Minor Sesquiterpene Lactones from *Centaurea pullata* and Their Antimicrobial Activity

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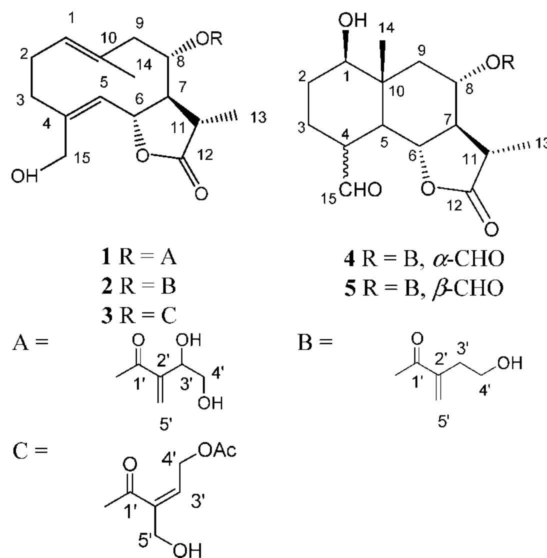
The aerial parts of *Centaurea pullata* afforded, in addition to the previously isolated sesquiterpene lactones, 11β,13-dihydrocnicin and 11β,13-dihydro-19-desoxycnicin, three minor sesquiterpene lactones, namely, a new germacranolide, 8α-*O*-(4-acetoxy-5-hydroxyangeloyl)-11β,13-dihydrocnicin, and two new eudesmanolides, 8α-*O*-(4-hydroxy-2-methylenebutanoyloxy)-11β,13-dihydrosonchucarpolide and 8α-*O*-(4-hydroxy-2-methylenebutanoyloxy)-11β,13-dihydro-4-*epi*-sonchucarpolide. The in vitro antimicrobial activity of all isolated sesquiterpene lactones was tested against six bacteria and eight fungal species, using a microdilution method. All compounds tested showed greater antibacterial and antifungal activities than the positive controls used.

The genus *Centaurea* L. (Asteraceae), with nearly 300 species,[1] traditionally has been considered problematic taxonomically. However, more recent analyses of the genus and particularly of the subtribe Centaureinae have enabled the limits of *Centaurea* to be established with greater confidence.[2–5] *Centaurea pullata* L., known under the common name "Achbet Ennegar",[6] is a biennial plant belonging to the section Melanoloma,[5,7] with large terminal pink flowers, very variable in height, distributed from Spain to France and North Africa.[8] In Algeria, it is a common edible herb used in the preparation with other plants of a local traditional dish called "El Hammama".

In continuation of our work on the chemical constituents of *Centaurea* spp.[9–15] we have investigated *C. pullata*, whose major sesquiterpene lactones, 11β,13-dihydrocnicin (**1**) and 11β,13-dihydro-19-desoxycnicin (**2**), have been described already.[16] The crude extracts of the aerial parts of *C. pullata* afforded sesquiterpene lactones **1** and **2**,[16] along with three new minor compounds, namely, 8α-*O*-(4-acetoxy-5-hydroxyangeloyl)-11β,13-dihydrocnicin (**3**), 8α-*O*-(4-hydroxy-2-methylenebutanoyloxy)-11β,13-dihydrosonchucarpolide (**4**), and 8α-*O*-(4-hydroxy-2-methylenebutanoyloxy)-11β,13-dihydro-4-*epi*-sonchucarpolide (**5**). The evaluation of the antibacterial and antifungal activities of all compounds isolated herein is also reported in this paper.



Compound **3** showed in its mass spectrum a pseudomolecular ion $[M + H]^+$ at *m/z* 439.1967, compatible with the molecular formula $C_{22}H_{30}O_9$ (calcd for 438.1881). The IR spectrum afforded absorption bands at 3600–3300 (OH), 1764 (*C*=O, γ-lactone, ester), and 1712 cm^{-1} (*C*=O, acetate). The ^{1}H NMR spectrum (Table 1) together with the COSY, HSQC, and HMBC data revealed the presence of a germacranolide with an α-methyl-γ-lactone moiety. Two olefinic protons at δ_H 4.96 (dd, J = 11.4, 4.5, H-1) and 4.71 (d, J = 9.8, H-5), along with the presence of a tertiary methyl group at δ_H 1.46, vicinal to the double bond (H-14), and finally four olefinic carbons, two methines (at δ_C 129.3, C-1 and 128.7, C-5), and two quaternary carbons (at δ_C 142.9, C-4 and 132.6, C-10) supported this assumption. The resonance of a secondary methyl group at δ_H 1.32 (d, J = 7.0 Hz) along with the chemical shift of the carbonyl group of the lactone ring (at δ_C 177.8) suggested the presence of an α-methyl-γ-lactone moiety instead of an α-methylene-γ-lactone group, which is usually present in sesquiterpene lactones isolated from other *Centaurea* spp.[10–16,18] This finding was further supported by a COSY experiment where the spin system H-13/H-11/H-7 was observed. As a result of the absence of a 11,13 exocyclic double bond, H-7 appeared shielded at δ_H 2.17 (m). A COSY experiment on **3** displayed the following spin systems: H-1/H-2a,b/H-3a,b (spin system A), H-5/H-6/H-7/H-8/H-9a,b (spin system B), H-7/H-11/H-13 (spin system C), and H-15a/H-15b (spin system D). Key HMBC correlations of C-10 with H-1, H-9, and H-14, as well as C-4 with H-3, H-5, H-6, and H-15 completed the sesquiterpene skeleton as shown. Furthermore, a diagnostic cross-peak between the acetyl group at δ_H 2.08 and H-4′a,b and the carbonyl resonance at δ_C 170.6 confirmed the acetylation site. NOE signals between CH$_3$-14/H-8/H-6 indicated that they have a common orientation in the molecule, while NOE interactions between H-7/CH$_3$-13 indicated them to be oppositely oriented (Figure 2). Accordingly, compound **3** was assigned as 8α-*O*-(4-acetoxy-5-hydroxyangeloyl)-11β,13-dihydrocnicin.

Compound **4** showed in its mass spectrum a pseudomolecular ion $[M + 1]^+$ at *m/z* 381.1908, indicating a molecular weight corresponding to a molecular formula of $C_{20}H_{28}O_7$. The IR spectrum afforded absorption bands typical of hydroxyl (3600–3300 cm^{-1}) and carbonyl groups [1776 cm^{-1} (*C*=O, γ-lactone, ester), 1717 cm^{-1} (*C*=O, aldehyde)]. The ^{1}H and ^{13}C NMR spectra (Table 1) of compound **4** exhibited typical signals that suggested a eudesmane skeleton.[18] The ^{13}C NMR spectrum displayed 20 carbons, which were assigned by HSQC, HMBC, and DEPT 135° experiments to

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **3**–**5**[a] (400 MHz, $CDCl_3$)

	3			4			5		
position	δ_H (J in Hz)	δ_C, mult.	HMBC	δ_H (J in Hz)	δ_C, mult.	HMBC	δ_H (J in Hz)	δ_C, mult.	HMBC
1	4.96, dd (11.4, 4.5)	129.3	10, 14	3.42, dd (11.0, 4.3)	76.9		3.57, dd (11.3, 4.7)	77.7	
2a	2.24–2.12[b]	26.0	1	1.86 *m*	28.1		1.72	27.6	
2b				1.5[b]			1.60		
3a	2.53[b]	34.5	1, 2, 4,	1.72[b]	24.1	5, 10	1.38[b]	23.6	5, 10
3b	1.92, td (12.1, 6.2)		15	1.54[b]			1.24[b]		
4		142.9		2.49[b]	47.6		2.75, t (5.1)	45.0	
5	4.71, d (9.8)	128.7	3, 7, 15	1.76, t (11.3)	47.7		1.89[b]	48.4	
6	5.0, t (11.7)	75.9	4, 8	3.99, t (11.0)	78.1		4.53, dd (11.7, 11.0)	75.7	
7	2.17[b]	57.9	6, 8, 9, 11, 13	1.94, dd (11.5, 10.9)	56.3	6, 8	1.91[b]	56.9	6, 8
8	5.28, td (9.4, 4.3)	73.6	1′	5.17, td (11.0, 4.7)	70.3		5.21, td (10.9, 4.3)	70.1	
9a	2.55–2.41[b]	49.0	8, 10	2.44, dd (12.3, 4.3)	42.3	2, 7, 8, 10,	2.36, dd (12.9, 4.1)	43.5	7, 8, 10
9b				1.34, dd (12.5, 1.7)			1.35		
10		132.6			40.5			40.8	
11	2.57[b]	39.8	7, 12, 13	2.53[b]	40.3		2.55[b]	40.1	
12		177.8			176.3			177.4	
13	1.32, d (7.0)	16.9	7, 11, 12	1.19, d (7.0)	13.8	7, 11, 12	1.19, d (7.0)	13.8	7, 11, 12
14	1.46, s	16.5	1, 9, 10	1.03, s	12.7	1, 5, 9, 10	0.90, s	13.9	1, 5, 9, 10
15a	4.27, d (14.5)	61.3	3, 4, 5	9.58, d (3.9)	202.0		9.93, s	201.7	4
15b	4.05, d (14.1)								
1′		164.5			166.1			165.9	
2′		131.5			136.6			137.0	
3′a, 3′b	6.36, dd (5.4, 5.1)	140.4		2.56, t (6.2)	35.1	1′, 2′, 4′, 5′	2.56, t (6.1)	35.0	1′, 2′, 4′, 5′
4′a, 4’b	5.06, d (5.4)	62.7	2′, 3′, –C=O (OAc)	3.76, t (6.2)	61.3	2′	3.76, t (6.3)	61.4	2′
5′a	4.43, t (13.7)	62.4	2′, 3′	6.23, brs	128.3	1′, 2′, 3′	6.23, brs	127.3	1′, 2′, 3′
5′b				5.73, brs			5.72, brs		
$\underline{CH_3}$C=O	2.08, s	20.7	–C=O (OAc)						
$CH_3\underline{C}$=O		170.6							

[a] Carbon resonances were assigned by HSQC and HMBC spectra. [b] Signal pattern unclear due to overlapping.

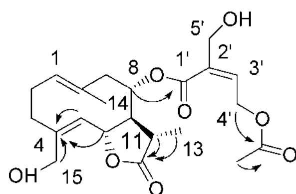

Figure 1. Key HMBC correlations for compound **3**.

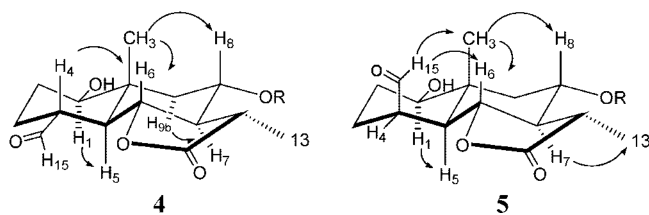

Figure 2. Key NOESY correlations for compounds **4** and **5**.

the resonances of four quaternary carbons, seven methines, six methylenes (one of them olefinic), and two methyls.

The presence of a secondary methyl group at δ_H 1.19 (d, J = 7.0 Hz) suggested the presence of an α-methyl-γ-lactone moiety in **4**. In the COSY spectrum, the H-7 resonance appeared coupled with signals of two vicinal protons (H-6 at δ_H 3.99 and H-8 at δ_H 5.17) attached to oxygenated carbons at δ_C 78.1 (C-6) and 70.3 (C-8). From the downfield shift of C-6, it was evident that lactonization occurred at this position. Furthermore, H-8 correlated with two methylene protons, which were assigned to H-9a and H-9b (at δ_H 2.44, dd, J = 12.3, 4.3 Hz and 1.34, dd, J = 12.5, 11.7 Hz, respectively), while H-6 coupled with one methine proton, which was assigned to H-5 (at δ_H 1.76, t, J = 11.3 Hz), and corresponded to an aliphatic carbon at δ_H 47.7 (C-5). The H-5 signal correlated to a methine proton at δ_H 2.49, which in turn coupled with two methylene protons (H-3a and H-3b), as well as with a low-field signal at δ_H 9.58 (d, 3.9 Hz) belonging to an aldehyde group (H-15). Further couplings between the geminal protons H-3/H-2 and H-2a,b/H-1 completed a decalin ring. The chemical shift of C-1 at δ_C 76.9 indicated the presence of an oxygen group at this position. In the COSY spectrum, a second spin system was observed belonging to a side-chain unit. The identity of a 4-hydroxy-2-methylenebutanoyloxy ester side chain was deduced from the chemical shifts of its protons. The H-2′a and H-2′b olefinic protons appeared coupled (J^4) with the H-3′a and H-3′b methylene protons centered at δ_H 2.56. The latter displayed couplings with two more methylene protons attached to an oxygen-bearing carbon at δ_C 61.3 (C-4′).

A NOESY experiment confirmed the presence of a *trans*-eudesmanolide ring in **4**. NOE signals between CH_3-14/H-8/H-6 and H-6/H-4 indicated that they have a common orientation in this molecule, while NOE interactions between H-1/H-2a, H-5, H-9b and H-7/H-9b, as well as H-7/CH_3-13, indicated them to be oriented in an opposite manner (Figure 2). Therefore, the structure of **4** was assigned as 8α-*O*-(4-hydroxy-2-methylenebutanoyloxy)-11β,13-dihydrosonchucarpolide.

The mass spectrum of compound **5** showed a pseudomolecular ion at *m/z* 403.1730 [M + Na]$^+$, indicating a molecular weight corresponding to a molecular formula of $C_{20}H_{28}O_7$. Its ^{1}H and ^{13}C NMR spectra showed typical signals that suggested a eudesmane framework with features common to those of compound **4**. NMR analysis (Table 1) showed that **5** has a eudesmanolide nucleus with an 8α-acyl side chain with identical functionalities and the same relative configuration as compound **4** except at C-4. The following differences were observed: H-5 was deshielded at δ_H 1.89 (vs δ_H 1.76 in compound **4**), while H-4 was deshielded at δ_H 2.75 as a triplet (J = 5.1 Hz), suggesting an equatorial orientation of this proton. The aldehyde proton, H-15, was determined to be axial and appeared as a singlet deshielded at δ_H 9.93 (vs in **4**, where it appeared as a doublet at δ_H 9.58). The above data suggested a change in the stereochemistry of C-4, which was further confirmed by NOESY experiment, where NOE cross-peaks between H-15 and H-6, CH_3-14 were observed. The conformation of the decalin skeleton was also confirmed by the NOESY spectrum (Figure 2).

Table 2. Minimum Inhibitory and Bactericidal Concentrations (MICs/MBCs, μg/mL) of Compounds **1**–**5**[a]

bacteria	1	2	3	4	5	streptomycin
M. flavus	0.2	0.2	0.2	0.2	0.2	0.5
	0.3	0.3	0.3	0.3	0.3	1.0
B. subtilis	0.2	0.2	0.2	0.2	0.2	0.5
	0.2	0.2	0.3	0.3	0.3	0.5
Ps. tolaasii	0.2	0.3	0.2	0.4	0.2	1.0
	0.3	0.3	0.3	0.5	0.3	2.0
S. epidermidis	0.4	0.4	0.4	0.4	0.4	1.0
	0.4	0.4	0.5	0.5	0.5	1.0
S. enteritidis	0.3	0.3	0.3	0.4	0.4	1.0
	0.3	0.3	0.3	0.4	0.4	1.0
E. coli	0.2	0.2	0.3	0.4	0.4	0.5
	0.3	0.2	0.3	0.4	0.4	1.0

[a] For a list of organisms and protocols used, see the Experimental Section.

Table 3. Minimum Inhibitory and Fungicidal Concentrations (MICs/MFCs μg/mL) of Compounds **1**–**5**[a]

fungal species	1	2	3	4	5	miconazole
A. flavus	0.1	0.1	0.2	0.2	0.2	0.5
	0.2	0.2	0.2	0.3	0.3	2.0
A. niger	0.2	0.2	0.2	0.2	0.2	1.5
	0.3	0.3	0.3	0.3	0.3	4.0
A .ochraceus	0.1	0.1	0.1	0.2	0.2	1.5
	0.2	0.2	0.2	0.3	0.3	4.0
P. funiculosum	0.2	0.2	0.2	0.3	0.3	2.0
	0.3	0.3	0.3	0.4	0.4	5.0
P. ochrachloron	0.1	0.1	0.1	0.3	0.3	2.0
	0.2	0.2	0.2	0.4	0.4	5.0
T. viride	0.2	0.2	0.2	0.3	0.4	2.0
	0.3	0.3	0.3	0.4	0.5	2.0
F. tricinctum	0.1	0.2	0.2	0.2	0.2	0.2
	0.2	0.3	0.3	0.3	0.3	1.0
A. alternata	0.1	0.2	0.2	0.2	0.2	0.2
	0.2	0.3	0.3	0.3	0.3	1.0

[a] For a list of organisms and protocols used, see the Experimental Section.

The structure of compound **5** was therefore established as 8α-*O*-(4-hydroxy-2-methylenebutanoyloxy)-11β,13-dihydro-4-*epi*-sonchucarpolide.

We also report here the revision of ^{13}C NMR spectroscopic assignments for compound **2**[16] as follows: δ 143.0 (C-4), 58.3 (C-7), 132.7 (C-10), and 40.1 (C-11).

From Table 2 and Table 3, it can be seen that all investigated compounds exhibited more potent antimicrobial activity than the positive controls used. The higher activity of the germacranolides can be related to their degree of lipophilicity required for such compounds to pass through the microbial cell wall. This supports the hypothesis of an inverse relationship between polarity and antimicrobial activity for sesquiterpene lactones, in general.[18,19] The present results support this hypothesis since, in terms of their retention times on a RP-18 column, eudesmanolides are more polar than germacranolides. It seems that although the presence of an α-methyl-γ-lactone moiety instead of an α-methylene-γ-lactone results in a slight decrease of activity, the final antimicrobial potential is not dramatically affected, among these sesquiterpene lactones.

Experimental Section

General Experimental Procedures. Polarimeter: Perkin-Elmer 341. FT-IR spectrometer: Perkin-Elmer Paragon 500. NMR: The 1D and 2D spectra (400 MHz) were recorded using Bruker DRX 400 and Bruker AC 200 spectrometers. Chemical shifts are reported in δ (ppm) values. COSY, HMQC, HSQC, HMBC, and NOESY (mixing time 950 ms) were performed using standard Bruker microprograms. MS: recorded on a PerSeptive Biosystems Voyager-DE time-of-flight mass spectrometer, ESI-Micromass Quattro LC triple quadrupole mass spectrometer (University of Notre Dame, Department of Chemistry and Biochemistry, South Bend, IN), and on a Thermo LTQ Orbitrap (FT-MS^n) (University of Florence, Italy). Vacuum liquid chromatography (VLC): silica gel (Merck; 43–63 μm). Column chromatography: silica gel (SDS; 40–63 μm), gradient elution with the solvent mixtures indicated in each case. HPLC support: preparative HPLC was performed using a C_{18} 25 cm × 10 mm Kromasil column using a JASCO HPLC system equipped with a RI detector. Fractionation was always monitored by TLC silica gel 60 F-254, Merck, art. 5554, with visualization under UV (254 and 365 nm) and spraying with anisaldehyde–sulfuric acid reagent on silica gel.

Plant Material. Aerial parts of *Centaurea pullata* L. were collected from Chréa Mountain in Blida (North Algeria) in April 2006 and authenticated by Mr. Beloued abd El Kader (Agronomic National Institute, Algiers). A voucher specimen has been deposited in the Herbarium of the Department of Biology, Environmental Laboratory, University of Annaba, under the code Ann-BV 2006/0010.

Extraction and Isolation. The fresh plant material (1.2 kg) was ground finely and extracted at room temperature with cyclohexane–Et_2O–MeOH (1:1:1) and MeOH–H_2O (5:1), successively. The first extract was washed with brine, with the aqueous layer re-extracted with EtOAc, and the organic layer dried with Na_2SO_4 and concentrated under reduced pressure. The latter residue (12.7 g) was prefractionated by VLC on silica gel, using cyclohexane–EtOAc–Me_2CO mixtures of increasing polarity as eluents to give 10 fractions (A_1–A_{10}). Column chromatography over silica gel of fraction A_6 (386.6 mg; eluted with EtOAc–Me_2CO, 90:10) afforded 15 fractions (B_1–B_{15}). Further purification of fraction B_{15} (225.9 mg; eluted with 100% MeOH) over silica gel using mixtures of CH_2Cl_2–MeOH of increasing polarity yielded fractions C_1–C_{10}. Fraction C_3 (88.9 mg; eluted with CH_2Cl_2–MeOH, 90:10) was subjected to RP-HPLC (MeOH–H_2O, 4:3) and afforded compounds **2** (11.4 mg; t_R = 33.5 min) and **3** (4.7 mg; t_R = 25.5 min). Further purification of fraction C_4 (33.4 mg; eluted with CH_2Cl_2–MeOH, 90:10) by RP-HPLC (MeOH–H_2O, 1:1) yielded compounds **1** (3.4 mg; t_R = 32.4 min), **4** (1.7 mg; t_R = 12.4 min), and **5** (2.7 mg; t_R = 14.5 min).

8α-*O*-(4-Acetoxy-5-hydroxyangeloyl)-11β,13-dihydrocnicin (3): oil; $[\alpha]^{20}_D$ +30.6 (0.12, MeOH); IR (CaF_2) ν_{max} 3600–3300 (OH), 1764 (*C*=O, γ-lactone, ester), 1712 (*C*=O, acetate) cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 439.1967 [M + 1]$^+$ (calcd for $C_{22}H_{30}O_9$ 438.1881).

8α-*O*-(4-Hydroxy-2-methylenebutanoyloxy)-11β,13-dihydrosonchucarpolide (4): oil; $[\alpha]^{20}_D$ +5.3 (*c* 0.17, MeOH); IR (CaF_2) ν_{max} 3600–3300 (OH), 1776 (*C*=O, γ-lactone, ester), 1717 (*C*=O, aldehyde) cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 381.1908 [M + 1]$^+$ (calcd for $C_{20}H_{28}O_7$ 380.1827).

8α-*O*-(4-Hydroxy-2-methylenebutanoyloxy)-11β,13-dihydro-4-*epi*-sonchucarpolide (5): oil; $[\alpha]^{20}_D$ +15.0 (*c* 0.12, MeOH); IR (CaF_2) ν_{max} 3600–3300 (OH), 1772, (*C*=O, γ-lactone, ester), 1721 (*C*=O, aldehyde) cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 403.1730 [M + Na]$^+$ (calcd for $C_{20}H_{28}O_7$ 380.1827).

Bioassays. The following Gram-negative bacteria were used: *Pseudomonas tolaasii* (isolated from *Agaricus bisporus*) and *Escherichia coli* (ATCC 35210). The following Gram-positive bacteria were used: *Bacillus subtilis* (ATCC 10907), *Micrococcus flavus* (ATCC 10240), and *Staphylococcus epidermidis* (ATCC 12228). For the antifungal bioassays, eight fungi were used: *Aspergillus niger* (ATCC 6275), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus flavus* (ATCC 9643), *Penicillium ochrochloron* (ATCC 9112), *Penicillium funiculosum* (ATCC 36839), *Trichoderma viride* (IAM 5061), *Fusarium tricinctum* (CBS 514478), and *Alternaria alternata* (DSM 2006). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia.

The micromycetes were maintained on malt agar and the cultures stored at 4 °C and subcultured once a month.[21] In order to investigate the antimicrobial activity of the isolated compounds, a modified microdilution technique was used.[22,23] Bacterial species were cultured overnight at 37 °C in Luria broth medium. The spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). 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 Müller-Hinton agar for

bacteria and solid malt agar for fungi to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. The compounds investigated were dissolved in DMSO (0.1–1 μg/mL) and added in broth medium (bacteria)/broth malt medium (fungi) with inocula. The microplates were incubated for 48 h at 37 °C or 72 h at 28 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs.

The minimum bactericidal concentrations (MBCs) and minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 μL sample into microtiter plates containing 100 μL of broth per well and further incubation for 48 h at 37 °C or 72 h at 28 °C. The lowest concentration with no visible growth was defined as MBC/MFC, respectively, indicating 99.5% killing of the original inoculum. DMSO was used as a negative control, while streptomycin and the commercial fungicide miconazole were used as positive controls (0.1–5 μL/mL).

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Supporting Information Available: Figures S1–S11. 1D (^{1}H NMR) and 2D NMR (COSY, HSQC, HMBC, NOESY) spectra for compounds **3**–**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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