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POTENTIAL ALLELOPATHIC SESQUITERPENE LACTONES FROM SUNFLOWER LEAVES*

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Key Word Index—*Helianthus annuus* cultivar; Compositae; sesquiterpene lactones; guaianolides; annuolides A, C, F, G; *trans,trans*-germacranolides; melampolides; heliangolides; *cis,cis*-germacranolides; annuithrin; helivypolides A-B; 1,2-anhydrido-4,5-dihydroniveusin A; allelopathy.

Abstract—Isolation, structure elucidation and allelopathic bioassays of 13 sesquiterpene lactones, four of them new, from the sunflower cultivar VYP® are described. Structures of the lactone assigned as 1,2-anhydrido-4,5-dihydroniveusin A, previously isolated from capitate glandular trichomes of *Helianthus annuus* and of annuithrin are corrected. The effect of a series of aqueous solutions at 10^{-4}–10^{-9} M of the sesquiterpene lactones was studied on the root and shoot lengths of *Lactuca sativa var. nigra, Lepidium sativum, Lycopersicon esculentum,* seedlings (dicotyledons) and *Hordeum vulgare* and *Triticum estivum* (monocotyledons). The guaianolides generally had no effect on the germination and growth of *L. sativum* and *L. esculentum*, except for C-10 epimers 8β-angeloyloxycumambranolide and annuolide G where inhibitory effects were found on the shoot length of *L. esculentum*. Both exhibit similar activity profiles, the most persistenty active compound on dilution was 8β-angeloyloxycumambranolide with an α-orientated hydroxyl group at C-10. The conformational changes due to functionalization within germacranolides influence principally root and shoot growth. Heliangolides have greater effect on root and shoot length of dicotyledon species, presumably due to conformational flexibility and the presence of electrophylic groups. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Farmers annually endure significant crop losses due to plant pathogenic bacteria and fungi, insects and weeds. Modern herbicides have improved yields and quality of many agricultural products, but have undesirable side-effects such as residual contamination, resistance, ecosystem impairments and waste generation [1]. Plants have physical adaptations for defence and produce a wide spectrum of secondary metabolites, some of which are active allelopathic agents [2]. The natural products are an attractive source of potential leads to new agrochemicals, because of the diversity and novelty of chemical structures produced by living organisms and the potential specificity of biological action and the greatly reduced likelihood of harmful bioaccumulation on soil and ground water residues [3].

In continuation of our systematic study of the allelopathic activity of different cultivars of *Helianthus annuus* L., we report the isolation, structural elucidation and allelopathic bioassay results of 13 sesquiterpene lactones, guaianolides **1–6**, germacranolides **7** and **15**, heliangolides **8–11**, **13**, the *cis,cis*-germacradienolide **14** and the melampolide **16**, from the sunflower cultivar VYP® which is in commercial use by KOYPE™. Annuolide F (**5**) and G (**6**), and helivypolides **A-B** (**14–16**) are new. The structure of a lactone previously assigned formula **12** [4] is corrected to **13**. Lactone **8** appears to be identical with the previously described [5] of undefined stereochemistry.

In order to evaluate the potential allelopathic activity of the lactones, we have studied the effect of a series of aqueous solutions at 10^{-4}–10^{-9} M on the root and shoot lengths of *Lactuca sativa* var. *nigra, Lepidium sativum, Lycopersicon esculentum,* seedlings (dicotyledons) and *Hordeum vulgare* and *Triticum aestivum* (monocotyledons).

RESULTS AND DISCUSSION

The extraction of the fresh leaf aqueous extract of *H. annuus* L. var. VYP® with dichloromethane gave material which was chromatographed on a silica gel

*Part 7 in the series 'Allelopathic studies in cultivar species' For part 6 see Proceedings of the International Symposium 'Allelopathy in Sustainable Agriculture, Forestry and Environment'. F.A. Macías, R.M. Varela, A. Torres and J.M.G. Molinillo 'Potentiality of Important Agricultural Commercial Crops as Source of Natural Herbicide Models: The Case of Cultivar Sunflowers' Chapter 4, New Delhi, India (January 1996).

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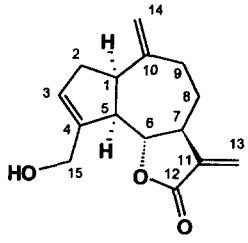

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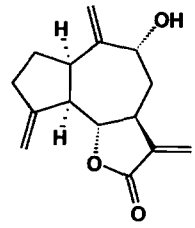

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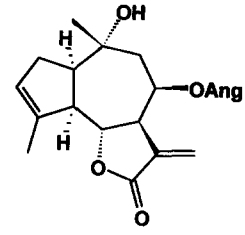

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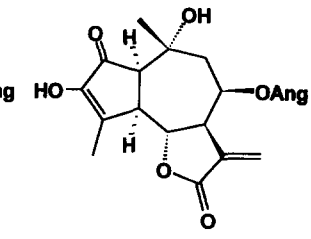

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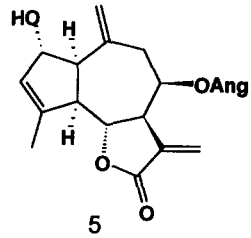

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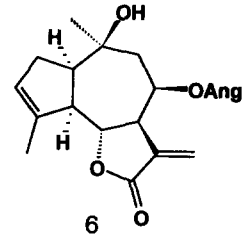

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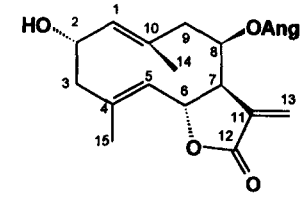

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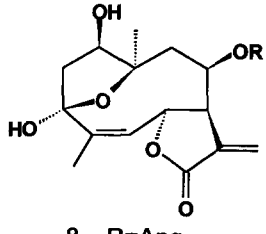
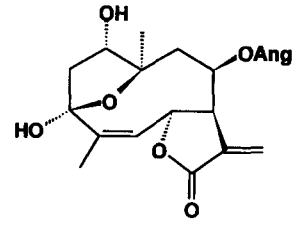


8 R=Ang
8a R=Meac



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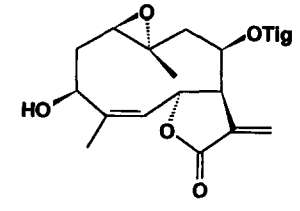

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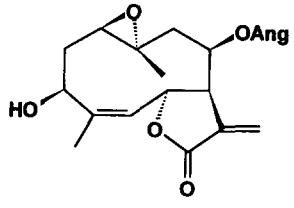

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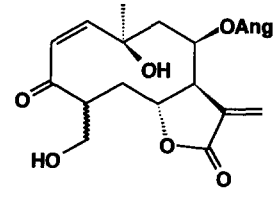

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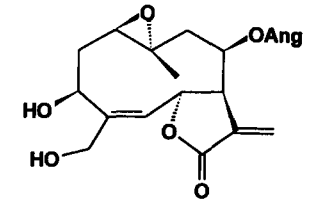

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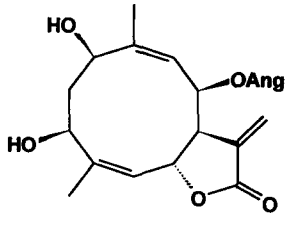

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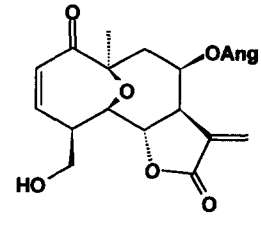

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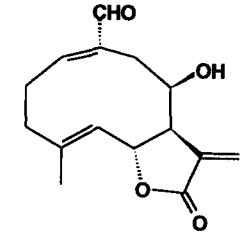

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column eluted with hexane–EtOAc mixtures of increasing polarity. Medium polar fractions yielded 13 sesquiterpene lactones, six guaianolides and seven germacranolides. Spectroscopic data of **1, 2** [6], **3** [7], **4** [8], **7** [9], **10** [10], **11** [11, 12] and **16** [13] were identical to those reported previously.

Annuolide F (**5**) was isolated as a gum, $[M]^+$ at *m/z* 344, which together with the ^1H NMR data (Table 1) was in agreement with the molecular formula $C_{20}H_{24}O_5$. The additional peak at *m/z* 326 $[M-18]^+$ in the mass spectrum and the absorptions at 3550 (hydroxyl group), 1760 (α,β-unsaturated-γ-lactone) and 1700 cm^{-1} (α,β-unsaturated ester) in the IR spectrum, showed that **5** was a sesquiterpene lactone with a hydroxyl group and an ester moiety. Additional mass spectral peaks at *m/z* 244 $[M-C_5H_8O_2]^+$, 83

Table 1. ^{1}H NMR spectra of lactones **5**, **6**, **8** and **13–15** (399.95 MHz, $CDCl_3$ signal of residual $CHCl_3$ centred at δ 7.25)*

H	5	6	8	13	14	15
1	2.93 *dd*	2.20 *ddd*	4.14 *dd*	2.80 *m*	4.82 *dd*	
2						6.33 *d*
2α		1.50 *dd*	2.10 *dd*	2.46 *dd*	2.23 *ddd*	
2β	4.79 *br s*	2.08 dd	2.57*dd*	1.77 *dd*	2.15 *ddd*	
3α	5.71 *br q*	5.68 *br s*		4.61 *dd*	4.55 *br s*	5.93 *dd*
3β				4.61 *dd*	4.55 *br s*	
4						3.09 *dddd*
5	3.06 *br dd*	2.49 *dd*	5.89 *d*	5.58 *d*	5.45 *br d*	4.23 *dd*
6	4.43 *dd*	4.31	5.50	6.61	5.93	5.14
7	3.18 *dddd*	4.07	4.18 *m*	2.91	3.16 *dddd*	3.41
8α	5.63 *ddd*	5.64	5.67	5.20 *m*	6.00 *dd*	5.78 *ddd*
9α	2.50 *dd*	1.60 *ddd*	1.85 *dd*	2.83	5.34 *br d*	2.14 *dd*
9β	2.68 *dd*	2.42 *ddd*	2.20 *dd*	1.32	5.34 *br d*	2.36 *dd*
13a	5.57 *d*	5.44	5.62	6.38	6.08	5.65
13b	6.31 *d*	6.27	6.27	5.78	6.29	6.33
14	4.95 *s*	1.28	1.24	1.48	1.82 *d*	1.45 *s*
14′	4.93 *s*					
15	1.94 *dd*	1.65 *s*	1.56	4.11 *m*	1.87 *d*	4.05 *m*
3′	6.06 *qq*	6.04	6.03	6.10	6.16	6.14
4′	1.91 *dq*	1.91	1.92	1.97	2.05	1.96
5′	1.71 *dq*	1.77	1.74	1.85	1.94	1.78

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

J (Hz). **5**: 1,2β=4.0; 1,5=8.0; 3,15=5,15=1.5; 5,6=10.5; 7,8α=3.0. 8α,9α=8α,9β=5.5; **6**: 1,2=6.5; 1,5=7.5; 2,2′=14; 5,6=11.3; 7,8α=4.3; 8α,9β=8.3; 8α,9α=12.4; **5,6**: 6,7=8.5; 9α,9β=14.1. **8**: 1β,2=6.2; 1β,2′=10.9; 2,2′=12.6; 5,6=4.1; 6,7=2.5; 7,8α=1.5; 7,13a=2.1; 7,13b=2.6; 8α,9α=4.8; 8α,9β=11.5; 9α,9β=15.2. **13**: 1,2α=4.5; 2α,2β=14.9; 2α,3=4.6; 2β,3=7,13a=2.3; 5,6=10.8; 6,7=2.2; 7,13b=2.0; 8α,9α=4.8; 8α,9β=4.0; 9α,9β=15.8. **14**:1,2=11.0; 1,2′=4.0; 2,2′=14.5; 2,3=3.0; 2′,3=4.0; 5,6=10.5; 6,7=10.5; 7,8α=6.0; 8α,9=10.5; 9,14=1.3. **15**: 6,7=10.0; 2,3=7,8α=8α,9β=3.0; 9α,9β=15.5; 8α,9β=3.5; 3,4=1.0; 4,5=1.5; 4,15=4,15′=6.0. **15**: 5,6=10.1. **5,6,8,13,14,15**: 3′,4′=7; 3′,5′=4′,5′=1.5. **5,6,14,15**: 7,13a=3.2; 7,13b=3.4

$[C_5H_7O]^+$ and 55 $[C_4H_7]^+$ as well as ^{1}H NMR resonances at δ 1.77 *dq*, 1.91 *dq*, and 6.06 *qq*, indicated the presence of an angelate moiety.

The ^{1}H NMR spectrum of **5** (Table 1) exhibited typical signals of a guaianolide sesquiterpene lactone with an exocyclic α-methylene-γ-lactone at δ 6.31 and 5.57 assigned to H-13b and H-13a; a methylene group at δ 4.95 and 4.93 assigned to H-14 and H-14′; and an olefinic proton at δ 4.78 (H-2). ^{1}H NMR 2D COSY spectrum of **5** showed the following series of coupling protons: H-13a (*d*, δ 5.57) and H-13b (*d*, δ 6.31) with H-7 (*dddd*, δ 3.18); H-7 with H-6 (*dd*, δ 4.43); H-6 with H-5 (*brdd*, δ 3.06); H-5 with H-1 (*dd*, δ 2.93); H-1 with H-2 (very *br s*, 4.79). The proton attached to C-3 (*m*, δ 5.71) coupled with H-5 and H-15 (*dd*, 3H, δ 1.94) as did H-15 with H-2 and H-8 (*ddd*, δ 5.63) with H-9α (*dd*, δ 2.50) and H-9β (*dd*, δ 2.68). The angeloxy moiety must be at C-8 since H-8 appeared at δ 5.63. The small coupling constant value ($J_{7,8\alpha}$ = 3 Hz) required an α-orientation of H-8, which was further substantiated by NOE difference experiments, that showed effects between H-7 and H-8. The β-orientation of H-2 was deduced from the deshielding observed in comparison with those of the epimeric compound [14] at C-2 of H-5 ($\Delta\delta$ = 0.4 ppm).

The spectroscopic data of annuolide G (**6**), $C_{20}H_{26}O_5$ were almost identical with those of **3**, previously reported from *Helianthus maximiliani* [15]. The β-orientation of the hydroxyl group at C-10 followed from the greater shielding of H-1 compared with epimer **3** (δ 2.20, $\Delta\delta$ = 0.5 ppm), H-5 (δ 2.49, $\Delta\delta$ = 0.22 ppm) and H-8α (δ 5.64, $\Delta\delta$ = 0.1 ppm). The ^{1}H NMR 2D COSY of **6** was in full agreement with the proposed structure.

Compound **8**, $C_{20}H_{26}O_7$, exhibited IR absorptions at 1750 and 1710 which suggested the presence of two carbonyl groups. Its ^{1}H NMR spectrum (Table 1) exhibited typical signals of an α-methylene-γ-lactone moiety at δ 6.27 (*d*, H-13b) and 5.62 (*d*, H-13a) and an angelate ester [δ 6.03 (*qq*, H-3′), 1.92 (*dq*, 3H, H-4′) and 1.74 (*dq*, 3H, H-5′)]. The ^{1}H and ^{13}C NMR data in Tables 1 and 2 and a careful study of the ^{1}H NMR 2D COSY spectra in $CDCl_3$ and C_6D_6 led to the structure and stereochemistry shown in **8**, with a β-oriented hydroxyl group on C-1. The ^{1}H NMR data essentially duplicated those of annuithrin [5] which was assigned an α-oriented hydroxyl group on C-1 and thought to be identical 'in most of the published data' with niveusin C (**9**) isolated more or less simultaneously from *Helianthus niveus* [16] and *H. maximiliani* [17]. The NMR data of **8** were also very similar to those of **8a** from *Calea zacatechichi* [18], if one interchanges the

Table 2. ^{13}C NMR of lactones **8** and **13** (100.23 MHz, $CDCl_3$ signal centred at δ 77.00)

C	8	13
1	207.2 *d*	
2	122.9 *d*	33.9 *t*
3	151.0 *d*	73.8 *d*
4	29.7 *d*	143.9 *s*
5	79.1 *d*	126.7 *d*
6	75.9 *d*	69.0 *d*
7	47.2 *d*	48.3 *d*
8	65.3 *d*	75.8 *d*
9	46.8 *t*	43.8 *t*
10	77.2 *s*	
11	134.0 *s*	137.1 *s*
12	175.8 *s*	169.8 *s*
13	122.9 *t*	125.1 *t*
14	32.4 *q*	19.6 *q*
15	64.0 *t*	65.3 *t*
1′	162.0 *s*	166.8 *s*
2′	127.4 *s*	126.9 *s*
3′	141.4 *s*	140.5 *d*
4′	19.2 *q*	15.8 *q*
5′	20.6 *q*	20.4 *q*

H-6 and H-2α assignments for H-8 and H-9β. Consequently annuithrin has the formula **8**.

The NMR data of known **11** and a lactone assigned structure **12** from the capitate glandular trichomes of *H. annuus* [4] were almost identical except that the methyl group at C-4 of **11** is replaced by a hydroxymethylene moiety in **12**. In ref. [4] the presence of a carbonyl group was inferred from what was described as a 'very small signal' in the ^{13}C NMR spectrum at δ 195.6. This suggests that structure **12** must be revised to **13**; the reassigned spectra are listed in Tables 1 and 2.

Helivypolide A (**14**) had the characteristic mass spectrum of a sesquiterpene lactone with an angelate ester of formula $C_{20}H_{26}O_6$. The molecular ion $[M]^+$ was not observed but the following peaks confirmed this hypothesis: *m/z* 344 $[M-H_2O]^+$, 279 $[M-C_5H_7O]^+$, 262 $[M-C_5H_8O_2]^+$, 261 $[M-C_5H_7O-H_2O]^+$, 244 $[M-H_2O-C_5H_8O_2]^+$, 83 $[C_5H_7O]^+$, 55 $[C_4H_7]^+$. Inspection of its ^1H NMR spectrum (Table 1) which displayed a 1H quartet of quartets at δ 6.16 (H-3′), a 3H doublet of quartets at δ 2.05 (H-4′) and a 3H doublet of quartets at δ 1.94 (H-5′), corroborated an angelate side chain in compound **14**. An IR absorption at 3447 cm^{-1}, together with signals at δ 4.82 and δ 4.55 in the ^1H NMR spectrum and the mass spectrum indicated the presence of two hydroxyl groups.

The ^1H NMR 2D COSY spectrum of **14** showed the following series of coupling protons: H-13a (*d*, 6.08) and H-13b (*d*, 6.29) with H-7 (*dddd*, 3.16), H-7 with H-6 (*dd*, 5.93), H-6 with H-5 (*brd*, 5.45) and H-5 with H-15 (*d*, 1.87). H-7 was also coupled with H-8 (*dd*, 6.00) and H-8 with H-9 (*brd*, 5.34). Finally H-1 (*dd*, 4.82) was coupled with H-2β (*ddd*, 2.23) and H-2α (*ddd*, 2.15) and H-2 were coupled with H-3 (*m*, 4.55). These couplings confirmed the presence of a germacranolide type skeleton with double bonds between C-4 and C-5, C-9 and C-10, and between C-11 and C-13. As in all the other compounds described the angelate moiety was at C-8 with a β-orientation. This was deduced from the chemical shift of H-8 (δ 6.00) and the small coupling constant $J_{7,8\alpha} = 6$ Hz that required an α-orientation of H-8.

The geometry of the double bonds (Δ^{4-5} and Δ^{9-10}) as well as the stereochemistry of H-1 and H-3 was established by NOE difference spectrometry (Fig. 1). Irradiation of the H-1 showed a significant effect on H-8. This accorded with a *Z*-geometry of the Δ^9 bond and a β-orientation of the hydroxyl group at C-3 as a result of the short distance between H-1 and H-8. The observed NOEs between H-5 and H-15, as well as H-3 and H-15, were in agreement with a *Z*-double bond between C-4 and C-5. An effect between H-5 and H-7

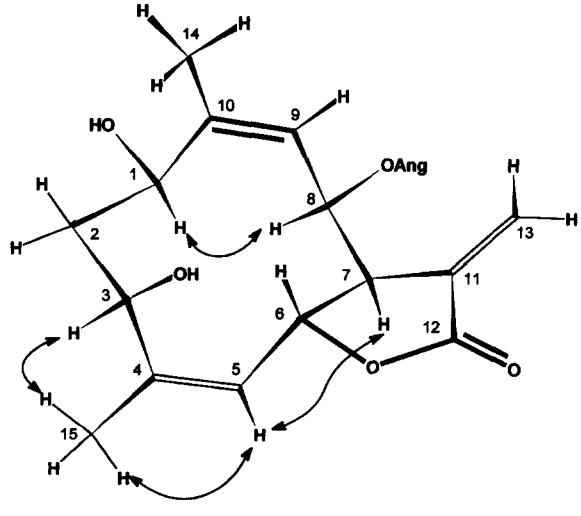

Fig. 1. Observed NOE effects for compound **14**.

indicated that H-5, the 4-methyl and H-3 lie below the plane. This fact can explain the chemical shift of H-6, which appeared at δ 5.93 due to the proximity of the hydroxyl group at C-3.

$J_{6,7}$ in heliangolides and (4,5,1,10)*cis,cis*-germacranolides is usually smaller than 3 Hz, presumably because the angle between H-6 and H-7 is approximately 90°. However, compound **14**, is of a different type, since the double bonds are at positions 4–5 and 9–10 instead of 4–5 and 1–10. To determine whether the derived structure **14** is in agreement with the most stable conformation, we carried out a conformational study using semiempirical calculations (PM3) [19]. The most stable conformations are shown in Fig. 2. The most stable one (the structure at lower right in Fig. 2) differs from that found in the majority of *cis,cis*-germacranolides. There is good agreement between the angles found for this conformation and the experimental coupling constants (Table 3). Also, this conformation explains the observed NOE's (Fig. 1), especially the one involving H-8 and H-1, which are 1.71 Å apart. The chemical shift of H-6 could be explained by the proximity (1.85 Å) of the hydroxyl group attached at C-3.

The mass spectrum of helivypolide B (**15**) showed ions at *m/z* 376 $[M]^+$, 358 $[M-H_2O]^+$ and 276 $[M-C_5H_8O_2]^+$ and exhibited the typical base peak at *m/z* 83 of a C_5-unsaturated ester side chain. The NMR and mass spectral data established the presence of

Table 3. Observed coupling constants for compound **14** *vs* Φ obtained for its most stable conformer

Protons	Calculated Φ	Experimental J (Hz)
1α–2α	68.7	4.0
1α–2β	176.8	1.1
2α–3α	49.8	4.0
2β–3α	64.2	3.0
5–6	178.6	10.5
6–7	160.4	10.5
7–8	48.3	6.0
8–9	166.5	10.5

angelic ester. The ^{1}H NMR spectrum contained typical signals of an α-methylene-γ-lactone moiety with two one-proton doublets at δ 6.33 (*d*, H-13b) and δ 5.65 (*d*, H-13a), both coupled to a signal at δ 3.41 (*dddd*, H-7). ^{1}H NMR 2D COSY studies indicated that H-7 was coupled to a doublet of doublets at δ 5.14 which was assigned to the lactonic proton at C-6, as well as H-6 with H-5 (δ 4.23, *dd*). The chemical shift of H-5 suggested the presence of an oxygen attached to C-5. H-5 was coupled with H-4 (δ 3.09, *dddd*) and H-4 with H-15 (δ 4.05, 2H, *m*), a hydroxymethylene moiety, and H-3 (δ 5.93, *dd*). Finally H-3 was coupled with H-2 (δ 6.33, *d*, $J_{2,3}$ = 3Hz).

The observed chemical shifts of H-4 (δ 3.09), H-2 (δ 6.33) and H-3 (δ 5.93) were consistent with the presence of an α,β-unsaturated carbonyl group at C-1.

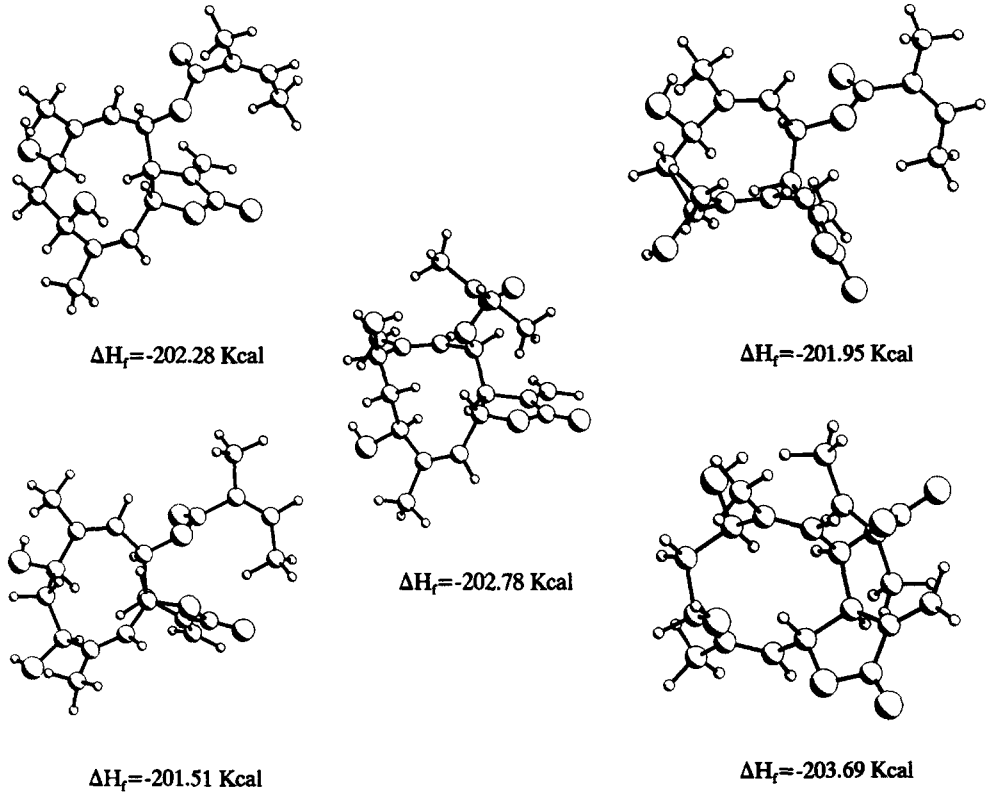

Fig. 2. Most stable conformers of compound **14**, obtained using PM3 calculations.

^{1}H NMR 2D COSY spectrum showed a second series of couplings: H-7 with H-8α (δ 5.78, *ddd*), H-8α with H-9α (δ 2.14, *dd*) and H-9β (δ 2.36, *dd*). The angelate moiety must be placed at C-8. The small value of $J_{7,8}$ (3 Hz) required an α-orientation of H-8. This was further substantiated by NOE difference experiments which showed effects between H-7 and H-8 and between H-7 and H-5, implying an α-orientation of H-5. The NOE effect observed between H-2 and H-3 confirmed a *cis*-arrangement for the C-2, C-3 double bond.

The chemical shift and the multiplicity of the signal of H-14 (δ 1.45, *s*) was in agreement with an oxygen attached to C-10. The molecular formula deduced from the mass spectrum implied an internal ether function between C-5 and C-10. This was in agreement with the observed coupling constant, $J_{2,3}$ (13 Hz) according with an unsaturated seven membered ring containing an electron withdrawing group as a substituent of the double bond [20–22]. The β-orientation for the hydroxymethylene group at C-4 was deduced from the small coupling constant $J_{4,5}$ (1.5 Hz).

An α-orientation for the methyl group attached at C-10 was assigned by comparison with the spectroscopic data of the analogous structures of chapliatrin and its derivatives [23, 24]. This was further substantied with the semiempirical calculations (PM3) of the most stable conformer that was in good accordance with the observed coupling constants for this stereostructure.

Bioassay results

There are several reports about the regulatory activity on the germination and plant growth of sesquiterpene lactones [27, 28]. This has been related to the presence of an α-methylene-γ-lactone moiety, other functionalizations and the different spacial arrangements that the molecule can adopt [27]. It seems that accessibility of groups which can be alkylated plays an important role in the activity.

In order to evaluate the potential allelopathic activity and to obtain information about the specific requirements needed for bioactivity, we have studied the effect of a series of aqueous solutions at 10^{-4}–10^{-9} M of isolated compounds on root and shoot lengths of *L. sativa* (Table 4), *L. sativum* (Table 6), *L. aesculentum* (Table 5), *H. vulgare* (Table 8) and *T. aestivum* (Table 7) seedlings.

The slightly functionalized guaianolides **1** and **2** showed a high inhibitory activity on the germination of *L. sativa* seeds in high as well as in low concentration (average -40 and -60%, respectively), and little or no effect on root and shoot length (Fig. 3). On the other hand, guaianolides **4** and **5** with a second α,β-unsaturated system, an angeloyl ester at C-8, showed stimulatory effects on the germination of lettuce (average 40%) and inhibitory effects on root (**4**, -33%, 10^{-5} M; -29%, 10^{-9} M; **5**, -25%, 10^{-7} M) and shoot length (**4**, -34%, 10^{-5} M; -34%, 10^{-7} M; -34%, 10^{-9} M; **5**, -24%, 10^{-5} M; -30%, 10^{-7} M). Compounds **1** and **2** inhibited germination, while **3–6** showed a

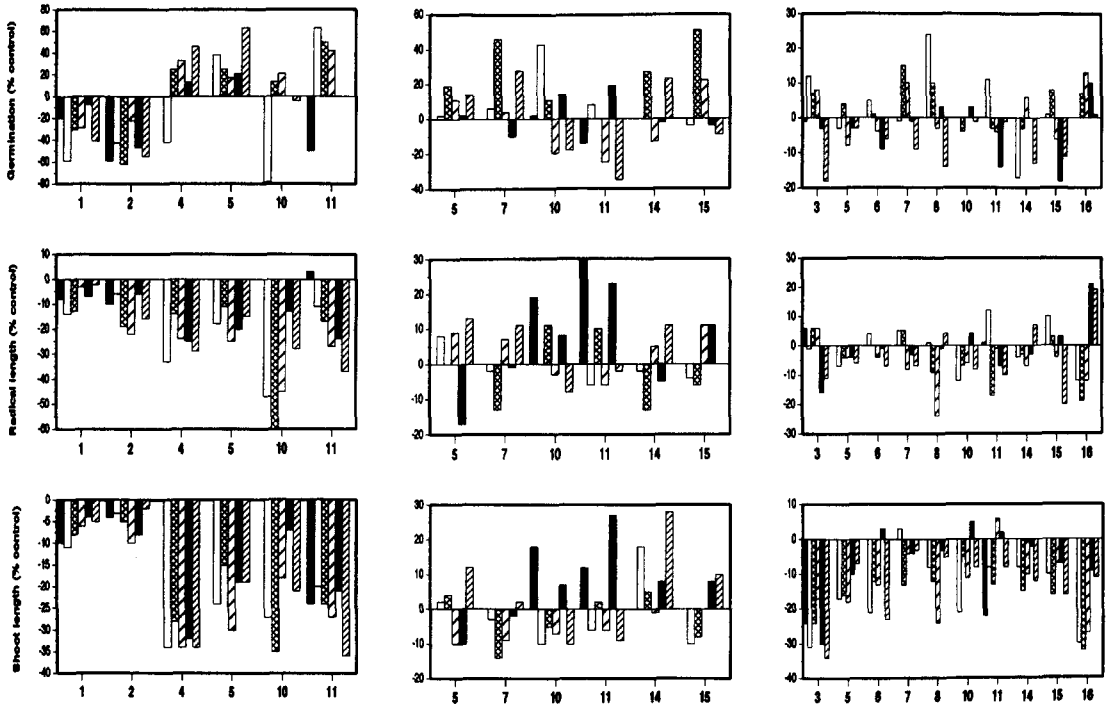

Fig. 3. Effect of lactones **1–8**, **10–11**, **14–16** on the germination, radicle and shoot length of Dicotyledon species *L. sativa* L. (left), *L. sativum* L. (centre) and *L. esculentum* L. (right). ■, 1×10^{-4} M; □, 1×10^{-5} M; ▩, 1×10^{-6} M; ▨, 1×10^{-7} M; ▦, 1×10^{-8} M; ▨, 1×10^{-9} M.

stimulatory effect on the germination and inhibitory effects on shoot and root lengths. The different activity profiles of compounds **1–2** and **3–6** can be attributed to the presence of an ester at C-8, which causes steric hindrance on the β-side of the molecule and, consequentely, less accessibility to the α-methylene-γ-lactone moiety.

Guaianolides generally have no effect on germination and growth of *L. sativum* and *L. esculentum*, except in the case of the C-10 epimers **3** and **6** which inhibit on the shoot length of *L. esculentum*. Both have similar activity profiles, the most persistantly active-compound on dilution (−30%) is **3**, with an α-orientated hydroxyl group at C-10.

The influence on germination and growth of monocotyledons was, again small except for **1** which inhibited germination of *H. vulgare* seeds and **5** which inhibited germination of *H. vulgare* and *T. aestivum* seeds (Fig. 4). The skeletons of germacranolides (**7**, **8**, **10** and **14–16**) are more flexible. Some of them have been tested previously. Compound **8** was shown to have antibacterial properties and a reduced growth in the *Avena* straight growth test [5] and **10** showed anti-auxin activity [28]. The most notable observed effects were the following: compounds **10** and **11** have related structures and both showed strong inhibitory effects at high concentration on the germination (**10**, −78%, 10^{-5} M; **11**, −50%, 10^{-4} M) and shoot (**10**, −35%, 10^{-6} M; **11**, −24%, 10^{-4} M) and root growth (**10**, −47%, 10^{-5} M; −60%, 10^{-6} M). Compound **10** inhibited shoot growth (−21%, 10^{-5} M) of *L. esculentum*. The effect on *H. vulgare* was, in general, stimulatory, especially at low concentrations. Compounds **14** and **15** inhibited germination (**14**, −53%, 10^{-9} M; **15**, −48%, 10^{-8} M) as well as shoot (**15**, −24%, 10^{-6} M) and root growth (**14**, −27%, 10^{-8} M, **15**, −22%, 10^{-8} M) of *T. aestivum*.

All the germacranolides tested possess an α-methylene-γ-lactone moiety, therefore the different profiles of activity might be attributed to the presence of a second or a third receptor site for alkylation, such as α,β-unsaturated carbonyl systems as well as to conformational changes due to the different functionalization, which will favor or hinder accessibility to the receptor sites. These effects influence fundamentally root and shoot growth, more than germination.

Compounds which possess a 4,5-*Z*-double bond (**10**, **11**, **14** and **16**) have greater effects on root and shoot length of dicotyledon species, perhaps due to conformational changes with increasing flexibility of the molecule. This is of more importance in germacranolides than in guaianolides. The presence of electrophilic groups and conformational changes could be considered the reasons for an increment in the bioactivity of these compounds.

EXPERIMENTAL

Plant material. Leaves of *H. annuus* L. var. VYP® commercialized by KOYPE™ (Spain) were collected in September 1990 during the third plant development

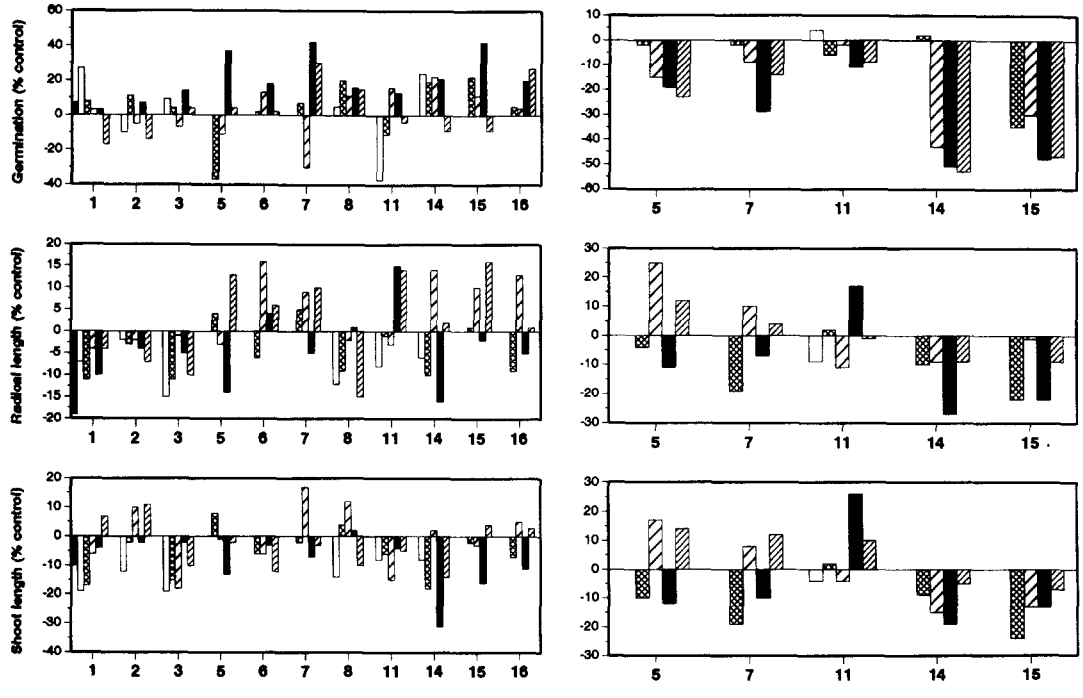

Fig. 4. Effect of lactones **1–3**, **5–8**, **11**, **14–16** on the germination, radicle and shoot length of Monocotyledon species *H. vulgare* L. (left) and *T. aestivum* L. (right). ■, 1×10^{-4} M; □, 1×10^{-5} M; ▩, 1×10^{-6} M; ▨, 1×10^{-7} M; ⬚, 1×10^{-8} M; ▨, 1×10^{-9} M.

Table 4. Statistical results of bioassays (using *L. sativa* L.) of lactones **1**, **2**, **4**, **5**, **10** and **11***

	(%) Germination						(%) Radicle 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	−59[a]	−31[a]	−29	−7	−41[a]	−8	−14[b]	−13[a]	−3	−7[b]	−2	−10[b]	−11	−8	−6	−4	−5
2	−59[a]	−43[a]	−62[a]	−22	−47[a]	−55[a]	−10[b]	−6	−19[a]	−22[a]	−6	−16[a]	−4	−3	−5	−10	−8	−2
4	–	−42[b]	+25	+33	+13	+46[a]	–	−33[a]	−14[b]	−24[b]	−25[b]	−29[b]	–	−34[a]	−28[a]	−34[a]	−32[a]	−34[a]
5	–	+38[b]	+25	+17	+21	+63[a]	–	−18	−11	−25[b]	−20[b]	−15[b]	–	−24[a]	−15[b]	−30[a]	−19[a]	−19[a]
10	–	−78[a]	+13	+21	0	−4	–	−47[b]	−60[a]	−45[a]	−13	−28	–	−27	−35[a]	−18	−7	−21
11	−50	+63	+50	+42[b]	0	0	+3	−11	−17[b]	−27[a]	−24[b]	−37[a]	−24[b]	−20[a]	−24[a]	−27[a]	−21[b]	−36[a]

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

Table 5. Statistical results of bioassays (using *L. esculentum* L.) of lactones **3**, **5–8**, **10**, **11**, **14–16***

	(%) Germination						(%) Radicle 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
3	−1	+12[b]	+7	+8	−3	−18	+6	−1	+6	+6	−16	−11	−24[b]	−31[b]	−24[a]	−17	−30[b]	−34[b]
5	–	−3	+4	−8	−3	−3	–	−7	−4	−4	−4	−6	–	−17[b]	−16[b]	−18[b]	−10	−7
6	–	+5	+1	−4	−9	−6	–	+4	0	−4	−1	−7	–	−21[b]	−12[b]	−13	+3	−23[b]
7	–	−1	+15[b]	+10	−1	−9	–	+5	+5	−8	−3	−7	–	+3	−13[b]	−4	−4	−3
8	–	+24[b]	+10	−3	+3	−14[b]	–	+1	−9	−24	−1	+4	–	−8	−12	−24	−3	−5
10	–	0	−4	0	+3	−1	–	−12	−7	−6	+4	−8	–	−21[b]	−5	−11	+5	−8
11	0	+11[b]	−3	−4	−14	−1	+1	+12[b]	−17[a]	−1	−7	−10	−22[a]	−8	−13	+6	+2	−8
14	–	−17	−3	+6	0	−13	–	−4	−3	−7	−3	+7[b]	–	−8	−15[b]	−10	−2	−12
15	–	+1	+8	−6	−18	−11	–	+10[b]	+3	−4	+3	−20[a]	–	−10	−16[b]	−7	−7	−16[b]
16	–	0	+7	+13	+10	+1	–	−12	−19[b]	−12	+21[b]	+19[b]	–	−30[a]	−32[b]	−27[b]	−9	−11[b]

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

Table 6. Statistical results of bioassays (using *L. sativum* L.) of lactones **5**, **7**, **10**, **11**, **14** and **15***

	(%) Germination						(%) Radicle 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
5	–	+2	+19	+11	+2	+14	–	+8	0	+9	−17[b]	+13	–	+2+4	−10	−10	+12	
7	–	+6	+46[b]	+4	−10	+28[b]	–	−2	−13	+7	−1	+11	–	−3	−14	−9	−2	+2
10	+2	+43	+11	−20	+14	−18	+19	0	+11	−3	+8	−8	+18	−10	−5	−7	+7	−10
11	−14	+8	0	−25	+19	−35[b]	+30[b]	−6	+10	−6	+23[b]	−2	+12	−6	+2	−6	+27[b]	−9
14	–	0	+27	−13	−2	+23	–	−2	−13	+5	−5	+11	–	+18	+5	−1	+8	+28[b]
15	–	−4	+51[b]	+22	−4	−9	–	−4	−6	+11	+11	0	–	−10	−8	0	+8	+10

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

Table 7. Statistical results of bioassays (using *T. aestivum* L.) of lactones **5**, **7**, **11**, **14** and **15***

	(%) Germination						(%) Radicle 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
5	–	–	−2	−15	−19	−23[b]	–	–	−4	+25[b]	−11	+12	–	–	−10	+17	−12	+14
7	–	–	−2	−9	−29	−14	–	–	−19[b]	+10	−7	+4	–	–	−19	+8	−10	+12
11	–	+4	−6	−2	−11	−9	–	−9	+2	−11	+17	−1	–	−4	+2	−4	+26[b]	+10
14	–	–	+2	−43	−51[b]	−53[a]	–	–	−10	−9	−27[b]	−9	–	–	−9	−15	−19	−5
15	–	–	−35	−30	−48[b]	−47[b]	–	–	−22[b]	−1	−22[b]	−9	–	–	−24[b]	−13	−13	−7

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

Table 8. Statistical results of bioassays (using *H. vulgare* L.) of lactones **1–3**, **5–8**, **11** and **14–16***

	(%) Germination						(%) Radicle 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	+27[b]	+8	+3	+3	−17	−19[a]	−7	−11[b]	−4	−10	−4	−10	−19[a]	−17[a]	−6	−4	+7
2	–	−10	+11	−5	+7	−14	–	−2	−3	−2	−4	−7	–	−12[b]	−2	+10	−2	+11
3	–	+9	+4	−7	+14	+4	–	−15[b]	−11	−1	−5	−10	–	−19[b]	−15[b]	−18[b]	−2	−10
5	–	–	−37	−11	+37	+4	–	–	+4	−3	−14	+13	–	–	+8	−1	−13	−2
6	–	–	+2	+13	+18	+2	–	–	−6	+16	+4	+6	–	–	−6	−6	−3	−12
7	–	–	+7	−30	+42	+30	–	–	+5	+9	−5	+10	–	–	−2	+17	−7	−3
8	–	+5	+20	+11	+16	+15	–	−12[b]	−9	−2	+1	−15	–	−14[b]	+4	+12	+2	−10
11	–	−37	−11	+16	+13	−4	–	−8	−1	−3	+15	+14	–	−8	−6	−15	−4	−5
14	–	+24	+19	+22	+21	−9	–	−6	−10	+14	−16	+2	–	−8	−18[b]	+2	−31[b]	−14
15	–	–	+22	+11	+42[a]	−9	–	–	+1	+10	−2	+16	–	–	−2	−3	−16	+4
16	–	–	+5	+4	+20	+27[b]	–	–	−9	+13	−5	+1	–	–	−7	+5	−11	+3

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

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 aq. leaf extracts corresponding to 4 different plant development stages.

Extraction and isolation. Fresh leaves (1.7 kg) were soaked in H_2O (wet plant: solvent volume 1:3) for 24 hr at 25° in the dark. The H_2O extracts were re-extracted (8 ×) with 1.0 l. of CH_2Cl_2 for each 1.2 l. of H_2O and the combined extracts were dried over Na_2SO_4 and evapd *in vacuo* to yield 24 g of crude extract termed H_2O–Cl_2CH_2 extract which was sepd by CC on silica gel using *n*-hexane–EtOAc mixts of increasing polarity yielding 192 × 50 ml frs which were reduced to 30 frs after comparison by CCF.

By following the bioactivity exhibited by the medium polar frs on *L. sativa* and *H. vulgare*, fr. 16 was chromatographed using silica gel (with N_2 pressure) and eluting with hexane–EtOAc (6:4), hexane–EtOAc (2:1) and $CHCl_3$–MeOH (1:1). Frs 92–97 were combined and chromatographed using HPLC with a Hibar Si60 (Merck) column, $CHCl_3$–EtOAc (3:1) as eluent, $\lambda = 250$ nm, 254 nm and 360 nm, with 3 ml min^{-1} flow, yielding **1** (9 mg), **2** (8 mg), **3** (7 mg), **4** (3 mg), **5** (3 mg), **6** (8 mg), **7** (6 mg), **8** (5 mg), **10** (6 mg), **11** (3 mg), **14** (3 mg), **15** (2 mg) and **16** (5 mg).

Annuolide F (**5**). $C_{20}H_{24}O_5$, oil, IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3550 (OH); 1760 (α,β-unsaturated-γ-lactone), 1700 (α,β-unsaturated ester). EI-MS (70 eV) *m/z* (rel. int.): 344 $[M]^+$ (0,3); 326 $[M-H_2O]^+$ (2); 244 $[M-C_5H_8O_2]^+$ (5); 83 $[C_5H_7O]^+$ (100); 55 $[C_4H_7]^+$ (85); ^1H NMR: Table 1.

Annuolide G (**6**). $C_{20}H_{26}O_5$, oil, IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3340 (OH); 1783 (α,β-unsaturated-γ-lactone); 1720 (α,β-unsaturated ester). EI-MS (70 eV) *m/z* (rel. int.). 347 $[M]^+$ (0,5); 328 $[M-H_2O]^+$ (1); 314 $[M-OH-CH_3]^+$ (4). 246 $[M-C_5H_8O_2]^+$ (3); 228 $[M-C_5H_8O_2-H_2O]^+$ (2); ^1H NMR: Table 1.

Helivypolide A (**14**). $C_{20}H_{26}O_6$, oil, IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3440 (OH); 1740 (α,β-unsaturated-γ-lactone); 1640, 1620 (double bonds); EI-MS (70 eV) *m/z* (rel. int.). 344 $[MH_2O]^+$ (1); 279 $[M-C_5H_8O]^+$ (1); 262 $[M-C_5H_7O_2]^+$ (2); 261 $[M-C_5H_8O-H_2O]^+$ (2); 244 $[M-H_2O-C_5H_8O_2]^+$ (3); 83 $[C_5H_7O]^+$ (85); 55 $[C_4H_7]^+$ (100), ^1H NMR: Table 1.

Helivypolide B (**15**). $C_{20}H_{24}O_7$, oil; IR $\gamma_{max}^{KBr,neat}$ cm^{-1}: 3420 (OH); 1775 (α,β-unsaturated-γ-lactone); 1720 (α,β-unsaturated ester); 1660 (α,β-unsaturated C=O); EI-MS (70 eV) *m/z* (rel. int.). 376 $[M]^+$ (2); 358 $[M-H_2O]^+$ (1); 276 $[M-C_5H_7O_2]^+$ (100); 55 $[C_4H_7]^+$ (70); ^1H NMR: Table 1; ^{13}C NMR: Table 2.

Lettuce, cress, tomato, wheat and barley seed germination bioassay. Seeds of *L. sativa* var. nigra, *L. sativum, L. esculentum, H. vulgare* and *T. aestivum* were obtained from Rancho La Merced, Junta de Andalucia, 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 dicotyledon seeds for 5 days (3 for germination and 2 for root and shoot growth) for lettuce, 3 days (1 for germination and 2 for root and shoot growth) for cress, 4.5 days (3 for germination and 1.5 for root and shoot growth) for tomato, and 5 monocotyledon seeds for 4 days in 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 dicotyledon, and 5 ml for monocotyledon seeds.

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 conc.

There were 3 replicates for dicotyledon and 19 for monocotyledon seeds of each treatment, and 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, the 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 Mann–Whitney's test being the differences between the experimental and the control, significant with a value of $P = 0.01$.

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