# Two New Sulfated Saponins from the Roots of *Gypsophila bermejoi*

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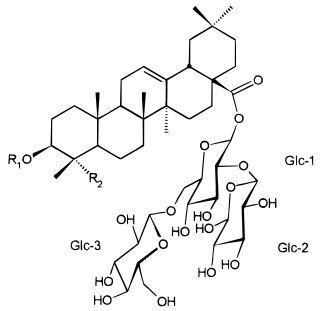
Two new sulfated saponins (**1** and **2**) were isolated from a butanol-soluble extract of the roots of *Gypsophila bermejoi* and were identified by a combination of chemical degradation and spectral methods as the 3*β*-sulfate ester of gypsogenin 28-*O*-*β*-D-glucopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→6)]-*β*-D-glucopyranoside (**1**), and the 23-sulfate ester of hederagenin 28-*O*-*β*-D-glucopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→6)]-*β*-D-glucopyranoside (**2**), respectively. Plants of the genus *Gypsophila* (Caryophyllaceae) are important industrially because of the capacity of their saponin constituents to behave like natural detergents.[1] Saponins from this genus are based on oleanolic acid (3-hydroxyolean-12-en-28-oic acid), with gypsogenin being the main pentacyclic triterpenoidal aglycon found. In general, this aglycon is substituted at the C-3 hydroxyl and/or C-28 carboxylic acid groups by saccharide units.[2]

*Gypsophila bermejoi* G. López is a Spanish endemic species found in Cuenca, Guadalajara, and Madrid. It grows in gypseum soils, at altitudes between 700 and 800 m.[4,5] The present study is the first general phytochemical investigation carried out on this species, and we report herein on the isolation and structure elucidation of two new sulfated saponins (**1** and **2**). Oleanolic acid C-3 sulfated glycosides have recently been reported for the first time from *Bupleurum rotundifolium*.[3,4]

The butanol-soluble extract of *G. bermejoi* was fractionated by Si gel column chromatography to give several fractions, of which two were further purified by medium-pressure liquid chromatography (MPLC), yielding two pure compounds (**1** and **2**).




**1** $R_1 = SO_3^-$ $R_2 = CHO$

**2** $R_1 = H$ $R_2 = CH_2OSO_3^-$

The $^1$H NMR spectrum of **1** was typical for a saponin, with the sugar region showing three anomeric proton doublets, with coupling constants characteristic of *β*-anomers ($J_{1,2}$ ca. 7.8 Hz). The three anomeric protons were labeled Glc-1−3 from low to high field. The rest of the glycosidic protons were resolved well enough to allow for most of the coupling constants to be measured. Assignment of the different resonances to each one of the three residues was achieved through COSY and TOCSY (mixing time = 85 ms) experiments. The large coupling constants found for protons H-2, H-3, and H-4 of the spin-systems of each residue were consistent with glucose moieties (see Table 2). Concerning the aglycon moiety, an olefinic triplet

**Table 1.** $^1$H and $^{13}$C NMR Chemical Shifts (*δ*) and Proton−Proton Coupling Constants (*J*, Hz) for the Aglycon Moieties of Compounds 1 and 2 in $CD_3OD$

| | **1** | | **2** | |
|---|---|---|---|---|
| position | $^1$H[a] | $^{13}$C | $^1$H | $^{13}$C |
| 1 | 1.92−1.33 | 39.5 | 1.81−1.18 | 39.6 |
| 2 | 2.32−1.97 | 24.7 | 1.86−1.78 | 27.2 |
| 3 | 4.70 (dd, *J* = 4.6, 11.9) | 80.5 | 3.86 | 72.3 |
| 4 | | 56.2 | | 52.2 |
| 5 | 1.56 | 48.3 | 1.44 | 48.0 |
| 6 | 1.71−1.12 | 21.7 | 1.77−1.58 | 18.6 |
| 7 | 1.72−1.47 | 33.7 | 1.73−1.50 | 33.6 |
| 8 | | 41.4[b] | | 40.8 |
| 9 | 1.92 | 48.9 | 1.82 | 49.0 |
| 10 | | 37.2 | | 38.0 |
| 11 | 2.14−2.11 | 24.8 | 2.12−2.08 | 24.4 |
| 12 | 5.45 (t, *J* = 3.5) | 123.7 | 5.43 (t, *J* = 3.5) | 123.6 |
| 13 | | 145.4 | | 146.1 |
| 14 | | 43.3[b] | | 43.3 |
| 15 | 1.94−1.22 | 30.1 | 1.96−1.24 | 30.1 |
| 16 | 2.20−2.04 | 23.9 | 2.20−2.03 | 23.7 |
| 17 | | n.d.[c] | | n.d.[c] |
| 18 | 3.04 (dd, *J* = 4.2, 13.6) | 42.9 | 3.03 (dd, *J* = 4.2, 13.5) | 42.6 |
| 19 | 1.91−1.34 | 47.5 | 1.90−1.33 | 47.2 |
| 20 | | 31.9 | | 31.5 |
| 21 | 1.60−1.41 | 35.2 | 1.60−1.40 | 34.6 |
| 22 | 1.91−1.82 | 33.4 | 1.92−1.80 | 33.5 |
| 23 | 9.50 (s) | 207.1 | 4.11−3.96 | 70.9 |
| 24 | 1.24 (s) | 10.2 | 0.92 (s) | 12.8 |
| 25 | 1.21 (s) | 16.5 | 1.17 (s) | 16.5 |
| 26 | 0.99 (s) | 18.1 | 0.99 (s) | 18.0 |
| 27 | 1.38 (s) | 26.7 | 1.34 (s) | 26.5 |
| 28 | | 178.5 | | 178.3 |
| 29 | 1.10 (s) | 33.8 | 1.10 (s) | 33.5 |
| 30 | 1.13 (s) | 24.5 | 1.13 (s) | 24.5 |

[a] Protons are multiplets, unless otherwise stated (s, singlet; dd, double doublet; t, triplet). When two values are given, the first corresponds to the equatorial proton and the second to the axial one, with the exception of those of the C-23 hydroxymethyl group in compound **2**. [b] These values may be interchanged. [c] n.d., not determined.

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**Table 2.** $^1$H and $^{13}$C NMR Chemical Shifts ($\delta$) and Proton−Proton Coupling Constants (Hz) for the Sugar Moieties of Compounds 1 and 2

| | **1** | | **2** | |
|---|---|---|---|---|
| | $^1$H | $^{13}$C | $^1$H | $^{13}$C |
| glucose 1 (Glc-1)[a] | | | | |
| 1 | 5.61 (d, $J_{1,2} = 8.2$) | 94.3 | 5.63 (d, $J_{1,2} = 8.0$) | 94.0 |
| 2 | 4.03 (dd, $J_{2,3} = 9.2$) | **78.4** | 4.01 (t, $J_{2,3} = 9.2$) | **78.1** |
| 3 | 3.84 (dd, $J_{3,4} = 9.7$) | 79.0 | 3.85 (t, $J_{3,4} = 9.2$) | 78.8 |
| 4 | 3.67 (dd, $J_{4,5} = 9.2$) | 71.1 | 3.67 (t, $J_{4,5} = 9.7$) | 70.9 |
| 5 | 3.70 (m, $J_{5,6a} = 2$, $J_{5,6b} = 4.9$) | 78.1 | 3.72 (m, $J_{5,6a} = 1.9$, $J_{5,6b} = 4.9$) | 77.8 |
| 6a | 4.29 (dd, $J_{6a,6b} = -11.6$) | **69.9** | 4.29 ($J_{6a,6b} = -11.6$) | **69.7** |
| 6b | 4.09 (dd) | | ca. 3.98 (dd) | |
| glucose 2 (Glc-2) | | | | |
| 1 | 4.98 (d, $J_{1,2} = 7.7$) | 104.0 | 4.99 (d, $J_{1,2} = 7.7$) | 103.8 |
| 2 | 3.37 (dd, $J_{2,3} = 9.4$) | 76.1 | 3.37 (dd, $J_{2,3} = 8.9$) | 75.2 |
| 3 | 3.53 (dd, $J_{3,4} = 8.8$) | 78.3 | 3.55 (dd, $J_{3,4} = 9.7$) | 78.1[b] |
| 4 | 3.30 (dd, $J_{4,5} = 10.8$) | 72.8 | ca. 3.48 | 72.6 |
| 5 | 3.44 (m, $J_{5,6a} = 1.6$, $J_{5,6b} = 7.0$) | 78.3[c] | ca. 3.48 | 77.8 |
| 6a | 4.08 (dd, $J_{6a,6b} = -11.6$) | 64.0 | 4.08 | 63.7 |
| 6b | 3.80 (dd) | | 3.83 | |
| glucose 3 (Glc-3) | | | | |
| 1 | 4.52 (d, $J_{1,2} = 7.6$) | 105.0 | 4.54 (d, $J_{1,2} = 7.9$) | 104.7 |
| 2 | 3.39 (dd, $J_{2,3} = 9.1$) | 75.5 | 3.41 (dd, $J_{2,3} = 9.2$) | 75.9 |
| 3 | 3.54 (dd, $J_{3,4} = 9.2$) | 78.4 | 3.55 (dd, $J_{3,4} = 9.7$) | 78.0[b] |
| 4 | 3.47 (dd, $J_{4,5} = 9.4$) | 71.9 | ca. 3.48 | 71.7 |
| 5 | ca. 3.49 | 78.5[c] | 3.43 | 77.8 |
| 6a | 4.04 | 63.1 | 4.04 | 62.8 |
| 6b | ca. 3.85 | | 3.85 | |

[a] The bold underlined values are glycosylation points. [b,c] These values may be interchanged.

(ca. 5.45 ppm, $J = 3.5$ Hz), probably due to a double bond at C-12, a double doublet (ca. 3.03 ppm, $J = 4.2$ Hz), characteristic of an axial (ring E) H-18 proton, and six methyl singlets were observed for **1**. The $^1$H NMR spectrum of **1** contained also an aldehyde proton (9.50 ppm) and a double doublet (4.70 ppm), with coupling constants indicating that this proton was geminal to an equatorial hydroxyl group. All of these observations were in good agreement with the characteristics of a gypsogenin moiety in the molecule of **1**.

The ROESY spectrum of **1** (mixing time = 400 ms), in addition to the expected intraresidue signals, gave the cross peaks H-1 (Glc-2)/H-2 (Glc-1), H-1 (Glc-3)/H-6a (Glc-1), and H-1 (Glc-3)/H-6b (Glc-1), suggesting that the Glc-2 and Glc-3 units are linked to Glc-1 at C-2 and C-6, respectively. However, due to the possible different conformations around the glycosidic bonds, the existence of NOEs between two protons does not guarantee the glycosylation site, although it was clear that a trisaccharide unit was linked to the aglycon moiety.

The $^{13}$C NMR spectrum of **1** gave 48 carbons and supported the deductions derived from the interpretation of the $^1$H NMR spectrum. Compound **1** showed two carbonyl peaks ($\delta$ 207.1, aldehyde, and $\delta$ 178.5, carboxylic). The rest of the $^{13}$C NMR chemical shifts were assigned from the HMQC spectrum (see Tables 1 and 2). The values for the aglycon moiety agreed with those published for similar saponins, and the value of the anomeric carbon for Glc-1 (ca. 94.0 ppm) demonstrated that it was linked as an ester to the C-28 carboxyl group of the aglycon.[6] In addition, C-2 and C-6 of the same unit gave values shifted downfield from those expected for unsubstituted glucopyranoses,[7] which indicated that Glc-1 was glycosylated at these positions by the other two glucose units (Glc-2 and Glc-3), thus supporting the ROESY results. Unequivocal demonstration of the type of glycosidic linkages was obtained from a HMBC experiment, which, in addition to expected intraresidue peaks for all different moieties (the aglycon and the three sugar residues), gave the cross peaks H-1 (Glc-2)/C-2 (Glc-1), H-1 (Glc-3)/C-6 (Glc-1), and C=O/H-1 (Glc-1). The HMBC experiment also corroborated the assignment of the chemical shifts for the quaternary carbons.

The $^1$H NMR spectrum of compound **2** did not show an aldehyde proton, but COSY and TOCSY experiments indicated the presence of two coupled protons (4.11 and 3.96 ppm, $J = 10.8$ Hz) and an additional signal at 3.86 ppm, probably arising from an axial H-3 proton, overlapping with those of the sugar rings. The lack of an aldehyde group and the presence of two additional downfield protons instead, were in agreement with the aglycon of **2** being hederagenin.[8] The downfield shift observed for H-3 in compound **1**, as compared with that in **2** (ca. 0.8 ppm), was indicative of the hydroxyl group being substituted in compound **1**. In compound **2**, the $^1$H NMR chemical shifts of the C-23 hydroxymethyl protons also appeared much more downfield than expected, suggesting that the hydroxyl group at this position was also substituted. Concerning the sugar protons of **2**, the pattern of the $^1$H NMR spectrum was almost identical to that observed in compound **1** (see Table 2).

The $^{13}$C NMR spectrum of **2** gave 48 carbons, but only one was carboxylic ($\delta$ 178.5). A secondary carbon at 70.9 ppm was assigned to C-23 through a HMBC spectrum. The chemical shifts of the sugar carbons were also almost identical to those of saponin **1** (Table 2).

Parallel hydrolytic experiments were carried out on **1** and **2**. On alkaline hydrolysis, saponin **1** produced gypsogenin[8] and a trisaccharide. Compound **2** afforded hederagenin[8] and the identical trisaccharide. On acidic hydrolysis, saponin **1** furnished gypsogenin and glucose, while saponin **2** yielded hederagenin and glucose, thus confirming the inferences from the NMR experiments.

As the three sugar moieties were linked to the carboxylic C-28 and no other substituent was deduced from the spectra that could justify the lowfield $^1$H NMR chemical shifts of the H-3 signal in saponin **1** and the resonances of the C-23 hydroxymethyl protons in saponin **2**, FABMS in the negative-ion mode were run for both compounds, which gave $[M - H]^-$ ions at $m/z$ 1035 and 1037, respectively,

corresponding to the molecular formulas $C_{48}H_{76}O_{22}S$ (**1**) and $C_{48}H_{78}O_{22}S$ (**2**). That suggested the presence of a sulfate group in both molecules. Acid hydrolysis of the saponins, followed by treatment with $BaCl_2$, gave white precipitates in both cases, thus demonstrating the existence of sulfate residues. Accordingly, saponins **1** and **2** were assigned as the 3*β*-sulfate ester of gypsogenin 28-*O*-*β*-D-glucopyranosyl-(1→2)-[*β*-D-glucopyranosyl (1→6)]-*β*-D-glucopyranoside (**1**) and the 23-sulfate ester of hederagenin 28-*O*-*β*-D-glucopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→6)]-*β*-D-glucopyranoside (**2**), respectively.

## Experimental Section

**General Experimental Procedures.** UV spectra were run in MeOH, on a Philips PU 8720 UV/vis spectrophotometer. IR spectra were recorded in KBr, on a Perkin−Elmer 681 spectrometer. NMR spectra were recorded in $CD_3OD$, on a Varian Unity 500 instrument at 25 °C. Chemical shifts refer to the MeOH-$d_4$ multiplet ($^1$H, 3.30 ppm; $^{13}$C, 49.0 ppm). FABMS were carried out in a VG AutoSpec (Fisons) mass spectrometer. Analytical TLC was carried out on Merck Si gel $F_{254}$ aluminum sheets, eluted with *n*-BuOH−AcOH−$H_2O$ (4:1:5), visualized with 1% vanillin in MeOH−$H_2SO_4$ (1:1). Sugars were identified by chromatographic comparison with an authentic sample of D-glucose. The eluents used were $CH_2Cl_2$−MeOH−$H_2O$ (50:25:5) and EtOAc−MeOH−AcOH−$H_2O$ (65:15:25:15), with the spray reagent being naphthoresorcinol−phosphoric acid. Analytical MPLC was carried out on a Merck LiChroprep RP-18 column.

**Plant Material.** *G. bermejoi* roots were collected in September 1995, in Cuenca, Spain, and identified by Dr. C. Bartolomé, Departamento de Biología Vegetal, Facultad de Ciencias, Universidad de Alcalá de Henares, Madrid, Spain. A voucher specimen has been deposited in Herbarium A. H. (no. 25.527), Universidad de Alcalá de Henares, Madrid, Spain.

**Extraction and Isolation.** Air-dried and powdered roots of *G. bermejoi* (900 g) were treated for 24 h at room temperature with 80% MeOH (9 L). The MeOH was removed in vacuo, and the resulting aqueous solution was extracted first with $CH_2Cl_2$ and then with *n*-BuOH. The *n*-BuOH was removed under vacuum, affording a solid residue (25.1 g). A portion (4.4 g) of this residue was chromatographed over a Si gel column, eluting with $CHCl_3$−MeOH−$H_2O$ mixtures of increasing $H_2O$ content (55:37:5, 55:37:6, 55:37:7), monitoring the fractions by TLC. Two saponin-containing fractions were each rechromatographed by MPLC with an elution gradient ($H_2O$ → MeOH 100%), from which two fractions were collected, giving **1** (33.6 mg) and **2** (8.1 mg) in pure form.

**3*β*-Sulfate ester of gypsogenin 28-*O*-*β*-D-glucopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→6)]-*β*-D-glucopyranoside (1):** amorphous powder, mp dec > 320 °C; $[\alpha]_D$ +20.6 °C *c* 0.50, MeOH); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 268 (2.9), 331 (2.6) nm; IR (KBr) $\nu_{max}$ 3400, 2900, 2820, 1730, 1710, 1620, 1220, 1050 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Tables 1 and 2; FABMS *m*/*z* [M − 1]$^-$ 1035.

**23-Sulfate ester of hederagenin 28-*O*-*β*-D-glucopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→6)]-*β*-D-glucopyranoside (2):** amorphous powder, mp 234−236 °C; $[\alpha]_D$ +6.5 °C *c* 1.50, MeOH); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 270 (3.2), 300 (sh) (2.9) nm; IR (KBr) $\nu_{max}$ 3380, 2900, 1730, 1620, 1050 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Tables 1 and 2; FABMS *m*/*z* [M − 1]$^-$ 1037.

**Alkaline Hydrolysis of 1 and 2.** Compounds **1** and **2** (2 mg) were each hydrolyzed with 0.2% KOH (4 mL) at 100 °C for 1.5 h and, after neutralization (HCl), extraction ($Et_2O$), and removal of the organic solvent in vacuo, the organic residues were chromatographed by TLC with $C_6H_6$−MeOH (50:6 v/v) and visualized with vanillin−$H_2SO_4$. The aglycons gypsogenin[8] (compound **1**) and hederagenin[8] (compound **2**) were obtained and identified by TLC comparison with authentic samples.

**Acid Hydrolysis of 1 and 2.** Compounds **1** and **2** (2 mg) were each refluxed with 10% HCl (4 mL) for 4 h. After extraction of the solution ($Et_2O$), the organic layers were chomatographed by TLC with $CHCl_3$−$CH_3COCH_3$ (50:6). Comparison with standard aglycons gave the same results as obtained by alkaline hydrolysis. The aqueous layers were neutralized with 10% *N*,*N*-dioctylmethylamine in $CHCl_3$ and concentrated in vacuo. The residues were compared (TLC) with standard sugars, and found to be D-glucose for both compounds **1** and **2**.

**Detection of a Sulfate Group in 1 and 2.** A 2-mg quantity of each sample was refluxed with 10% HCl (4 mL) for 4 h and then extracted with $Et_2O$. An aliquot of the aqueous layer of each was treated with 70% $BaCl_2$ to give a white precipitate ($BaSO_4$). The remainder of the aqueous layer was concentrated under reduced pressure. The residues were chromatographed by TLC (cellulose) with EtOH−$H_2O$ (7:3) and sprayed first with a solution of $BaCl_2$ in 70% MeOH and then with a solution of sodium rhodizonate.[3] A positive reaction revealed the presence of sulfate groups in both compounds **1** and **2**.

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