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Fungicidal activity of natural and synthetic sesquiterpene lactone analogs

D.E. Wedge[a,*], J.C.G. Galindo[b], F.A. Macías[b]

[a]*United States Department of Agriculture, Agricultural Research Service, Natural Products Utilization Research Unit, P.O. Box 8048, University, MS 38677, USA*

[b]*Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Cádiz, c/ República Saharahui s/n, Apdo. 40, 11520 Puerto Real, Cádiz, Spain*

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Abstract

Fungicidal activity of 36 natural and synthetic sesquiterpene lactones with guaianolide, *trans*, *trans*-germacranolide, *cis*, *cis*-germacranolide, melampolide, and eudesmanolide carbon skeletons was evaluated against the phytopathogenic fungi *Colletotrichum acutatum, C. fragariae, C. gloeosporioides, Fusarium oxysporum, Botrytis cinerea*, and *Phomopsis* sp. Dose-response data for the active compounds dehydrozaluzanin C, dehydrocostuslactone, 5α-hydroxydehydrocostuslacone, costunolide, and zaluzanin C are presented. A new 96-well microbioassay procedure for fast and easy evaluation of antifungal activity was used to compare these compounds with commercial fungicide standards. Some structure-activity conclusions are also presented.

Keywords: Fungicides; Dehydrozaluzanin C; Zaluzanin C; Dehydrocostuslactone; Costunolide; 5α-Hydroxydehydrocostuslactone; Sesquiterpene lactones; *Colletotrichum acutatum*; *Colletotrichum fragariae*; *Colletotrichum gloeosporioides*; *Fusarium oxysporum*; *Botrytis cinerea*; *Phomopsis* sp

1. Introduction

Owing to the continuing development of microbial resistance in medicine and agriculture, discovery of new antimicrobial substances is an important, if not urgent, research objective. In addition, the desire for safer agrochemicals with less environmental and mammalian toxicity is a major concern. Particularly desirable is the discovery of novel prototype antimicrobial agents representing new chemical classes that operate by different modes of action than existing antifungal agents and, consequently, lack cross-resistance to chemicals currently used (McChesney, 1993; Kirst et al., 1992). Following natural product leads offers an efficient approach to discovering and optimizing new agrochemicals for disease control. This study evaluates several sesquiterpene lactones for their potential use as disease control agents for pathogenic fungi.

Filamentous fungi of the genera *Colletotrichum, Botrytis, Fusarium* and *Phomopsis* species, all considered major plant pathogens worldwide (Farr, Bills, Chamuris & Rossman, 1989) were selected as test organisms. Failure to control these fungi can result in serious economic losses to both US and worldwide agriculture. Anthracnose (caused by *Colletotrichum* sp.), *Phomposis* and *Botrytis* diseases are serious problems for strawberry fruit and plant production in many areas of the world but are especially serious in the southeastern US (Maas, 1998). New shortened crop production cycles and increased growing pressure to meet the rapidly expanding mass market of orchids has facilitated *Fusarium oxysporum* in becoming a serious fungal pathogen often uncontrolled by commercial fungicides.

Sesquiterpene lactones constitute a large family of

* Corresponding author. Tel.: +1-601-232-1134; fax: +1-601-232-1035.

E-mail address: dwedge@olemiss.edu (D.E. Wedge).

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more than 5000 compounds (Fraga, 1996, 1997, 1998) mainly isolated from members of the Compositae. Sesquiterpenes possess a wide spectrum of biological activity (Fischer, 1991; Robles, Aregullin, West & Rodríguez, 1995; Marles, Pazos-Sanou, Compadre, Pezzuto, Bloszyk & Arnason, 1995) in which they appear to play a role in plant defense mechanisms. Due to their toxicity, some sesquiterpene lactones with eudesmanolide (Sabanero, Quijano, Ríos & Trejo, 1995), pseudoguaianolide, and *trans,trans*-germacranolide (Picman, 1984) skeletons have been evaluated for fungicidal activity. Sesquiterpene lactones have not been reported as phytoalexins or other induced antifungal metabolites. This paper describes the antifungal characteristics of 36 natural and semi-synthetic sesquiterpene lactones evaluated by direct TLC-bioautography and 96-well microtiter plate assays. Compounds tested belong to guaianolide (**1**–**17**), *trans,trans*-germacranolide (**18**–**23**), melampolide (**24**–**27**), *cis,cis*-germacranolide (**28**), or eudesmanolide (**29**–**36**) classes.

2. Results and discussion

The three chloro derivatives **7** (42%), **8** (20%) and **16** (5%) were prepared by treatment of a dry pyridine solution of **2** with freshly distilled mesyl chloride in dry pyridine at 0°C. Compound **7** shows an EIMS with a molecular ion at *m/z* 264:266, in accordance with the molecular formula $C_{15}H_{19}O_2Cl$. The IR spectrum shows an absorption band at 813 cm^{-1} for the (C–Cl) functional group. The ^{1}H-NMR spectrum was also very close to that of the starting product **2** (Macías, Galindo & Massanet, 1992). Major differences were observed for signals corresponding to the lactonic proton and the exomethylene at C4–C15. The lactonic proton resonated at δ 4.11 (1H, *dd*, $J_{5,6} = J_{6,7} = 9.5$ Hz, H-6), downfield in comparison with its equivalent in **2** (δ 3.83, 1H, *dd*, $J_{5,6} = J_{6,7} = 9.5$ Hz, H-6); the exomethylene protons appeared at δ 5.56 (1H, *dd*, $J_{3\alpha,15} = J_{5,15} = 2.1$ Hz, H-15) and δ 5.50 (1H, *dd*, $J_{3\alpha,15'} = J_{5,15'} = 2.1$ Hz, H-15′). Both signals were shifted upfield due to the introduction of the chlorine atom with a β orientation. A 2D COSY experiment confirmed the structure of **7**.

Compound **8** had the same IR and EIMS spectra as **7**. Major differences were observed in the ^{1}H-NMR spectrum, where the signal corresponding to H-3β in **8** appeared downfield (δ 4.89, 1H, *dd*, $J_{2\alpha,3\beta} = 6.8$ Hz, $J_{2\beta,3\beta} = 2.5$ Hz); the signal of H-6 was shifted to a higher field (δ 3.93, 1H, *dd*, $J_{5,6} = J_{6,7} = 9.2$ Hz) because of the α-oriented chlorine atom.

Compound **16** exhibited an EIMS spectrum close to those of **7** and **8**, showing a molecular ion at *m/z* 264:266 suggesting the molecular formula $C_{15}H_{19}O_2Cl$. The ^{1}H-NMR spectroscopic data were very close to those reported for the natural guaianolide annuolide A (Macías, Varela, Torres & Molinillo, 1993). The main differences were observed in the position of the H-3 (δ 5.99, 1H, *br s*) and H-15 (δ 4.28, 1H, *br s*, H-15; δ 4.36, 1H, *br s*, H-15′) signals, which were shifted upfield with respect to annuolide A. Analysis of 2D ^{1}H–^{1}H COSY and ^{13}C-NMR spectroscopic data confirmed the proposed structure. Substitution of primary and secondary allylic hydroxyl groups by chloride ion in reactions with chloride derivatives possessing good leaving groups have been reported (Collington & Meyers, 1971). Since both the C-3 epimers and the rearrangement product were obtained, the mechanism must have a strong SN^1 component, since the formation of the intermediate carbocation is favored by the use of aprotic and polar solvents such as pyridine.

Compound **2** was protected as the tetrahydropyranyl ether 9 (99%) by stirring a dry THF solution of **2** with an excess of dihydropyran (DHP) and a catalytic amount of *p*-toluene sulfonic acid. The structure of **9** was confirmed by its EIMS spectrum (*m/z* 330), consistent with the molecular formula $C_{20}H_{26}O_4$ in addition to analysis of its and ^{1}HNMR (Table 1) and ^{13}C-NMR (Table 2) spectral data.

Acetonide derivative **11** was prepared by refluxing an acetone solution of **6** for 24 h with a catalytic amount of *p*-toluene sulfonic acid. The structure was confirmed by its EIMS spectrum (molecular ion at *m/z* 318, according to the molecular formula $C_{18}H_{22}O_5$) and ^{1}H- (Table 1) and ^{13}C-NMR (Table 2) spectral data: δ 1.49 (3H, *s*, Me); δ 41.54 (3H, *m*, Me); δ 113.2 (*s*, C-16); δ 28.6 (*q*, C-17); δ 28.3 (*q*, C-18).

Oxidation of **12** with SeO_2 and *tert*-butyl hydroperoxide (Macías et al., 1992) afforded **14** (62%) as the major product. Its EIMS gave a molecular ion at *m/z* 264, consistent with the molecular formula $C_{15}H_{18}O_4$. Comparison of the ^{1}H- and ^{13}C NMR spectroscopic data of **12** and **14** showed a new signal in the ^{1}H-NMR spectrum at δ 4.63 (1H, *dd*, $J_{2\alpha,3\beta} = 6.8$ Hz, $J_{2\beta,3\beta} = 6.9$ Hz) for H-3β and the anticipated deshielding of C-3 (**12**: δ 30.6, **14**; δ 74.4). An α-orientation was proposed for the C-3 hydroxyl group on the basis of previous observations with similar compounds (Macías et al., 1992; Kalsi, Kaur, Sharma & Talwar, 1984).

Reduction of **1** with diisobutylaluminum hydride (DIBAL) in dry toluene (−70°C, N_2 atmosphere) yielded the corresponding lactol characterized by the lack of the γ-lactone absorption band and the presence of a strong –OH absorption band at 3396 cm^{-1} in the IR spectrum. Notable changes in the ^{1}H-NMR spectrum are the lactol proton at δ 5.71 (1H, *br d*, $J_{12,13a} = 1.8$ Hz, H-12), and the upfield signals corresponding to the lactonic exomethylene protons (δ 5.26, 1H, *dd*, $J_{7,13a} = 2.7$ Hz, $J_{12,13a} = 1.8$ Hz, H-13a; δ 5.05 1H, *dd*, $J_{7,13b} = 3.1$ Hz, $J_{12,13b} = 1.3$ Hz, H-13b).

Table 1
^{1}H-NMR spectral data for compounds **7**–**9**, **11**, **16**–**17**, and **26** (399.95 MHz, $CDCl_3$)[a]

H	7	8	9	11	14	16	17	26
1	2.96 *dd*	3.27	3.07 *m*	–	3.01	3.26 *dd*	2.90	5.59
2	α2.15 *ddd*	α2.38	2.17 *m*	α2.65 *dd*	α2.15 *ddd*	α2.52 *m*	α1.72 *ddd*	α1.99[b] *m*
	β2.47 *ddd*	β2.21	1.83 *m*	β2.06 *dd*	β1.83 *ddd*	β2.52 *m*	β2.05 *m*	β2.15[b] *m*
3	α4.71 *ddd*	β4.89	β4.68 *dd*	β4.51	β4.63	β5.99 *s*	α1.96 *m*	α1.99
							β2.42 *m*	β2.15
5	2.84 *dd*	3.18	3.07 *m*	–	3.06	3.10 *dd*	2.73	5.00 *d*
6	4.11 *dd*	3.93	3.91	4.01 *d*	3.88 *dd*	4.06	3.26	4.58
7	2.82 *m*	2.87	2.85	3.32	2.37 *dddd*	2.84 *m*	2.33	2.43
8	α2.25 *dddd*	α2.27	2.07 *m*	α1.45 *ddd*	α2.10 *m*	α2.27 *dddd*		α2.36 *m*
	β1.47 *dddd*	β1.42	2.23 *m*	β2.16 *dd*	β1.27 *dddd*	β1.44 *dddd*	β1.49 *m*	β1.57
9	α2.15 *dddd*	α2.14	2.52 *ddd*	α2.54 *m*	α2.45 *ddd*	α2.14 *dd*		α1.97 *m*
	β2.56 *dddd*	β2.50	1.40 *ddd*	β2.22 *m*	β1.99 *ddd*	β2.53 *m*		β2.03
12	–	–	–	–	–	–	4.12 *d*	–
							4.02 *d*	
13	a5.49 *d*	a5.50	a5.48	a5.48	3.96 *dd*	a5.47 *d*	a5.14 *s*	a5.46 *d*
	b6.21 *d*	b6.23	b6.21	b6.20	3.68 *dd*	b6.19 *d*	b5.10 *s*	b6.18 *d*
14	5.01 *s*	4.96	4.91	5.36	4.86	4.93	4.85	4.13 *d*
	4.96 *s*	4.82	4.75	5.15	4.68	4.89	4.74 *dd*	3.99 *d*
15	5.56 *dd*	5.63	5.44 *br s*	5.75	5.35 *dd*	4.36 *d*	5.13 *br s*	1.84 *d*
	5.50 *dd*	5.51	5.25 *br s*	5.55	5.29 *dd*	4.28 *d*	4.97 *br s*	
2′			5.54 *m*	1.54 *s*				
3′				1.49 *s*				
6′			3.92 *dd*					
			3.52 *m*					

[a] Coupling constants (Hz): **7** 1,2β = 2β,3α = 6; 2α,2β = 12; 2α,3α = 2; 2β,3α = 8; 3α,15 = 2; 5,6 = 6,7 = 10; 8α,8β = 12; 8α,9α = 8β,9α = 8β,9β 8α,9β = 8; 9α,9β = 14; 7,13α = 3; 7,13β = 4; 5,15′ = 2. **8** 1,2α = 7; 1,2,β = 7; 1,5 = 10; 2α,2β = 14; 2α,3β = 7; 2β 3β = 2; 5,6 = 6,7 = 9; 7,8α = 9; 7,8β = 10; 8α,8β = 9α,9β = 13; 8α,9α = 8β,9β = 8β,9α = 8α,9β = 8; 8α,8β = 12; 9α,9β = 14; 7,13a = 7,13b = 3. **9** 5,6 = 6,7 = 9; 5′6′ = 8; 7,13a = 7,13b = 3. **11** 2α,2β = 14; 2α,3α = 2α,3β = 7; 3β,15 = 2; 3β,15′ = 2; 6,7 = 9; 7,13a = 3; 7,13b = 4. **14** 1,2α = 4; 1,2β = 7; 1,5 = 2; 2α,2β = 13; 2α3β = 7; 2β,3β = 7; 5,6 = 6,7 = 9; 7,8β = 9; 8α,8β = 13; 8α,9α = 8β,9β = 4; 8α,9β = 8β,9α = 12; 9α,9β = 13; 11,13 = 3; 11,13′ = 12; 15′,1 = 15′,3β = 2; 15,1 = 15,3β = 2. **16** 1,2β = 16; 1,5 = 10; 5,6 = 9, 6,7 = 10; 7,8α = 5; 7,8β = 10; 8α,8β = 13; 8α,9α = 5; 8α,9β = 11; 8β,9α = 10; 8β,9β = 5; 9α,9β = 13; 7,13α = 7,13b = 3. **17** 1,2β = 2β,3α = 10; 1,5 = 8; 2α,2β = 13; 5,6 = 6,7 = 10; 8α,8β = 15; 12,12′ = 121. **26** 1,2α, 1,2β = 8; 5,6 = 10; 5,15 = 1; 6,7 = 9; 7,13a = 7,13b = 3; 14,14′ = 11.
[b] Signals in the same column may be interchanged.

Table 2
^{13}C-NMR data of compounds **9**, **11**, **16**, **26**, and lactol (50.00 MHz, $CDCl_3$)[a]

	9	11	14	16	Lactol	26
1	50.2 *d*	88.8 *s*	49.6 *d*	48.0	48.8	130.4
2	37.6 *t*	45.4	38.0	34.0	30.5	22.8
3	73.7 *d*	71.8	74.4	131.7	32.1*t*	24.4
4	150.1 *s*	153.4	154.4	140.5	151.9	139.6
5	44.6 *d*	91.7 *s*	49.3 *d*	43.4 *d*	48.2	125.3
6	85.1 *d*	82.7	85.9	84.1	83.0	80.3
7	45.9 *d*	39.7	43.6[b]	46.5	52.5	45.4
8	30.7[b] *t*	30.6	32.4	30.9	32.7	25.1
9	30.7[b] *t*	29.4	39.7	36.5	32.3	38.1
10	138.8 *s*	140.1	149.1	139.0	149.9	137.6
11	147.8 *s*	147.8	43.1[b] *d*	148.5 *s*	153.5	138.2
12	169.0 *s*	169.3	177.4		97.4 *d*	170.2 *s*
13	119.8 *t*	120.3	58.6	120.2	108.2	119.2
14	112.8 *t*	116.2	112.4	52.6	110.9	48.9
15	113.5 *t*	115.7	112.4	114.0	108.1	17.2 *q*

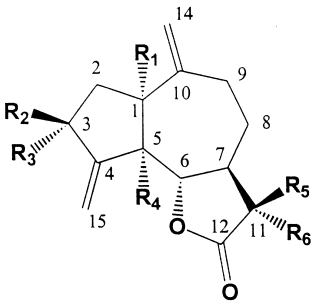
[a] **9**: δ 95.9 (*s*, C-2′), δ 62.2 (*t*, C-6′), δ 26.2 (*t*, C-3′, C-5′), δ 19.9 (*t*, C-4′); **11**: δ 113.2 (*s*, C-1′), δ 28.6 (*q*, C-2′), δ 28.3 (*q*, C-3′).
[b] Signals in the same column may be interchanged.

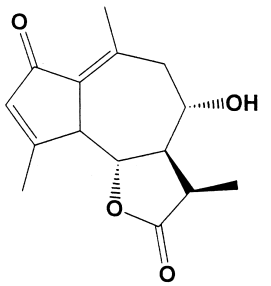
Further reduction of the lactol with $NaBH_4$ yielded **17** (80%). Its EIMS spectrum showed a molecular ion at *m/z* 234, corresponding to the molecular formula $C_{15}H_{22}O_2$. A strong hydroxyl band at 3250 cm^{-1} (–OH st.) was detected in the IR spectrum of **17**. The ^{1}H-NMR spectrum shows signals corresponding to a hydroxymethylene group at δ 4.12, 4.02 (1H, both *d*, $J_{12,12'} = 12.1$ Hz, H-12).

The chlorinated derivative **26** was obtained from **24** by treatment with an excess of tosyl chloride in dry pyridine. Its EIMS spectrum showed a molecular ion at *m/z* 266, according to the molecular formula $C_{15}H_{19}O_2Cl$. No important changes appeared in the ^{1}H-NMR spectrum compared to **24**, differences arising from the ^{13}C-NMR spectrum, where the signal corresponding to C-14 was strongly shifted downfield for **24** (**24**: δ 66.1, *t*, C-14; **26**; δ 49.2, *t*, C-14). The halogen displacement of an hydroxyl group by a halogen derivative is similar to that reported for compounds **7**, **8**, and **9** (Collington & Meyers, 1971). No traces of the C_1-chloro rearrangement product were found. This could be easily explained since the reaction involved a

primary hydroxyl group where the mechanism must necessarily be of the SN^2-type.

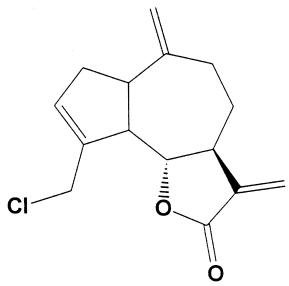
Antifungal pre-screening of 36 sesquiterpene lactones (Figs. 1 and 2) in a matrix format was conducted directly on TLC plates sprayed with a spore suspension. Bioautography indicated that six sesquiterpene lactones had antifungal activity against either *C. acutatum, C. fragariae, Phomopsis* sp. *or Botrytis cinerea.* (Table 3). Commercial fungicides (vinclozolin and thiabendazole) to which many fungi have developed resistance demonstrated small or no zone of inhibition with a diffuse margin. Compounds possessing strong antifungal activity produced a clear zone of inhibition bounded by a sharp margin regardless of the size of the inhibitory zone. Active compounds in decreasing order of activity were **10** (dehydrozaluzanin C), **1** (dehydrocostuslactone), **4** (5α-hydroxydehydrocostuslacone), **18** (costunolide), **23** (parthenolide), and **3** (zalu-



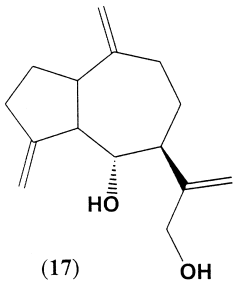


Hydroxyachillin (**15**)

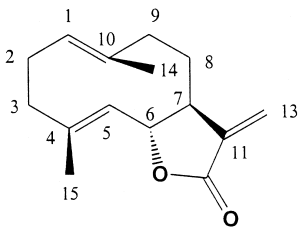
R_1	R_2	R_3	R_4	R_5	R_6	
H	H	H	H	$=CH_2$		(**1**)
H	H	OH	H	$=CH_2$		(**2**)
H	OH	H	H	$=CH_2$		(**3**)
H	H	H	OH	$=CH_2$		(**4**)
H	H	OH	OH	$=CH_2$		(**5**)
OH	H	OH	OH	$=CH_2$		(**6**)
H	Cl	H	H	$=CH_2$		(**7**)
H	H	Cl	H	$=CH_2$		(**8**)
H	H	-OTHP	H	$=CH_2$		(**9**)
H	O	O	H	$=CH_2$		(**10**)
R_1-R_4=O-CMe_2-O; R_2=H R_3=OH				$=CH_2$		(**11**)
H	H	H	H	H	CH_2OH	(**12**)
H	H	H	H	CH_2OH	OH	(**13**)
H	H	OH	H	H	CH_2OH	(**14**)



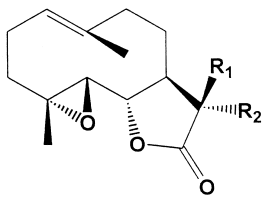
(**16**)



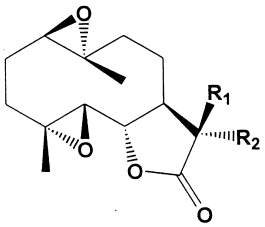
(**17**)



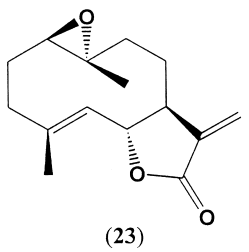
(**18**)



$R_1 = R_2 = CH_2$ (**19**)
$R_1 = H$; $R_2 = CH_3$ (**20**)



$R_1 = R_2 = CH_2$ (**21**)
$R_1 = H$; $R_2 = CH_3$ (**22**)



(**23**)

Fig. 1. Guaianolides **1**–**17** and *trans, trans*-germacranolides **18**–**23** tested for antifungal activity.

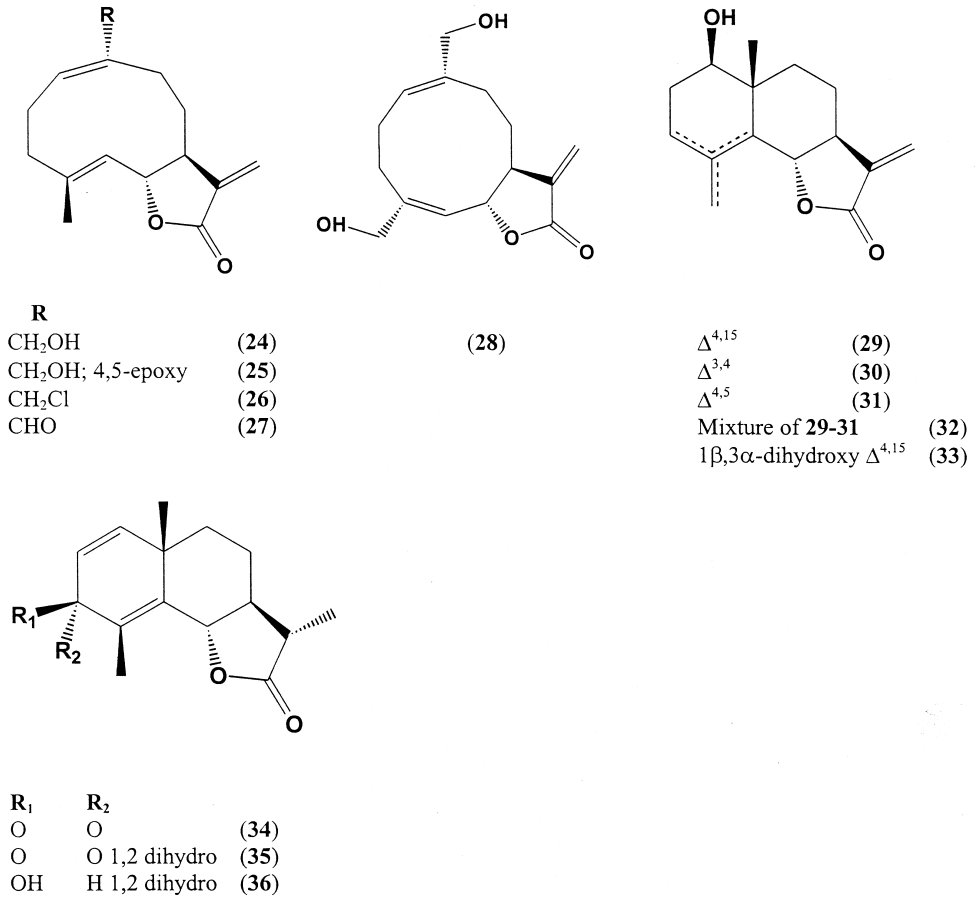


Fig. 2. Melampolides **24**–**27**, *cis, cis*-germacranolide **28**, and eudesmanolides **29**–**36** tested for antifungal activity.

Table 3
Fungicidal activity of sesquiterpene lactones **1**–**36** on the autobiography assay by visual evaluation of the clear inhibitory antifungal zone[a]

Test compound	*C. acutatum*	*C. fragariae*	*Phomopsis* sp.	*B. cinerea*
1	WA (7)	WA (8)	–	–
2	–	–	–	–
3	WA (4)	WA (4)	–	–
4	WA (7)	WA (6)	–	–
5–9	–	–	–	–
10	SA (7)	SA (8)	SA (11)	SA (7)
11–17	–	–	–	–
18	VW (4)	VW (6)	–	–
19–22	–	–	–	–
23	VW (4)	WA (4)	–	–
24–36	–	–	–	–
Vinclozolin	VW (10)	VW (3.5)	VW (7.5)	–
Chlorothalonil	SA (7)	SA (11)	SA (15.5)	SA (10)
Thiabendazole	–	–	SA (16.5)	–

[a] **a**: Vinclozolin; **b**: Chlorothalonil; **c**: Thiabendazole; **dashes**: No Activity; **VW**: Very Weak activity; **WA**: Weak Activity; **SA**; Strong Activity. Letters indicate zone clairity and numbers indicate zone diameter (mm). Mean zone diameter from two experiments is expressed in parentheses.

zanin C). Bioautography indicated that guaianolides **10** and **1** appeared to be the most effective against *C. acutatum* and *C. fragariae*, and **10** inhibited growth of all four fungi. None of these compounds, except parthenolide, have previously reported fungicidal activity, and parthenolide was previously tested only against *Phytophthora capsici* (Gennari, Abbattista-Gentile & Cugudda, 1987).

The five most active antifungal compounds identified by bioautography (**1**, **3**, **4**, **10**, and **18**) were subsequently evaluated using a 96-well microbioassay system (Wedge & Kuhajek, 1998) for activity against *B. cinerea, C. acutatum, C. fragariae, C. gloeosporioides* (Figs. 3–6), and *F. oxysporum* (data not shown). The 96-well microbioassay system used a liquid broth culture system in a dose-response format to evaluate each compound in more detail. Secondary screening of active compounds using this microbioassay system identified one compound (**10**) as having significant antifungal properties, one compound (**1**) with moderate antifungal activity, and eliminated three weakly active compounds (**3**, **4**, and **18**). Microbioassay results indicated that the antifungal activity of sesquiterpene lactones (**1** and **10**) appear to be species-dependent and

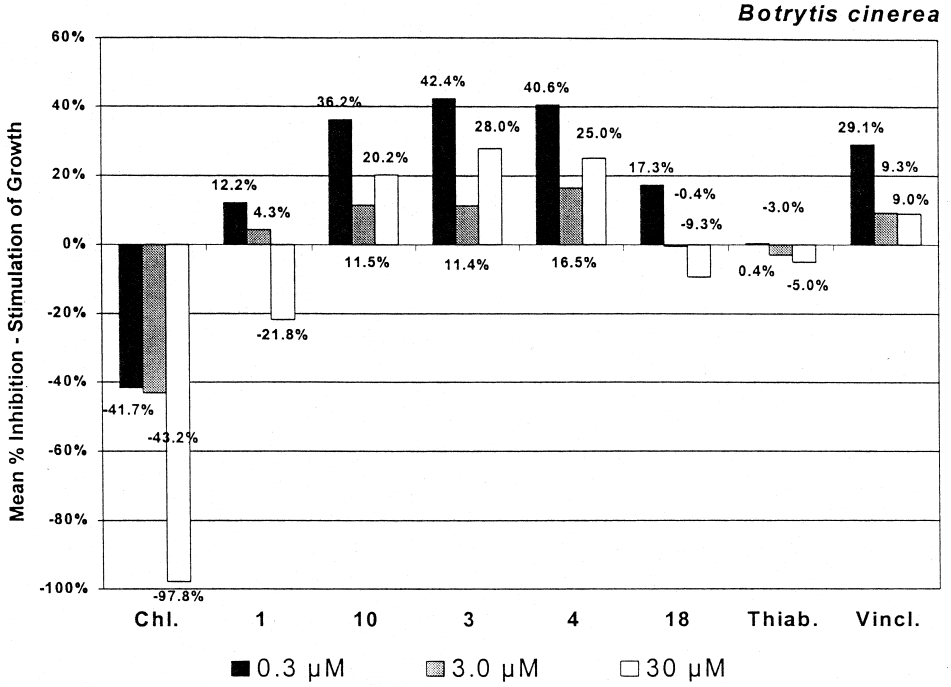


Fig. 3. Antifungal activity of compounds **1**, **10**, **3**, **4**, and **18** against *Botrytis cinerea* growth. Commercial standard fungicides: **Chl.**: chlorothalonil, **Thiab.**: thiabendazole, **Vincl.**: vinclozolin. Least Square Means for percent inhibition of each sesquiterpene lactone is listed for each of three concentrations ($n = 4$) by fungal species. Positive values indicate growth stimulation and negative values indicate growth inhibition relative the untreated control ($n = 32$).

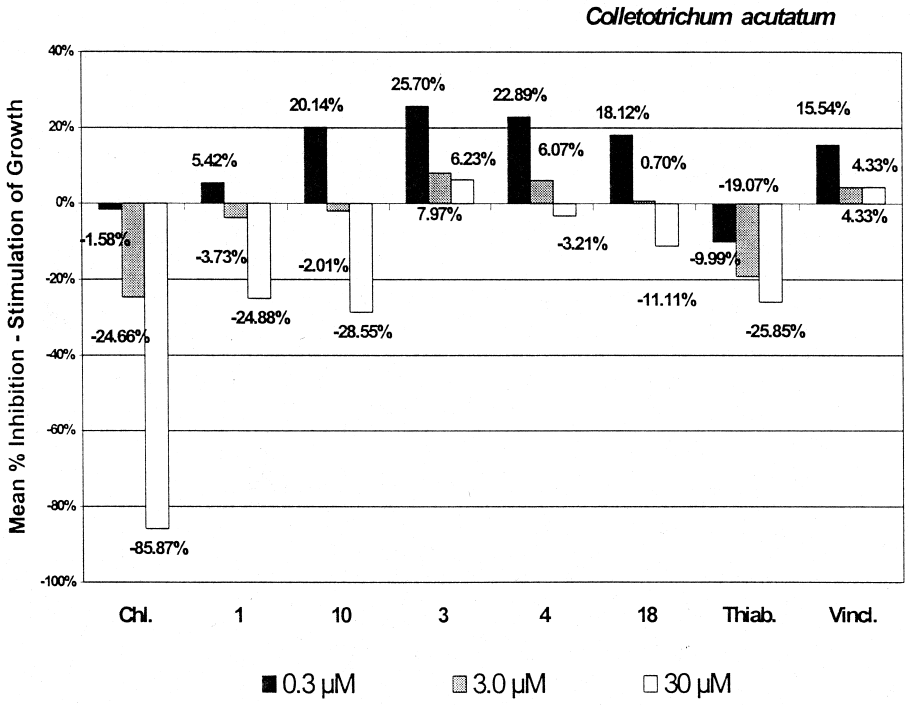


Fig. 4. Antifungal activity of compounds **1**, **10**, **3**, **4**, and **18** against *Colletotrichum acutatum* growth. Commercial standard fungicides: **Chl.**: chlorothalonil, **Thiab.**: thiabendazole, **Vincl.**: vinclozolin. Least Square Means for percent inhibition of each sesquiterpene lactone is listed for each of three concentrations ($n = 4$) by fungal species. Positive values indicate growth stimulation and negative values indicate growth inhibition relative the untreated control ($n = 32$).

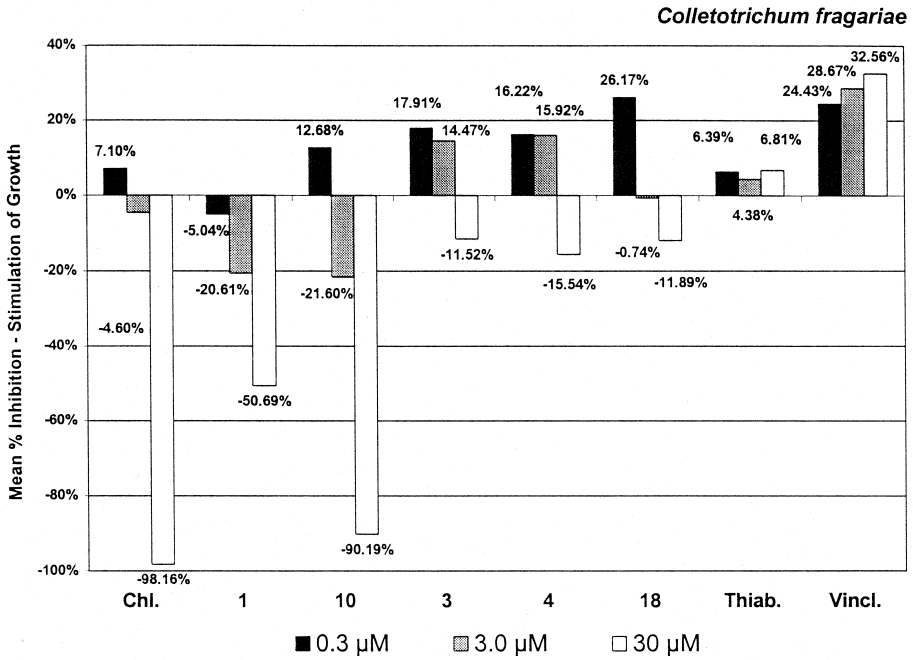


Fig. 5. Antifungal activity of compounds **1**, **10**, **3**, **4**, and **18** against *Colletotrichum fragariae* growth. Commercial standard fungicides: **Chl.**: chlorothalonil, **Thiab.**: thiabendazole, **Vincl.**: vinclozolin. Least Square Means for percent inhibition of each sesquiterpene lactone is listed for each of three concentrations ($n = 4$) by fungal species. Positive values indicate growth stimulation and negative values indicate growth inhibition relative the untreated control ($n = 32$).

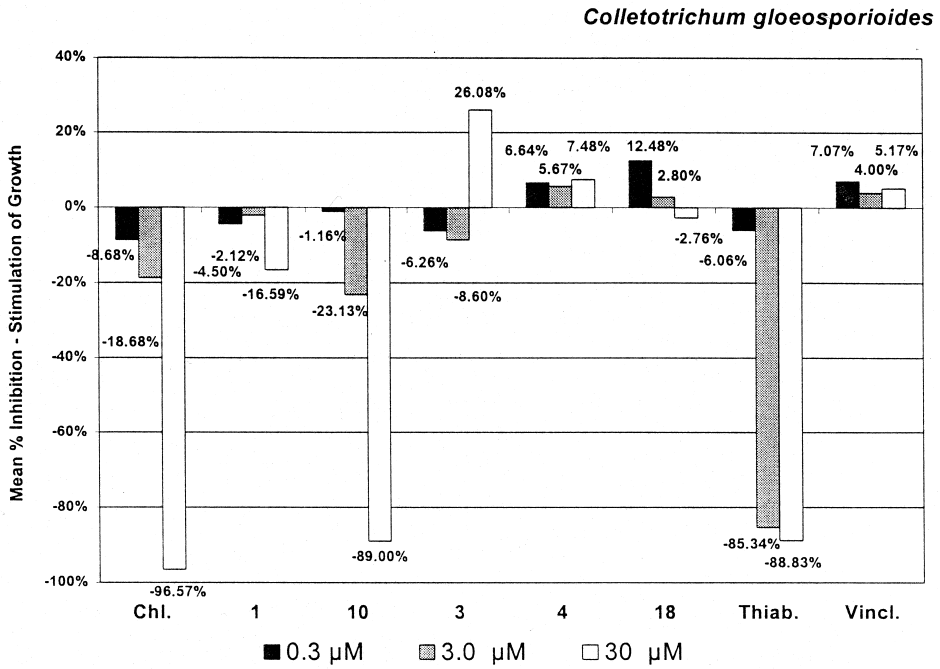


Fig. 6. Antifungal activity of compounds **1**, **10**, **3**, **4**, and **18** against *Colletotrichum gloeosporioides* growth. Commercial standard fungicides: **Chl.**: chlorothalonil, **Thiab.**: thiabendazole, **Vincl.**: vinclozolin. Least Square Means for percent inhibition of each sesquiterpene lactone is listed for each of three concentrations ($n = 4$) by fungal species. Positive values indicate growth stimulation and negative values indicate growth inhibition relative the untreated control ($n = 32$).

that the biological activity depends upon the type of carbon skeleton. Furthermore, the results demonstrated that our microbioassay test system was sufficiently sensitive to make biological predictions about structure-activity relationships.

The most active compound in bioautography, **10** (dehydrozaluzanin), exhibited selective activity against *Colletotrichum* species (Figs. 4–6). Dehydrozaluzanin at 30 μM reduced *C. fragariae* growth by 90% (Fig. 5), *C. gloeosporioides* by 89% (Fig. 6), and *C. acutatum* by 29% (Fig. 4). Compound **10** was nearly as effective as the commercial fungicide chlorothalonil against *C. fragariae* and *C. gloeosporioides*, and was more effective than thiabendazole and vinclozolin. However, at 30 μM, **10** was ineffective against *B. cinerea* (Fig. 3) or *F. oxysporum* (data not shown). *Colletotrichum acutatum* showed less sensitivity than *C. fragariae* or *C. gloeosporioides* to **10**. The effective concentration against *C. acutatum* appears to be about 10-fold higher in comparison to the other two *Colletotrichum* species.

Compound **1** had the second highest fungicidal activity of the compounds tested in bioautography. At 30 μM, growth inhibition of 51% was observed in *C. fragariae* (Fig. 5), 25% in *C. acutatum* (Fig. 4), 17% in *C. gloeosporioides* (Fig. 6), and 22% in *B. cinerea* (Fig. 3). There was no significant inhibition of *F. oxysporum* (data not shown) at 30 μM. Compound **1** was the only compound that showed any significant ($p < 0.05$) activity against *B. cinerea*. Compounds **4** (zaluzanin C), **18** (costunolide), and **23** (parthenolide) all showed weak activity in the bioautography assay but were ineffective as antifungal agents in the liquid broth microbioassay when compared to commercially available fungicides. None of the sesquiterpene lactones tested showed significant inhibition against *F. oxysporum*. Antifungal activity of several sesquiterpene lactones against *Colletotrichum* species suggested that these compounds might have a plant defense role in maintaining leaf integrity by inhibiting foliar pathogens. Further research into the use of **10** and **1** as protectant fungicides is warranted. However, most of these compounds with an exocyclic methylene function are strongly allergenic (Type IV allergy) and may induce dermatoses (Hausen and Schmalle, 1985; Schaeffer, Talaga, Stampf & Benezra, 1990). Ultimately, it is expected that the use of these compounds as antifungal agents will be limited by their phytotoxicity rather than their allergenic nature.

It has been reported that the biological activity of sesquiterpene lactones is generally due to the α-methylene-γ-lactone system (Hall, Lee, Starnes, Muraoka, Sumida & Waddell, 1980). However, this does not appear to be the only requisite in fungi (Marles et al., 1995) or other living systems (Rodríguez, Enriz, Santagata, Jáuregui, Pestchanker & Giordano, 1997). Other requirements such as molecular accessibility seem to play an important role (Macías, et al., 1992; Rodríguez et al., 1997). In this study, we observed that several compounds possessing the α-methylene-γ-lactone ring did not exert any inhibition of mycelial growth (**2**, **5**–**9**, **19**, **21**, **24**–**33**; Table 3). Some of these compounds also have other reactive groups like epoxides, but this does not appear to improve activity. Comparison between compounds **1**, **3**, **9**, **11** shows that the introduction of steric impediments in the form of bulky groups can cause the loss of antifungal activity in compounds **9** and **11** with respect to those of **1** and **3**; these results are in agreement with previous reports (Macías et al., 1992). Lipophilicity is another factor to be considered (Beekman et al., 1997); compounds **5** and **6**, with an increasing number of hydroxyl groups, presented no activity in comparison with mono-hydroxylated compounds **3** and **4**, or with the weekly polar compounds **1** and **10**. The fact that **1**, **3**, **4**, and **10** were toxic to all fungi tested but with different profiles of activity may be indicative of different modes of action from those of the commercial fungicidal standards.

Compound **10** represents a special case, since the inhibition of mycelial growth was greater than that shown by the rest of guaianolides and sesquiterpene lactones tested, even with species having fungicidal resistance (*C. acutatum, C. fragariae* — thiabendazole; *B. cinerea* — thiabendazole, vinclozolin; *F. oxysporum* — vinclozolin).

Previous results (unpublished) showed that the phytotoxicity of **10** was greater than that of other guaianolides and sesquiterpene lactones without an additional α,β-unsaturated carbonyl group. A different mode of action has been proposed for **10** due to this difference (Galindo, Dayan, Hernández, Macías and Duke, 1999). No similar effects have been reported in fungi yet, except in the case of encelin, a *cis*-7,8-eudesmanolide with a double α,β-unsaturated carbonyl group, which has been reported to exert a detergent action on the growth of fungal cells of *Mucor rouxii* (Robles et al., 1995). Altered cellular permeability and quantitative changes in protein synthesis could also be detected in both the carbonyl containing encelin, and in the dihydro-derivative 3-hydroxyencelin (Trejo, Gaytan, Mendoza & Sabanero, 1996).

Though further investigation is needed, it is reasonable to postulate that the difference in the activity between **10** and the rest of the sesquiterpene lactones tested relies on the presence of the α,β-unsaturated carbonyl group in the cyclopentanone ring. Nevertheless, the group needs to be accessible since reaction through a Michael addition mechanism is also viable (Galindo et al., 1999).

Hydroxyachillin (**15**) has no exomethylene group in the lactone ring, but has an enone group with two double bonds conjugated to the carbonyl group. How-

ever, compound **15** had no activity, probably because the two double bonds are hindered (β-methyl groups) and the accessibility to allow nucleophilic additions is extremely restricted, especially with nucleophiles such as bulky biomolecules.

With respect to germacranolide type sesquiterpene lactones, only **18** and its 1,10-epoxyderivative **23** produced weak mycelial growth inhibition. Parthenolide (**19**), which has some fungicidal activities (Picman, 1984), did not show any activity with the fungi in our assays. Other germacranolides did not demonstrate any antifungal activity. Although several fungicidal activities were reported for eudesmanolides (Marles et al., 1995; Trejo et al., 1996), none of the eudesmanolides tested (compounds **29**–**36**) showed any activity.

3. Experimental

3.1. Starting materials

Dehydrocostuslactone (**1**) and costunolide (**18**) were obtained from crude Costus Resin Oil (Pierre Chauvet S. A.) by CC purification and recrystallization from hexane:EtOAC mixt. Parthenolide (**19**) was obtained from *Magnolia grandiflora* L. leaves, and was purified by CC and recrystallization from hexane:EtOAc mixt. Hydroxyachillin (**15**) was obtained from *Artemisia lanata* Wild. extract and purified by CC. Structures were verified by comparison of spectroscopic data (MS, IR, ^{1}H and ^{13}C-NMR) with those reported in the literature. An: Refs. α-Santonin (**34**) was purchased from Sigma Co (st. Louis, MO), and the commercial fungicides vinclozolin, chlorothalonil and thiabendazole from Chem. Service, West Chester PA.

3.2. Synthesis of guaianolides

Compounds **2**–**6** were synthesized according to the previously described method (Macías et al., 1992). Chlorinated derivatives **7**, **8** and **16** were obtained by treatment of **2** with freshly distilled mesyl chloride in dry pyridine at 0°C during 24 h. Reaction mixt. was then filtered and pyridine was evapd. under reduced pressure using cyclohexane. HPLC purification (hexane–AcOEt, 1:9) yielded **7** (42%), **8** (20%), and **16** (5%). Treatment of **2** with anhydrous, freshly distilled dihydropyran and *p*-toluene sulfonic acid as catalyst in dry THF for 16 h, followed by neutralization with K_2CO_3, filtration and evapn. under reduced pressure, yielded a racemic mixture of 3α-*O*-tetrahydropyranyl derivatives **9** (95%). Dehydrozaluzanin C **10** was synthesized as previously described (Macías, Galindo, Molinillo & Castellano, 2000b).

Acetonide **11** was prepd. from **6** by refluxing in Me_2CO with *p*-toluene sulfonic acid for 24 h. The reaction mixt. was then evapd. under reduced pressure and purified by CC [hexane–AcOEt (1:4)] to give **11** (99% yield). Compounds **12** and **13** were synthesized from **1** by refluxing with hexamethylphosphoric triamide (HMPA) and 20% aqueous $CaCO_3$ solution for 7 days according to the published method (Macías, Galindo, Massanet & Gómez-Madero, 2000a). Oxidation of **12** with SeO_2 and *tert*-butyl hydroperoxide (Macías et al., 1992) afforded **14** as the major product (62% yield). A dry toluene solution of **1** was stirred at −70° under N_2 with a DIBAL/toluene solution (1:1.1) for 1.5 h, following which MeOH and H_2O were sequentially added. The resulting gelatinous crude was homogenized and extracted several times with EtOAc and H_2O. The two-phase mixt. was then separated and the mother liquors extracted with EtOAc (×3). All organic phases were dried over Na_2SO_4 and evapd. under reduced pressure to yield 61% of the lactol derivative of **1**. Further reduction of this lactol derivative with a methanolic solution of $NaBH_4$ at 0°C for 1 h produced **17** (80% yield).

3.3. Synthesis of germacranolides

Germacranolides **20**–**23** and melampolides **24**, **25**, and **27** were obtained according to the published methods (Rodrígues, García & Rabi, 1978; Fischer, Weidenhamer, Riopel, Quijano & Menelaou, 1990; Umbrier & Sharpless, 1977). *Cis,cis*-germacranolide **28** was synthesized according to (Macías et al., 1992). Compound **26** was synthesized from **24** by treatment with excess of tosyl chloride in dry pyridine, and stirring for 24 h. Reaction mixt. was then filtered and pyridine was evapd. under reduced pressure using cyclohexane. After purification using CC (hexane:EtOAc, 1:4), compound **26** was obtained in a yield of 80%.

3.4. Synthesis of eudesmanolides

Eudesmanolides **29**–**31** were synthesized as previously described (Rodrígues et al., 1978) while **35**–**36** were obtained from **34** according to (Macías, Aguilar, Molinillo, Massanet & Fronczek, 1994).

3.5. Pathogen production

Isolates of *Colletotrichum acutatum* Simmonds, *Colletotrichum fragariae* Brooks, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz., and a *Phomopsis* sp. were obtained from B. J. Smith, USDA, ARS, Small Fruit Research Station, Poplarville, MS. The three *Colletotrichum* species were isolated from strawberry (*Fragaria x ananassa* Duchesne) and the *Phomopsis* sp. was isolated from muscadine grape (*Vitis rotundifolia* Michx.). *Botrytis cinerea* Pers.:Fr was iso-

lated from commercial grape (*Vitis vinifera*) and *Fusarium oxysporum* Schlechtend:Fr from orchid (*Cynoches* sp.). *Colletotrichum, Phomopsis* and *Botrytis* species were used in the bioautography as a prescreen for active antifungal compounds. *Colletotrichum, Botrytis* and *Fusarium* species were used in the 96-well microbioassay because of suitable in vitro growth and economic importance. Commercial fungicides vinclozolin, chlorothalonil, and thiabendazole (Chem, Service, West Chester, PA) were used as validation controls in all microbioassay tests.

3.6. Inoculum preparation

Conidial suspensions were prepared according to published procedures (Wedge & Kuhajek, 1998). Conidial concentrations were determined spectro photometrically (Espinel-Ingrof & Kerkerin, 1991; Wedge & Kuhajek, 1998) from a standard curve, and suspensions were then adjusted with sterile distilled water to a concentration of 1.0×10^6 conidia ml^{-1}.

3.7. Bioautography

Inhibition of fungal growth on chromatographic plates was evaluated by modifications of thin layer chromatography (TLC) bioautographic assays (Homans & Fuchs, 1970; Osborne, Chase, Lunness, Scott & Daniels, 1994). Sesquiterpene lactones were dissolved in Me_2CO and commercial fungicide standards in 95% EtOH. Each test compound was placed on the TLC plate from stock solutions to achieve a final amount of 2 μg using a disposable glass micropipette for each sample. To detect biological activity directly on the TLC plate, silica gel plates with a fluorescent indicator (250 μg, Silica Gel GF Uniplate, Analtech, Newark, DE) were sprayed with a spore suspension. Aliquots of 25–50 ml of inoculum spray solution (ca. 3×10^5) were prepared for each test fungus with liquid potato dextrose broth (PDB) containing 12 g/500 ml (PDB), 0.1% bacto agar, and 0.1% Tween-80. Using a 50 ml chromatographic sprayer, each plate was sprayed lightly (to a damp appearance) three times with conidial suspension. Inoculated plates were placed in a $30 \times 13 \times 7.5$ cm moisture chamber (398-C, Pioneer Plastics, Dixon, KY) and incubated in a growth chamber at $24 \pm 1^{\circ}C$ and 12 h photoperiod under 60 ± 5 μmol light. Inhibition of fungal growth was measured 4 days after treatment. Sensitivity of each fungal species to test compounds and a fungicide standard at 2 μg was determined by comparing size of inhibitory zones.

3.8. Microtiter assay

A 96-well microtiter assay was used to determine sensitivity of *B. cinerea, C. acutatum, C. fragariae, C. gloeosporioides*, and *F. oxysporum* to the various sesquiterpene lactones in comparison with known fungicidal standards (Wedge & Kuhajek, 1998). Vinclozolin, chlorothalonil, and thiabendazole, which represent three different modes of action, were used as standards in this experiment. Each fungus was challenged in a dose-response format using test compounds where the final treatment concentrations were 0.3, 3.0 and 30.0 μM. Microtiter plates (Nunc MicroWell (untreated), Roskilde, Denmark) were covered with a plastic lid and incubated in a growth chamber as described previously for fungal growth. Growth was then evaluated by measuring absorbance of each well at 620 nm using a microplate photometer (Packard Spectra Count, Packard Instrument, Downers Grove, IL).

3.9. Microbioassay experimental design

Each fungus was challenged using a 96-well plate microbioassay format in duplicate. Each chemical was evaluated in duplicate in a dose-response format where the final assay concentrations were 0.3, 3.0 and 30.0 μM. Eight wells containing broth and inoculum served as positive controls; eight wells containing solvent at the appropriate concentration and broth without inoculum were used as negative controls. Mean absorbance values and standard errors were used to evaluate fungal growth at 48 h. Analysis of variance of Least Square Means for percent inhibition of each fungus at each dose of test compound ($n = 4$) relative the untreated controls ($n = 32$) were used to evaluate fungal growth inhibition. The experiment used a Repeated Measures Design and was repeated once. Data presented were pooled from two independent experiments.

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