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POTENTIAL ALLELOPATHIC GUAIANOLIDES FROM CULTIVAR SUNFLOWER LEAVES, VAR. SH-222*

FRANCISCO A. MACÍAS,† ROSA M. VARELA, ASCENSIÓN TORRES and JOSÉ MARÍA G. MOLINILLO

Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Cádiz. Apdo. 40, 11510 Puerto Real, Cádiz, Spain

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Key Word Index—*Helianthus annuus*; Asteraceae; Heliantheae; sunflower; sesquiterpene lactones; guaianolides; allelopathy; *Lactuca sativa*; *Hordeum vulgare*.

Abstract—The leaf aqueous extracts of cultivar sunflower (*Helianthus annuus* L.) var. SH-222® afforded, from the medium polar fractions, five new guaianolides, the annuolides A–E which have been tested as allelochemicals. Their structures and stereochemistry were elucidated by spectroscopic analysis. A series of aqueous solutions at 10^{-4}–10^{-9} M of annuolides A–E were tested for their effects on the germination and growth of the dicotyledon *Lactuca sativa* and the monocotyledon *Hordeum vulgare* species. All five guaianolides posses potential allelopathic activity, in particular over dicotyledon species, and are likely to be significantly involved in the allelopathic action of cultivar sunflowers.

INTRODUCTION

Cultivation of sunflowers is predominantly performed to produce oil and plays an important role in southern parts of Spain and France. Sunflower has become more and more important as an oilseed crop and ranks as the second most important source of vegetable oil in the world [1].

Biochemical investigations on sunflower reveal that this species is a rich source of sesquiterpenoids [2–6] and other plant metabolites with a wide spectrum in biological activities [7–9]. In spite of the sunflower's world wide economical importance, little is known about the function of its compounds. Recent investigations have shown that sunflowers can actively influence the growth of surrounding plants [10, 11], but the mechanism of these so-called allelopathic effects is unresolved.

In continuation of our systematic allelopathic activity studies of the different varieties of *Helianthus annuus* L., we have analysed *H. annuus* L. var. SH-222® leaf aqueous extract. This variety is in commercial use by 'Semillas Pacífico' and is widely employed as an oil production crop in the Andalusia region (Spain). From the medium polar active fractions, we isolated five new guaianolides which were identified by spectroscopic techniques (MS, IR and NMR). The structure elucidation of the new sesquiterpene lactones, which were named annuolides A–E is described below.

In order to evaluate their potential allelopathic activity, we have studied the effect of a series of aqueous solutions at 10^{-4}–10^{-9} M of the annuolides A–E on root and shoot lengths of *Lactuca sativa* var. *nigra* seedlings (dicotyledon) and *H. vulgare* L. seedlings (monocotyledon).

RESULTS AND DISCUSSION

Extraction of the fresh leaf aqueous extract of *H. annuus* L. var SH-222® with methylene dichloride afforded, after chromatography, guaianolide-type sesquiterpene lactones of increasing polarity, annuolides A–E. This is the first report of guaianolides from *H. annuus* L.

Annuolide A (**1**) is a gum with a molecular ion at *m/z* 246 which together with the ^{1}H and ^{13}C NMR data (Tables 1, 2) was in agreement with the molecular formula $C_{15}H_{18}O_3$. The additional peak at *m/z* 228 $[M-18]^+$, the absorptions at 3419 (hydroxyl group), 1751 cm^{-1} (α,β-unsaturated-γ-lactone) in the IR spectrum and the signal at δ61.2 in the ^{13}C NMR spectrum showed that it is a sesquiterpene lactone with a hydroxyl group.

The ^{1}H NMR data of **1** (Table 1) exhibited typical signals of a guaianolide sesquiterpene lactone with an exocyclic α-methylene-γ-lactone at δ6.21 and 5.50 assigned to H-13a and H-13b; a methylene group at δ4.91 and 4.86 assigned to H-14 and H-14', a hydroxymethylene group at δ4.28 as a broad singlet (2H) assigned to H-15, and a vinyl proton at 5.86 (H-3).

The ^{1}H NMR 2D COSY spectrum of **1** showed two series of proton couplings, H-6 (*dd*, 4.08) with H-5 (*dd*, 3.08) and H-7 (*dddd*, 2.85), the C-5 proton with H-1 (*ddd*, 3.19), H-1 with the two H-2 (*ddd*, 2.54), H-2 with H-3 (*dd*, 5.96). The C-7 proton with H-8α (*ddd*, 2.12), H-8β (*ddd*,

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†Author to whom correspondence should be addressed.

Table 1. ^{1}H NMR of annuolides **1**–**5** (399.95 MHz, $CDCl_3$, signal of residual $CHCl_3$ centred at δ7.25)*

H		1	2	3	4	5
1		3.19(*ddd*)	3.15	3.44	3.41	3.40
2α		2.54(*ddd*)	2.51	1.82(*dddd*)	1.81	1.79
2β		2.54(*ddd*)	2.51	2.03(*dddd*)	2.02	2.01
3		5.86(*dd*)	5.83	2.55(*ddd*)	2.55	2.53
5		3.08(*dd*)	3.00	2.90(*dddd*)	2.84	2.86
6		4.08(*dd*)	4.01	3.87	3.84	3.99
7		2.85(*dddd*)	1.89	3.21(*ddddd*)	2.33(*dddd*)	3.82
8α		2.12(*dddd*)	1.98	1.61(*ddd*)	1.52	1.55(*dddd*)
8β		1.44(*dddd*)	1.35	2.31(*ddd*)	2.21	2.01
9α		2.27(*ddd*)	2.13	—	—	—
9β		2.54(*ddd*)	2.51	4.55(*dd*)	4.51	4.55
11		—	2.21(*qd*)	—	2.21	2.68
13	13a	5.50(*d*)	1.22	5.50	1.25	1.15
	13b	6.21(*d*)		6.23		
14		4.91(*s*)	4.88	5.06	5.01	5.00
14′		4.86(*s*)	4.82	4.86	4.82	4.82
15		4.28(*brs*)	4.24(*s*)	5.24(*ddd*)	5.16	5.18
15′				5.05(*ddd*)	5.02	5.04

*Multiplicities are not repeated if identical with those in the preceding column.

J (Hz): **1**: 1,2α = 7.9; 1,2β = 4.8; 6,7 = 10.3; 7,8α = 3.3; 7,13a = 7, 13*b* = 3.2; 8α,9α = 5.3; 8β,9α = 9.1; 8β,9β = 5.2. **2**: 1,2α = 8.9; 1,2β = 5.8; 6,7 = 9.0; 7,8α = 3.8; 7,11β = 13.6; 8α,9α = 8β,9β = 4.4; 11β,13 = 7.5. **1,2**: 1,5 = 9.0: 2α,3 = 2β,3 = 1.6; 5,6 = 9.0; 7,8β = 8.8; 8α,8β = 13.1; 9α,9β = 13.0. **3**: 1,2α = 3.3: 1,2β = 9.0; 2α,3α = 2α,3β = 2β,3α = 2β,3β = 7.5; 2α,2β = 15.7; 1,5 = 5,6 = 6,7 = 9.1; 7,8α = 3.3; 7,8β = 11.0; 7,13α = 3.1; 7,13β = 3.4; 8α,8β = 13.9; 8α,9β = 8β,9β = 3.8; 15,3α = 15,3β = 15,5 = 2.6; 15′,3α = 15′,3β = 15′,5 = 2.3. **4**: 2α,2β = 14.3; 7,8α = 2.9; 7,8β = 9.5; 7,11α = 11.9; 11α,13 = 7.0; 8α,8β = 14.1; 15,3α = 15, 3β = 15,5 = 15′,3α = 15′,3β = 15′,5 = 2.6. **5**: 2α,2β = 13.5; 7,8α = 3.6; 7,8β = 11.7; 7,11 = 11,13 = 7.7; 8α,8β = 13.3; 15,3α = 15,3β = 15,5 = 15′,3α = 15′,3β = 15′,5 = 1.9. **4,5**: 1,2α = 4.4; 1,2β = 8.7; 1,5 = 8.5; 2α,3α = 2α,3β = 2β,3α = 2β,3β = 8.1; 5,6 = 6,7 = 9.5; 8α,9β = 8β,9β = 3.4.

1.44), H-13a (*d*, 5.50) and H-13b (*d*, 6.21), as well as H-8α and H-8β with H-9α (*ddd*, 2.27) and H-9β (*ddd*, 2.54). These findings required that the hydroxyl group had to be at C-15 and the double bond placed between C-3 and C-4. The ^{13}C NMR spectrum of **1** (Table 2) was assigned with the aid of heteronuclear multipulse APT experiment, 2D COSY and ^{1}H–^{13}C correlations.

Annuolide B (**2**) ^{1}H NMR spectrum was similar to that of **1** except for the absence of the exocyclic methylene proton signals and the appearance of a doublet proton signal of H-13 at δ1.22. From these data, **2** was assumed to be the dihydro derivative of **1**, and this was supported by ^{13}C NMR data (Table 2). The observed coupling of $J_{7,11}$ (13 Hz) showed that the hydrogens at C-11 and C-7 had a *trans*-arrangement [12].

Annuolide C (**3**), $C_{15}H_{18}O_3$, showed in its IR spectrum strong absorptions at 3440 (hydroxyl group), 1746 cm^{-1} (α,β-unsaturated-γ-lactone). In addition to signals of an exocyclic α-methylene-γ-lactone at δ6.23 (H-13b) and 5.50 (H-13a), signals due to two exomethylene groups at δ5.24 (H-15), 5.06 (H-14), 5.05 (H-15′) and 4.86 (H-14′) were observed in the ^{1}H NMR spectrum (Table 1). The ^{1}H and ^{13}C NMR spectra suggested a guaianolide-type skeleton with a hydroxyl group at the seven member ring portion of the molecule.

A 2D COSY study showed the following series of coupling protons for the seven member ring moiety: H-6β (*dd*, 3.87) to H-7α (*ddddd*, 3.21), H-7α with H-8α (*ddd*, 1.61) and H-8β (*ddd*, 2.31) and these protons with H-9β (*dd*, 4.55). The stereochemistry of the hydroxyl group attached at C-9 must be α-orientated since the signals of H-1 were shifted down-field approximately 0.3 ppm from the usual value [13–16]. This stereochemistry was further substantiated by comparison with 9α-acyloxy guaianolide derivatives [16, 17]. The ^{13}C NMR spectral signals (Table 2) were assigned by the use of APT experiments.

Annuolides D (**4**) and E (**5**), $C_{15}H_{20}O_3$, were isolated as gums. Comparison of the ^{1}H and ^{13}C NMR spectra of **4** and **5** with that of the previously described lactone **3** showed one significant difference: the absence of the α-methylene-γ-lactone proton signals and the appearance of a doublet proton signal at δ1.25 and 1.15 (H-13), respectively. Consequently, **4** and **5** must be dihydro derivatives of **3**. The large coupling constant $J_{7\alpha,11\beta}$ = 11.9 Hz for **4** required a β-orientation of H-11, and the observed coupling constant $J_{7\alpha,11\alpha}$ = 7.7 Hz is in accordance with the α-orientation of H-11 for **5**. The other parameters in the ^{1}H and ^{13}C NMR spectra of **4** and **5** were very similar to **3** and were assigned on the basis of 2D COSY and APT experiments (Tables 1, 2).

Table 2. ^{13}C NMR of annuolides 1–5 (100.23 MHz, $CDCl_3$, signal centred at δ77.00)*

C	1†	2	3	4	5
1	46.9 *d*	46.6	40.5	38.4	39.0‡
2	37.1 *t*	36.9‡	29.7	29.7	29.8
3	128.5 *d*	128.4	32.9 *t*	32.7	32.9
4	151.9 *s*	149.4	151.0	151.0	151.0
5	53.4 *d*	53.1	51.5	51.7	51.9
6	85.0 *d*	84.6	86.3	85.5	85.6
7	45.8 *d*	50.9	45.7	44.0	38.9‡
8	31.2 *t*	32.9	38.9	39.8	36.1
9	35.8 *t*	36.7‡	74.0 *d*	74.4	74.3
10	146.8 *s*	143.4	141.9	142.4	130.9
11	130.4 *s*	41.9 *d*	129.7 *s*	41.7	38.4
12	169.0 *s*	178.0	171.0	177.9	177.9
13	120.7 *t*	13.2 *q*	120.4 *t*	13.1 *q*	11.4
14	113.4 *t*	112.7	112.8	112.4	112.1
15	61.2 *t*	61.4	109.1	108.8	108.9

* Degree of protonation was obtained by APT heteronuclear multipulse programs; multiplicities are not repeated if identical with those in the preceding column.

† The assignments were obtained by ^{13}C–^{1}H correlation.

‡ Paired values in the same column may be interchanged.

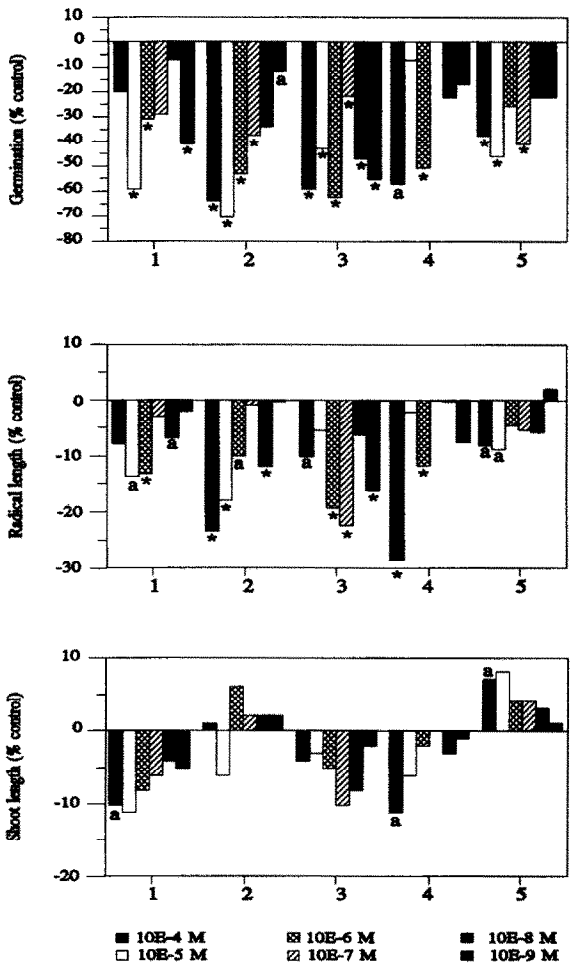
There are several contributions about the regulatory activity on the germination and plant growth of sesquiterpene lactones [18, 19]. These have been related to the presence of an α-methylene-γ-lactone moiety [18]. Nevertheless, recent studies have shown that the activity is clearly affected by the conformation of the molecules and the accessibility of groups which can be alkylated [20].

As observed with the fraction from which they were isolated, **2** and **3** showed (Fig. 1) a high inhibitory activity on the germination of *L. sativa* seeds in high and low concentrations (10^{-5} M **2**: −71%; 10^{-6} M **3**: −62%). The effects on the radical and shoot length are, in general, of little or no significance. The most powerful inhibitory effects on the radical length are those showed by α-orientated methyl dihydro derivatives (10^{-4} M **2**: −23%; 10^{-4} M **4**: −28%).

These compounds have a small effect on the germination and growth of *Ho. vulgare* seeds (Fig. 2), except for **1** and **4**. Compound **1** has an inhibitory effect on the radical length (10^{-4} M, −19%) and there are stimulatory effects on germination promoted by **1** (10^{-5} M, 27%) and **4** (10^{-5} M, 17%; 10^{-6} M, 23%).

These data confirm that the bioactivity of these compounds is not exclusively related to the presence of an α-methylene-γ-lactone moiety as shown by **2**, but this group seems to permit the effect at higher dilutions.

The above findings suggest that the guaianolides **1–5** are likely to be significantly involved in the allelopathic action of cultivar sunflowers with a certain specificity over dicotyledon species.



* Values are significantly different with $P < 0.01$ for Student's t test
a: Values significantly different with $0.01 < P < 0.05$.

Fig. 1. Effect of annuolides **1–5** on the germination, radicle and shoot length of *Lactuca sativa*.

EXPERIMENTAL

Plant material. Leaves of *H. annuus* L. var. SH-222® commercialized by 'Semillas Pacífico' (Spain) were collected in August 1991 during the third plant development stage (plants 1.2 m tall with flowers, 1 month before harvest) and were provided by Rancho de la Merced, Agricultural Research Station, Junta de Andalucía, Jerez, Spain. The collection period was established on the basis of bioactivity exhibited by the different leaf aq. extracts corresponding to 4 different plant development stages [11].

Extraction and isolation. Fresh leaves (6.0 kg) were soaked with H_2O (wt plant: V. solvent 1:3) for 24 hr at 25° in the dark. The H_2O extracts were re-extracted (8 ×) with 0.5 l of CH_2Cl_2 for each 1.0 l of H_2O, and the combined extracts were dried over Na_2SO_4 and evapd *in vacuo* to yield 16.0 g of crude extract termed H_2O–CH_2Cl_2 extract which was sepd by CC on silica gel using *n*-hexane–EtOAc mixs of increasing polarity yielding 170 × 50 ml frs which were reduced to 17 frs after comparison by CCF.

Table 3. Statistical results of allelopathic bioassays (using *L. sativa*) of annuolides 1–5*

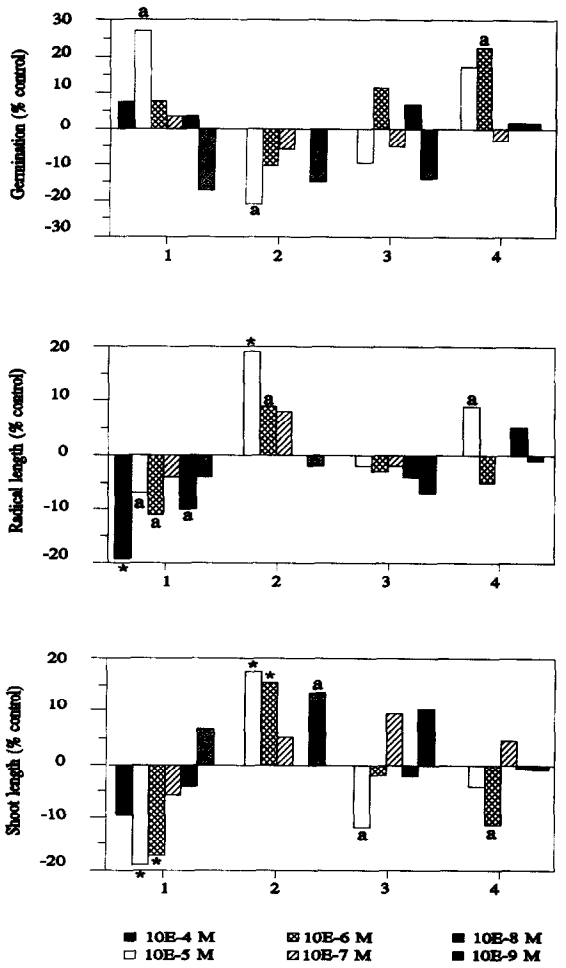
	(%) Germination						(%) Radical length						(%) Shoot length					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
1	−20[b]	−59	−31	−29[b]	−7[b]	−41	−8[b]	−14[a]	−13	−3[b]	−7[a]	−2[b]	−10[a]	−11[b]	−8[b]	−6[b]	−4[b]	−5[b]
2	−64	−71	−53	−38	−34[b]	−12[a]	−23	−18	−10[a]	−1[b]	−12	0	+1[b]	−6[b]	+6[b]	+2[b]	+2[b]	+2[b]
3	−59	−43	−62	−22	−47	−55	−10[a]	−6[b]	−19	−22	−6[b]	−16	−4[b]	−3[b]	−5[b]	−10[b]	−8[b]	−2[b]
4	−57[a]	−7[b]	−51	—	−22[b]	−17[b]	−28	−2[b]	−12	—	0	−8[b]	−11[a]	−6[b]	−2[b]	—	−3[b]	−1[b]
5	−38	−43	−25[b]	−41	−22[b]	−22[b]	−10[a]	−9[a]	−5[b]	−5[b]	−6[b]	+2[b]	+7[a]	+8[b]	+4[b]	+4[b]	+3[b]	+1[b]

*Values are expressed as percentage from the control and are significantly different with $P<0.01$ for Student's t-test.
[a]Values significantly different with $0.01<P<0.05$.
[b]Values significantly different with $P>0.05$.

Table 4. Statistical results of allelopathic bioassays (using *Ho. vulgare*) of annuolides 1–4*

	(%) Germination						(%) Radical length						(%) Shoot length					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
1	+7[b]	+27[a]	+ 8[b]	+3[b]	+3[b]	−17[b]	−19	−7[a]	−11[a]	−4[b]	−10[a]	−4[b]	−10[b]	−19	−17	−6[b]	−4[b]	+7[b]
2	—	−21[a]	−10[b]	−6[b]	—	−15[b]	—	+19	+9[a]	+8[b]	—	−2[b]	—	+18	+16	+5[b]	—	+14[a]
3	—	−10[b]	+11[b]	−5[b]	+7[b]	−14[b]	—	−2[b]	−3[b]	−2[b]	−4[b]	−7[b]	—	−12[a]	−2[b]	+10[b]	−2[b]	+11[b]
4	—	+17[b]	+23[a]	−3[b]	+2[b]	+2[b]	—	+9[a]	−5[b]	0	+5[b]	−1[b]	—	−4[b]	−12[a]	+5[b]	−1[b]	−1[b]

*Values are expressed as percentage from the control and are significantly different with $P<0.01$ for Student's t-test.
[a]Values significantly different with $0.01<P<0.05$.
[b]Values significantly different with $P>0.05$.



* Values are significantly different with $P < 0.01$ for Student's t test
a: Values significantly different with $0.01 < P < 0.05$.

Fig. 2. Effect of annuolides 1–4 on the germination, radicle and shoot length of *Hordeum vulgare*.

By following the bioactivity exhibited by the medium polar frs on *L. sativa* and *Ho. vulgare*, fr. 8 was chromatographed using silica gel (with N_2 pressure), and eluting with $CHCl_3$–*t*-BuOH (2 l), EtOAc (1 l) and MeOH (1 l). Frs 12–25 were combined and chromatographed using HPLC with a Hibar Si60 (Merck) column, *n*-hexane–EtOAc (3:1) as éluent, and 4 ml min^{-1} flow yielding **1** (10 mg), **2** (5 mg), **3** (8 mg), **4** (3 mg) and **5** (3 mg).

Annuolide A (**1**). $C_{15}H_{18}O_3$, colourless oil; IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3419 (OH), 1751 (α,β-unsaturated-γ-lactone), 1635 (double bonds); EIMS (70 eV) *m/z* (rel. int.): 246 $[M]^+$ (5), 228 $[M-H_2O]^+$ (14); ^1H NMR; Table 1; ^{13}C NMR: Table 2.

Annuolide B (**2**). $C_{15}H_{20}O_3$, colourless oil; IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3408 (OH), 1758 (γ-lactone), 1631 (double bonds); EIMS (70 eV) *m/z* (rel. int.): 248 $[M]^+$ (8), 233 $[M-Me]^+$, 230 $[M-H_2O]^+$ (14); ^1H NMR: Table 1; ^{13}C NMR: Table 2.

Annuolide C (**3**). $C_{15}H_{18}O_3$, colourless oil; IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3440 (OH), 1746 (α,β-unsaturated-γ-lactone), 1637 (double bonds); EIMS (70 eV) *m/z* (rel. int.) :246 $[M]^+$ (4), 228 $[M-H_2O]^+$ (10); ^1H NMR: Table 1; ^{13}C NMR: Table 2.

Annuolide D (**4**). $C_{15}H_{20}O_3$, colourless oil; IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3440 (OH), 1757 (γ-lactone), 1637 (double bonds); EIMS (70 eV) *m/z* (rel. int.): 248 $[M]^+$ (9), 233 $[M-Me]^+$ (6), 230 $[M-H_2O]^+$ (9); ^1H NMR: Table 1; ^{13}C NMR: Table 2.

Annuolide E (**5**). $C_{15}H_{20}O_3$, colourless oil; $[\alpha]_D^{25} = +6°$ ($CHCl_3$; *c* 0.11); IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3440 (OH), 1755 (γ-lactone), 1634 (double bonds); EIMS (70 eV) *m/z* (rel. int.) :248 $[M]^+$ (5), 233 $[M-CH_3]^+$ (3), 230 $[M-H_2O]^+$ (10); ^1H NMR: Table 1; ^{13}C NMR: Table 2.

Lettuce and barley seed germination bioassay. Seeds of lettuce, *L. sativa* var. nigra and *Ho. vulgare* 1991 crop, were obtained from Rancho La Merced, Junta de Andalucía, Jerez, Spain. All undersized and damaged seeds were discarded and the assay seeds were selected for uniformity of size.

Germination bioassays consisted of germinating 25 lettuce seeds for 5 days (3 for germination and 2 for root and shoot growth) and 5 *Ho. vulgare* seeds for 3 days, in the dark at 25° in 9-cm plastic Petri dishes containing a 10-cm sheet of Whatman no. 1 filter paper and 10 ml of a test of control soln for lettuce, and 5 ml for *Ho. vulgare*. Test solns (10^{-4} M) were prepd using DMSO (0.1% v/v) as initial solubilizing agent. Test solns 10^{-5}–10^{-9} M were obtained by diluting the previous soln. Parallel controls consisted of deionized H_2O with the same DMSO concn. There were 3 replicates for *L. sativa* and 19 for *Ho. vulgare* of each treatment, and of parallel controls. The number of seeds per replicate, time and temp. of germination were chosen in agreement with a number of preliminary experiments, varying the number of seeds, vol. of test soln per dish and the incubation period. All the pH values were adjusted to 6.0 before the bioassay using MES (2-[N-Morpholino] ethanesulfonic acid, 10 mM).

Statistical treatment. The germination, root and shoot length values were tested by the Student's *t*-test, being the differences between the experiment and the control, significant with a value of $P = 0.01$.

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