New Polyphenol Glycosides from *Ramonda myconi*†

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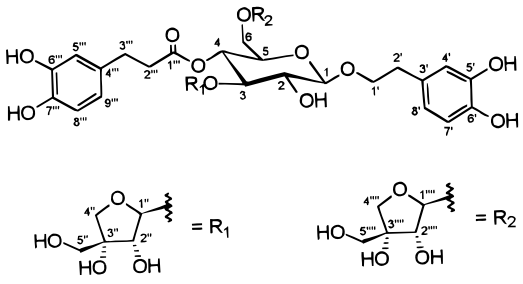
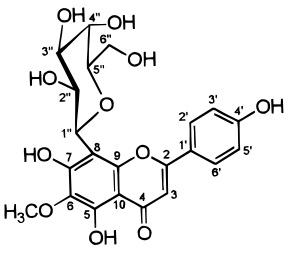
Two new polyphenol glycosides, myconoside (**1**) and hispidulin-8-*C*-*β*-D-galactopyranoside (**2**), and the known disaccharide sucrose have been isolated and identified from a MeOH extract of *Ramonda myconi* leaves. Structures were determined mainly using homonuclear and inverse-detected 2D NMR experiments.

Ramonda myconi Reichenbach (Gesneriaceae) is a plant endemic to Northeastern Iberian peninsula. Its dried leaves are used in Spain in traditional medicine to treat cough, bronchitis, and the common cold.[1]

In a previous work we have described the chemical composition of its mucilage, whose main polysaccharide showed a pectin-like structure with α-1,4-polygalacturonic acid as the main chain.[2] As a result of our continued studies on the phytochemistry of this plant, we report here the isolation and structure elucidation of two new polyphenol glycosides: **1**, for which the trivial name *myconoside* is proposed, and **2**, hispidulin-8-*C*-*β*-D-galactopyranoside, as well as the known disaccharide sucrose.



1



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The leaves of *R. myconi* were extracted sequentially with petroleum ether, $CHCl_3$, and MeOH. Separation of the MeOH extract was achieved on a polyamide column, eluting with mixtures of Me_2CO-H_2O of decreasing polarity. By means of further flash chromatography (Si gel), column chromatography (Si gel, polyamide, Sephadex LH-20), and MPLC (Si gel), compounds **1** and **2** and sucrose were isolated from the resulting fractions.

The negative-ion FABMS spectrum of myconoside (**1**) showed a $[M - H]^-$ peak at *m*/*z* 743, corresponding to a molecular weight of 744. Its IR spectrum displayed a strong band at 3413 cm^{-1}, indicating the presence of hydroxyl groups. When the ^1H NMR spectrum of **1** was recorded at 27 °C, the presence of broad hydroxy signals rendered its analysis difficult. The ^1H NMR spectrum of **1** obtained at −33 °C showed four sharp singlets between 8.3 and 8.5 ppm, characteristic of phenolic protons. This was in accordance with the UV data of **1**, which showed bathochromic shifts with the addition of basic reagents (NaOMe, NaOAc). In addition, the bathochromic shifts observed in the UV spectra of **1** after the addition of $NaOAc + H_3BO_3$ and $AlCl_3$, as well as the suppression of the shift observed in the $AlCl_3$ spectrum when HCl was added, suggested the presence of aromatic *o*-dihydroxy groups.

After acid hydrolysis of **1** and acetylation, glucose was the only sugar detected by GC−FID. In addition, the methylation of **1**, followed by hydrolysis, acetylation and GC−MS analysis showed that the glucose hydroxyl groups in the positions 1, 3, and 6 were substituted. Due to the methylation method used, however, it was not possible to detect the ester-linked substitutions. The presence of a *β*-glucose unit was elucidated by TOCSY-1D experiments, pulsing selectively the anomeric proton signal (*δ* 4.38, *J* = 7.8 Hz) and varying the mixing time from 16 to 95 ms. In the glucose subspectrum of **1** (TOCSY-1D), only one broad hydroxylic proton (*δ* 4.6 at 27 °C), belonging to position 2, was observed, which confirmed the substitution pattern on positions 1, 3, and 6 and also indicated that position 4 was substituted.

In the decoupled ^{13}C NMR spectrum of **1**, 33 carbons were observed, the multiplicity of which was analyzed from the DEPT spectrum (Table 1). A carbonyl carbon appearing at *δ* 172.6 was in accord with the IR band at 1733 cm^{-1}. In addition, 12 carbons belonging to two benzene rings were observed in the region ranging from *δ* 115 to 146. The remaining signals (*δ* 30−111) were attributable to aliphatic carbons.

In the HMBC, the H-1 showed a key cross-peak with the methylene C-1′ carbon at *δ* 71.5, in addition to the

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Table 1. NMR Data (Acetone-d_6) for **1**[a]

position[b]	δ_H	multiplicity ($J_{H/H}$, Hz)	δ_C	long-range heteronuclear correlations[c]
1	4.38	d (7.8)	103.6	74.1, 80.6, **71.5**
2	3.39	t (7.6)	75.4	103.6, 80.6
3	3.70	dd (9.5, 7.5)	80.6	70.4, 75.4, **111.0**
4	4.78	dd (9.5, 10.0)	70.4	67.7, 74.1, 80.6, **172.6**
5	3.62	ddd (2.5, 6.4, 10.0)	74.1	67.7, 70.4, 80.6, 103.6
6	3.55	dd (11.5, 2.5)	67.7	70.4, 74.1, **110.5**
	3.36	dd (11.5, 6.4)		70.4, 74.1, **110.5**
1′	3.95	ddd (6.9, 7.7, 9.7)	71.5	36.2, **103.6**, 131.1
	3.68	ddd (7.0, 7.7, 9.5)		36.2, **103.6**, 131.1
2′	2.75	m	36.2	71.5, 116.3, 121.0, 131.1
3′			131.1	
4′	6.75	d (2.1)	116.3	36.2, 121.0, 144.2
5′			145.6	
6′			144.2	
7′	6.70	d (8.0)	116.1	131.1, 144.2, 145.6
8′	6.58	dd (2.1,8.0)	121.0	36.2, 116.3, 144.2
1″	5.35	d (1.9)	111.0	74.8, 77.6, 80.1, **80.6**
2″	3.88	broad signal	77.6	overlapped
3″			80.1	
4″	3.85	d (9.5)	74.8	65.7, 77.6
	3.69	d (9.5)		77.6, 80.1, 111.0
5″	3.53	broad signal	65.7	overlapped
1‴			172.6	
2‴	2.65	m	36.8	30.7, 133.3, 172.6
3‴	2.80	m	30.7	36.8, 116.3, 120.3, 133.3, 172.6
4‴			133.3	
5‴	6.73	d (2.1)	116.2	30.6, 120.3, 133.3, 144.2, 145.7
6‴			145.7	
7‴			144.2	
8‴	6.71	d (8.0)	115.9	133.3, 144.2, 145.7
9‴	6.55	dd (2.1, 8.0)	120.3	30.7, 116.3, 144.2
1⁗	4.90	d (2.0)	110.5	**67.7**, 77.7, 80.1
2⁗	3.90	broad signal	77.7	overlapped
3⁗			80.1	
4⁗	3.91	d (9.5)	74.6	65.5, 77.7
	3.72	d (9.5)		77.7, 80.1, 110.5
5⁗	3.58	broad signal	65.5	overlapped
hydroxyl groups[d]				
2-OH	5.22	d (4.0)		
2″-OH	4.96	d (5.4)		
3″-OH	4.35	s		
5″-OH	4.64	t (5.9)		
2⁗-OH	4.95	d (5.7)		
3⁗-OH	4.33	s		
5⁗-OH	4.52	t (5.8)		

[a] Chemical shifts in δ (ppm), δ_H (acetone-d_6) = 2.2 ppm, δ_C (acetone-d_6) = 30.2 ppm. [b] Numbering according to the formula **1**. [c] δ_C (ppm) showing cross peaks with each proton signal in the HMBC spectrum. The more significant correlations are boldface. [d] Data from the spectrum recorded at −33 °C. In addition, at −33 °C, the phenolic protons 5′-OH, 6′-OH, 6‴-OH, and 7‴-OH were observed as four singlets between 8.3 and 8.5 ppm, which were not individually assigned.

intraglucose cross-peaks. The reverse interaction of the corresponding methylene protons (δ 3.95 and 3.68) with C-1 was also observed. Following the structural analysis from the concerted use of COSY, HMQC, and HMBC data, 2-(3,4-dihydroxyphenyl)ethanol was established as the aglycon structure linked to the position 1 of the glucose.

On the other hand, the H-3 proton showed a HMBC cross peak with the methynic C-1″ carbon at δ 111.0, which was directly bonded to the proton resonating at δ 5.35. The NOE effect between H-3 and H-1″ also confirmed these results. The concerted use of COSY, HMQC, and HMBC data confirmed a β-apiosyl structure linked to position 3 of the glucose. Its NMR data were in accordance with those reported in literature.[3,4] Following the same procedure, another β-apiosyl moiety was found to be linked to position 6 of glucose. NOE effects from the H-1⁗ and H-6 protons, as well as HMBC cross-peaks between H-1⁗/C-6, H-6α/C-1⁗, and H-6β/C-1⁗, confirmed this linkage.

Finally, an intense cross-peak in the HMBC spectrum appeared between the deshielded H-4 proton (δ 4.78) and the carbonyl carbon at δ 172.6 (C-1‴). Otherwise, this carbonyl carbon was long-range coupled with H-2‴ and H-3‴ protons, corresponding to different methylene groups (multiplets at δ 2.65 and δ 2.80, respectively), that in addition showed mutual COSY signal. The detailed analysis of HMQC and HMBC spectra allowed the establishment of a dihydrocaffeoyl structure linked to position 4 of glucose.

The mass spectra were in accordance with the proposed structure. Two consecutive losses of 132 mass units (m/z at 630 and 498, respectively), due to the cleavage of the apiosyl residues, followed by a loss of 154 units (m/z at 344), corresponding to the (dihydroxyphenyl)ethyl group, were observed in the DCIMS. A

Table 2. NMR Data for **2** in Methanol-d_4[a]

position	δ_C	δ_H	multiplicity ($J_{H/H}$, Hz)
2	166.8		
3	102.4	6.62	s
4	184.4		
5	153.5		
6	132.3		
7	158.7		
8	105.5		
9	153.4		
10	105.1		
1′	122.8		
2′, 6′	130.8	8.21	d (8.5)
3′, 5′	116.8	6.83	d (8.5)
4′	162.7		
6-OCH_3	60.8	3.86	s
1″	76.1	4.94	d (9.5)
2″	70.1	4.46	t (9.5)
3″	76.9	3.66	dd (9.5, 3.2)
4″	71.1	4.06	d (3.2)
5″	81.8	3.72	dd (7.6, 4.6)
6″	63.4	3.79	dd (7.6, 11.9)
		3.85	dd (4.6, 11.9)

[a] Chemical shifts in δ (ppm), δ_H (methanol-d_4) = 3.5 ppm, δ_C (methanol-d_4) = 49.3 ppm.

loss of 132 mass units (*m/z* at 611, apiosyl residue), followed by one of 152 units (*m/z* at 459, (dihydroxyphenyl)ethyl group), as well as a loss of 164 mass units (*m/z* 579, cleavage of the dihydrocaffeoyl group) were found in the negative FABMS. Thus, the structure of **1** (myconoside) was established as β-[(3,4-dihydroxyphenyl)ethyl]3,6-di-*O*-β-D-apiofuranosyl-4-*O*-α,β-dihydrocaffeoyl-*O*-β-D-glucopyranoside.

The positive-ion FABMS of **2** showed a $[M + H]^+$ peak at *m/z* 463, corresponding to a molecular weight of 462, which was confirmed by the negative-ion FABMS ($[M - H]^-$ peak at *m/z* 461). A loss of 162 mass units, characteristic of an hexose unit, was observed in the negative-ion FABMS (*m/z* at 299).

The UV spectrum showed bands at 273 and 335 nm and bathochromic shifts with NaOMe, $AlCl_3$, $AlCl_3$/HCl, NaOAc, and Na_3BO_3, indicating that **2** may be a 6-substituted 5,7,4′-trihydroxyflavone.[5,6] This structure was confirmed by the analysis of the ^1H NMR at 0 °C, which also evidenced a substitution in the position 8. Protons of a methoxy group (δ 3.86, s) and of the hexose moiety (δ 3.7−5.0) were also observed. A HMBC cross-peak between the methoxy singlet and the quaternary carbon at δ 132.3 confirmed this substituent for the position 6.

The resistance of **2** to acidic (TFA and HCl) hydrolysis suggested a C-glycoside structure. The hexose residue was identified as β-galactopyranose. The β-linkage was established by the large *J* (9.5 Hz) between H-1″ and H-2″ protons. In addition, sequential analysis of the sugar protons showed that all of them had an axial disposition except the H-4″ (doublet at δ 4.6, J = 3.2 Hz). The galactose C-1″ chemical shift at δ 76.1 was in accordance with a C-glycosidic linkage. Finally, the HMBC cross-peaks between the galactose H-1″ proton and the quaternary carbons at δ 105.5, 153.3, and 158.7 (corresponding to the aglycon C-8, C-9, and C-7, respectively) confirmed that the galactose was 8-C-linked.

The flavone glycoside **2** was therefore identified as hispidulin 8-C-β-D-galactopyranoside. The analysis of the HMQC and HMBC spectra allowed the complete assignment of the ^{13}C NMR signals (Table 2).

The pure disaccharide isolated was identified as β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside (sucrose) by chemical (hydrolysis and acetylation) and spectroscopic methods (FABMS, DCIMS, ^1H NMR, ^{13}C NMR), co-TLC with an authentic sample, as well as by comparison of its spectroscopic data with literature reports.[7−9]

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a Perkin-Elmer Model 550 S UV/vis spectrophotometer in MeOH, using the shift reagents described by Mabry *et al.*[5] IR spectra were taken in a Perkin-Elmer Model 1600 FTIR spectrophotometer. FABMS and DCIMS spectra were obtained on a Finnigan MAT instrument, Model MAT 95.

All NMR spectra were recorded in a Bruker AC-400 spectrometer operating at 400.16 and 100.62 MHz for proton and carbon, respectively. One-dimensional TOCSY spectra[10] were performed using a 80 ms Gaussian-shaped pulse, followed by a MLEV-17 sequence with additional 2.5 ms trim pulses for spin-lock. The one-bond $^1H-^{13}C$ chemical shift correlated spectra (HMQC)[11] and the multiple bond $^1H-^{13}C$ chemical shift correlated spectra (HMBC)[12] were acquired and processed with a nonshifted sine-bell window in both dimensions and a one-level zero-filling in the t_1 dimension prior Fourier transformation.

Column chromatography (CC) stationary phases were Polyamide CC-6 (Macherey Nagel, Düren, Germany), Si gel 60 0.040−0.063 mm (E. Merck, Darmstadt), and Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden). Flash chromatography[13] was over Si gel 60 0.040−0.063 mm (E. Merck, Darmstadt) and MPLC over Si gel 60 25−40 μm (LiChroprep^R, E. Merck, Darmstadt). TLC was over Si gel 60 F_{254}, aluminum sheets (E. Merck, Darmstadt), using the following solvent systems: $CHCl_3$−MeOH−H_2O (65:35:5) (S-1), AcOEt−MeOH−H_2O (77:15:8) (S-2), AcOEt−HCOOH−AcOH−H_2O (100:11:11:27) (S-3), and AcOEt−EtCOMe−AcOH−H_2O (5:31:1:1) (S-4).

Hydrolysis and Acetylation. The hydrolysis of the isolated substances was done with 2 M TFA at 121 °C for 90 min. The released monosaccharides were converted in their corresponding alditol acetates, according to the method of Blakeney *et al.*[14] These derivatives were analyzed by GC−FID using an OV 225 capillary column and an oven temperature of 207 °C (isotherm).

Plant Material. The leaves of *R. myconi* were collected near Olot (Girona, Spain) in September 1987. A voucher specimen (no. 35062) was deposited in the BCF Herbarium (Faculty of Pharmacy, University of Barcelona).

Extraction and Isolation. The air-dried leaves (750 g) were successively extracted in a soxhlet extractor with petroleum ether and $CHCl_3$. The marc was macerated with MeOH (2 × 7.5 L, 24 h for each extraction) at room temperature. Evaporation of the MeOH extracts yielded 99.7 g, 30 g of which were chromatographed on a polyamide column (100 × 6 cm). Mixtures of Me_2CO−H_2O of decreasing polarity (3:7−1:0) were used as eluents to give 19 fractions (1−19). Fractions 1 and 2 were submitted to flash chromatography over Si gel eluting with $CHCl_3$−MeOH−H_2O (65:45:5) in a column of 60 × 4 cm. From the fractions obtained, sucrose (777

mg) was obtained by means of MPLC and CC over Si gel, both eluted with $CHCl_3$−MeOH−H_2O (65:45:5), and CC on Sephadex LH-20 using MeOH−H_2O (1:1) as eluent. Fraction 3 (15 g) was purified on a polyamide column (100 × 6 cm), and elution with MeOH−H_2O (2:8) afforded 10 g of **1**. Finally, fractions 9 (74 mg) and 10 (132 mg) were rechromatographed over Sephadex LH-20 with MeOH as eluent, giving 7 and 27 mg, respectively, of **2**.

Myconoside (1): R_f 0.45 (S-1), 0.92 (S-2), and 0.37 (S-3); $[\alpha]^{24}_D$ −74° (*c* 1.0, MeOH); UV λ max nm (log ϵ) (MeOH) 280 (3.78), 327 (2.24); (NaOMe) 292, 325 (sh), 382; ($AlCl_3$) 288, 360; ($AlCl_3$ + HCl) 280, 320 (sh); (NaOAc) 280, 322 (sh); (NaOAc + H_3BO_3) 288, 325 (sh); IR (KBr) ν max 3413 (br), 1733 (sh), 1607, 1528, 1448, 1363, 1285, 821 cm^{-1}; negative FABMS *m/z* $[M - H]^-$ 743, 611, 579, 459, 447; positive FABMS *m/z* $[M + Na]^+$ 767; DCIMS *m/z* $[M + NH_4]^+$ 762, 630, 612, 498, 344. NMR spectra were recorded on a 20 mg sample of **1** dissolved in 0.5 mL of acetone-d_6; see Table 1.

Methylation of **1** was performed according to the method of Harris *et al.*[15] The permethylated derivatives were hydrolyzed and the released sugars converted to their corresponding alditol acetates, which were analyzed by GC−MS, using a DB 1701−30W capillary column.

Hispidulin 8-C-*β*-D-galactopyranoside (2): R_f 0.67 (S-1) and 0.72 (S-4); $[\alpha]^{24}_D$ +9° (*c* 0.2, MeOH); UV λ max nm (log ϵ) (MeOH) 273 (4.12), 335 (4.24); (NaOMe) 277, 328, 395; ($AlCl_3$) 267 (sh), 280, 303, 361; ($AlCl_3$ + HCl) 264 (sh), 290 (sh), 302, 355; (NaOAc) 276, 300 (sh), 380; (NaOAc + H_3BO_3) 281, 318 (sh), 400 (sh); positive FABMS *m/z* $[M + H]^+$ 463; negative FABMS *m/z* $[M - H]^-$ 461, 299; ^1H and ^{13}C NMR data, see Table 2.

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References and Notes

(1) Font Quer, P. *Plantas Medicinales*; Labor: Barcelona, 1962; pp 631−633.
(2) Cañigueral, S.; Müller, B. M.; Salvía, M. J.; Iglesias, J.; Franz, G. *Planta Med.* **1994**, *60*, 73−76.
(3) Kim; J.; Kinghorn, A. D. *Tetrahedron Lett.* **1987**, *28*, 3655−3658.
(4) Hamburger, M.; Gupta, M.; Hostettmann, K. *Phytochemistry* **1985**, *24*, 2689−2692.
(5) Mabry, T. J.; Markham, K. R.; Thomas, M. B. *The Systematic Identification of Flavonoids*; Springer: Berlin, 1970.
(6) Voirin, B. *Phytochemistry* **1983**, *22*, 2107−2145.
(7) Pfeffer, P. E.; Valentine, K. M.; Parrish, F. W. *J. Am. Chem. Soc.* **1979**, *101*, 1265−1274.
(8) Morris, G. A.; Hall, D. L. *J. Am. Chem. Soc.* **1981**, *103*, 4703−4711.
(9) Bremser, W.; Ernst, L.; Fachinger, W.; Gerhards, R.; Hardt, A.; Lewis, P. *Carbon-13 NMR Spectral Data: A Living COM-microfiche Collection of Reference Material*; Weinheim VCH: New York, 1987.
(10) Davis, D. G.; Bax, A. *J. Am. Chem. Soc.* **1985**, *107*, 7197−7198.
(11) Bax, A.; Subramanian, S. *J. Magn. Reson.* **1986**, *67*, 565−569.
(12) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.
(13) Still, W. C.; Kahn, M.; Mitra, A. *J. Org Chem.* **1978**, *43*, 2923−2925.
(14) Blakeney, A.; Harris, P.; Henry, R.; Stone, B. *Carbohydr. Res.* **1983**, *113*, 291−299.
(15) Harris, P. J.; Henry, R. J.; Blakeney, A. B.; Stone, B. A. *Carbohydr. Res.* **1984**, *127*, 59−73.

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