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Hypocoprins A–C: New Sesquiterpenoids from the Coprophilous Fungus *Hypocopra rostrata*

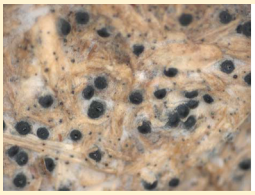
Dinith R. Jayanetti,[†] Qun Yue,[‡] Gerald F. Bills,[‡] and James B. Gloer*[,†]

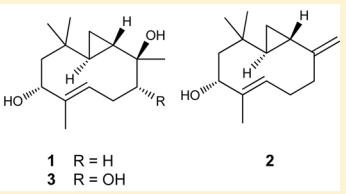
[†]Department of Chemistry, University of Iowa, Iowa City, Iowa 52242, United States

[‡]Texas Therapeutics Institute, The Brown Foundation Institute of Molecular Medicine, University of Texas Health Science Center at Houston, 1881 East Road, Houston, Texas 77054, United States

Supporting Information

ABSTRACT: Studies of the coprophilous fungus *Hypocopra rostrata* (TTI-0009, NRRL 66178) isolated from a sample of horse dung collected in Texas led to the isolation of three new sesquiterpenoids that we named hypocoprins A–C (**1**–**3**), together with the known fungal metabolite helvolic acid. The new metabolites have a distinctive ring system consisting of fused cyclopropane and cyclodecene units not previously reported from a fungal source. Compounds **1** and **3** moderately inhibited growth of *Staphylococcus aureus*. The structures of these metabolites were assigned mainly by analysis of 2D NMR and HRESITOFMS data. Relative and absolute configurations were assigned by interpretation of NMR *J*-values and NOESY data and by application of Mosher's method. These results represent the first report of chemistry from any strain of the genus *Hypocopra*.





Coprophilous fungi are those that colonize and reproduce in animal dung.[1−3] These fungi are distinctly adapted to complete their life cycle in their substrata, so they are largely exclusive to the dung environment. Our initial studies of such organisms as sources of bioactive natural products were fruitful,[4−6] but far from comprehensive, and while limited studies of coprophilous isolates by some other research groups have also afforded new metabolites, these fungi remain relatively unexplored from a chemical standpoint.[6] As part of an initiative to further explore the potential of these fungi, we investigated an isolate identified as *Hypocopra rostrata* (TTI-0009). Fungi of the genus *Hypocopra* are a distinctive group of about 25 species of the Xylariaceae characterized by their adaptations to grow and disperse in dung of herbivorous animals.[7,8] Anatomically, they are distinguished from the other genera of dung-inhabiting Xylariaceae (*Poronia* and *Podosordaria*) by their sessile ascomata embedded directly in the dung surface.

Cultured specimens of *Hypocopra* species have rarely been reported, possibly because, like many other dung-inhabiting fungi, their ascospores require specific environmental cues to activate germination. Thus, nothing is known about their chemistry and little about their phylogenetic affinities. *H. rostrata* was originally described from a collection on horse dung from Tucson, Arizona, USA, in 1901.[9] To the best of our knowledge, this is the first report of a conidial stage of a *Hypocopra* species and represents only the second known living isolate of *H. rostrata* aside from CBS 722.68 from Nebraska, USA. Investigation of the secondary metabolites from cultured mycelium of our strain of *H. rostrata* led to the isolation of compounds **1**–**4**.

An initial small-scale fermentation extract contained the known antibiotic helvolic acid as the major component (**4**), but displayed evidence of other unidentified metabolites, and subsequent studies of a scaled-up fermentation extract afforded three new sesquiterpenoids (**1**–**3**). The structures of these metabolites were assigned mainly by analysis of 2D NMR and HRESITOFMS data and supported by the results of chemical derivatization experiments. Details of the isolation and structure elucidation of compounds **1**–**3** are presented here.

RESULTS AND DISCUSSION

The fungus (TTI-0009, NRRL 66178) formed patches of white mycelial pseudostromata on the dung surface that were accompanied by bright orange conidial masses (sporodochia) (Figure S1; Supporting Information). Isolation of the fungus from the sporodochial conidia resulted in cultures identical to those from the ascospores, and fertile perithecia and sporodochia formed in ascospore and conidial isolates (Figure S1). The fungus was identified as *H. rostrata* based on the combination of the white mycelial pseudostromata from which aggregated black, shiny ascomatal necks protruded. We also examined the type specimen (BPI 581328) of *H. rostrata* and observed that the ascospores were of similar size and shape to those in our specimen. In our specimen, the ascospores ranged from 27 to 32 μm long to 14–18 μm wide with a lateral germ slit about half the length of the spore[7−9] (Figure S1). Database matching with the ITS rDNA sequence (www.ncbi.nlm.nih.gov;

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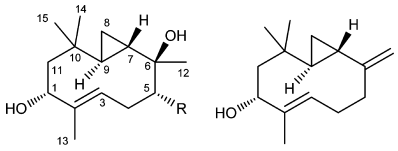
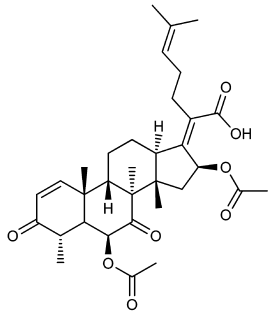


1 R = H
3 R = OH

2



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www.fungalbarcoding.org) yielded a very high sequence similarity (99%) to one of the few known strains of *Hypocopra*, *H. anomala* CBS 124649, thus indicating that the strains were congeneric. Although *H. anomala* and *H. rostrata* have been previously noted to be very similar taxa based on the nature of the white pseudostroma,[7,8] our Texas specimen appeared to represent the latter species because of its larger ascospore size. Database matching with the 28S rDNA retrieved a large number of sequences of *Xylaria* species in the range of 96−99% identity. Neighbor-joining analysis of the ITS sequences of dung-inhabiting species and other species of Xylariaceae indicated that *H. rostrata* is highly related to other coprophilous species and several undefined environmental isolates of the Xylariaceae (Figure S2).

Methyl ethyl ketone extracts from liquid cultures of *H. rostrata* were partitioned between hexanes and MeCN to remove lipids, and the MeCN-soluble material was subjected to silica gel column chromatography followed by separation over Sephadex LH-20, leading to the isolation of **1**−**3**. Hypocoprin A (**1**) was isolated as a pale yellow solid. Two of the initial Sephadex column fractions contained **1** (fractions 5 and 6). However, when fraction 5 was stored in $CDCl_3$ after ^1H NMR analysis, it showed signs of degradation. Use of acetone-d_6 as the solvent for subsequent NMR analysis of fraction 6 avoided (or at least substantially slowed) this degradation process. The molecular formula of **1** was determined to be $C_{15}H_{26}O_2$ (3 unsaturations) on the basis of HREITOFMS and NMR data. The ^1H NMR spectrum (Table 1) displayed signals characteristic of a trisubstituted olefin and an oxymethine unit, along with four methyl singlets and four exceptionally upfield multiplets between 0.1 and 0.8 ppm that suggested the presence of a cyclopropane unit. ^{13}C and DEPT-135 NMR data also supported the presence of the olefin unit and an oxygenated methine and further showed the presence of two aliphatic methines, four aliphatic methylenes, and two non-protonated sp^3 carbons, of which one was oxygenated. The gross structure of **1** was determined by analysis of COSY and HMBC data (Figure 1). Mutual COSY correlations among H-7, H-8, and H_2-9 enabled assembly of the cyclopropane unit. Other COSY correlations were consistent with the presence of the H-1/H_2-11 and H-3/H_2-4/H_2-5 spin-systems. HMBC

Table 1. NMR Spectroscopic Data for Hypocoprins A (1) and C (3) in Acetone-d_6

	1		3	
position	δ_C[a]	δ_H, mult (J in Hz)[b]	δ_C[a]	δ_H, mult (J in Hz)[b]
1	75.7	4.21, dd (3.2, 13)	75.6	4.21, br d (11)
2	136.7		138.3	
3	127.6	5.34, br d (12)	122.9	5.36, br dd (4.2, 10)
4	25.0	2.00, br d (12)	32.7	2.28, m
		2.30, dq (4.2, 12)		
5	44.8	1.78, m[c]	79.4	3.61, dd (9.6, 6.6)
6	72.7		75.5	
7	32.4	0.80, dt (9.2, 5.2)	28.7	0.71, dt (9.3, 5.5)
8	5.2	0.10, dt (9.2, 5.2)	5.5	0.18, dt (9.3, 5.5)
		0.36, dt (9.6, 5.2)		0.44, dt (9.6, 5.5)
9	28.3	0.47, dt (9.6, 5.2)	27.9	0.54, dt (9.6, 5.5)
10	31.3		32.4	
11	50.1	1.53, dd (3.2, 13)	50.6	1.52, dd (3.0, 13)
		1.78, m[c]		1.82, dd (13, 14)
12	20.9	0.71, s	15.1	0.67, s
13	10.6	1.67, s	10.8	1.68, s
14	20.1	0.64, s	19.9	0.63, s
15	34.4	0.90, s	34.4	0.91, s

[a]Data were collected at 600 MHz (^1H). [b]Data were collected at 100 MHz (^{13}C). [c]Overlapping signals.

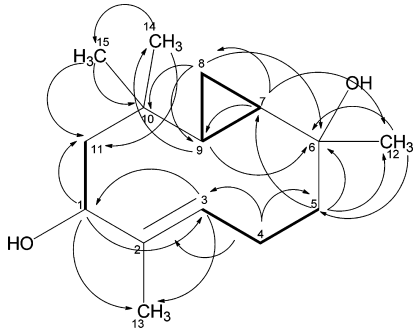

Figure 1. Key COSY (boldfaced bonds) and HMBC correlations (arrows) for **1**.

correlations from H-7 to C-10, from H_2-8 to C-6 and C-10, and from H-9 to C-6 established the connectivity of the cyclopropane unit to quaternary carbons C-6 and C-10. HMBC correlations from both H_3-14 and H_3-15 to C-15 and C-14, respectively, and to C-9, C-10, and C-11 placed these two methyl groups on C-10 and linked C-10 to C-11. HMBC correlations from H_3-13 to C-1, C-2, and C-3 confirmed its attachment to the olefin unit and linked oxygenated carbon C-1 to olefinic C-2. Further correlations from H_2-4 to C-3 and C-5 supported the presence of the C-3C-4/C-5 unit, while correlations from H_3-12 to C-5, C-6, and C-7 enabled the completion of the cyclodecene ring-containing structure. Additional HMBC correlations observed were fully consistent with the assignment. The chemical shift of the H_3-12 signal (δ 0.71) was significantly upfield relative to that expected for a typical methyl group linked to an oxygenated sp^3 carbon, but was rationalized by its proximity to the cyclopropane ring and is consistent with shifts observed for other structures having an analogous structural subunit.[10] Treatment of **1** with acetic anhydride afforded a monoacetate displaying a significantly downfield shifted signal for H-1, confirming the presence of a free secondary alcohol group and ruling out the possibility of an

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ether linkage between C-1 and C-6. This enabled completion of the gross structure of **1**, for which we propose the name hypocoprin A.

The relative configuration of **1** was assigned on the basis of NOESY correlations (Figure 2). A strong NOESY correlation

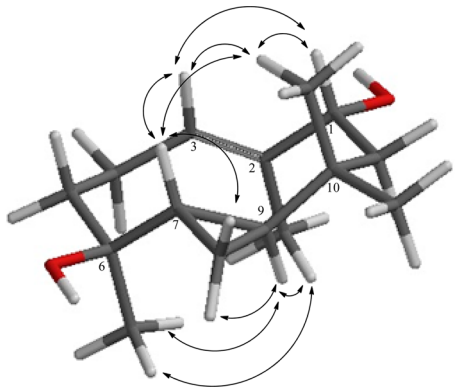

Figure 2. Selected NOESY correlations for **1**.

between H-1 and H-3, together with the chemical shift of C-13, indicated the *E*-geometry for the olefin unit. A NOESY correlation between H-7 and one of the diastereotopic C-8 protons placed these on the same face of the cyclopropane ring. The other C-8 proton signal showed a NOESY correlation to H-9, indicating that these two protons are oriented on the same face of the cyclopropane ring and opposite that of H-7. A NOESY correlation between H-7 and H_3-14 indicated that H_3-14 lies on the same face of the cyclodecene ring as H-7. H_3-14 also correlated with H-1, indicating that both H-1 and H-7 are on the same face. A correlation between H-9 and H_3-12 oriented H_3-12 on the same face as H-9. These correlations allowed the assignment of the relative configuration at the four stereocenters of compound **1**. An energy-minimized model having this relative configuration was constructed using Spartan'10, and the major conformer was consistent with the observed NOESY correlations, including a correlation of olefinic H-3 with H-7. Mosher ester analysis[11,12] of **1** enabled the assignment of the absolute configuration. The most significant $\Delta\delta$ ($\delta_S - \delta_R$) values were observed for H_3-13 (−0.14 ppm), H_3-15 (+0.03 ppm), and the pseudoequatorial proton on C-11 (+0.09 ppm). These values led to assignment of the absolute configuration of **1** as shown (1*R*, 6S, 7S, 9S).

Hypocoprin B (**2**) was present in relatively limited quantities in the scale-up extract, though it was more abundant in the initial screening extract based on NMR analysis. The Sephadex LH-20 column fraction from which **2** was ultimately obtained initially contained nearly pure **1**. However, upon standing in $CDCl_3$ for 4 days, it was evident that approximately 50% of the sample of **1** had degraded, as the ^{1}H NMR spectrum showed the appearance of two new singlets at δ 4.17 and 4.56, while other signals were doubled. An attempt to separate the components by chromatography on Sephadex LH-20 was not successful. Therefore, the mixture was simply allowed to stand in $CDCl_3$ to foster further conversion to the product. After 6 days, the ^{1}H NMR spectrum showed ca. 90% conversion, enabling acquisition of useful spectra. The molecular formula of the product (**2**) was deduced to be $C_{15}H_{24}O$ (4 unsaturations) by analysis of HREITOFMS and NMR data, suggesting that **2** might be a dehydration product of **1**. The ^{1}H, ^{13}C, and DEPT NMR data for **2** (Table 2) indicated the presence of a 1,1-

Table 2. NMR Spectroscopic Data for Hypocoprin B (2) in $CDCl_3$

position	δ_C[a]	δ_H, mult (*J* in Hz)[b]
1	76.4	4.29, dd (3.5, 12)
2	137.6	
3	128.5	5.56, br ddd (1.0, 3.5, 12)
4	30.1	2.13, m
		2.40, m
5	40.0	2.10, m
		2.43, m
6	153.6	
7	25.2	0.85, dt (8.0, 5.5)
8	11.5	0.51, m
		0.60, m
9	36.4	0.47, m
10	32.4	
11	49.0	1.58, dd (3.5, 14)
		1.87, dd (12, 14)
12	103.6	4.17, br s
		4.56, br s
13	10.6	1.61, br s
14	19.5	0.64, s
15	33.2	0.94, s

[a]Data were collected at 500 MHz (^{1}H). [b]Data were collected at 100 MHz (^{13}C).

disubstituted olefin unit and lacked signals corresponding to the oxygenated quaternary sp^3 carbon and the associated methyl group found in **1**. Analysis of COSY and HMBC data confirmed these observations, independently confirming that **2** differed from **1** by elimination of a molecule of water to afford a C6−C12 olefin. HMBC correlations from both C-12 protons to C-5, C-6, and C-7 confirmed the location of the 1,1-disubstituted olefin unit. Additional HMBC correlations verified that the remainder of the structure was intact. As was the case for the C-12 methyl group in **1**, the terminal olefin ^{1}H signals (δ 4.17 and 4.56) are somewhat upfield-shifted relative to those of typical units of this type, and this observation is consistent with NMR data reported for other structures having a cyclopropane unit in a similarly adjacent position.[10] Analysis of NOESY data and energy-minimized models as described for **1** enabled assignment of the expected relative configuration at the three remaining stereogenic centers of **2**, providing independent support for the configuration originally assigned to precursor **1**. The absolute configuration of **2** is presumed to be analogous to that of **1**. Clearly, simple dehydration of **1** at the tertiary alcohol functionality would afford **2**. An acid-catalyzed process would be consistent with its occurrence in $CDCl_3$ due to the presence of possible traces of DCl. The limited amount of **2** evident in the NMR spectrum of the initial extract was somewhat surprising given the facility of this process. Interestingly, the process preferentially yielded disubstituted exocyclic olefin **2**, rather than the trisubstituted endocyclic olefin, which was not observed. This is presumably due to energetic differences between the possible products. An analogous process (and regiochemical preference) has been observed upon attempted acetylation of a marine diterpenoid with a similar moiety,[13] but no literature precedent was found for spontaneous occurrence of the process in $CDCl_3$.[10,13]

Hypocoprin C (**3**) was isolated as a colorless solid with the molecular formula $C_{15}H_{26}O_3$ (3 unsaturations), as determined by analysis of HRESITOFMS and NMR data. To prevent

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possible decomposition analogous to that observed for **1**, NMR analyses for **3** were carried out using acetone-d_6. Key differences between the ^{1}H and ^{13}C NMR data of **1** and **3** (Table 1) included replacement of one aliphatic methylene unit with an additional oxygenated methine, suggesting that one of the methylene units in **1** is oxidized to a secondary alcohol group in **3**. The gross structure of **3** was again elucidated by analysis of HSQC, COSY, and HMBC data. COSY data, as well as HMBC correlations from the additional oxymethine proton in **3** to C-4, C-6, C-7, and C-12, enabled location of this proton at C-5. Treatment of **3** with acetic anhydride afforded a diacetate with downfield-shifted oxymethine signals, confirming the presence of two secondary alcohol groups in **3** and eliminating the possibility of an ether linkage between any two of the three oxygenated carbons. The relative configuration of **3** was again established on the basis of NOESY data (Figure 2). Similarity of the NOESY correlations observed for **1** and **3** enabled the assignment of the relative configuration at the four common stereocenters shared by **1** and **3**. An additional NOESY correlation of H-7 with H-5 placed these hydrogens on the same face of the system, thus enabling the assignment of the relative configuration at the additional stereogenic center as shown in **3**. The absolute configuration of **3** was assigned as shown by analogy to that of **1**.

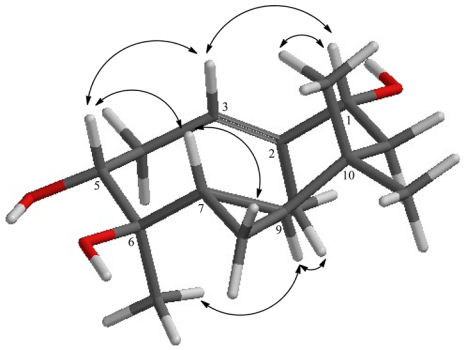

Figure 3. Selected NOESY correlations for **3**.

The closest known structural analogues to **1**−**3** are a group of marine invertebrate-derived diterpenoids consisting of palmatol (from the Mediterranean octocoral *Alcyonium palmatum*)[13] and pacifins (from the Formosan soft coral *Naphathea pacifica*).[10] These previously reported compounds all have an additional prenyl unit forming more elaborate side-chain moieties attached to the cyclodecene ring in place of one of the geminal methyl groups present in the structures reported here. Most of them have other additional features not found in **1**−**3**, but the cyclopropane unit and adjacent quaternary stereocenter (where present) share the same relative configuration found in **1**−**3**.

Hypocoprin A (**1**) showed antibacterial activity in disk assays against the Gram-positive bacteria *Staphylococcus aureus* (ATCC 29213) and *Bacillus subtilis* (ATCC 6051), affording 13−14 mm inhibitory zones at 200 μg/disk (a gentamicin standard gave similar results at 20 μg/disk), but no effects against *Escherichia coli* (ATCC 25922) or *Candida albicans* (ATCC 14053) at this level. Hypocoprin C (**3**) showed only a 9 mm zone in the assay against *Staph. aureus*, while hypocoprin B (**2**) was inactive in all four assays. Hypocoprins A−C (**1**) were also tested for cytotoxicity against a human osteosarcoma cell line (U2OS), but were found to be inactive (IC_{50} > 10 μM). Helvolic acid (**4**),[14] a well-known fungal metabolite, was found to be the major component in the original screening extract, but was only a minor constituent of the scaled-up extract. Crude MEK extracts of TTI-0009 dissolved in DMSO were strongly inhibitory to *S. aureus*, a result consistent with the presence of helvolic acid, which is known to show broad spectrum activity against both Gram-positive and Gram-negative bacteria as well as phytotoxic and antifungal effects.[15]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on an AUTOPOL III automatic polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). ^{1}H and ^{13}C NMR spectra were recorded using Bruker AVANCE-400, 500, or 600 spectrometers. Chemical shift values were referenced to residual solvent signals for acetone-d_6 (δ_H/δ_C, 2.05/29.9, 206.7), $CDCl_3$ (δ_H/δ_C, 7.24/77.2), or pyridine-d_5 (δ_H/δ_C, 7.22, 7.58, 8.74/123.9, 135.9, 150.3). HSQC, HMBC, and NOESY data were recorded using the Bruker AVANCE-600 instrument. All standard NMR data were processed using the NUTS 2007 software, while 2D-NMR data were processed on the Avance-600 computer. HREITOFMS data were recorded using a Waters Micromass GCT Premier mass spectrometer. HRESITOFMS data were obtained using a Waters Premier QTOF instrument.

Producing Fungus and Its Characterization. Partially decomposed horse dung was collected in Jack Brookes Gregory Park, Hitchcock, Galveston Co., Texas. The dung was rehydrated in deionized H_2O and incubated in deep-dish Petri plates lined with filter paper to simulate natural dung decomposition. The strain of *H. rostrata* (TII-0009) was isolated by germinating ascospores or conidia on cornmeal dextrose agar amended with 50 μg/mL chlortetracycline and streptomycin sulfate. Conidial isolates germinated directly on the agar, while ascospore germination was stimulated by overnight incubation at 44 °C. The ascospore isolate was used for all experiments. A subculture was deposited at the USDA NRRL Culture Collection at the National Center for Agricultural Utilization Research, Peoria, IL, USA, as NRRL 66178.

To reconstruct the approximate phylogenetic position of strain TTI-0009, genomic DNA was extracted from mycelia grown on malt-yeast extract agar. The rDNA region containing the ITS region and the partial sequence of 28S rDNA containing D1 D2 D3 variable domains was amplified with primers ITS1 and LR7.[16,17] Purified PCR products were cloned in the pJET1.2/blunt cloning vector (Thermo Fisher Scientific, MA, USA) and sequenced using primer LR0R[18] and sequencing primers supplied by CloneJET PCR cloning kit (Thermo Fisher Scientific) in GENEWIZ, Inc. (South Plainfield, NJ, USA). Partial sequences obtained in sequencing reactions were assembled with Genestudio 2.1.1.5 (Genestudio, Inc.). The sequence was deposited in GenBank (KM067909). Sequence alignments and a neighbor joining estimate of phylogenetic relationships were assembled with Mega v6.0.[19]

Fermentation. Strain TTI-0009 was initially grown in 50 mL of Sabouraud maltose broth supplemented with yeast extract and dilute agar (Difco neopeptone 10 g, maltose 40 g, yeast extract 10 g, agar 4 g, deionized H_2O 1000 mL) in a 250 mL Erlenmeyer flask for 4 d at 23 °C and 220 rpm. A 1 mL aliquot of this seed culture was transferred to each of 20 250 mL Erlenmeyer flasks each containing 50 mL of MMK2 medium (mannitol 40 g, yeast extract 5 g, Murashuge & Skoog salts [Sigma-Aldrich M5524] 4.3 g, deionized water 1000 mL) at 23 °C at 220 rpm for 14 d.

Bioassays. Antimicrobial activity was evaluated by agar disk diffusion assays using standard protocols.[20,21] Cytotoxicity was evaluated with the CellTiter-Blue Cell (Promega, WI, USA) viability assay with an osteosarcoma cell line (U2OS). A total of 250 cells were plated in each well of a 384-well plate and incubated overnight. Individual wells were treated with 50 nL of a 10 mM solution of each compound in DMSO (in duplicate) and incubated for 48 h at 37 °C, and 10 μL of CellTiter-Blue reagent was added. After further incubation for 22 h at 37 °C, absorbance was read at 530/580 nm.

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Extraction and Isolation. The combined 1 L of whole fermentation culture was extracted by addition of methyl ethyl ketone (1 L) followed by agitation at 220 rpm for 2 h. The organic phase was collected and filtered, and the organic solvent and residual H_2O were removed by vacuum evaporation to afford ca. 1 g of crude extract. The crude extract was partitioned between hexanes and MeCN to obtain fractions weighing 328 and 671 mg, respectively. The MeCN-soluble partition was subjected to silica gel column chromatography using a stepwise gradient of hexanes, EtOAc, and MeOH to afford 14 fractions. Fraction 6 (96 mg), eluted with 60% EtOAc in hexanes, was then chromatographed over a Sephadex LH-20 column using 4:1 CH_2Cl_2–hexanes, 3:2 CH_2Cl_2–acetone, 1:4 CH_2Cl_2–acetone, and 100% MeOH to afford eight fractions, among which fraction 3 (32 mg) consisted of compound **1**.

Fraction 5 (187 mg), eluted with 50% EtOAc in hexanes, was subjected to Sephadex LH-20 chromatography employing the same solvent sequence to obtain eight fractions, among which fraction 2 contained an additional sample of **1** (33 mg). Upon standing in $CDCl_3$ over time, **1** showed signs of degradation. Another Sephadex column failed to separate **2** from **1**. Therefore, the recovered mixture of **1** and **2** was stored again in $CDCl_3$. After 6 days, conversion of **1** to **2** was more than 90% complete based on NMR integration. Compound **2** was also evident in fraction 3 from the initial silica gel column, but was not purified from that source.

Fraction 8 (40 mg), which eluted with 80% EtOAc in hexanes, was similarly chromatographed over Sephadex LH-20 to afford four fractions. Fraction 2 (10 mg), eluted with 4:1 CH_2Cl_2–hexanes, consisted of compound **3**. Known compound **4** was present as a minor constituent in this extract, but was more abundant in the initial screening extract (obtained from a single Erlenmeyer flask containing 50 mL of MMK2 medium cultured, extracted, and partitioned as above). The MeCN-soluble fraction (49 mg) was subjected to silica gel column chromatography using a stepwise gradient of hexanes, EtOAc, and MeOH to afford eight fractions. Fraction 2 (13 mg) was subjected to reversed-phase (C_{18}) HPLC using a gradient of 20–100% MeCN in H_2O to afford **4** (3.6 mg), which was identified by NMR comparison to literature data.[15]

*Hypocoprin A (**1**):* pale yellow solid; $[\alpha]^{20}_D$ +18 (*c* 2.3, MeOH); ^1H and ^{13}C NMR data, see Table 1; HMBC data (acetone-d_6) H-1 → C-3, 11, 13; H-3→ C-1, 13; H_2-4 → C-2, 3, 5; H-7 → C-5, 8, 9, 10, 12; H_2-8 → C-6, 9, 10; H-9 → C-6, 8, 11, 14; H_2-11 → C-1, 2, 14; H_3-12 → C-5, 6, 7; H_3-13 → C-1, 2, 3; H_3-14 → C-9, 10, 11, 15; H_3-15 → C-9, 10, 11, 14; HREITOFMS *m*/*z* 220.1833 $[M - H_2O]^+$ (calcd for $C_{15}H_{26}O_2 - H_2O$, 220.1821).

*Hypocoprin B (**2**):* colorless solid; $[\alpha]^{20}_D$ +13 (*c* 0.58, MeOH); ^1H and ^{13}C NMR data, see Table 2; HMBC data ($CDCl_3$) H-1 → C-3, 11, 13; H-3→ C-1, 4, 13; H_2-4 → C-2, 3, 5, 6; H_2-5 → C-3, 4, 6, 7, 12; H-7 → C-5, 9, 10, 12; H_2-8 → 6, 7, 9, 10; H-9 → C-6, 7, 8, 10; H_2-11 → C-1, 2, 14, 15; H_2-12 → C-5, 7; H_3-13 → C-1, 2, 3; H_3-14 → C-9, 10, 11, 15; H_3-15 → C-9, 10, 11, 14; HREITOFMS *m*/*z* 220.1826 $[M]^+$ (calcd for $C_{15}H_{24}O$, 220.1821).

*Hypocoprin C (**3**):* colorless solid; $[\alpha]^{20}_D$ +22 (*c* 0.49, MeOH); ^1H and ^{13}C NMR data, see Table 1; HMBC data (acetone-d_6) H-1 → C-3, 13; H-3→ C-5, 13; H_2-4 → C-2, 3, 5, 6; H-5 → C-6, 7, 12; H-7 → C-5, 10, 12; H_2-8 → C-6, 9, 10; H-9 → C-6, 8, 11, 14; H_2-11 → C-1, 2, 9, 14, 15; H_3-12 → C-5, 6, 7; H_3-13 → C-1, 2, 3; H_3-14 → C-9, 10, 11, 15; H_3-15 → C-9, 10, 11, 14; HRESITOFMS *m*/*z* 277.1780 $[M + Na]^+$ (calcd for $C_{15}H_{26}O_3$, 277.1773).

Preparation of Mosher Esters of 1.[11,12] A solution of **1** (0.5 mg) in pyridine-d_5 (500 μL) was combined with a solution of pyridine-d_5 (6.6 μL) and *R*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (*R*-MTPA-Cl, 6.5 μL). The resulting mixture was stirred with a Teflon-coated magnetic stir bar for 24 h at room temperature in a Teflon-lined screw-cap vial, resulting in formation of the crude *S*-MTPA ester. The solution was placed in an NMR tube for direct analysis. Analogous treatment of an additional 0.5 mg portion of **1** using *S*-MTPA-Cl afforded the *R*-MTPA ester. Well-resolved ^1H NMR signals (400 MHz, pyridine-d_5) showing significant (>0.01 ppm) $\Delta\delta$ ($\delta_S - \delta_R$) values: *S*-MTPA ester δ 1.66 (s, H_3-13), 0.95 (s, H_3-15), 1.84 (dd, J = 14, 3.2, H-11$_{eq}$); *R*-MTPA ester: δ 1.80 (s, H_3-13), 0.92 (s, H_3-15), 1.75 (dd, J = 14, 3.2, H-11$_{eq}$).

*Acetylation of **1**.* A solution of **1** (1 mg) in pyridine (0.2 mL) was treated with acetic anhydride (0.2 mL), and the reaction mixture was stirred at room temperature for 24 h followed by evaporation to dryness to obtain the monoacetylated product (1 mg). ^1H NMR data (acetone-d_6, 400 MHz) δ 0.14 (dt, 9.2, 4.8 Hz, H_a-8), 0.40 (dt, 9.2, 4.8 Hz, H_b-8), 0.51 (m, H-9), 0.70 (s, H_3-14 or 12), 0.71 (s, H_3-12 or 14), 0.85 (m, H-7), 0.93 (s, H_3-15), 1.50 (dd, 3.2, 14 Hz, H_a-11), 1.70 (t, 1.2 Hz, H_3-13), 1.80 (m, 2H, H_a-5 and H_b-11), 1.96 (s, acetyl CH_3), 2.01 (m, H_a-4), 2.30 (m, H_b-4), 5.35 (dd, 3.2, 12 Hz, H-1), 5.52 (br d, 12 Hz, H-3).

*Acetylation of **3**.* A solution of **3** (1 mg) in pyridine (0.2 mL) was treated with acetic anhydride (0.4 mL), and the reaction mixture was stirred at room temperature for 24 h followed by evaporation to dryness to obtain the diacetylated product (1 mg). ^1H NMR data (acetone-d_6, 400 MHz) δ 0.25 (dt, 9.6, 5.2 Hz, H_a-8), 0.47 (dt, 9.6, 5.2 Hz, H_b-8), 0.60 (dt, 9.6, 5.2 Hz, H-9), 0.73 (s, H_3-14 or H_3-12), 0.77 (s, H_3-12 or H_3-14), 0.87 (m, H-7), 0.95 (s, H_3-15), 1.54 (dd, 3.6, 14 Hz, H_a-11), 1.73 (t, 1.2 Hz, H_3-13), 1.94 (m, H_b-11), 1.97 (s, acetyl CH_3), 2.00 (s, acetyl CH_3), 2.29 (m, H-4$_a$), 2.40 (m, H-4$_b$), 4.95 (dd, 4.8, 12 Hz, H-5), 5.37 (dd, 3.2, 12 Hz, H-1), 5.60 (br d, 12 Hz, H-3).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR data for compounds **1**–**3**, along with details about the organism and its taxonomic placement. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 319-335-1361. E-mail: james-gloer@uiowa.edu.

Notes

The authors declare no competing financial interest.

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■ DEDICATION

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■ REFERENCES

(1) Richardson, M. J. *Mycol. Res.* **2001**, *105*, 387–402.

(2) Wicklow, D. T; Carroll, G. C. *The Fungal Community: Its Organization and Role in the Ecosystem*, 2nd ed.; M. Dekker: New York, 1992; p 715.

(3) Richardson, M. J.; Watling, R. *Keys to Fungi on Dung*; British Mycological Society: Scotland, 1997; p 7.

(4) Gloer, J. B. In *The Mycota: Environmental and Microbial Relationships*, 2nd ed.; Kubicek, C. P.; Druzhinina, I. S., Eds.; Springer: New York, 2007; pp 257–286.

(5) Weber, H. A.; Baenziger, N. C.; Gloer, J. B. *J. Am. Chem. Soc.* **1990**, *112*, 6718–6719.

(6) Bills, G. F.; Gloer, J. B.; An, J. *Curr. Opin. Microbiol.* **2013**, *16*, 549–565.

(7) Doveri, F. *Fungi Fimicoli Italici*; Associazione Micologica Bresadola: Trento, Italy, 2004.

DOI: 10.1021/np5007718
J. Nat. Prod. 2015, 78, 396–401

(8) Krug, J. C.; Benny, G. L.; Keller, H. W. Coprophilous fungi. In *Biodiversity of Fungi, Inventory and Monitoring Methods*; Mueller, G.; Bills, G. F.; Foster, M. S., Eds.; Elsevier Academic Press: Oxford, 2004; pp 467−499.
(9) Griffiths, D. *Mem. Torrey Bot. Club* **1901**, *11*, 1−134.
(10) El-Gamal, A. H.; Wang, S.-K.; Dai, C.-F.; Chenwa, I.-G.; Duh, C.-Y. *J. Nat. Prod.* **2005**, *68*, 74−77.
(11) Hoye, T. R.; Jeffery, C. S.; Shao, F. *Nat. Protoc.* **2007**, *2*, 2451−2458.
(12) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092−4096.
(13) Zubia, E.; Spinella, A.; Giusto, G. B.; Crispino, A.; Cimino, G. *Tetrahedron Lett.* **1994**, *35*, 7069−7072.
(14) Yamakawa, K.; Ishibashi, N.; Arakawa, K. *Chem. Pharm. Bull.* **1964**, *12*, 121−124.
(15) Tschen, J. S.-M.; Chen, L. L.; Hsieh, S.-T.; Wu, T.-S. *Bot. Bull. Acad. Sin.* **1997**, *38*, 251−256.
(16) White, T. J.; T. Bruns, T.; Lee, S.; Taylor, J. In *PCR Protocols: A Guide to Methods and Applications*; Innis, N.; Gelfand, D.; Sninsky, J.; White, T., Eds.; Academic Press: New York, 1990; pp 315−322.
(17) Vilgalys, R.; Hester, M. *J. Bacteriol.* **1990**, *172*, 4238−4246.
(18) Cubeta, M. A.; Echandi, E.; Abernethy, T.; Vilgalys, R. *Phytopathology* **1991**, *81*, 1395−1400.
(19) Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. *Mol. Biol. Evol.* **2013**, *30*, 2725−2729.
(20) Bauer, A. W.; Kirby, W. M.; Sherris, J. C.; Turck, M. *Am. J. Clin. Pathol.* **1966**, *45*, 493−496.
(21) Wagenaar, M. M.; Clardy, J. *J. Nat. Prod.* **2001**, *64*, 1006−1009.

DOI: 10.1021/np5007718
J. Nat. Prod. 2015, 78, 396−401