*O*-β-D-glucopyranosyl-(1→4)-α-L-arabinopyranoside. A comparison of the ^{13}C-NMR spectrum of **3** with that of the saponin **1** revealed a loss of 12 resonance signals in addition to the disappearance of two anomeric signals at δ = 95.75 and 104.62 ppm. It could, therefore, be suggested that **1** had a dissaccharide chain composed of two glucose units bonded to the C-28 carboxyl group by an ester linkage. Furthermore, the disaccharide moiety was considered to be 6-*O*-glucosylglucopyranoside (gentiobiose) from the fact that the C-6 resonance signal (δ = 69.59 ppm) of a glucose molecule connected to the C-28 carboxyl group exhibited a characteristic low field shift due to alkyl substitution (11–13).

The prosapogenin **3** on partial acid hydrolysis yielded the monoside **4** and D-glucose. The presence of L-arabinose in the hydrolysate of **3** indicated undoubtedly that this sugar was attached to hederagenin at position C-3.

These results suggested that **1** was a bidesmoside which contained one molecule of arabinose and three molecules of glucose. The ^{13}C-NMR spectrum of **1** showed significant glycosidation shifts of signals for the C-3 (+10.11 ppm) of the aglycone, for the C-4′ (+10.7 ppm) of the arabinopyranosyl moiety (5), and for the C-6‴ (+7.09 ppm) of the glucopyranosyl moiety, in comparison to the reported values for hederagenin (8) and methyl pyranoside (14, 15) due to glycosidation in these positions, in addition to the two terminal glucose moieties. Consequently, **1** was suggested to possess a glucopyranosyl residue attached to the 4′-OH function in the arabinopyranosyl moiety.

In the COSY spectrum (7) of **1** strong cross peaks were observed for H-1′ (δ = 4.33) with H-2′ (δ = 3.55). The assignments for H-3′ (δ = 3.60) and H-4′ (δ = 3.90) hydroxy methine protons were also confirmed from the COSY-45° spectrum. In the heteroCOSY spectrum H-4′ (δ = 3.90) showed cross peak with C-4′ (δ = 79.84). This disclosed that C-1″ was coupled with C-4′ and shows (1→4) linkage between glucose and arabinose. Similarly COSY interactions were also observed between the anomeric methine H-1‴ (δ = 5.35) with the vicinal methine H-2‴ (δ = 3.31) as well as with the H-3‴ methine (δ = 3.35). The hydroxyl bearing methine H-2⁗ (δ = 3.20) on the other hand showed coupling with the anomeric methine H-1⁗ at δ = 4.36 ppm.

In the light of the above observations the structure of **1** was established as 3-*O*-[β-D-glucopyranosyl-(→4)-α-L-arabinopyranosyl]-hederagenin 28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester. A literature search revealed that **1** is a new natural product.

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