Sapogenins of *Allium porrum* L.†

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A chemical study of the sapogenin content has been conducted on leek, *Allium porrum* L., sown and collected at different times. As a result of this investigation, a variability in the sapogenin composition depending on the climate was observed. In addition, a novel sapogenin named porrigenin C (**1a**) and its 25*S* epimer (**1b**) have been isolated, together with the previously described sapogenins **2**−**5** and the known compounds agigenin, diosgenin, *β*-chlorogenin, and 24-ethylcholesta-(6-acyl)-3-*O*-*β*-D-glucoside. The chemical structure of the novel compound was elucidated by a detailed spectroscopic analysis based mainly on 1D and 2D NMR techniques. Porrigenin C showed a considerable antiproliferative activity on four tumor cell lines in vitro.

Keywords: *Allium porrum L.; Liliaceae; spirostanol sapogenin; NMR; antiproliferative activity*

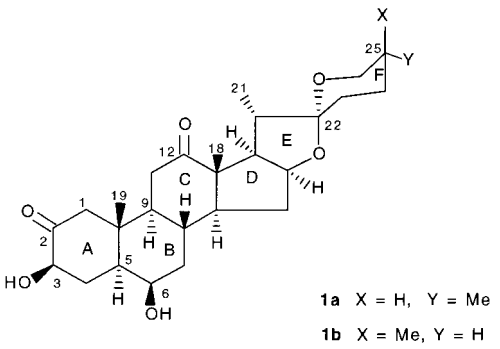
INTRODUCTION

Allium porrum L. is a bulbous perennial plant cultivated worldwide and used as a food, commonly named leek (Stearn, 1980; Uphof, 1968). Diuretic, hypotensive, and digestive properties are attributed to this plant, and its fresh juice is bactericide (Schauenberg and Paris, 1977). The first citation about its use in folk medicine as a remedy "to make good the voice", is reported in the first century A.D. by Plinius the Elder in his *Historiae naturalis*.

Scientific investigation on the chemical composition of *A. porrum* L. started with the work of Block on the apolar fractions of several *Allium* species (Block et al., 1992). This work led to the discovery of the chemical structures, biosynthesis, and reactivity of thiosulfinates, enzymatic breakdown products of alkylcysteine sulfoxides responsible for flavor, and of some bioactivities attributed to *Allium*.

Only recently some samples of *A. porrum* have been analyzed for the characterization of novel polar bioactive compounds. On this material, a quite interesting secondary metabolism was found that elaborates a number of sapogenins (**2**−**5**; Chart 1) with antiproliferative activity on IGR-1 (human melanoma), J-774 (murine monocyte), WEHI-164 (murine fibrosarcoma), and P-388 (murine leukemia) cell lines in vitro (Carotenuto et al., 1997a,b). In addition, a recent investigation reported the isolation and characterization of three new dibenzofurans (Carotenuto et al., 1998) showing antifungal activity toward *Fusarium culmorum*.

The very interesting pharmacological activities of these isolated metabolites and their stability during food processing prompted us to investigate the variation of the free sapogenin content in samples of *A. porrum* L. sown and then collected at four different periods of the year. Preliminary interesting results were obtained and are reported in this paper. In addition, this investigation led to the isolation of a further sapogenin, named porrigenin C (**1a**), and of small quantities of its 25*S* isomer neoporrigenin C (**1b**). We report herein their isolation, structure elucidation, and biological activity.



1a X = H, Y = Me

1b X = Me, Y = H

MATERIALS AND METHODS

General Methods. High-resolution mass spectra (HRMS) were obtained by electron impact (EI) at 65 eV on a VG Prospec Fisons mass spectrometer. Optical rotations were determined on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 10-cm microcell.

^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker AMX-500 spectrometer in $CDCl_3$ solution. Proton chemical shifts were referenced to the residual solvent signal (δ 7.26); ^{13}C NMR signals were referenced to the center peak of the quintet at 77.0 ppm. 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 experiment (Hull, 1994) was performed in the phase-sensitive mode (TPPI) using the MLEV-17 (mixing time = 120 ms) sequence for mixing. ^{1}H−^{13}C connectivities were determined with 2D HMQC experiments (Griesinger et al., 1994), with a BIRD pulse sequence followed by a delay of 0.5 s before each scan to suppress the signal from protons not directly bonded to ^{13}C.

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Chart 1

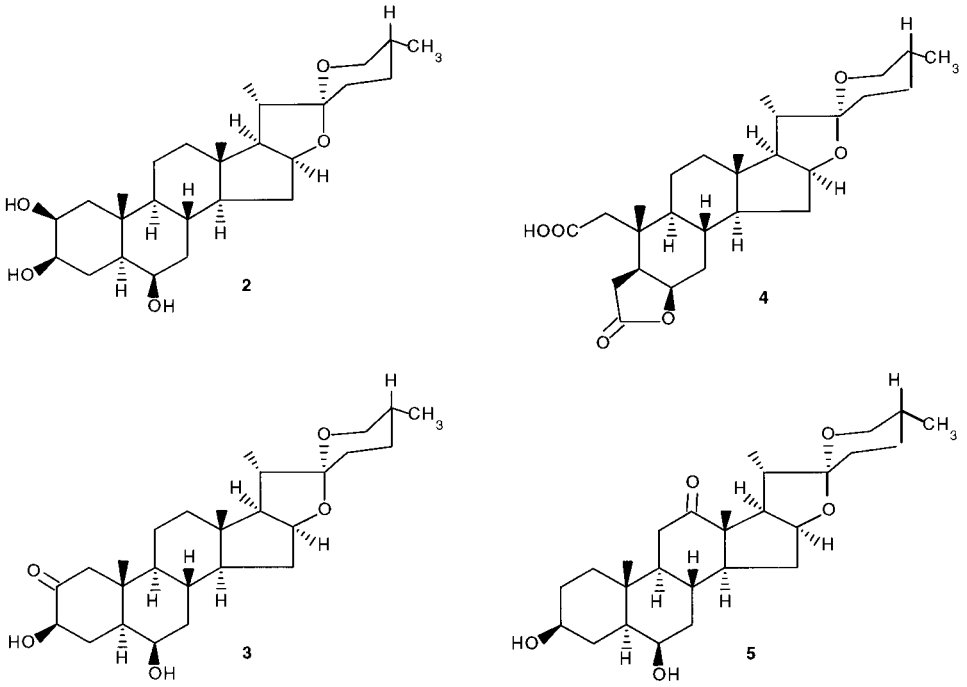

Table 1. ^{1}H and ^{13}C NMR Assignments ($CDCl_3$) for Compound 1a

position	δ_C (multiplicity)	δ_H (multiplicity, *J* [Hz])	position	δ_C (multiplicity)	δ_H (multiplicity, *J* [Hz])
1α	52.1 (CH_2)	2.05 (d, 13.0)	15α	31.1 (CH_2)	2.12 (m)
β		2.32 (d, 13.0)	*β*		1.51[a]
2	212.0 (C)		16	79.0 (CH)	4.35 (bq, 7.5)
3	76.2 (CH)	4.18 (dd, 8.5, 11.2)	17	53.4 (CH)	2.55 (dd, 7.3, 8.8)
4α	36.6 (CH_2)	1.97[a]	18	16.0 (CH_3)	1.05 (s)
β		2.27[a]	19	16.2 (CH_3)	1.10 (s)
5	46.7 (CH)	1.79[a]	20	42.2 (CH)	1.76[a]
6	70.0 (CH)	4.05 (bs)	21	13.2 (CH_3)	1.06 (d, 7.1)
7α	39.1 (CH_2)	1.30 (td, 5.0, 12.0)	22	110.5 (C)	
β		1.96[a]	23*β*	32.4 (CH_2)	1.65[a]
8	29.3 (CH)	2.25[a]	α		1.65[a]
9	55.9 (CH)	1.42 (dt, 2.0, 7.5)	24α	28.2 (CH_2)	1.45[a]
10	37.0 (C)		*β*		1.60[a]
11*β*	37.2 (CH_2)	2.52 (t, 13.4)	25	31.2 (CH)	1.62[a]
α		2.16 (dd, 5.0, 13.4)	26α	67.1 (CH_2)	3.33 (t, 11.0)
12	213.5 (C)		*β*		3.49 (ddd, 1.5, 3.5, 11.0)
13	55.4 (C)		27	17.1 (CH_3)	0.80 (d, 6.9)
14	56.2 (CH)	1.45[a]			

[a] Overlapped with other signals.

The interpulse delays were adjusted for an average $^1J_{CH}$ of 130 Hz. Two- and three-bond heteronuclear $^1H-^{13}C$ connectivities were determined with 2D HMBC experiments (Griesinger et al., 1994), optimized for $^{2,3}J_{CH}$ of 8 Hz. Nuclear Overhauser effect (NOE) measurements were performed by 2D ROESY experiments.

Medium-pressure liquid chromatography (MPLC) was performed on a Büchi 861 apparatus using SiO_2 (230−400 mesh) and RP-18 columns. High-performance liquid chromatography (HPLC) in isocratic mode was performed on a Varian apparatus equipped with an RI-3 refractive index detector. Hibar LiChrospher SiO_2 columns were used.

Plant Material. Plants of the same variety of *A. porrum* L. were collected in the same place near Benevento (Italy) in different periods of the year: January 1997 (I), May 1997 (II), July 1997 (III), and September 1997 (IV); each sample was sown ~6 months before its collection. Reference specimens have been deposited at the Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli, Italy.

Extraction and Isolation. The plants were air-dried immediately after collection under controlled temperature (22 °C) and without exposure to 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. The $CHCl_3$/MeOH (9:1) extracts obtained from the four different collections were filtered and then concentrated in vacuo to afford four 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/H_2O (8:2) were rechromatographed by MPLC on SiO_2 columns using sequential mixtures of increasing polarity from $CHCl_3$ to $CHCl_3$/MeOH (8:2). Fractions were collected and analyzed by TLC on SiO_2. Fractions eluted with $CHCl_3$/MeOH (95:5) were purified by HPLC on a Hibar LiChrospher Si60 column with mobile phase $CHCl_3$/MeOH (95:5), to give crude sapogenins. The yield of the total sapogenin fraction for each collection amounts to an average value of 0.3 mg/g of dry bulbs. Crude sapogenins were further purified on HPLC [eluent of *n*-hexane/EtOAc (1:9)] affording the pure compounds. Sapogenin percentage composition of the four samples is reported in Table 2.

*Porrigenin C (**1a**)* was obtained as a colorless powder: yield, see Table 2; $[\alpha]^{25}_D = -20°$ (*c* 0.03, $CHCl_3$); ^{1}H and ^{13}C NMR spectra, see Table 1; HREIMS (65 eV) obsd *m*/*z* 460.2834, $C_{27}H_{40}O_6$ calcd *m*/*z* 460.2814.

Table 2. Sapogenin Percentage Composition of Four Samples[a] of *A. porrum* L.

sapogenin	I (%)	II (%)	III (%)	IV (%)
agigenin	10.0	32.0	26.3	26.5
β-chlorogenin	2.0	9.7	8.0	7.9
diosgenin			2.4	2.3
porrigenin A (**2**)	6.0	10.7	22.7	22.9
porrigenin B (**3**)	2.0	7.7	18.4	18.0
2,3-secoporrigenin (**4**)		2.5		
12-ketoporrigenin (**5**)		2.7	3.0	3.2
porrigenin C (**1**)			14.0	14.2
24-ethylcholesta-(6-acyl)-3-*O*-β-D-glucoside	80.0	34.7	5.2	5.0

[a] I, collected in January 1997; II, collected in May 1997; III, collected in July 1997; IV, collected in September 1997.

Neoporrigenin C (***1b***) was obtained as a colorless powder: $[\alpha]^{25}_D = -35°$ (*c* 0.001, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 4.36 (1H, bq, *J* = 7.5 Hz, H-16), 4.18 (1H, dd, *J* = 8.5, 11.2 Hz, H-3), 4.05 (1H, bs H-6), 3.90 (1H, ddd, *J* = 1.5, 4.0, 11.0 Hz, Hβ-26), 3.30 (1H, dd, *J* = 3.5, 11.0 Hz, Hα-26), 2.55 (1H, dt, *J* = 8.8, 7.3 Hz, H-17), 2.50 (1H, t, *J* = 13.4 Hz, Hα-11), 2.32 (1H, d, *J* = 13.0 Hz, Hβ-1), 2.27 (1H, overlapped, Hβ-4), 2.26 (1H, overlapped, H-8), 2.17 (1H, dd, *J* = 13.4, 5.0 Hz, Hβ-11), 2.12 (1H, m, Hβ-15), 2.05 (1H, d, *J* = 13.0 Hz, Hα-1), 1.97 (1H, overlapped, Hα-4), 1.95 (1H, overlapped, Hβ-7), 1.79 (1H, overlapped, H-5), 1.77 (1H, overlapped, H-20), 1.66 (2H, ovelapped, H_2-23), 1.64 (1H, overlapped, Hβ-24), 1.47 (1H, overlapped, H-15), 1.46 (1H, overlapped, Hα-24), 1.45 (1H, overlapped, H-25), 1.45 (1H, overlapped, H-14), 1.40 (1H, dt, *J* = 2.0, 7.5 Hz, H-9), 1.30 (1H, td, *J* = 5.0, 12.0 Hz, Hα-7), 1.15 (3H, d, *J* = 7.1 Hz, H_3-21), 1.10 (3H, s, H_3-19), 1.05 (3H, s, H_3-18), 1.00 (3H, d, *J* = 7.0 Hz, H_3-27); ^{13}C NMR ($CDCl_3$) δ 52.1 (t, C-1), 212.0 (s, C-2), 76.2 (d, C-3), 36.6 (t, C-4), 46.7 (d, C-5), 70.0 (d, C-6), 39.2 (t, C-7), 29.3 (d, C-8), 55.9 (d, C-9), 37.0 (s, C-10), 37.2 (t, C-11), 213.5 (s, C-12), 55.4 (s, C-13), 56.2 (d, C-14), 31.1 (t, C-15), 79.2 (d, C-16), 53.0 (d, C-17), 16.0 (q, C-18), 16.2 (q, C-19), 43.3 (d, C-20), 12.8 (q, C-21), 111.0 (s, C-22), 27.0 (t, C-23), 25.4 (t, C-24), 30.9 (d, C-25), 65.8 (t, C-26), 16.2 (q, C-27); HREIMS (65 eV) obsd *m*/*z* 460.2829, $C_{27}H_{40}O_6$ calcd *m*/*z* 460.2814.

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 units/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 (2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). IGR-1 cells (human melanoma cell line) were grown in adhesion on Petri dishes with Minimum Essential Medium Eagle (MEM) supplemented with 10% FBS, 25 mM HEPES, penicillin (100 units/mL), and streptomycin (100 μg/mL). P388 cells (murine leukemia cell line) was grown in adhesion on Petri dishes with L-15 (Leibovitz) medium supplemented with 10% FBS, 25 mM HEPES, penicillin (100 units/mL), and streptomycin (100 μg/mL). All reagents for cell culture were from Cellbio; MTT and 6-mercaptopurine were from Sigma.

Proliferation Assay. WEHI 164, J774, IGR-1, and P-388 (1×10^4 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, a 75 μL aliquot of 1.2 v/v serial dilution of test compound was added, and then the cells were incubated for 96 h. In some experiments 6-mercaptopurine (6-MP) was added. The cell's viability was assessed through a 3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2*H*-tetrazolium bromide (MTT) conversion assay (Mosman, 1983). Briefly, 25 μL of MTT (5 mg/mL) was added and the cells were incubated for an 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) SDS with an adjusted pH of 4.5 (Opipari et al., 1992). The optical density (OD) of each cell 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 compound **1a** and 6-MP was calculated as follows: % dead cells = 100 − (OD treated/OD control) × 100. The results are expressed as IC_{50} (the concentration that inhibited the cell growth by 50%). All of the measurements were repeated on triplicate samples; the data reported are the mean of them.

Chemicals. The reagents (Carlo Erba, Milan, Italy) were of analytical or HPLC grade, as required.

RESULTS AND DISCUSSION

Chromatographic separations of the four $CHCl_3$/MeOH (9:1) extracts of *A. porrum* L. yielded a series of pure sapogenins. The sapogenin compositions obtained for each collection are listed in Table 2, reported as percentage relative to the total sapogenin fraction.

The chemical structures of the known compounds agigenin (Carotenuto et al., 1997a), β-chlorogenin (Agrawal et al., 1985), diosgenin (Agrawal et al., 1985), porrigenin A (**2**) (Carotenuto et al., 1997a), porrigenin B (**3**) (Carotenuto et al., 1997a), 2,3-secoporrigenin (**4**) (Carotenuto et al., 1997b), 12-ketoporrigenin (**5**) (Carotenuto et al., 1997b), and 24-ethylcholesta-(6-acyl)-3-*O*-β-D-glucoside (Eichenberg, 1977) were assigned by analysis of their spectral data in comparison with those reported in the literature.

During the analysis of the third and fourth collections (July and September 1997) of *A. porrum* L., we isolated a new sapogenin, named porrigenin C (**1a**), along with minor amounts of its 25*S* epimer (**1b**).

The chemical study of porrigenin C (**1a**) started when the HREIMS spectrum was in our hands. It shows a molecular ion peak at *m*/*z* 460.2834 in accordance with the empirical formula $C_{27}H_{40}O_6$. The ^{13}C NMR and DEPT NMR spectra confirmed the presence of 27 carbon atoms (Table 1), including two quaternary sp^2 carbons (carbonyls at δ 212.0 and 213.5), and thus indicated the hexacyclic nature of the molecule, on the basis of the formal unsaturations implied by the molecular formula. The most striking feature of the proton NMR spectrum of **1a** (Figure 1, assignment shown in Table 1) is the presence of four methyl signals [two singlets at δ 1.10 and 1.05, and two doublets at δ 0.80 (*J* = 6.9 Hz) and 1.06 (*J* = 7.1 Hz)] and three methine (δ 4.05, 4.18 and 4.35) and one methylene (at δ 3.33 and 3.49) signal resonating in the region of the spectrum characteristic of protons on sp^3 carbons linked to heteroatoms. These data, together with the presence of a ketal carbon (singlet at δ 110.5) in the ^{13}C NMR spectrum, indicate a hydroxylated spirostane skeleton for porrigenin C (**1a**) (Carotenuto et al., 1997a).

A comparative analysis of ^1H and ^{13}C NMR spectra of **1a** with those of known porrigenins (Carotenuto et al., 1997a,b) suggested that **1a** possesses actually the same structure for the D−F rings, the differences being confined to the A−C rings, which must contain the two carbonyl functionalities. One of them was located at C-2 as indicated by the chemical shift and splitting pattern of ^1H NMR signal of H_2-1, which appears as an AB system resonating at δ 2.05 and 2.32, the signal at δ 2.05 being four-bond coupled (as evidenced by the 2D COSY) with the methyl signal at δ 1.10 (H_3-19), a typical feature of the axial proton at C-1 in steroidal compounds. In addition, the downfield chemical shift of the C-1 evident in the ^{13}C NMR spectrum of **1a** (δ 52.1),

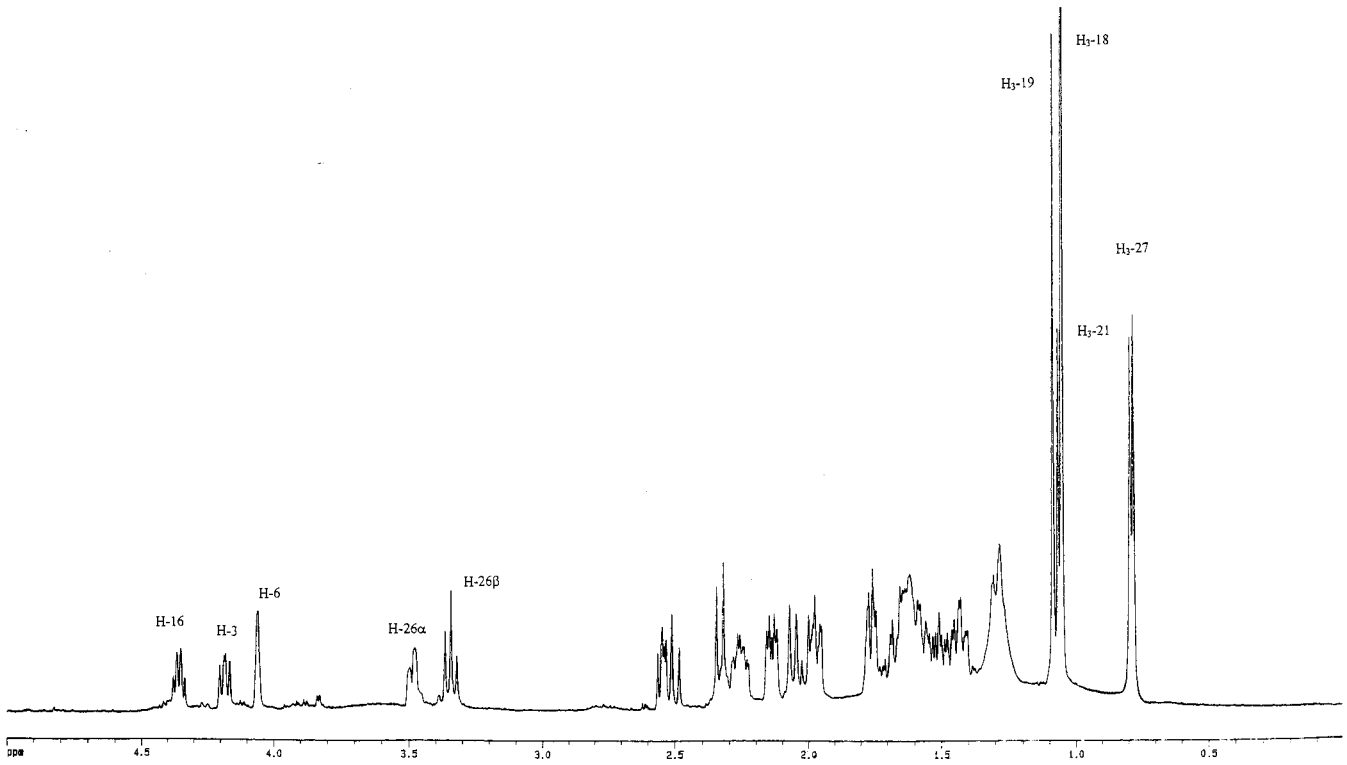

Figure 1. ^{1}H NMR (500 MHz) spectrum of porrigenin C (**1a**) in $CDCl_3$.

assigned by a 2D HMQC experiment, was in agreement with the carbonyl function located on the adjacent C-2. These data fit very well with those of porrigenin B (**3**), thus strengthening the proposed substructure. The chemical shift observed for H_3-18 at unusually low fields (δ 1.05 instead of the usual δ 0.85) suggested the location of the other ketone group at position 12. This was confirmed by the ^{1}H NMR data of H_2-11 (double doublet at δ 2.16 and triplet at δ 2.52) and by ^{13}C NMR resonances of C-11 (δ 37.2) and C-13 (δ 55.4), which are similar to the values found in 12-ketoporrigenin (**5**) (Carotenuto et al., 1997b). Correlation peaks observed in the HMBC experiment between C-12 and H_2-11 and between C-12 and H-9 fully confirmed the proposed structure for ring C.

Using as a starting point the signal at δ 4.18 (H-3), accurate analysis of 2D COSY and HOHAHA spectra allowed us the complete assignment of all the proton resonances of the A−C rings. In particular, the signal at δ 4.05 was attributed to H-6, thus determining the position of the remaining hydroxyl group; its β orientation was deduced by the small coupling constants with H-5 and H-7. The resonances of all the carbons in the ^{13}C NMR spectrum were assigned as reported in Table 1 by accurate inspection of 2D HMQC spectrum, which allowed us to correlate all of the proton signals to the relevant carbon atoms, whereas the assignment of quaternary carbons was based on the interpretation of the HMBC spectrum.

Intense NOE contacts, evidenced by a ROESY spectrum, of H-1α and H-3, H-1α and H-5, and H-9 and H-5 established the β orientation of the hydroxyl group linked to C-3 and the α orientation of H-3, H-5, and H-9, defining the trans junction of the A and B rings, thus fully determining the chemical structure of compound **1a**.

Neoporrigenin C (**1b**) displayed in the HREIMS a molecular peak at *m/z* 460.2829 in accordance with the empirical formula $C_{27}H_{40}O_6$. Comparison of the ^{1}H and ^{13}C NMR values of **1b** (reported under Materials and Methods) with those of **1a** (Table 1) suggested that the differences must be due to an isomeric relationship between them; in fact, the most significant differences were found in the ^{1}H and ^{13}C resonances of the F ring atoms. In particular, the upfield shifts of all the F ring carbon atoms in **1b** is characteristic of an inversion at the chiral center C-25 (Agrawal et al., 1985). Among these values, the C-23 resonance exhibited a dramatic shielding of 5.4 ppm (δ 27.0 in **1b** versus δ 32.4 in **1a**) due to the existence of γ-gauche interactions. From these data neoporrigenin C (**1b**) could be identified as the 25*S* epimer of porrigenin C (**1a**).

Compound **1a** was tested for its antiproliferative activity on four tumor cell lines in vitro. It inhibited the growth of all cell lines, evaluated at 96 h, at the following concentrations expressed as IC_{50}: J-774 (murine monocyte/macrophage), 35 ± 5 μg/mL; WEHI-164 (murine fibrosarcoma), 120 ± 12 μg/mL; P-388 (murine leukemia), 20 ± 3 μg/mL; IGR-1 (human melanoma), 60 ± 9 μg/mL. Values are expressed as mean ± SEM of three separate experiments in triplicate. These results indicate that porrigenin C exhibits a higher activity on all cell lines compared to 12-ketoporrigenin (Carotenuto et al., 1997b), whereas the antiproliferative activity is similar to that exhibited by porrigenin B (**3**) (Carotenuto et al., 1997a), with the exception of P-388 cell line against which compound **1a** is considerably more active (IC_{50} = 20 ± 3 versus 74 ± 22). Comparison of these preliminary pharmacological results led to the hypothesis that the carbonyl functionality in position 12 is not so important for activity as that in position 3.

CONCLUSION

The sapogenin content of four samples of the same variety of leek, *A. porrum* L., grown at different times,

has been explored. This investigation led to preliminary results showing a variability in the sapogenin composition which seems to parallel that observed in the thiosulfinate composition depending on the climate previously reported in *Allium* species (Block et al., 1992). Our results appear of interest because the isolated sapogenins constitute a new class of *Allium* bioactive compounds that are relatively stable within the food during and after processing.

Concerning the spirostanol-type sapogenins, the novel antiproliferative porrigenin C (**1a**) has been isolated together with small quantities of its 25*S* isomer (**1b**) in the third and fourth collections. In all of the samples examined, agigenin is the most abundant compound of this type, followed by porrigenin A and porrigenin B. It is worthy of note that the spirostanol sapogenin content is least in sample I (collected January 1997), in which on the contrary, 24-ethylcholesta-(6-acyl)-3-*O*-*β*-D-glucoside represents the major compound. In spring (collection II) its quantity decreases, reaching minimum values in summer collections (III and IV), when its amount is smaller than that of almost all the spirostanol sapogenins. The acylsterylglycosides have been previously reported for their growth-promoting effects (Kojima et al., 1989).

The data here presented are only preliminary results. To best define the changes in the sapogenin ratios of *A. porrum* L., a more extensive study will be conducted using well-identified cultivation and correlating the sapogenin variations with growing conditions and other controlled variables.

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