STRUCTURE ELUCIDATION OF A GLYCOSIDE OF 2β,3β,23-TRIHYDROXY-16-OXOOLEAN-12-EN-28-OIC ACID FROM *BELLIS BERNARDII* USING MASS SPECTROMETRY FOR THE SUGAR SEQUENCE DETERMINATION

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ABSTRACT.—The structure of a novel saponin, possessing an unusual aglycone named bellisonic acid and isolated from whole plants of *Bellis bernardii*, has been elucidated by modern ms and nmr techniques as 3-O-α-L-rhamnopyranosyl-2β,3β,23-trihydroxy-16-oxoolean-12-en-28-oic acid 28-α-L-rhamnopyranosyl(1→3)-O-β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-β-D-fucopyranoside [1]. Ms procedures of general applicability allowed unambiguous sugar sequence determination from very small amounts of material.

In previous papers we have reported the structures of several triterpenoid saponins from *Bellis perennis* and *B. sylvestris* (1–5). The present paper describes the isolation and structure elucidation of a novel glycoside obtained from *Bellis bernardii* Boiss. et Reuter. (Asteraceae), which is native to the Mediterranean island of Corsica.

The saponin **1** was isolated from the mild alkaline hydrolysate of a saponin mixture obtained from the whole plants of *B. bernardii*.

A four-stage ms procedure, of general applicability, was used to establish the nature of this compound using only a small amount of compound (<0.1 mg). In the first stage the isotopic composition of compound **1** was determined by high-resolution liquid
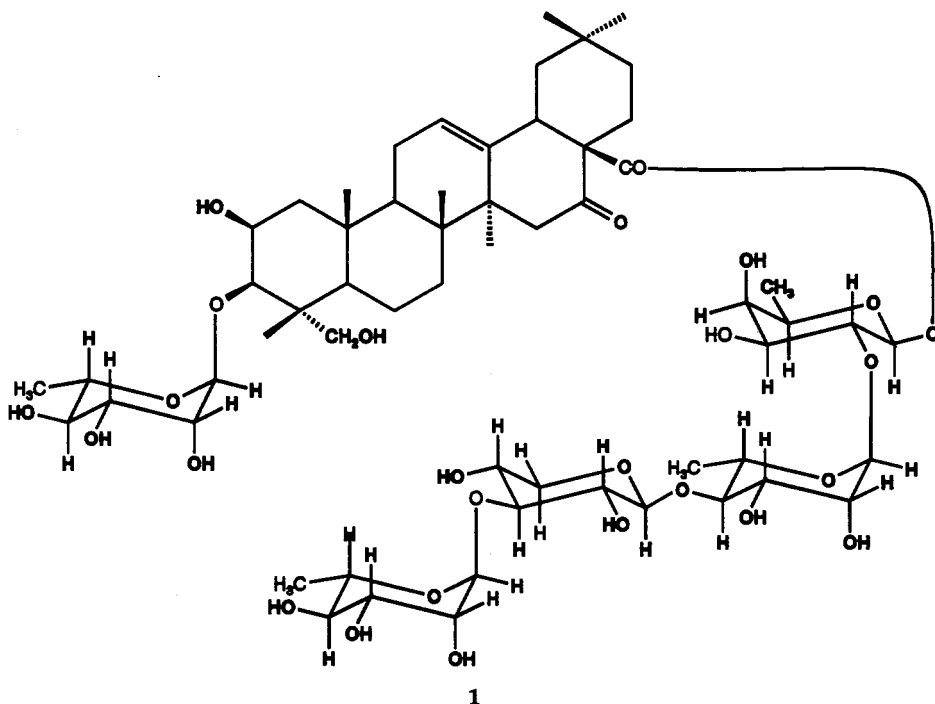
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secondary ion mass spectrometry (lsims) and gave a protonated molecular ion at m/z 1219.6071 daltons (calculated 1219.6111 daltons) corresponding to an elemental formula of $C_{59}H_{94}O_{26}$. In a second step, an electrospray ionization mass spectrum (esims) gave the equivalent molecular ion as the sodium adduct at m/z 1241 $[M+Na]^+$. This ion was selected as parent for ms/ms experiments. After collision-induced decompositon (cid) a daughter ion at m/z 1197 $[M-CO_2+Na]^+$ was detected, together with a series of further daughter ions generated by the repetitive loss of monosaccharide residues leaving the OH group on the respective fragments at m/z 1051 $[M-CO_2-dhex+Na]^+$, m/z 919 $[M-CO_2-dhex-pent+Na]^+$, m/z 905 $[M-CO_2-dhex; -dhex+Na]^+$, m/z 773 $[M-CO_2-dhex-pent-dhex+Na]^+$, m/z 627 $[M-CO_2-dhex-pent-dhex+Na]^+$, and m/z 463 $[M-CO_2-dhex-pent-dhex-dhex; -dhex-H_2O+Na]^+$. This fragmentation pattern indicated the presence of two terminal deoxyhexoses. While one of them is bound directly to the aglycone the other occurs as the terminal sugar of the tetrasaccharide unit consisting of a dhex-pent-dhex-dhex. The latter was confirmed by appearance of a complementary ion series generated by the respective eliminated oligosaccharides at m/z 593 $[dhex+pent+dhex+dhex-H_2O+Na]^+$, 447 $[dhex+pent+dhex-H_2O+Na]^+$, 301 $[dhex+pent-H_2O+Na]^+$, and 169 $[dhex-H_2O+Na]^+$. From these data it is evident that the mol wt and elemental composition of the aglycone are 502 daltons and $C_{30}H_{46}O_6$, respectively. The loss of CO_2 from the Na-adduct is characteristic of this aglycone and was not observed in other systems. It arises from skeletal rearrangement of the molecule: transfer of the tetrasaccharide from the carboxyl group at C-28 to the neighboring ketone (enol) group at C-16 with subsequent elimination of CO_2. The nmr data below confirmed this rationale as the original linkage of the tetrasaccharide to the carboxyl group and the presence of a ketone at C-16 was unequivocally demonstrated.

In a third stage, methylation analysis, by derivatization of the carbohydrate moiety of **1** to the methylated alditol acetates followed by gc-ms analysis, yielded 1,5,-di-O-acetyl-2,3,4-tri-O-methyl-rhamnitol, 1,3,5-tri-O-acetyl-2,4-di-O-methyl-xylitol, 1,4,5-tri-O-acetyl-2,3-di-O-methyl-rhamnitol and 1,2,5-tri-O-acetyl-3,4-di-O-methyl-fucitol, identified by their retention times and characteristic fragmentation patterns (6), in a ratio of 2:0.7:1:1. These results indicated the presence of two terminal rhamnose residues, a 3-substituted xylose, a 4-substituted rhamnose, and a 2-substituted fucose.

Finally, in a fourth step, reductive elimination of the tetrasaccharide, followed by permethylation, gave a molecular ion at m/z 767 $[dhex_2 pent\ dhex\text{-}ol+Na]^+$, which by esims gave a fragmentation pattern by ms/ms compatible with dhex-pent-dhex-dhex-ol, confirming the sugar sequence. Methylation analysis of this reduced tetrasaccharide gave 2-O-acetyl-1,3,4,5-tetra-O-methylfucitol instead of the tri-acetylated fucitol derivative described above demonstrating the presence of fucose at the reducing end of the tetrasaccharide and establishing unequivocally the sequence $rha^1{\rightarrow}^3xyl^1{\rightarrow}^4rha^1{\rightarrow}^2fuc$.

The above-described ms procedures obviously do not yield information about the anomericity of the monosaccharide residues, nor is a complete structural characterization of the aglycone possible using exclusively ms techniques. However, it is possible to determine differences in the aglycone or the sugar moiety in a series of compounds with similar basic structures using ms techniques alone. Additionally, all the described mass spectrometric techniques have the advantage of requiring only very small amounts of material and are applicable to mixtures and impure samples.

Nmr data allowed the further characterization of the nature of the aglycone and sugar moieties. The presence of signals at 210.15, 173.43, 141.16, and 126.18 ppm in the ^{13}C-nmr spectrum indicated that compound **1** possesses a ketone function, an esterified carboxylic group, and a double bond. By means of 1D, ^{1}H-, ^{13}C-, DEPT 2D

COSY, HMQC, and HMBC nmr spectra all the proton and carbon signals of **1** could be assigned as shown in Tables 1 and 2. Coupling constants of 3.5 Hz of H-2 (4.26 ppm) and H-3 (3.72 ppm) in the ^{1}H-nmr spectrum demonstrated the relative stereochemistry at C-2 and C-3. The positions of a rhamnose at C-3 and of the tetrasaccharide at C-28 were deduced from the HMBC (2D long-range ^{13}C-^{1}H correlation) spectrum. Cross-peaks were observed between H-1 (4.90 ppm) of one rhamnose (rha C) and C-3 of the aglycone (82.3 ppm) and C-1 (104.17 ppm) of rha C and H-3 (3.72 ppm) of the aglycone, and between H-1 (5.37 ppm) of fucose and C-28 (173.43 ppm) of the aglycone. The same spectra confirmed the sugar sequence. The β-anomeric $^4C_1$ configurations of xylose and fucose and the α-anomeric $^1C_4$ configurations of the rhamnoses were evident from the ^{1}H-^{1}H and ^{1}H-^{13}C coupling constants. The preparation and glc of the L-cysteine methyl ester derivatives prepared according to (7) indicated that rhamnose is present as the L-enantiomer and that xylose and fucose are present as D-enantiomers.

Hence, **1** (bernardioside B$_4$) has the structure 3-O-α-L-rhamnopyranosyl-2β,3β,23-trihydroxy-16-oxoolean-12-en-28-oic acid 28-O-α-L-rhamnopyranosyl(1→3)-β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-β-D-fucopyranoside.

A keto function at position 16 occurs very rarely among triterpenes with the oleanane skeleton. Although 16-oxo derivatives of oleanan-29-oic acids (8,9), oleanan-30-oic acid (10) and of oleanane sapogenols (11) have been obtained from plants, the aglycone of bernardioside B$_4$ [**1**] is the first genuine 16-oxoolean-12-en-28-oic acid. It

TABLE 1. ^{1}H-Nmr Data of **1**.

Proton	Aglycone [δ J (Hz)]		Proton	Sugars [δ J (Hz)]
H-1A	2.08	Rhamnose A	H-1	5.17 (1.8)
H-1B	1.23	(chain-end)	H-2	4.00 (3.4, 1.8)
H-2	4.25 (3.5, 3.5, 3.5)		H-3	3.76 (9.5, 3.4)
H-3	3.72 (3.5)		H-4	3.44 (9.6, 9.6)
H-5	1.59		H-5	4.04 (9.6, 6.2)
H-6A	1.59		H-6	1.31 (6.2)
H-6B	1.56	Xylose	H-1	4.50 (7.8)
H-7A	1.62		H-2	3.38
H-7B	1.35		H-3	3.50 (8.9, 8.9)
H-9	1.59		H-4	3.57
H-11A	2.14 (18.6, 11.5, 3.1)		H-5A	3.92 (11.5, 5.4)
H-11B	2.06		H-5B	3.25 (11.6, 10.3)
H-12	5.59 (3.8, 3.8)	Rhamnose B	H-1	5.37 (1.9)
H-15A	2.83 (14.8)	(internal)	H-2	3.94 (3.3, 1.8)
H-15B	2.03 (14.7)		H-3	3.83 (9.5, 3.4)
H-18	ca. 1.59		H-4	3.54 (9.4, 9.4)
H-19A	1.47		H-5	3.69 (9.5, 6.2)
H-19B	1.27		H$_3$-6	1.29 (6.5)
H-21A	1.51	Fucose	H-1	5.45 (8.1)
H-21B	1.29		H-2	3.82 (9.1, 8.1)
H-22A	2.23 (13.8, 3.8, 3.0)		H-3	3.71 (9.1, 3.5)
H-22B	1.61		H-4	3.61 (3.5, 1.0)
H-23A	3.37 (11.3)		H-5	3.74 (6.5, 1.1)
H-23B	3.31 (11.3)		H$_3$-6	1.28 (6.5)
H$_3$-24	0.96	Rhamnose C	H-1	4.90 (1.6)
H$_3$-25	1.39	(at C-3)	H-2	3.96 (3.4, 1.7)
H$_3$-26	0.97		H-3	3.83 (9.5, 3.4)
H$_3$-27	1.25		H-4	3.42 (9.5, 9.5)
H$_3$-29	0.91		H-5	3.85 (9.5, 6.3)
H$_3$-30	0.98		H$_3$-6	1.28 (6.5)

TABLE 2. ^{13}C-Nmr Data of **1** in CD$_3$OD.

Aglycone (δ)				Sugar (δ)						
C-1	44.97	C-16	210.14	Rha A	C-1	102.56	Fuc	C-1	95.68
C-2	71.71	C-17	60.44		C-2	72.20[a]		C-2	74.99
C-3	82.30	C-18	48.26		C-3	72.80[a]		C-3	76.40
C-4	43.42	C-19	47.41		C-4	74.03		C-4	73.44
C-5	47.92	C-20	31.43		C-5	70.06		C-5	72.20
C-6	18.76	C-21	35.56		C-6	18.41		C-6	16.56
C-7	33.30	C-22	27.48	Xyl	C-1	107.17	Rha C	C-1	104.17
C-8	41.36	C-23	65.50		C-2	76.40		C-2	72.20[a]
C-9	47.92	C-24	14.79		C-3	84.52[b]		C-3	72.80[a]
C-10	37.67	C-25	17.50		C-4	69.91		C-4	74.03
C-11	24.73	C-26	18.03		C-5	67.17		C-5	70.38
C-12	126.18	C-27	27.81	Rha B	C-1	101.41		C-6	17.86
C-13	141.16	C-28	173.43		C-2	71.90			
C-14	49.24	C-29	33.30		C-3	72.20[a]			
C-15	46.88	C-30	23.79		C-4	84.54[b]			
					C-5	69.04			
					C-6	17.98			

[a-b]Assignments bearing the same superscript may be interchanged.

seems that saponins of 2β,3β,23-trihydroxy-16-oxoolean-12-en-28-oic acid are a distinct feature of *B. bernardii*, as glycosides of this aglycone (bellisonic acid) have so far not been found in *B. perennis* or *B. sylvestris*.

Experimental details including general procedures, plant material, extraction, and isolation, may be obtained from the senior author.

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