Sesquiterpene Lactones as Allelochemicals

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Received February 7, 2006

Six new sesquiterpene lactones, annuolide H (**3**), helivypolides F, H−J (**4**, **11**−**13**), and helieudesmanolide A (**6**), together with known compounds, were isolated from polar bioactive fractions of *Helianthus annuus* cv. SH-222 and Stella fresh leaf water extracts. Spectroscopic analysis of the new data for 1,2-anhydroniveusin A and 1-methoxy-4,5-dihydroniveusin A corrects some previous assignments. The compounds were tested using the etiolated wheat coleoptile bioassay, and the most active compounds were assayed in standard target species (STS) (*Lepidium sativum, Allium cepa, Lactuca sativa, Lycopersicon esculentum,* and *Triticum aestivum*) from 5×10^{-4} to 10^{-5} M. The most phytotoxic compounds were helivypolide F and 15-hydroxy-3-dehydrodeoxyfruticin, both of which have a carbonyl group at C-3 conjugated with two double bonds.

Sesquiterpene lactones are widely distributed in plants. More than 7000 structures have been described.[1] These, together with diterpenes, are the most abundant chemicals isolated from the genus *Helianthus*.[2] The sesquiterpene lactones isolated display germacranolide, heliangolide, melampolide, eudesmanolide, and secogermacranolide skeletons, the most common being germacranolides and heliangolides (including furaneheliangolides), presenting in most cases an angelate substituent at C-8 with β orientation. These compounds present a wide spectrum of biological activities, including potential allelopathy.[3] We have continued our systematic studies of the allelopathic activity of different varieties of *Helianthus annuus* by analyzing water fresh leaf extracts of cv. SH-222 and Stella. Herein, we report the isolation and structural elucidation of six new sesquiterpene lactones, annuolide H (**3**), helivypolides F, H−J (**4**, **11**−**13**), and helieudesmanolide A (**6**), in addition to the known compounds: **1**, **2**, **5**, **7**, **8**, **9**, **10**, and **14**. The spectroscopic data of **8** and **10** correct some previous assignments.

Results and Discussion

The polar bioactive fractions of *Helianthus annuus* var. cv. SH-222 and Stella fresh leaf water extracts yielded compounds **1**−**14**. Spectroscopic data of **1**, **2**, **5**, **7**−**10**, and **14** were identical to those previously reported for annuolide A (**1**),[4] 8β-angeloyloxycumambranolide (**2**),[5] 11βH-dihydrochamissonin (**5**),[6] niveusin B (**7**),[7] l,2-anhydroniveusin A (**8**),[8] 15-hydroxy-3-dehydrodeoxyfruticin (**9**),[9] 1-methoxy-4,5-dihydroniveusin A (**10**),[8] and argophyllin A (**14**).[10] Some spectroscopic data of **8** and **10** have been corrected with respect to assignments reported previously.

Compound **3** was isolated as a colorless oil, with a molecular ion at *m*/*z* 362.1729 that agrees with the molecular formula $C_{20}H_{26}O_6$. Its ^{1}H NMR spectrum showed signals typical of a guaiane-type sesquiterpene lactone with an α-methylene-γ-lactone moiety at δ 6.26 (d, $J = 3.7$ Hz) and 5.48 (1H, dd, $J = 3.2$ Hz). The signal at δ 5.56 (brs, H-3) was assigned to an olefinic proton. Two methyl groups were evident, one bonded to a double bond, according to the chemical shift at δ 1.94 (3H, brs, H-15), and another at δ 1.33 (3H, s, H-14), attached to a quaternary carbon bonded to oxygen. The ^{1}H NMR-2D-COSY spectrum showed correlation of the exocyclic methylene with a signal at δ 3.69 corresponding to H-7. This proton was coupled with the signal at δ 4.40 (dd, $J = 10.7$, 9.0 Hz) assigned to H-6 that showed correlation with H-5 (δ 3.08, dd, $J = 9.0$, 8.1 Hz).

Absence of the angular methyl group, together with the correlation of H-5 with the proton at δ 2.41 (dd, $J = 7.8$, 5.4 Hz, H−l), confirmed the presence of a guaiane-type skeleton. H-l correlated with the signal at δ 4.79 assigned to H-2. The chemical shift suggested that a hydroxyl group was at C-2. H-2 also correlated with a signal at δ 5.56, which corresponded to the olefinic proton at C-3 (δ 129.7, CH), according to the gHSQC spectrum. H-3 (δ 5.56, brs) showed coupling with the methyl bonded to C-4 at δ 1.94 (3H, m, H-15). Finally, H-8, at δ 5.69 (ddd, $J = 6.5$, 5.9, 3.4 Hz), correlated with both C-9 protons at δ 2.29 (dd, $J = 14.1$, 6.5 Hz, H-9α) and 2.01 (dd, $J = 14.1$, 5.9 Hz, H-9β).

The peak in the MS at *m*/*z* 83, as well as signals in the ^{1}H NMR spectrum at δ 1.76 (3H, dq, $J = 1.5$, 1.5 Hz, H-5′), 1.90 (3H, dq, $J = 7.3$, 1.5 Hz, H-4′), and 6.04 (qq, $J = 7.3$, 1.5 Hz, H-3′) indicated the presence of an angeloyloxy moiety. This ester group was placed at C-8 with β-orientation, based on the shift and multiplicity of H-8 (δ 5.69, ddd, $J = 6.5$, 5.9, 3.4 Hz). A clear nOe effect between H-14 and H-6 indicated β-orientation of the C-14 methyl. This was corroborated with the nOe effects among the signals corresponding to H-2, H-14, and H-6, establishing β-orientation for H-2. The β-orientation of H-8 was confirmed by a nOe effect between H-7 and H-8. These data allowed for assignment of the structure, 8β-angeloyloxy-2α,10α-dihydroxy-guaiano-3-(4),11(13)dien-6α,12-olide, which is described for the first time, and the compound was named annuolide H.

Compound **4** was isolated as colorless oil. The mass spectrum showed peaks at *m*/*z* 358.1414 $[M]^+$, 275 $[M - C_5H_7O]^+$, and 83 $[C_5H_7O]^+$. These data are in accord with a molecular formula $C_{20}H_{22}O_6$ and suggested the presence of an angelate ester in its structure. The ^{1}H NMR-2D-COSY spectrum presented two doublet signals corresponding to an exocyclic methylene-γ-lactone moiety at δ 5.62 (d, $J = 3.2$ Hz, H-13a) and 6.29 (d, $J = 3.4$ Hz, H-13b), coupled with a proton at δ 3.41 (dddd, $J = 9.7$, 3.4, 3.2, 1.7 Hz) assigned to H-7. This signal was correlated with another at δ 5.11, corresponding to H-6, which showed coupling with H-5 at δ 4.54. The chemical shift of H-5 indicated the presence of oxygen at this position, and C-4 must be a quaternary carbon because H-5 was only coupled with H-6.

The ^{1}H NMR-2D-COSY spectrum of **4** showed a second correlation series: H-7 coupled with H-8α, and H-8α with H-9β and H-9α. The angelate moiety should be located at C-8 as deduced

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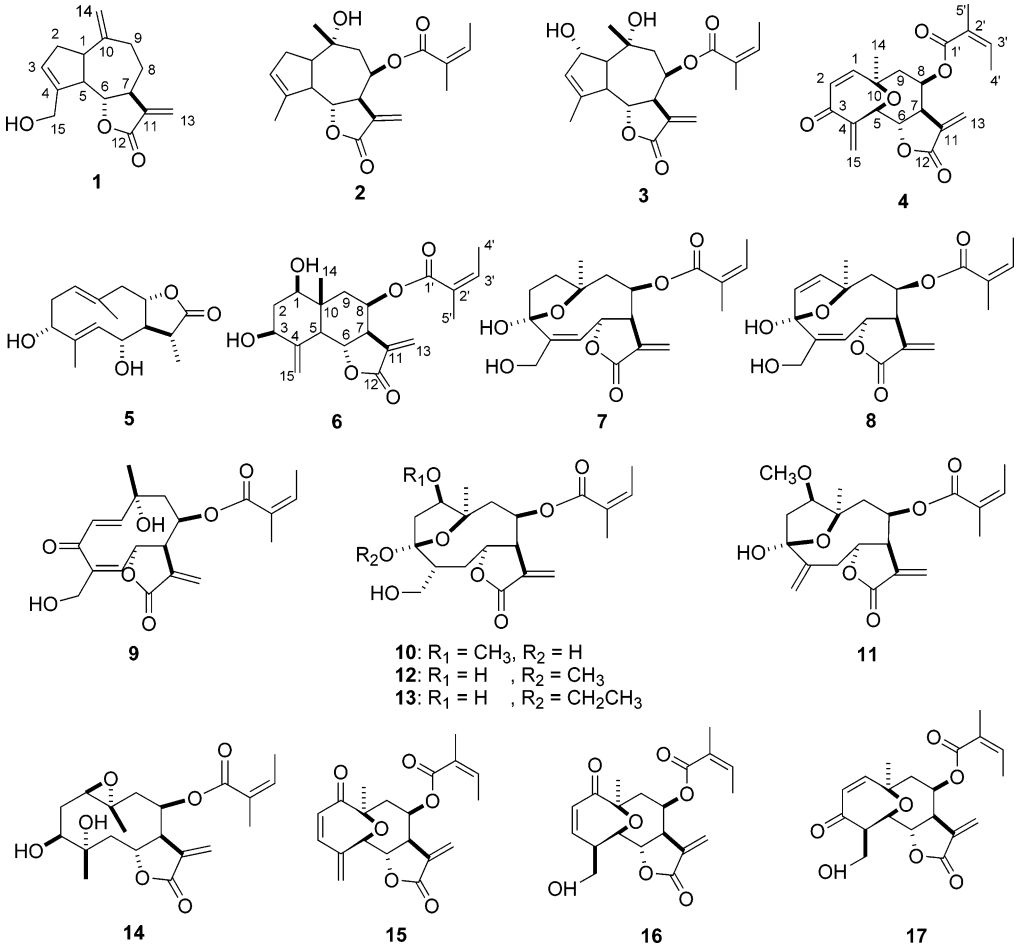
10.1021/np060056s CCC: $33.50
Published on Web 04/27/2006

Chart 1



from the chemical shift of H-8 (δ 5.75). On the other hand, the chemical shifts of the 14 methyl (δ 1.40) and C-10 (δ 80.4) agree with an oxygen function at C-10.

The molecular formula ($C_{20}H_{22}O_6$) and the chemical shifts for C-5 and C-10 imply the presence of an ether union between these carbons. This explains the value obtained for the coupling constant between H-1 and H-2 (d, J = 13.1 Hz). On the other hand, these protons are observed as an isolated AB system that infers the presence of a ketone at C-3 according to its chemical shift (δ 189.4) in the ^{13}C NMR spectrum. The double bond between C-l and C-2 was confirmed by the nOe effect observed between the signals of the 14 methyl and H-l (δ 6.33, d, J = 13.1 Hz). Absence of an assignable signal to H-4, as well as the presence in the ^{1}H NMR spectrum of two isolated doublets (δ 5.60, J = 1.5 Hz, H-15a and δ 6.35, J = 1.5 Hz, H-15b) and a triplet in the ^{13}C NMR spectrum at δ 128.5 (CH_2), indicated the presence of an exocyclic double bond between C-4 and C-15. The gHMBC experiment confirmed the carbon skeleton of this compound.

The α-orientation for the C-10 methyl group was assigned by comparison with the spectroscopic data of chapliatrin and its derivatives.[11] This was further supported by the nOe effects observed between CH_3-14 and H-9α and H-9β. These effects can be explained because the methyl group is located in the bisector of the angle formed by H-9α and H-9β in the most stable conformer (Figure 1). The low value of the coupling constant between H-7 and H-8 (J = 1.8 Hz) indicates an α-orientation for H-8, which was corroborated by the corresponding nOe experiment. When irradiating the signal of H-7, effects on H-7, H-8, and H-5 were observed. Thus, **4** was established as 8β-angeloyloxy-5β,10β-epoxy-3-oxo-germacrane-1*Z*,4(15),11(13)-trien-6α,12-olide and was named as helivypolide F.

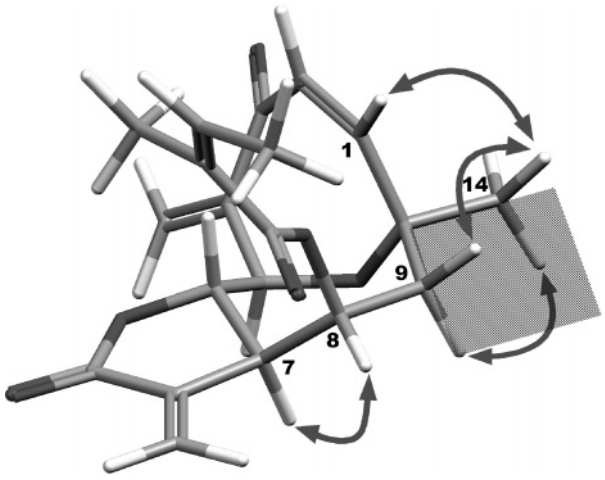


Figure 1. nOe effects observed for the most stable conformer of helivypolide F (**4**) using PM3 calculations.

The NMR data of **4** were identical to those published for 4,15-anhydrohelivypolide (**15**).[12] This suggests that structure **15** must be revised to **4**. Those authors suggested that this compound could be an artifact of helivypolide B. The structure assigned for helivypolide B was **16**.[12] Comparison of spectra of helivypolide B and **4** suggest that the structure of **16** should be revised to **17**. This is further supported by the absence of coupling between the signal at δ 6.33, which should be assigned to H-3 in structure **16**, and H-4 at δ 3.09. This also agrees with the usual oxidation pattern for heliangolides and furaneheliangolides previously isolated from the genus *Helianthus*.[2]

Compound **6** was isolated as a colorless oil. The molecular ion at *m*/*z* 362.1729 indicated molecular formula $C_{20}H_{26}O_6$. Its IR spectrum showed bands at 3400 (hydroxyl), 1750 and 1650 (α,*β*-unsaturated lactone), and 1700 cm^{-1} (α,*β*-unsaturated ester). The ^{1}H NMR spectrum showed signals characteristic of a sesquiterpene lactone with a eudesmane skeleton: an angular methyl at *δ* 0.96 (H-14); an exocyclic methylene at *δ* 6.18 (H-13b) and 5.50 (13a) conjugated with H-7, and H-6. An exocyclic double bond belonging to C-4 was observed as two broad singlets at *δ* 5.35 and 5.12. The ^{1}H NMR-2D-COSY experiment showed that H-6 was coupled with signals at *δ* 2.14 and 2.84 assigned to H-5 and H-7, respectively, and H-7 also showed correlation with the signal at *δ* 5.81 (H-8α). The signal assigned to H-8 showed coupling with H-9a and H-9b. Signals were observed in the ^{1}H NMR spectrum at *δ* 4.10, 3.58, 1.58, and 2.25, assigned to H-1, H-3, H-2*β*, and H-2α, respectively. The chemical shifts of C-1, C-2, and C-3 in the ^{13}C NMR spectrum at *δ* 69.5 (CH), 39.9 (CH_2), and 76.7 (CH) indicated that there were hydroxyl groups bonded to C-1 and C-3. The peak in the mass spectrum at *m*/*z* 83 $[C_5H_7O]^+$, as well as signals at *δ* 1.84 (dq, *J* = 1.5, 1.5 Hz, H-5′), 1.97 (dq, *J* = 7.0, 1.5 Hz, H-4′), and 6.01 (qq, *J* = 7.0, 1.5 Hz, H-3′) indicated the presence of an angeloyloxy moiety. This was placed at C-8, with *β* orientation, based on the chemical shift and multiplicity of the H-8 signal. The orientation of H-5 was established according to coupling constant with H-6 (*J* = 11.2 Hz) and assuming *β* orientation for H-6 and α for the H-7 and H-8. The relative stereochemistry at C-l and C-3 is based on the coupling constants of H−l (dd, *J* = 11.6, 5.4 Hz) and H-3 (dd, *J* = 11.6, 4.6 Hz) that implies axial disposition for both protons. Thus, **6** was assigned the structure 8*β*-angeloyloxy-l*β*,3*β*-dihydroxy-eudesmane-4(15),11(13)-dien-6α,12-olide and was named helieudesmanolide A.

Compound **8** was previously isolated from *Helianthus annuus.*[8] The spectroscopic data are identical to those published except for the chemical shifts and assignments for C-l, C-4, and C-5. These have been corrected according to the correlations observed in the two-dimensional ^{1}H-^{13}C NMR g-HSQC (Table 2).

Analytical data for compound **10** suggested the structure shown, and the spectroscopic data agree with those for l-methoxy-4,5-dihydroniveusin A previously isolated from *Helianthus annuus* and published with unknown stereochemistry at C-1 and C-4.[8] Irradiation of the signal corresponding to H-14 produced an nOe effect on the signal of H-1 indicating α orientation of H-1. Irradiation of the signal of H-15 gave an nOe effect with the signal of H-2, indicating that the C-4 hydroxymethylene has α-orientation. The bidimensional ^{1}H-^{13}C NMR gHSQC experiment allowed assigment of the signal at *δ* 86.7 (CH) to C-1 and the signal at *δ* 77.5 (CH) to C-6, changing these values with respect to those previously described.

Compound **11** was isolated as a colorless oil. Its ^{1}H NMR spectrum indicated that it was a sesquiterpene lactone with an angelate ester and an additional methoxyl group. Its spectroscopic data were very similar to those of compound **10**, suggesting that it was a furaneheliangolide-type lactone. One of the methyl groups was modified as an olefinic methylene appeared at *δ* 5.38 (brs, H-15a) and 5.30 (brs, H-15b), attached at C-4. Two doublet signals coupled each other at *δ* 6.29 (H-13a) and 5.59 (H-13b) were correlated with a signal at *δ* 3.92 (H-7). Data comparison of **10** with those for l-methoxy-4,5-dihydroniveusin A, showed similarity of the structures, except for disappearance of the hydroxymethylene group at C-15, which appears as an exocyclic methylene in **10**. The stereochemistry of the methoxyl group bonded to C-1 was deduced from the nOe effect between H−l and H-14, suggesting *β*-orientation for this methoxyl group. Thus, **11** is 8*β*-angeloyloxy-3*β*,10*β*-epoxy-3α-hydroxy-1*β*-methoxy-germacrane-4(15),11(13)-dien-6α,12-olide and was named helivypolide H.

Compound **12** was isolated as a colorless oil, and its MS indicated the molecular formula $C_{21}H_{30}O_8$. Additional peaks corresponding

Table 1. ^{1}H NMR Data (*δ*) of Compounds **3**, **4**, **6**, **11**, **12**, and **13** in $CDCl_3$ (400 MHz)[a]

no.	3	4	6	11	12	13
1	2.41, dd (7.8, 5.4)	6.33 d (13.1)	4.10 dd (11.6, 5.4)	3.78 dd (8.5, 9.0)	4.32 dd (9.5, 8.5)	4.32 dd (9.5, 8.5)
2	4.79, m	5.99 d (13.1)	2.25 ddd (11.6, 5.4, 4.6) α	2.44 dd (13.7, 9.0) a	2.22 dd (14.0, 9.7) α	2.22 dd (14.2, 9.5) α
			1.58 ddd (11.6,11.6,11.6)*β*	2.69 dd (13.7, 8.5) b	2.30 dd (14.2, 8.3) *β*	2.30 dd (14.2, 8.5) *β*
3	5.56, brs		3.58 dd (11.6, 4.6)			
4					2.37 m	2.40 m
5	3.08, dd (9.0, 8.1)	4.54 d (9.8)	2.14 d (11.2)	2.67 dd (12.7, 6.4) α	2.05 ddd (17.2, 10.1, 4.4)	2.05 ddd (15.5,10.1, 4.4) a
				2.86 d (12.7) *β*	2.21 m	2.21 m b
6	4.40, dd (9.0, 10.7)	5.11 dd (9.7, 9.8)	4.56 dd (10.8, 11.2)	4.45 dd (6.4, 9.0)	4.61 ddd (10.5, 4.0, 2.3)	4.61 ddd (10.5, 4.0, 2.3)
7	3.69, m	3.41 dddd(1.7,3.2,3.4,9.7)	2.84 ddd (10.8, 2.9, 3.3)	3.92 dddd(9.0,2.7,2.9, 3.4)	4.45 ddd (9.0, 4.0, 2.8,)	4.47 ddd (6.0,3.2, 3.4, 3.0)
8	5.69, ddd (6.5, 5.9, 3.4) α	5.75 ddd (2.8, 3.8, 1.7)	5.81 ddd (2.9, 3.7, 2.5)	5.61 ddd (2.7, 4.9, 11.5)	5.75 ddd (2.8, 6.8, 10.0)	5.75 ddd (9.3, 3.2, 3.0) α
9	2.29, dd (14.1, 6.5) α	2.34 dd (15.4, 2.8) α	2.40 dd (15.3, 2.5)	1.54 dd (14.4, 11.5) *β*	1.76 dd (11.7, 10.0)	1.77 m
	2.01, dd (14.1, 5.9) *β*	2.16 dd (15.4, 3.8) *β*	1.59 dd (15.3, 3.7)	1.94 dd (14.4, 4.9) α	1.78 dd (11.7, 6.8)	
13	5.48, d (3.2) a	5.62 d (3.2) a	5.50 d (2.9) a	5.59 d (2.9) a	5.37 d (3.2) a	5.38 d (3.2) a
	6.26, d (3.7) b	6.35 d (3.4) b	6.18 d (3.3) b	6.29 d (3.4) b	6.16 d (3.7) b	6.16 d (3.4) b
14	1.33, s	1.40 s	0.96 s	1.50 s	1.53 s	1.54 s
15	1.94, brs	5.60 d (1.5)	5.12 brs	5.30 s	3.60 dd (11.0, 6.8) a	3.60 dd (11.0, 7.1) a
		6.40 d (1.5)	5.35 brs	5.38 s	3.52 dd (11.0, 4.0) b	3.52 dd (11.0, 7.1) b
3′	6.04, qq (7.3, 1.5)	6.15 qq (7.2, 1.5)	6.01 qq (7.0, 1.5)	6.03 qq (7.3, 1.5)	6.06 qq (7.2, 1.5)	6.07 qq (7.3, 1.5)
4′	1.90, dq (7.3, 1.5)	1.98 dq (1.5, 7.2)	1.97 dq (7.0, 1.5)	1.88 dq (7.3, 1.5)	1.92 dq (7.2, 1.5)	1.92 dq (7.3, 1.5)
5′	1.76, dq (1.5, 1.5)	1.80 dq (1.5, 1.5)	1.84 dq (1.5, 1.5)	1.74 dq (1.5, 1.5)	1.77 dq (1.5, 1.5)	1.77 dq (1.5, 1.5)
OCH_3				3.37 s	3.23 s	
OCH_2CH_3						3.50 q (7.1)
OCH_2CH_3						1.18 t (7.1)

[a] Spectra recorded in $CDCl_3$, *J* in Hz at 400 MHz. Assignments aided by COSY, HSQC, HMBC, and NOESY experiments.

Table 2. ^{13}C NMR Data (δ) of Compounds **3**, **4**, **6**, **8**, **10**, **11**, **12**, and **13** in $CDCl_3$ (100 MHz)

no.	3	4	6	8	10	11	12	13
1	63.2	150.0	69.5	126.7	86.7	86.9	78.3	78.3
2	77.7	127.3	39.9	140.0	37.6	42.7	38.3	38.4
3	129.7	189.4	76.7	108.6	106.7	103.4	109.3	109.3
4	146.6	143.8	144.4	141.3	43.3	150.5	40.4	40.9
5	54.5	79.3	50.6	134.7	31.9	39.9	31.8	31.8
6	80.6	76.2	74.7	76.5	77.5	81.8	77.6	77.6
7	47.3	47.1	52.3	47.8	46.7	48.1	46.5	46.5
8	66.1	65.4	65.3	74.0	46.3	69.3	66.4	66.4
9	40.4	46.8	40.3	43.6	35.6	32.9	35.6	35.6
10	73.1	80.4	42.7	87.7	81.7	82.2	82.0	81.9
11	134.7	134.5	134.3	138.7	136.9	169.1	137.0	137.0
12	169.4	168.3	169.6	169.5	169.5	137.0	169.5	169.5
13	121.9	122.6	119.9	124.5	119.2	122.0	119.2	119.1
14	29.7	32.9	13.5	31.3	25.8	24.0	24.2	24.3
15	17.4	128.5	107.5	66.2	65.6	117.6	65.4	65.6
1′	167.0	166.1	166.8	166.1	166.8	166.8	166.9	166.9
2′	127.2	122.4	127.0	126.7	127.3	127.3	127.3	127.3
3′	139.1	141.2	139.7	140.9	139.2	139.0	139.2	139.2
4′	15.8	15.8	15.9	15.8	15.9	15.7	15.9	15.9
5′	20.5	20.5	20.6	20.5	20.5	20.4	20.5	20.5
OCH_3					58.6	58.9	48.6	
OCH_2CH_3								56.7
OCH_2CH_3								15.3

to the presence of an unsaturated five-carbon ester were observed. This was identified as an angeloyloxy group, based on the signals observed in the ^1H NMR spectrum. The ^1H NMR spectrum of **12** was similar to that of **10**, except for the chemical shifts of the signals corresponding to the methoxyl group, H-1 and, H-4. The differences suggested that **12** must be an isomer of **10**, with the methoxyl group attached at a different position but having the same stereochemistry. The chemical shift of C-3 (δ 109.3) was too high with respect to that of a carbon bonded to a hydroxyl group. Comparison of the chemical shifts of H-1 (δ 4.32) and of C-1 (δ 78.3) indicated they were similar to those corresponding to H-1 and C-1 in 4,5-dihydroniveusin A. This suggested that the methoxyl group was at C-3. The nOe effects observed between H-1, H-14, and the methoxyl group indicated α-orientation for H-l and the methoxyl group at C-3. The nOe effect observed between H-15a and the methoxyl group suggested α-orientation for the hydroxymethylene group at C-4. Thus, compound **12** is 8β-angeloyloxy-3β,10β-epoxy-1β,15-dihydroxy-3α-methoxy-4β-H-germacran-11(13)-en-6α,12-olide and was named helivypolide I.

Compound **13** was presented a molecular ion at *m/z* 424.2090, agreeing with the molecular formula $C_{22}H_{32}O_8$. The following bands were observed in the IR spectrum: 3406 (hydroxyl) and 1760 cm^{-1} (γ-lactone). The ^1H NMR spectrum of **13** was similar to that of **12**. The ethoxy group was deduced from the absence of the singlet signal corresponding to a methoxyl group present in the compound **12** and the appearance of signal at δ 3.50 (q, $J = 7.1$ Hz) and a signal at δ 1.18 (t, $J = 7.1$ Hz) in the ^1H NMR spectrum and signals at δ 56.7 and 15.3 in the ^{13}C NMR spectrum. Assignment of relative stereochemistry was based on nOe experiments. Irradiation of the H-14 signal induced nOe effects on H-1 and the signal assigned to the CH_2 of the ethyl group. The hydroxymethylene group at C-4 presented α-orientation on the basis of the nOe effect observed between H-15a and the CH_3 of the ethyl group. Thus, **13** was determined to be 8β-angeloyloxy-3β,10β-epoxy-3α-ethoxy-1β,15-dihydroxy-4β-H-germacran-11(13)-en-6α,12-olide and was named helivypolide J.

Compounds **1**−**14** (except **10**, due to the low amount obtained) were tested using the etiolated wheat coleoptile bioassay[14] in a range from 10^{-3} to 10^{-6} M. Compound **11** was tested starting at 10^{-4} M due the low amount isolated. (Figure 2) This is a fast bioassay (≤24 h), and it is sensitive to a wide range of bioactive substances including plant growth regulators, herbicides,[12] antimicrobials, mycotoxins, and assorted pharmaceuticals.[16] Compounds **2** and **4**

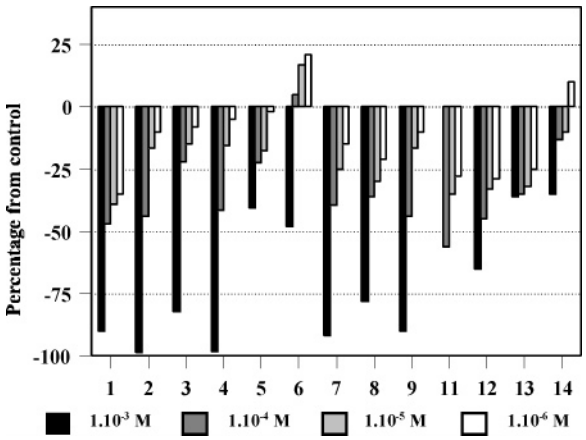


Figure 2. Effect of compounds **1**−**14** on growth of wheat etiolated coleoptiles.

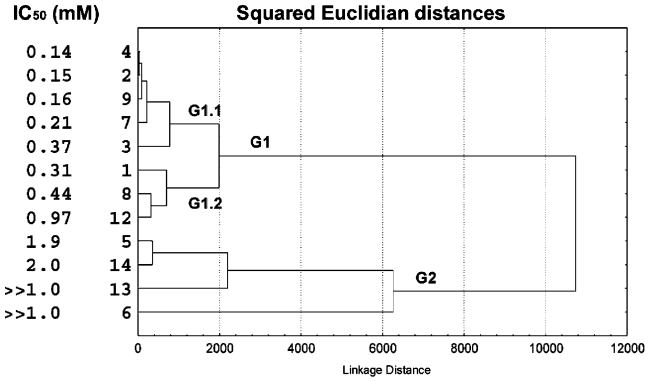


Figure 3. Cluster analysis of compounds **1**−**14** based on the on their activity on wheat etiolated coleoptiles.

gave total inhibition of growth at 10^{-3} M. (Figure 3) Cluster analysis of the data allowed us to split the sesquiterpene lactones into two different groups, namely **G1** and **G2**. Group **G1** contains those compounds that present IC_{50} values lower than 10^{-3} M, and **G2** contains compounds that are less active. Group **G2** is formed by the less-active compounds **5**, **14**, **13**, and **6**. Compound **5** was the only compound in this group with the lactone group located between C-7 and C-8. Compound **6** was the only eudesmane, and it was the only compound showing a stimulating effect at concentrations lower than 10^{-3} M. It is unusual for a natural product to be a plant growth promoter, and a search of the literature showed that this is a rare phenomenon. The usual natural product plant growth promoters are IAA, gibberellins, cytokinins, ethylene,[17] and brassinosteroids.[18] The behavior of **6** was similar to that of IAA, which inhibits the growth of etiolated wheat coleoptiles at 10^{-3}M, and promotes growth at lower concentrations.[19] Promoting plant growth in crops is of significant importance in global food production. Anytime crop production is increased, or sped up, economic savings may be realized. The shorter period of time that a crop is in the field also means reduced time of exposure to phytopathogens.

Among the guaianolides, compound **2** was the most active (−98% at 10^{-3} M, $IC_{50} = 0.15$ mM); whereas compound **3**, with a second hydroxyl group at C-2, showed lesser activity (−82%). Compound **1**, which lacks an angeloyloxy group at C-8, showed good activity at high concentrations (−90% at 10^{-3} M, $IC_{50} = 0.37$ mM) and significant effects at the lowest test concentration (−35% at 10^{-6} M).

With regard to the furaneheliangolides, compound **7** had the highest activity (−92% at 10^{-3} M), whereas the closely related compound **8**, having a double bond between C-1 and C-2, had slightly lower activity levels at higher concentrations. The group attached to C-3 appears to play an important role in the activity.

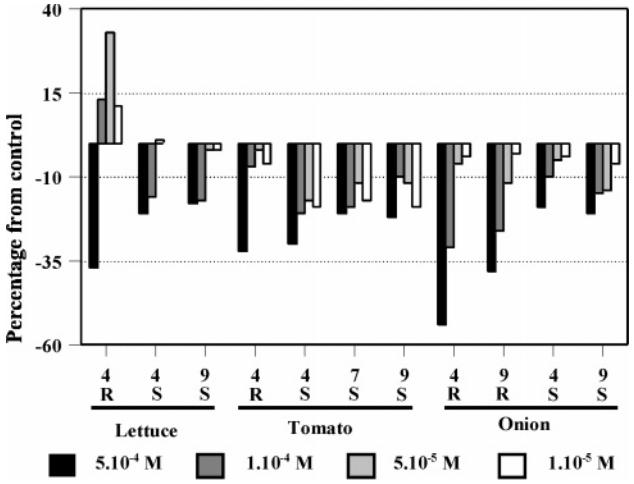


Figure 4. Selected effects of compounds **4**, **7**, and **9** on growth of standard target species. R = root, S = shoot.

Regarding phytotoxic activity, the four most active compounds in the previous bioassay (**2**, **4**, **7**, and **9)** were assayed using standard target species (STS) *Lepidium sativum* L., *Allium cepa* L., *Lactuca* sativa L., *Lycopersicon esculentum* Will., and *Triticum aestivum* L.[20] at concentrations from 5×10^{-4} to 10^{-5} M. The results obtained are summarized in Figure 4.

The most significant values obtained on lettuce were found for root or shoot growth with compounds **4** and **9**. There were few effects on tomato germination, but a significant effect for tomato root growth was found for compound **4** (−32%). Compounds **4**, **7**, and **9** also showed inhibitory effects (around 20% at 5×10^{-4} M) for the shoot development in this species.

Higher phytotoxicity was observed with onion. Thus, compounds **4** and **9** showed significant inhibitory effects on root growth (−54% at 5×10^{-4} M) which remained upon dilution. Compound **4** also showed effects on shoot growth (−20% at 5×10^{-4} M).

In all species, the most phytotoxic compounds were **4** and **9**, which present, in both cases, a carbonyl group at C-3 conjugated with two double bonds. It is also interesting to note that **4** had selective growth-promoting properties in lettuce roots but did not exhibit this property in tomato or onion.

Experimental Section

General Experimental Procedures. IR spectra (KBr) were recorded on a Perkin-Elmer FT-IR Spectrum 1000, Matton 5020 spectrophotometer. NMR spectra were run on Varian INOVA-400 and Varian INOVA 600 spectrometers. Chemical shifts are given in ppm (δ) with respect to residual $CHCl_3$ or $CDCl_3$ signals (δ 7.25 and 77.00, respectively). Optical rotations were determined using a Perkin-Elmer polarimeter model 241 (sodium D line). FABMS and HRMS were carried out on VG 1250 and VG AUTOESPEC mass spectrometers (70 eV).

Plant Material. *H. annuus* cv. Stella (commercialized by SENASA) and cv. SH-222 (commercialized by Semillas Pacífico) were collected during the third plant development stage[21] (plants 1.2 m tall with flowers, 1 month before harvest) and were provided by Rancho de la Merced, Agricultural Research Station (CIFA), Junta de Andalucía, Jerez, Spain.

Extraction and Isolation. Fresh leaves of *H. annuus* cv. Stella (4 Kg) were extracted in water (12 L) for 24 h at room temperature in the dark. The aqueous solution was extracted with CH_2Cl_2 (DCM) and then with EtOAc at room temperature. The solvent was removed in vacuo, yielding two fractions of 17.6 (DCM-A) and 7 g (EtOAc-A), respectively. The plant residue was dried at room temperature and re-extracted with CH_2Cl_2 and MeOH yielding, after solvent removal, 64.0 (DMC-P) and 69.0 g (MeOH-P), respectively. These extracts were bioassayed using wheat etiolated coleoptiles. DCM-P extract was the most active. This was chromatographed over silica gel using hexanes−EtOAc mixtures of increasing polarity. Those fractions eluted between hexanes−EtOAc 7:3 and 5:5 yielded compounds **2** (8 mg), **4** (45 mg), and **7** (50 mg). The fraction eluted with hexanes−EtOAc 4:6 yielded compounds **3** (9 mg), **5** (8 mg), and **9** (55 mg).

Fresh leaves of *H. annuus* cv. SH-222 (6 Kg) were treated in a similar way. The DCM-A extract (16.0 g) was chromatographed over silica gel using a hexane−EtOAc mixtures of increasing polarity, and finishing with acetone. Those fractions eluted between hexanes−EtOAc 5:5 and 3:7 yielded compounds **8** (7 mg) and **1** (4.3 mg). The fraction eluted with acetone yielded **6** (5 mg), **10** (1 mg), **11** (1 mg), **12** (2 mg), **13** (5 mg), and **14** (4 mg).

Annuolide H (3): colorless oil; $[\alpha]^{25}_D$ +23.0 (*c* 1.0, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}; 3485 (OH), 1775 (γ-lactone, α,β-unsaturated), 1720 (ester α,β-unsaturated). ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS *m/z* (rel int.): 362 $[M]^+$, 279 $[M - C_5H_7O]^+$ (15); HREIMS *m/z* 362.1729 (calcd for $C_{20}H_{26}O_6$, 362.1729).

Helivypolide F (4): colorless oil; IR ν_{max} (KBr) cm^{-1}; 3468 (OH), 1776 (γ-lactone, α,β-unsaturated), 1720 (ester α,β-unsaturated). ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS *m/z* (rel int.): 358 $[M]^+$ (3), 83 $[M - C_5H_7O]^+$ (100), 55 $[C_4H_7]^+$; HREIMS *m/z* 358.1414 (calcd for $C_{20}H_{22}O_6$, 358.1416).

Helieudesmanolide A (6): colorless oil; $[\alpha]^{25}_D$ −12.0 (*c* 1.0, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}; 3410 (OH), 1776 (γ-lactone, α,β-unsaturated). ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HREIMS *m/z* 362.1729 (calcd for $C_{20}H_{26}O_6$, 362.1729).

Helivypolide H (11): colorless oil; $[\alpha]^{25}_D$ +53.6 (*c* 1.0, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}; 3480 (OH), 1780 (γ-lactone, α,β-unsaturated), 1720 (ester α,β-unsaturated). ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HREIMS *m/z* 392.1848 (calcd for $C_{21}H_{28}O_7$, 392.1835).

Helivypolide I (12): colorless oil; $[\alpha]^{25}_D$ +17.1 (*c* 1.0, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}; 3360 (OH), 1760 (γ-lactone, α,β-unsaturated), 1710 (ester α,β-unsaturated), 1600 (C═C),1150 (C−O−C). ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HREIMS *m/z* 410.1941 (calcd for $C_{21}H_{30}O_8$, 410.1941).

Helivypolide J (13): colorless oil; $[\alpha]^{25}_D$ +45.4 (*c* 1.0, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}; 3380 (OH), 1760 (γ-lactone, α,β-unsaturated), 1720 (ester α,β-unsaturated), 1600 (C═C),1130 (C−O−C). ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HREIMS *m/z* 424.2090 (calcd for $C_{22}H_{32}O_8$, 424.2097).

Coleoptile Bioassay. Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in 15 cm diameter Petri dishes moistened with water and grown in the dark at 22 ± 1 °C for 3 days.[14] Roots and caryopses were removed from the shoots. The latter were placed in a Van der Weij guillotine, and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for bioassay. All manipulations were performed under a green safelight.[22] Compounds were predissolved in DMSO and diluted to the final bioassay concentration with a maximum of 0.1% DMSO. Parallel controls were also run.

Crude extracts, fractions, or pure compounds to be assayed for biological activity were added to test tubes. The assays were duplicated. Phosphate−citrate buffer (2 mL) containing 2% sucrose[22] at pH 5.6 was added to each test tube. Following the placement of five coleoptiles in each test tube (three tubes per dilution), the tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at 22 °C in the dark. The coleoptiles were measured by the digitalizing of their images. Data were statistically analyzed using Welch's test.[23] Data are presented as percentage differences from control. Thus, zero represents the control, positive values represent stimulation of the studied parameter, and negative values represent inhibition.

Phytotoxicity Bioassays. The selection of target plants was based on an optimization process made by us in our search for a standard phytotoxicity bioassay.[20] Several STS were proposed, including monocots *Triticum aestivum* L. (wheat) and *Allium cepa* L. (onion) and dicots *Lycopersicon* esculentum Will. (tomato), *Lepidium sativum* L. (cress), and *Lactuca sativa* L. (lettuce), which were assayed for this study.

Bioassays were conducted using Petri dishes (90 mm diameter), with one sheet of Whatman No.1 filter paper as support. Germination and growth were conducted in aqueous solutions at controlled pH by using 10^{-2} M 2-[*N*-morpholino]ethanesulfonic acid (MES) and NaOH 1 M (pH 6.0). Compounds to be assayed were dissolved in DMSO (0.1, 0.02, 0.01, and 0.002 M), and these solutions were diluted with buffer (5 μL DMSO solution/mL buffer) so that test concentrations for each compound (5×10^{-4}, 10^{-4}, 5×10^{-5}, and 10^{-5} M) were reached. This procedure facilitated solubility of the assayed compounds. The number

of seeds in each Petri dish depended on the seed size. Twenty five seeds were used for tomato, lettuce, cress, and onion, and 10 seeds were used for wheat. Treatment, control, or internal reference solution (5 mL) was added to each Petri dish. Four replicates were used for tomato, cress, onion, and lettuce (100 seeds); 10 replicates (100 seeds) were used for wheat.

After seeds and aqueous solutions were added, Petri dishes were sealed with Parafilm to ensure closed-system models. Seeds were further incubated at 25 °C in a Memmert ICE 700 controlled-environment growth chamber in the dark. Bioassays took 4 days for cress, 5 days for lettuce, tomato, and wheat, and 7 days for onion. After growth, plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process.

The commercial herbicide Logran, a combination of *N*-(1,1-dimethylethyl)-*N*′-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (Terbutryn, 59.4%) and 2-(2-chloroethoxy)-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2yl)amino]carbonyl]benzene sulfonamide (Triasulfuron, 0.6%) was used as an internal reference, according to a comparison study reported previously.[20] It was used at the same concentrations (5×10^{-4}, 10^{-4}, 5×10^{-5}, and 10^{-5} M) and in the same conditions as those reported. Control samples (buffered aqueous solutions with DMSO and without any test compound) were used for all of the plant species assayed.

Evaluated parameters (germination rate, root length, and shoot length) were recorded by using a Fitomed system[24] that allowed automatic data acquisition and statistical analysis using its associated software. Data were analyzed statistically using Welch's test, with significance fixed at 0.01 and 0.05. Results are presented as percentage differences from the control. Zero represents control, positive values represent stimulation, and negative values represent inhibition.[20,24] Once the germination and growth data were acquired, cluster analysis was used to group compounds with similar phytotoxicity behaviors and associate them with their molecular structure. Complete Linkage was used as amalgamation rule and the distance measurement was based on Squared Euclidean Distances,[25] given by this equation:

$$d(x,y) = \sum_i (x_i - y_i)^2$$

Where $d(x,y)$ is the Squared Euclidean Distance (i-dimensional), i represents the number of variables, and x and y the observed values. The cluster was obtained by using Statistica v. 5.0 software.

IC_{50} values were obtained after adjusting phytotoxicity data to concentration (logarithmic scale) to a sigmoidal dose−response curve defined by the equation:

$$Y = Y_{\min} + \frac{Y_{\max} - Y_{\min}}{1 + 10^{\log EC50 - X}}$$

Where X indicates the logarithm of concentration, Y indicates the response (phytotoxicity), and $Y_{\max}$ and $Y_{\min}$ are the maximum and minimum values of the response, respectively. Goodness of fit is described by determination coefficient (r^2). The adjustment and the r^2 were obtained by using GraphPad Prism software v. 4.00.

Acknowledgment. This research was supported by the Ministerio de Ciencia y Tecnología, Spain (MCYT; Project No. PPQ2002-03595). A Postdoctoral fellowship from FAPESP, Brazil (P.L.C.A.A.), is also gratefully acknowledged.

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NP060056S