Cytotoxic Saponins from Bulbs of *Allium porrum* L.[†]

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An extensive phytochemical analysis of the saponin content has been undertaken on leek, *Allium porrum* L., sown and collected at different seasons. As a result of this investigation, eight saponins (**1**−**8**) have been isolated, four of them (**5**−**8**) being novel compounds. Compounds **5** and **6**, possessing the same tetrasaccharide moiety of compounds **1** and **3**, display very unusual spirostane aglycones, 12-ketoporrigenin and 2,12-diketoporrigenin (named porrigenin C), respectively, recently isolated for the first time as free sapogenin in the same plant. Compounds **7** and **8** are rare cholestane bidesmosides possessing a di- and trisaccharide residues linked to a polyhydroxycholesterol aglycone, respectively. The structures of the isolated compounds have been determined by nondegradative spectroscopic analysis, mainly based on NMR. All the eight saponins isolated from leek were tested for their cytotoxic activity against two different cell lines in vitro, and compounds **1**, **2**, and **6** resulted particularly active.

Keywords: *Allium porrum L.; Liliaceae; saponins; NMR; cytotoxic activity*

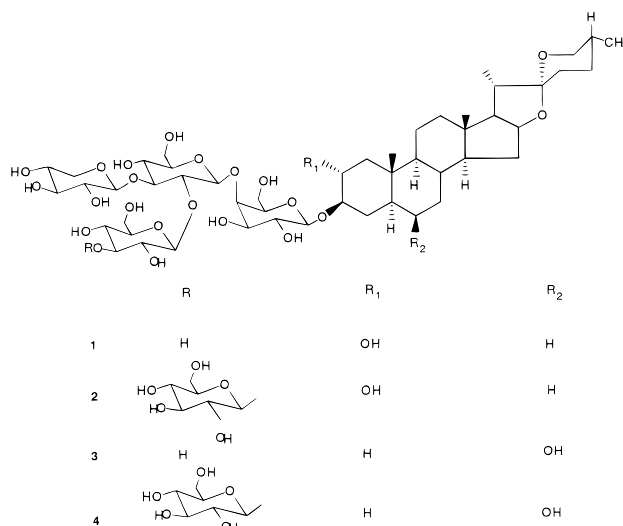
INTRODUCTION

Allium porrum L. (Liliaceae), commonly named leek, is a biennial herb, closely related to garlic and onion, that is known only in cultivation but is believed to be derived from a wild Eurasian plant, *A. ampeloprasum* L. It commonly grows as an annual crop for its mildly pungent succulent linear leaves and especially its thick cylindrical stack consisting of blanched leafstalks and a small simple bulb (Stearn, 1980).

The plant is used worldwide as an ingredient of many recipes, and its fresh juice is claimed to possess antibacterial, antifungal, and cancer-preventing properties (Schauenberg and Paris, 1977). This was the reason prompting us to start the analysis of the bioactive polar chemical constituents of this species. Our investigation led to the isolation of a number of unusual spirostane sapogenins (Carotenuto et al., 1997a, 1997b; Fattorusso et al., 1998) with antiproliferative activity on tumor cell lines in vitro, whose percentage in the plant was found to be affected by the sampling season (Fattorusso et al., 1998).

Our next target was then to analyze if these compounds may be also present in the complex form of saponin. As a first result of this investigation we have recently isolated four antifungal spirostanol saponins (**1**−**4**, Chart 1); two of them, compounds **3** and **4**, based on a β-chlorogenin aglycone, were previously unknown

Chart 1



(Carotenuto et al., 1999). We have now continued the saponin analysis of two samples of the same variety of leek, grown at different times. This paper refers to the isolation and structure elucidation of four further new saponins. Compounds **5** and **6** (Chart 2) are the tetrasaccharide derivatives of 12-ketoporrigenin and 2,12-diketoporrigenin (named porrigenin C), respectively. Compounds **7** and **8** (Chart 3) are cholestane bidesmosides possessing di- and trisaccharide residues, respectively. All the isolated saponins were subjected to pharmacological assays using two different cell lines, *J-774* (murine monocyte/macrophage) and *WEHI-164* (murine fibrosarcoma).

MATERIALS AND METHODS

General Methods. FABMS (recorded in a glycerol matrix) were measured on a Prospec Fisons mass spectrometer. Optical

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Chart 2

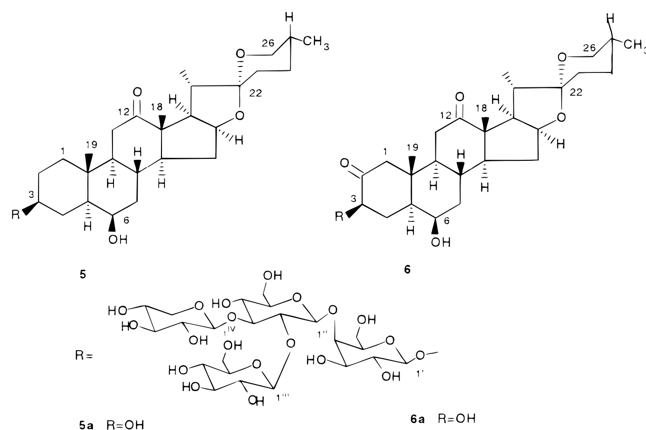
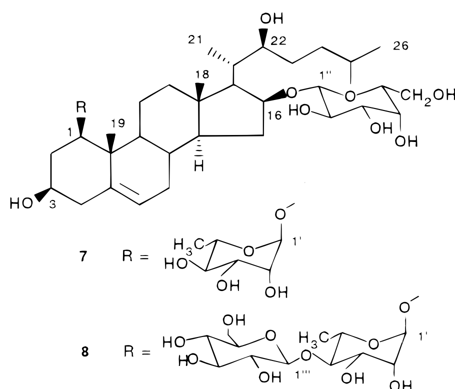


Chart 3



rotations were determined on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and 10-cm microcell.

^{1}H and ^{13}C NMR spectra were recorded at 500.13 and 125.795 MHz, respectively, on a Bruker AMX-500 spectrometer. Chemical shifts were referenced to the residual solvent signal (CD_3OD, δ_H 3.34, δ_C 49.0; $CDCl_3$, δ_H 7.26, δ_C 77.0). The multiplicities of ^{13}C resonances were determined by DEPT experiments. ^{1}H connectivities were determined by using COSY and HOHAHA experiments; the 2D HOHAHA experiments were performed in the phase-sensitive mode (TPPI) using the MLEV-17 (mixing time 125 ms) sequence for mixing. One-bond heteronuclear ^{1}H−^{13}C connectivities were determined with 2D HMQC experiments, with a BIRD pulse 0.5 s before each scan to suppress the signal from protons not directly bonded to ^{13}C. The interpulse delays were adjusted for an average $^{1}J_{CH}$ of 125 Hz. Two- and three-bond heteronuclear ^{1}H−^{13}C connectivities were determined with 2D HMBC experiments, optimized for $^{2,3}J_{CH}$ of 8 Hz. Nuclear Overhauser effect (nOe) measurements were performed by 2D ROESY experiments. MPLC was performed on a Buchi 861 apparatus using LiChroprep RP-18 (40−63 μm) columns. HPLC in isocratic mode was performed on a Varian apparatus equipped with an RI-3 refractive index detector. Hibar LiChrospher RP18 and μ-Bondapack C18 columns were used.

Plant Material. Plants of the same variety of *Allium porrum* L. were collected in the same place near Benevento, Italy, in two different months of the year, January 1997 and September 1997, each sample being sown about 6 months before its collection. Reference specimens have been deposited at the Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli Federico II, Napoli, Italy.

Extraction and Isolation. The plants were air-dried immediately after collection under controlled temperature (22 °C) and without exposure of light. They were chopped and then exhaustively extracted at room temperature with the following solvents: *n*-hexane; $CHCl_3$; $CHCl_3$/MeOH (9:1); MeOH. Each extraction was repeated four times using 500 mL of solvent, under stirring (dry weight of the plants after extraction: January 107 g; September 79 g) The butanol soluble fractions of the MeOH extracts obtained from the two different collections (2.1 and 1.5 g, respectively) were filtered and then concentrated in vacuo to afford two crude organic extracts, which were separately chromatographed by MPLC on RP-18 columns using a gradient solvent system from H_2O to MeOH.

Fractions eluted with MeOH from the September collection were purified by HPLC on a μ-Bondapack C-18 (7.8 × 300 mm) column with a mobile phase MeOH−H_2O (7:3) to give a crude saponin fraction. This was further purified by HPLC on a μ-Bondapack C-18 (3.9 × 300 mm) with a mobile phase MeOH−H_2O (7:3) to give fractions containing compounds **5** and **6**. Both fractions were further purified on a μ-Bondapack C-18 (3.9 × 300 mm) with a mobile phase MeOH−H_2O (6:4) finally affording pure compounds **5** (3.1 mg, yield 0.004%) and **6** (6.5 mg, 0.008%). Fractions of the samples collected in January eluted with MeOH−H_2O (8:2) were purified by HPLC on a μ-Bondapack C-18 (7.8 × 300 mm) with a mobile phase MeOH−H_2O (6:4). The obtained fractions were further purified on a μ-Bondapack C-18 (3.9 × 300 mm) with a mobile phase MeOH−H_2O (1:1) to give pure compound **7** (13.7 mg, yield 0.013%). Fractions eluted with MeOH−H_2O (7:3), again from the January collection, were purified by HPLC on a μ-Bondapack C-18 (7.8 × 300 mm) with a mobile phase MeOH−H_2O (6:4) and then on a μ-Bondapack C-18 (3.9 × 300 mm) with a mobile phase MeOH−H_2O (6:4), to give pure compound **8** (2.7 mg, yield 0.002%).

*(25R)-3β,6β-Dihydroxy-5α-spirostan-12-one3-O-{O-β-D-glucopyranosyl-(1→2)-O-[β-D-xylopyranosyl-(1→3)]-O-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (**5**):* $[\alpha]_D^{25}$ −16° (c = 0.001 in MeOH); FABMS (negative ion) *m/z* 1063 $[M-H]^-$; HRFABMS found *m/z* 1063.5050, calcd for $C_{50}H_{79}O_{24}$ *m/z* 1063.5039; ^{1}H and ^{13}C NMR data are reported in Table 1.

*(25R)-3β,6β-Dihydroxy-5α-spirostan-2,12-dione-3-O-{O-β-D-glucopyranosyl-(1→2)-O-[β-D-xylopyranosyl (1→3)]-O-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (**6**):* $[\alpha]_D^{25}$ −13° (c = 0.003 in MeOH); FABMS (negative ion) *m/z* 1077 $[M-H]^-$; HRFABMS found *m/z* 1077.4844, calcd for $C_{50}H_{78}O_{25}$ *m/z* 1077.4831; ^{1}H and ^{13}C NMR data are reported in Table 1.

22S-Cholest-5-ene-1β,3β,16β,22-tetrol 1-O-α-L-rhamnopyranosyl 16-O-β-D-Galactopyranoside (7): $[\alpha]_D^{25}$ −25° (c = 0.004 in MeOH); FABMS (negative ion) *m/z* 741 $[M-H]^-$; HRFABMS found *m/z* 741.4515, calcd for $C_{39}H_{66}O_{13}$ *m/z* 741.4502; ^{1}H and ^{13}C NMR data are reported in Table 2.

*Acetylation of Saponin **7**.* Acetylation was performed with Ac_2O and pyridine (1:1) overnight at room temperature The concentrated reaction mixture was subjected to HPLC (column, Hibar LiChrospher Si-60 5 mm; eluent, 3:7 hexane/EtOAc). ^{1}H NMR ($CDCl_3$) of the peracetyl derivative of **7**: δ 3.40 (1H, dd, J = 7.5, 1.3 Hz, H-1), 1.56 (1H, overlapped, H_a-2), 2.17 (1H, overl., H_b-2), 4.57 (1H, m, H-3), 2.32 (2H, overl. H_2-4), 5.60 (1H, d, J = 5.5 Hz, H-6), 1.97 (2H, overl., H_2-7), 1.44 (1H, overl., H-8), 0.97 (1H, overl., H-8), 0.97 (1H, overl., H-9), 1.05 (1H, overl., H_a-11), 2.07 (1H, overl., H_b-11), 0.83 (1H, overl., H_a-12), 1.45 (1H, overl., H_b-12), 1.62 (1H, overl., H-14), 1.02 (1H, overl., H_a-15), 2.06 (1H, overl., H_b-15), 4.27 (1H, ddd, J = 4.9, 7.9, 8.5 Hz, H-16), 1.56 (1H, overl., H-17), 1.08 (3H, s, H_3-18), 1.26 (3H, s, H_3-19), 2.02 (1H, overl., H-20), 0.94 (3H, d, J = 6.9 Hz, H-21), 5.02 (1H, overl., H-22), 1.32 (1H, overl., H_a-23), 1.67 (1H, overl., H_b-23), 1.18 (1H, m, H_a-24), 1.05 (1H, m, H_b-24), 1.51 (1H, m, H-25), 0.88 (6H, d, J = 6.6 Hz, H_3-26 and H_3-27), 4.88 (1H, s, H-1′), 5.17 (1H, bd, J = 2.1 Hz, H-2′), 5.25 (1H, dd, J = 2.1, 7.3 Hz, H-3′), 5.04 (1H, t, J = 7.3 Hz, H-4′), 3.81 (1H, qd, J = 6.5, 7.3 Hz, H-5′), 1.22 (3H, d, J = 6.5 Hz, H_3-6′), 4.56 (1H, d, J = 7.5 Hz, H-1″), 5.18 (1H, overl., H-2″), 4.97 (1H, dd, J = 1.5, 7.1 Hz, H-3″), 5.37 (1H, d, J = 1.5 Hz, H-4″), 3.92 (1H, dt, J = 1.5, 7.2 Hz, H-5″), 4.08 (1H, dd, J = 7.2, 10.2 Hz, H_a-6″), 4.16 (1H, dd, J = 7.2, 10.2 Hz, H_b-6″).

*22S-Cholest-5-ene-1β,3β,16β,22-tetrol 1-O-[O-β-D-Glucopyranosyl-(1→4)-α-L-rhamnopyranoside] 16-O-β-D-Galactopyranoside (**8**):* $[\alpha]_D^{25}$ −2° (c = 0.002 in MeOH); FABMS (negative

Table 1. ^{13}C and ^{1}H NMR Data for Compounds 5 and 6 (Measured in CD_3OD)

	5		6	
pos.	δ_C (mult.)	δ_H (mult., *J* in Hz)	δ_C (mult.)	δ_H (mult., *J* in Hz)
1α	41.6 (CH_2)	1.02 (dt, 3.7, 12.5)	53.6 (CH_2)	2.21[a]
1β		1.52[a]		2.26[a]
2	32.5 (CH_2)	1.58,[a] 1.92[a]	212.8 (C)	
3	74.8 (CH)	3.77[a]	76.7 (CH)	4.67 (m)
4	35.0 (CH_2)	1.71,[a] 1.72[a]	37.8 (CH_2)	2.07 (m) 2.28[a]
5	51.5 (CH)	1.19[a]	48.8 (CH)	1.88 (m)
6	65.5 (CH)	3.84 (bs)	65.8.(CH)	4.00 (m)
7α	42.5 (CH_2)	1.28[a]	42.7 (CH_2)	1.35 (m)
7β		1.94[a]		2.00 (td, 3.0, 13.2)
8	33.2 (CH)	2.38 (m)	33.3 (CH)	2.33 (m)
9	59.8 (CH)	1.21[a]	61.2 (CH)	1.53 (m)
10	38.5 (C)		38.2 (C)	
11α	41.2 (CH_2)	2.21 (dd, 5.1, 14.0)	41.5 (CH_2)	2.13 (dd, 4.9, 14.2)
11β		2.59 (t, 14.0)		2.62 (t, 14.2)
12	214.2 (C)		214.4 (C)	
13	56.1 (C)		56.3 (C)	
14	59.6 (CH)	1.53[a]	59.5 (CH)	1.55[a]
15α	34.5 (CH_2)	1.56[a]	34.6 (CH_2)	1.57[a]
15β		2.17 (t, 7.4)		2.19[a]
16	84.1 (CH)	4.37 (bq, 7.1)	84.3 (CH)	4.38 (m)
17	58.1 (CH)	2.51 (dd, 7.4, 7.6)	58.4 (CH)	2.51 (dd, 7.4, 7.6)
18	17.5 (CH_3)	1.17 (s)	17.6 (CH_3)	1.12 (s)
19	17.9 (CH_3)	1.15 (s)	17.0 (CH_3)	1.08 (s)
20	46.7 (CH)	1.82 (m)	46.5 (CH)	1.82 (m)
21	16.5 (CH_3)	1.05 (d, 6.6)	16.3 (CH_3)	1.06 (d, 6.6)
22	110.1 (C)		110.4 (C)	
23α	35.2 (CH_2)	1.61[a]	35.5 (CH_2)	1.64[a]
23β		1.74 (dd, 4.4, 13.2)		1.73 (dd, 4.4, 13.8)
24α	28.5 (CH_2)	1.45 (ddd, 4.4, 9.2, 11.8)	28.4 (CH_2)	1.45 (ddd, 4.4, 9.2, 11.8)
24β		1.63[a]		1.63[a]
25	32.3 (CH)	1.64[a]	32.3 (CH)	1.65[a]
26α	71.1 (CH_2)	3.38[a]	71.1 (CH_2)	3.37[a]
26β		3.49 (dd, 3.7, 13.2)		3.51 (m)
27	19.8 (CH_3)	0.82 (d, 6.6)	19.6 (CH_3)	0.80 (d, 6.6)
1′	106.8 (CH)	4.43 (d, 7.4)	107.1 (CH)	4.43 (d., 7.4)
2′	76.2 (CH)	3.68 (dd, 7.4, 8.1)	76.5 (CH)	3.76 (dd, 7.4, 8.1)
3′	78.2 (CH)	3.53[a]	78.3 (CH)	3.57[a]
4′	82.6 (CH)	4.07 (bd, 2.2)	82.8 (CH)	4.03 (bd, 2.2)
5′	65.6 (CH)	3.94[a]	65.4 (CH)	3.95[a]
6′a,b	65.0 (CH_2)	3.55[a]	65.0 (CH_2)	3.55[a]
1″	107.4 (CH)	4.61 (d, 7.3)	107.6 (CH)	4.58 (d, 7.3)
2″	83.5 (CH)	3.78 (dd, 7.3, 8.1)	83.5 (CH)	3.79[a]
3″	90.2 (CH)	3.75 (dd, 7.3, 8.1)	90.4 (CH)	3.77[a]
4″	73.8 (CH)	3.32[a]	73.9 (CH)	3.31[a]
5″	69.3 (CH)	3.30[a]	69.4 (CH)	3.35[a]
6″a,b	64.1 (CH_2)	3.55,[a] 3.98[a]	64.2 (CH_2)	3.62,[a] 3.98[a]
1‴	107.2 (CH)	4.94 (d, 7.3)	107.4 (CH)	4.94 (d, 7.3)
2‴	79.2 (CH)	3.22 (dd, 8.1, 10.3)	79.4 (CH)	3.20 (dd, 8.1, 10.3)
3‴	81.1 (CH)	3.38[a]	81.2 (CH)	3.39[a]
4‴	69.8 (CH)	3.86 (m)	69.7 (CH)	3.87 (dd, 8.5, 9.3)
5‴	79.6 (CH)	3.93[a]	79.7 (CH)	3.93[a]
6‴ab	64.8 (CH_2)	3.62,[a] 3.68[a]	64.7 (CH_2)	3.91[a]
1^{IV}	107.5 (CH)	4.63 (d, 7.4)	107.8 (CH)	4.63 (d, 7.4)
2^{IV}	74.6 (CH)	3.28[a]	78.6 (CH)	3.28 (dd, 7.4, 7.0)
3^{IV}	78.8 (CH)	3.55[a]	74.7 (CH)	3.56[a]
4^{IV}	73.0 (CH)	3.61 (ddd, 2.1, 7.5, 8.8)	73.2 (CH)	3.66[a]
$5^{IV}_{a,b}$	69.1 (CH_2)	3.45,[a] 3.92[a]	69.2 (CH_2)	3.39,[a] 3.79 (dd, 10.5, 9.5)

[a] Overlapped with other signals.

ion) *m/z* 903 [M − H]$^-$; HRFABMS found *m/z* 903.5050, calcd for $C_{45}H_{76}O_{18}$ *m/z* 903.5031; ^1H and ^{13}C NMR data are reported in Table 2.

Cells. *WEHI 164* cells (murine fibrosarcoma cell line) were maintained in adhesion on Petri dishes with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, penicillin (100 U/mL), and streptomycin (100 μg/mL). *J774* cells (murine monocyte/macrophage cell line) were grown in suspension culture, in Techne stirrer bottles, spun at 25 rpm, and incubated at 37 °C in DMEM medium supplemented with 10% FBS, 25 mM Hepes, glutamine (2mM), penicillin (100U/mL), and streptomycin (100 μg/m).

Cytotoxicity Assay. *WEHI 164* and *J774* cells were plated on 96-well microliter plates and allowed to adhere at 37 °C in 5% CO_2/95% air for 2 h. Thereafter the medium was replaced with 50 μL of fresh medium, and 75 μL aliquot of 1.2 v/v serial dilution of each test compound **1**−**8** was added. Then the cells were incubated for 72 h. In some experiments 6-mercaptopurine (6-MP) was added. The cells viability was assessed through an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2*H*-tetrazolium bromide] conversion assay. Briefly, 25 μL of MTT (5 mg/mL) was added and the cells were incubated for additional 3 h. Following this time the cells were lysed and the dark blue crystals solubilized with 100 μL of a solution containing 50% (v:v) *N,N*-dimethylformamide and 20% (w:v)

Table 2. ^{13}C and ^{1}H NMR Data for Compounds 7 and 8 (Measured in CD_3OD)

	7		**8**	
pos.	δ_C (mult.)	δ_H (mult., J in Hz)	δ_C (mult.)	δ_H (mult., J in Hz)
1	85.7 (CH)	3.36[a]	85.6 (CH)	3.36[a]
2	38.1 (CH_2)	1.48,[a] 2.26[a]	38.3 (CH_2)	1.47,[a] 2.26[a]
3	71.9 (CH)	3.45[a]	71.9 (CH)	3.44 (m)
4	46.3 (CH_2)	2.27[a]	46.6 (CH_2)	2.27[a]
5	142.1 (C)		142.1 (C)	
6	130.2 (CH)	5.62 (d, 5.7)	130.3 (CH)	5.62 (d, 5.7)
7	34.9 (CH_2)	2.00 (m)	35.1 (CH_2)	1.99 (m)
8	34.8 (CH)	1.46[a]	34.6 (CH)	1.47[a]
9	55.1 (CH)	1.08[a]	55.6 (CH)	1.08[a]
10	45.1 (C)		45.3 (C)	
11α	28.8 (CH_2)	1.59,[a] 2.22[a]	28.6 (CH_2)	1.58,[a] 2.21[a]
12	44.7 (C)	1.22,[a] 2.00[a]	44.7 (C)	1.22,[a] 2.00[a]
13	44.8 (C)		44.8 (C)	
14	57.2 (CH)	1.60[a]	57.4 (CH)	1.60[a]
15	40.2 (CH_2)	2.24[a]	40.7 (CH_2)	2.24[a]
16	86.4 (CH)	4.20 (ddd, 4.0, 7.9, 8.5)	86.8 (CH)	4.20 (td, 4.0, 8.3)
17	61.8 (CH)	1.59[a]	61.7 (CH)	1.58[a]
18	20.0 (CH_3)	0.99 (s)	20.1 (CH_3)	0.97 (s)
19	20.2 (CH_3)	1.10 (s)	20.3 (CH_3)	1.09 (s)
20	46.4 (CH)	2.16 (m)	46.5 (CH)	2.15 (m)
21	18.2 (CH_3)	0.95[a]	18.2 (CH_3)	0.95[a]
22	77.1 (CH)	3.73 (bt, 7.2)	77.1 (CH)	3.73[a]
23	36.7 (CH_2)	1.48,[a] 1.42[a]	36.9 (CH_2)	1.48,[a] 1.42[a]
24	40.3 (CH_2)	1.29[a]	40.2 (CH_2)	1.28[a]
25	28.5 (CH)	1.45[a]	28.2 (CH)	1.48[a]
26−27	27.5 (CH_3)	0.94[a]	27.3 (CH_3)	0.94[a]
1′	101.2 (CH)	4.94 (bs)	101.4 (CH)	4.91 (bs)
2′	74.5 (CH)	3.88 (bd, 3.1)	74.5 (CH)	3.81 (bd, 3.1)
3′	74.6 (CH)	3.68 (dd, 3.1, 9.6)	73.1 (CH)	3.92 (dd, 3.1, 9.6)
4′	76.4 (CH)	3.42[a]	79.6 (CH)	3.63 (t, 9.6)
5′	72.3 (CH)	3.60 (qd, 6.3, 9.0)	72.5 (CH)	3.70[a]
6′	18.8 (CH_3)	1.27 (d, 6.3)	18.9 (CH_3)	1.37 (d, 6.5)
1″	111.3 (CH)	4.17 (d, 7.5)	111.5 (CH)	4.17 (d, 7.5)
2″	78.8 (CH)	3.52 (dd, 7.5, 9.1)	78.9 (CH)	3.50 (dd, 7.5, 9.3)
3″	78.1 (CH)	3.48[a]	78.3 (CH)	3.48[a]
4″	73.2 (CH)	3.88 (bd, 3.1)	73.3 (CH)	3.88 (bs)
5″	75.8 (CH)	3.50[a]	75.9 (CH)	3.50[a]
6″a,b	64.8 (CH_2)	3.74[a]	64.5 (CH_2)	3.74[a]
1‴			111.0 (CH)	4.58 (d, 7.6)
2‴			79.2 (CH)	3.26 (t, 7.6)
3‴			81.3 (CH)	3.38 (t, 7.6)
4‴			69.9 (CH)	3.89[a]
5‴			70.1 (CH)	3.73[a]
6‴a,b			64.8 (CH_2)	3.34[a]

[a] Overlapped with other signals.

SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with compounds **1**−**8** was calculated as % dead cells = 100 − (OD treated/OD control) × 100.

RESULTS AND DISCUSSION

Two samples of the same variety of leek, *Allium porrum* L., collected at different seasons (summer and winter) were exhaustively extracted with *n*-hexane, $CHCl_3$, $CHCl_3$/MeOH (9:1), and MeOH. Both MeOH extracts were separated by sequential chromatography, by using column and HPLC techniques (see Materials and Methods). Samples collected in September, in addition to the already reported compounds **1**−**4** (Mimaki et al., 1995, Carotenuto et al., 1999), contained compounds **5** and **6**, while those collected in January afforded compounds **7** and **8**, together with traces of the above spirostane saponins. This distribution of the isolated saponins appears quite interesting, if compared with our recently report on the sapogenin content of the same samples of leek, which resulted dependent from the season of sowing and collection (Fattorusso et al., 1998). We should now note that the saponin content of *A. porrum* strictly reflects the sapogenin composition, and this is particularly evident in the January collection where the spirostane sapogenin percentage is the least and in a parallel manner spirostane saponins are almost absent, being replaced by cholestane saponins. On the other hand, these two class of compounds are probably biosynthetically related: polyhydroxycholesterol glucosides have been unambiguously demonstrated as the precursors of spirostanol sapogenins, as gitogenin and diosgenin, in several plants (Tschesche and Hulpke, 1966; Tomita and Uomori, 1971).

Compound **5**, an amorphous solid possessing the molecular formula $C_{50}H_{80}O_{24}$, as determined by high-resolution negative-ion FABMS $[M - H]^-$ (m/z 1063), was shown to be a tetrasaccharide derivative of 12-ketoporrigenin (**5a**), previously found by our research group as a free sapogenin in the same plant (Carotenuto et al., 1997b).

The ^{13}C NMR spectrum (CD_3OD) of **5** showed two key signals in the low-field region: a singlet at δ 110.1, attributed to the ketal carbon C-22, and a signal at δ 214.2, indicating a ketone functionality. Although the high-field region of the ^{1}H NMR spectrum of **5** contained

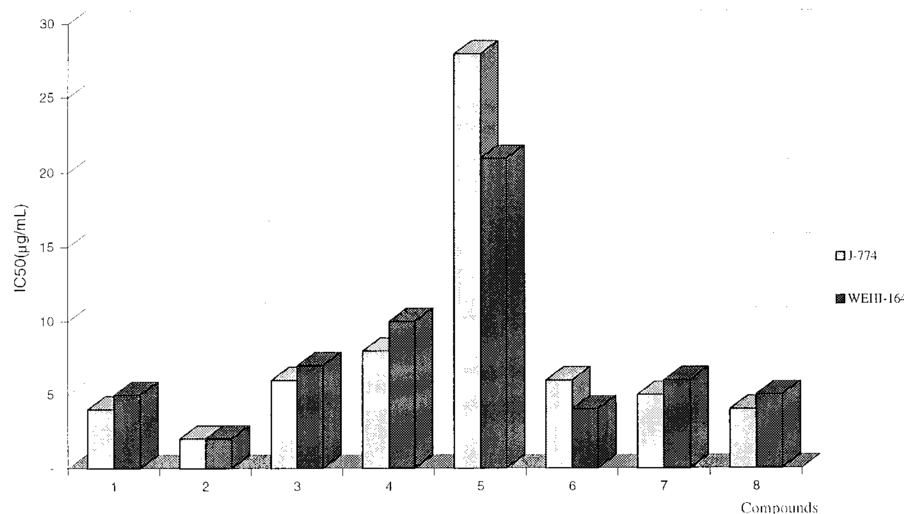


Figure 1. Cytotoxic activity of the isolated saponins.

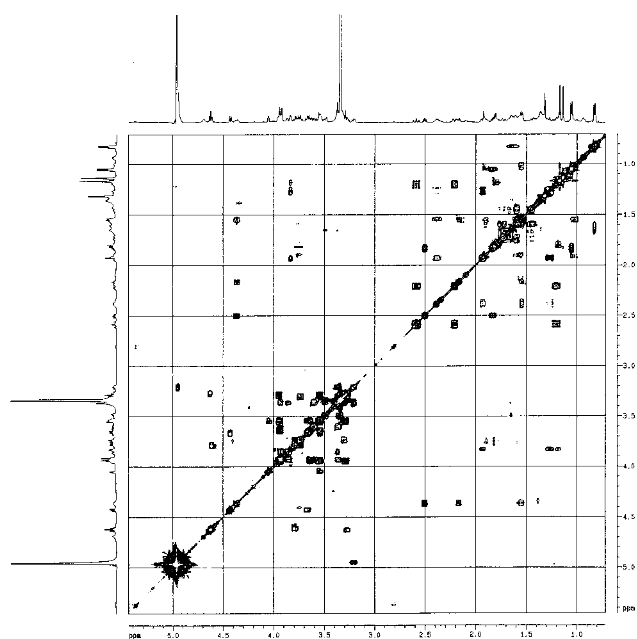


Figure 2. COSY spectrum of compound **5** ($CDCl_3$).

several overlapping signals, analysis of homonuclear COSY (shown in Figure 2) and HOHAHA 2D experiments allowed us to assign all the proton resonances of the aglycone moiety. These signals resulted to belong to two different spin systems, the first one extending from the protons of ring A to those of ring E and the second one including the protons of ring F. Within these spin systems, the presence of three oxymethines (δ 3.77, 3.84, and 4.37) and of an oxymethylene (δ 3.38 and 3.49) was revealed. In addition, two further methyl proton signals were present at δ 1.15 (s, H_3-19) and 1.17 (s, H_3-18; note the relatively low field). All these resonances were associated with those of directly attached carbon signals in the ^{13}C NMR spectrum by using HMQC experiment (assignment of ^1H and ^{13}C NMR signals shown in Table 1), and the analysis of the whole series of these data was in perfect agreement with a (25*R*), 12-ketoporrigenin structure.

The first step for the analysis of the saccharide part of the molecule was the association of the anomeric carbons detected in the ^{13}C NMR spectrum (δ 106.8, 107.4, 107.5, and 107.2) with the relevant anomeric proton signals (δ 4.43, 4.61, 4.63, and 4.94), through the HMQC experiment. The nature of the single monosaccharides and their sequence were determined by combined analysis of COSY, HOHAHA, ROESY, and HMQC spectra. Starting from the anomeric proton of each sugar unit, all the proton signals within each spin system were delineated using COSY (Figure 2), with the aid of a HOHAHA spectrum. Having in our hand the proton assignments, we inferred the ^{13}C NMR resonances within each sugar unit by cross-peaks present in the HMQC spectrum. The obtained data were in accordance with the presence of three hexoses and one pentose, all of them in the pyranose form. The relative stereochemistry of these monosaccharides was deduced by considering the coupling constants and some diagnostic ROESY cross-peaks (for example H-1′−H-3′, H-3′−H-5′ and analogous couplings for the other sugars), allowing us to identify them as one galactose (directly attached to the aglycone), two glucoses, and one xylose, respectively. In addition, the β-anomeric configuration of all these sugars was judged from their large $J_{H1,H2}$ coupling constants ($J = 7-8$ Hz). Furthermore, strong ROESY cross-peaks between H-4′ (δ 4.07) and H-1″ (δ 4.61), H-2″ (δ 3.78) and H-1‴ (δ 4.94), and H-3″ (δ 3.75) and H-1^{IV} (δ 4.63) allowed us to deduce the sugar sequence within the oligosaccharide chain of **5**, which resulted to be identical to that of **1** and **3**. The same sugar sequence has been also reported for other saponins isolated from *Allium*, as the recently investigated *A. jesdianum* (Mimaki et al., 1999). Finally, the cross-peak between H-3 (δ 3.77) and H-1′, present in the ROESY spectrum of **5**, indicated the C-3 of the aglycone as the glycosylation site. Thus, saponin **5** was determined as (25*R*)-3β,6β-dihydroxy-5α-spirostan-12-one-3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}.

Compound **6** possesses the molecular formula $C_{50}H_{78}O_{25}$, determined by negative-ion FABMS $[M - H]^-$ (*m/z* 1077) and in accordance with ^{13}C NMR data. This spectrum showed the resonances of two ketone carbonyls at δ 212.8 and 214.4, of a ketal carbon at δ 110.4 (C-22), and of four anomeric carbons at δ 107.8, 107.6, 107.4, and 107.1. Inspection of COSY and HOHAHA spectra of **6** allowed us to follow the scalar couplings within the pattern of aglycone and sugar protons and to assign all their resonances (Table 1). ^1H NMR spectrum of **6** appeared quite similar to that of **5**, the

main differences being confined to the ring A of the spirostane aglycone. The location of one of the ketone functionalities at C-2 of compound **6** was indicated by the chemical shift of H_2-1, resonating as an AB system at δ 2.26 and 2.21, and by the relatively low field resonances of H-3 (δ 4.67), H_2-4 (δ 2.28 and 2.07), and H-5 (δ 1.55). Furthermore, the downfield chemical shift of C-1 in the ^{13}C NMR spectrum of **6** (δ 5.28) was in perfect agreement with the location of the carbonyl function on the adjacent C-2. Finally, all the ^{1}H NMR signals attributed to rings B−F of the aglycone resulted quite similar to the corresponding signals exhibited by saponin **5**. These evidences indicated 2,12-diketoporrigenin (porrigenin C) as the aglycone of **6**. Porrigenin C (**6a**) has been recently isolated by our group as free sapogenin from bulbs of *A. porrum* (Fattorusso et al., 1998).

As far as the sugar portion of compound **6**, its ^{1}H and ^{13}C (associated by HMQC) NMR signals (Table 1) appeared almost superimposable with those above determined for compound **5**, and so the same sugar portion of **5** was easily assigned to **6**. This was further confirmed by ROESY cross-peaks between H-4′ (δ 4.03) and H-1″ (δ 4.58), H-2″ (δ 3.79) and H-1‴ (δ 4.94), and H-3″ (δ 3.77) and H-1^{IV} (δ 4.63). Finally, an intense NOE contact between signals at δ 4.67 (H-3) and 4.43 (anomeric proton of galactose), present in the ROESY spectrum, confirmed also the tetraglycoside linkage position at C-3 of the aglycone. By these data, saponin **6** was formulated as (25*R*)-3β,6β-dihydroxy-5α-spirostan-2,12-dione-3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}.

Compound **7** was isolated as an amorphous solid, with the molecular formula $C_{39}H_{66}O_{13}$, deduced from high-resolution negative-ion FABMS (calculated *m*/*z* 741.4502, found *m*/*z* 741.4515) and also confirmed by NMR evidence. Some diagnostic resonances of the ^{1}H NMR spectrum of **7** (in CD_3OD, Table 2) were those attributed to two tertiary methyl (δ 1.10 and 0.99), three secondary methyl (δ 0.95, 0.94 × 2), one olefinic (δ 5.62), and two anomeric (δ 4.94 and 4.17) protons. The ^{13}C NMR spectrum (Table 2) showed 39 resonance lines, thus supporting the molecular formula deduced from MS: 27 of them were attributed to the aglycone part and 12 to two monosaccharides. Interpretation of the COSY (Figure 3), HOHAHA, HMQC, and HMBC spectra of **7** allowed us to assign all the signals of its aglycone moiety, which was identified as a tetrahydroxylated steroid of the cholestane type. In particular, ring A constitutes an isolated spin system possessing two hydroxyl groups in positions 1 and 3 (H-1 at δ 3.36, C-1 at δ 85.7; H-3 at δ 3.45, C-3 at δ 71.9). The diaxial spatial coupling of H-1 with H-3, evidenced by a cross-peak in the ROESY spectrum, indicated the relative orientation of the OH groups of ring A. The two remaining hydroxyl groups implied from the molecular formula were placed at positions 16 and 22, respectively, by following the series of scalar connections in ring D and within the side chain. In particular, the relatively low field H_2-23 (δ 1.48 and 1.42) were coupled with a carbinol proton resonating at δ 3.73 (H-22). Going on, H-22 showed a small coupling constant with H-20 (δ 2.16), and this was coupled with H-17 (δ 1.59), in turn coupled with H-16, whose ^{1}H NMR resonance at δ 4.20 (and that of the corresponding carbon at δ 86.4, in the ^{13}C NMR spectrum) allowed us to position the remaining hydroxyl group at C-16. The β orientation of the 16-OH was deduced by comparison with reported literature data (Kawashima et al., 1991) and by considering the relatively low-field ^{1}H NMR resonance of the spatially close H_3-18 (δ 0.99). Furthermore, we have also assumed the *S* stereochemistry for the chiral C-22 by comparison with data of analogous compounds. In particular, as already reported for other 22*S*-OH steroids, the ^{1}H NMR spectrum of **7** exhibited a particularly low (about 1 Hz) H-22/H-20 coupling constant. This points to a preferential conformation in which the dihedral angle formed by H-20 and H-22 is near to 90°, and as demonstrated by molecular dynamic calculations, this angle is formed only by the *S*-isomer at C-22 (Cafieri et al., 1999). The aglycone of compound **7** was therefore defined as 22*S*-cholest-5-ene-1β,3β,16β,22-tetrol.

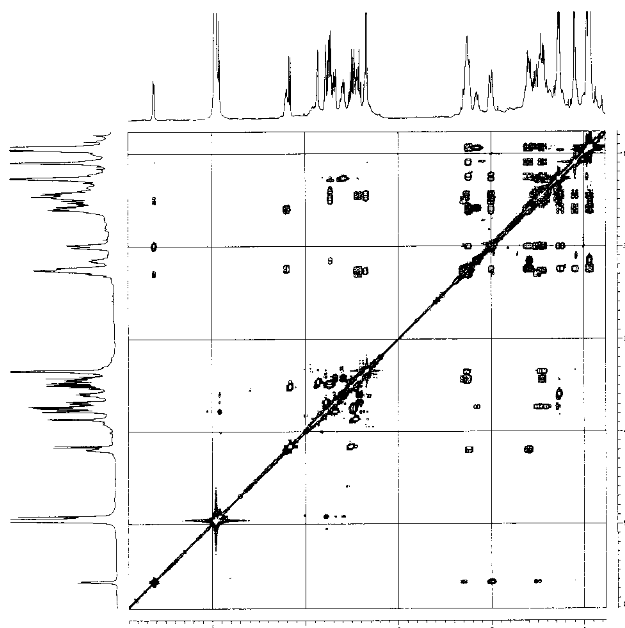


Figure 3. COSY spectrum of compound **7** ($CDCl_3$).

Concerning the sugar portion of the molecule, the presence of two monosaccharides, as above stated, was deduced on the basis of the ^{13}C NMR spectrum of **7**, which showed the resonances of two anomeric carbon atoms (δ 111.3 and 101.2, respectively), correlated to the relevant protons through the HMQC experiment. One of these sugar unit was confidently placed at C-1 of ring A by interpretation of the key HMBC correlation peak between H-1′ (δ 4.94) and C-1 (δ 85.7), this junction being also confirmed by the strong ROESY peak between H-1′ and H-1 (δ 3.36). Analogously, the second monosaccharide was linked at C-16, by considering the HMBC cross-peak between C-16 (δ 85.4) and H-1″ (δ 4.17) and the ROESY cross-peak between this latter signal and H-16 (δ 4.20).

Analysis of COSY, HOHAHA, and ROESY spectra of **7** permitted the unambiguous assignment of the signals belonging to the sugar portion of the molecule. Starting from the signal at δ 4.17, we identified the sequence of a hexose unit and, within its spin system, the axial−axial H-1″/H-2″ and H-2″/H-3″ and the axial−equatorial H-3″/H-4″ relationships were deduced by measurement of the coupling constants, whereas the axial position of H-5″ was indicated by the ROESY correlation peak with H-3″. This evidence identified the “eastern” monosaccharide as a β-galactopyranose, as depicted in the figure. With the same type of analysis, the C-1 linked sugar

unit was identified as a 6-deoxyhexose in the pyranose form in which the axial−axial couplings H-3′/H-4′ and H-4′/H-5′ and the axial−equatorial relationships between H-2′ and H-3′ led to the assignment as a rhamnopyranose. In accordance with data reported in the literature (Agrawal, 1992; Sang et al., 1999) the α-anomeric configuration of this sugar was judged by the chemical shift of C-5′ (δ 72.3).

The ^{1}H NMR resonances of the peracetyl derivative of **7** (see Materials and Methods Section) showed a particularly favorable dispersion of the sugar signals (without any superimposition), allowing a surer measurement of the coupling constants. The obtained results offered further support for the assigned relative stereochemistry of both monosaccharides. Thus, compound **7** has been assigned as 22*S*-cholest-5-ene-1β,3β,16β,22-tetrol 1-*O*-α-L-rhamnopyranosyl 16-*O*-β-D-galactopyranoside.

Compound **8**, $C_{45}H_{76}O_{18}$, deduced from high-resolution negative-ion FABMS (calculated *m*/*z* 903.5031, found *m*/*z* 903.5050), was isolated in low amounts from more polar fractions. The presence of three sugars in **8** was apparent from the three anomeric proton signals at δ 4.91, 4.58, and 4.17, associated with the relevant carbon atoms in the ^{13}C NMR spectrum (δ 101.4, 111.0, and 111.5, respectively) through the HMQC spectrum. Comparison of the molecular formula, and of ^{1}H and ^{13}C NMR spectra of **8** (assigned by 2D NMR spectroscopy and reported in Table 2) with parallel data arising from **7**, evidenced the presence of an additional hexose monosaccharide. Furthermore, while resonances of the aglycone and of galactopyranose appeared almost identical with those of **7**, some slight modifications were detected among the resonances of the rhamnose. In particular, signals of C-4′ (δ 79.6) and of H-4′ (δ 3.63) were downfield shifted (+ 3.2 ppm and + 0.21 ppm, respectively), accompanied by a slight upfield shift of C-3′ (−1.5 ppm), as compared with those of **7**. These data suggested C-4′ as the glycosylated position to which the additional monosaccharide is linked. This was also confirmed by the ROESY cross-peak of H-1‴ (δ 4.58) with H-4″ (δ 3.63). The same H-1‴ constituted the starting point to deduce, through COSY and HOHAHA, the sequence of the further hexose unit. Within its spin system, the large coupling constants of all H-1‴ to H-5‴ signals, indicative of axial−axial relationships, pointed for the β-glucopyranoside nature of this monosaccharide. Consequently, the structure of **8** was formulated as 22*S*-cholest-5-ene-1β,3β,16β,22-tetrol 1-*O*-[*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside] 16-*O*-β-D-galactopyranoside.

Cholestane bidesmosides are somewhat rare in nature; however, some saponins related to **7** and **8** have been found in *Allium* species native to central Asia, as *A. schubertii* (Kawashima et al., 1991) and *A. albopilosum* (Mimaki et al., 1993) and in *Nolina recurvata* (Agavaceae) stems (Takahashi et al., 1995).

The use of nondestructive methods for the structural elucidation of compounds **1**−**8** allowed us to preserve the required amounts for the pharmacological screening. All the eight saponins isolated from *A. porrum* were tested for cytotoxic activity on two different cell lines (*J-774*, murine monocyte/macrophage, and *WEHI-164*, murine fibrosarcoma) in vitro. Results expressed as IC_{50} (μg/mL, the concentration that inhibited the cell growth by 50%) against *J-774* (A) and *WEHI-164* (B) are the following: (**1**) 3.7 (A) and 4.8 (B); (**2**) 2.1 (A) and 1.9 (B); (**3**) 5.7 (A) and 6.5 (B); (**4**) 7.6 (A) and 10.0 (B); (**5**) 27.9 (A) and 21.1 (B); (**6**) 5.8 (A) and 4.3 (B); (**7**) 4.6 (A) and 5.8 (B); (**8**) 4.0 (A) and 5.4 (B). These results, summarized in Figure 1, indicate a quite good activity for almost all the compounds under investigation, and this is possibly related with some of the pharmacological activities ascribed to *A. porrum* (Schauenberg and Paris, 1977).

The most active saponins of the series are **1**, **2**, and **6** (values ranging from 1.9 to 5.8 μg/mL) bearing spirostanol aglycones of different types. Interestingly, saponin **5** is considerably less active than **6**, even if their structures are quite similar, differing only for the ketone group on C-2. This result appears even more interesting if we consider that the corresponding free sapogenins, 12-ketoporrigenin and porrigenin C, respectively, exhibited a not so marked difference in their cytotoxic activities (Fattorusso et al., 1998). Finally, cholestane glycosides **7** and **8** exhibited a good activity with IC_{50} values from 4.0 to 5.8 μg/mL. These results do not agree with some recent data (Mimaki et al., 1999) reporting the lack of activity for cholestane glycosides and a markedly low activity for 6-OH spirostanol saponins, if compared with those possessing a gitogenin aglycone.

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