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Structural characterization of phytotoxic terpenoids from *Cestrum parqui*

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Abstract

Isolation, chemical characterization and phytotoxicity of nine polyhydroxylated terpenes (five C_{13} *nor*-isoprenoids, two sesquiterpenes, a spirostane and a pseudosapogenin) from *Cestrum parqui* L'Herr are reported. In this work we completed the phytochemical investigation of the terpenic fraction of the plant and described the structural elucidation of polar isoprenoids using NMR methods. All the configurations of the compounds have been assigned by NOESY experiments. Four new structures have been identified as (3*S*,5*R*,6*R*,7*E*,9*R*)-5,6,9-trihydroxy-3-isopropyloxy-7-megastigmene, 5α-spirostan-3β,12β,15α-triol, and 26-*O*-(3′-isopentanoyl)-β-D-glucopyranosyl-5α-furost-20(22)-ene-3β,26-diol, and as an unusual tricyclic sesquiterpene.

The compounds have been assayed for their phytotoxicity on lettuce at the concentrations ranging between 10^{-4} and 10^{-7} M. The activities of some compounds were similar to that of the herbicide pendimethalin.

Keywords: *Cestrum parqui* L'Herr; Solanaceae; Phytotoxicity; Spectroscopic analysis; Terpenoids; Spirostane; Pseudosapogenin

1. Introduction

To ensure the survival in the ecosystem, plants produce secondary metabolites with original chemical or biological features. If these compounds, introduced in the environment, interfere with the development of other vegetal organisms, they are called allelochemicals. Among the secondary metabolites, terpenoids show a wide spectrum of biological activities including potential allelopathy (Calera et al., 1995; Skaltsa et al., 2000; Cangiano et al., 2002; Macías et al., 2002).

In the search for bioactive natural products from Mediterranean spontaneous plants and weeds (DellaGreca et al., 2003, 2005), we investigated *Cestrum parqui*. This plant, commonly named green cestrum, has been introduced from South America for use as an ornamental shrub in gardens. It is now naturalised and widely distributed in the Mediterranean area as one of the major weeds. It grows in dense masses, crowding out other species and it is noted for its extreme toxicity to farm animals.

The phytochemical study of the leaves of the plant has already afforded the isolation of 12 C_{13} *nor*-isoprenoids, identified by spectroscopic means and chemical correlations. These compounds showed phytotoxic effect on the germination and growth of *Lactuca sativa* L. (D'Abrosca et al., 2004a,b).

In this study we have completed the phytochemical investigation of the terpenic fraction of the plants and described the isolation, characterization and the phytotoxicity of more polar novel isoprenoids.

2. Results and discussion

The hydroalcoholic extract of the leaves of *C. parqui* was concentrated and performed in a separator funnel, first

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with CH_2Cl_2 and then with EtOAc. The chemical study of the first extract led to the isolation of the 12 C_{13} *nor*-isoprenoids (D'Abrosca et al., 2004a,b). Phytochemical investigation of the EtOAc extract led to the identification of nine terpenoids. The use of NMR experiments (COSY, TOCSY, HSQC, HMBC, NOESY, ROESY) and mass spectrometry techniques led to identification of four new compounds **3**, **7**, **8**, and **9** and five known compounds: the C_{13} *nor*-isoprenoids **1** (Macias et al., 2004), and **2** (Calis et al., 2002), the glucosides **4**, and **5** (Takeda et al., 1997) and the sesquiterpene **6** (Jakupovic et al., 1988).

Compound **3** (Fig. 1) showed a molecular formula $C_{16}H_{30}O_4$ as suggested by spectral data and elemental analysis. The ^1H NMR revealed the presence of two olefin protons, as a doublet at δ 6.16 and a double doublet at δ 5.78, three multiplets at δ 4.34, 4.20 and 4.18; four diastereotopic protons of two methylenes as two double doublets were also present, at δ 1.81/1.59 and 2.04/1.70, both correlated in a COSY experiment with the methine at δ 4.18. Furthermore, we identified six methyl: three singlets at δ 0.91, 1.11 and 1.22, a doublet at δ 1.26, correlated with the methine at δ 4.34 in the COSY spectrum, and two coincident doublets at δ 1.35, showing cross peak with the methine at δ 4.20. The ^{13}C NMR showed 15 signals in accordance with C_{13} *nor*-isoprenoids with an ethereal isopropyl group. In fact, besides the methine carbinol already mentioned, there were two trisubstituted carbinols at δ 77.8 and 69.4. These data were in accordance with a 3,5,6,9-tetrahydroxy-7-megastigmene. In fact, the methyls at δ 1.35 were both correlated, in the HMBC experiment, with the carbon at δ 68.2, the proton at δ 4.18, assigned to the H-3 proton, was heterocorrelated with the carbon at δ 69.9 and with the methyls at δ 21.6. The NOESY experiment showed a NOE between the H-3 proton and the H-13 and H-12 methyls, indicating its α orientation. The NMR data of compound **3** were in accordance with those reported for (3*S*,5*R*,6*R*,7*E*,9*R*)-3,5,6,9-tetrahydroxy-7-megastigmene, suggesting an *R* configuration for the C-5 and C-6 chiral carbons. The absolute configuration to the C-9 carbon has been confirmed by Mosher's method (D'Abrosca et al., 2004a,b).

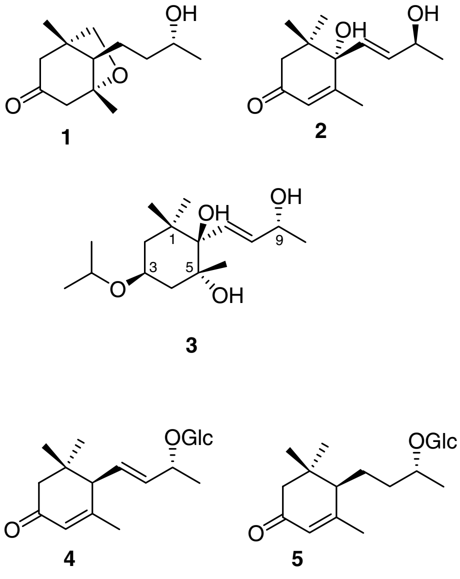
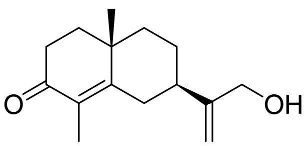


Fig. 1. Polar C_{13} *nor*-isoprenoids isolated from *C. parqui*.

Compound **7** (Fig. 2) had an unusual sesquiterpenic skeleton. It had a molecular formula $C_{15}H_{22}O_2$ indicating the presence of five unsaturations in the molecule.

The ^{13}C NMR (Table 1) showed 15 carbon signals identified, on the basis of a DEPT experiment, as three methyls, four methylenes, four methines, and four tetrasubstituted carbons, whose values allowed us to identify them as a carbonyl (δ 200.5), an olefinic carbon (δ 160.1), a carbinol carbon (δ 76.1) and an aliphatic sp^3 carbon at δ 40.1. The ^1H NMR data (Table 1), together with those derived from an HSQC experiment, showed a singlet olefinic proton at δ 5.82 bonded to the carbon at δ 122.2, a methylene as singlet at δ 2.45, correlated with the carbon at δ 50.0, and two diasterotopic protons at δ 2.43 and 2.29 both correlated with the carbon at δ 50.2. In the region ranging from 2.05 to 1.00 ppm a doublet methine at δ 1.72 (bonded to the carbon at δ 57.9), two singlet methyls at δ 1.32, and 1.15, correlated with the carbons at δ 28.0 and 28.4, respectively, and a doublet methyl at δ 1.03 ($J = 6.8$) bonded to the carbon at δ 12.2 were identifiable. The ^1H–^1H COSY and TOCSY experiments showed the following correlations: the doublet methyl correlated with the methine at δ 1.40, which showed cross peak with the methine at δ 1.90. This proton was correlated with the doublet methine at δ 1.72 and with the methylene protons at δ 1.48 and 1.98, bonded to the carbon at δ 30.4, which were both correlated with the methylene protons at δ 1.64 and 1.73, bonded to the carbon at δ 39.9. The HMBC experiment furnished useful data for solving the structure (Table 1). In fact, the carbonyl was correlated with both the methylene protons at δ 2.29 and 2.43 which in turn were correlated with the carbons at δ 39.9, 40.1 and 57.9. This last heterocorrelated with the methylene protons at δ 1.48 and 1.98, with the methines at δ 1.40 and 1.90, and with the methylene at δ 2.45, and with the methyl at δ 1.15. Finally, the latter methylene protons showed interactions with the carbinol and both the olefinic carbons. These data were in accordance with an unusual tricyclic structure of a sesquiterpene as shown in Fig. 2. The coupling constant of the H-10 methine (10.7 Hz) indicated a *trans* orientation with the H-9 proton. The relative configurations at the chiral carbons have been determined by a NOESY experiment (Fig. 3). In the Figs. 2 and 3 compound **7** has been drawn as one of its enantiomer. The H-14 methyl give NOE with the H-15 methyl, the H-8 methine and with one of the H-6 protons, which was also correlated with the H-10 proton. The H-15 methyl showed correlations with the H-9 and the H-11 proton at δ 1.48. Finally, the H-13 methyl gave NOE with the H-10 methine and the H-2 proton at δ 2.43.

Compound **8** (Fig. 2) has been identified as 5α-spirostane-3β,12β,15α-triol. Its molecular formula was $C_{27}H_{44}O_5$ as suggested by the EI-MS spectrum, showing



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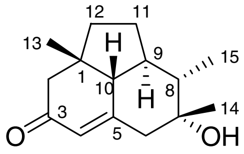

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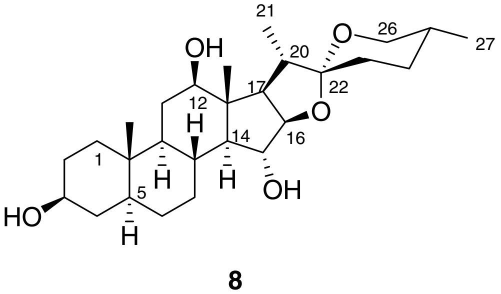

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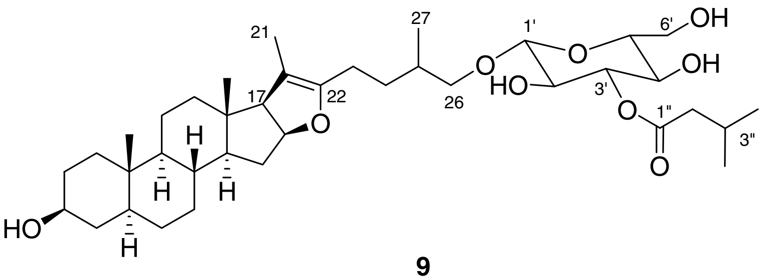

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Fig. 2. Sesquiterpenes and spirostanes isolated from *C. parqui*.

Table 1
NMR data of compound **7** in $CDCl_3$

Position	δ_C	DEPT	δ_H	HMBC (C → H)
1	40.1	C	–	H2α, H2β, H13
2	50.2	CH_2	2.29 *d* (15.6 Hz) 2.43 *d* (15.6 Hz)	H11
3	200.5	C	–	H2α, H2β
4	122.2	CH	5.82 s	H2β, H6, H10
5	160.1	C	–	H6, H10
6	50.0	CH_2	2.45 *s*	H14
7	76.1	C	–	H6, H14, H15
8	46.6	CH	1.40 *dq* (6.8, 9.8 Hz)	H6, H14, H15
9	48.8	CH	1.90 *m*	H10, H15
10	57.9	CH	1.72 *d* (10.7 Hz)	H2α, H2β, H6, H8, H9, H11,H13
11	30.4	CH_2	1.48 *m* 1.98 *m*	H8
12	39.9	CH_2	1.64 *m* 1.73 *m*	H2α, H2β, H10, H11, H13
13	28.4	CH_3	1.15 *s*	H2α, H2β, H12
14	28.0	CH_3	1.32 *s*	H6, H7, H8
15	12.2	CH_3	1.03 *d* (6.8 Hz)	H8, H9

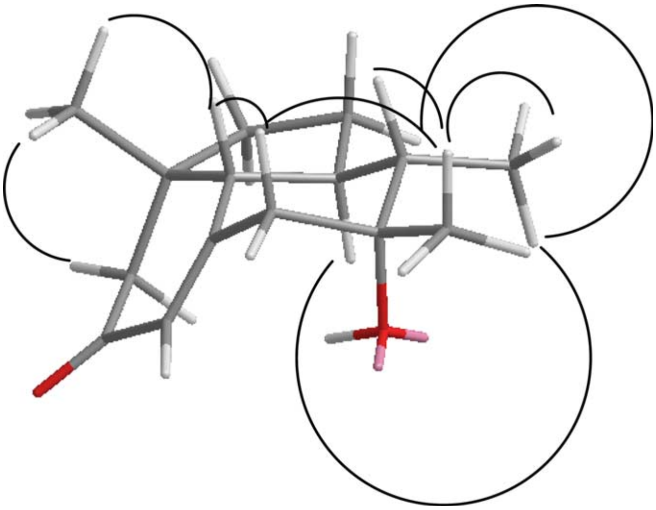

Fig. 3. Selected NOE observed in the NOESY experiment of sesquiterpene **7**.

the molecular peak at *m*/*z* 448 and by the elemental analysis.

The ^1H NMR spectrum showed six protons geminal to an oxygenated function, four as a double doublets at δ 4.34, 4.12, 3.52, and 3.28, a multiplet at δ 3.59, and a triplet at δ 3.39. In the upfield region, two singlet methyls at δ 0.99 and 0.86 and two doublet methyls at δ 1.02 and 0.81 were evident. The ^{13}C NMR showed five carbinol signals at δ 82.2, 80,5, 71.2, 69.7, and at δ 67.3, apart from an acetal carbon at δ 110.3. In the HMBC spectrum, this signal showed correlations, with the protons at δ 1.88 (H-20), and the methylene at δ 3.52 and 3.39. The ^{1}H–^{1}H COSY and TOCSY experiments showed correlations between the doublet methyl at δ 1.02 and the methine at δ 1.88. This latter correlated with the methine at δ 2.12, which showed cross peak with the proton at δ 4.34. Finally, this latter proton was correlated with the proton at δ 4.12. These data were in accordance with a 3β-hydroxyspirostane skeleton with an hydroxyl group at the H-15 carbon. The correlations, in the HMBC experiment, between the proton at δ 3.28 with the methyl at δ 12.7, bonded to protons at δ 0.99, and between this methyl with the carbinol at δ 80.5 localized another hydroxyl at the C-12 carbon. The comparison of ^{13}C NMR with those present in the literature (Wawer et al., 2001), allowed us to define an α configuration for the H-5, H-17 and for the methyls H-21 and H-27 and a *R* configuration for the C-22 carbon. The stereochemistry of C-12 and C-15 carbons was defined by a NOESY experiment. In fact, the H-18 methyl showed a NOE effect with the H-15 proton, justifying an α orientation for the hydroxyl group, while the proton H-12 at δ 3.28 which showed a NOE effect with the 17α proton justifying a β orientation for the hydroxyl group on the C-12 carbon.

Compound **8** has been described for the first time, although spirostanol glycosides have been already isolated from *C. parqui* (Baqui et al., 2001).

Compound **9** was identified as the pseudosapogenin glucoside 26-*O*-(3-isopentanoyl)-β-D-glucopyranosyl-5α-furost-20(22)-ene-3β,26-diol. It had a molecular formula $C_{38}H_{62}O_9$ as suggested by the ^{13}C NMR and the MALDI-MS experiments, which showed the pseudomolecular ion at *m/z* 663 $[M+1]^+$.

The ^{1}H NMR spectrum showed 11 protons geminal to oxygen. The presence of a doublet at δ 4.34 and a methylene at δ 3.92 and 3.81 suggested a monosaccharide moiety in the molecule. A multiplet at δ 4.70, another methylene as two double doublets at δ 3.77 and 3.35, and a multiplet at δ 3.57 were also evident. The ^{1}H–^{1}H COSY and TOCSY experiments confirmed this hypothesis, showing correlations between the anomeric proton at δ 4.34 and a double doublet at δ 3.49 (H-2′), which gave cross peaks with an downshifted double doublet at δ 4.91. This latter showed correlations with a multiplet at δ 3.41, which gave cross peak with the diasterotopic methylene protons. A multiplet at δ 4.70, another methylene as two double doublets at δ 3.77 and 3.35, and a multiplet at δ 3.59 were also evident. In the upfield region three singlet methyls at δ 0.64, 0.81 and 1.56 and three doublet methyls δ 0.99 (2×) and 0.91 were identifiable. A signal was due to a methyl at δ 0.91 and another due to two coincident methyls at δ 0.99. The ^{13}C NMR revealed signals due to 38 carbons, which were identified, on the basis of a DEPT experiment, as six methyls, 13 methylenes, 14 methines and five tetrasubstituted carbons. The HSQC experiment allowed the attribution of the protons to the corresponding carbons.

The H-3′ proton of the sugar moiety was heterocorrelated, in a HMBC experiment, with a carboxyl carbon at δ 175.1, which showed correlations with a doublet methylene at δ 2.28 and a methine at δ 2.21, bonded to the carbons at δ 43.5 and 25.9, respectively. These last showed interactions with two coincident methyls at δ 0.99, bonded to the carbon at δ 22.3. These data suggested the presence of an isopentanoic acid esterificated to the hydroxyl group bonded at C-3′ carbon of the sugar. The data were supported by the MALDI-MS experiment that showed the fragmentation peak at *m/z* 417, due to loss of 3′-isopentanoyl-β-D-glucopyranosyl group from the molecule. The comparison of the remaining carbon signals with those of compound **8** and with other related compounds (Tobari et al., 2000), suggested a pseudotigogenin structure for the aglicone. The HMBC experiment showed correlation between the H-27 methyl at δ 0.91 with the C-24, C-25 and C-26 carbons at δ 30.6, 34.9, and 75.3, respectively. Correlations between the H-17 methine at δ 2.44 and the C-16, C-18, C-20, C-22 carbons were also evident. Furthermore, the correlation between the anomeric proton and the C-26 carbon suggested the presence of the pseudotigogenin linkage of the 3′-isopentanoyl-β-D-glucopyranosyl moiety at the C-26 hydroxyl group of the aglicone. The NMR data suggested the presence of glucose in the molecule. This hypothesis was confirmed by the GC comparison of the alditole acetate, obtained from hydrolyzed compound **9**, with an authentic sample of acetylated glucitol.

In order to evaluate the potential phytotoxicity, we have studied the effects of aqueous solutions of the isolated compounds ranging from 10^{-4} to 10^{-7} M on germination, root and shoot elongation of *L. sativa* L. (lettuce). The results of the bioassays are reported in Fig. 4.

With the exception of compound **2**, no significant effects were observed on lettuce germination. The C_{13} *nor*-isoprenoids **1**, **4**, and **5** had a similar activity, showing a moderate phytotoxicity on both root (Fig. 4B) and shoot elongations (Fig. 4C). On the contrary, compound **2** showed a good activity on the germination (Fig. 4A), root and shoot elongation at all the tested concentrations. The aglicones of glucosides **4** and **5** have been tested on *L. sativa* (D'Abrosca et al., 2004a,b) showed no significant effects: the values included between ±10% from control. The presence of the sugar moiety enhances the phytotoxicity on the plant development by inhibiting, in particular, the shoot elongation. In fact, their values achieved about 40% inhibition at the higher concentrations. Sesquiterpene **6** was inactive, while the spirostane **8** was the most active compound inhibiting, the root and shoot elongations at 10^{-4} M, about 60%. At this concentration, slight effects of over 30% were observed on the seed germination too.

The most phytotoxic compounds **1**, **2** and **8**, have been compared with the Pendimethalin, a commercial postemergency herbicide for their effects on seedling growth (Fig. 5). Literature data reported that this herbicide acts as an inhibitor for cell division and elongation (Hess and Bayer, 1977; Richard and Hussey, 1999). Furthermore,

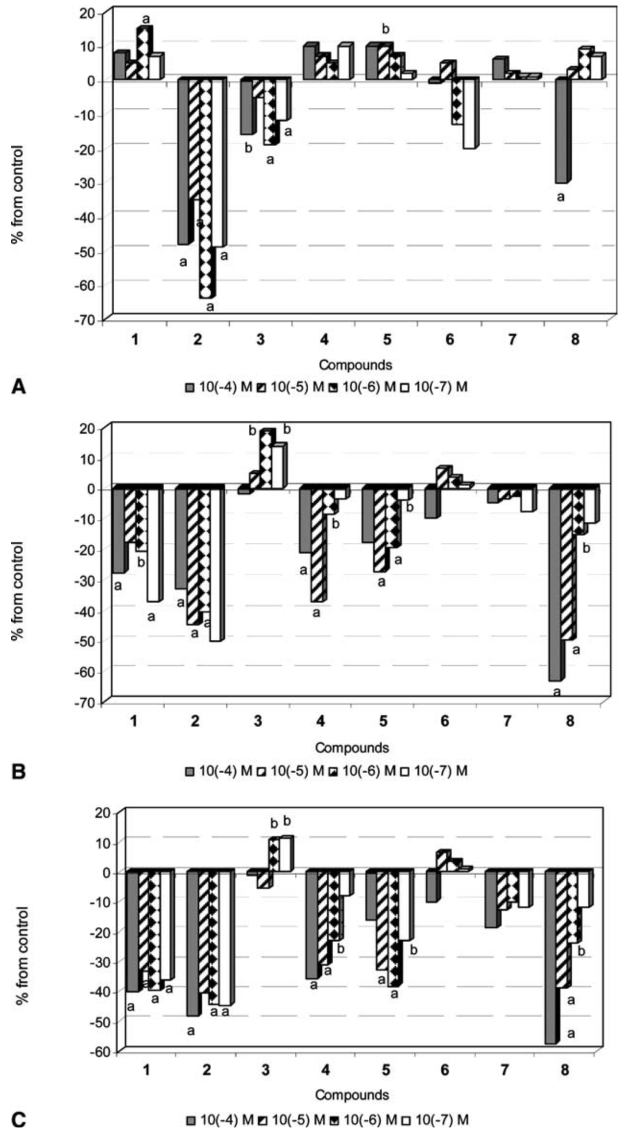

Fig. 4. Effect of terpenoids from *C. parqui* on germination (A), root length (B) and shoot length (C) of *Lactuca sativa* L. Value presented as percentage differences from control and are not significantly different with $P > 0.05$ for Student's *t*-test. (a) $P < 0.01$; (b) $0.01 < P < 0.05$.

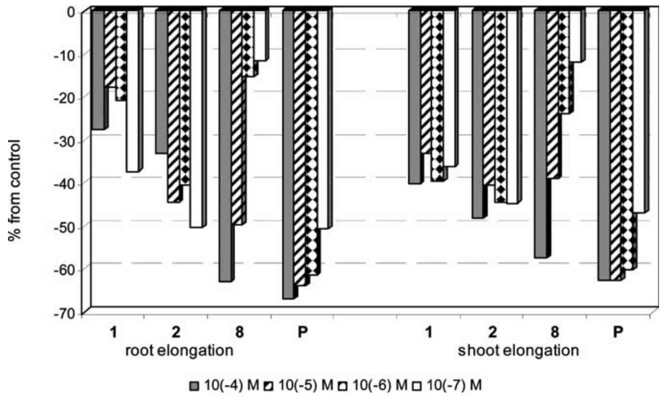

Fig. 5. Comparison of phytotoxicity of compounds **1**, **2** and **8** with the post-emergency herbicide Pendimethalin (**P**) on *Lactuca sativa* L. Value presented as percentage differences from control.

studies on the alga *Protosiphom botryoides* indicated that growth rate, cell number, chlorophyl level and dry weight decrease with increasing Pendimethalin concentration (Shabana et al., 2001).

All the compounds showed a toxic effect similar to herbicide, especially at the lowest concentration for the terpene **2**.

In conclusion the terpenes isolated from *C. parqui*, may, play a role in the phytotoxicity of the extract on the lettuce (D'Abrosca et al., 2004a,b). The less polar ones seem to have no relevant effects. The introduction of polar group **1**–**3** or sugar moiety (**4**, **5**) improve their phytotoxicity, probably for the enhanced solubility in aqueous solution. Also spirostanol **8** was active, especially at the higher concentrations used in the bioassays. This compound showed a good correspondence between concentration and effect for all the measured parameters.

3. Experimental

3.1. General experiment procedures

NMR spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a Varian 500 spectrometer Fourier transform NMR, in $CDCl_3$ or CD_3OD solutions at 25 °C. Proton-detected heteronuclear correlations were measured using HMQC (optimised for $^1J_{HC} = 145$ Hz) and HMBC (optimised for $^nJ_{HC} = 8$ Hz). Optical rotations were measured on a Perkin–Elmer 343 polarimeter. IR spectra were determined in $CHCl_3$ solutions on a FT-IR Perkin–Elmer 1740 spectrometer. Electronic ionization mass spectra (EI-MS) were obtained with a HP 6890 instrument equipped with a MS 5973 N detector. Matrix assisted laser desorption ionization (MALDI) mass spectra were recorded using a Voyager-DE MALDI-TOF mass spectrometer. The HPLC apparatus consisted of a pump (Shimadzu LC-10AD), a refractive index detector (Shimadzu RID-10A) and a Shimadzu Chromatopac C-R6A recorder. Preparative HPLC was performed using RP-8 (Luna 10 μm, 250 × 10 mm i.d., Phenomenex), RP-18 (Luna 10 μm, 250 × 10 mm i.d., Phenomenex) or SiO_2 (Maxsil 10 silica, 10 μm, 250 × 10 mm i.d., Phenomenex), columns. Analytical TLC was performed on Merk Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm layer thickness. Spots were visualized by UV light or by spraying with H_2SO_4–AcOH–H_2O (1:20:4). The plates were then heated for 5 min at 120 °C. Prep. TLC was performed on Merck Kieselgel 60 F_{254} plates, with 0.5 or 1 mm film thickness. Flash column chromatography (FCC) was performed on Merck Kieselgel 60 (230–400 mesh) at medium pressure. Column chromatography (CC) was performed on Merck Kieselgel 60 (70–240 mesh), Baker Bond Phase C18 (0.040–0.063 mm), Fluka Reversed phase silica gel 100 C8 (0.040–0.063 mm) or on Sephadex LH-20® (Pharmacia).

3.2. Plant material

Plants of *C. parqui* were collected, in the vegetative state, in Sant'Agata de' Goti, near Caserta (Italy), and identified by Dr. Assunta Esposito of the Second University of Naples. A voucher specimen (CE125) has been deposited at the Herbarium of the Dipartimento di Scienze della Vita of Second University of Naples.

3.3. Extraction and isolation

Fresh leaves of *C. parqui* (30 kg) were frozen at −80 °C, powdered and extracted with MeOH–H_2O (1:9) for 48 h at 10 °C. The hydroalcoholic solution, after the evaporation of the MeOH, was extracted in a separator funnel first using CH_2Cl_2 and then with EtOAc. Both the organic fractions were dried with Na_2SO_4 and concentrated under vacuum, yielding 8.0 and 9.2 g of crude residue respectively. Both the organic extracts have been stored at −80 °C until purification.

3.3.1. CH_2Cl_2 extract fractionation

The CH_2Cl_2 extract was chromatographed on silica gel, with $CHCl_3$ and EtOAc solutions, to give four fractions A–E.

Fraction A, eluted with $CHCl_3$, was rechromatographed on RP-18 silica eluting with MeOH–MeCN–H_2O (2:1:1) and collecting fractions of 10 mL. The fractions 14–24 were purified on TLC eluting with $CHCl_3$–Me_2CO (17:3) to have pure **7** (2 mg). Fraction B, eluted with EtOAc–$CHCl_3$ (1:19) was rechomatographed on Sephadex LH-20 eluting with hexane–$CHCl_3$–MeOH (3:1:1) and collecting fractions of 5 ml. The fractions 21–34 were purified by HPLC on RP-18 semipreparative column eluting with MeOH–MeCN–H_2O (2:1:2) to obtain pure **1** (2 mg) and **6** (5 mg). Fraction C, eluted with EtOAc–$CHCl_3$ (1:9), was purified on RP-18 silica gel, eluting with MeOH–MeCN (3:2) to have pure **8** (7 mg). Fraction D, eluted with EtOAc–$CHCl_3$ (1:1), was rechromatographed by FCC on SiO_2, eluting with the lower phase of the biphasic solution constituted by $CHCl_3$–MeOH–H_2O (13:7:6). We obtained a crude fraction which was purified by HPLC on RP-18 column, eluting with MeOH–MeCN–H_2O (3:1:1) to have pure **2** (2 mg). Fraction E, eluted with EtOAc, was rechromatographed by CC on RP-18 eluting with MeOH–MeCN–H_2O (2:1:2) to obtain a fraction which was purified by TLC, eluting with the lower phase of the biphasic solution obtained by $CHCl_3$–MeOH–H_2O (13:7:6), to give pure **9** (8 mg).

3.3.2. EtOAc extract fractionation

The EtOAC extract was chromatographed on silica gel, with $CHCl_3$ and Me_2CO solutions, to give two fractions F–G.

Fraction F, eluted with Me_2CO–$CHCl_3$ (1:1), was chromatographed on Sephadex LH-20, eluting with EtOH–H_2O (3:1) to have a crude fraction which was purified by TLC, eluting with the lower phase of the biphasic solution obtained by $CHCl_3$–MeOH–H_2O (13:7:6), to obtain pure **3** (8 mg). Fraction G, eluted with Me_2CO–$CHCl_3$ (3:2), was chromatographed on Sephadex LH-20, eluting with EtOH–H_2O (1:1) to have a crude fraction which was purified by TLC, eluting with the upper phase of the biphasic solution obtained by EtOAc–MeOH–H_2O (79:10:11), to have pure **4** (5 mg) and **5** (11 mg).

3.3.3. Compound characterization

*3.3.3.1. (3S,5R,6R,7E,9R)-5,6,9-Trihydroxy-3-isopropyloxy-7-megastigmene (**3**).* Colourless oil; $[\alpha]_D^{25}$ −11.7° (MeOH, c 0.02); ^1H NMR (500 MHz, CD_3OD): δ 6.16 (1H, *d*, $J = 15.6$ Hz, H-7), 5.78 (1H, *dd*, $J = 15.6$ and 6.3 Hz, H-8), 4.34 (1H, *m*, H-9), 4.20 (1H, *m*, *i*-PrO), 4.18 (1H, *m*, H-3), 2.04 (1H, *dd*, $J = 14.1$ and 3.3 Hz, $H4_{eq}$), 1.81 (1H, *dd*, $J = 14.1$ and 3.3 Hz, $H2_{ax}$), 1.70 (1H, *ddd*, $J = 14.1$, 4.5 and 1.8 Hz, H-4_{ax}), 1.59 (1H, *dd*, $J = 14.1$ and 4.8 Hz, H-2_{eq}), 1.35 (6H, *d*, $J = 6.6$ Hz, *i*-PrO), 1.26 (3H, *d*, $J = 6.3$ Hz, H-10), 1.22 (3H, *s*, H-13), 1.11 (3H, *s*, H-12), 0.91 (3H, *s*, H-11). ^{13}C NMR (125 MHz, CD_3OD): δ 135.4 (C-8), 131.4 (C-7), 77.8 (C-6), 69.9 (*i*-PrO), 69.4 (C-5), 69.1 (C-9), 68.2 (C-3), 47.6 (C-2), 44.2 (C-4), 38.7 (C-1), 28.9 (*i*-PrO), 27.8 (C-11), 26.0 (C-12), 24.0 (C-13), 21.6 (C-10). EI-MS: *m*/*z* 286 $[M]^+$, 271 $[M - Me]^+$, 268 $[M - H_2O]^+$; elemental analysis – found: C, 67.32; H, 10.65. Calc. for $C_{16}H_{30}O_4$: C, 67.10; H, 10.56%.

*3.3.3.2. 1,2,2a,3,6,7,8,8a-Octahydro-7-hydroxy-2a,7,8-trimethylacenaphthylen-4(4H)-one (**7**).* In describing this compound we do not use the IUPAC numeration but we have adopted that reported in Fig. 2. Colourless oil; $[\alpha]_D^{25}$ +27.1° ($CHCl_3$, c 0.02); UV λ^{MeOH} nm (logε): 244 (3.7); IR ν^{CHCl_3} cm^{-1}: 3634, 2926, 1663; ^1H NMR: see Table 1; ^{13}C NMR: see Table 1; EI-MS: *m*/*z* 234 $[M]^+$, 216 $[M - H_2O]^+$; elemental analysis – found: C, 76.44; H, 9.63. Calc. for $C_{15}H_{22}O_2$: C, 76.88; H, 9.46%.

*3.3.3.3. 5α-Spirostan-3β,12β,15α-triol (**8**).* White powder; $[\alpha]_D^{25}$ −19.3° (CH_2Cl_2, c 0.07); IR ν^{CHCl_3} cm^{-1}: 3675, 2926; ^1H NMR: (500 MHz, $CDCl_3$): 4.34 (1H, *dd*, $J = 9.3$ and 5.4 Hz, H-16), 4.12 (1H, *dd*, $J = 5.4$ and 3.6 Hz, H-15), 3.59 (1H, *m*, H-3), 3.52 (2H, *dd*, $J = 11.1$ and 4.5 Hz, H-26), 3.39 (1H, *t*, $J = 11.1$ Hz, H-26), 3.28 (1H, *dd*, $J = 9.3$ and 5.4 Hz, H-12), 2.12 (1H, *t*, $J = 5.4$ Hz, H-17), 1.88 (1H, overlapped, H-20), 1.02 (3H, *d*, $J = 6.9$ Hz, H-21), 0.99 (3H, *s*, H-18), 0.86 (3H, *s*, H-19), 0.81 (3H, *d*, $J = 6.3$ Hz, H-27); ^{13}C NMR: see Table 2; EI-MS: *m*/*z* 448 $[M]^+$, 433 $[M - Me]^+$, 430 $[M - H_2O]^+$; elemental analysis – found: C, 72.46; H, 9.24. Calc. for $C_{27}H_{44}O_5$: C, 72.28; H, 9.89%.

*3.3.3.4. 26-O-(3′-Isopentanoyl)-β-D-glucopyranosyl-5α-furost-20(22)-ene-3β,26-diol (**9**).* White powder; IR ν^{CHCl_3} cm^{-1}: 3682, 2923, 1717; ^1H NMR: (500 MHz, CD_3OD): 4.91 (1H, *dd*, $J = 9.3$ and 8.7 Hz, H-3′), 4.70 (1H, *m*, H-16), 4.34 (1H, *d*, $J = 7.8$ Hz, H-1′), 3.92 (1H, *dd*, $J = 12.0$ and 3.6 Hz, H-6′), 3.81 (1H, *dd*, $J = 12.0$ and 5.1 Hz,

Table 2
^{13}C NMR data of spirostane **8** and glucoside **9** in $CDCl_3$

Position	**8**		**9**	
	DEPT	δ_C	DEPT	δ_C
1	CH_2	38.1	CH_2	38.1
2	CH_2	31.3	CH_2	27.0
3	CH	71.2	CH	71.3
4	CH_2	38.1	CH_2	38.1
5	CH	45.1	CH	44.8
6	CH_2	30.6	CH_2	30.0
7	CH_2	36.9	CH_2	32.4
8	CH	30.3	CH	34.9
9	CH	53.7	CH	54.3
10	C	35.8	C	35.6
11	CH_2	28.5	CH_2	23.0
12	CH	80.5	CH	39.7
13	C	46.3	C	39.7
14	CH	59.1	CH	54.8
15	CH	69.7	CH	31.5
16	CH	82.2	CH	84.3
17	CH	60.2	CH	64.2
18	CH_3	12.7	CH_3	14.1
19	CH_3	12.2	CH_3	12.3
20	CH	42.9	C	103.7
21	CH_3	13.6	CH_3	11.6
22	C	110.3	C	151.3
23	CH_2	31.3	CH_2	21.2
24	CH_2	29.7	CH_2	30.6
25	CH	30.2	CH	34.9
26	CH_2	67.3	CH_2	75.3
27	CH_3	17.2	CH_3	18.7
1′	–	–	CH	103.3
2′	–	–	CH	75.7
3′	–	–	CH	78.0
4′	–	–	CH	69.9
5′	–	–	CH	72.0
6′	–	–	CH_2	62.3
1″	–	–	C	175.1
2″	–	–	CH_2	43.5
3″	–	–	CH	25.9
4″	–	–	CH_3	22.3
5″	–	–	CH_3	22.3

H-6′), 3.77 (1H, *dd*, $J = 9.3$ and 6.0 Hz, H-26β), 3.65 (1H, *t*, $J = 9.3$ Hz, H-4′), 3.57 (1H, *m*, H-3), 3.49 (1H, *dd*, $J = 8.7$ and 7.8 Hz, H-2′), 3.41 (1H, *m*, H-5′), 3.35 (1H, *dd*, $J = 9.3$ and 7.2 Hz, H-26α), 2.44 (1H, *d*, $J = 10.5$ Hz, H-17), 2.28 (2H, *d*, $J = 6.6$ Hz, H-2″), 2.21 (1H, *m*, H-3″), 1.56 (3H, *s*, H-21), 0.99 (9H, *d*, $J = 6.6$ Hz, H-4″, H-5″), 0.91 (3H, *d*, $J = 6.6$ Hz, H-27), 0.81 (3H, *s*, H-18), 0.64 (3H, *s*, H-19). ^{13}C NMR: see Table 2. MALDI-MS: *m/z* 663 $[M + H]^+$, 417 $[M - C_{11}H_{17}O_6]^+$; elemental analysis – found: C, 68.87; H, 9.36. Calc. for $C_{38}H_{62}O_9$: C, 68.85; H, 9.43%.

3.3.4. Preparation of the alditol acetate of 9

A sample of **9** (2 mg) was hydrolysed with 2 N TFA (250 μl) for 1 h at 120 °C. Two hundred and fifty microlitres of *iso*PrOH were added to the mixture and kept under nitrogen for 1 h. To the dried residue, dissolved in deionised water (200 μl), 2 mg of $NaBH_4$ were added in magnetic stirring. After 1 h two drops of AcOH were added to eliminate the excess of hydride and the solution was dried under N_2. The residue was kept overnight on P_2O_5 at room temperature. Successively, 250 μl of pyridine dry and 100 μl of Ac_2O were added and the mixture was kept in magnetic stirring for 30 min at 120 °C. After the removal of the solvent the residue was dissolved in water (0.5 ml), extracted with CH_2Cl_2 (0.5 ml) and analysed by GLC.

3.4. Bioassays

Seeds of *L. sativa* L. (cv Napoli V. F.) collected during 2003, were obtained from Ingegnoli S.p.a. All undersized or damaged seeds were discarded and the assay seeds were selected for uniformity. Bioassays used Petri dishes (50 mm diameter) with one sheet of Whatman No. 1 filter paper as support. In four replicate experiments, germination and growth were conducted in aqueous solutions at controlled pH. Test solutions (10^{-4} M) were prepared using (2-[*N*-morpholino]ethanesulfonic acid (MES; 10 mM, pH 6) and the rest (10^{-5}–10^{-7} M) were obtained by dilution. Parallel controls were performed. After adding 25 seeds and 5 ml test solutions, Petri dishes were sealed with Parafilm® to ensure closed-system models. Seeds were placed in a growth chamber KBW Binder 240 at 25 °C in the dark. Germination percentage was determined daily for five days (no more germination occurred after this time). After growth, plants were frozen at −20 °C to avoid subsequent growth until the measurement process. Data are reported as percentage differences from control in the graphics and tables. Thus, zero represents the control; positive values represent the stimulation of the parameter studied and negative values represent inhibition.

3.5. Statistical treatment

The statistical significance of differences between groups was determined by a Student's *t*-test, calculating mean values for every parameter (germination average, shoot and root elongation) and their population variance within a Petri dish. The level of significance was set at $P < 0.05$.

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