Sesquiterpene Lactones from *Centaurea achaia*, a Greek Endemic Species: Antifungal Activity

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The aerial parts of *Centaurea achaia* afforded, in addition to several known sesquiterpene lactones and sesquiterpene hydroxyesters, a new germacranolide and a new elemanolide. Their structures were determined as the 8α-O-(4,5-dihydroxy-tigloyloxy) esters of salonitenolide and of 11,13-dihydromelitensin, respectively. The *in vitro* antifungal activity of most compounds was tested against nine fungal species using the micro-dilution method. All the tested compounds showed strong antifungal activity.

Introduction

The large genus *Centaurea* (Asteraceae – Cardueae) comprises about 500 species, which are predominately distributed around the Mediterranean area and in W Asia (Mabberley, 1997). Continuing our research on the chemical constituents of *Centaurea* sp. (Skaltsa *et al.*, 1999; Cardona *et al.*, 1997), we have investigated *C. achaia*, a greek endemic taxon, belonging to the section Acrocentron (Cass.) D. C.

We now report the isolation of nine germacranolides (**1**–**9**), two elemanolides (**10**, **11**), two eudesmanolides (**13, 14**) and the hydroxyester derivatives **12** and **15**. Compounds **7** and **11** are new naturally occurring sesquiterpene lactones and their structures were elucidated by extensive high-field NMR studies. Compounds **2**–**4**, **7**, **8**, **10**–**15** were tested against several micromycetes.

Results and Discussion

Chromatographic separation of the ethyl acetate extract of *C. achaia* (aerial parts) yielded salonitenolide (**1**) (Cardona *et al.*, 1997) and its 8α-acyl derivatives onopordopicrin (**2**) (Cardona *et al.*, 1989), **3** (El-Masry *et al.*, 1985) **4** (El-Masry *et al.*, 1985), **5** (Lazari *et al.*, 1998), **6** (Lazari *et al.*, 1998) and the new compound **7**; 11β,13-dihydrosalonitenolide (**8**) (Marco *et al.*, 1992) and its acyl derivative **9** (Lazari *et al.*, 1998); the dehydromelitensin derivative **10** (Cardona *et al.*, 1989) and the new compound **11**; elemacarmanin (**12**) (Cardona *et al.*, 1989); the eudesmanolides **13** (Rustaiyan *et al.*, 1986) and **14** (Rustaiyan *et al.*, 1986); 4-*epi*-carmanin (**15**) (Rustayian *et al.*, 1986). All compounds were isolated in sufficient amounts to allow structure elucidation (Fig. 1) and most of them were tested against several micromycetes. We also report the ^{13}C NMR for compound **15**.

Compound **7** was identified as an 8α-acyl derivative of salonitenolide, since its ^{1}H and ^{13}C NMR spectra were similar to those of compounds **1**–**6**. Its molecular formula ($C_{20}H_{26}O_7$) was deduced from its IR, ^{13}C NMR, and EIMS spectral data. The IR spectrum afforded intense absorptions at 3600–3300 (OH), 1750 (C=O, γ-lactone) and 1720 cm^{-1} (C=O, ester). The analysis of the ^{13}C NMR and DEPT experiments (Table I) indicated that **7** contained twenty carbons: one CH_3, seven CH_2 (one exomethylene, three hydroxymethyl and three highfield methylenes), six CH (three sp^2 methines, two carbinols and a saturated methine),

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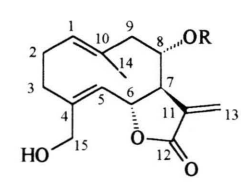

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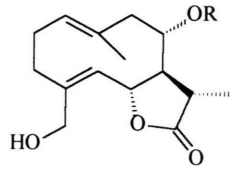

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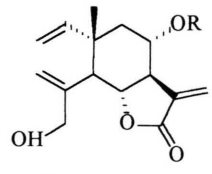

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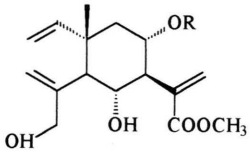

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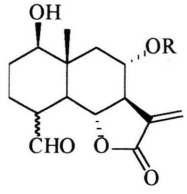

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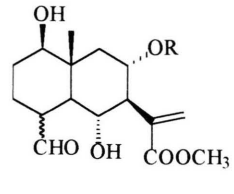

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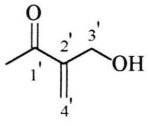
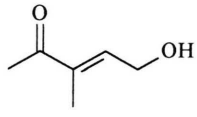
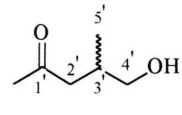
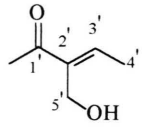
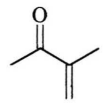
A = 

C = 

E = 

B = 

D = 

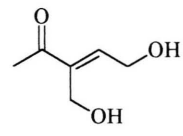
F = 

Fig. 1. Structures of the isolated compounds.

as well as four olefinic quaternary carbons and two carbonyls (δ 167.5, γ-lactone; δ 172.5, ester). From those data a molecular formula $C_{20}H_{26}O_7$ was suggested, which is in accordance with the peak at *m/z* 378 $[M]^+$ in the mass spectrum. In its ^1H NMR spectrum (Table I) the broadened doublet at δ 4.97 ($J = 8.7$ and 9.1 Hz) and the broadened doublet at δ 4.81 ($J = 9.5$ Hz) were assigned to H-1 and H-5, respectively. In the ^{13}C NMR spectra the signals at δ 131.1, 145.7, 130.1 and 133.0 were assigned to C-1, C-4, C-5 and C-10, respectively. The chemical shifts of the methyl group (*s*, δ 1.48 in the ^1H NMR, δ 17.4 in the ^{13}C NMR) were the usual values for a C-10 methyl group in germacranolides. From a pair of doublets at δ 4.28 and 4.06 ($J = 14.1$ Hz) a hydroxymethyl group (at δ 61.1 in the ^{13}C NMR spectrum) as substituent at C-4 was evident. The presence of an oxygenated group at C-6α (δ 78.9 in the ^{13}C NMR) was inferred from the double doublet at δ 4.99 ($J = 7.9$ and 9.1 Hz),

Table I. ^{1}H NMR of compound **7** and **11** (400 MHz, $CDCl_3$) and ^{13}C NMR of compound **7** (50.3 MHz, CD_3OD) and **15** (50.3 MHz, $CDCl_3$) [HMQC allowed assignments of protonated carbons].

H	7	11	C	7	15
1	4.97 *dd* (8.7/ 9.1)	5.74 *dd* (10.8/17.0)	1	131.1	77.1
2a	2.30–2.10 *m*	5.03 *d* (10.8)	2	27.4	27.8
2b	2.30–2.10 *m*	4.99 *d* (17.0)	3	35.4	24.0
3a	2.55 *ddd* (2.9/7.5/11.6)	5.39 *s*	4	145.7	48.5
3b	1.97 *ddd* (6.6/11.2/11.6)	4.95 *s*	5	130.1	50.8
5	4.81 *d* (9.5)	2.56 *d* (11.6)	6	78.9	70.9
6	4.99 *dd* (7.9/9.1)	4.23 *t* (11.6)	7	54.2	55.9
7	3.06 *m*	2.94 *tt* (2.9/11.2)	8	74.8	70.5
8	5.14 br *dd* (8.3/9.1)	5.26 *td* (3.7/11.2)	9	48.9	41.3
9a	2.52 br *d* (12.0)	1.65 *dd* (11.6/12.4)	10	133.0	38.8
9b	2.47 br *dd* (11.2/12.0)	2.02 *dd* (4.2/12.8)	11	137.6	137.2
13a	6.25 *d* (2.9)	6.12 *d* (2.9)	12	167.5	166.9
13b	5.76 *d* (2.5)	5.57 *d* (2.9)	13	125.9	129.3
14	1.48 *s*	1.15 *s*	14	17.4	12.0
15a	4.28 *d* (14.1)	4.05 *d* (13.7)	15	61.1	202.7
15b	4.06 *d* (14.1)	3.98 *d* (13.7)	1′	172.5	165.2
3′	6.99 *t* (5.8)	6.99 *t* (5.8)	2′	133.7	139.1
4′	4.46 *d* (5.8)	4.45 *d* (5.8)	3′	147.2	62.3
5′	4.36 *s*	4.38 *s*	4′	59.7	125.9
			5′	57.0	–
			$OOCH_3$	–	52.1

which showed coupling with the signal of H-5 (δ 4.81) and H-7 (δ 3.06). From the two doublets for H-13 and H-13′ at δ 6.25 (J = 2.9 Hz) and 5.76 (J = 2.5 Hz), which showed coupling with H-7, an α-methylene-γ-lactone was also evident [1760 cm^{-1} in the IR spectrum; a methylene at δ 137.6 (C-11) and 125.9 (C-13) in the ^{13}C NMR]. An ester moiety was located at C-8α (δ 74.8) as was deduced from the position and pattern of the signal at δ 5.14 (*br dd*, J = 8.3 and 9.1). The identity of the ester side chain was inferred from a two-proton singlet at δ 4.36, and a doublet at δ 4.46 (2H, J = 5.8 Hz), which was coupled to a triplet at δ 6.99 (1H). The chemical shifts and the pattern of these signals strongly suggested the presence of a 4,5-dihydroxytiglate moiety (de Hernández *et al.*, 1999). This was confirmed by the signals at δ 167.5 (C), 133.7 (C), 147.2 (CH), 59.7 (CH_2) and 57.0 (CH_2) in the ^{13}C NMR spectrum (de Hernández *et al.*, 1999), and the peaks at *m/z* 247 [M–$C_5H_7O_4$, M–RCOO]$^+$ and *m/z* 131 [$C_5H_7O_4$, RCOO]$^+$ in the mass spectrum. Consequently, **7** is the new 8α-(4,5-dihydroxytigloyloxy)-salonitenolide.

The ^{1}H NMR spectrum of compound **11** (Table I) showed typical low-field signals that clearly indicated the presence of an elemanolide, and exhibited common features with those of compound **10**, an 8α-acyl derivative of 11,13-dehydromelitensin (Cardona *et al.*, 1989). Its mass spectrum presented peaks which suggested the same molecular mass and acyl side chain than compound **7** (*m/z* 378 [M]$^+$, *m/z* 247 [M–RCOO]$^+$ and *m/z* 131 [RCOO]$^+$), and its structure was elucidated by the analysis of the ^{1}H NMR spectrum. The signals at δ 5.74 *dd*, 5.03 *d*, 4.99 *d*, 5.39 *s* and 4.95 *s* were assigned to H-1, H-2a, H-2b, H-3a and H-3b, respectively. An α-methylene-γ-lactone was evident for the two doublets for H-13 at δ 6.12 and 5.57 which showed coupling (J = 2.9 Hz) with the triplet of triplets at δ 2.94 for H-7. A typical doublet at δ 2.56 (J = 11.6 Hz) for H-5, the signal at δ 4.23 (*t*, J = 11.6 Hz) for H-6 and the lowfield double triplet at δ 5.26 corresponding to H-8 indicated a *trans*-disposition of H-5/H-6, H-6/H-7 and H-7/H-8 and so the oxygenated functions at C-6 and C-8 should be α-oriented. From a pair of doublets at δ 4.05 and 3.98 a hydroxymethyl group as substituent at C-4 was also evident. The identity of the ester side chain was deduced from the chemical shifts of its protons, almost identical to those of compound **7**. We have assigned to compound **11** the structure of the new 8α-(4, 5-dihydroxytigloyloxy)-11,13-dehydromelitensin.

C. achaia is characterised by the total absence of guaianolides, common metabolites of other *Centaurea* species (Fraga, 1992; 1993; 1994; 1995; 1996; 1997; 1998; 1999).

When its chemical profile is compared to previously studied *Centaurea* sp. belonging to the same section, appears quite deviating, as their main constituents are guaianolides: *C. clementei* (González *et al.*, 1986); *C. nicolai* (Vajs *et al.*, 1999), *C. ornata* (Navarro *et al.*, 1990); *C. ornata* subsp. *ornata* (Bastos *et al.*, 1994); *C. ragustina* subsp. *ragustina* (Mahmoud *et al.*, 1986); *C. salonitana* (Daniewski *et al.*, 1992; Daniewski *et al.*, 1993).

Moreover, our results indicate that *C. achaia* comes close to many *Onopordum* species (Fraga; 1992; 1993; 1994; 1995; 1996; 1997; 1998; 1999), whose main compounds are onopordopicrin and closely related sesquiterpene lactones.

Compound **7** and onopordopicrin (**2**) were found to be the principal constituents of the lactone mixture, but both of them decompose rapidly.

All the tested compounds possess great antifungal activity. From the observed results (Table II), the lowest minimum inhibitory concentrations (nmol/ml), which inhibited the fungal growth, and the minimum fungicidal concentrations (nmol/ml), which killed the fungi, are for compounds **3** and **2**, while compound **15** showed the greatest MICs and MFCs values, so, it possesses the lowest antifungal potential. MICs and MFCs for all tested compounds except **15** are 0.1–3.0 and 0.75–6.0 nmol/ml, respectively, while MICs and MFCs for miconazole are 0.06–4.0 and 4.0–10.0 nmol/ml, indicating that most compounds investigated possessed greater antifungal potential as this commercial fungicide, which was used as a control (Table II). *Penicillium ochrochloron, P. funiculosum* and *Trichoderma viride* were the most resistant fungal species. It can be seen (Table II) that germacranolides (**2**–**4**, **7**, **8**) possess greater antifungal potential than the other groups, elemanolides (**10**, **11**) eudesmanolides (**13**, **14**) and the hydroxyester derivatives (**12**, **15**).

The biological activity of sesquiterpene lactones is generally attributed to the alkylating property of the α-methylene-γ-lactone moiety, and the presence of other alkylating sites (epoxides and conjugated carbonyl groups) may enhance their biological activities. Moreover, their lipophilicity values seem to play an important role in antifungal activity (Inoue *et al.*, 1995; Barrero *et al.*, 2000). Since the chemical composition of the fungal cell walls is highly lipophilic, they generally represent strong barriers for the penetration of hydrophobic compounds, and the transport of polar compounds through the outer lipid layer of fungi is retarded. Our results support the hypothesis of an inverse relationship between polarity and antifungal activ-

Table II. Minimum inhibitory concentrations (MICs, nmol/ml) and minimum fungicidal concentrations (MFCs, nmol/ml) of different compounds.

Micromycetes	2 MIC (MFC)	3 MIC (MFC)	4 MIC (MFC)	7 MIC (MFC)	8 MIC (MFC)	10 MIC (MFC)	11 MIC (MFC)	12 MIC (MFC)	13 MIC (MFC)	14 MIC (MFC)	15 MIC (MFC)	Miconazole MIC (MFC)
A. niger	0.1 (0.75)	0.4 (0.75)	0.4 (0.75)	0.4 (0.75)	0.5 (1.0)	0.4 (0.75)	0.4 (0.75)	0.4 (1.5)	0.75 (1.5)	0.4 (0.75)	1.5 (3.0)	3.0 (8.0)
A. ochraceus	0.1 (0.75)	0.2 (0.4)	0.4 (1.5)	0.4 (0.75)	0.5 (2.0)	0.4 (0.75)	0.4 (0.75)	0.4 (1.5)	0.75 (1.5)	0.4 (0.75)	1.5 (3.0)	3.0 (8.0)
A. versicolor	0.4 (1.5)	0.2 (0.4)	0.75 (1.5)	1.5 (1.5)	2.0 (4.0)	1.5 (1.5)	1.5 (3.0)	0.75 (1.5)	1.5 (3.0)	0.75 (1.5)	3.0 (6.0)	4.0 (8.0)
A. flavus	0.4 (1.5)	0.4 (0.75)	0.75 (1.5)	0.4 (0.75)	1.0 (2.0)	0.75 (1.5)	1.5 (3.0)	0.75 (1.5)	1.5 (3.0)	0.75 (1.5)	6.0 (12.0)	1.0 (8.0)
P.ochrochloron	0.75 (1.5)	0.75 (0.75)	1.5 (3.0)	0.4 (1.5)	2.0 (4.0)	0.75 (1.5)	1.5 (3.0)	0.75 (1.5)	3.0 (6.0)	1.5 (3.0)	6.0 (12.0)	2.0 (10.0)
P. funiculosum	1.5 (3.0)	0.75 (1.5)	1.5 (3.0)	1.5 (3.0)	2.0 (4.0)	1.5 (1.5)	3.0 (6.0)	0.75 (1.5)	3.0 (6.0)	1.5 (3.0)	6.0 (12.0)	4.0 (10.0)
T. viride	1.5 (3.0)	0.75 (1.5)	1.5 (3.0)	0.75 (3.0)	2.0 (4.0)	1.5 (1.5)	3.0 (6.0)	1.5 (3.0)	3.0 (6.0)	1.5 (3.0)	6.0 (12.0)	4.0 (4.0)
C. cladosporioides	0.4 (0.75)	0.4 (0.75)	0.75 (1.5)	0.75 (1.5)	1.0 (2.0)	0.4 (0.75)	0.75 (1.5)	0.4 (1.5)	0.75 (1.5)	0.75 (1.5)	1.5 (3.0)	0.06 (0.06)
A. alternata	0.75 (1.5)	0.75 (1.5)	1.5 (1.5)	0.75 (1.5)	1.0 (2.0)	0.75 (1.5)	0.75 (1.5)	0.75 (1.5)	0.75 (1.5)	0.75 (1.5)	0.75 (1.5)	1.0 (1.0)

ity for sesquiterpene lactones in general (Barrero *et al.*, 2000), since, regarding the retention times of our compounds on a RP-18 column, their polarity is decreasing from eudesmanolides > elemanolides > germacranolides.

Experimental

Plant material

Aerial parts of *Centaurea achaia* Boiss. & Heldr. were collected on Mount Pateras (Attiki – central Greek mainland), in June 1994 and authenticated by Dr. Th. Constantinidis (Institute of Systematic Botany, University of Patras). A voucher specimen is deposited in the Herbarium of the above-mentioned Institute under the number Constantinidis 2180 (UPA).

Extraction and isolation

The fresh plant material (2.0 kg) was finely ground and extracted at room temp. with cyclohexane–Et_2O–MeOH (1:1:1 v/v). The extract was washed with brine, the aq. layer re-extracted with EtOAc, and the organic layer dried with Na_2SO_4 and concd. under red. pressure. The residue 19.3 g was prefractionated by VLC on silica gel, using cyclohexane–EtOAc–Me_2CO mixts. of increasing polarity as eluents to give several frs. Frs B (hexane–EtOAc, 1:1 v/v), C (hexane–EtOAc, 1:4 v/v), D (EtOAc) and E (EtOAc–acetone, 4:1 v/v) were subjected to further chromatographic separations as described below.

CC of fr B (CH_2Cl_2–MeOH, 10:0 to 9:1 v/v) followed by HPLC (MeOH–H_2O, 3:2) allowed the isolation of **5** (3.9 mg). VLC of fr C (CH_2Cl_2–MeOH, 10:0 to 1:1 v/v) followed by HPLC (MeOH–H_2O, 2:1 and 4:3 v/v) allowed the isolation of **2** (9.6 mg) and **10** (1.0 mg). CC of fr D (CH_2Cl_2–MeOH, 10:0 to 8:2 v/v) followed by several HPLC (MeOH–H_2O, 3:2, 4:3 and 1:1) allowed the isolation of **1** (20.4 mg), a mixt. of **1** and **8** (*ca.* 12.1 mg, not separated), **8** (14.0 mg), **2** (103.1 mg), **3** (42.8 mg), **4** (4.5 mg), **6** (5.2 mg), **9** (1.1 mg), **10** (5.1 mg), **12** (17.2 mg), **13** (31.1 mg) and **14** (4.3 mg). VLC of fr E (CH_2Cl_2–MeOH, 10:0 to 7:3) followed by further CC and HPLC (MeOH–H_2O, 1:1 v/v) allowed the isolation of **2** (4.8 mg), **7** (177.3 mg), **10** (1.9 mg), **12** (11.3 mg), **14** (10.2 mg), **15** (11.5 mg), **11** (1.3 mg) and a mixt. of **7** and **11** (*ca.* 12.5 mg, not separated).

Chromatography

Vacuum liquid chromatography (VLC): silica gel (Merck; 43–63 μm), CC: silica gel (SDS; 40–63 μm), gradient elution with the solvents mixts indicated in each case; HPLC: CE 1100 Liquid Chromatography Pumb Techsil 10-C18 (250 × 10 mm).

Absorbents for TLC: Merck RP 18 F_{254s}; Merck silica gel 60 F_{254s}; solvents for TLC: mixt. of CH_2Cl_2 with MeOH; mixt. of cyclohexane with EtOAc. Detection on TLC-plates: UV-light, spray reagent (anisaldehyde–H_2SO_4 on silica gel).

Spectroscopic data

NMR: 400 MHz (1D and 2D), 200 MHz (^{1}H) and 50.3 MHz (^{13}C). The NMR spectra were recorded using Bruker Avance 400 and Bruker AC 200. Chemical shifts are reported in δ (ppm) values. MS: Hewlett-Packard mod. 5973; 70eV. FT-IR Spectrometer: Perkin-Elmer Paragon 500. Polarimeter: Perkin-Elmer 341. The optical rotation values were determined at 25 °C at 589 nm in $CHCl_3$ (Uvasol).

Compound **7**: Unstable colorless oil; $[\alpha]_D^{20}$ +0.18° ($CHCl_3$ c, 2.84); IR ν_{max}^{KBr} cm^{-1}: 3600–3300, 1750, 1720; EIMS *m/z* (rel.int.): 378 $[M]^+$ (3), 247 $[M-\mathbf{FO}]^+$ (10), 131 $[\mathbf{FO}]^+$ (30). ^{1}H NMR and ^{13}C NMR spectral data: see Table I.

Compound **11**: Colorless oil; $[\alpha]_D^{20}$ +19.6° ($CHCl_3$ c, 0.09); IR ν_{max}^{KBr} cm^{-1}: 3600–3300, 1760, 1710, 1640; EIMS *m/z* (rel.int.): 378 $[M]^+$ (5), 350 $[M-CO]^+$ (10), 247 $[M-\mathbf{FO}]^+$ (12), 230 (15), 131 $[\mathbf{FO}]^+$ (100). ^{1}H NMR spectral data: see Table I.

Bioassays

For the bioassays nine fungi were used: *Aspergillus niger* (ATCC 6275), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus flavus* (ATCC 9643), *Penicillium ochrochloron* (ATCC 9112), *Penicillium funiculosum* (ATCC 36839), *Trichoderma viride* (IAM 5061), *Cladosporium cladosporioides* (ATCC 13276) and *Alternaria alternata* (DSM 2006).

The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological research "Sinisa Stankovic", Belgrade, Yugoslavia.

The micromycetes were maintained on malt agar (MA) and the cultures were stored at +4 °C and subcultured once a month (Booth, 1971).

In order to investigate the antifungal activity of 11 compounds the modified microdilution technique was used (Hanel and Raether, 1988; Daouk *et al.*,1995). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (vol/vol). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µl per well. The inocula were stored at +4 °C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentrations (MICs) determination was performed by a serial dilution technique using 96-well microtitre plates. Extracts of compounds investigated were dissolved in malt medium broth with fungal inoculum to achieve concentrations of 0.1–12.0 nmol/ml. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth (at the binocular microscope) was defined as concentrations which completely inhibited fungal growth (MICs). The minimum fungicidal concentrations (MFCs) was determined by serial subcultivation of a 2 µl into microtitre plates containing 100 µl of broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating = 99.5% killing rate of the original inoculum. Miconazole, a commercial fungicide, was used as a control (0.06–10.0 nmol/ml).

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