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Structure elucidation and phytotoxicity of C_{13} *nor*-isoprenoids from *Cestrum parqui*

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Abstract

Twelve C_{13} *nor*-isoprenoids have been isolated from the leaves of *Cestrum parqui* (Solanaceae). The structure (2*R*,6*R*,9*R*)-2,9-dihydroxy-4-megastigmen-3-one has been assigned to the new compound. All the structures have been determined by spectroscopic means and chemical correlations. The compounds showed phytotoxic effect on the germination and growth of *Lactuca sativa* L.

Keywords: *Cestrum parqui*; Solanaceae; Spectroscopic analysis; Phytotoxicity; C_{13}-*Nor*-isoprenoids

1. Introduction

In a study of the potential allelopathic effects of spontaneous plants present in Italy, we recently reported that some metabolites isolated from *Sambucus nigra* inhibited the germination and growth of some mono- and dicotiledones (D'Abrosca et al., 2001).

Continuing the phytochemical study of common weeds from the Mediterranean area, we investigated *Cestrum parqui* L'Herrit.1788 (Solanaceae).

This plant, commonly named green cestrum, is a perennial shrub indigenous to South America, naturalised and now 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 (McLennan and Kelly, 1984). Pearce et al. (1992) revealed the presence of *ent*-kaurene glycosides, named parquin and carboxyparquin, from the alcoholic extract of *C. parqui*.

2. Results and discussion

Leaves of *C. parqui* were infused in aq. 10% MeOH for 48 h. The aq. soln. was extracted with methylene chloride in a separator funnel. A combination of various chromatographic separations of the organic extract, afforded the C_{13} *nor*-isoprenoids **1**–**13** (Fig. 1).

The new compound **1** was identified as (2*R*,6*R*,9*R*) 2,9-dihydroxy-4-megastigmen-3-one. Its EI mass spectrum showed the molecular ion at *m*/*z* 226, which, together with the elemental analysis was in good agreement with the molecular formula of a bisnorsesquiterpene $C_{13}H_{22}O_3$.

In the ^{1}H NMR spectrum (Table 1) were present two methyls at δ 0.88 and 1.23 as singlets, two methyl doublets at δ 1.22 and 2.02, five aliphatic protons as two multiplets ranging from 1.27 to 2.05 ppm, two methine protons geminal to hydroxyls as a multiplet at δ 3.78 and a singlet at δ 4.18, and an olefinic proton as singlet at δ 5.90. The ^{13}C NMR spectrum (Table 1) showed 13 carbon signals, identified, by a DEPT experiment, as four methyls, two methylenes, three aliphatic methines, two olefinic carbons, one of them tetrasubstituted, a quaternary carbon and a carbonyl carbon. All the carbons were correlated to the corresponding protons on the basis of a HMQC experiment.

The COSY experiment showed a correlation series beginning with the carbinol methine at δ 3.78, assigned to H-9, which was coupled with the doublet methyl at δ 1.22 assigned to H-10, and with the H-8 methylene signals at δ 1.58. These latter protons were correlated to the H-7 protons at δ 1.55 and 1.27, which were coupled with the methine at δ 2.05 attributed at the H-6. The

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signal at δ 4.18, in the ^{1}H NMR spectrum indicated the presence of a further hydroxyl group in the molecule. It was positioned at the C-2 on the basis of heterocorelation observed in the HMBC experiment, that showed the correlations of the H-2 proton with the carbons C-1, C-3, C-11, C-12, C-4, and between the carbinol carbon at δ 76.0 with the protons H-4, H-11 and H-12.

The absolute configurations at the carbinol carbons have been established as follows: compound **1** was converted into the diasteromeric MTPA diesters, and the Mosher's method (Dale and Mosher, 1973) was applied for the configuration of the C-9 carbon. Comparison of the chemical shifts of the signals due to protons H-8 and H-10 in both the *R* and the *S* derivatives and the calculation of the corresponding differences, expressed as $\Delta\delta_{R-S}$, were in agreement with a *R* configuration for C-9. For the C-2 carbon a modified Mosher method was utilised (Ohtani et al., 1991). The positive and the negative $\Delta\delta_{R-S}$ values for the H-4 and the H-11 and H-12 protons were found, respectively, on the right and the left sides of the MTPA plane (Fig. 2) indicating an *R* configuration for C-2 carbon, too.

Compound **2** was identified as (6*R*,9*R*) 9-hydroxy-4-megastigmen-3-one. Its spectral data were in agreement with those isolated from Greek tobacco (Aasen et al., 1974). The MTPA esters of **2** indicated an *R* configuration at the C-9 carbon. The configuration *R* at the C-6 carbon was established by MnO_2 oxidation to the corresponding diketone, which had the same rotation as (6*R*)-4-megastigmen-3,9-dione (Aasen et al., 1974).

Compound **3** showed NMR spectra identical with those reported for (3*R*,6*R*,7*E*)-3-hydroxy-4,7-megastigmadien-9-one, a C-13 *nor*-terpene isolated from *Vilburnum dilatatum* (Machida and Kikuchi, 1996). The CD spectrum showed a positive Cotton effect, $\Delta\epsilon_{239.5\ nm}$ +20.9, confirming an *R* configuration at C-6 (Harada and Nakanishi, 1982). The *R* configuration at C-3 carbon was confirmed by Mosher's method.

Compound **4**, (6*R*,7*E*,9*R*)-9-hydroxy-4,7-megastigmadien-3-one, was identified as the aglycone of a glucoside isolated from *Rubus idaeus* (Pabst et al., 1992). The *R* configuration at the C-9 carbon was attributed by Mosher's method, and the *R* configuration at the C-6 carbon was confirmed by the CD spectrum, which showed a positive Cotton effect, $\Delta\epsilon_{251.7\ nm}$ +193.6.

Compound **5** has been identified as (3*R*,6*R*,7*E*,9*R*)-3,9-dihydroxy-4,7-megastigmadiene, already obtained from the Greek tobacco (Behr et al., 1978). The configurations at the C-3 and C-9 carbons have been attributed by Mosher's method. The positive Cotton effect in the CD spectrum ($\Delta\epsilon_{231.9\ nm}$ +77.1) indicated a *R* configuration for the C-6 as the correlated compounds **3** and **4**.

Compound **6** was identified as (3*S*,7*E*,9*R*)-3,9-dihydroxy-5,7-megastigmadiene. It was previously isolated as glucoside from *Bunias orientalis* leaves (Dietz and Winterhalter, 1996). The spectroscopic data were in agreement with those of the compound described by Perez et al. (1996), and the configurations at the C-3 and C-9 carbons were established by Mosher's method.

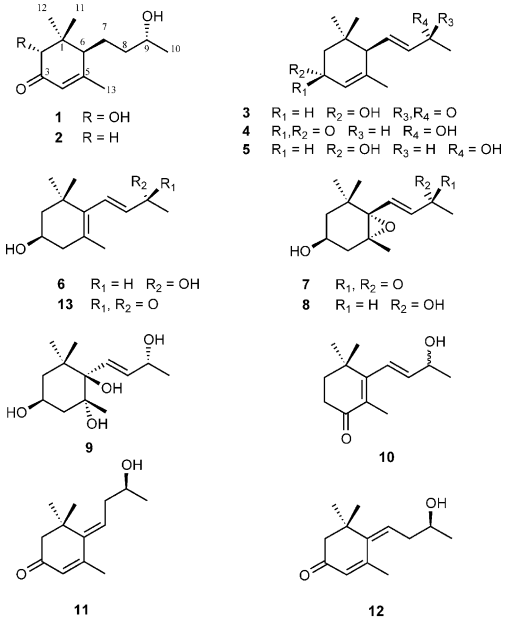

Fig. 1. C_{13} *nor*-isoprenoids **1**–**13**.

Table 1
NMR data for compound **1**[a]

Position	δ_C	DEPT	δ_H	HMBC (C→H)
1	42.0	C	—	2, 6, 11, 12
2	76.0	CH	4.18 *s*	4, 6, 11, 12
3	198.8	C	—	2, 4
4	122.4	CH	5.90 *s*	2, 6, 13
5	166.1	C	—	4, 7, 13
6	52.9	CH	2.05 *m*	2, 4, 8, 11, 12
7	24.7	CH_2	1.55 *m*	8, 9
			1.27 *m*	
8	38.8	CH_2	1.58 *m*	6, 7, 9, 10
9	67.6	CH	3.78 *m*	7, 8, 10
10	23.8	CH_3	1.22 *d* (6.0)	9, 8
11	23.8	CH_3	0.88 *s*	2, 6
12	21.2	CH_3	1.23 *s*	2, 6
13	24.3	CH_3	2.02 *d* (1.2)	4, 6

[a] Values were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C in $CDCl_3$ with *J* values in Hz in parentheses.

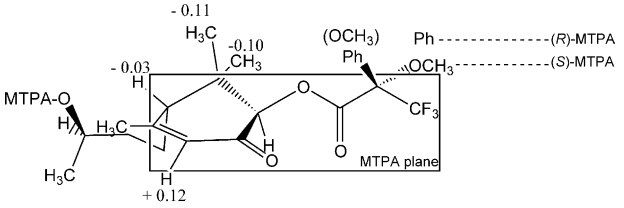

Fig. 2. MTPA plane of diester **1**.

Compound **7** was identified as (3*S*,5*R*,6*S*,7*E*)-5,6-epoxy-3-hydroxy-7-megastigmen-9-one. The NMR data were in good agreement with annuionone D, isolated from sunflower leaves (Macías et al., 1999). A comparison of the optical rotation of this compound with the literature data (Broom et al., 1992) suggested the enantiomeric structure for **7**. According with the assigned structure, oxidation of **5** with MnO_2 gave **13** and the following treatment with MCPBA led to compound **7** as the main product.

Also compound **8** was characterised by an oxirane ring at the 5,6 carbons, but the spectral data indicated the presence of a hydroxyl group at the C-9 position. Like compound **6**, it had already been isolated as 3-*O*-glucoside from *Pistia stratiotes* (DellaGreca et al., 1995).

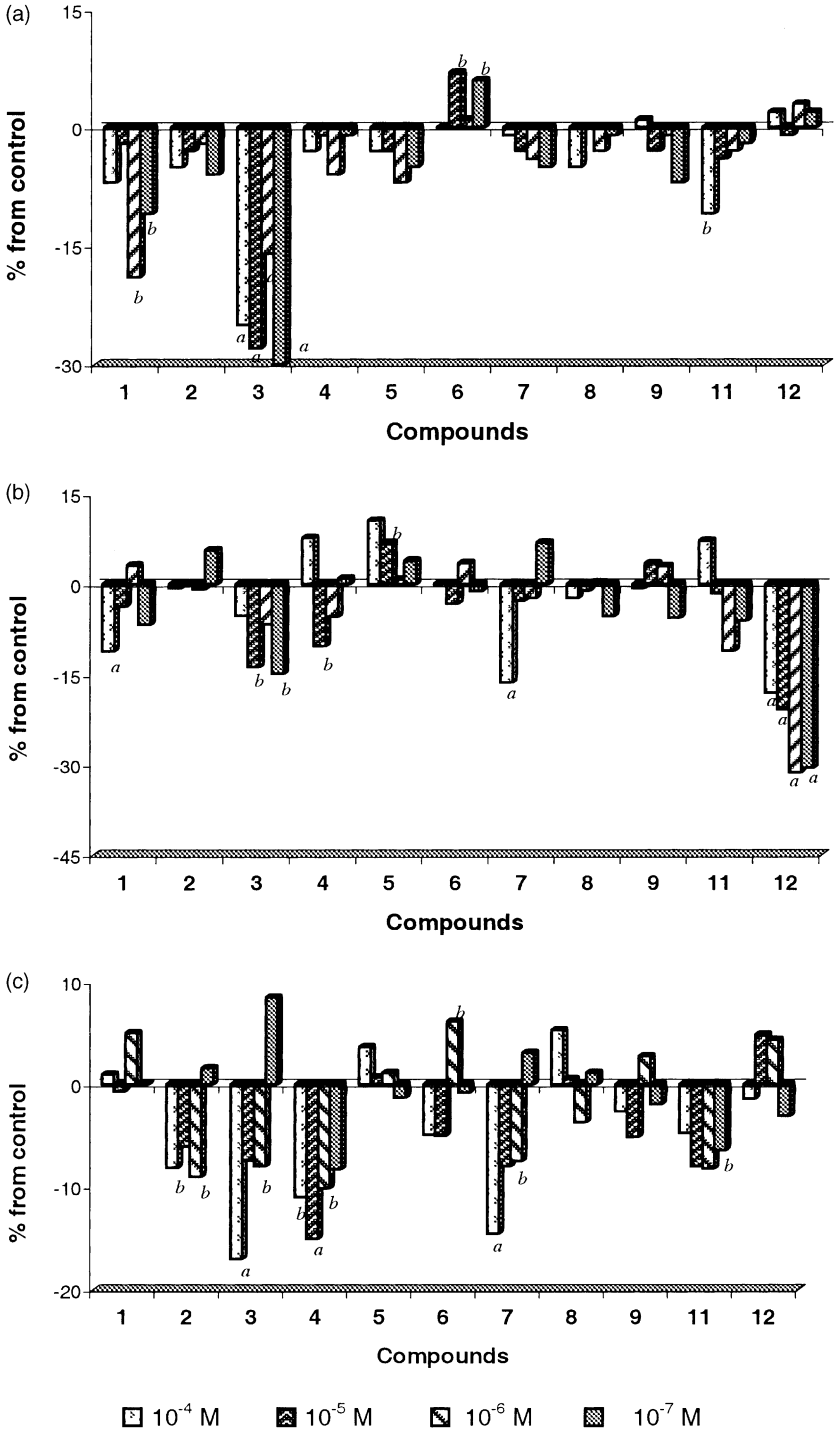

Fig. 3. Effect of C13-norisoprenoids on germination (A), root length (B) and shoot lenght (C) of *Lactuca sativa* L. Value presented as percentage differences from control and are not significantly different with $P > 0.05$ for Student's *t*-test. (a) $P < 0.01$; (b) $0.01 < P < 0.05$.

Compound **9,** was identified as the (3*S*,5*R*,6*R*,7*E*,9*R*)-3,5,6,9-tetrahydroxy-7-megastigmene. Like the previous compound, it was isolated as 3-*O*-glucosides from the aquatic plant *Pistia stratiotes* (DellaGreca et al., 1996).

Compound **10** had spectral data identical to the 4-oxo-β-ionol obtained from the biotransformation of β-ionone by *Cunninghamella blakesleeana* (Hartman et al., 1988).

Compounds **11** and **12** were identified as geometric isomers at the Δ^6 double bond. They were obtained by chemical synthesis (Ito et al., 1997) and now is the first time that they have been reported as natural products. The MTPA esters of both the *nor*-terpenes indicated an *S* configuration for the C-9 carbon.

In order to evaluate their potential phytotoxicity, all the compounds were tested on the seeds of *Lactuca sativa* L. (Macias et al., 2000), with exception of **10** because of an insufficiency samples. The results are reported in Fig. 3. Aq. solns. of *nor*-terpenes, ranging between 10^{-4} and 10^{-7} M, were tested on germination, root and shoot length of the lettuce. With the exception of the C-13 *nor*-terpene **3**, the compounds had no effect on germination, but they had a moderate inhibitory profile on root and shoot growth.

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, with 0.05 M solns. in $CDCl_3$ or CD_3OD at 37 °C. Proton-detected heteronuclear correlations were measured using HMQC (optimised for $^1J_{HC} = 145$ Hz) and HMBC (optimised for $^1J_{HC} = 7$ Hz). Optical rotations were measured on a Perkin-Elmer 343 polarimeter. CD spectra were obtained in $CHCl_3$ solns. on a Jasco J-715 Spectrophotometer Polarimeter. IR spectra were determined in $CHCl_3$ solns. on a FT-IR Perkin-Elmer 1740 spectrometer. UV spectra were obtained on a Perkin-Elmer Lambda 7 spectrophotometer in $CHCl_3$ or EtOH solns. Electronic impact mass spectra (EI-MS) were obtained with a HP 6890 spectrometer equipped with a MS 5973 N detector. 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 110 °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) or on Sephadex LH-20® (Pharmacia).

3.2. Plant material

Plants of *Cestrum parqui* were collected 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 aq. soln. obtained was extracted in a separator funnel using methylene chloride. The organic layer was dried with Na_2SO_4 and concentrated under vacuum yielding 8 g of residual material.

3.3.1. Organic extract fractionation

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

Fraction A, eluted with $CHCl_3$–EtOAc (19:1) was rechromatographed by FCC on SiO_2, to give two fractions: the first was purified on HPLC using an RP-18 preparative column, and eluting with MeOH–MeCN–H_2O (2:1:2), to have **10** (2 mg); the second fraction was purified on prep HPLC using an RP-18 preparative column, and eluting with MeOH–MeCN–H_2O (3:2:5), to have **3** (5 mg).

Fraction B, eluted with $CHCl_3$–EtOAc (9:1), was rechromatographed on Sephadex LH-20 eluting with hexane–$CHCl_3$–MeOH (3:1:1), to produce two fractions: the first was purified on HPLC using an RP-18 preparative column, and eluting with MeOH–MeCN–H_2O (2:1:2) to give pure **1** (15 mg) and **2** (21 mg); the second fraction was chromatographed on C-18 reverse phase silica with MeOH–MeCN–H_2O (3:2:5) and then purified on SiO_2-HPLC, eluting with $CHCl_3$–*iso*-PrOH (49:1) to give pure isomers **5** (12 mg) and **6** (15 mg).

Fraction C, eluted with $CHCl_3$–EtOAc (4:1) was rechromatographed on C-18 reverse phase silica with MeOH–MeCN–H_2O soln. to have three fractions. The first, obtained with MeOH–MeCN–H_2O (1:1:3), was chromatographed on prep. TLC eluting with toluene–Me_2CO (3:2) to give **7** (7 mg); the second fraction, obtained with MeOH–MeCN–H_2O (1:1:2), was purified on SiO_2-HPLC, eluting with $CHCl_3$–Me_2CO (9:1) to

Table 2
Selected ^{1}H NMR data for compounds **2**–**12**[a]

	2	3	4	5	6	7	8	9	10	11	12
2	*eq* 2.39 *d* (16.9)	*eq* 1.40 *dd* (13.4, 6.4)	*eq* 2.06 *d* (16.8)	*eq* 1.36 *dd* (13.5, 6.3)	*eq* 1.77 *ddd* (12.0, 3.5, 2.5)	*eq* 1.30 *dd* (12.2, 10.2)	*eq* 1.37 *dd* (12.3, 10.1)	*eq* 1.59 *dd* (11.4, 4.2)	1.85 *t* (6.2)	2.36 *s*	2.30 *s*
	ax 2.04 *d* (16.9)	*ax* 1.84 *dd* (13.4, 5.8)	*ax* 2.32 *d* (16.8)	*ax* 1.82 *dd* (13.5, 5.8)	*ax* 1.45 *t* (12.0)	*ax* 1.60 *dd* (12.2, 4.5)	*ax* 1.60 *m*	*ax* 1.30 *m*			
3	–	4.27 *brs*	–	4.21 *brs*	3.97 *m*	3.90 *m*	3.89 m	3.74 *m*	2.50 *t* (6.2)	-	-
4	5.84 *s*	5.63 *brs*	5.89 *s*	5.53 *brs*	*eq* 2.34 *dd* (16.5, 5.5) *ax* 2.01 *dd* (16.5, 10.0)	*eq* 2.39 *dd* (9.2, 5.2) *ax* 1.65 *dd* (11.5, 9.2)	*eq* 2.36 *dd* (9.3, 5.2) *ax* 1.60 *m*	*eq* 2.27 *dd* (9.3, 5.0) *ax* 1.62 *dd* (11.5, 9.3)	–	5.91 *s*	5.92 *s*
6	overlapped	2.49 *d* (10.0)	2.54 *d* (8.2)	2.32 *d* (8.1)	–	–	–	–	–	–	–
7	overlapped	6.55 *dd* (15.8, 10.0)	5.54 *dd* (15.5, 8.2)	5.38 *dd* (15.5, 8.1)	6.01 *d* (16.5)	7.03 *d* (15.5)	5.90 *d* (16.5)	5.90 *dd* (15.5, 1.5)	6.22 *d* (15.7)	6.06 *t* (7.1)	5.74 *t* (7.0)
8	overlapped	6.09 *d* (15.8)	5.56 *dd* (15.5, 5.6)	5.59 *dd* (15.5, 5.4)	5.50 *dd* (16.5, 6.3)	6.29 *d* (15.5)	5.76 *dd* (16.5, 6.5)	5.66 *dd* (15.5, 6.0)	5.70 *dd* (15.7, 6.0)	2.61 *m*	2.48 *m*
9	3.78 *m*	–	4.34 *m*	4.33 *m*	4.38 *m*	–	4.38 *m*	4.28 *m*	4.46 *m*	3.99 *m*	3.94 *m*
10	1.21 *d* (6.3)	2.26 *s*	1.28 *d* (6.6)	1.28 *d* (6.5)	1.31 *d* (6.0)	2.28 *s*	1.28 *d* (6.5)	1.22 *d* (6.5)	1.36 *d* (6.5)	1.29 *d* (6.3)	1.25 *d* (6.0)
11	1.07 *s*	1.03 *s*	1.03 *s*	1.00 *s*	1.04 *s*	1.20 *s*	1.12 *s*	1.12 *s*	1.15	1.31 *s*	1.18 *s*
12	1.02 *s*	0.89 *s*	0.97	0.84 *s*	1.03 *s*	0.98 *s*	0.97 *s*	0.96 *s*	1.15	1.30 *s*	1.18 *s*
13	2.00 *d* (1.2)	1.62 *s*	1.88 *d* (1.2)	1.61 *s*	1.69 *s*	1.56 *s*	1.19 *s*	1.18 *s*	1.80 *s*	2.08 *d* (1.5)	2.23 *d* (0.5)

[a] Values were recorded at 500 MHz, in $CDCl_3$, with *J* values in Hz in parentheses.

give pure **4** (12 mg) and **11** (5 mg) besides **2**; the third fraction, obtained with MeOH–MeCN–H_2O (1:1:1), was purified on HPLC, using an NH_2 preparative column and eluting with hexane–Me_2CO (9:1) to give pure **12** (6 mg) besides **2** and **4**.

Fraction D, eluted with $CHCl_3$–EtOAc (3:1), was rechromatographed on SiO_2 by FCC. The fraction obtained by eluting with $CHCl_3$–MeOH (19:1) was first chromatographed on C-8 silica with MeOH–MeCN–H_2O (3:2:1), and then purified by prep. TLC with toluene–Me_2CO (3:2) to give pure **8** (15 mg) and a mixture that was purified by RP-8 HPLC eluting with MeOH–MeCN–H_2O (3:2:5) to give pure **9** (4 mg).

3.3.2. Compound characterization

*(2R,6R,9R)-2,9-Dihydroxy-4-megastigmen-3-one (**1**).* Colourless oil; $[\alpha]_D^{25}$ +102.7° (CH_2Cl_2, *c* 0.56); UV ($CHCl_3$) λ_{max} (log ϵ): 245 (3.94); ^{1}H NMR and ^{13}C NMR: see Table 1; EI-MS: *m*/*z* 226 $[M]^+$, 208 $[M-H_2O]^+$; elemental analysis: found: C, 69.01; H, 9.83. $C_{13}H_{22}O_3$ requires: C, 68.99; H, 9.80%.

*(6R,9R)-9-Hydroxy-4-megastigmen-3-one (**2**).* Colourless oil; $[\alpha]_D^{25}$ +83.6° (CH_2Cl_2, *c* 0.61); UV ($CHCl_3$) λ_{max} (log ϵ): 243 (3.48); ^{1}H NMR: see Table 2; ^{13}C NMR: see Table 3; EI-MS: *m*/*z* 210 $[M]^+$, 192 $[M-H_2O]^+$; elemental analysis: found: C, 74.41; H, 10.13. Calc. for $C_{13}H_{22}O_2$: C, 74.24; H, 10.54%.

*(3R,6R,7E)-3-Hydroxy-4,7-megastigmadien-9-one (**3**).* Colourless oil; $[\alpha]_D^{25}$ +37.1° (CH_2Cl_2, *c* 0.21); CD ($CHCl_3$) +20.9 (239.5 nm); UV ($CHCl_3$) λ_{max} (log ϵ): 207 (3.95); ^{1}H NMR: see Table 2; ^{13}C NMR: see Table 3; EI-MS: *m*/*z* 208 $[M]^+$, 190 $[M-H_2O]^+$; elemental analysis: found: C, 74.58; H, 9.73. Calc. for $C_{13}H_{20}O_2$: C, 74.96; H, 9.68%.

*(6R,7E,9R)-9-Hydroxy-4,7-megastigmadien-3-one (**4**).* Colourless oil; $[\alpha]_D^{25}$ +292.0° (CH_2Cl_2, *c* 0.42); CD ($CHCl_3$) +193.6 (251.7 nm); UV ($CHCl_3$) λ_{max} (log ϵ): 245 (4.06); ^{1}H NMR: see Table 2; ^{13}C NMR: see Table 3; EI-MS: *m*/*z* 208 $[M]^+$, 190 $[M-H_2O]^+$; elemental analysis: found: C, 74.72; H, 9.65. $C_{13}H_{20}O_2$ requires: C, 74.96; H, 9.68%.

*(3R,6R,7E,9R)-3,9-Dihydroxy-4,7-megastigmadiene (**5**).* Colourless oil; $[\alpha]_D^{25}$ +25.9° (CH_2Cl_2, *c* 0.53); CD ($CHCl_3$) +77.1 (231.9 nm); UV ($CHCl_3$) λ_{max} (log ϵ): 207 (3.93); ^{1}H NMR: see Table 2; ^{13}C NMR: see Table 3; EI-MS: *m*/*z* 210 $[M]^+$, 192 $[M-H_2O]^+$; elemental analysis: found: C, 74.31; H, 10.68. Calc. for $C_{13}H_{22}O_2$: C, 74.24; H, 10.54%.

*(3S,7E,9R)-3,9-Dihydroxy-5,7-megastigmadiene (**6**).* Colourless oil; $[\alpha]_D^{25}$ −97.9° (CH_2Cl_2, *c* 0.48); UV ($CHCl_3$) λ_{max} (log ϵ): 243 (3.63); ^{1}H NMR: see Table 2; ^{13}C NMR: see Table 3; EI-MS: *m*/*z* 210 $[M]^+$, 192 $[M-H_2O]^+$; elemental analysis: found: C, 74.31; H, 10.68. $C_{13}H_{22}O_2$ requires: C, 74.24; H, 10.54%.

*(3S,5R,6S,7E)-5,6-Epoxy-3-hydroxy-7-megastigmen-9-one (**7**).* Colourless oil; $[\alpha]_D^{25}$ −43.7° (CH_2Cl_2, *c* 0.39);

UV ($CHCl_3$) λ_{max} (log ϵ): 243 (3.76); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3; EI-MS: *m/z* 224 $[M]^+$, 206 $[M-H_2O]^+$; elemental analysis: found: C, 70.08; H, 9.04. Calc. for $C_{13}H_{20}O_3$: C, 69.61; H, 8.99%.

*(3S,5R,6S,7E,9R)-5,6-Epoxy-3,9-dihydroxy-7-megastigmene (**8**)*. Colourless oil; $[\alpha]_D^{25}$ −53.9° (CH_2Cl_2, *c* 0.47); UV ($CHCl_3$) λ_{max} (log ϵ): 245 (3.95); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3; EI-MS: *m/z* 226 $[M]^+$, 208 $[M-H_2O]^+$; elemental analysis: found: C, 68.84; H, 9.74. $C_{13}H_{22}O_3$ requires: C, 68.99; H, 9.80%.

*(3S,5R,6R,7E,9R) - 3,5,6,9 - Tetrahydroxy - 7 - megastigmene (**9**)*. Colourless oil; $[\alpha]_D^{25}$ 33.8° (MeOH, *c* 0.53); UV (EtOH) λ_{max} (log ϵ): 207 (3.95); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3; EI-MS: *m/z* 244 $[M]^+$, 226 $[M-H_2O]^+$; elemental analysis: found: C, 63.98; H, 9.87. $C_{13}H_{24}O_4$ requires: C, 63.91; H, 9.90%.

*(7E,9ξ)-9-Hydroxy-5,7-megastigmadien-4-one (**10**)*. Colourless oil; $[\alpha]_D^{25}$ +101.1° (CH_2Cl_2, *c* 0.39); UV ($CHCl_3$) λ_{max} (log ϵ): 264 (4.03); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3; EI-MS: *m/z* 208 $[M]^+$, 190 $[M-H_2O]^+$; elemental analysis: found: C, 74.85; H, 9.71. Calc. for $C_{13}H_{20}O_2$: C, 74.96; H, 9.68%.

*(6E,9S)-9-Hydroxy-4,6-megastigmadien-3-one (**11**)*. Colourless oil; $[\alpha]_D^{25}$ +4.7° (CH_2Cl_2, *c* 0.42); UV ($CHCl_3$) λ_{max} (log ϵ): 285 (4.68); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3; EI-MS: *m/z* 208 $[M]^+$, 190 $[M-H_2O]^+$; elemental analysis: found: C, 74.85; H, 9.87. Calc. for $C_{13}H_{20}O_2$: C, 74.96; H, 9.68%.

*(6Z,9S)-9-Hydroxy-4,6-megastigmadien-3-one (**12**)*. Colourless oil; $[\alpha]_D^{25}$ +28.5° (CH_2Cl_2, *c* 0.37); UV ($CHCl_3$) λ_{max} (log ϵ): 287 (4.65); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3; EI-MS: *m/z* 208 $[M]^+$, 190 $[M-H_2O]^+$; elemental analysis: found: C, 74.59; H, 9.54. Calc. for $C_{13}H_{20}O_2$: C, 74.96; H, 9.68%.

3.3.3. Preparation of (S) and (R)-MTPA diesters of 1

(*R*)-(−)-MTPA chloride (5 μl, 26 μmol) was added to a soln. of pure **1** (1.5 mg, 6.6 μmol) in dry pyridine (50 μL). After 6 h under magnetic stirring at room temperature, EtOAc (5 ml) and H_2O (5 ml) were added to the reaction mixture. The organic layer, separated by centrifugation at 4000 rpm for 10 min, gave a crude extract which was purified by prep. TLC eluting with hexane–EtOAc (4:1). The (*S*)-MTPA diester had the ^1H NMR spectral data (500 MHz, $CDCl_3$): δ 1.00 (3H, *s*, H-11), 1.05 (3H, *s*, H-12), 1.39 (3H, *d*, *J* = 6.0 Hz, H-10), 1.85 (3H, *s*, H-13), 2.00 (1H, *m*, H-6α), 3.59 (6H, *s*, OMe), 5.12 (1H, *m*, H-9), 5.42 (1H, *s*, H-2α), 5.83 (1H, *s*, H-4), 7.38 (3H, *m*), 7.44 (3H, *m*), 7.52 (2H, *m*), 7.66 (2H, *m*). The (*R*)-MTPA diester was prepared, from (*S*)-(+)-MTPA chloride, using the same procedure. ^1H NMR (500 MHz, $CDCl_3$): δ 0.90 (3H, *s*, H-11), 0.94 (3H, *s*, H-12), 1.32 (3H, *d*, *J* = 6.0 Hz, H-10), 1.94 (3H, *d*, *J* = 1.0 Hz, H-13), 1.97 (1H, *m*, H-6α), 3.51 (3H, *s*, OMe), 3.67 (3H, *s*, OMe), 5.11 (1H, *m*, H-9), 5.56 (1H, *s*, H-2α), 5.90 (1H, *s*, H-4), 7.41 (6H, *m*), 7.51 (2H, *m*), 7.78 (2H, *m*).

3.3.4. Preparation of (S) and (R)-MTPA esters of 2

(*R*)-(−)-MTPA chloride (2.5 μl, 13 μmol) was added to a soln. of pure **2** (1.5 mg, 7.1 μmol) in dry pyridine (25 μl). After 6 h under magnetic stirring at room temperature, EtOAc (5 ml) and H_2O (5 ml) were added to the reaction mixture. The organic layer, separated by centrifugation at 4000 rpm for 10 min, gave a crude extract which was purified by prep. TLC eluting with hexane–EtOAc (4:1). The (*S*)-MTPA ester had the ^1H NMR spectral data (500 MHz, $CDCl_3$): δ 0.91 (3H, *s*, H-12), 0.96 (3H, *s*, H-11), 1.35 (3H, *d*, *J* = 6.5 Hz, H-10), 1.86 (3H, *d*, *J* = 1.5 Hz, H-13), 1.95 (1H, *d*, *J* = 17.5 Hz, H-2α), 2.17 (1H, *d*, *J* = 17.5 Hz, H-2β), 3.59 (3H, *s*, OMe), 5.09 (1H, *m*, H-9), 5.76 (1H, *s*, H-4), 7.40 (3H, *m*), 7.53 (2H, *m*). The (*R*)-MTPA ester was prepared from (*S*)-(+)-MTPA chloride, using the same procedure. ^1H NMR (500 MHz, $CDCl_3$): δ 0.99 (3H, *s*, H-12), 1.00 (3H, *s*, H-11), 1.29 (3H, *d*, *J* = 6.0 Hz, H-10), 1.93 (3H, *s*, H-13), 2.01 (1H, *d*, *J* = 17.0 Hz, H-2α), 2.27 (1H, *d*, *J* = 17.0 Hz, H-2β), 3.50 (3H, *s*, OMe), 5.08 (1H, *m*, H-9), 5.82 (1H, *s*, H-4), 7.41 (3H, *m*), 7.55 (2H, *m*).

Table 3
^{13}C NMR data for compounds **2–12**[a]

	2	3	4	5	6	7	8	9	10	11	12
1	36.2	33.9	36.0	33.4	36.8	35.3	34.9	36.4	35.4	38.1	40.9
2	47.1	44.0	47.4	44.4	48.2	40.8	40.8	42.1	34.3	53.6	53.0
3	199.6	65.6	199.0	65.8	65.0	64.2	64.2	65.0	37.2	198.9	199.1
4	125.1	126.0	125.7	124.6	42.2	47.0	47.0	48.5	192.0	130.9	129.0
5	165.8	135.4	161.7	137.4	125.8	67.5	66.3	68.5	160.1	143.1	144.6
6	51.1	54.3	55.3	54.0	136.2	69.7	69.5	71.6	140.3	154.8	155.9
7	26.2	147.0	126.6	129.1	126.6	142.6	124.9	139.6	125.3	125.4	126.6
8	38.6	133.5	138.5	137.6	138.5	132.8	137.8	126.4	140.3	39.1	39.6
9	68.0	199.0	68.2	68.7	69.3	197.6	68.2	69.1	68.9	67.9	68.2
10	24.6	27.0	23.5	23.6	23.6	28.5	23.6	24.3	23.5	23.4	23.5
11	27.2	29.2	27.0	29.4	30.0	29.6	29.5	30.5	28.6	28.9	28.0
12	28.8	24.7	27.8	24.0	28.4	25.2	24.7	25.4	27.3	29.0	28.1
13	23.7	22.8	23.4	22.6	21.2	20.0	19.8	20.6	19.9	22.3	24.8

[a] Values were recorded at 125 MHz in $CDCl_3$.

3.3.5. Oxidation of ***2***

Active MnO_2 (60 mg, 0.69 mmol) was added to a soln. of **2** (6 mg, 28 μmol) in $CHCl_3$ (0.8 ml). The reaction mixture was kept under magnetic stirring at room temperature. After 6 h, the reaction was filtered on SiO_2 with $CHCl_3$–Me_2CO (19:1), and the residue was purified on prep TLC eluting with $CHCl_3$–Me_2CO (19:1) to obtain pure (6*R*)-4-megastigmen-3,9-dione (2 mg): ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 1.02 (3H, *s*, H-12), 1.07 (3H, *s*, H-11), 2.00 (3H, *d*, *J* =1.0, H-13), 2.05 (1H, *d*, *J*=17.5 Hz, H-2_{ax}), 2.16 (3H, *s*, H-10), 2.37 (1H *d*, *J*=17.5 Hz, H-2_{eq}), 5.83 (1H, *s*, H-4).

3.3.6. Preparation of (S) and (R)-MTPA esters of ***3***

The (*S*) and (*R*)-MTPA esters of **3** were prepared using the same procedure described for the compound **2.** The (*S*)-MTPA ester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 0.92 (3H, *s*, H-12), 0.96 (3H, *s*, H-11), 1.62 (1H, *dd*, *J*=4.0 and 14.3 Hz, H-2α), 1.65 (3H, *s*, H-13), 1.94 (1H, *dd*, *J*=6.0 and 14.3 Hz, H-2β), 2.26 (3H, *s*, H-10), 2.46 (1H, *d*, *J*=10,0 Hz, H-6), 3.55 (3H, *s*, OMe), 5.57 (1H, *m*, H-3), 5.60 (1H, *brs*, H-4), 6.08 (1H, *d*, *J*=15.8 Hz, H-8), 6.53 (1H, *dd*, *J*=10.0 and 15.8 Hz, H-7), 7.41 (3H, *m*), 7.54 (2H, *m*). The (*R*)-MTPA ester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 0.88 (3H, *s*, H-12), 0.88 (3H, *s*, H-11), 1.49 (1H, *dd*, *J*=3.8 and 14.5 Hz, H-2α), 1.68 (3H, *s*, H-13), 1.87 (1H, *dd*, *J*=6.0 and 14.5 Hz, H-2β), 2.27 (3H, *s*, H-10), 2.46 (1H, *d*, *J*=10,0 Hz, H-6), 3.56 (3H, *s*, OMe), 5.57 (1H, *m*, H-3), 5.65 (1H, *brs*, H-4), 6.08 (1H, *d*, *J*=15.8 Hz, H-8), 6.52 (1H, *dd*, *J*=10.0 and 15.8 Hz, H-7), 7.40 (3H, *m*), 7.53 (2H, *m*).

3.3.7. Preparation of (S) and (R)-MTPA esters of ***4***

The (*S*) and (*R*)-MTPA esters of **4** were prepared using the same procedure described for the compound **2.** The (*S*)-MTPA ester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 0.90 (3H, *s*, H-12), 1.00 (3H, *s*, H-11), 1.43 (3H, *d*, *J*=6.0 Hz, H-10), 1.83 (3H, *s*, H-13), 2.05 (1H, *d*, *J*=16.5 Hz, H-2α), 2.23 (1H, *d*, *J*=16.5 Hz, H-2β), 2.48 (1H, *d*, *J*=8.0 Hz, H-6α), 3.56 (3H, *s*, OMe), 5.55 (1H, *dd*, *J*=6.0 and 15.5 Hz, H-8), 5.57 (1H, *m*, H-9), 5.60 (1H, *dd*, *J*=8.0 and 15.5 Hz, H-7), 5.89 (1H, *s*, H-4), 7.39 (3H, *m*), 7.53 (2H, *m*). The (*R*)-MTPA ester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 0.93 (3H, *s*, H-12), 1.02 (3H, *s*, H-11), 1.38 (3H, *d*, *J*=6.0 Hz, H-10), 1.86 (3H, *s*, H-13), 2.09 (1H, *d*, *J*=17.0 Hz, H-2α), 2.29 (1H, *d*, *J*=17.0 Hz, H-2β), 2.53 (1H, *d*, *J*=9.0 Hz, H-6α), 3.52 (3H, *s*, OMe), 5.58 (1H, *m*, H-9), 5.65 (1H, *dd*, *J*=6.5 and 15.5 Hz, H-8), 5.72 (1H, *dd*, *J*=9.0 and 15.5 Hz, H-7), 5.91 (1H, *s*, H-4), 7.40 (3H, *m*), 7.54 (2H, *m*).

3.3.8. Preparation of (S) and (R)-MTPA diesters of ***5***

The (*S*) and (*R*)-MTPA esters of **5** were prepared using the same procedure described for the compound **1.** The (*S*)-MTPA diester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 0.82 (3H, *s*, H-12), 0.88 (3H, *s*, H-11), 1.42 (3H, *d*, *J*=6.0 Hz, H-10), 1.51 (1H, *dd*, *J*=4.0 and 14.1 Hz, H-2α), 1.58 (3H, *s*, H-13), 1.84 (1H, *dd*, *J*=6.0 and 14.1 Hz, H-2β), 2.25 (1H, *d*, *J*=8.0 Hz, H-6α), 3.55 (3H, *s*, OMe), 3.56 (3H, *s*, OMe), 5.44 (1H, *dd*, *J*=6.0 and 15.5 Hz, H-8), 5.47 (1H, *dd*, *J*=8.0 and 15.5 Hz, H-7), 5.49 (1H, *m*, H-3), 5.52 (1H, br*s*, H-4), 5.57 (1H, *m*, H-9), 7.40 (6H, *m*), 7.52 (4H, *m*). The (*R*)-MTPA ester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 0.81 (3H, *s*, H-12), 0.83 (3H, *s*, H-11), 1.36 (3H, *d*, *J*=6.5 Hz, H-10), 1.44 (1H, *dd*, *J*=4.0 and 14.5 Hz, H-2α), 1.64 (3H, *s*, H-13), 1.82 (1H, *dd*, *J*=6.5 and 14.5 Hz, H-2β), 2.28 (1H, *d*, *J*=8.5 Hz, H-6α), 3.57 (6H, *s*, OMe), 5.49 (1H, *dd*, *J*=6.5 and 15.0 Hz, H-8), 5.50 (1H, *m*, H-3), 5.52 (1H, *dd*, *J*=8.5 and 15.0 Hz, H-7), 5.55 (1H, *m*, H-9), 5.56 (1H, *s*, H-4), 7.40 (3H, *m*), 7.43 (3H, *m*), 7.52 (2H, *m*), 7.63 (3H, *m*).

3.3.9. Preparation of (S) and (R)-MTPA diesters of ***6***

The (*S*) and (*R*)-MTPA diesters of **6** were prepared using the same procedure described for the compound **1.** The (*S*)-MTPA diester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 1.01 (3H, *s*, H-11), 1.10 (3H, *s*, H-12), 1.47 (3H, *d*, *J*=6.1 Hz, H-10), 1.59 (1H, *dd*, *J*=4.0 and 14.1 Hz, H-2ax), 1.82 (1H, *dd*, *J*=6.1 and 14.1 Hz, H-eq), 1.70 (3H, *s*, H-13), 2.23 (1H, *dd*, *J*=16.6, 10.2 Hz, H-4ax), 2.48 (1H, *dd*, J=16.6, 5.5 Hz, H-4eq), 5.31 (1H, *m*, H-3), 5.35 (1H, *dd*, *J*=6.0 and 15.5 Hz, H-8), 3.55 (6H, *s*, OMe), 5.62 (1H, *m*, H-9), 6.10 (1H, *dd*, *J*=8.0 and 15.5 Hz, H-7), 7.39 (6H, *m*), 7.52 (4H, *m*). The (*R*)-MTPA diester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 1.04 (3H, *s*, H-11), 1.11 (3H, *s*, H-12), 1.39 (3H, *d*, *J*=6.1 Hz, H-10), 1.66 (1H, *m*, H-2ax), 1.88 (1H, *m*, H-2eq), 1.67 (3H, *s*, H-13), 2.12 (1H, *dd*, *J*=16.4, 9.9 Hz, H-4ax), 2.43 (1H, *dd*, J=16.4, 5.6 Hz, H-4eq), 5.30 (1H, *m*, H-3), 5.39 (1H, *dd*, *J*=6.0 and 15.5 Hz, H-8), 3.55 (6H, *s*, OMe), 5.59 (1H, *m*, H-9), 6.15 (1H, *dd*, *J*=8.0 and 15.5 Hz, H-7), 7.39 (6H, *m*), 7.50 (4H, *m*).

3.3.10. Preparation of compound ***7*** *from* ***6***

Oxidation of ***6***. Active MnO_2 (25 mg, 0.29 mmol) was added to a soln. of **6** (5 mg, 24 μmol) in $CHCl_3$ (0.5 ml). The reaction mixture was kept under magnetic stirring at room temperature. After 2 h, the reaction was filtered on SiO_2 with $CHCl_3$–*iso*-PrOH (24:1) and the residue was purified on prep TLC eluting with $CHCl_3$–*iso*-PrOH (24:1) to obtain pure (3*R*,7*E*)-3-hydroxy-5,7-megastigmadien-9-one (2 mg). The oxidation product **13** had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 1.13 (3H, *s*, H-12), 1.14 (3H, *s*, H-11), 1.50 (1H, *t*, *J*=12,2 Hz, H-2_{ax}), 1.79 (3H, *s*, H-13), 1.80 (1H, *m*, H-2_{eq}), 2.10 (1H, *dd*, *J*=10.1 and 16.5 Hz, H-4_{ax}), 2.32 (3H, *s*, H-10), 2.46 (1H, *dd*, *J*=8.0 and 16.5 Hz, H-4_{eq}), 4.04 (1H, *m* H-3), 6.13 (1H, *d*, *J*=16.5 Hz, H-8), 7.22 (1H, *d*, *J*=16.5 Hz, H-7).

*Epoxidation of **13***. 3-Chloroperbenzoic acid (4.2 mg, 31.4 μmol) was added to a soln. of **13** (2 mg, 9.5 μmol) in $CHCl_3$ (0.5 ml). The reaction mixture was kept under magnetic stirring at room temperature. After 4 h 5% $NaHCO_3$ was added and the mixture was extracted with CH_2Cl_2. The organic layer was washed with 10% Na_2SO_3, 5% Na_2SO_3 and, finally with H_2O until neutrality. The residue was chromatographed on prep. TLC [toluene–Me_2CO (3:1)] to give compound **7**, as the main product along with a small amount of the isomeric (3*R*,5*S*,6*R*,7*E*)-5,6-epoxy-3-hydroxy-7-megastigmen-9-one.

*3.3.11. Preparation of (S) and (R)-MTPA esters of **8***

The (*S*) and (*R*)-MTPA esters of **8** were prepared using the same procedure described for the compound **1.** The (*S*)-MTPA diester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 0.92 (3H, *s*, H-12), 0.95 (3H, *s*, H-11), 1.14 (3H, *s*, H-13), 1.28 (1H, *dd*, $J=9.0$ and 13.5 Hz, H-2_{ax}), 1.42 (1H, *d*, $J=6.0$ Hz, H-10), 1.68 (1H, *dd*, $J=6.0$ and 13.5 Hz, H-2_{eq}), 1.86 (1H, *dd*, $J=7.5$ and 14.5 Hz, H-4_{ax}), 2.46 (1H, *dd*, $J=5.5$ and 14.5 Hz, H-4_{eq}), 3.54 (3H, *s*, OMe), 3.56 (3H, *s*, OMe), 5.16 (1H, *m*, H-3), 5.62 (1H, *m*, H-9), 5.65 (1H, *dd*, $J=7.0$ and 14.5 Hz, H-8), 5.89 (1H, *d*, $J=14.5$ Hz, H-7), 7.40 (6H, *m*), 7.51 (4H, *m*). The (*R*)-MTPA ester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 0.95 (3H, *s*, H-12), 1.04 (3H, *s*, H-11), 1.10 (3H, *s*, H-13), 1.36 (1H, *d*, $J=6.0$ Hz, H-10), 1.40 (1H, *dd*, $J=9.0$ and 13.5 Hz, H-2_{ax}), 1.74 (1H, *dd*, $J=2.5$ and 13.5 Hz, H-2_{eq}), 1.77 (1H, *dd*, $J=7.0$ and 14.5 Hz, H-4_{ax}), 2.40 (1H, *dd*, $J=5.0$ and 14.5 Hz, H-4_{eq}), 3.53 (6H, *s*, OMe), 5.18 (1H, *m*, H-3), 5.60 (1H, *m*, H-9), 5.71 (1H, *dd*, $J=7.0$ and 15.5 Hz, H-8), 5.99 (1H, *d*, $J=15.5$ Hz, H-7), 7.40 (6H, *m*), 7.51 (4H, *m*).

*3.3.12. Preparation of (R) and (S)-MTPA esters of **11***

The (*R*) and (*S*)-MTPA esters of **11** were prepared using the same procedure described for the compound **2.** The (*S*)-MTPA diester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 1.25 (6H, *s*, H-11 and H-12), 1.36 (3H, *d*, $J=6.0$ Hz, H-10), 2.03 (3H, *s*, H-13), 2.25 (2H, *s*, H-2), 2.79 (2H, *m*, H-8), 3.55 (3H, *s*, OMe), 5.30 (1H, *m*, H-9), 5.94 (1H, *t*, $J=6.9$ Hz, H-7), 5.93 (1H, *s*, H-4), 7.40 (6H, *m*), 7.52 (4H, *m*). The (*R*)-MTPA diester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 1.25 (3H, *s*, H-11), 1.26 (3H, s, H-12), 1.44 (3H, *d*, $J=6.0$ Hz, H-10), 2.23 (2H, *s*, H-2), 2.02 (3H, *s*, H-13), 2.75 (2H, *m*, H-8), 3.52 (3H, *s*, OMe), 5.32 (1H, *m*, H-9), 5.84 (1H, *t*, $J=6.9$ Hz, H-7), 5.89 (1H, *s*, H-4), 7.39 (6H, *m*), 7.52 (4H, *m*).

*3.3.13. Preparation of (R) and (S)-MTPA esters of **12***

The (*R*) and (*S*)-MTPA esters of **12** were prepared using the same procedure described for the compound **2.** The (*S*)-MTPA diester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 1.12 (3H, *s*, H-11), 1.13 (3H, s, H-12), 1.33 (3H, *d*, $J=6.0$ Hz, H-10), 2.17 (3H, *s*, H-13), 2.19 (2H, *s*, H-2), 2.66 (2H, *m*, H-8), 3.52 (3H, *s*, OMe), 5.28 (1H, *m*, H-9), 5.62 (1H, *t*, $J=6.9$ Hz, H-7), 5.92 (1H, *s*, H-4), 7.39 (6H, *m*), 7.52 (4H, *m*). The (*R*)-MTPA diester had the ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 1.06 (3H, *s*, H-11), 1.09 (3H, s, H-12), 1.40 (3H, *d*, $J=6.0$ Hz, H-10), 2.16 (2H, *s*, H-2), 2.17 (3H, *s*, H-13), 2.62 (2H, *m*, H-8), 3.55 (3H, *s*, OMe), 5.26 (1H, *m*, H-9), 5.53 (1H, *t*, $J=6.9$ Hz, H-7), 5.90 (1H, *s*, H-4), 7.40 (6H, *m*), 7.52 (4H, *m*).

3.4. Bioassays

Seeds of *Lactuca sativa* L. (cv Parella) collected during 2001, were obtained from Blumen® (Milan, Italy). All undersized or damaged seeds were discarded and the assay seeds were selected for uniformity.

Bioassays used Petri dishes (90 mm diameter) with one sheet of Whatman N° 1 filter paper as support. In four replicate experiments, germination and growth were conducted in aq. solns. at controlled pH. Test solns. (10^{-4} M) were prepared using MES (2-[*N*-morpholino]ethanesulfonic acid, 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 solns, 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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