Pergamon 0031-9422(95)00749-0 Phytochemistry, Vol. 41, No. 6, pp. 1579–1582, 1996

0031-9422/96 $15.00 + 0.00

MINOR CONSTITUENTS FROM *BUPLEURUM FRUTICOSUM* ROOTS

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(*Received in revised form* 3 *August* 1995)

Key Word Index—*Bupleurum fruticosum*; Apiaceae; roots; coumarins; triterpenoid saponin.

Abstract—A new triterpenoid saponin, 23-acetoxy,16α-hydroxy-13,28-epoxyolean-11-en-3β-yl-[β-D-glucopyranosyl-(1 → 2)]-[β-D-glucopyranosil(1↦3)]-β-D-fucopyranoside, has been isolated from the roots of *Bupleurum fruticosum*, along with eight coumarins, scopoletin, scoparone, prenyletin, capensin, fraxetin, aesculetin,7-(3-methyl-2-butenyloxy-6-methoxycoumarin, 7-(2-hydroxy-3-methyl-3-butenyloxy)-6-methoxycoumarin and 5,7-dihydroxy-6-methoxy-8-(3-methyl-2-butenyl)coumarin. The last compound has not been previously encountered in nature. The structures were determined by analysis of NMR spectral data including two-dimensional techniques.

INTRODUCTION

Bupleurum fruticosum L. is an evergreen wild shrub, typical of many zones of the Mediterranean area. Bupleuri Radix [dried roots of *Bupleurum* spp.] is one of the most frequently occurring crude drugs in the prescriptions of Chinese traditional medicine. In this system it is used as an antiinflammatory and antihepatotoxic agent [1, 2]; roots and stems of this species are used in Sardinian folk medicine as an antirheumatic remedy [3].

Previous investigations of this species led to the isolation of several saponins from a fraction of a methanolic extract of the roots [4] and two phenylpropanoids from the *n*-hexane extract of the leaves [5]. We now communicate the complete results of our research on the roots of this species, which include the characterization of one additional saponin and two new coumarins.

RESULTS AND DISCUSSION

Elemental analysis of compound **1** yielded values consistent with the formula $C_{50}H_{80}O_{19}$. This was supported by the FAB-mass spectrum (glycerol, positive ion mode) which contained ion species at *m/z* 1023 $[M + K]^+$ and 514 $[aglycone]^+$. The substance exhibited IR bands at 3480 (OH), 1725 ($COCH_3$) and 1640 (C=C) cm^{-1}. The ^1H NMR spectrum of **1**, in CD_3OD, showed six tertiary methyl singlets between δ0.75 and 1.12, one sugar methyl at δ1.28 (*d*, $J = 6.5$ Hz), a methyl of an acetyl group at δ2.05 (3H, *s*), three anomeric protons at δ4.48 (*d*, $J = 7.5$ Hz), 4.60 (*d*, $J = 7.0$ Hz) and 4.84 (*d*, $J = 7.7$ Hz), and two olefinic signals at δ5.37 and 5.95 (H-11 and H-12, respectively). The ^{13}C NMR data confirmed the above and revealed the presence of a pair of olefinic carbons (δ130.3 and 134.3, methines), one acetyl group (δ21.2, 172.8) and three anomeric carbons (δ103.4, 104.8, and 105.3). The number and chemical shifts of the tertiary methyl functions and quaternary carbons suggested that **1** was an oleanene-type triterpene triglycoside with an acetyl group bonded to the aglycone. This was also confirmed by the FAB-MS data which showed by the loss of 470 amu, corresponding to the trisaccharide moiety (two hexose and one deoxyhexose units). Acid hydrolysis of the saponin gave glucose and fucose as sugar components. Comparison of ^{13}C NMR data of **1** with those of the saponins previously extracted from the same source [4], suggested the identity of the sugar portion, while the ^1H and ^{13}C NMR parameters revealed very similar signals with the aglycone moiety of saikogenin F, except that the C-23 signal was deshielded (+3.5 ppm) to δ68.25; this shift resulted from the presence of an acetyl group at the C-23 position of the aglycone, supported by the relative upfield chemical shift (δ0.75) of the highest methyl and by the absence of effects of the adjacent nucleus owing to the nonhindered equatorial position of the acetoxyl function [5]. Therefore, the structure of **1** was 23-acetoxy-16β-hydroxy-13,28-epoxyolean-11-en-3β-yl-[β-D-glucopyranosyl(1↦2)]-[β-D-glucopyranosyl (1↦3)]-β-D-fucopyranoside.

From the *n*-hexane, $CHCl_3$ and $CHCl_3$–MeOH extracts of *B. fruticosum*, after chromatographic fractionation, two new derivatives, **2** and **3**, were extracted. They produced a violet coloration on treatment with hydroxylamine and ferric chloride, i.e. a reaction typical for coumarins [6].

Compound **2** was isolated from the $CHCl_3$ extract as an oil and the EI-mass spectrum contained an $[M]^+$ peak at *m/z* 276 in accord with a molecular formula of $C_{15}H_{16}O_5$. The ^1H and ^{13}C NMR spectra, recorded in CD_3OD, confirmed the presence of the coumarin

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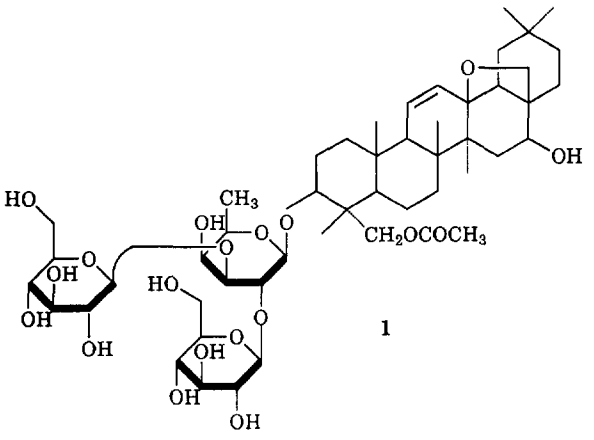

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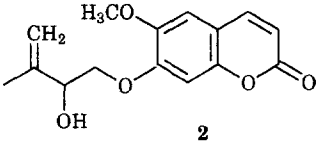

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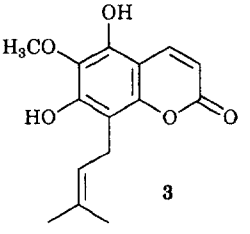

3

skeleton (δ6.26 (1H, *d*, J = 9.5 Hz, H-3), 7.88 (1H, *d*, J = 9.5 Hz, H-4) and 113.7 (C-3), 145.8 (C-4)). The two singlets in the proton spectrum at δ6.99 and 7.15 were in agreement with two substituents at C-6 and C-7 of the B-ring, while a singlet (3H) at δ3.89 was due to a methoxy group. In the NOESY spectrum, cross-peaks were observed between this signal (δ3.89) and H-5 (δ7.15), and between H-5 and H-4 (δ7.88), indicating that the MeO group was attached to C-6. The other substituent at C-7 was identified as an oxyprenyl chain by the chemical shifts, J couplings and splittings of the resonances in the proton spectrum. A methyl singlet (δ1.83, *br s*) and two signals at δ4.97 and 5.12 (each 1H, *br s*) suggested a $CH_2=C(CH_3)$ fragment, while the signals at δ4.05 (1H, *dd*, J = 8.7 and 9.7 Hz), 4.14 (1H, *dd*, J = 1.7 and 8.7 Hz) and 4.46 (1H, *dd*, J = 1.7 and 9.7 Hz) suggested a O–CH–CH_2–O system. The ^{13}C and DEPT data (see Table 1) were in agreement with the structure of **2** as 7-(2-hydroxy-3-methyl-3-butenyloxy)-6-methoxycoumarin. This compound was isolated for the first time from *Pterocaulon virgatum* [7], but this is the first report of its presence in the genus *Bupleurum* and in the Apiaceae.

Compound **3** was isolated from the $CHCl_3$–MeOH extract. Its UV spectra exhibited adsorptions between 325 and 330 nm, typical of 5,7-dioxygenated and 5,6,7-trioxygenated coumarins [8,9], while the absence of bathochromic shifts, owing to the formation of a complex with aluminium chloride, showed that compound **3** was not an *ortho*-dihydroxy coumarin [8].

The EI-mass spectra (see Experimental) gave peaks corresponding to a compound with a molecular formula $C_{15}H_{16}O_5$. This was confirmed by the further ^{13}C NMR and DEPT spectra. The ^{13}C NMR spectrum showed 15 signals, that were attributed, on the basis of DEPT experiments, to: Me × 2, MeO × 1, CH_2 × 1, =CH × 3, =C × 7, and C=O × 1 (Table 1). The complete structure elucidation was carried out on the basis of the chemical shifts and J values of the ^{1}H NMR spectrum and from detailed spectral analyses of NOESY, HETCOR and COLOC.

In the ^{1}H NMR spectrum of **3** (recorded in CD_3OD), the doublets at δ6.19 and 7.95 (J = 9.7 Hz) were attributable to H-3 and H-4 and the low-field nature of the chemical shift of H-4 suggested the presence of an oxygenated group at C-5 [8].

A multiplet at δ5.06 (1H), a doublet at δ3.53 (2H, J = 6.5 Hz) and two singlets at δ1.82 and 1.68 (3H each) indicated the presence of a C-prenyl side-chain, which was confirmed by the ^{13}C NMR data (24.8 ppm for C-1'). The methoxyl singlet at δ3.77 and the absence of other signals in the aromatic region of the ^{1}H NMR spectrum showed that the three oxygenated substituents were at C-5, C-6 and C-7. NOESY experiments revealed no correlations between the methoxyl singlet and the signals of the prenyl moiety; thus, the methoxyl group was linked to C-5 or C-6 of the coumarin nucleus. The position of the methoxyl was assigned to C-6 by the UV spectral data in the presence of aluminium chloride, and by COLOC experiments (three-bond correlation between the singlet at δ3.77 and the carbon at 145.3). Thus, on the basis of these results and by comparison the carbon resonances with those reported in the literature for similar compounds [10–12], **3** was identified as 5,7-dihydroxy-6-methoxy-8-(3-methyl-2-butenyl) coumarin, a new natural product of coumarin series.

The known coumarins: scopoletin [12], prenyletin [13], 7-(3-methyl-2-butenyloxy)-6-methoxycoumarin [14], scoparone [15,16], capensin [14,17], aesculetin [12,18], fraxetin [19], were also isolated and identified by comparison of the ^{1}H and ^{13}C NMR data with those reported in the literature.

EXPERIMENTAL

General. UV: MeOH; IR: nujol mulls; ^{1}H and ^{13}C NMR: 200 and 50 MHz, respectively, in MeOH-d_4 (TMS as an int. standard). Carbon multiplicities were determined by DEPT 90° or 135° pulse sequence. NOESY and COLOC were performed using standard Bruker software. Delays of 2D ^{13}C–^{1}H shift correlations by long range coupling (COLOC) were adjusted to an average CH coupling of 7 Hz to obtain maximum polarization transfer. FAB-MS: positive-ion mode, glycerol as matrix; EI-MS: VG TRIO 2000 (70 eV); TLC: silica gel

Table 1. ^1H and ^{13}C NMR spectral data of compounds **2** and **3**

	2		**3**	
Position	δH (*J* in Hz)	δC	δH (*J* in Hz)	δC
2		163.8		163.5
3	6.26 *d* (9.5)	113.7	6.15 *d* (9.7)	111.7
4	7.88 *d* (9.5)	145.8	7.95 *d* (9.7)	144.0
4a		113.1		110.7
5	7.15 *s*	110.0		142.6
6		148.2		145.3
7		153.9		144.7
8	6.99 *s*	102.2		124.2
8a		151.1		144.0
1′	4.05 (H_a) *dd* (8.7; 9.7) 4.14 (H_b) *dd* (1.7; 8.7)	73.5	3.53 (2H) *br d* (6.5)	24.8
2′	4.46 *dd* (1.7; 9.7)	74.2	5.06 *m*	124.5
3′		145.7		132.9
4′	4.97 (H_a) *m* 5.11 (H_b) *m*	113.1	1.82 (3H) *s*	25.7
5′	1.83 *br s*	19.0	1.68 (3H) *s*	18.1
OMe	3.89 *s*	56.88		61.51

and RP-8 plates (Merck); CC: Lobar RP-8 (Merck) with Duramat pump.

Plant material. B. fruticosum was collected in November 1991 in Italy and a voucher specimen is deposited in the Herbarium of the Istituto di Botanica Farmaceutica, Università di Sassari.

Isolation of saponin. The pulverized material (235 g) was extracted in a Soxhlet with *n*-hexane, $CHCl_3$ and $CHCl_3$–MeOH (9:1), then with MeOH at room temp. The MeOH residue (14.25 g) was chromatographed on Sephadex LH-20 using MeOH to give 10 frs. Fr. 1 (7.4 g), after precipitation with Et_2O, yielded to 16β,23-dihydroxyl-13,28-epoxyolean-11-en-3β-yl-[β-D-glucopyranosyl-(1 → 2)]-[β-D-glucopyranosil(1 ↦ 3)]-β-D-fucopyranoside (73 mg), identified by comparison with an authentic sample [4]; the filtrate was subjected to Lobar RP-8 eluted with MeOH–H_2O (7:3) to give compound **1** (15 mg).

Saponin (**1**). α_D^{20} − 18.1 (Py; *c* 0.007). TLC RP-8, MeOH–H_2O (7:3): R_f 0.54. Found: C, 59.45; H, 8.92%; $C_{50}H_{80}O_{19}$ requires: C, 60.97; H, 8.13%. IR ν_{max} cm^{-1}: 3480, 2920, 1640, 900; ^1H NMR (MeOH-d_4): δ0.75 − 1.12 (each 3H, each *s*, tert-Me × 6), 1.28 (3H, *d*, *J* = 6.5 Hz), 2.05 (3H, *s*, $\underline{CH_3}CO$), 4.48 (1H, *d*, *J* = 7.5 Hz), 4.60 (*d*, *J* = 7 Hz), 4.84 (*d*, *J* = 7.7 Hz), 5.37 (1H, *dd*, *J* = 3.2 and 10.2), 5.95 (1H, *d*, *J* = 10.2); ^{13}C NMR (MeOH-d_4): for aglycone: δ39.2 (C-1), 26.4 (C-2), 84.2 (C-3), 44.5 (C-5), 18.1 (C-6), 31.2 (C-7), 42.9 (C-8), 52.2 (C-9), 35.8 (C-10), 130.3 (C-11), 134.3 (C-12), 85.5 (C-13), 46.5 (C-14), 34.5 (C-15), 65.3 (C-16), 47.3 (C-17), 53.9 (C-18), 39.2 (C-19), 32.0 (C-20), 33.9 (C-21), 26.4 (C-22), 68.2 (C-23), 12.5 (C-24), 18.8 (C-25), 20.2 (C-26), 20.7 (C-27), 73.2 (C-28), 33.9 (C-29), 23.8 (C-30), 21.2 (COMe), 172.8 (COMe); for sugar moiety: δ104.8 (F-1), 76.3 (F-2), 85.5 (F-3), 72.7 (F-4), 71.2 (F-5), 16.9 (F-6), 103.5 (G-1′), 76.0 (G-2′), 77.9 (G-3′), 72.3 (G-4′), 78.1 (G-5′), 63.5 (G-6′), 105.3 (G-1″), 75.3 (G-2″), 78.0 (G-3″), 71.3 (G-4″), 78.3 (G-5″), 62.3 (G-6″). Positive FAB-MS: *m/z* 1024 $[M + K]^+$, 514 $[M - 470]^+$, 179, 163.

Acid hydrolysis of saponin. A mixture containing 1 ml 1 N HCl, 1 ml dioxane and 10 mg **1** was heated in a sealed tube at 90° for 4 hr, then 5 ml H_2O were added and the aglycone was removed by extraction with 10 ml $CHCl_3$. The aq. layer was neutralized with Amberlite IRA 400 (OH^- type) and evapd to dryness. The sugar samples were directly analysed by TLC: glucose and fucose were identified by comparison with authentic samples.

Extraction and isolation of coumarins. The residue (11.3 g) obtained on evaporation of the *n*-hexane extract was partitioned between *n*-hexane and 90% aq. MeOH. The MeOH layer (3.65 g) was subjected to Sephadex LH-20 gel filtration (MeOH–$CHCl_3$, 9:1) and then chromatographed on Lobar RP-8 to yield: scopoletin (17 mg), prenyletin (23 mg) and a mixture of two compounds. The mixture was further purified by flash chromatography with $CHCl_3$–Et_2O (4:1) as eluent to yielded 6 mg of 7-(3-methyl-2-butenyloxy)-6-methoxycoumarin and 8 mg scoparone.

The $CHCl_3$ extract (4.5 g) was chromatographed on Sephadex LH-20 with MeOH–$CHCl_3$ (9:1) followed by flash chromatography over silica gel, eluting with $CHCl_3$–MeOH mixts of increasing polarity, to give capensin (8 mg) and 7-(2-hydroxy-3-methyl-3-butenyloxy)-6-methoxycoumarin **2** (5 mg).

The $CHCl_3$–MeOH residue was chromatographed on a Sephadex LH-20 column eluted with MeOH–$CHCl_3$ (9:1) to give seven (A-G) fractions. Fr. B was subjected to Lobar RP-8 chromatography eluted with MeOH–H_2O (4:3), yelding 7 mg aesculetin. Fraxetin (13 mg) was obtained from fr. D (100 mg) after prep. TLC over silica gel ($CHCl_3$–MeOH–toluene 45:4:1). From frs. F-G (0.87 g) was obtained compound **3** (10 mg) (5,7-dihydroxy-6-methoxy-8-(3-methyl-2-butenyl)coumarin) by Sephadex

LH-20 column, flash chromatography and final purification with Lobar RP 8.

Known compounds were characterized by comparison of their physical and spectral properties with those of authentic samples and with those reported in the literature.

Compound **2**. TLC silica gel, $CHCl_3$–Et_2O (4:1): R_f 0.14; UV λ_{max}^{MeOH} nm; 342.3, 294.9, 231.0; EI-MS *m/z*: 276 [M] $^+$, 192, 177, 164, 69, 43; ^{1}H NMR (MeOH-d_4): δ1.83 (1H, *br s*, H-5′), 3.89 (3H, *s*, OMe), 4.05 (1H, *dd*, J = 8.7 and 9.7 Hz, H-1′a), 4.14 (1H, *dd*, J = 1.7 and 8.7 Hz, H-1′b), 4.46 (1H, *dd*, J = 1.7 and 9.7 Hz, H-2′), 4.97 (1H, *m*, H-4′a), 5.11 (1H, *m*, H-4′b), 6.26 (1H, *d*, J = 9.5 Hz, H-3), 6.99 (1H, *s*, H-8), 7.15 (1H, *s*, H-5); ^{13}C NMR (MeOH-d_4): δ19.0 (C-5′), 56.9 (OMe), 73.5 (C-1′), 74.2 (C-2′), 102.2 (C-8), 110.0 (C-5), 113.1 (C-4′ and C-4a), 113.7 (C-3), 145.7 (C-3′), 145.8 (C-4), 148.2 (C-6), 151.1 (C-8a), 153.9 (C-7), 163.8 (C-2).

Compound **3**. TLC RP-8, MeOH–H_2O (7:3): R_f 0.28; UV λ_{max}^{MeOH} nm: 329, 260, $AlCl_3$: 335, 260; EI-MS *m/z*: 276 [M] $^+$, 233, 221, 69, 41; ^{1}H NMR (MeOH-d_4): δ1.68 (3H, *s*, Me-5′), 1.82 (3H, *s*, Me-4′), 3.53 (2H, *br d*, J = 6.5 Hz, H_2-1′), 3.81 (3H, *s*, OMe), 5.06 (1H, *m*, H-2′), 6.15 (1H, *d*, J = 9.7 Hz, H-3), 7.95 (1H, *d*, J = 9.7 Hz, H-4); ^{13}C NMR (MeOH-d_4): ^{13}C NMR: δ18.1 (C-5′), 24.8 (C-1′), 25.7 (C-4′), 61.5 (OMe), 110.7 (C-4a), 111.7 (C-3), 124.2 (C-8).

Acknowledgement—This work was supported by a grant from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (40%).

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