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REVISED STRUCTURES OF TRITERPENOID SAPONINS FROM THE FLOWERS OF *CALENDULA OFFICINALIS*

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ABSTRACT.—From the flowers of *Calendula officinalis* six saponins (glycosides A–D, D_2, and F), previously studied by Kasprzyk *et al.*, were isolated. Their structures were definitively established by fabms, fabmikems, ^{13}C-nmr spectroscopy, and chemical methods (acid and basic hydrolysis). Oleanolic acid 3-*O*-β-D-glucuronide is a basic unit of all these compounds. In glycosides A and B, β-D-galactopyranosyl and β-D-glucopyranosyl substituents are bound to glucuronic acid at positions 3 and 2, respectively.

Calendula officinalis L. (Compositae) is an annual plant which is cultivated in Mediterranean countries. It is reputed for its use as an emmenagogue, anti-inflammatory, and cicatrizing drug (1,2). It was previously reported (3–5) that in flowers of *C. offficinalis* there are six saponins related to oleanolic acid (glycosides A–D, D_2, and F). It was assumed that the structures of three of them, containing sugars only at the 3β position of oleanolic acid, were the β-D-glucuronoside (glycoside F), the β-D-galactopyranosyl(1↦3 or 2)-β-D-glucuronoside (glycoside D), and the β-D-glucopyranosyl(1↦4)-[β-D-galactopyranosyl(1↦3 or 2)]-β-D-glucuronoside (glycoside B). The remaining three saponins (D_2, C, and A) were the 28-β-D-glucopyranosyl ester of glycosides F, D, and B, respectively. However, among these proposed structures, only those of glycosides F and D_2 were clearly established by comparison with calenduloside F (6).

In the course of a pharmacological study of *C. officinalis* we isolated the six saponins previously studied by Kasprzyk and co-workers (3). In the present paper, we report the isolation and the structural study of these compounds. Their structures were definitively established by fabms, fabmikems, and ^{13}C nmr.

The dried flowers of *C. officinalis* were extracted successively with $CHCl_3$, EtOAc, EtOAc/MeOH and MeOH/H_2O. The hydromethanolic extract was purified by low pressure chromatography. The isolation of pure glycosides A–D, D_2, and F was carried out by low and medium pressure chromatography. Alkaline hydrolysis of glycosides A, C, and D_2 liberated glucose and glycosides B, D and F, respectively. Further evidence for monodesmosidic vs. bidesmosidic structures was obtained by ^{13}C-nmr spectra where the free carboxyl group of the aglycone appeared at δ ca. 180 ppm whereas when esterified with a sugar chain, it resonated at δ ca. 176.5 ppm (7). Acid hydrolysis of glycosides B, D, and F afforded, besides oleanolic acid, glucose, galactose, and glucuronic acid for glycoside B, galactose and glucuronic acid for glycoside D, and glucuronic acid for glycoside F.

The molecular formulae $C_{54}H_{86}O_{24}$, $C_{48}H_{76}O_{19}$, $C_{48}H_{76}O_{19}$, $C_{42}H_{66}O_{14}$, $C_{42}H_{66}O_{14}$, and $C_{36}H_{56}O_9$, for glycosides A–D, D_2 and F, respectively, were determined by fabms and by DEPT ^{13}C nmr (Table 1).

The fabms spectrum of glycoside A showed a quasi molecular peak at *m/z* 1117 and signals at *m/z* 955, 455, corresponding respectively to the loss of one hexose moiety, three hexose, and one

TABLE 1. ^{13}C-nmr Data for Saponins **1–6** (in pyridine-d_5; in ppm; TMS as internal reference).

Carbon		Compound						DEPT
		Glycoside A	Glycoside B	Glycoside C	Glycoside D	Glycoside D_2	Glycoside F	
β-D-glucuronic acid	1	104.9[a]	105.2	106.2[a]	106.3[a]	106.5	106.7	CH
	2	79.0[b]	79.3	74.5[b]	74.0	76.2	76.0	CH
	3	87.6	87.8	87.6	87.6	78.0	78.0	CH
	4	72.1[c]	71.8	71.9[c]	71.3	74.0[a]	74.2	CH
	5	76.1[d]	77.2[a]	75.9	77.0	75.3	75.8	CH
	6	—	171.7	—	171.6	176.5	174.3	C
β-D-galactose	1	104.6[a]	105.2	106.0[a]	106.0[a]			CH
	2	72.8[e]	73.0	72.8	72.7			CH
	3	75.4	75.4	75.0	74.8			CH
	4	70.8	70.1	70.6	69.2			CH
	5	77.3[f]	77.3[a]	77.2	77.0			CH
	6	62.8	62.0	62.6[d]	61.9			CH_2
β-D-glucose	1	103.7	103.8					CH
	2	76.4[d]	76.3					CH
	3	77.6[f]	77.7[a]					CH
	4	72.9[e]	72.8					CH
	5	78.6[b]	78.6					CH
	6	63.7	63.5					CH_2
28-β-D-glucose	1	95.9		95.9		95.7		CH
	2	74.3		74.3[b]		74.1[a]		CH
	3	79.2[b]		79.2		79.0		CH
	4	71.7[c]		71.6[c]		71.5		CH
	5	79.0[b]		79.0		78.8		CH
	6	62.8		62.7[d]		62.5		CH_2

[a–f]Data in the same column with the same superscript may be interchanged.

glucuronic acid moiety. Glycosides B and C yielded a quasi molecular peak at *m/z* 955 and signals at *m/z* 793, 455 in agreement with the loss of one hexose moiety, two hexose, and one glucuronic acid moiety, respectively. Glycoside D showed a quasi molecular peak at *m/z* 793 and signals at *m/z* 631 and 455, corresponding respectively to the loss of one hexose, one hexose, and one glucuronic acid moiety. In addition, the mike spectrum of the peak at *m/z* 793, for glycosides A–D, showed an ion at *m/z* 631 resulting from the loss of one hexose fragment. This fact indicated that oleanolic acid 3-β-D-glucuronide is a basic structure of glycosides A–D, D_2, and F.

The identification and the β-D-configuration of the sugar chains were deduced from ^{13}C-nmr spectra (Table 1) by comparison with literature data (8–14). The important downfield shift in glycosides A–D, D_2, and F for C-3 of the aglycone indicated that the sugar chains were linked at this position (14–16). Downfield shifts for C-2 and C-3, in glycosides A and B, and C-3, in glycosides C and D, of the glucuronic acid moiety, clearly indicated that sugar residues are linked at these positions (14). From these results and from the well established biosynthetic reactions of the sugar chains of *C. officinalis* glycosides (4,17), it is evident that in glycosides A–D the β-D-galactopyranosyl group is attached to glucuronic acid at position 3, whereas the β-D-glucopyranosyl substituent in glycosides A and B is at position 2.

Finally, the structures of glycosides A–D were established as glycoside A: 3-*O*-{*O*-β-D-galactopyranosyl(1→3)*O*-[β-D-glucopyranosyl(1→2)]β-D-glucuronopyranosyl} oleanolic acid-28-*O*-β-D-glucopyranoside; glycoside B: 3-*O*-{*O*-β-D-galactopyranosyl(1→3)-*O*-[β-D-glucopyranosyl(1→2)]β-D-glucuronopyranosyl} oleanolic acid; glycoside C: 3-*O*-[*O*-β-D-galactopyranosyl(1→3)-*O*-β-D-glucuronopyranosyl] oleanolic acid-28-*O*-β-D-glucopyranoside; and glycoside D: 3-*O*-[*O*-β-D-galactopyranosyl(1→3)-*O*-β-D-glucuronopyranosyl] oleanolic acid. Glycosides D_2 and F were identified, respectively, as 3-*O*-β-D-glucuronopy-

ranosyl oleanolic acid-28-*O*-β-D-glucopyranoside and 3-*O*-β-D-glucuronopyranosyl oleanolic acid on the basis of their ^{13}C-nmr spectra.

Among these saponins, glycoside B was previously described from *Hemsleya chinensis* (18), glycosides C and D from *Calendula arvensis* (7), and glycoside F from *Lonicera nigra* (19) and *Beta vulgaris* (20).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were recorded on a Bruker AM-200 multinuclear spectrometer; the chemical shifts were measured in pyridine-d_5 solutions and TMS was used as an internal standard; carbon multiplicities were determined by the DEPT pulse sequence; fab mass spectra were obtained on a VG Micromass ZAB-HF mass spectrometer in the negative ion mode in a thioglycerol matrix; low pressure chromatography was performed on a Jobin Yvon chromatospac prep 10; medium pressure chromatography was performed on a Buchi B680 chromatograph. Hplc retention times were obtained on a Waters Model 6000 A pump equipped with a U6K injector and a UV Model 490 detector.

ISOLATION AND IDENTIFICATION.—The dried flowers of *C. officinalis* (1 kg) were extracted successively with $CHCl_3$, EtOAc, EtOAc-MeOH (50:50) and MeOH-H_2O (50:50). The hydromethanolic extract is concentrated in vacuo until complete elimination of MeOH and lyophilized. The extract (220 g) was subjected to preparative low pressure chromatography on an RP 18 reversed-phase column (Merck 15–25 μm) with MeOH-H_2O (60:40 for **1**, 80:20 for **2**). These fractions were submitted to medium chromatography on a silica column (Merck 230–400 mesh) with $CHCl_3$-MeOH-H_2O (61:32:5) to yield pure glycoside A (541 mg), glycoside B (167 mg), glycoside C (471 mg), glycoside D (141 mg), glycoside D_2 (21 mg), and glycoside F (23 mg).

ANALYTICAL TLC.—Analytical tlc was carried out on Si gel (Merck F_{254}, 0.25 nm). Tlc system 1 employed *n*-BuOH–HOAc–H_2O (4:1:5). Tlc system 2 used C_6H_6-MeOH (50:6). The spots were visualized by spraying with H_2SO_4 and then heating (110°, 5 min). Tlc system 3 used iPrOH 1/30 M-H_3BO_3 (80:15). The sugars were visualized by spraying the aniline hydrogen phthalate and then heating (110°, 10 min).

ACID HYDROLYSIS.—Acid hydrolysis was performed in a sealed tube at 100° for 4 h with 3 mg of saponin in 2 ml of concentrated HCl-H_2O (10:90). The aqueous residue was extracted with Et_2O. It was then neutralized with *N,N*-dioctylamine (10% in $CHCl_3$) and lyophilized. Oleanolic acid was detected in the organic layer, while sugars were identified in the aqueous layer.

ALKALINE HYDROLYSIS.—Saponin (3 mg) in 2 ml of KOH (15%) was heated at 100° in a sealed tube for 75 min. After acidification with HCl (pH 5), the monodesmoside was extracted with *n*-BuOH.

3-*O*-{*O*-β-D-GALACTOPYRANOSYL(1↦3)-*O*-[β-D-GLUCOPYRANOSYL(1↦2)]β-D-GLUCURONOPYRANOSYL} OLEANOLIC ACID-28-*O*-β-D-GLUCOPYRANOSIDE (GLYCOSIDE A).—Hplc retention time 5.85 min; tlc R_f (system 1) 0.33; mp 227–229° (dec); $[\alpha]_D$ +12° (H_2O, c = 0.5); fabmikems (negative ion mode, thioglycerol matrix) *m/z* $[M-H]^-$ 1117, $[M-H-162]^-$ 955, $[M-H-162-162]^-$ 793, $[M-H-162-2\times162]^-$ and $[M-H-162-162-162]^-$ 631, $[M-H-162-162-(162+176)]^-$, $[M-H-162-(2\times162+176)]^-$, and $[M-H-162-(2\times162)-176]^-$ 455.

3-*O*-{*O*-β-D-GALACTOPYRANOSYL(1↦3)-*O*-[β-D-GLUCOPYRANOSYL(1↦2)]β-D-GLUCURONOPYRANOSYL} OLEANOLIC ACID (GLYCOSIDE B).—Hplc retention time 12.92 min; tlc R_f (system 1) 0.33; mp 242–244° (dec); fabmikems (negative ions mode, thioglycerol matrix) *m/z* $[M-H]^-$ 955, $[M-H-162]^-$ 793, $[M-H-162-162]^-$ and $[M-H-2\times162]^-$ 631, $[M-H-162-162-176]^-$ and $[(M-H)-2\times162-176]^-$, and $[(M-H-162)-172-166]^-$ 455.

3-*O*-[*O*-β-D-GALACTOPYRANOSYL(1↦3)-*O*-β-D-GLUCURONOPYRANOSYL] OLEANOLIC ACID-28-*O*-β-D-GLUCOPYRANOSIDE (GLYCOSIDE C).—Hplc retention time 6.89 min; tlc R_f (system 1) 0.38; mp 199–201° (dec); fabmikems (negative ion mode, thioglycerol matrix) *m/z* $[M-H]^-$ 955, $[M-H-162]^-$ 793, $[M-H-162-162-176]^-$ 455.

3-*O*-[*O*-β-D-GALACTOPYRANOSYL(1↦3)-*O*-β-D-GLUCURONOPYRANOSYL] OLEANOLIC ACID (GLYCOSIDE D).—Hplc retention time 18.38 min; tlc R_f (system 1) 0.46; mp 117–119° (dec); fabmikems (negative ion mode, thioglycerol matrix) *m/z* $[M-H]^-$ 793, $[M-H-162]^-$ 631, $[M-H-162-176]^-$ 455.

3-*O*-β-D-GLUCURONOPYRANOSYL OLEANOLIC ACID-28-*O*-β-D-GLUCOPYRANOSIDE (GLYCOSIDE D_2).—Hplc retention time 8.09 min; tlc R_f (system 1) 0.58; mp 197–199° (dec); fabmikems (negative ion mode, thioglycerol matrix) *m/z* $[M-H]^-$ 793, $[M-H-162]^-$ 631, $[M-H-162-176]^-$ 455.

3-*O*-β-D-GLUCURONOPYRANOSYL OLEANOLIC ACID (GLYCOSIDE F).—Hplc retention

time 22.45 min; tlc R_f (system 1) 0.68; mp 243–245° (dec); fabmikems (negative ion mode, thioglycerol matrix) *m/z* $[M-H]^-$ 631, $[M-H-176]^-$ 455.

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