

Sesquiterpene Lactones with Potential Use as Natural Herbicide Models. 2. Guaianolides[†]

Francisco A. Macías,* Juan C. G. Galindo, Diego Castellano, and Raúl F. Velasco

Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Cádiz, c/ República Saharaui s/n, Apdo. 40, 11510-Puerto Real, Cádiz, Spain

A structure–activity study to evaluate the effect of 17 guaianolide sesquiterpene lactones (in a range of 100–0.001 μM) on the growth and germination of several mono- and dicotyledon target species is accomplished. Results are compared with those obtained in the same bioassay with an internal standard, the commercial herbicide Logran, to validate the results with a known active formulation and to compare the results with a commercial product to test their potential use as natural herbicide models. Specific conditions for the selective mono- or polyhydroxylation of guaianolides using the SeO_2 /*tert*-butyl hydroperoxide system are presented and discussed. The high regio- and stereoselectivities of the reaction are explained through the specific structural requirements of the bulky first adduct formed during the ene reaction. These compounds appear to have deeper effects on the growth of either monocots or dicots than the previously tested germacranolides. Otherwise, the lactone group seems to be necessary for the activity, though it does not necessarily need to be unsaturated. However, the presence of a second and easily accessible unsaturated carbonyl system greatly enhances the inhibitory activity. Lipophilicity and the stereochemistry of the possible anchoring sites are also crucial factors for the activity. Finally, the levels of growth inhibition obtained with some compounds on dicots or monocots are totally comparable to those of Logran and allow proposing them as lead compounds.

Keywords: *Guaianolide*; *dehydrocostuslactone*; *isozaluzanin C*; *dehydrozaluzanin C*; *Logran*; *allylic oxidation*; *phytotoxicity*; *allelopathy*; *herbicide*; *Lactuca sativa L.*; *Lycopersicon esculentum L.*; *Lepidium sativum L.*; *Allium cepa L.*; *Triticum aestivum L.*

INTRODUCTION

Guaianolides and the closely structurally related pseudoguaianolides are an especially bioactive group of sesquiterpene lactones (SLs). They have been reported to have several activities such as cytotoxic (Yuuya et al., 1999), antifungal (Wedge et al., 2000), larvaecidal (Neves et al., 1999), antiplasmodial (Fournet et al., 1993), and phytotoxic (Galindo et al., 1999; Macías et al., 2000a). SLs have been reported as allelopathic agents in many plants of the family Compositae (Macías et al., 1993; Pandey, 1994), being the guaianolide- and pseudoguaianolide-like compounds most frequently tested.

In continuation of our systematic study on SLs as potential lead herbicides (Macías et al., 1999), herein we present the results of a structure–activity relationship (SAR) study accomplished with 17 SLs bearing the guaianolide backbone. Most of them have been synthesized from the readily available dehydrocostuslactone (**1**) (compounds **2–18**) (Macías et al., 1992, 2000b,c; Wedge et al., 2000) (Figure 1), while 8 α -hydroxyachillin (**19**) and 8 α -acetoxyachillin (**20**) (Figure 1) have been

obtained from natural sources. Compounds have been tested in our phytotoxic bioassay system (Macías et al., 2000b) using monocot and dicot species as targets and the commercial herbicide Logran as internal standard.

Water solubility is one of the main objections made to the use of these compounds and their possible mechanism of action. However, a certain degree of lipophilicity is needed to cross the membranes, and depending on their target site, amphipathic compounds are desirable. Thus, a methodology to introduce in consecutive steps different hydroxyl groups in selected positions has been developed using the soft oxidant selenium dioxide/*tert*-butylhydroperoxide or the hexamethylphosphoric triamide (HMPA)/ Na_2CO_3 system, and their effect on the phytotoxicity is discussed.

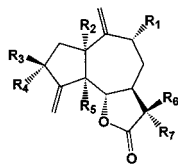
Since the mode of action of these compounds is yet unknown, no attempts to establish any further comparison between the mode of action of Logran and the SLs are made. The study has been focused on germination and root and shoot elongation effects. They have been accepted as indirect measures of the effects caused by the chemicals on their target sites.

MATERIALS AND METHODS

Dehydrocostuslactone (**1**) was obtained from crude costus resin oil (*Saussurea lappa*) by previous column chromatography (CC) separation and then purified by crystallization from hexane/ethyl acetate mixtures. 8 α -Hydroxyachillin (**19**) and 8 α -acetoxyachillin (**20**) were obtained from *Artemisia lanata* Wild extract by CC. All the structures of the natural compounds were confirmed by comparison of their spectroscopic

* To whom correspondence should be addressed. E-mail: famacias@uca.es. Phone: +34-956-016370. Fax: +34-956-016288, +34-956-016193.

[†] Natural Products as Allelochemicals. 13. Part 12: Macías, F. A.; Simonet, A. M.; Pacheco, P. C.; Barrero, A. F.; Cabrera, E.; Jiménez-González, D. Natural and synthetic podolactones with potential use as natural herbicide models. *J. Agric. Food Chem.* **2000**, *48*, 3003–3007.



R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	
H	H	H	H	H	-CH ₂ -		1
H	H	H	OH	H	-CH ₂ -		2
H	H	OH	H	H	-CH ₂ -		3
H	H	H	H	OH	-CH ₂ -		4
H	H	H	OH	OH	-CH ₂ -		5
H	OH	H	OH	OH	-CH ₂ -		6
OH	H	H	OH	OH	-CH ₂ -		7
H	H		O	H	-CH ₂ -		8
H	H	H	H	H	CH ₂ OH	H	10
H	H	H	H	H	H	CH ₂ OH	11
H	H	H	H	H	CH ₂ OH	OH	12
H	H	H	OH	H	H	CH ₂ OH	13
H	H	H	OH	OH	H	CH ₂ OH	14
H	H	H	H	OH	H	CH ₂ OH	15
H	H	H	OH	OH	H	CH ₃	17
H	OH	H	OH	OH	H	CH ₃	18

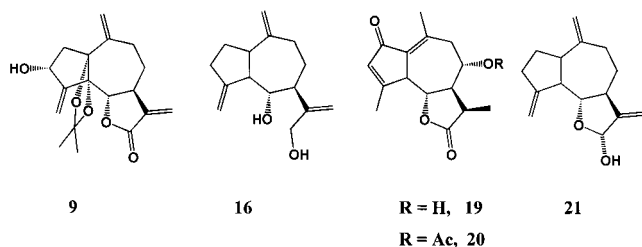


Figure 1. Guaianolides tested.

data (IR, EIMS, ¹H and ¹³C NMR) with those in the literature. Logran (terbutryn (59.4%) + triasulfuron (0.6%)) was supplied by Novartis.

General Experimental Procedures. ¹H NMR and ¹³C NMR spectra were recorded at 399.952 and 100.577 MHz, respectively, on a Varian UNITY-400 spectrometer using CDCl₃ as solvent. The resonances of residual chloroform at δ_H 7.25 ppm in the ¹H spectra and of CDCl₃ at δ_C 77.00 ppm in the ¹³C spectra were used as internal references. Mass spectra were obtained by using a VG 1250 or a Kratos MS-80-RFA instrument at 70 eV. The infrared (IR) spectra were recorded on a Bio-Rad FTS-7. Column chromatography was performed on silica gel (35–75 mesh), and TLC analysis was carried out using aluminum-packed precoated silica gel plates. For HPLC, LiChrosorb silica 60 was used in the normal-phase mode using differential refractometer (RI) and UV detectors, with a Hitachi L-6020A HPLC instrument. All solvents were spectral grade or distilled from glass prior to use.

Monohydroxylation of SLs 1 and 11. Compound **1** (500 mg, 2.17 mmol, 1 equiv) was dissolved in CHCl₃ (75 mL, 0.03 M solution) and strongly stirred at room temperature with selenium dioxide (SeO₂, 60 mg, 0.54 mmol, 0.5 equiv). Then, *tert*-butyl hydroperoxide (TBHP; 2 mL, 2 equiv) was added dropwise at a rate of 0.2 mL/min and allowed to react for 10 min. Filtering the reaction mixture through silica gel stopped the reaction, and the solvent was then evaporated under vacuum. The crude product of the reaction was purified by CC using a hexane/ethyl acetate mixture as eluent, yielding **2** (294 mg, 55%), **4** (150 mg, 28%), **5** (74 mg, 13%), and trace amounts of **3** (Macias et al., 1992). Treatment of **11** under the same conditions yielded **13** (335 mg, 63%) and **14** (68 mg, 12%).

Compound 4. Colorless crystals; IR (neat, KBr) ν_{max} 3401 (OH), 1754 (carbonyl group) cm⁻¹; EIMS *m/z* (rel intens) 246

[M]⁺ (1), 228 [M - H₂O]⁺ (3), 218 [M - CO]⁺ (6); ¹H NMR, see Table 2; ¹³C NMR, see Table 3; HREIMS (M⁺) found 246.1250, C₁₅H₁₈O₃ requires 246.1256.

Compound 13. Colorless crystals; IR (neat, KBr) ν_{max} 3450 (OH), 1746 (carbonyl group) cm⁻¹; EIMS *m/z* (rel intens) 264 [M]⁺ (2), 246 [M - H₂O]⁺ (5), 228 [M - 2H₂O]⁺ (4); ¹H NMR, see Table 2; ¹³C NMR, see Table 3; HREIMS (M⁺) found 264.1370, C₁₅H₂₀O₄ requires 264.1362.

Compound 14. Colorless crystals; IR (neat, KBr) ν_{max} 3428 (OH), 1752 (carbonyl group) cm⁻¹; EIMS *m/z* (rel intens) 280 [M]⁺ (1), 262 [M - H₂O]⁺ (7), 244 [M - 2H₂O]⁺ (4); ¹H NMR, see Table 2; ¹³C NMR, see Table 3; HREIMS (M⁺) found 280.1319, C₁₅H₂₀O₅ requires 280.1311.

Dihydroxylation of SLs 1 and 11. Compound **1** (240 mg, 1.04 mmol, 1 equiv) was dissolved in CHCl₃ (10 mL, 0.1 M) and strongly stirred at room temperature. Then, SeO₂ (27 mg, 0.24 mmol, 1 equiv) and TBHP (1 mL, 4 equiv) were added. The reaction was stopped 2 h later by filtering through silica gel and the solvent evaporated under vacuum. CC of the crude product of the reaction yielded **2** (38 mg, 15%) and **5** (164 mg, 60%). Treatment of **11** (200 mg) under the same conditions yielded **13** (38 mg, 18%), **14** (109 mg, 51%), and trace amounts of **15**.

Compound 15. Colorless crystals; IR (neat, KBr) ν_{max} 4332 (OH), 1745 (carbonyl group) cm⁻¹; EIMS *m/z* (rel intens) 264 [M]⁺ (3), 246 [M - H₂O]⁺ (5), 228 [M - 2H₂O]⁺ (8); ¹H NMR, see Table 2; ¹³C NMR, see Table 3; HREIMS (M⁺) found 264.1359, C₁₅H₂₀O₄ requires 264.1362.

Hydroxylation of 5. Compound **5** (100 mg, 0.38 mmol, 1 equiv) was dissolved in CHCl₃ (3.8 mL, 0.1 M) and strongly stirred at room temperature. Then, SeO₂ (34 mg, 0.31 mmol, 2 equiv) and TBHP (35 mL, 1 equiv) were added. After 12 h, the reaction was stopped as usual, yielding **6** (30 mg, 28%) as the unique product (Macias et al., 1992).

Hydroxylation of 1 under Reflux. Compound **1** (2.17 mmol, 1 equiv) was dissolved in CHCl₃ (0.1 M solution) with SeO₂ (120 mg, 1.08 mmol, 1 equiv) and TBHP (2 mL, 1 equiv). The reaction mixture was refluxed over 90 min and then stopped as usual, yielding **5** (42%), **6** (16%), and **7** (10%).

Compound 7. Colorless crystals; IR (neat, KBr) ν_{max} 3280 (OH), 1756 (carbonyl group) cm⁻¹; EIMS *m/z* (rel intens) 278 [M]⁺ (1), 260 [M - H₂O]⁺ (3), 242 [M - 2H₂O]⁺ (5), 224 [M - 3H₂O]⁺ (4); ¹H NMR, see Table 2; ¹³C NMR, see Table 3; HREIMS M⁺, found 278.1162, C₁₅H₁₈O₅ requires 278.1154.

Hydroxylation of 1 with HMPA/Na₂CO₃. Compound **1** (150 mg, 0.65 mmol, 1 equiv) was dissolved in HMPA (15 mL), and then, an aqueous solution of Na₂CO₃ (20% (w/v)) was added dropwise until the mixture remained unclear. The reaction mixture was strongly stirred and heated at 90–95 °C over 7 days. Workup: The reaction mixture was extracted with AcOEt (5×), and the combined organic phases were washed consecutively with aqueous HCl (1 N) (3×), aqueous Na₂CO₃ (20% (w/v)) (3×), and brine (3×). The organic phase was dried over anhydrous sodium sulfate, the solvent evaporated under vacuum, and the crude product of the reaction purified by CC (hexane/ethyl acetate mixture), yielding **10** (72 mg, 30%), **11** (60 mg, 21%), and **12** (11 mg, 6%), with 45 mg (30%) of starting material **1** remaining.

Sodium Borohydride (NaBH₄) Reductions. A 2 mL methanolic solution with 15 mg each of compounds **5** and **6** was kept in a Dewar glass at 0 °C. While the solution was stirring, NaBH₄ (1.4 equiv) was added during the first 5 min of reaction. After 1 h, the reaction was stopped by addition of 2 mL of distilled water. Extraction with AcOEt and HPLC purification (hexane/ethyl acetate mixture) yielded dihydro derivatives **17** and **18** (average 90%).

Compound 17. White powder; IR (neat, KBr) ν_{max} 3220 (OH), 1756 (carbonyl group) cm⁻¹; EIMS *m/z* (rel intens) 264 [M]⁺ (2), 248 [M - H₂O]⁺ (5); ¹H NMR, see Table 2; ¹³C NMR, see Table 3; HREIMS (M⁺) found 264.1355, C₁₅H₂₀O₄ requires 264.1362.

Compound 18. White powder; IR (neat, KBr) ν_{max} 3430 (OH), 1756 (carbonyl group) cm⁻¹; EIMS *m/z* (rel intens) 280 [M]⁺ (2), 262 [M - H₂O]⁺ (5), 244 [M - 2H₂O]⁺ (4), 226 [M - 3H₂O]⁺

Table 1. Resume of the Bioactivity Data of Compounds 1–9 and 11–20^a

	lettuce			cress			tomato			wheat			onion			
	G	R	S	G	R	S	G	R	S	G	R	S	G	R	S	
1	–	0	(+)	–, +	=	=	0	0	0	=, +	=	–	–	0	=	=
2	(+)	0	(–)	=	=, ++	=, ++	–	=	=	–	=	–	–	0	=	=
3	0	+	+	(–)	(–)	0	0	(+)	0	(+)	–	–	0	0	0	
4	0	+	0	(+)	–, ++	–, ++	0	(–)	0	0	0	(+)	(+)	0	–	(–)
5	+	(–)	–	=	=, ++	=, ++	0	=	=	0	=	–	–	0	=	=
6	0	(+)	+	0	(+)	0	0, (+)	0	0	0	(+)	(+)	0	0	0	0
7	0	+	(+)	0	0	0	(+)	0	0	0	+	+	0	0	0	0
8	=	=	=	=	=	=	=	=	=	=	=	=	=	=	=	=
9	(+)	+	0	+	++	++	0	0	0	0	(–)	(–)	0	0	0	0
11	(+)	–	0	(–)	=	=	0	=	=	–, (+)	–	–	0	=	=	=
12	0	0	(+)	0	0	0	(+)	0	0	(+)	0	0	0	0	0	0
13	(–)	+	++	0	–	(+)	0	0	0	0	0	0	0	0	0	0
14	–	+	++	0	=	–	0	0	0	(+)	0	0	0	0	0	0
16	0	0	(+)	0	0	0	+	(–)	0	0	+	(+)	0	0	0	0
17	0	(+)	+	0	0	0	0	0	0	0	0	–	0	0	0	0
18	–	++	++	0	(+)	(+)	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	(+)	0	(–)	0	0	0	0	0	0	0
20	0	(–)	(–)	0	0	0	0	0	(–)	–	0	(–)	0	0	0	0
L	=	=	=	–	=	=	0	=	=	–	–	–	0	=	=	=

^a Key: 0, no activity; (+), (–), stimulatory or inhibitory values below 20%; +, –, stimulatory or inhibitory values between 20% and 40%; ++, =, stimulatory or inhibitory values higher than 40%. G = germination, R = root length, and S = hypocotyl length. Values of Logran activity presented here correct the previous ones in Macías et al. (1999).

Table 2. ¹H NMR Data for Compounds 4, 7, 13–15, 17, 18, and 21 (400 MHz in CDCl₃, Signal of the Residual CHCl₃ Centered at δ 7.25 ppm)^a

	4	7	13	14	15	17	18	21
1	2.69 dd	3.25	3.01 m	2.79 dd	2.70 m	2.81 dd	–	2.84 m
2_α	1.82 dd	2.08 m	2.15 ddd	2.10 m	1.82 dddd	2.25 m	2.52 dd	–
2_β	2.19 dd	2.08 m	1.83 ddd	2.10 m	2.20	2.17 ddd	2.03 dd	–
3_α	2.57 dd	–	–	–	2.54 ddd	–	–	–
3_β	2.77 dd	4.63	4.63	4.60 br dd	2.70 m	4.70 br s	4.57 dddd	–
5	–	–	3.06 m	–	–	–	–	2.76
6	6.18 dd	3.97 d	3.88 dd	4.05 d	4.23	4.09	4.05 d	3.72 dd
7	3.26 dddd	3.48	2.37	2.44 m	2.69	2.36 dddd	2.39 m	2.41
8_α	2.19 ddd	2.25	2.10 m	1.32 dddd	1.40	2.05 m	2.00	1.33 m
8_β	1.45 m	1.53 ddd	1.27 dddd	2.10 m	2.17 m	1.33 m	1.32	–
9_α	2.24 m	–	2.45 ddd	2.43 m	2.17 m	2.12	2.08	–
9_β	2.48 m	4.46 dd	1.99 ddd	2.37 m	2.70 m	2.53 ddd	2.54	–
11_β	–	–	2.33 ddd	2.73	2.42 m	2.26 dq	2.23	–
12	–	–	–	–	–	–	–	–
13_a	5.52 d	5.51	3.96 dd	3.84	3.80 dd	1.25 d	1.28	5.71 br d
13_b	6.24 d	6.15	3.68 dd	3.73	3.94 dd	–	–	5.04
14	4.92 s	5.02	4.86	4.87	4.81 br s	4.93	4.91	4.81 dd
14'	4.82 s	4.76	4.68	4.75	4.91 br s	4.72	4.75	4.72
15	5.41 dd	5.61 d	5.35 dd	5.53 d	5.34 s	5.61 d	5.59	5.20 m
15'	5.10 dd	5.40 d	5.29 dd	5.34 d	5.08 s	5.43 d	5.40	5.00 m

^a Multiplicities are not repeated if identical with those of the preceding column. *J* (Hz): (**4**) 1,2_α = 2_α,3_α = 2_α,2_β = 5.1; 1,2_β = 7.9; 2_α,3_β = 9.2; 2_α,2_β = 13.2; 3_α,3_β = 11.3; 6,7 = 8.8; 7,13_a = 3.2; 7,13_b = 3.6; 7,8_α = 4.7; 8_α,9_α = 2.2; 8_α,9_β = 8.3; 8_α,8_β = 10.6; 15,3_α = 15,3_β = 2.4; 15',3_α = 15',3_β = 2.1; (**7**) 1,2_α = 4.3; 1,2_β = 8.5; 2_α,2_β = 14.1; 2_α,3_β = 2_β,3_β = 7.6; 3_β,15 = 1.8; 3_β,15' = 1.6; 6,7 = 8.8; 7,8_α = 3.2; 8_α,8_β = 10.1; 8_α,9_β = 8_β,9_β = 6.6; 7,13_a = 3.1; 7,13_b = 3.4; (**13**) 1,2_α = 3.8; 1,2_β = 7.3; 1,5 = 1.7; 2_α,2_β = 13.2; 2_α,3_β = 6.8; 2_β,3_β = 6.9; 5,6 = 6.7 = 9.3; 8_α,8_β = 12.6; 8_α,9_α = 8_β,9_α = 4.1; 8_α,9_β = 12.3; 8_β,9_α = 11.8; 7,8_β = 8_β,9_β = 4.6; 9_α,9_β = 12.9; 11,13_a = 3.3; 11,13_b = 4.4; 13_a,13_b = 11.8; 15,1 = 15,3_β = 1.9; 15',1 = 15',3_β = 1.8; (**14**) 1,2_α = 3.6; 1,2_β = 7.2; 2_α,3_β = 2_β,3_β = 7.6; 3_β,15 = 2.2; 3_β,15' = 1.8; 6,7 = 9.7; 7,11 = 8.8; 11,13_a = 11,13_b = 4.1; 13_a,13_b = 11.6; (**15**) 1,2_α = 2_α,3_α = 4.0; 2_α,2_β = 13.2; 2_α,3_β = 4.9; 6,7 = 9.7; 7,8_α = 5.8; 8_α,9_α = 11.3; 8_α,8_β = 14.9; 9_α,9_β = 16.3; 11,13_a = 6.1; 11,13_b = 4.3; 13_a,13_b = 11.4; 15,2_α = 15,2_β = 2.3; 15',2_α = 15',2_β = 2.4; (**17**) 1,2_α = 3.3; 1,2_β = 8.8; 2_α,2_β = 15.4; 2_β,3_β = 6.8; 6,7 = 9.6; 7,8_α = 2.4; 7,8_β = 11.5; 7,11 = 11.5; 9_α,9_β = 11.0; 8_α,9_β = 8_β,9_β = 5.6; 11,13 = 3.5; 15,3_β = 1.8; 15',3_β = 1.5; (**18**) 2_α,2_β = 15.4; 2_β,3_β = 6.8; 6,7 = 9.6; 7,11 = 11.2; 9_α,9_β = 13.2; 8_α,9_β = 8_β,9_β = 6.4; 11,13 = 3.5; 15,3_β = 1.6; 15',3_β = 1.4; (**21**) 5,6 = 6,7 = 9.2; 7,13_a = 2.7; 7,13_b = 3.1; 12,13_a = 1.8; 12,13_b = 1.3; 1,14' = 9,14' = 1.4.

(7); ¹H NMR, see Table 2; ¹³C NMR, see Table 3; HREIMS (M⁺) found 280.1308, C₁₅H₂₀O₅ requires 280.1311.

Diisobutylaluminum Hydride (DIBALH) Reduction. A dry toluene solution of **1** (100 mg, 0.43 mol, 1 equiv) was stirred with a DIBALH toluene solution (1.1 equiv) in a Dewar glass (–70 °C, N₂ atmosphere, 1.5 h). Workup: MeOH and distilled water were added consecutively; the gelatinous precipitate was homogenized and extracted with AcOEt. All organic phases were dried over anhydrous Na₂SO₄, and the solvent was evaporated under vacuum to yield **21** (62 mg, 61%).

Compound 21. Colorless oil; IR (neat, KBr) ν_{max} 3220 (OH), 1756 (carbonyl group) cm^{–1}; EIMS *m/z* (rel intens) 232 [M]⁺ (4), 214 [M – H₂O]⁺ (18); ¹H NMR, see Table 2; ¹³C NMR, see

Table 3; HREIMS (M⁺) found 232.1455, C₁₅H₂₀O₂ requires 232.1463.

Molecular Modeling. Minimum-energy conformations and molecular properties were obtained using MMX and PM3 calculations (PCModel version 6.0, Serena Software, Bloomington, IN; MOPAC version 6.00). Conformers were obtained using the randomize command in PCModel, and the local minimum energy structures obtained were used for further semiempirical minimization with MOPAC using the PM3 method. For semiempirical calculations the parameters PRECISE, GEOM-OK, and T = 86400 were used. Theoretical Δ*H*_f values produced with MOPAC allowed us to discriminate and to obtain the minimum-energy conformer in each case.

Table 3. ^{13}C NMR Data for Compounds **4**, **13–15**, **17**, **18**, and **21** (100 MHz in CDCl_3 , Signal of CDCl_3 Centered at δ 77.00 ppm)^a

	4	7	13	14	15	17	18	21
1	55.4 d	45.5	49.6	58.4	55.0	52.7	80.1 s	48.8 d
2	28.2 t	38.9	38.0	30.7	28.2	38.6	44.8	30.5
3	31.2 t	73.9 d	74.4	71.6	31.5 t	74.9 d	71.5	32.1 t
4	154.2 s	150.1	154.4	152.5	154.5	157.0	155.5	151.9
5	81.2 s	79.1	49.3 d	83.6 s	81.3	80.3 s	82.7	48.2 d
6	85.8 d	86.0	85.9	83.0	86.4	85.8	83.5	83.0
7	40.2 d	35.6	43.6	44.4	38.4	41.6	39.9	52.5
8	31.5 t	37.6	32.4	32.2	32.7	32.9	31.4	32.7
9	37.0 t	73.3 d	39.7 t	46.5	37.9	38.9	38.5	32.3
10	139.5 s	139.2	149.1	150.3	148.3	147.8	147.7	149.9
11	148.2 s	155.7	43.1 d	49.6	49.0	44.2	43.8	153.5
12	176.9 s	170.8	177.4	177.2	177.3	178.4	177.3	97.4 d
13	121.0 t	120.7	58.6	59.2	60.1	13.2 q	14.1	108.2 ^b t
14	110.7 t	113.2 t	112.4	116.4	113.2	113.5	115.7	110.9
15	113.7 t	115.0	112.4	114.7	110.3	114.0	117.9	108.1 ^b

^a Multiplicities are not repeated if identical with those of the preceding column. ^b Values may be interchanged.

Germination and Seedling Growth Bioassays. Seeds of *Lactuca sativa* L. cv. Roman, *Lycopersicon esculentum* L. cv. Tres Cantos, *Lepidium sativum* L. cv. Común, *Allium cepa* L. cv. Valenciana, and *Triticum aestivum* L. cv. Cortex were obtained from FITO, S.L. (Barcelona, Spain). All undersized or damaged seeds were discarded, and the assay seeds were selected for uniformity. Bioassays were carried out in 9 cm \varnothing plastic Petri dishes, using Whatman No. 1 filter paper as support.

Bioassay Methodology. The general procedure was as follows: *L. sativum*, 25 seeds per dish, 5 mL of test solution, 4 days in the dark, 25 °C, and four replicates of each concentration; *L. sativa* and *L. esculentum*, 25 seeds per dish, 5 mL of test solution, 5 days in the dark, 25 °C, and four replicates of each concentration; *A. cepa*, 25 seeds per dish, 5 mL of test solution, 7 days in the dark, 25 °C, and four replicates of each concentration; *T. aestivum*, 10 seeds per dish, 5 mL of test solution, 5 days in the dark, 25 °C, and 10 replicates of each concentration (Macías et al., 2000a).

Test solutions (10^{-3} or 10^{-4} M) were prepared using MES (2-[N-morpholino]ethanesulfonic acid) (10 mM), and the rest were obtained by dilution. Parallel controls were performed. All pH values were adjusted to 6.0 before bioassay with MES. All products were purified prior to the bioassay using HPLC equipped with a refractive index detector. The minimum degree of purity was 99% as extracted from the chromatograms.

Data are presented as percentage differences from control in graphics (Figures 5 and 6). Thus, zero represents the control; positive values represent stimulation of the studied parameter, and negative values represent inhibition.

Statistical Treatment. Germination and root and shoot length values were tested by Welch's test (Zar, 1984), the differences between test solutions and controls being significant with $P \leq 0.01$.

RESULTS

Selective mono- and dihydroxylations are obtained depending upon the reaction conditions (Figure 2). Compounds **2**, **3**, and **5** spectral data (MS, IR, ^1H and ^{13}C NMR) were fully consistent with those in the literature. Moreover, X-ray analysis of compounds **2** and **5** confirmed the structures and the stereochemistry proposed for the hydroxyl groups. For the newly reported compound **4**, the IR and the EIMS data confirmed the incorporation of a hydroxyl group, whereas the position and stereochemistry of the new group was established as C-5 α through the analysis of its ^1H NMR spectrum and comparison with data obtained for **5**.

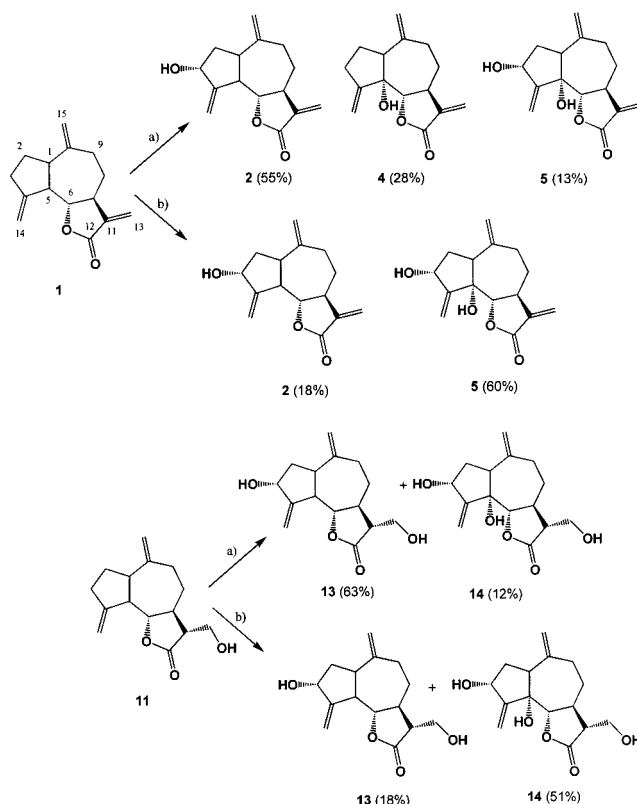


Figure 2. (a, top) 0.03 M in CHCl_3 ; $1/\text{SeO}_2/\text{TBHP}$ (1:0.5:2); room temperature; 30 min; (b, bottom) 0.1 M in CHCl_3 ; $1/\text{SeO}_2/\text{TBHP}$ (1:1:4); 2 h.

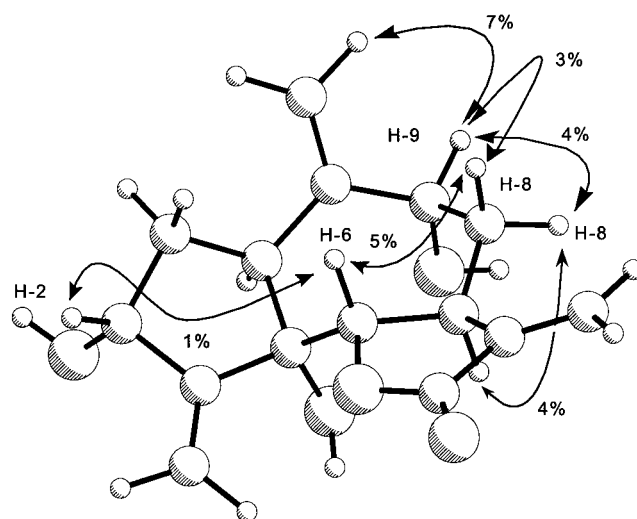


Figure 3. Selected NOE effects on the minimum-energy conformer of compound **7** obtained by using PM3 calculations.

Interestingly, while hydroxylation at room temperature of **5** led to the 1,3,5-trihydroxyl derivative **6** as a unique product, treatment under reflux of **1** led to the mixture of **5**, **6**, and the 1,3,9-trihydroxyl derivative **7**. The position and stereochemistry of the hydroxyl group as C-9 α was unambiguously established through ^1H - ^1H COSY and NOE experiments (Figure 3). The minimum-energy conformation (MEC) that explained all the NOE effects observed was fully coincident with that obtained using the PM3 calculation. The trihydroxylated compounds **6** and **7** were obtained in similar yields. No trace of compound **7** could be detected when **5** was treated at room temperature.

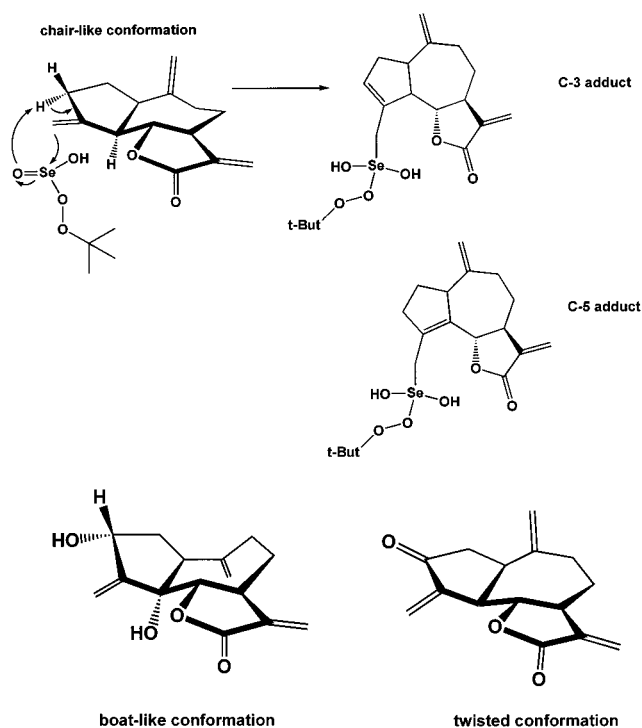


Figure 4. Selected comparison of minimum-energy conformers obtained by using PM3 calculations.

In view of these results, the order of reactivity seems to be C-3 > C-5 > C-1 > C-9. This order does not seem to change when the starting material is compound **11**, lacking the hydroxymethylene moiety in the lactone ring.

Mono- and dihydroxyl derivatives at the C-11 and C-13 positions were obtained by treatment with aqueous $\text{Na}_2\text{CO}_3/\text{HMPA}$ according to the previously published methods (Macías et al., 2000c). New compounds **13–15** were obtained from compound **2** using the same methodology as described above and their structures and stereochemistry unequivocally established through comparison of their spectral data.

Bioassay results are presented in Figures 5 and 6. Table 1 presents a resume of the main trends shown by every compound tested on each target species. Data corresponding to phytotoxic activities shown by compound **8** have already been reported (Macías et al., 2000b), but they have been introduced in Table 1 by means of making suitable comparisons.

L. sativa L. (Figure 5). Main inhibitory effects on the germination can be observed for compounds **14** and **18**, both of them lacking the exomethylene system in the lactone ring, and compound **8**. However, the effects observed for the rest of the compounds are moderate or active, even though some of them have been tested at 1 mM.

The most important effects are those observed on the growth (radicle and hypocotyl). In most of the compounds, there is a general trend to stimulate growth. The results obtained are especially high in compounds **13**, **14**, and **18** in the range between 0.001 and 10 μM . Only compound **11** shows an important inhibitory effect on radicle growth, while **5** shows a good dose–response curve for both parameters, although the values of activity are moderate. All of these data are in good accordance with previous results obtained for the root-promoting activity of some guaianolides and some other sesquiterpenes (Kalsi et al., 1981, 1983).

L. sativum L. (Figure 5). Main inhibitory effects on germination are observed for **2** (1 mM), **5** (1 mM and 100 μM), and **12** (10–100 μM). Moderate effects are obtained for compounds **9** and **11**, the rest remaining inactive.

Important inhibitory effects on radicle and hypocotyl growth with good dose–response curves are obtained for compounds **2**, **5**, **11**, **12**, and **14**, turning **2**, **4**, and **5** to stimulatory with the dilution. The acetone derivative **9** acts as a strong growth promoter.

L. esculentum L. (Figure 5). Tomato is usually the less sensitive dicot plant species, and this is well correlated with the results obtained in germination, where no strong inhibitory or promoting activities are obtained.

Similar results are obtained in radicle and hypocotyl growth, where only compounds **2**, **5**, and **11** (1 mM) present important inhibitions on root and shoot elongation.

T. aestivum L. (Figure 6). The effects observed are less intense than with the dicots assayed. Germination is poorly affected with main inhibitory effects for compounds **2** (1 mM), **11** (0.1 and 1 mM), and **20** (0.1 and 1 mM). Compounds **2**, **3**, **5**, **11**, and **17** inhibit radicle and hypocotyl growth, while compounds **7** and **16** enhance growth.

A. cepa L. (Figure 6). Onion is a more sensitive species, and the growth is deeply inhibited by **2**, **5**, and **11** (1 mM) and compound **4** (0.1 mM). Germination is not affected by any of the compounds.

DISCUSSION

The proposed mechanism of the allylic oxidation with SeO_2/TBHP proceeds through an ene reaction of the *t*-ButOO– SeO_2H adduct and the double bond of the lactone followed by a [2,3]-sigmatropic rearrangement (Haruna and Ito, 1981). Among the three possible sites of reaction, the *exo*-methylene group in the lactone ring is electron-deficient and thus a low reactive position. MMX calculations show that the adduct of the ene reaction at the C-10–C-14 positions is of higher energy than that resulting from the attack at C-4–C-15. This would explain the preferred order of reactivity C-3–C-5 > C-1 > C-9. Parametrization of the selenium atom in PCModel was not possible, and a silicon atom was selected as a suitable and reasonable approach to calculate and to compare the energy of the possible conformers.

Moreover, among all possible conformers at C-14, those with the selenium atom lying down or aligned with the plane of the molecule and the *tert*-butyl hydroperoxide moiety coming out from the molecule are of lower energy. In these cases, the hydroxyl groups of the selenium atom have to approach the double bond from the α -face to give the [2,3]-sigmatropic rearrangement (Figure 4). There is also a slight difference of energy between the two adducts with the double bond at C-3–C-4 and C-4–C-5 that favors the former. This is also in agreement with the higher reactivity obtained at the C-3 position. This situation is reproduced with the two possible C-14 adducts that lead to the α -hydroxyl derivatives at C-1 and C-9, thus explaining the high regioselectivity obtained in the reaction, where the bulky *tert*-butyl hydroperoxide moiety is the driving force.

The preferred α -approximation and disposition of the adduct in compounds **1**, **2**, and **5** can be explained

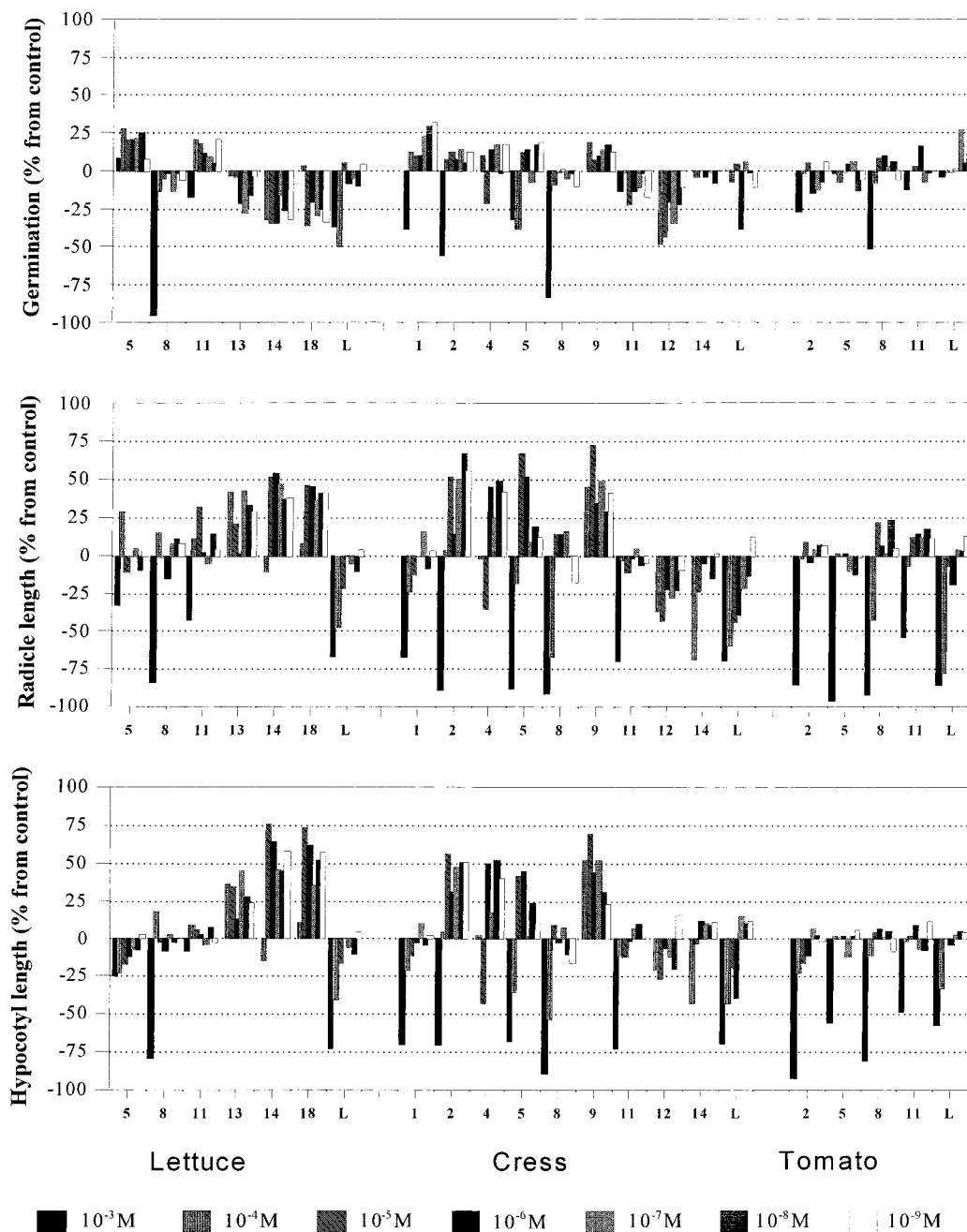


Figure 5. Germination and growth effects of selected compounds on lettuce, cress, and tomato. Values are expressed as percentage differences from the control. L = Logran.

through the chairlike conformation of the seven-membered ring, which forces the C-4–C-15 double bond to bend to the same face of the lactone ring. Thus, any bulky group, such as the TBHP–SeO₂ adduct, has to approach from the less hindered α -face.

Bioassay Discussion. From the results shown in Table 1 and Figures 5 and 6, four general observations can be made.

First, not all the compounds tested present activity, even though many of them present an *exo*-methylene moiety in the lactone ring, which has been usually claimed to be responsible for the activity observed. Moreover, compound 11, which is one of the most active, does not present this group. The most active compounds are 2, 5, and 11, followed by 4, 9, and 14.

Second, the different mode of action shown by these compounds depends on whether the target species

belongs to monocotyledons or dicotyledons, or even on the species within the same family. This was already reported in an earlier paper for *trans,trans*-germacranolides (Macías et al., 1999). Guaianolides, when active on monocots, are growth inhibitors, but never show an enhancing effect at low concentrations. Germination, except in two cases, remains untouched. On the other hand, active guaianolides are inhibitory to the germination of dicots, although their main effects remain on growth. They are usually inhibitory at high concentrations (0.1–1.0 mM) and turn to stimulatory at low concentrations (10–0.01 μ M).

Third, tomato is again the less sensitive plant species, which is in good accordance with previous results (Macías et al., 1999, 2000). However, intense growth inhibition is obtained with some compounds at 1 mM.

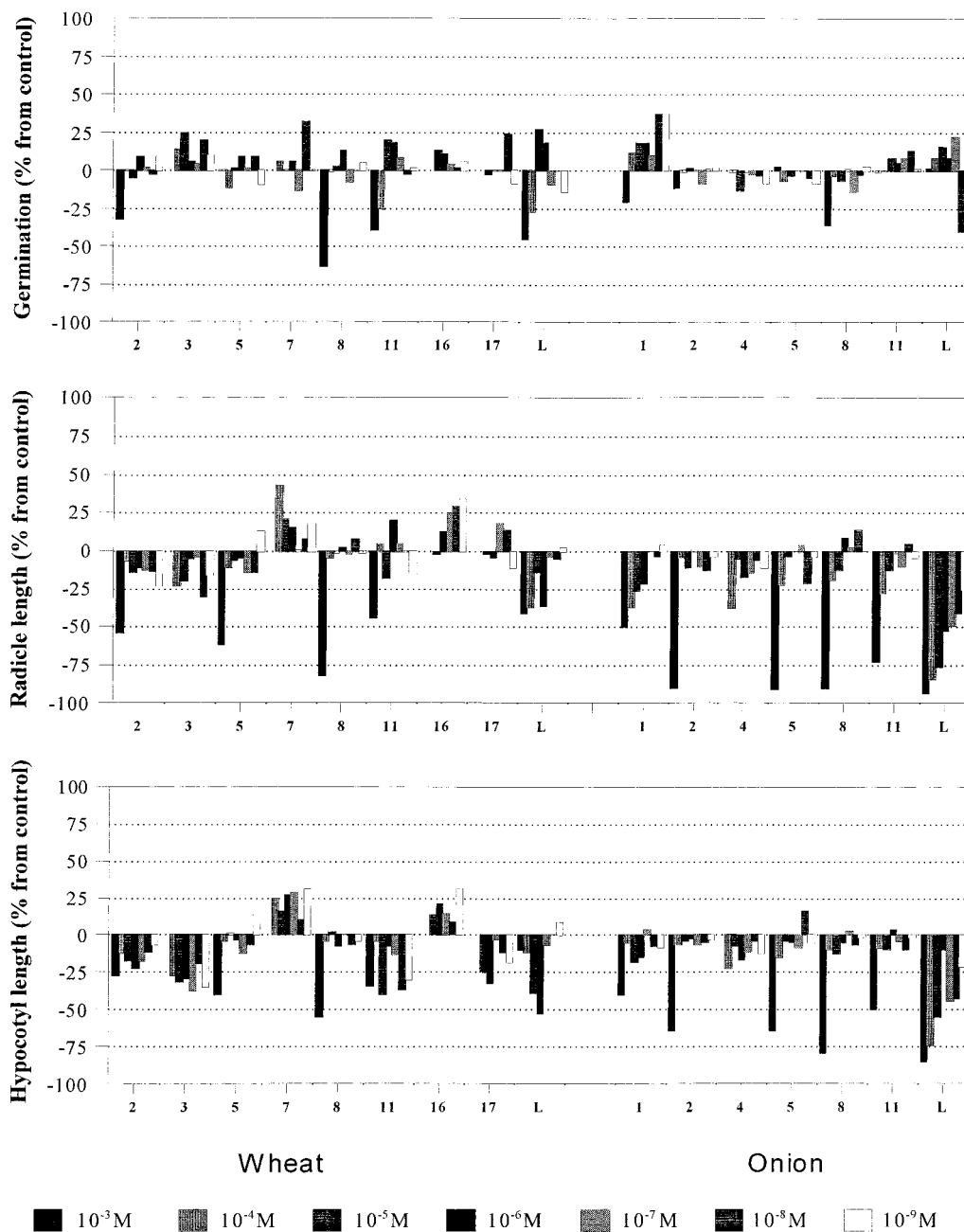


Figure 6. Germination and growth effects of selected compounds on wheat and onion. Values are expressed as percentage differences from the control. L = Logran.

Finally, comparison of the values and profiles of activity shown by these lactones and those of the herbicide Logran reveals that they are as effective or more effective than the commercial product on dicotyledons, but in many cases with opposite profiles of activities. They show similar values of activity on monocotyledons.

Since a wide range of concentrations has been chosen, a dose–response curve should be expected if no other factors such as solubility are going to affect the bioactivity. Previously (Macías et al., 1999) it has been proposed that SLs probably do not act as phytohormones and an allelopathic or/and antifeedant defensive role is most reasonable (Picman, 1986; Castillo et al., 1998). From this point of view, molecules can fall into one of two classifications: (a) Inhibitors. If the range of concentrations is correctly chosen, bioactivity usually exhibits a dose–response format; compounds tend to act

as inhibitors at the highest concentrations, activity decreasing with dilution. In many cases, the activity may turn into stimulatory (phytohormone-like activity) as the test solution is diluted. (b) The test compound is not a powerful inhibitor, or the range of concentrations chosen falls out of the “inhibitory” zone. Then, low or no activity at the higher concentrations should be observed (no inhibition), and enhancing activities are obtained as the test solutions are more diluted. Once the maximum of stimulatory activity has been reached, the activity decreases again, tending to zero.

It is striking that the guaianolides tested may fill up each of the two categories depending on whether the target species is a monocot or a dicot: growth of the monocot species is never stimulated, while most dicot growth is enhanced at low concentrations. This could be indicative of different mechanisms of binding to the targets, or even of different targets.

Table 4. Selected Theoretical Molecular Properties Obtained by Using PM3 Calculations for Some Compounds Correlated with Their Biological Activity^a

	preferred conformation	dipole moment, D	activity (dicots)	activity (monocots)
1	chair	4.432		
2	chair	4.937	-, +	=
3	chair	4.929		
4	boat	3.628	(-), +	-
5	boat	2.565	-, +	=
6	boat	4.131		
7	chair	1.896		
8	twist chair	5.188	=	=
9	boat	4.434	(+)	
10	boat	9.875		
11	chair	4.140	-	-
12	chair	9.849	-	
13	boat	2.666	(+)	
14	boat	2.982	-, +	
17	boat	2.426		

^a Key: empty cells, activity below 20% or no activity; -, +, stimulatory or inhibitory activities between 20% and 40%; =, ++, stimulatory or inhibitory activities higher than 40%.

Activity may depend on the following factors: the presence or not of certain functionalizations, preferred conformation, water solubility, and lipophilicity, among others.

Regarding the influence of different functional groups, the effect of one or two α,β -unsaturated carbonyl systems (cyclopentenones **8**, **19**, and **20**, compounds **1–7** and **9**, or lactol **21**), the presence of hydroxyl groups in different positions, and the introduction of bulky groups (**9**) are discussed. Compounds **1–9**, **19**, and **21** fill the first requirement. However, not all of them show growth inhibitory activity. It has been demonstrated that unsaturated γ -lactones and cyclopentanone carbonyl groups react with sulfhydryl groups of important biomolecules such as glutathione (GSH) (Galindo et al., 1999; Schmidt, 1997), the last one being up to 10 times more reactive than the lactone group (Schmidt, 1997).

In a recent report, two different modes of action have been proposed for **8**: one which proceeds through the cyclopentenone group and causes plasma membrane leakage, and one which has been attributed to the lactone ring (Galindo et al., 1999). The main difference among cyclopentenones **8**, **19**, and **20** is that the first one presents an *exo*-methylene group in the cyclopentane ring, while the methyl group hinders the double bond in compounds **19** and **20**, which also lack the exocyclic double bond at the lactone ring. Thus, a steric factor in the cyclopentenone ring could be proposed to explain the lack of activity of **19** and **20**.

In view of the results presented here, other factors should be considered to explain the lack of inhibition shown by compounds **3**, **4**, **6**, **7**, and **9**. All of them present the α -methylene- γ -lactone ring, the reactivity of this group to GSH in compound **2** also being demonstrated (Galindo et al., 1999). There are two extreme conformational possibilities for the double ring system in these compounds: chairlike and boatlike conformations (Figure 4). Molecular mechanics calculations using PM3 methods (MOPAC, 1990) lead to a chairlike minimum-energy conformer for compounds **1–3** and **7**, a boatlike conformer for compounds **4–6** and **9**, and a twisted conformation for compound **8**. The boat conformation induces a certain difficulty of access to the double bond by the upper (β) face. However, no correlation between the preferred conformation and the activity could be established (Table 4). As can be observed,

conformational effects on pseudorigid systems are of much less importance than on flexible rings such as germacranolides (Macías et al., 1992; Velasco, 2000).

Moreover, the activities of some compounds without the α -methylene- γ -lactone group (**10–20**) show very good levels of activity on dicots and monocots for some of them. Actually, compound **11** presents as good of levels of growth inhibition as compounds **1**, **2**, **5**, and **8**; compounds **12** and **14** also present good levels of growth inhibition on dicots, while they are inactive on monocots. However, when another hydroxyl group is added (compounds **13** and **15**), no inhibition is again observed. Finally, the lactone-ring-lacking compound **16** presents no activity on any of the target plants. With all of these data in mind, it can be advanced that, even though the lactone ring seems to be necessary for the activity, the presence of a conjugated exocyclic double bond does not necessarily mean growth inhibition. Moreover, when active, modes of action can differ depending on whether the lactone ring is saturated or unsaturated, or even on the presence of other groups (e.g., hydroxyl groups) in the lactone ring.

It was previously reported that some 11,13-dihydrogermacranolides without the double bond in the lactone ring presented good levels of inhibitory activity (Macías et al., 1999). Nevertheless, in this collection, compounds **17** and **18** present growth-enhancing activity, while their unsaturated precursors (compounds **5** and **6**) are inhibitors or weak growth stimulators, respectively.

Which other factors could be involved to explain the order of bioactivity? It has been proposed that a certain degree of lipophilicity is necessary to cross the cell membranes (Reynolds, 1987) or to concentrate inside them and thus to exert a biological effect. On the other hand, and depending on the threshold level of activity in each compound, water solubility is also needed: too polar compounds would not be allowed to cross the membranes and extremely low water soluble compounds would not be soluble enough. In this study, compound **1**, which represents the simplest molecule of this collection, presents a good degree of activity on cress and onion growth. The inhibition is enhanced when an α -hydroxyl group is introduced (**2** and, in a lower extension, **4**). The introduction of a β -hydroxyl group (**3**) results in no activity. The activity of the dihydroxylated derivative **5** is similar to that of **2**. However, a third hydroxyl group dramatically results in no activity (**6** and **7**). Then, two possible hypotheses can be advanced: (a) One is to postulate that a third hydroxyl group introduces too much charge in the molecule, thus making crossing the membranes more difficult. (b) Hydroxyl groups need a specific stereochemistry to fit the requirements for binding in the appropriate orientation, thus allowing a better approach of the active part of the molecule to the reactive center of the target; the best orientations are obtained with 3 α - and 3 $\alpha,5\alpha$ -dihydroxyl derivatives, while 1 α - and 9 α -hydroxyl derivatives would place the molecule in the wrong orientation. Obviously, either hypothesis is in need of further substantiation.

Finally, some conclusions can be obtained from these results in terms of inhibitory activity.

(a) The lactone group is necessary for the activity, but it does not seem to be necessary for it to be unsaturated.

(b) The introduction of a second and easily accessible α,β -unsaturated carbonyl system results in an enhanced activity.

(c) The introduction of an increasing number of hydroxyl groups leads to the loss of activity when polarity reaches a value that makes their transport through the membranes difficult. Until this limit, the activity is enhanced.

(d) The hydroxyl groups need a specific orientation and placement in the molecule to provoke the appropriate conformational changes that correctly orient the active part toward the reactive center of the target.

(e) The levels of activity of some of these compounds are similar to those of Logran on monocotyledons and dicotyledons. Thus, compounds such as dehydrocostuslactone (**1**), isozaluzanin C (**2**), 5 α -hydroxyisozaluzanin C (**5**), dehydrozaluzanin C (**8**), and its 11 β ,13-dihydro-13-hydroxydehydrocostus lactone (**11**) can be proposed as lead compounds for the development of new models of herbicides.

(f) Main stimulatory effects are observed for the growth of the dicots cress and lettuce (in decreasing order of activity) at low concentrations. Such effects are especially notorious for compounds **13**, **14**, and **18** (lettuce) and **2**, **4**, **5**, and **9** (cress).

(g) It is striking the lack of important growth-promoting activities on monocots, even though significant levels of growth inhibition are obtained. The differences between dose–response curves in monocots and dicots could be due to different mechanisms of binding to the targets.

ABBREVIATIONS

DCM, dichloromethane; GSH, glutathione; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; SAR, structure–activity relationship; TBHP, *tert*-butyl hydroperoxide.

ACKNOWLEDGMENT

We thank FITÓ, S.A. and Novartis for providing seeds and herbicides, respectively.

LITERATURE CITED

- Castillo, M.; Martínez-Pardo, R.; Garcera, M. D.; Couillaud, F. Biological activities of natural sesquiterpene lactones and the effect of synthetic sesquiterpene derivatives on insect juvenile hormone biosynthesis. *J. Agric. Food Chem.* **1998**, *46*, 2030–2035.
- Fournat, A.; Muñoz, V.; Roblot, F.; Hocquemiller, R.; Cave, A.; Gantier, J. C. Antiprotozoal activity of dehydrozaluzanin C, a sesquiterpene lactone isolated from *Muzonia maronii* (Asteraceae). *Phytother. Res.* **1993**, *7* (2), 111–115.
- Galindo, J. C. G.; Hernández, A.; Dayan, F. E.; Téllez, M. R.; Macías, F. A.; Paul, R. N.; Duke, S. O. Dehydrozaluzanin C, a natural sesquiterpenolide, causes rapid plasma membrane leakage. *Phytochemistry* **1999**, *52*, 805–813.
- Haruna, M.; Ito, K. Regio- and stereospecific allylic oxidation of germacrane-type sesquiterpene lactones with selenium dioxide and *t*-butyl hydroperoxide. *J. Chem. Soc., Chem. Commun.* **1981**, 483–485.
- Kalsi, P. S.; Talwar, K. K. A dramatic effect of stereochemistry on the plant growth activity of the cadinane group of terpenoids. *Phytochemistry* **1981**, *20*, 511–513.
- Kalsi, P. S.; Sood, V. B.; Masih, A. B.; Gupta, D.; Talwar, K. K. Structure and plant growth activity relationship in terpenoid lactones. *Phytochemistry* **1983**, *22*, 1387–1392.
- Macías, F. A.; Castellano, D.; Molinillo, J. M. G. In the search for a standard bioassay for allelopathic studies of phytotoxicity. Selection of standard target species (STS). *J. Agric. Food Chem.* **2000a**, *48*, 2512–2521.
- Macías, F. A.; Galindo, J. C. G.; Massanet, G. M. Potential allelopathic activity of several sesquiterpene lactone models. *Phytochemistry* **1992**, *21*, 1969–1977.
- Macías, F. A.; Galindo, J. C. G.; Molinillo, J. M.; Castellano, D. Dehydrozaluzanin C, a potent plant growth regulator with potential use as natural herbicide template. *Phytochemistry* **2000b**, *54*, 165–171.
- Macías, F. A.; Galindo, J. C. G.; Massanet, G. M.; Gómez-Madero, J. F.; Fronczek, F. R. Synthesis of 13-hydroxylated and 11,13-dihydroxylated sesquiterpene lactones: Michael addition of a nucleophilic hydroxyl group. *Synthesis* **2000c** (submitted for publication).
- Macías, F. A.; Galindo, J. C. G.; Castellano, D.; Velasco, R. F. Sesquiterpene lactones with potential use as natural herbicide models (I): *trans,trans*-Germacranolides. *J. Agric. Food Chem.* **1999**, *47*, 4407–4414.
- Macías, F. A.; Varela, R. M.; Torres, A.; Molinillo, J. M. G. Potential allelopathic guaianolides from cultivar sunflower leaves, var. SH-222. *Phytochemistry* **1993**, *34*, 669–674.
- MOPAC 6.0: Stewart, J. J. P.; Seiler, F. J., 1990.
- Neves, M.; Morais, R.; Garner, S.; Stoeckli-Evans, H.; Hostettmann, K. New sesquiterpene lactones from the portuguese liverwort *Targionia lorbeeriana*. *Phytochemistry* **1999**, *50*, 967–972.
- Pandey, D. K. Inhibition of salvinia (*Salvinia molesta* Mitchell) by parthenium (*Parthenium hysterophorus* L.). 1. Effect of leaf residue and allelochemicals. *J. Chem. Ecol.* **1994**, *20*, 3111–3122.
- Picman, A. K. Biological activities of sesquiterpene lactones. *Biochem. Syst. Ecol.* **1986**, *3*, 255–281.
- Schmidt, T. J. Helenanolide-type sesquiterpene lactones-III. Rates and stereochemistry in the reaction of helenanin and related helenanolides with sulfhydryl containing biomolecules. *Bioorg. Med. Chem.* **1997**, *5*, 645–653.
- Reynolds, T. Comparative effects of alicyclic compounds and quinones on inhibition of lettuce fruit germination. *Ann. Bot. (London)* **1987**, *60*, 215–223.
- Velasco, R. F. Chemical transformations in germacranolides, leads for natural herbicides. M.S. Dissertation, University of Cádiz, Spain, 2000.
- Wedge, D.; Galindo, J. C. G.; Macías, F. A. Fungicidal activity of several natural and synthetic sesquiterpene lactones. *Phytochemistry* **2000**, *53*, 747–757.
- Yuuya, S.; Hagiwara, H.; Suzuki, T.; Ando, M.; Yamada, A.; Suda, K.; Kataoka, T.; Nagai, K. Guaianolides as immunomodulators: Synthesis and biological activities of dehydrocostuslactone, mokko lactone, eremanthin, and their derivatives. *J. Nat. Prod.* **1999**, *62*, 22–30.
- Zar, J. H. *Statistical analysis*. Prentice Hall Inc.: Upper Saddle River, NJ, 1984.

Received for review April 28, 2000. Revised manuscript received July 24, 2000. Accepted July 28, 2000. This research was supported by the Dirección General de Investigación Científica y Técnica, Spain (DGICYT; Project No. AGF 97-1230-C02-02).

JF0005364