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ARVENSOSIDE A AND B, TRITERPENOID SAPONINS FROM *CALENDULA ARVENSIS*

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Key Word Index—*Calendula arvensis*; Compositae; structure determination; spectroscopic methods; oleanolic acid-28-*O*-β-D-glucopyranoside-3-β-*O*-(*O*-β-D-galactopyranosyl(1 → 3)-β-D-glucopyranoside); oleanolic acid 3-β-*O*-(*O*-β-D-galactopyranosyl(1 → 3)-β-D-glucopyranoside).

Abstract—Two new triterpenoid glycosides from the aerial parts of *Calendula arvensis* were identified as oleanolic acid-28-*O*-β-D-glucopyranoside-3-β-*O*-(*O*-β-D-galactopyranosyl(1 → 3)-β-D-glucopyranoside) and oleanolic acid 3-β-*O*-(*O*-β-D-galactopyranosyl(1 → 3)-β-D-glucopyranoside) by FAB, FAB MIKE mass spectrometry and ^{13}CNMR spectroscopy.

INTRODUCTION

Calendula arvensis L. is an annual plant which grows in mediterranean countries especially in Tunisia between Hammam-Lif and Sfax. It is reputed for its use as an emmenagogue [1] and an anti-inflammatory [2–4] drug. However, no chemical work appears to have been done on this plant. In the course of our study of *Calendula arvensis*, we have isolated two new glycosidic substances which we named arvensoside A (1) and arvensoside B (2). The former shows anti-inflammatory activity [5]. In this paper we wish to report the structural investigation of these compounds.

RESULTS AND DISCUSSION

A methanolic extract of the fresh aerial parts of *Calendula arvensis* on repeated chromatographic purification yielded the two glycosides 1 and 2. Acid hydrolysis of these compounds afforded the same aglycone, identified as oleanolic acid by comparison with an authentic sample (TLC, ^{13}C NMR), as well as glucose and galactose identified by TLC. Basic hydrolysis of 1 gave 2.

Mass spectrometry of 1 with fast atom bombardment yielded a quasi molecular peak at m/z 977 $[M-H+2H_2O]^-$ indicating a molecular weight of 978 in agreement with the formula $C_{48}H_{78}O_{18}+4H_2O$ found by elemental analysis. Similarly we obtained the formula of 2 as $C_{42}H_{68}O_{13}+4H_2O$.

The sugar sequence of the saponins were established by FAB MS [6]. The mass spectrum of 1 (thioglycerol matrix, negative-ion mode) showed peaks at m/z 779 $[M-H-2H_2O-162]^-$, 617 $[M-H-2H_2O-324]^-$ and 455 $[M-H-2H_2O-486]^-$ corresponding to the loss of one, two and three hexose moieties, respectively. In addition the MIKE spectrum [7] of the peak at m/z 977 showed an ion at m/z 779 resulting from the loss of 162 amu (hexose). The peaks in the MIKE spectrum of the ion at m/z 779 were m/z 735, 617, 599 and 455 resulting from the loss of 44 amu (CO_2), 162 amu (hexose), 180 amu (hexose + H_2O), and 324 amu (2 hexose), respectively. These data indicated that the ion at m/z 779, which was the base peak of the mass spectrum of 1, corresponds to

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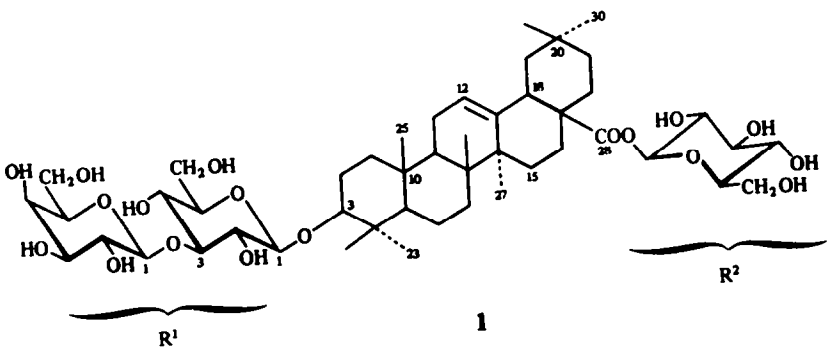

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the elimination from the $[M-H]^-$ ion of one hexose at the ester group [8]. It was further inferred that a dihexose moiety was linked to the aglycone at C-3. This result was confirmed by the MIKE spectrum of the ion at m/z 617 showing two ions at m/z 455 and 393 corresponding to the loss of 162 amu (hexose) and 224 amu (hexose + H_2O + CO_2). In the mass spectrum of **2** a quasimolecular ion appeared at m/z 815 $[M-H+2H_2O]^-$ and a molecular ion at 779 $[M-H]^-$. Signals at m/z 617 $[M-H-162]^-$, 599 $[M-H-162-H_2O]^-$ and 455 $[M-H-324]^-$ correspond to the successive loss of two hexose residues as indicated by the MIKE spectra of the peak at m/z 779 (peak at m/z 617) and peak at m/z 617 (peak at m/z 455). Furthermore, peaks at m/z 763 $[M-H-OH]^-$, 735 $[M-H-CO_2]^-$ and 455 in the MIKE spectrum of the $[M-H]^-$ ion indicated clearly that **2** contained a free carboxyl group and thus a dihexose moiety linked to the aglycone at C-3.

The interglycosidic linkages as well as the nature of the sugar chains linked to the aglycone in the saponins were established by ^{13}C NMR spectroscopy. The data were obtained from a proton noise decoupled spectrum and from DEPT [9] experiments that allowed us to recognize the number of CH, CH_2 and CH_3 carbons unequivocally. They are summarized in Table 1.

The identification of ^{13}C NMR signals belonging to each monosaccharide residue relied mostly on comparison with those of model compounds. Experience indicates that the chemical shifts of monosaccharide units within polysaccharide chains are similar to those of monosaccharides except for substituent effects. These effects cause an increase in the chemical shift of the carbon directly involved in the linkage. This is usually accompanied by a decrease of smaller magnitude in the chemical shifts of the neighbouring β-carbon atom. Finally, this approach led to a number of simple, general rules [10, 11]: (a) the anomeric carbon atoms in pyranoses and in their derivatives resonate at lowest field (90–110 ppm), (b) carbon atoms bearing secondary hydroxyl groups in pyranoses give signals at 65–85 ppm; signals of alkoxylated carbon atoms are shifted 5–10 ppm to lower field when compared with the corresponding hydroxy-substituted carbon atoms, (c) carbon atoms carrying primary hydroxyl groups are found at 60–64 ppm, (d) acylation of oxygen leads to smaller (1.5–4 ppm) high frequency shifts of the β-carbon atom.

Table 1. ^{13}C NMR chemical shifts of arvensoside A and arvensoside B

Aglycone moieties					Sugar moieties						
							Arvensoside A			Arvensoside B	
							R^1		R^2	R^1	
Atoms	Multiplicity	Oleanolic acid Me ester	Arvensoside A	Arvensoside B	Atoms	Multiplicity	β-D-Gal	β-D-Glc	β-D-Glc	β-D-Gal	β-D-Glc
1	CH_2	38.5	38.2	38.1	1	CH	104.7[i]	104.6[i]	94.1	104.7[n]	104.5[n]
2	CH_2	27.1	27.2[a]	27.2[d]	2	CH	71.1	72.7[j]	72.4	71.0	72.7[o]
3	CH	78.7	88.8	88.4	3	CH	72.9[j]	88.2	77.7	72.9[o]	88.1
4	C	38.7	38.7[b]	38.6[e]	4	CH	68.4[k]	68.2[k]	69.7	68.4[p]	68.2[p]
5	CH	55.2	55.1	55.0	5	CH	75.7[l]	76.1[l]	76.7	75.7[q]	76.1[q]
6	CH_2	18.3	17.8	17.8	6	CH_2	61.0[m]	60.8[m]	60.5	60.9[r]	60.5[r]
7	CH_2	32.6	32.3	32.4[f]							
8	C	39.3	38.3[b]	38.4[e]							
9	CH	47.6	47.1	47.1							
10	C	37.0	36.3	36.3							
11	CH_2	23.1	23.0	22.9[g]							
12	CH	122.1	121.7	121.5							
13	C	143.4	143.5	143.8							
14	C	41.6	41.3	41.3							
15	CH_2	27.7	27.6[a]	27.5[d]							
16	CH_2	23.4	22.6	22.6[g]							
17	C	46.6	45.6	45.4							
18	CH	41.3	40.8	40.8							
19	CH_2	45.8	45.9	45.7							
20	C	30.6	30.3	30.3							
21	CH_2	33.8	33.3	33.3							
22	CH_2	32.3	31.6	32.1[f]							
23	Me	28.1	27.6	27.5							
24	Me	15.6	15.1	15.0							
25	Me	15.3	16.4[c]	16.4[h]							
26	Me	16.8	16.7[c]	16.8[h]							
27	Me	26.0	25.2	25.5							
28	CO	177.9	175.2	178.5							
29	Me	23.6	23.4	23.3							
30	Me	33.1	32.7	32.8							

Data with identical superscript may be reversed.

The important glycosylation shift (+9.8) [12] observed in both glycosides 1 and 2 for C-3 of the aglycone moiety revealed [13–15] that a sugar chain is linked at this carbon.

Signals at 99.1, 77.7, 76.7, 72.4, 69.7 and 60.5 in the spectrum of 1 were absent in the spectrum of 2; they are relavent to the pyranoside linked at C-28 of the aglycone and indicated a β-D-glucopyranosyl group [16]. The negative glycosylation shift at C-1 of the glucose moiety (−2.6) [17, 18] clearly indicated that this group was attached at this position to the acid group. Thus a disaccharide moiety was linked to the aglycone at C-3 in both saponosides.

The β-D-configuration of glucose and galactose as well as the interglycosidic linkage were determined by comparison of measured chemical shifts with those reported for laminarin [19], α,β-laminaribiose, α,β-nigerose [10], α-gal(1 → 3)-α-glc-OMe and β-gal(1 → 3)-α-glc-OMe [11, 20, 21].

In both saponins 1 and 2, signals at 104.7, 104.6 and 104.5 ppm were attributed to the anomeric carbon of each hexose unit in the disaccharide moiety [22]. The absorption at 88.2 ppm clearly indicated that the inter-glycosidic linkage is 1 → 3 [19]. The peaks at 76.1 and 76.7 ppm were attributed to C-5 of both sugars [10]. These data are in agreement with a β-interglycosidic linkage and a β-conformation of the hexose residue bound to the aglycone at C-3 [20, 23]. Effectively, for glucose and galactose in the α-conformation, anomeric carbons involved in the glycosidic linkage resonate between 95 and 100 ppm and C-5 carbons give signals between 71 and 72 ppm [21].

In the case of a β-D-glucopyranosyl-(1 → 3)-β-D-galactopyranosyl group one can predict the chemical shift of the hexose C-3 atom involved in the interglycosidic linkage, based on the glycosylation shift (+7.9) previously established by Kochetkov *et al.* [24]. This value (83.5 ppm), is quite different from the measured one in 1 and 2 thus precluding a terminal glucose unit. We conclude that the disaccharide chain at C-3 of the aglycone in both saponins is a β-D-galactosyl-(1 → 3)-β-D-glucosyle group. The enzymatic hydrolysis of 1 and 2 with β-D-galactosidase confirms this result.

Finally we identified arvensoside A and arvensoside B, respectively, as oleanolic acid-28-*O*-β-D-glucopyranoside-3-β-*O*-(*O*-β-D-galactopyranosyl(1 → 3)-β-D-glucopyranoside) and oleanolic acid 3-β-*O*-(*O*-β-D-galactopyranosyl(1 → 3)-β-D-glucopyranoside).

EXPERIMENTAL

Mps are uncorr. Analytical TLC was carried out on silica gel (Merck F_{254}, 0.25 mm). TLC system 1 employed *n*-BuOH–HOAc–H_2O (4:1:5). TLC system 2 was performed with C_6H_6–MeOH (50:6) as developing solvent. The spots were visualized by spraying with conc. H_2SO_4 and then heating (110°, 5 min). TLC system 3 used *i*-PrOH–H_3BO_3 M/30 (85:15). The compounds were visualized by spraying with aniline hydrogen phthalate and then heating (110°, 10 min).

IR spectra were taken in KBr pellets. ^{13}C NMR spectra were recorded at 200 MHz in DMSO-d_6. The chemical shifts are given in ppm; TMS was used as internal standard.

Acid hydrolysis was performed in a sealed tube at 100° for 6 hr with 25 mg of saponin in 2 ml of conc. HCl–H_2O (10:90). The aq. residue was extracted with Et_2O. The aq. layer was neutralized with *N*,*N*-dioctylamine (10% in $CHCl_3$) and lyophilized. Oleanolic acid was detected in the organic layer whilst sugars were identified in the aq. layer.

Alkaline hydrolysis was performed in a sealed tube at 100° for 75 min with 15 mg of saponoside in 5 ml of KOH (15%). After acidification with HCl (pH 5) the monodesmoside was extracted with *n*-BuOH.

Enzymatic hydrolysis [25] was carried out with 10 ml of saponin in 0.1 ml of β-D-galactosidase (1500 U/ml) and 5 ml of phosphate buffer (0.1 M, pH 7) during 12 h at 25°. After extraction with *n*-BuOH, galactose was identified in the aq. phase.

Extraction of saponins. The fresh aerial parts of *Calendula arvensis* were treated with hot EtOH. The extract was purified by CC using neutral alumina with MeOH as eluant. The purified mixture of saponisides was subjected to preparative RP8 reversed-phase column liquid chromatography (Jobin Yvon Chromatospac Prep 10; Merck columns RP8; MeOH–H_2O, 65:35 for 1; MeOH–H_2O, 73:27 for 2). Four saponins were obtained two of which were arvensoside A and B.

Arvensoside A. Mp 198–200° (decomp.); TLC system 1: R_f 0.50 (violet spot), TLC system 2: oleanolic acid, R_f 0.45, (violet spot), TLC system 3: glucose R_f 0.65, galatose R_f 0.52 (brown). (Found: C, 56.85; H, 8.56; O, 34.69. Calc. for $C_{48}H_{78}O_{18} \cdot 4H_2O$: C, 56.80; H, 8.48; O, 34.71%.) IR ν^{KBr} cm^{-1}: 3400 (OH sugars), 1745 (CO ester), 1635 (aglycone insaturation), 1200–950 (CO sugars).

Arvensoside B. Mp 230–232° (decomp.); TLC system 1: R_f 0.57 (violet spot), TLC system 2: oleanolic acid, R_f 0.45 (violet spot), TLC system 3: glucose R_f 0.65, galactose R_f 0.52 (brown). (Found: C, 59.68; H, 8.96; O, 31.46. Calc. for $C_{42}H_{68}O_{13} \cdot 4H_2O$: C, 59.15; H, 8.92; O, 31.92%.) IR ν^{KBr} cm^{-1}: 3400 (OH sugars), 1692 (CO acid).

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