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Screening Study of Lead Compounds for Natural Product-Based Fungicides: Antifungal Activity and Biotransformation of 6α,7α-Dihydroxy-β-himachalene by *Botrytis cinerea*

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Eleven β-himachalene derivatives were tested, using the poisoning food technique, for their potential antifungal activity against the phytopathogen *Botrytis cinerea*. Compounds **1**−**11** displayed moderate activity, whereas the 6,7-diol derivative (**12**) produced an inhibition of 91% after 6 days. The microbial transformation of **12** was investigated and yielded four new compounds hydroxylated at positions C-5 (**13**), C-2 (**14**), C-4 (**15**), and C-12 (**16**). The structures were established on the basis of their spectroscopic data including two-dimensional NMR analysis (HMQC, HMBC, nOesy) and nOes. The results obtained from biotransformation experiments shed further light on the detoxification mechanism of the phytopathogenic fungus against this compound and give an indication of the structural modifications that may be necessary if substrates of this type are to be further developed as selective fungal control agents for *B. cinerea*.

KEYWORDS: Himachalene derivatives; antifungal activity; biotransformation; *Botrytis cinerea*

INTRODUCTION

Application of synthetic fungicides to control fungal diseases in major agricultural crops is standard farming practice throughout Europe. Although modern fungicides have reached a considerable level of efficacy associated with increasingly reduced toxicity, some environmental problems remain, as many of these fungicides are persistent enough to be detected after several weeks in vegetables (*1*) and soil (*2*) and even after vinification (*3*, *4*). In addition, several fungal species, such as the plant pathogen *Botrytis cinerea* (*5*−*7*), have developed resistance to some commercial fungicides (*8*−*10*). Consequently, there is great interest in developing novel, nonpersistent, and rational antifungal agents, especially those with activity against particularly damaging and resistant fungi such as *B. cinerea*.

Recent progress in the identification of the pathogenicity factors of this harmful fungus has opened significant options for major innovations in the control of plant disease (*11*). Thus, the putative role of the phytotoxins excreted by *B. cinerea* in the infection mechanism has been reported (*12*, *13*). A new and rational alternative to synthetic fungicides has been obtained by using compounds that are structurally analogous to the biosynthetic intermediate of the naturally expressed toxins of the fungus (*14*, *15*). Studies on the structure−activity relationships of substrates that inhibited the growth of *B. cinerea* and their relationships to the botryane metabolites produced by the fungus revealed structural similarities between them (*16*, *17*). In particular, the distance between the hydrophobic geminal dimethyl group and the hydrophilic hydroxyl group seems to mimic the analogous distances found in botryanes (*16*).

β-Himachalene (**1**) is the main constituent of the essential oil of *Cedrus* species. In addition to its basic skeleton of two fused rings, which is of interest for our purposes, some derivatives have also been found to possess the same key distance between the hydrophobic geminal dimethyl group and the hydrophilic hydroxyl group as found in the aforementioned phytotoxins, as well as in the major secondary metabolites of *B. cinerea* (*18*). Furthermore, both antimicrobial and insecticidal activities have been reported for different himachalene oxygenated derivatives (*19*, *20*). The further development of this promising class of compounds as fungistatic agents against *B. cinerea*, however, requires the evaluation of a certain number of derivatives with a himachalane skeleton and different patterns of oxidation and substitution.

In this paper we present an evaluation of the fungicidal activity against *B. cinerea* of several himachalane derivatives, along with their metabolism by *B. cinerea*.

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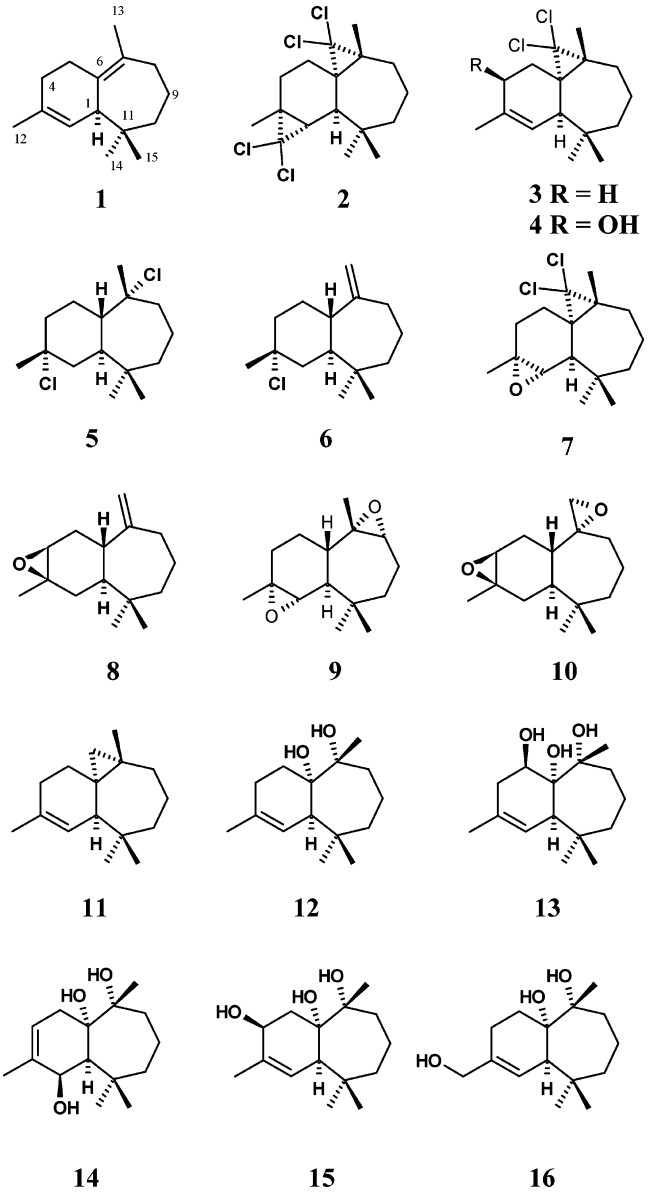

Figure 1. Structures of compounds **1**–**16**.

MATERIALS AND METHODS

Chemical Analysis. Optical rotations were determined with a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Mattson Genesis spectrophotometer, series FTIR. ^{1}H and ^{13}C NMR measurements were obtained on Varian Gemini 300 and Varian Unity 400 NMR spectrometers with $SiMe_4$ as the internal reference. Mass spectra were recorded on Fisons MD 800 and Finnigan MAT95 S spectrometers. HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with an UV−vis detector (L 4250) and a differential refractometer detector (RI-71). TLC was performed on Merck Kieselgel 60 F_{254}, 0.2 mm. Silica gel (Merck) was used for column chromatography. Purification by means of HPLC was accomplished using a silica gel column (Hibar 60, 7 m, 1 cm wide, 25 cm long). Chemicals were products of Fluka or Aldrich. All solvents used were freshly distilled.

Microorganism and Antifungal Assays. The culture of *B. cinerea* employed in this work, *B. cinerea* 2100, was obtained from the Centro Español de Cultivos Tipos (CECT), Facultad de Biologia, Universidad de Valencia, Spain, where a culture of this strain is deposited. Bioassays were performed by measuring the inhibition of radial growth on an agar medium in a Petri dish. The test compound was dissolved in acetone to give final compound concentrations of 25−200 mg L^{-1}. Solutions of the test compound were added to glucose−malt−peptone−agar medium [61 g/L of glucose (20 g), malt (20 g), peptone (1 g), agar (20 g), pH 6.5−7.0]. The final acetone concentration was identical in both control and treated cultures. The medium was poured into sterile plastic Petri dishes measuring 9 cm in diameter, and a mycelial disk of *B. cinerea* cut from an actively growing culture and measuring 5 mm in diameter was placed in the center of the agar plate.

Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control (*I*%) and was measured for 6 days. The assays were carried out in triplicate, and the results are shown as mean values of three replicates of colony diameters (± SD) (**Figure 2**). The commercial fungicide dichlofluanid was used throughout as a standard for comparison. The IC_{50} value was determined by calculating a linear regression of the probit of the test fungus percentage inhibition and the log of the studied compound concentrations. The IC_{50} was the average of three replications.

Table 1. ^{13}C NMR (100 MHz) of Compounds **12**–**16**

C	12	13	14	15	16
C1	53.8	55.5	51.6	55.2	53.8
C2	122.7	124.7	72.8	125.8	124.7
C3	135.1	135.8	136.4	136.9	136.2
C4	27.2	35.0	121.1	69.1	22.9
C5	26.1	68.3	32.4	36.4	25.7
C6	78.5	79.7	80.0	80.3	78.8
C7	77.7	78.4	77.7	77.8	78.1
C8	33.0	32.7	39.9	32.7	32.9
C9	19.9	20.0	18.1	20.0	20.0
C10	40.3	40.3	43.9	40.1	40.3
C11	37.1	37.1	34.6	36.8	37.1
C12	23.3	20.7	20.7	19.5	67.1
C13	28.2	28.5	27.0	28.2	28.3
C14	34.1	34.3	32.9	34.4	34.2
C15	21.6	20.8	25.9	21.3	21.6

Compounds 1−12. β-Himachalene (**1**) was isolated from the essential oil of the Atlas cedar (*Cedrus atlantica*) collected in Morocco. Compounds **2**−**12** (**Figure 1**) were synthesized following the procedures previously reported (*21*−*28*).

Biotransformation by *B. cinerea*. Twenty Erlenmeyer flasks (500 mL) were filled with 200 mL of Czapek−Dox medium: glucose (50 g), yeast extract (1 g), KH_2PO_4 (5 g), $NaNO_3$ (2 g), $MgSO_4$ (0.5 g), and $FeSO_4$ (10 mg) per liter of distilled water. The pH was adjusted to 7.0 with aqueous NaOH, and the flasks were inoculated with a suspension (2.7 mL) of *B. cinerea* conidia (7×10^6 conidia/mL). The flasks were incubated at 25 °C for 3 days and stirred at 180 rpm; the mycelium was then filtered and transferred into 18 (500 mL) flasks containing 200 mL of Czapek−Dox medium (with only 25 g of glucose) and 60 ppm of the substrate (1*S*,6*S*,7*R*)-3,7,11,11-tetramethylbicyclo-[5,4,0]undec-2-ene-6,7-diol (**12**) per flask. The remaining two flasks were used as the control. Three days after inoculation, the mycelium was filtered and the fermentation broth was extracted three times with ethyl acetate. The extract was dried over anhydrous sodium sulfate, and the solvent was then evaporated. Fractionation of the extract (320 mg) was carried out by means of column chromatography on silica gel (SiCC), eluting with petroleum ether/ethyl acetate (85:15). Final purification was carried out by means of semipreparative HPLC (hexane/ethyl acetate 97:3; 2.5 mL min^{-1}) to afford new compounds (1*S*,5*R*,6*S*,7*R*)-3,7,11,11-tetramethylbicyclo[5,4,0]undec-2-ene-5,6,7-triol (**13**) (2.3 mg, 1.6%), (1*S*,2*R*,6*S*,7*R*)-3,7,11,11-tetramethylbicyclo-[5,4,0]undec-3-ene-2,6,7-triol (**14**) (3 mg, 2%), (1*S*,4*S*,6*S*,7*R*)-3,7,11,11-tetramethylbicyclo[5,4,0]undec-2-ene-4,6,7-triol (**15**) (25 mg, 16.3%), and (1*S*,6*S*,7*R*)-7,11,11-trimethylbicyclo[5,4,0]undec-2-ene-6,7,12-triol (**16**) (32 mg, 21%). Their structures were established on the basis of their spectroscopic data including two-dimensional NMR analysis (HMQC, HMBC, and nOesy) and nOe studies.

(1*S*,5*R*,6*S*,7*R*)-3,7,11,11-Tetramethylbicyclo[5,4,0]undec-2-ene-5,6,7-triol (13): $[\alpha]^{20}_D$, +57.1° (*c* 0.17, $CHCl_3$); IR (film) ν_{max} 3399, 1456, 1364, 1163, 1053, 855 cm^{-1}; ^{1}H and ^{13}C NMR data, see **Tables 1** and **2**; MS (*m*/*z*), 254 ($M^{\bullet+}$, 1), 236 ($M^{\bullet+} - H_2O$, 2), 221 [$M^{\bullet+} - (H_2O + CH_3)$, 7], 218 ($M^{\bullet+} - 2H_2O$, 2), 203 [$M^{\bullet+} - (2H_2O + CH_3)$, 5], 151 (21), 149 (21), 109 (100); HRMS (*m*/*z*), M^+ calcd for $C_{15}H_{26}O_3$, 254.1881; found 254.1878.

(1*S*,2*R*,6*S*,7*R*)-3,7,11,11-Tetramethylbicyclo[5,4,0]undec-3-ene-2,6,7-triol (14): $[\alpha]^{20}_D$, −34.55° (*c* 0.136, $CHCl_3$); IR (film) ν_{max} 338,

Table 2. ^{1}H NMR (400 MHz) of Compounds **12**–**16**

^{1}H	12	13	14	15	16
1	2.05 d (5.3)	2.2 d (5.5)	2.21 s	2.18 d (4.7),	2.26 d (5.4)
2	5.43 dd (5.3, 1.5)	5.58 dd (1, 5.3)	3.91 s	5.58 dd (2, 4.7)	5.78 d (5.4)
4	2.05 m	1.97 m	5.43 s	4.23 dd (8, 9)	2.2 m
5	1.7 m	3.9 d (4.2)	2.16 m	β1.67 m; α2.15 d (2)	1.7–1.82 m
8	1.73 m	1.73 m	α1.89 ddd (4.2, 8, 15); β1.52 m	1.69 m	1.73 m
9α	1.72 m	1.73 m	1.66 m	1.71 m	1.72 m
9β	1.53 m	1.59 m	1.45 m	1.53 m	1.53 m
10α	2.05 m	2.05 m	1.77 dd (9.7, 4.5)	2.06 m	2.1 m
10β	1.30 m	1.38 m	1.23 m	1.37 ddd (3, 6.5, 14)	1.35 m
12	1.7 d (1.5)	1.88 s	1.85 d (1.8)	1.84 d (0.4)	4.8 s
13	1.23 s	1.33 s	1.24 s	1.3 s	1.28 s
14	0.95 s	1.01 s	1.09 s	1.0 s	1.02 s
15	0.77 s	0.77 s	0.68 s	0.9 s	0.81 s

1459, 1370, 1154, 1047 cm^{-1}; ^{1}H and ^{13}C NMR data, see **Tables 1** and **2**; MS (*m*/*z*), 254 ($M^{\bullet+}$, 0.2), 236 ($M^{\bullet+} - H_2O$, 2), 221 [$M^{\bullet+} - (H_2O + CH_3)$, 3], 218 ($M^{\bullet+} - 2H_2O$, 2), 203 [$M^{\bullet+} - (2H_2O + CH_3)$, 3], 109 (100); HRMS (*m*/*z*), M^+ calcd for $C_{15}H_{26}O_3$, 254.1881; found 254.1875.

(1*S*,4*S*,6*S*,7*R*)-3,7,11,11-Tetramethylbicyclo[5,4,0]undec-2-ene-4,6,7-triol (15): $[\alpha]^{20}_D$, −2.35° (*c* 0.68, $CHCl_3$); IR (film) ν_{max} 3416, 1459, 1361, 1169, 1033 cm^{-1}; ^{1}H and ^{13}C NMR data, see **Tables 1** and **2**; MS (*m*/*z*), 254 ($M^{\bullet+}$, 2), 236 ($M^{\bullet+} - H_2O$, 1), 221 [$M^{\bullet+} - (H_2O + CH_3)$, 9], 218 ($M^{\bullet+} - 2H_2O$, 2), 203 [$M^{\bullet+} - (2H_2O + CH_3)$, 6], 151 (68), 149 (29), 109 (100); HRMS (*m*/*z*), M^+ calcd for $C_{15}H_{26}O_3$, 254.1881; found 254.1883.

(1*S*,6*S*,7*R*)-7,11,11-Trimethylbicyclo[5,4,0]undec-2-ene-6,7,12-triol (16): $[\alpha]^{20}_D$, +38.6° (*c* 0.36, $CHCl_3$); IR (film) ν_{max} 3398, 1461, 1361, 1050 cm^{-1}; ^{1}H and ^{13}C NMR data, see **Tables 1** and **2**; MS (*m*/*z*), 236 ($M^{\bullet+} - H_2O$, 6), 221 [$M^{\bullet+} - (H_2O + CH_3)$, 25], 218 ($M^{\bullet+} - 2H_2O$, 5), 203 [$M^{\bullet+} - (2H_2O + CH_3)$, 5], 109 (100); HRMS (*m*/*z*), [$M^{\bullet+} - H_2O$] calcd for $C_{15}H_{24}O_2$, 236.1776; found 236.1806.

RESULTS AND DISCUSSION

The reactivity of himachalene sesquiterpenes has been extensively studied. Compounds **2**−**12** (**Figure 1**) were obtained following procedures previously described (*21*−*28*). The "poisoned food technique" was used to determine the antifungal properties of the compounds under study (*29*, *30*). The results of the antifungal activity screening test on *B. cinerea* show that, in general, himachalene derivatives **1**−**11** displayed weak antifungal activity, whereas compound **12** was active against the fungus.

It is worth noting that this latter compound possesses the aforementioned key distance between the hydrophobic geminal dimethyl group on C-11 and the hydrophilic tertiary hydroxyl group on C-6.

Thus, as can be seen in **Figure 2**, the dihydroxyhimachalene derivative **12** displayed total inhibition of *B. cinerea* for 2 and 3 days at 150 and 200 ppm, respectively, retaining an inhibition percentage of 91% after 6 days. The IC_{50} for this compound was 80.8 mg L^{-1}. Nevertheless, the inhibitory effect of compound **12** diminished with time, a fact which suggests that the fungus possesses a detoxification mechanism. To study this mechanism, the substrate (1*S*,6*S*,7*R*)-3,7,11,11-tetramethylbicyclo-[5,4,0]undec-2-ene-6,7-diol (**12**) was incubated with *B. cinerea* for 3 days on a shaken culture (see Materials and Methods). The mycelium was filtered, and the fermentation broth was extracted with ethyl acetate and purified by means of column chromatography and HPLC. The structures of the newly isolated compounds were established after extensive NMR and MS studies. Compounds **13**−**16** had the molecular formula $C_{15}H_{26}O_3$, as deduced from their mass spectra (M^+ at *m*/*z* 254). The locations of the additional oxygen functions on the himachalene skeleton were established after an analysis of the spectroscopic data (**Tables 1** and **2**).

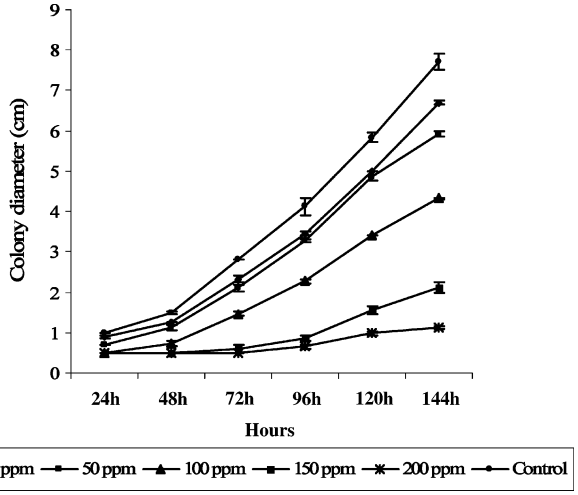

Figure 2. Antifungal effect of compound **12** on in vitro grown of *B. cinerea* 2100. Results are shown as median values of three replicates of micelium diameter; bar = ± SD.

The ^{1}H NMR spectrum of product **13** showed a new secondary alcohol resonance at δ 3.9 (1H, d), a fact that suggests the introduction of a new hydroxyl group at one of the methylene groups in the himachalene molecule; this hypothesis is further bolstered by the presence of a low-field hydroxyl-bearing methyne signal at δ 68.3. The position of the newly introduced hydroxyl was established by comparing the ^{13}C NMR spectrum of **13** with that of himachalene **12**. The methylene signal assigned to C-5 in **12** was not present in the ^{13}C NMR spectrum of **13,** indicating that the hydroxyl group was inserted at C-5. The stereochemistry at C-5 was determined by examining the correlations observed in the nOesy experiment. The correlation between H-2 and H-1 and between H-14 and H-12 indicated a conformation for **13** where the carbons C-2, C-3, and C-12 are oriented toward the α face of the molecule. The proton H-12 showed a correlation with H-5, which indicates an α disposition for the proton and a β disposition for the hydroxyl group on C-5. This stereochemistry is supported by the multiplicity of the signal at H-5, a broad doublet with J = 4.2 Hz, which indicates that the proton is in an axial (α) position in a twisted-boat conformation for the cyclohexene ring.

The ^{1}H NMR spectra of compound **14** and its isomer **15** each display a downfield signal for oxygen-bearing methyne protons at δ 3.91 and 4.23, respectively. In addition, the DEPT spectra show the disappearance of a CH_2 signal and the appearance of

a CH signal at δ 72.8 and 69.1, respectively, confirming that both compounds are hydroxylated metabolites of **12**.

The HMBC spectrum of **14** showed three-bond connectivity between the new proton signal at δ 3.91 and carbons 4, 11, 6, and 12, suggesting that the hydroxyl group is located at C-2 and that the double bond is located between C-3 and C-4. The stereochemistry of the hydroxyl group at C-2 was determined to be β on the basis of nOesy data, which showed nOe correlations between H-2_{eq} and H-1_{ax}.

Examination of the HMQC, HMBC, and COSY data of **15** revealed that the resonances of carbons 3 and 5 are shifted significantly downfield to δ 125.8 ($\Delta\delta$ +3.1) and 36.4 ($\Delta\delta$ +10.3), respectively, whereas carbon 12 exhibits upfield shifts from δ 23.3 to 19.5 ($\Delta\delta$ −3.8) in comparison with those observed for **12.** These data indicate that the new hydroxyl group is at C-4. Irradiation of the signal corresponding to H-4 (δ 4.23) led to positive nOe enhancements at H-1, which is α-oriented; accordingly, the relative stereochemistry of 4-OH was confirmed as having a β-disposition. On the basis of all this evidence, the structure of **15** was determined to be (1*S*,4*S*,6*S*,7*R*)-3,7,11,11-tetramethylbicyclo[5,4,0]undec-2-ene-4,6,7-triol.

The absence of the methyl group signal on the double bond in the ^{1}H and ^{13}C NMR spectra of compound **16**, along with the appearance of a new hydroxymethyl resonance (δ_H 4.08; δ_C 67.1), suggested that **16** was monohydroxylated in the methyl group at C-3. This was confirmed by the downfield shift of the signal assigned to C-3.

The antifungal properties of biotransformation products **13**−**16** against *B. cinerea* were tested, showing that all new metabolites obtained from biotransformation were inactive at 100 and 200 ppm. These results confirm that *B. cinerea* has a mechanism to detoxify compound **12** by hydroxylating positions C-2, C-4, C-5, and C-12. The structures of these metabolites suggest compounds that might be targets for future study as inhibitory agents.

In conclusion, according to the results described herein, the most promising antifungal agent for the control of the fungus *B. cinerea* as judged by its consistency in inhibiting growth of the fungus was the dihydroxyhimachalene derivative **12**. The results obtained from biotransformation experiments shed further light on the detoxification mechanism of the phytopathogenic fungus against this compound and give an indication of the structural modifications that may be necessary if substrates of this type are to be further developed as selective fungal control agents for *B. cinerea*.

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