*Journal of Natural Products*
*Vol. 50, No. 5, pp. 927-931, Sep-Oct 1987*



# PLANT METABOLITES. TRITERPENOID SAPONINS FROM *CALENDULA ARVENSIS*

COSIMO PIZZA,* ZHOU ZHONG-LIANG, and NUNZIATINA DE TOMMASI

*Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli, via L. Rodinò 22, I-80138 Napoli, Italy*

*Calendula arvensis* L. (Compositae) is an herbaceous plant used in Italian folk medicine as an antiinflammatory and antipyretic. In a recent pharmacologic study, the methanolic extract of aerial parts showed antiinflammatory activity (1). In previous work we reported the isolation and structure determination of a sesquiterpene glycoside from *C. arvensis* (2). In the present paper, we report the isolation of four triterpenoid saponins [**1-4**].

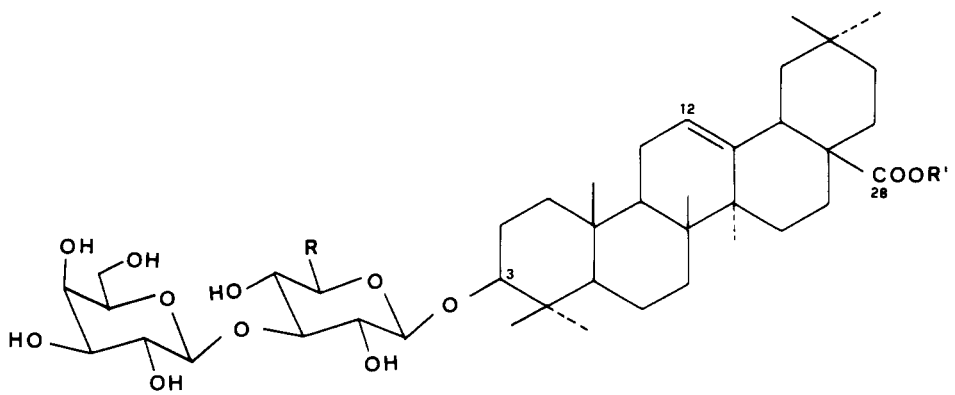
The aerial parts of air-dried plant material were extracted successively with light petroleum ether, $CHCl_3$ and MeOH. The MeOH extract was chromatographed on Sephadex LH-20, and the saponin-containing fractions were further purified by droplet counter current chromatography (dccc) (10) and hplc to obtain saponins **1-4**.

The molecular formulae $C_{48}H_{78}O_{18}$, $C_{42}H_{68}O_{13}$, $C_{48}H_{76}O_{19}$, $C_{42}H_{66}O_{14}$, for saponins **1-4**, respectively, were determined by DEPT $^{13}C$ nmr (Table 1) and fabms, negative ion mode, in a thioglycerol-glycerol matrix (see Experimental).

Acid methanolysis liberated methyl galactoside and methyl glucoside (molar ratio 1:2) from **1**, methyl galactoside and methyl glucoside (1:1) from **2**, methyl galactoside, methyl glucuronide, and methyl glucoside (1:1:1) from **3**, and methyl glucuronide and methyl galactoside (1:1) from **4**. The methylated sugars were analyzed by glc.

Signals in fabms of **3** and **4** at *m/z* 631 and 455, corresponded to the subsequent loss of a galactosyl moiety and a glucuronic moiety and indicated that glucuronic acid was attached to the aglycone. The aglycone (molecular formula $C_{30}H_{48}O_3$, deduced from fabms) for saponins **1-4**, was identified as oleanolic acid on the basis of $^1H$-, $^{13}C$- and DEPT $^{13}C$-nmr spectral data and comparison with literature data (3,4).

The β-D-pyranosyl configuration of galactose, glucose, and glucuronic acid in saponins **1-4** was deduced from $^1H$- and $^{13}C$-nmr spectra (see Table 1 and




| | R | R' |
|---|---|---|
| **1** | $-CH_2OH$ | β-D-glucopyranosyl |
| **2** | $-CH_2OH$ | H |
| **3** | -COOH | β-D-glucopyranosyl |
| **4** | -COOH | H |

TABLE 1. $^{13}$C-nmr Data for Saponins **1-4** in $CD_3OD$

| Carbon | | Compounds | | | | DEPT |
|---|---|---|---|---|---|---|
| | | **1** | **2** | **3** | **4** | |
| Aglycone | 1 | 39.9 | 39.9 | 39.9 | 40.0 | $CH_2$ |
| | 2 | 27.0 | 27.0 | 26.9 | 27.1 | $CH_2$ |
| | 3 | 90.8 | 90.8 | 90.6 | 90.7 | CH |
| | 4 | 40.1 | 40.2 | 40.2 | 40.1 | C |
| | 5 | 57.1 | 57.2 | 57.1 | 57.1 | CH |
| | 6 | 19.3 | 19.4 | 19.4 | 19.4 | $CH_2$ |
| | 7 | 33.2 | 33.5 | 33.2 | 33.2 | $CH_2$ |
| | 8 | 40.8 | 40.9 | 40.8 | 40.9 | C |
| | 9 | 48.1[a] | 48.1[a] | 48.1[a] | 48.1[a] | CH |
| | 10 | 37.9 | 38.0 | 37.9 | 37.9 | C |
| | 11 | 24.6 | 24.6 | 24.6 | 24.6 | $CH_2$ |
| | 12 | 123.8 | 123.6 | 123.9 | 123.9 | CH |
| | 13 | 144.8 | 145.4 | 144.8 | 145.3 | C |
| | 14 | 43.0 | 43.1 | 43.0 | 43.1 | C |
| | 15 | 28.9 | 29.0 | 28.9 | 28.9 | $CH_2$ |
| | 16 | 24.0 | 24.3 | 24.1 | 24.2 | $CH_2$ |
| | 17 | 48.1[a] | 48.1[a] | 48.1[a] | 48.1[a] | C |
| | 18 | 42.6 | 42.9 | 42.7 | 42.9 | CH |
| | 19 | 47.3 | 47.5 | 47.3 | 47.4 | $CH_2$ |
| | 20 | 31.5 | 31.6 | 31.5 | 31.6 | C |
| | 21 | 34.9 | 35.1 | 35.0 | 35.0 | $CH_2$ |
| | 22 | 34.1 | 34.2 | 34.1 | 34.1 | $CH_2$ |
| | 23 | 28.7 | 28.6 | 28.6 | 28.5 | $CH_3$ |
| | 24 | 17.1 | 17.0 | 17.0 | 17.0 | $CH_3$ |
| | 25 | 16.0 | 15.9 | 16.0 | 16.0 | $CH_3$ |
| | 26 | 17.8 | 17.9 | 17.9 | 17.9 | $CH_3$ |
| | 27 | 26.4 | 26.4 | 26.3 | 26.3 | $CH_3$ |
| | 28 | 178.1 | 182.2 | 178.2 | 182.2 | C |
| | 29 | 33.5 | 33.5 | 33.4 | 33.4 | $CH_3$ |
| | 30 | 24.0 | 24.0 | 24.0 | 24.0 | $CH_3$ |
| Glucose I | 1 | 95.7 | | 95.8 | | CH |
| | 2 | 73.9 | | 74.1 | | CH |
| | 3 | 78.5 | | 78.5 | | CH |
| | 4 | 71.3 | | 71.4 | | CH |
| | 5 | 78.4 | | 78.4 | | CH |
| | 6 | 62.8 | | 62.7 | | $CH_2$ |
| Glucose II | 1 | 105.7 | 105.8 | | | CH |
| | 2 | 74.8 | 74.8 | | | CH |
| | 3 | 88.2 | 88.3 | | | CH |
| | 4 | 70.4 | 70.4 | | | CH |
| | 5 | 77.2 | 77.3 | | | CH |
| | 6 | 62.9 | 63.0 | | | $CH_2$ |
| Glucuronic | 1 | | | 105.2 | 105.3 | CH |
| Acid | 2 | | | 74.9 | 74.8 | CH |
| | 3 | | | 86.2 | 86.2 | CH |
| | 4 | | | 72.3 | 72.2 | CH |
| | 5 | | | 77.2 | 77.2 | CH |
| | 6 | | | 176.4 | 176.3 | C |
| Galactose | 1 | 106.2 | 106.2 | 106.3 | 106.2 | CH |
| | 2 | 73.1 | 73.1 | 72.8 | 72.9 | CH |
| | 3 | 75.0 | 75.0 | 75.1 | 75.0 | CH |
| | 4 | 70.4 | 70.4 | 70.6 | 70.5 | CH |
| | 5 | 77.1 | 77.1 | 77.1 | 77.0 | CH |
| | 6 | 62.8 | 62.6 | 62.7 | 62.7 | $CH_2$ |

[a]Under $CD_3OD$ signal.

Experimental section). The anomeric proton signal at δ 5.41 (1H, d, $J$=7.5 Hz) in the $^1$H-nmr spectra and the anomeric carbon signals at δ 95.7 and 95.8 in the $^{13}$C-spectra of **1** and **3**, respectively, were attributed to a β-glucose unit linked to the 28-carboxyl group of the aglycone through an ester bond.

Basic hydrolysis of **1** gave saponin **2**, while basic hydrolysis of **3** gave **4**.

In saponins **2** and **4** the free carboxyl group of the aglycone appeared at δ 182.2 (δ 179.8 in pyridine-$d_5$ for **2**), whereas, when esterified with the glucosyl moiety in saponins **1** and **3**, it resonated at δ 178.1 (δ 176.2 in pyridine-$d_5$ for **1**) (5,6). The C-3 of the aglycone as glycosidation site and the interglycosidic linkages in saponins **1-4** were derived from $^{13}$C-nmr data (Table 1). The $^{13}$C-nmr sugar signals of **1** and **2** indicated that the β-D-galactopyranosyl is the terminal unit and that this sugar is attached at the position 3 of the inner glucosyl unit (4,7,8).

Thus, the structure of saponin **1** is established as 3β-*O*-[β-D-galactopyranosyl-(1-3)-β-D-glucopyranosyl] oleanolic acid-28-*O*-β-D-glucopyranoside and that of **2** as 3β-*O*-[β-D-galactopyranosyl-(1-3)-β-D-glucopyranosyl] oleanolic acid. Saponins **1** and **2** are new natural compounds.

In saponins **3** and **4** the terminal sugar is the β-D-galactopyranosyl, attached at the position 3 of the β-D-glucopyranosyluronic acid (Table 1) (6). The structure of saponin **3** is established as 3β-*O*-[β-D-galactopyranosyl-(1-3)-β-D-glucopyranosyluronic acid] oleanolic acid-28-*O*-β-D-glucopyranoside, and that of **4** as 3β-*O*-[β-D-galactopyranosyl-(1-3)-β-D-glucopyranosyluronic acid] oleanolic acid.

Saponins **3** and **4** were earlier reported from *Calendula officinalis* L. (9). However, the present communication further confirms the assigned structure from spectral information and from hydrolytic studies.

## EXPERIMENTAL

INSTRUMENTAL.—$^1$H- and $^{13}$C-nmr spectra were recorded on a Bruker WM-250 spectrometer. Chemical shifts are reported relative to TMS. The DEPT experiments were made by using polarization transfer pulse of 90° and 135°, respectively, obtaining in the first case only CH groups and in the other case positive signals for CH and $CH_3$ and negative ones for $CH_2$ groups. Polarization transfer delays were adjusted to an average CH coupling of 135 Hz. Fab-mass spectra were recorded on a Kratos MS-50 mass spectrometer equipped with a Kratos fab source. The spectra were obtained by dissolving the samples in a thioglycerol-glycerol matrix and placing them on a copper probe tip prior to bombardment with Ar atoms of energy 2-6 Kv. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. Dccc separations were performed on a DCC-A apparatus manufactured by Tokyo Rikakikai Co., equipped with 300 tubes. Hplc separations were performed on a Waters Model 6000A pump equipped with a U6K injector and differential refractometer Model 401 detector. Glc analyses were performed with a Carlo Erba Fractovap 2900 capillary column.

ACIDIC METHANOLYSIS OF **1-4**, SUGAR ANALYSIS.—A solution of each saponin (0.5, 1 mg) in anhydrous 2 M HCl-MeOH (0.1 ml) was heated at 80° in a stoppered reaction vial for 10 h. After being cooled, the reaction mixture was neutralized with $Ag_2CO_3$ and centrifuged; the supernatant was evaporated to dryness. The residue was dissolved in TRISIL Z [0.05 ml; *N*-(trimethylsilyl) imidazole in pyridine, Pierce Chemical Co.], left at room temperature for 15 min, and analyzed by glc (25 m SE-30 capillary column, 146°). Glc peaks in the silylated hydrolysate co-eluted with those in silylated standards.

BASIC HYDROLYSIS.—The saponins **1** (20 mg) and **3** (10 mg) in 0.5 M aq. KOH (1 ml) were heated at 110° in a stoppered reaction vial for 2 h. The reaction mixture was adjusted to pH 7 and then extracted with BuOH. The organic phase was evaporated to dryness, dissolved in $CD_3OD$, and analyzed by $^1$H nmr and $^{13}$C nmr.

EXTRACTION AND ISOLATION.—Plants of *C. arvensis* were collected near Naples, Italy, in the Spring 1985; a sample has been deposited in Dipartimento di Chimica delle Sostanze Naturali, University of Naples. The aerial parts of the air-dried plant material (800 g) were extracted successively with light petroleum ether (40°-70° bp) (8.6 g), $CHCl_3$ (7.5 g), and with MeOH (15 g); 2.2 g of the MeOH extract was chromatographed on Sephadex LH-20 (3×60 cm; MeOH; 8-ml fractions were collected) to yield 900 mg (fractions 39-43) of the saponin mixture.

Purification was continued by dccc with $CHCl_3$-MeOH-$H_2O$ (7:13:8) in which the stationary phase consisted of lower phase; ascending mode; flow 12 ml/h; 4-ml fractions were collected to yield a mixture I containing the more polar glycosides **1** and **3** (300 mg) in the fractions 52-63 and a mixture II containing **2** and **4** (200 mg) in the fractions 71-83. Both mixtures I and II were submitted to hplc on a $C_{18}$ μ-bondapak column (30 cm×7.8 mm i.d.) with MeOH-$H_2O$ (65:35), to yield pure **1** (98 mg), **2** (30 mg), **3** (15 mg), and **4** (10 mg).

3-*O*-[β-D-GALACTOPYRANOSYL-(1-3)-β-D-GLUCOPYRANOSYL] OLEANOLIC ACID-28-*O*-β-D-GLUCOPYRANOSIDE [**1**].—Hplc retention time 12 min; $[\alpha]_D$=5° (MeOH); fabms, negative ions, glycerol-thioglycerol matrix, *m/z* 941 [(M-H)$^-$], 779 ([(M-H)−162]$^-$), 617 ([(M-H)−(162×2)]$^-$), 455 ([(M-H)−(162×3)]$^-$); $^{13}$C nmr in $CD_3OD$ see Table 1; $^1$H nmr in $CD_3OD$ (aglycone) singlets at δ 0.833, 0.874, 0.949, 0.968, 0.988, 1.095, 1.195 (each 3H), δ 5.28 (1H, m, H-12); (sugars) δ 4.41 (1H, d, *J*=7.5 Hz, H-1 of galactose unit), δ 4.58 (1H, d, *J*=7.6 Hz, H-1 of glucose II unit), δ 5.41 (1H, d, *J*=7.5 Hz, H-1 of glucose I unit).

$^{13}$C-nmr in pyridine-$d_5$ δ's of the aglycone corresponded to those of oleanolic acid (4); sugar signals: glucose I 95.6 (C-1), 73.9 (C-2), 78.7 (C-3), 71.2 (C-4), 78.9 (C-5), 62.1 (C-6); glucose II 105.9 (C-1), 74.8 (C-2), 89.0 (C-3), 70.0 (C-4), 77.5 (C-5), 62.6 (C-6); galactose 106.1 (C-1), 72.8 (C-2), 74.9 (C-3), 69.8 (C-4), 77.1 (C-5), 62.3 (C-6).

Basic hydrolysis of **1** afforded the saponin **2** (comparison of $^1$H- and $^{13}$C-nmr spectra in $CD_3OD$). Acidic methanolysis of **1** afforded methyl glucoside (×2) and methyl galactoside.

3β-*O*-[β-D-GALACTOPYRANOSYL-(1-3)-β-D-GLUCOPYRANOSYL] OLEANOLIC ACID [**2**].—Hplc retention time 47 min, $[\alpha]_D$=8° (MeOH); fabms, negative ions, 779 ([M-H]$^-$), 617 ([(M-H)−162]$^-$), 455 ([(M-H)−(162×2)]$^-$); $^{13}$C nmr in $CD_3OD$ see Table 1; $^1$H nmr in $CD_3OD$ (aglycone) singlets at δ 0.852, 0.873, 0.936, 0.973, 0.986, 1.09, 1.19 (each 3H), δ 5.28 (1H, m, H-12); (sugars) δ 4.41 (1H, d, *J*=7.5 Hz, H-1 of galactose unit), δ 4.58 (1H, d, *J*=7.6 Hz, H-1 of glucose unit). $^{13}$C nmr in pyridine-$d_5$ δ's of the aglycone corresponded to those of oleanolic acid (4); sugar signals: see δ's values reported for glucose II and galactose sugar units of **1** (±0.2 ppm).

3β-*O*-[β-D-GALACTOPYRANOSYL-(1-3)-β-D-GLUCOPYRANOSYLURONIC ACID] OLEANOLIC ACID-28-*O*-β-D-GLUCOPYRANOSIDE [**3**].—Hplc retention time 9 min, $[\alpha]_D$=15° (MeOH); fabms, negative ions, 955 ([M-H]$^-$), 793 ([(M-H)−162]$^-$), 631 ([(M-H)−(162×2)]$^-$), 455 ([(M-H)−(324+176)]$^-$); $^{13}$C nmr in $CD_3OD$ see Table 1; $^1$H nmr in $CD_3OD$ (aglycone) singlets at δ 0.827, 0.86, 0.943, 0.962, 0.974, 1.072, 1.184 (each 3H), δ 5.28 (1H, m, H-12); (sugars) δ 4.41 (1H, d, *J*=7.5 Hz, H-1 of galactose unit), δ 4.61 (1H, d, *J*= 7.6 Hz, H-1 of glucuronic acid unit), δ 5.41 (1H, d, *J*=7.5 Hz, H-1 of glucose unit).

Basic hydrolysis of saponin **3** afforded saponin **4** (comparison of $^1$H-nmr spectra in $CD_3OD$). Acidic methanolysis of **3** afforded methyl glucoside, methyl galactoside, and methyl glucuronide.

3β-*O*-[β-D-GALACTOPYRANOSYL-(1-3)-β-D-GLUCOPYRANOSYLURONIC ACID] OLEANOLIC ACID [**4**].—Hplc retention time 43 min, $[\alpha]_D$=12° (MeOH); fabms, negative ions, *m/z* 973 ([(M-H)$^-$]), *m/z* 631 ([(M-H)−162]$^-$), 455 ([(M-H)−(162+176)]$^-$); $^{13}$C nmr in $CD_3OD$ see Table 1; $^1$H nmr in $CD_3OD$ (aglycone) singlets at δ 0.852, 0.873, 0.936, 0.973, 0.986, 1.072, 1.187 (each 3H), δ 5.28 (1H, m, H-12); (sugars) δ 4.41 (1H, d, *J*=7.5, H-1 of galactose unit), δ 4.60 (1H, d, *J*=7.6 Hz, H-1 of glucuronic acid unit).

## ACKNOWLEDGMENTS

This study was supported by M.P.I. (ROMA). We thank Prof. Francesco Capasso, Dipartimento di Farmacologia Sperimentale, Università di Napoli, for collection and classification of the plants. We also thank Dott. Alfredo Milone of the "Servizio di Spettrometria di massa del CNR e dell'Università di Napoli" for fabms spectra. The assistance of the staff is gratefully acknowledged.

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*Received 20 November 1986*