

Heimiomyces A–C and Calamenens from the African Basidiomycete *Heimiomyces* sp.Tian Cheng,¹ Clara Chepkirui,¹ Cony Decock, Josphat C. Matasyoh, and Marc Stadler*Cite This: <https://dx.doi.org/10.1021/acs.jnatprod.0c00486>

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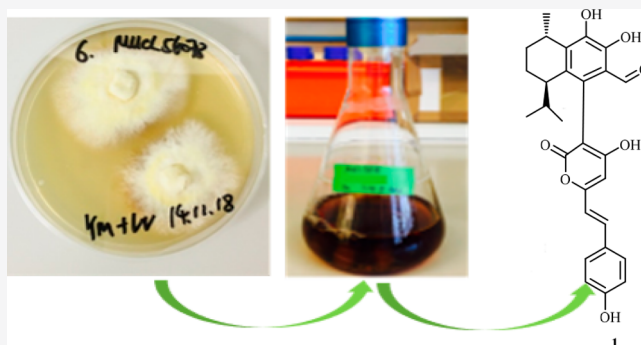


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ABSTRACT: Three previously undescribed compounds named heimiomycin A–C (1–3), featuring a unique scaffold with calamenene connected to a hydroxystyryl-pyrone moiety, along with the new calamenene derivatives 4 and 5 and phenanthridine derivative (6) were obtained from a culture of a *Heimiomyces* sp. This is the first report of the occurrence of calamenene-type terpenoids in fungi. Compound 3 exhibited antimicrobial activity against Gram-positive bacteria and *Mucor hiemalis*. Compounds 1 and 3 displayed moderate cytotoxicity against KB 3.1 and L929 cell lines, respectively.



Tropical rainforests are one of the richest and most diverse ecosystems,¹ but the vast majority of its biodiversity and natural chemical library remains unexploited. Basidiomycetes, which are known to produce diverse classes of bioactive molecules, thrive in this ecosystem.² The constant chemical innovation among tropical species may be driven by the active and constant evolutionary race.³ Even though the tropical basidiomycetes hold great potential as a source for novel secondary metabolites, the vast majority have not been described nor has their chemistry.³ In the last years, we have been studying different basidiomycetes originating from Kenya with the aim of documenting both their diversity and their rich chemistry. This study has resulted in the discovery of novel structurally diverse bioactive secondary metabolites. These include the biofilm inhibitors from *Microporus* sp.,⁴ antimicrobial metabolites from two *Sanghuangporus* strains^{5,6} and antibacterial agents from both liquid cultures and fruiting bodies of *Skeletocutis* sp.^{7,8}

In this study, the strain *Heimiomyces* sp. (MUCL 56078) that was collected from Mount Elgon National Reserve in Kenya was studied for the production of secondary metabolites. The genus *Heimiomyces* was first described in 1962 and is related to *Xeromphalina* (order Agaricales).⁹ Today it contains seven species and belongs to the family Mycenaceae,¹⁰ even though the evaluation of its genetic boundaries still remains to be clarified.^{11,12} In this paper we report the isolation and structure elucidation of the novel heimiomyces A–C, featuring a calamenene moiety connected to a hydroxystyryl-pyrone moiety. These compounds also represent the first secondary metabolites reported from this genus.

RESULTS AND DISCUSSION

The strain MUCL56078 was identified as *Heimiomyces* sp. by comparison of morphological characteristics and sequencing of the 5.8S/ITS nrDNA. An initial BLAST search of ITS nucleotide sequences from the NCBI database (<http://www.ncbi.nlm.nih.gov/>) gave MF100953.1 (*Heimiomyces tenuipes*) as the closest hit with query cover 98%, and the next closest hit was KM975407.1 (*Heimiomyces neovelutipes*) with 97%. The comparison of the 20 closest ITS BLAST results showed the relationship between MUCL56078, *Heimiomyces* species, and *Xeromphalina* species (Figure S46). Further taxonomic studies including comparisons with type specimens of related species are ongoing to characterize this strain to the species level.

The strain (MUCL56078) was selected for large-scale fermentation after its extract from a small-scale fermentation in YMG media presented activity against *Bacillus subtilis* (at a concentration of 18.8 µg/mL compared to the positive control ciprofloxacin at a concentration of 3.1 µg/mL). The compounds were purified by extensive preparative high-performance liquid chromatography (HPLC), resulting in six previously undescribed compounds (Figure 1).

Compound 1 was isolated as a yellow solid from the mycelial extracts. Its molecular formula C₂₈H₂₈O₇ was assigned on the basis of HRESIMS data, requiring 15 degrees of unsaturation.

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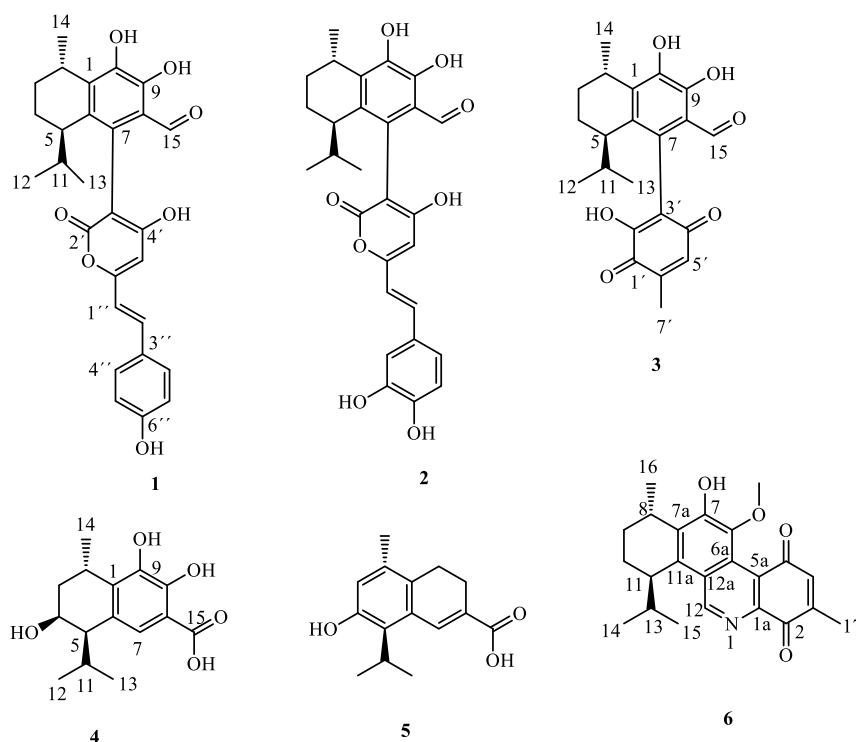


Figure 1. Chemistry structures of compounds 1–6.

Three methyl group doublets at δ 0.73 (H_3 -13), 0.77 (H_3 -12), and 1.21 (H_3 -14), two singlets at δ 6.43 (H -4') and 9.81 (H -15), two olefinic doublets, and four aromatic doublet proton signals were recorded in the 1H NMR spectrum (data in Table 1). The ^{13}C NMR and HSQC spectra attributed 28 carbon resonance signals to three methyls, two methylenes, 11 methines, and 12 nonprotonated carbons.

Analysis of the COSY data established correlations network of H -2 to H_3 -14/ H_2 -3, H_2 -4 to H_2 -3/ H -5, and H -11 to H -5/ H_3 -12/ H_3 -13, giving the first spin system in the molecule (Figure 2). HMBC correlations of H_3 -14 to C -1/ C -2/ C -3, H_3 -12/- H_3 -13 to C -5/ C -11, H -5 to C -3/ C -4/ C -6/ C -7, and H -2 to C -4/ C -1/ C -6/ C -10 were recorded. In addition, the aldehyde group proton H -15 (δ 9.81) showed HMBC correlations to C -7/ C -8/ C -9, while the OH -9 showed correlations to C -8/ C -9/ C -10. A hydroxy group was assigned to C -10 based on the chemical shift (δ 142.8). These COSY and HMBC correlations unambiguously established the 1-methyl-4-(propan-2-yl)-1,2,3,4-tetrahydronaphthalene part of the molecule.

The hydroxystyryl moiety (4'-hydroxy-6'-[(*E*)-2''-(6''-hydroxyphenyl)ethenyl]-pyran-2-one) was deduced from the HMBC correlations of H -5'' to C -3''/ C -6''/ C -7'', H -4'' to C -2''/ C -5''/ C -6''/ C -8'', H -1'' to C -2''/ C -3''/ C -5'', and H -5' to C -3''/ C -4''/ C -6''/ C -1''. This was further supported by the COSY correlations of H -1'' to H -2'' and H -4''/ H -8'' to H -5''/ H -7''. Carboxyl and hydroxy groups were attached to C -2' (δ 159.1) and C -4' (δ 166.4), respectively, based on their chemical shifts. Thus far, 14 degrees of unsaturation had been accounted for, and the ring closure to C -6' to form the pyrone was proposed. The downfield shift of C -6' (δ 159.7) further supported these assignments. The C -1''– C -2'' olefinic bond was assigned an *E*-configuration because of the large coupling constant (J = 15.9 Hz) recorded. The two parts of the molecule were connected via C -7, because this was the only open position. The structure of 1 was concluded to be 9,10-dihydroxy-7-(4'-hydroxy-6'-

((*E*)-6''-hydroxystyryl)-2'-oxopyran-3'-yl)-8-isopropyl-5-methyl-2,3,4,5-tetrahydronaphthalene-8-carbaldehyde.

Compound 2, which was obtained as a yellow solid from the mycelial extracts, was assigned the molecular formula $C_{28}H_{28}O_8$ from the HRESIMS data. The 1D and 2D NMR data of 2 revealed a similar structure to 1 with the exception of an additional hydroxy group at C -5'' (δ 146.3). This was confirmed by the HMBC correlations of H -4'' to C -2''/ C -5''/ C -6''/ C -8'', H -7'' to C -3''/ C -5''/ C -6'', and H -8'' to C -2''/ C -4''/ C -6''. Also, the presence of a doublet of doublets signal for H -8'' (δ 7.07) and a doublet signal for H -4'' (δ 7.19, J = 2.15 Hz) in the 1H NMR spectrum supported the 1,3,4-trisubstituted benzene moiety.

Compound 3 was obtained as an orange solid from the supernatant extracts. The molecular formula of 3 was assigned as $C_{22}H_{24}O_6$ on the basis of the HRESIMS and NMR data (Table 2). The NMR spectroscopic data of this compound displayed similarity to the 1-methyl-4-(propan-2-yl)-1,2,3,4-tetrahydronaphthalene moiety in 1. However, the hydroxystyryl moiety was missing, and instead a benzoquinone moiety was elucidated for 3. This was deduced based on the HMBC cross-peaks between H_3 -7' and C -4'/ C -5'/ C -6' and H -6' and C -2'/ C -1'/ C -4'/ C -7'. The two moieties were connected via the available carbons C -3' and C -7. The structure of 3 was therefore elucidated as 9,10-dihydroxy-7-(3'-hydroxy-5'-methyl-1,4-dioxocyclohexa-2,5-dien-2-yl)-5-isopropyl-2-methyl-2,3,4,5-tetrahydronaphthalene-8-carbaldehyde.

Compound 4, which was obtained as a yellow solid, was assigned the molecular formula $C_{15}H_{20}O_5$ by the positive HRESIMS data. The NMR data for this compound showed similarity to the naphthalene moiety of the above-described compounds with the difference being the hydroxy methine at C -4 (δ 65.1) and the absence of the aldehyde group, which was replaced by the carboxylic acid moiety at C -15 (δ 172.5). Two methyl singlets at δ 1.01 (H_3 -12), 0.41 (H_3 -13) and δ 1.47

Table 1. ^1H (700 MHz) and ^{13}C (175 MHz) NMR Data for 1–3 in Acetone- d_6

no.	1		2		3	
	δ_{C}	δ_{H} [J (Hz)]	δ_{C}	δ_{H} [J (Hz)]	δ_{C}	δ_{H} [J (Hz)]
1	138.4, C		139.1, C		139.4, C	
2	27.9, CH	3.40, m	28.6, CH	3.39, m	29.1, CH	3.40, m
3	25.7, CH_2	1.48, m	26.5, CH_2	1.47, m	26.9, CH_2	1.47, m
		2.08, m		2.08, m		2.06, m
4	19.2, CH_2	1.86, m	19.9, CH_2	1.85, m	20.3, CH_2	1.83, m
						189, m
5	39.6, CH	2.68, m	40.4, CH	2.67, m	40.6, CH	2.67, m
6	132.8, C		133.5, C		133.4, C	
7	126.5, C^a		126.3, C		125.5, C	
8	117.4, C		118.2, C		118.2, C	
9	147.7, C^a		147.9, C		148.5, C^a	
10	142.8, C^a		143.3, C		143.9, C	
11	33.4, CH	1.81, m	34.2, CH	1.81, m	34.9, CH	1.60, nonet (6.43)
12	21.4, CH_3	0.77, d (7.10)	22.3, CH_3	0.76, d (6.88)	22.7, CH_3	0.71, d (6.43)
13	19.9, CH_3	0.73, d (7.10)	20.7, CH_3	0.71, d (6.88)	20.8, CH_3	0.66, d (6.43)
14	21.3, CH_3	1.21, d (6.88)	22.1, CH_3	1.20, d (6.88)	22.7, CH_3	1.20, s (6.24)
15	198.4, CH	9.81, s	199.2, CH		199.1, CH	9.64, s
1'					189.8, C	
2'	159.1, C		160.6, C		118.4, C	
3'	99.9, C		100.9, C		154.9, C	
4'	166.4, C^a		166.9, C		184.6, C	
5'	100.5, CH	6.43, s	101.1, CH	6.39, s	143.1, C	
6'	159.7, C		160.6, C		135.8, CH	6.70, m
7'					15.6, CH_3	2.11, d (1.29)
1''	116.6, CH	6.79, d (15.92)	117.4, CH	6.74, d (15.92)		
2''	135.1, CH	7.40, d (15.92)	136.3, CH	7.33, d (15.92)		
3''	127.5, C		128.9, C			
4''	129.5, CH	7.57, d (8.48)	114.8, CH	7.19, d (2.15)		
5''	116.0, CH	6.90, d (8.48)	146.3, C			
6''	159.1, C		147.9, C			
7''	116.0, CH	6.90, d (8.48)	116.4, CH	7.53, d (8.17)		
8''	129.5, CH	7.57, d (8.48)	121.8, CH	7.07, dd (2.15, 8.17)		
10	OH	11.84, s			OH	11.72, s

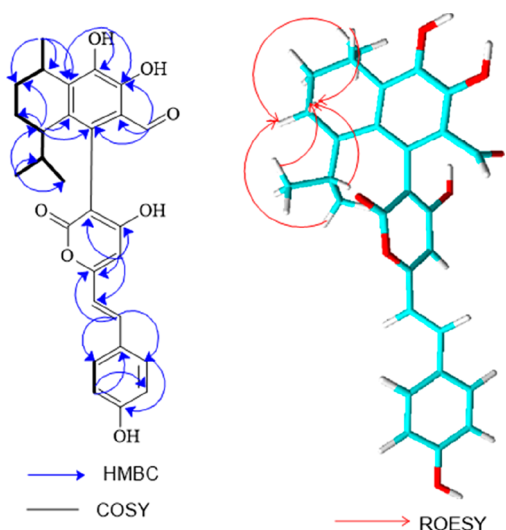
^aChemical shifts read from HMBC.

Figure 2. HMBC, COSY, and ROESY correlations of compound 1.

(H_3 -14), and one aromatic singlet at δ 7.99 (H-7) were recorded in the ^1H NMR. COSY correlations of H-2 to H_3 -14/ H_2 -3, H-4 to H_2 -3/H-5, and H-11 to H-5/ H_3 -12/ H_3 -13 were

recorded. The cross-peaks in the HMBC spectrum between H_3 -12/13 and C-5/C-11, H_3 -14 and C-1/C-2/C-3, H-2 and C-14/C-3/C-4/C-6/C-1/C-10, H-5 and C-4/C-6/C-11/C-12/C-13, and H-7 and C-1/C-5/C-8/C-9/C-15 were observed. Hydroxy groups were assigned to C-9 (δ 147.4) and C-10 (δ 142.4) based on their chemical shifts and the molecular formula. This led to the establishment of the structure of 4 as 4,9,10-trihydroxy-5-isopropyl-2-methyl-2,3,4,5-tetrahydronaphthalene carboxylic acid.

Compound 5, with the molecular formula $\text{C}_{15}\text{H}_{18}\text{O}_3$ deduced from the HRESIMS data, was isolated as a yellow solid from the supernatant extracts. The 1D and 2D NMR data of 5 suggested a similar scaffold to that in 4 with the difference being the indices of hydrogen deficiency in the cyclohexane moiety. In the ^{13}C spectrum, the C-9 and C-10 nonprotonated carbons were also missing and two methylene carbons at δ 22.8 (C-9) and 25.48 (C-10) were observed. The H_2 -10 showed HMBC correlations to C-1/C-2/C-6/C-8/C-9.

Compound 6, with the molecular formula $\text{C}_{23}\text{H}_{25}\text{NO}_4$, which was assigned on the basis of the HRESIMS data, requiring 12 indices of hydrogen deficiency, was isolated from the mycelial extracts as a yellow solid. The ^{13}C and HSQC NMR spectral data revealed the presence of 23 carbons in the molecule, which were further assigned as 5 methyls, 2

Table 2. ^1H (700 MHz) and ^{13}C (175 MHz) NMR Data for **4** in DMSO- d_6 , **5** in Methanol- d_4 , and **6** in Acetone- d_6

no.	4		5		6	
	δ_{C}	δ_{H} [J (Hz)]	δ_{C}	δ_{H} [J (Hz)]	δ_{C}	δ_{H} [J (Hz)]
1	135.4, C		128.4, C			
1a					141.5, C	
2	28.4, CH	3.20, m	134.3, C		183.7, C	
3	34.5, CH ₂	α : 1.48, m β : 1.92, m	120.2, CH	6.58, s	145.3, C	
4	65.1, CH	4.06, dt (4.38, 12.05)	155.2, C		134.9, CH	7.08, q (1.51)
5	49.4, CH	2.60, m	131.3, C		187.7, C	
5a					129.3, C	
6a					124.3, C	
6	128.4, C		132.1, C		138.6, C	
7	120.2, CH	6.99, s	136.2, CH	7.99, s	148.1, C	
7a					141.8, C	
8	109.8, C		129.9, C		29.0, CH	3.41, m
9	147.4, C		22.8, CH ₂	2.43, dt (1.29, 8.39)	25.9, CH ₂	1.51, m
						2.04, m
10	142.4, C		25.5, CH ₂	2.66, t (8.60)	19.1, CH ₂	2.02, m
						1.61, m
11	25.5, CH	2.22, m	28.3, CH	3.50, m	41.4, CH	3.00, m
11a					141.9, C	
12a					123.8, C	
12	24.9, CH ₃	1.01, d (6.88)	21.8, CH ₃	1.35, d (7.10)	149.9, CH	9.46, s
13	21.5, CH ₃	0.41, d (6.88)	21.8, CH ₃	1.35, d (7.10)	32.7, CH	1.46, m
14	21.9, CH ₃	1.17, d (6.88)	19.6, CH ₃	2.18, br s	21.5, CH ₃	0.31, d (6.67)
15	172.5, C		171.3, C		18.6, CH ₃	0.38, d (6.67)
16					22.8, CH ₃	1.25, s (6.88)
17					15.4, CH ₃	2.13, d (1.51)
18					62.3, OCH ₃	3.88, s
					OH	10.21, s

methylenes, 5 methines, and 11 nonprotonated carbons. In the ^1H NMR spectrum, one methyl singlet at δ 1.25 (H₃-16), three methyl doublets at δ 0.31 (H₃-14), 0.38 (H₃-15), and 2.13 (H₃-17), and a methoxy group at δ 3.88 (OCH₃) were observed.

The first spin system in the molecule was deduced from the COSY correlation networks established between H-8 and H₃-16/H₂-9, H₂-10 and H₂-9/H-11, and H-13 and H-11/H₃-14/H₃-15. The structure of **6** was assembled from the HMBC correlations of H₃-16 to C-8/C-9/C-7a, H₃-14/H₃-15 to C-11/C-13, H-11 to C-9/C-10/C-11a, H-12 to C-6a/C-12a/C-1a, H₃-17 to C-2/C-3/C-4, and H-4 to C-2/C-3/C-17/C-5a. Furthermore, the OCH₃ protons showed HMBC correlations to C-6 (δ 138.6), while the OH-7 showed correlations to C6/C7/C-7a. The chemical shifts of C-2 (δ 183.7) and C-5 (δ 187.7) were consistent with those of naphthalene-1,4-dione moieties, and hence, the ketone group was attached to C-5. The structure of **6** was therefore concluded to be 7-hydroxy-11-isopropyl-6-methoxy-16,17-dimethyl-8,9,10,11-tetrahydrobenzophenanthridine-2,5-dione.

The relative configuration of **1–4** and **6** was established through analysis of the ROESY correlations (Figure 2) and ^1H – ^1H coupling constant. Similar ROESY correlations were observed for the above-mentioned compound in the cyclohexane moiety. Using compound **4** as a representative of these compounds, ROESY correlations of H₃-14 to H₂-3 α /H-4 and H₃-12 to H-5/H₂-3 α were observed, placing these protons on the same face of the ring. On the other hand, H₂-3 β showed ROESY correlations to H₃-13/H-11. The α orientation assigned to H-4 was further supported by the large coupling

constant of H-4 (J = 4.38, 12.05 Hz). Therefore, the relative stereochemistry of **4** was assigned as 2*S**,4*S**,5*R**.

To determine the absolute configuration, **4** was derivatized to its corresponding *R*- and *S*-MTPA esters with both *R*- and *S*-MTPA chloride.¹³ The difference in chemical shifts $\Delta\delta^{\text{SR}} = (\delta_{\text{S}} - \delta_{\text{R}})$ for protons neighboring C-4 gave positive values of +0.035, +0.146, +0.156, and +0.071 for H-5, H-11, H₃-12, and H₃-13, respectively. H₃-14, H-2, H₂-3 α , and H₂-3 β gave negative values of −0.06, −0.018, −0.02, and −0.013, respectively (Figure 3). The absolute stereochemistry for **4** was therefore assigned as 2*S*,4*S*,5*R*. Since the compounds **1–3** and **6** showed similar ROESY correlations to **4**, along with the fact that these compounds were obtained as congeners and are likely to be biosynthesized via the same pathway, a 2*S*,5*R* configuration is proposed. Furthermore, the ECD spectra of **1**, **3**, and **4** showed a similar negative Cotton effect at 256 nm and

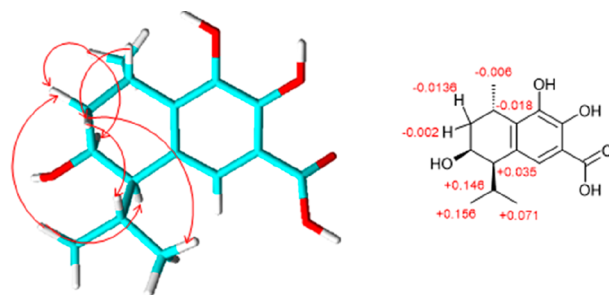


Figure 3. ROESY correlations of compound **4** and the chemical shift difference between the *S*- and *R*-MTPA esters of **4**.

positive Cotton effect between 233 and 245 nm (Figure 4). Some compounds closely related to 4 are the antimicrobial

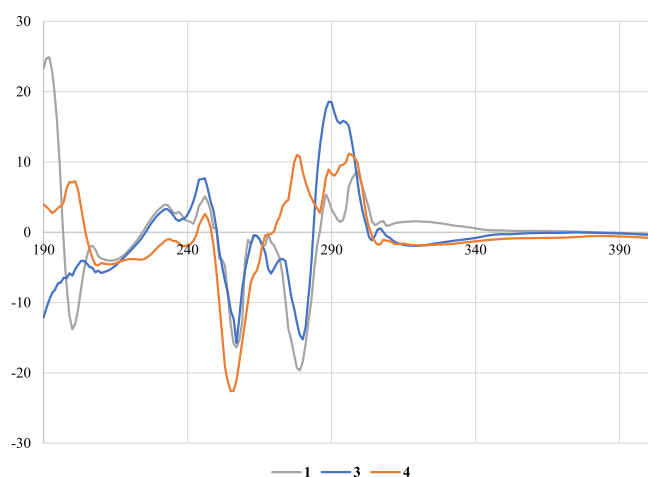


Figure 4. ECD spectra for compounds 1, 3, and 4 in ethanol.

hydroxycalamenenes isolated from the plants *Guardiola platyphylla*,¹⁴ *Subergorgia hicksoni*,¹⁵ and *Dysoxylum acutangulum*.¹⁶ Other related metabolites are the serrulatane diterpenoids,¹⁷ which were reported to possess the 2*S*,5*R* configuration. The optical rotations of the isolated compounds were also in the same range as those reported for hydroxycalamenenes ($[\alpha]_D^{20}$ +40 to +51).^{14,16}

Compounds 1–6 were evaluated for antimicrobial and cytotoxic activities (Table 3). Compound 3 displayed moderate activity against *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, and *Mucor hiemalis*. The highest activity of this compound was recorded against *M. luteus* with an MIC value of 3.1 μ g/mL. Also, compound 6 showed activity against *M. luteus* and *B. subtilis* with an MIC value of 6.3 μ g/mL. 1 and 3 were cytotoxic against KB 3.1 and L929 cell lines at IC_{50} 7.6 μ M and 5.5 μ M, respectively.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were recorded with a Bruker Avance III 700 spectrometer with a 5 mm TXI cryoprobe (1H 700 MHz, ^{13}C 175 MHz, ^{15}N 71 MHz) and

Bruker AV III-600 (1H 500 MHz, ^{13}C 150 MHz) spectrometers (sample temperature 298 K). All the NMR spectra were referenced to the solvent used. HRESIMS data were recorded with an Agilent 1200 series HPLC-UV system (column 2.1 $\times$ 50 mm, 1.7 μ m, C₁₈ Acquity UPLC BEH (waters), solvent A: H₂O + 0.1% FA; solvent B: AcCN + 0.1% FA, gradient: 5% B for 0.5 min increasing to 100% in 19.5 min and then maintaining 100% B for 5 min, flow rate 0.6 mL/min, UV/vis detection 200–600 nm) combined with ESI-TOF-MS (Maxis, Bruker) (scan range 100–2500 m/z , capillary voltage 4500 V, dry temperature 200 $^{\circ}C$). UV spectra were recorded using a Shimadzu UV-2450 UV/vis spectrophotometer.

Fungal Material. The fungal specimen was collected by C. Decock and J. C. Matasyoh from Mount Elgon National Reserve (1 $^{\circ}$ 7'6" N, 34 $^{\circ}$ 31'30" E) in Kenya. A dried specimen and corresponding cultures were deposited at MUCL, Louvain-la-Neuve, Belgium, under the accession number MUCL56078. The genus was identified by morphological studies and comparison of the internal transcribed spacer (ITS)-nrDNA sequences with others from Basidiomycota deposited in GenBank. The ITS sequence has been deposited in GenBank, with accession no. MT755873.

Sequencing. DNA extraction was performed using the EZ-10 spin column genomic DNA miniprep kit (Bio Basic Canada Inc., Markham, Ontario, Canada) following a previously published protocol.¹⁸ A Precellys 24 homogenizer (Bertin Technologies, France) was used for cell disruption at a speed of 6000 rpm for 2 $\times$ 40 s. The DNA regions were amplified using standard primers. For ITS, the PCR product was purified directly, using the Nucleo spin gel and PCR clean up kit (Macherey-Nagel, Düren, Germany). PCR products were sequenced at the Department of Genome analytics of the Helmholtz Centre for Infection Research, Braunschweig, Germany.

Small-Scale Fermentation. *Heimiomyces* sp. MUCL56078 was cultivated in three different liquid media: YMG 6.3 (10 g/L malt extract, 4 g/L yeast extract, 4 g/L D-glucose; pH 6.3), Q6 1/2 (10 g/L glycerol, 2.5 g/L D-glucose, 5 g/L cotton seed flour; pH 7.2), and ZM 1/2 (5 g/L molasses, 5 g/L oat meal, 4 g/L sucrose, 1.5 g/L glucose, 1.5 g/L calcium carbonate, 0.5 g/L lactalbumin hydrolysate, 0.5 g/L ammonium sulfate; pH 7.2). A well-grown culture from a YMG agar plate (YMG supplemented with 1.5% agar, pH = 6.3) was cut into small pieces using a cork borer (7 mm), and 5 pieces were inoculated into a 500 mL Erlenmeyer flask containing 200 mL of media. The cultures were incubated at 24 $^{\circ}C$ on a rotary shaker (140 rpm). The growth of the fungus was monitored by checking the amount of free glucose with Medi-test glucose (Macherey-Nagel). The fermentation was terminated 2 days after glucose depletion.

Scale-up of Fermentation. The masses of the LC-MS detected peaks and their molecular formula obtained from HRESIMS data

Table 3. Antimicrobial and Cytotoxic Activities of Compounds 1–6

test organism	MIC (μ g/mL) ^a						positive control
	1	2	3	4	5	6	
<i>Bacillus subtilis</i> DSM10	– ^b	100	12.5	–	–	6.3	9.2 ^c
<i>Mycobacterium smegmatis</i> DSM44200	–	–	100	–	–	–	0.6 ^d
<i>Micrococcus luteus</i> DSM1790	25	12.5	3.1	100	50	6.3	0.3 ^c
<i>Staphylococcus aureus</i> DSM346	100	100	50	100	100	50	9.2 ^e
<i>Mucor hiemalis</i> DSM2656	100	100	25	50	25	50	9.2 ^f
<i>Candida albicans</i> DSM1665	–	–	100	–	–	100	9.2 ^f
<i>Pichia anomala</i> DSM6766	100	100	100	–	100	100	4.6 ^f
cell line	cytotoxicity (IC_{50} μ M)						ref ^g
	1	2	3	4	5	6	
L929	–	–	5.5	–	–	–	2.6 $\times$ 10 ^{–3}
KB3.1	7.6	–	–	–	–	–	8.9 $\times$ 10 ^{–5}

^aFor MIC, 20 μ L of 1.5 mg/mL stock solution (start concentration 100 μ g/mL). Cell density was adjusted to 6.7 $\times$ 10⁵ cells/mL; 20 μ L of methanol was used as negative control. For the cytotoxicity assay, 6 $\times$ 10³ cells/well were selected in 96-well microtiter plates and treated with compounds for 5 days. ^b–, no activity. ^cOxytetracyclin. ^dKanamycin. ^eGentamicin. ^fNystatin. ^gEpothilone B.

were compared with those in the Dictionary of Natural Products (<http://dnp.chemnetbase.com>).¹⁹ Several possible new compounds were detected in the extracts from mycelia and supernatants in the three media. Initiation results suggested that the fungus grew and produced best in YMG medium; therefore, this was selected for scale-up fermentation. The mycelia were incubated in 25 flasks following the small-scale screening protocol.

Preparation of the Extracts. Supernatant and biomass from small-scale fermentation were separated by filtration. The supernatant was extracted with an equal amount of ethyl acetate and filtered through anhydrous sodium sulfate. The resulting ethyl acetate extract was evaporated to dryness by a rotary evaporator. The mycelia were extracted with 200 mL of acetone in an ultrasonic bath for 30 min and filtered, and the filtrate was evaporated. The remaining water phase was suspended in an equal amount of distilled water, extracted with an equal amount of ethyl acetate, and dried.

The mycelia and supernatant from the large-scale fermentation were separated via vacuum filtration. The mycelia were extracted with 500 mL of acetone using an ultrasonic bath for 30 min four times. The extracts were combined, and the solvent was evaporated. The remaining water phase was mixed with 200 mL of distilled water and extracted with 3 × 500 mL of ethyl acetate. The resulting organic layer was filtered through anhydrous sodium sulfate and evaporated to complete dryness, leaving a brown solid (376.2 mg). The supernatant was extracted by adding 2% Amberlite XADTM-16N adsorbent (Rohm & Haas Deutschland GmbH, Frankfurt am Main, Germany) and stirred for 4 h. The Amberlite resin was then harvested and eluted with 4 × 500 mL of acetone. The resulting acetone extract was evaporated, and the remaining water phase was extracted three times with an equal amount of ethyl acetate. The organic phase was dried to yield a brown crude extract (331.3 mg).

Isolation of Compounds 1–6. The extract was filtered through an SPME Strata-X 33 u Polymeric RP cartridge (Phenomenex, Inc., Aschaffenburg, Germany). The supernatant and mycelia crude extracts were fractionated by preparative reversed-phase liquid chromatography (Gilson, Middleton, USA), using a VP Nucleodur 100-5 C₁₈ ec column (150 × 40 mm, 7 μm, Macherey-Nagel); solvent A (deionized water (Milli-Q, Millipore, Schwalbach, Germany) with 0.1% FA) and solvent B (acetonitrile with 0.1% FA) were used as the mobile phase. Elution gradient used was 5–100% solvent B in 63 min and thereafter isocratic conditions at 100% solvent B for 5 min. UV detection was carried out at 210, 254, and 350 nm, and the flow rate was 40 mL/min. Fourteen fractions were collected according to the observed peaks (F1–F14) from supernatant extracts; 13 fractions (F1–F13) were collected from the mycelial extracts.

Fraction F7 of the supernatant was further purified by reversed-phase HPLC (solvent A: H₂O + 0.05% TFA/solvent B: ACN + 0.05% TFA, elution gradient: 10% B isocratic for 3 min, followed by 10–80% B over 40 min, afterward increased to 100% B in 5 min, and thereafter isocratic conditions at 100% for 5 min with a preparative Nucleodur phenyl hexyl column (Macherey-Nagel, Düren, Germany; 250 × 21 mm, 5 μm) and a flow rate of 12 mL/min) to afford compound 4 (2.59 mg). A VP Nucleodur 100-10 C₁₈ column (Macherey-Nagel, Düren, Germany; 250 × 10 mm) with a flow rate of 5 mL/min was used to isolate compounds 1 (1.42 mg) and 2 (4.89 mg). Compound 1 was purified from mycelia fraction F9 with an elution gradient: 30% B isocratic for 3 min, 30–75% B in 40 min, thereafter increased to 100% B in 5 min, and isocratic conditions of 100% B for 5 min. Supernatant fraction F11 and mycelium fraction F8 were combined and purified to obtain compound 2 with an elution gradient: 55% B for 3 min, increased to 75% B in 60 min, thereafter 75–100% B in 5 min, and 100% B isocratic conditions for 5 min. A preparative HPLC column (Kromasil, MZ Analysentechnik, Mainz, Germany; 250 × 20 mm, 7 μm C₁₈) with a flow rate of 12 mL/min was used to isolate compounds 3 (3.21 mg), 5 (1.84 mg), and 6 (0.69 mg). Supernatant fractions F10 and F12 were combined and purified with an elution gradient: 60% B for 3 min, followed by 60–80% B for 63 min, thereafter increased to 100% B in 5 min, and isocratic conditions of 100% B for 10 min to afford compound 3. Compound 5 was purified from supernatant fraction F9 with a gradient: 35% B for 3

min, 35–70% B for 40 min, afterward increased to 100% B in 5 min, and isocratic conditions of 100% B for 5 min. Compound 6 was purified from a combination of mycelium fractions F4 and F5 with an elution gradient: 66% B for 3 min, followed by 66–72% B in 35 min, thereafter increased to 100% B in 5 min, and isocratic conditions of 100% B for 5 min.

Preparation of the (R)- and (S)-MTPA Ester Derivatives of 4.

A 1 mg amount of compound 4 was dissolved in 0.6 mL of deuterated pyridine and transferred into two vials (0.3 mL for each). (R)-(-)-α-Methoxy-α-(trifluoromethyl) phenylacetyl chloride (2 μL) was added to one vial, and (S)-(-)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (2 μL) was added into another vial. The mixtures were stirred at 25 °C overnight before measurement of ¹H NMR. Unfortunately, it was very difficult to assign the resonance signals because of the strong signals from the pyridine solvent signals and some residual Mosher acids. The reaction products were therefore purified by HPLC using a VP Nucleodur 100-10 C₁₈ec column (Macherey-Nagel, Düren, Germany; 250 × 10 mm) as the stationary phase and the elution gradient of 20% to 100% solvent B (acetonitrile) for 25 min and thereafter isocratic conditions at 100% solvent B for 10 min. The ¹H and COSY NMR spectra of the purified products were recorded.

Antimicrobial Assay. Compounds 1–6 were evaluated for antimicrobial activity (minimum inhibition concentrations, MIC) in serial dilution assays against several microorganisms using a previously reported method.⁴ The assays were conducted in 96-well plates in Mueller-Hinton broth (MHB) media for bacteria and YMG media for filamentous fungi and yeasts.

Cytotoxicity Assay. In vitro cytotoxicity was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test in 96-well plates, as described by Mulwa et al.,²⁰ against the mouse fibroblast cell line L929 and the cervix carcinoma cell line KB3.1.

Heimiomycin A (1): yellow solid; [α]_D²⁰ +44.1 (c 1.0, acetone); UV (acetone, c 0.1) $\lambda_{\max}$ (log ϵ) 222 (4.169); HRESIMS *m/z* 499.1737 [M + Na]⁺, 459.1808 [M + H – H₂O]⁺, 477.1907 [M + H]⁺, calcd for C₂₈H₂₉O₇ 477.1913.

Heimiomycin B (2): yellow solid; [α]_D²⁰ +42.4 (c 1.0, acetone); UV (acetone, c 0.1) $\lambda_{\max}$ (log ϵ) 222 (4.153); HRESIMS *m/z* 515.1677 [M + Na]⁺, 475.1749 [M + H – H₂O]⁺, 493.1869 [M + H]⁺, calcd for C₂₈H₂₉O₈ 493.1862.

Heimiomycin C (3): orange solid; [α]_D²⁰ +53.4 (c 0.83, acetone); UV (acetone, c 0.17) $\lambda_{\max}$ (log ϵ) 216 (4.041); HRESIMS *m/z* 407.1466 [M + Na]⁺, 367.1537 [M + H – H₂O]⁺, 385.1644 [M + H]⁺, calcd for C₂₂H₂₅O₆ 385.1651.

4,9,10-Trihydroxy-5-isopropyl-2-methyl-2,3,4,5-tetrahydronaphthalene carboxylic acid (4): yellow solid; [α]_D²⁰ +30.9 (c 1.19, acetone); UV (acetone, c 0.119) $\lambda_{\max}$ (log ϵ) 218 (3.830); HRESIMS *m/z* 303.1201 [M + Na]⁺, 263.1276 [M + H – H₂O]⁺, 281.1382 [M + H]⁺, calcd for C₁₅H₂₁O₅ 281.1388.

4-Hydroxy-5-isopropyl-2-methyl-9,10-dihydronaphthalene carboxylic acid (5): yellow solid; UV (acetone, c 0.1) $\lambda_{\max}$ (log ϵ) 220 (3.711), 300 (3.457); HRESIMS *m/z* 269.1151 [M + Na]⁺, 229.1222 [M + H – H₂O]⁺, 247.1329 [M + H]⁺, calcd for C₁₅H₁₉O₃ 247.1334.

7-Hydroxy-11-isopropyl-6-methoxy-16,17-dimethyl-8,9,10,11-tetrahydrobenzophenanthridine-2,5-dione (6): yellow solid; [α]_D²⁰ +47.8 (c 1.0, acetone); UV (acetone, c 0.1) $\lambda_{\max}$ (log ϵ) 220 (3.717); HRESIMS *m/z* 781.3454 [2M + Na]⁺, 380.1858 [M + H]⁺, calcd for C₂₃H₂₆NO₄ 380.1856.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00486>.

Experimental procedures, 1D and 2D NMR data, HRESIMS data, 5.8S/ITS DNA sequence of the producing organism (PDF)

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Notes

The authors declare no competing financial interest.

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