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BIOACTIVE NORSESQUITERPENES FROM *HELIANTHUS ANNUUS* WITH POTENTIAL ALLELOPATHIC ACTIVITY*

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Key Word Index—*Helianthus annuus*; Compositae; norsesquiterpenes; norbisabolenes; bisnorsesquiterpenes; ionane derivatives; annuionones A–C; helinorbisabone; allelopathy.

Abstract—Six bioactive norsesquiterpenes have been isolated from *Helianthus annus* (sunflower) var. SH-222® and VYP®. Three new ionone type bisnorsesquiterpenes and a new norbisabolene are potential allelopathic agents. Their structures were determined through the analysis of their homo- and hetero-nuclear 2D-NMR spectral data. On the basis of combined studies of the theoretical conformations and NOEDIFF data the relative stereochemistry is proposed. These compounds exhibited clear selectivity (parameters and species) with monocotyledon species with an average of inhibition of −45% on the germination of *Allium cepa* and an average of stimulation of 50% on the root growth of *A. Cepa* and *Hordeum vulgare* in a range of concentrations of 10^{-5}–10^{-9} M.

INTRODUCTION

The indiscriminate use of herbicides has resulted in (a) an increasing incidence of resistance in weeds to some herbicide classes such as triazines and dinitroanilines, (b) shifts in weed populations to species that are more closely related to the crop they infest, and (c) environmental pollution and associated health hazards. Studies on Allelopathic interactions may help in overcoming such problems through the development of crop varieties having a greater ability to smother weeds, the use of natural phytotoxins from plants or microbes as herbicides and the use of synthetic derivatives of natural products as herbicides [1].

Plants have their own defence mechanisms and allelochemicals are, in fact, natural herbicides. One way to use allelopathy in agriculture is through the isolation, identification and synthesis of active compounds from allelopathic plant species.

Sunflower species are allelopathic in nature, indeed some of them, such as *Helianthus rigidus*, exhibit autotoxicity. Cultivated sunflower (*Helianthus annuus*) has great allelopathic potential and inhibits weed-seedling growth of velvet leaf, thorn apple, morning glory, wild mustard and other weeds [2]. Field studies have demonstrated that the weed biomass is equally reduced in sunflower plots with or without herbicides treatment [3, 4].

With this concept in mind and with the notion that allelopathic compounds have a wide diversity of skeletal types, we have initiated systematic allelopathic activity studies on sunflower cultivars in order to evaluate their potential as a source of allelopathic agents and consequently as natural herbicide templates. As results of these studies, we have described and characterized, in addition to simple phenolics, triterpenes and steroids [5], sesquiterpene lactones, mainly germacranolides and simple guaianolides [6, 7], flavonoids [8], diterpenes [9] and a novel family of sesquiterpenes named heliannuols [10, 11].

In continuation of our systematic allelopathic activity studies of sunflower cultivars, we now report the isolation and structural elucidation of three new bioactive ionone type bisnorsesquiterpenes, annuionones **A–C** (**3–5**), and the new norbisabolene helinorbisabone (**6**) from the medium polar active fractions. Their potential allelopathic activity is discussed and consequently their potential use as members of a new generation of natural agrochemicals.

Compounds with 13 carbon atoms are neither abundant nor usual as natural products. This represent, to our acknowledge, the first report of norsesquiterpenes from *Helianthus annuus* L.. Their origin is still uncertain; nevertheless, abscisic acid [12, 13], by oxidative removal of the two terminal carbon atoms as in blu-

* Part 10 in the series "Allelopathic studies in cultivar" for part 9 see ref. [8].

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menols A and B [14, 15], or carotenoids [16, 17] have been proposed as precursors. It is important to note that ionone-type bisnorsesquiterpenes have been related to the inhibitory activity of the extracts of several species as *Oryza sativa* [18] or *Bunias orientalis* [19].

RESULTS AND DISCUSSION

The dichloromethane extracts of the aqueous extracts of fresh leaves of *H. annuus* var VYP and SH-222 was chromatographed on columns of silica gel using hexane-ethyl acetate mixtures of increasing polarity. The medium polar bioactive fractions [20] yielded compounds **1–6** (Fig. 1). The spectroscopic data of **1** [21] and **2** [22] were identical to those previously reported.

Annuionone A (**3**) was isolated as a colourless oil. Its IR spectrum showed the presence of two carbonyl groups (1714 and 1698 cm^{-1}). The EI and FAB mass spectra both showed a molecular ion at m/z 224, which together with the ^{13}C NMR data (Table 3) were in good agreement with the molecular formula $C_{13}H_{20}O_3$.

The ^1H NMR 2D COSY spectrum showed two correlations series. The first one began with a multiplet at δ2.36 (3H) assigned to H-2, H-2′ and H-4, which was coupled with H-4′ (δ2.22, *d*, $J_{4,4'}=17.7$ Hz) and H-11′ (δ3.54, *dd*, $J_{11,11'}=7.9$, $J_{4,11'}=2.9$ Hz) and, finally, H-11′ with H-11 (δ3.61, *dd*, $J_{11,11'}=7.9$ Hz). In the second correlation series H-8 and H-8′ (δ2.63, 2H; *brdd*, $J_{7,8}=J_{7',8}=8.0$ Hz) were coupled with H-7 (δ1.80, *dddd*, $J_{7,7'}=15.9$, $J_{7,8}=J_{7,8'}=J_{7,6}=8.0$ Hz) and H-7′ (δ1.61, *m*), which overlaps with H-6 (δ1.61, *dd*, $J_{6,7'}=6.8$, $J_{6,7}=8.0$ Hz).

These correlations, together with the presence of three methyl groups at δ2.16 (H-10), δ1.05 and 1.29 (H-12 and H-13), and chemical shifts assigned to H-2, H-2′, H-4, H-4′, H-8, H-8′ and H-10 led us to propose an ionane skeleton with two carbonyl groups at positions 3 and 9.

The absence of absorptions at 3300 cm^{-1} in the IR spectrum, the chemical shift of the H-11 and H-11′ signals, and those corresponding with C-11 (δ78.3) and C-5 (δ83.4) in the ^{13}C NMR spectrum (Table 3), and the molecular formula indicated the presence of an oxirane ring between C-5 and C-11.

Annuionone B (**4**) was isolated as a colourless oil. Its IR spectrum showed the presence of a carbonyl group (1714 cm^{-1}) and an α,β-unsaturated ketone (1675 cm^{-1}). The MS and ^1H NMR spectra (Table 1) suggest the molecular formula $C_{13}O_3H_{18}$, ie a dehydro-derivative of the previously described **3**. The differences between their respective ^1H NMR spectra were as follows: the disappearance of the signals cor-

Table 1. ^1H-NMR data for compounds **3-6** (400 MHz, $CDCl_3$)

H	3	4	5	6
2	2.39 *d*	2.43 *m*	2.76 *dd*	——
2′	2.34 *d*	2.43 *m*	2.44 *d*	——
3	——	——	——	6.58* *s*
4	2.37 *dd*	2.43 *m*	2.81 *dd*	——
4′	2.22 *d*	2.43 *m*	2.62 *d*	——
6	1.61 *m*	2.50 *bd*	5.66 *d*	6.59* *s*
7	1.80 *dddd*	6.71 *m*	5.53 *dd*	3.24 *dq*
7′	1.61 *m*	—— *dd*	——	——
8	2.63 *ddd*	6.39	4.28 *dq(m)*	4.74 *ddd*
8′	2.63 *m*	—— *d*	——	——
10	2.16 *s*	2.29	1.22 *d*	6.84 *dd*
11	3.61 *d*	3.78 *s*	1.24 *s*	6.35 *dd*
11′	3.54 *dd*	3.68 *d*	——	——
12	1.29* *s*	1.27* *d*	1.19* *s*	2.28 *s*
13	1.05* *s*	1.04* *s*	1.14* *s*	1.35 *d*
14	——	—— *s*	——	2.20 *s*

*These values may be interchanged.

J (*Hz*): **3**: 2,2′ = 17.6; 4,4′ = 17.7; 4,11′ = 2.9; 6,7 = 8.0; 6,7′ = 6.8; 7,7′ = 15.9; 7,8 = 7,8′ = 7′,8 = 8,8′ = 80; 11,11′ = 7.9. **4**: 4,11′ = 2.8; 6,7 = 10.4; 7,8 = 15.5; 11,11′ = 8.3. **5**: 2,2′ = 17.3; 2,4 = 3.1; 4,4′ = 18.6; 7,8 = 16.0; 7,9 = 1.3; 8,9 = 5.7; 9,10 = 1.2. **6**: 7,8 = 7,13 = 6.8; 8,9 = 7.6; 8,10 = 1.2; 9,10 = 15.9.

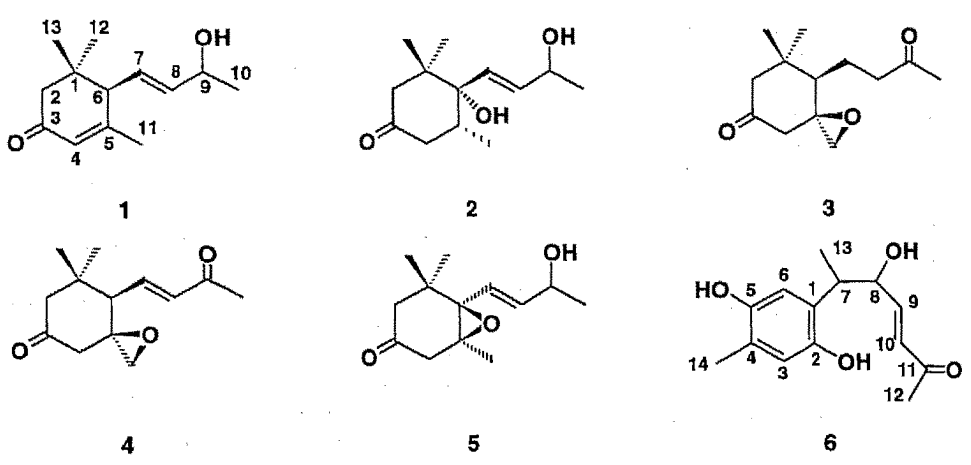

Fig. 1. Structures of norsesquiterpenoids isolated from *H. annuus*.

responding to H-7 and H-8, the deshielding effect observed over the signal assigned to H-6 (δ2.50) and the presence of two olefinic signals assigned to H-7(δ6.71, *dd*, $J_{6,7}=10.4$, $J_{7,8}=15.5$ Hz) and H-8(δ6.39, *d*, $J_{7,8}=15.5$ Hz).

These data led us to propose the structure **4** for annuionone B. With the purpose of determining the relative stereochemistry of the centres 5 and 6, various NOE experiments were carried out, from among those which we observed there was a clear effect between H-6 and H-11, as well as between H-7 and H-11, and between H-11 with one of the methyl groups attached at C-1. These effects, however, were not conclusive.

We carried out a conformational study of the two possible diastereoisomers using semiempirical calculations (PM3) [23]. The most relevant results are summarized in Table 2 (Fig. 2). Only conformation I could explain the effects between H-11 and H-6 as well as between H-11 and one of the methyl groups attached at C-1. This conformation with *trans* stereochemistry is the most stable conformation. Nevertheless, the differences in energy between the conformers of both configurations did not allow us to discard the possibility that a chemical equilibrium could account for all the observed effects. With these data, we proposed stereochemistry and conformation I as the most probable for compound **4**. Similar deductions can be made for compound **3**.

Annuionone C (**5**) was isolated as a colourless oil.

Table 2. Theoretical data for minimum energy conformers of compound 4 using PM3 calculations

Conformer	Calculated ΔHf (Kcal·mol^{-1})	Distance H-6, H-11 (Å)	Distance H-11, H_3C-C-1* (Å)
I	−91.01	2.49	2.80
II	−89.51	3.23	4.50
III	−89.69	2.62	4.46
IV	−89.36	3.88	2.86

*Distance between H-11 and the closest hydrogen attached at C-12 or C-13

Its IR spectrum showed the presence of hydroxyl groups (3417 cm^{-1}) and one carbonyl group (1697 cm^{-1}). The EI and FAB mass spectra both showed a molecular ion at *m/z* 224, which was in good agreement with the molecular formula $C_{13}H_{20}O_3$. Its ^{1}H NMR spectrum (Table 1) was very similar to that of **4**, except for the following differences: the disappearance of the signal corresponding to H−11 and H-11′, to be replaced by a singlet (3H) at δ1.24; the disappearance of the signal corresponding to H-6; and the presence of a new signal at δ4.28 assigned to H-9, geminal to an hydroxyl group. These data were accompanied by the corresponding modifications of H-7, which appeared in **4** as a doublet at δ5.66; H-8,

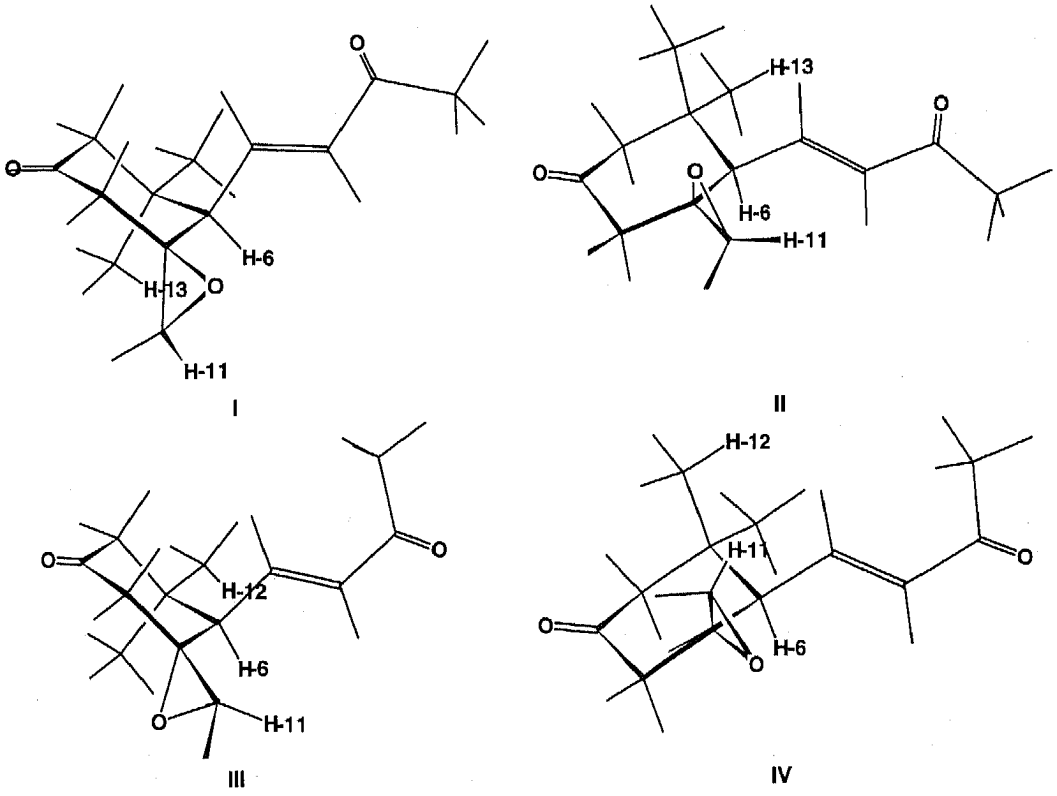

Fig. 2. Minimum energy conformers of compound 4.

Table 3. ^{13}C NMR data (100 MHz) for compounds 1, 4 and 5 ($CDCl_3$)[a].

C	3	6
1	43.4 *s*	129.3
2	49.4 *t*	151.0 *s*
3	208.7* *s*	111.3 *d*
4	48.6 *t*	123.5 *s*
5	83.4 *s*	148.1
6	53.1 *t*	110.7
7	18.7 *t*	43.2
8	42.6 *t*	88.9
9	207.2* *s*	143.6
10	30.0 *q*	130.0
11	78.3 *t*	194.2 *s*
12	24.9† *q*	27.6
13	20.8† *q*	18.5
14	—	16.1 *q*
	—	

[a] Degree of protonation and assignments were obtained by ^1H-^{13}C NMR correlations; multiplicities are not repeated if identical with those in preceeding column.

*, † These values may be interchanged.

doublet of doublets at δ5.53; and H-10, doublet at δ1.22. The molecular formula indicated the presence of an oxirane ring between C-5 and C-6. The NOE effect observed between H-11 and H-7 suggested a *cis* stereochemistry for this epoxide.

Helinorbisabone (**6**) was obtained as a yellowish oil. The ^{13}C NMR (Table 3) and mass spectra (*m/z* 250) suggested the presence of 14 carbon atoms with the molecular formula $C_{14}H_{18}O_4$. The molecular ion [M^+] was not observed in its HRMS but a peak at *m/z* 232.1091 (rel. int. 100) [$M-H_2O$]$^+$ indicated the presence of an hydroxyl group that is easily eliminated. The IR spectrum showed absorptions at 3400 (hydroxyl group) and 1680 (α,β-unsaturated ketone).

Inspection of its ^1H NMR spectrum (Table 1), which displayed two singlets at δ6.59 and 6.58, indicated the presence of two protons attached to a tetrasubstituted aromatic ring at positions 1 and 4. Two other signals at δ6.35 and 6.84 can be assigned to two olefinic protons with *E* geometry ($J_{9,10}$ = 15.9 Hz) conjugated with an electron withdrawing group. Two singlets (3H each) at δ2.28 and 2.20 were attributable to a methyl group attached to an aromatic ring or a methyl ketone moiety. The presence of a methyl ketone moiety was corroborated by the peaks in the mass spectrum at *m/z* 43 [CH_3CO]$^+$ (29.4) and 189 [$M-H_2O-CH_3CO$]$^+$ (40).

The ^1H NMR 2D COSY spectrum showed the following series of coupled protons: H-13 (δ1.35, *d*, $J_{7,13}$ = 6.8 Hz) with H-7 (δ3.24, *dq*, $J_{7,8} = J_{7,13}$ = 6.8 Hz); H-7 with H-8 (δ4.74, *ddd*, $J_{7,8}$ = 6.8, $J_{8,10}$ = 1.3, $J_{8,9}$ = 7.6 Hz); H-8 with H-9 (δ6.84, *dd*, $J_{8,9}$ = 7.6, $J_{9,10}$ = 15.9 Hz); and H-9 with H-10 (δ6.35, *dd*, $J_{8,10}$ = 1.3, $J_{9,10}$ = 15.9 Hz). These correlations allowed us to establish the structure of the side chain linked to the aromatic ring. The COSY spectrum showed a coupling between H-3 and H-14, which indicated that H-14 had to be assigned to the methyl group attached to the aromatic ring.

The molecular formula indicated the presence of two additional hydroxyl groups; these had to be located on the aromatic ring.

The ^{13}C NMR spectrum (Table 3) showed the following signals: δ194.2 (C-11, carbonyl group), 151.0 and 148.1 (C-2 and C-5, aromatic carbons with hydroxyl group), 143.6 and 130 (C-9 and C-10, olefinic carbons), 129.3, 123.5, 111.3, 110.7 (C-1, C-4, C-3 and C-6, aromatic carbons) and 88.9 (C-8, carbon attached to hydroxyl group). These assignments were made with the aid of HMQC experiments and the program ^{13}C NMR-Module of Hoening [24] for 2-*sec*-butyl-5-methyl-1,4-benzenediol.

The arrangement of substituents was assigned on the basis of the oxidation pattern and skeleton of bisabolene isolated from *H. annuus* [25]; a compound which could be considered as precursor. This was corroborated by HMBC experiments. This skeleton has been reported previously from *Senecio digitalifolius* [26]. This is the second time it has been reported.

Bioassay results

In order to evaluate their potential allelopathic activity, we studied the effect of a series of aqueous solutions (10^{-4}–10^{-9} M) of the isolated compounds on root and shoot length of *Lactuca sativa*, *Lepidium sativum*, *Allium cepa*, and *Hordeum vulgare*.

The most significant effects found for the dicotyledon species (*L. sativa* and *L. sativum*) were obtained with **4**, which stimulated root growth of *L. sativum* at low concentration (10^{-8} M, 47%; 10^{-9} M, 32%), and **6**, which showed a strong inhibitory effect on the germination of *L. sativa* at all tested concentrations (average −50%).

Clear selectivity (parameters and species) was observed with the monocotyledon species. **1** and **3** inhibited (**1**, 10^{-4} M, −38%; **3**, 10^{-4} M, −47%) the germination of *Allium cepa*, but stimulated (**1**, 10^{-4} M, 63%; 10^{-8} M, 54%; **3**, 10^{-4} M, 42%; 10^{-5} M, 48%; 10^{-6} M, 49%) root growth. However, only stimulatory effects on root and shoot growth of *Hordeum vulgare* were observed. In this case, **5** and **6** stimulated root growth by an average of 35% for and 40%, respectively, over the concentration range of 10^{-5}–10^{-9} M. Only **6** had an effect on shoot growth of *H. vulgare* (average of 30%).

The above findings suggest that compounds **1**—**6** are likely to be significantly involved in the allelopathic action of sunflower cultivars over monocotyledon species. Consequently, on the bases of these activities, we can conclude that **1** and **3**, and particularly **6**, are good candidates as potential herbicide templates with potential use as new generation of natural agrochemicals for the control of weeds in monocotyledon crops.

EXPERIMENTAL

^{1}H NMR and ^{13}C NMR: Varian UNITY-400. An asterisk indicates interchangeable signals. Low resolution MS: VG 1250 spectrometer; HRMS: Kratos MS 80RFA spectrometer. All solvents were distilled from glass prior to use.

Extraction and isolation. Leaves of *H. annuus* L. var. SH-222®, commercialized by Semillas Pacifico (Spain), and var. VYP®, commercialized by KOYPE (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 the bioactivity exhibited by leaf aq. extracts corresponding to 4 different plant development stages.

Fresh leaves (6.0 kg) were soaked in H_2O (18 L) for 24 hr at 25° in the dark. The H_2O extracts were 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 evaporated *in vacuo* to yield 16.0 g of crude extract which was separated by CC on silica gel using hexane-EtOAc mixtures of increasing polarity yielding 170 × 50 ml fractions which were reduced to 16 after comparison by CCF.

By following the bioactivity exhibited by the medium polar frs on *L. sativa* and *H. vulgare*, fr. **O** was chromatographed on silica gel under pressure N_2, and eluting with $CHCl_3$-t-BuOH (2 L), EtOAc (1 L) and Me OH (1 L). Frs 5-8 were combined and subjected to HPLC [Hibar Si60 (Merck) column, *n*-hexane-EtOAc (7:3) as eluent, and 3 mL min^{-1} flow] to give **1** (4 mg), **3** (5 mg), **4** (2 mg), **5** (2 mg) and **6** (2 mg).

Annuionone A (**3**). $C_{13}H_{22}O_3$; colourless oil; $[\alpha]_D^{25}$ +12.3° ($CHCl_3$, c = 0.4); **IR** $\nu^{KBr,neat}$ cm^{-1}: 1714 and 1698 (two C=O), 1271 (C-O-C); **FABMS** *m/z* (rel. int.): 224 $[M]^+$ (56.7); **EIMS** (70 eV) *m/z* (rel. int.): 224 $[M]^+$ (7), 181 $[M\text{-}CH_3\text{-}C{\equiv}O]^+$ (2.1), 167 $[M\text{-}CH_3\text{-}CH_2\text{-}C{\equiv}O]^+$ (6.4); **^{1}H NMR**: Table 1; **^{13}C NMR**: Table 3.

Annuionone B (**4**). $C_{13}H_{20}O_3$; colourless oil; $[\alpha]_D^{25}$ -13.5° ($CHCl_3$, c = 0.1); **IR** $\nu^{KBr,neat}$ cm^{-1}: 1715 and 1672 (two C=O), 1630 (double bonds); 1257 (C-O-C); **FABMS** *m/z* (rel. int.): 224 $[M]^+$ (46.8); **EIMS** (70 eV) *m/z* (rel. int.): 207 $[M\text{-}CH_3]^+$ (0.4), 179 $[M\text{-}CH_3\text{-}C{\equiv}O]^+$ (0.6), 43 $[CH_3\text{-}C{\equiv}O]^+$(100); ^{1}H NMR: Table 1.

Annuionone C (**5**). $C_{13}H_{22}O_3$; colourless oil; $[\alpha]_D^{25}$-8° ($CHCl_3$, c = 0.1); **IR** $\nu^{KBr,neat}$ cm^{-1}: 3417 (OH), 1697 (C=O), 1649 (double bonds); 1130 (C-O-C); **FABMS** *m/z* (rel. int.): 224 $[M]^+$ (41.8); **EIMS** (70 eV) *m/z* (rel. int.): 207 $[M+1\text{-}H_2O]^+$ (2.8), 152 $[M\text{-}CH_3\text{-}CH{=}CH\text{-}CH_2OH]^+$ (9.7); **^{1}H NMR**: Table 1.

Helinorbisabone (**6**). $C_{14}H_{18}O_3$; yellow oil; $[\alpha]_D^{25}$ +3° ($CHCl_3$, c = 0.1); **IR** $\nu^{KBr,neat}$ cm^{-1}: 3406 (OH), 1680 (α-β-unsaturated C=O), 1649 (double bonds); **EIMS** (70 eV) *m/z* (rel. int.): 250 $[M]^+$ (0.7), 43 $[CH_3\text{-}C{\equiv}O]^+$ (100); **HREIMS** Obsd. *m/z*: 232.1091 $[M\text{-}H_2O]^+$, requires *m/z* 232.3091, 189 $[M\text{-}H_2O\text{-}CH_3CO]^+$ (40), 43 $[CH_3\text{-}C{\equiv}O]^+$ (29.4); **^{1}H NMR**: Table 1; **^{13}C NMR**: Table 3.

Lettuce, cress, onion and barley seed germination bioassay. Seeds of lettuce, *L. sativa* var. nigra and *H. vulgare*, were obtained from Rancho La Merced, Junta de Andalucía, Jerez, Spain. Seeds of *Lepidium sativum* and *Allium cepa* were obtained from Fitó S.L. All undersized and damaged seeds were discarded and the assay seeds were selected for uniformity of size.

The bioassay consisted of germinating 25 seeds for 5 days (3 for germination and 2 for root and shoot growth) for lettuce and onion, 3 days (1 for germination and 2 for root and shoot growth) for cress and 5 barley seeds for 4 days in the dark at 25° in 9 cm plastic Petri dishes containing a 10 cm sheet of Whatman no. I filter paper and 10 ml of a test of control solution, except for barley (5 ml).Stock solns (10^{-4} M) were prepared. Test solutions (10^{-5}–10^{-9} M) were obtained by diluting the stock solutions. Parallel controls consisted of deionized H_20.

There were 3 replicates, except for barley (19 replicates), of each treatment, and parallel controls. The number of seeds per replicate and time and temp. of germination were based on a number of preliminary experiments which examined the effects of 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 Mann-Whitney's test being the differences between the experiment and the control, significant with a value of P = 0.01.

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