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Antibacterial Spirobisnaphthalenes from the North American Cup Fungus *Urnula craterium*

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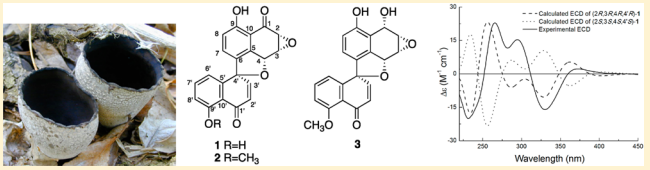
Supporting Information

ABSTRACT: Urnucratins A−C (**1**−**3**), which possess an unusual bisnaphthospiroether skeleton with one oxygen bridge and one C−C bridge and represent a new subclass of bisnaphthalenes, were isolated from the North American cup fungus *Urnula craterium*. Their structures, including absolute configurations, were determined by means of HRMS, NMR, and quantum chemical CD calculations. Urnucratin A (**1**) was found to be active against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, and *Streptococcus pyogenes* with MIC values of 2, 1, and 0.5 μg/mL, respectively.



Antibiotic-resistant bacteria continue to cause many harmful infections around the world. New anti-infective drugs are needed to treat these infections, and searching for compounds from diverse natural resources has been a key starting point in the development of new antimicrobials.[1] As part of our recent screening efforts, about 350 species of wild North American mushrooms and other macrofungi were examined for antibacterial activity, and two series of compounds with modest potencies were previously described.[2,3]

In additional work, the crude extract obtained from the saprobic fungus *Urnula craterium* ("devil's urn") showed promising antibacterial activity. *U. craterium* is a species of cup fungus in the family Sarcosomataceae (Ascomycota) that parasitizes oak and other hardwoods. The distribution of *U. craterium* includes eastern North America, Europe, and Asia. Few studies have examined the bioactive metabolites produced by *U. craterium*, although a previous investigation identified several simple lactone-type compounds and one naphthalone.[4]

In the present study, bioassay-guided isolation of secondary metabolites from *U. craterium* led to the purification and structure elucidation of three novel spirobisnaphthalenes, urnucratins A−C (**1**−**3**) (Figure 1), with broad activity against Gram-positive bacteria. These compounds contained an

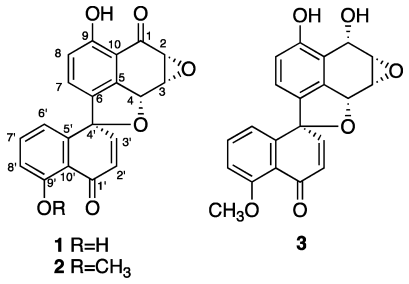


Figure 1. Structures of compounds **1**−**3**.

unusual bisnaphthospiroether skeleton and could be classified as a new chemical subclass of the spirodioxynaphthalenes that contain two 1,8-dihydroxynaphthalene (DHN)-derived spiroether units bridged through a spiroether linkage. A related series of compounds containing a dimeric naphthalene structure, such as daldinol and its analogues, has been described previously.[5] Additionally, since the metabolite MK3018 was first isolated in 1989 from the fungus *Tetroploa aristata*,[6] more

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of Compounds 1−3 in $CDCl_3$

	urnucratin A (1)		urnucratin B (2)		urnucratin C (3)	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	195.1, C		196.1, C		69.2, CH	5.52 brs
2	51.7, CH	4.18 dd (1.0, 4.0)	51.9, CH	4.20 d (3.6)	55.4, CH	3.72 d (4.5)
3	53.7, C	3.66 d (4.0)	53.8, CH	3.66 d (4.2)	52.0, CH	3.90 d (4.5)
4	79.8, CH	6.04 brs	80.2, CH	6.05 s	80.9, CH	5.79 s
5	140.0, C		139.6, C		134.5, C	
6	132.8, C		133.5, C		132.3, C	
7	130.7, CH	7.09 d (8.5)	130.9, CH	7.08 d (8.4)	123.2, CH	6.73 d (8.0)
8	119.2, CH	6.93 d (8.5)	119.3, CH	6.91 d (8.4)	119.0, CH	6.79 d (8.0)
9	159.3, C		159.4, C		156.0, C	
10	110.2, C		110.2, C		114.4, C	
1′	189.7, C		184.0, C		184.6, C	
2′	127.6, C	6.39 d (10.0)	129.8, CH	6.33 d (9.6)	129.0, CH	6.26 d (10.0)
3′	149.1, C	6.79 d (10.0)	145.2, CH	6.67 d (9.7)	146.5, CH	6.65 d (9.5)
4′	86.5, C		87.4, C		86.6, C	
5′	143.9, C		146.5, C		147.5, C	
6′	117.6, CH	6.80 d (8.0)	118.5, CH	6.96 d (7.8)	118.8, CH	6.95 d (7.5)
7′	136.1, CH	7.42 dd (8.0, 8.0)	134.5, CH	7.48 dd (7.8, 8.4)	134.3, CH	7.45 dd (7.5, 8.5)
8′	118.1, CH	6.97 d (8.0)	112.3, CH	7.01 d (8.4)	111.7, CH	6.96 d (8.5)
9′	162.4, C		160.5, C		160.2, C	
10′	113.7, C		118.9, C		118.5, C	
9-OH		9.28 s		9.26 s		
9′−OH		12.35 s				
9′-OCH_3			56.5, CH_3	3.99 s	56.2, CH_3	3.96 s

than 100 members of the bisnaphthospiroether class of compounds have been discovered, and they show a variety of biological activities, including antitumor,[7] antibacterial,[8] antifungal,[9] antileishmanial,[10] and enzyme inhibitory effects.[11] A review of the structure, bioactivities, biosynthesic relationship, and chemical synthesis of spirodioxynaphthalenes has been published by Cai et al.[12] This type of structure was divided into three subclasses by Munro in 2003:[13] (1) compounds with two oxygen bridges, such as palmarumycin CP1 and its analogues,[14] (2) compounds with three oxygen bridges, such as preussomerin A,[15] and (3) compounds with two oxygen bridges and one C−C bridge, such as spiroxin.[16]

Here, we report on a new subclass of the spirobisnaphthalenes, the urnucratins A−C (**1**−**3**), that possess one oxygen bridge and one C−C bridge. We also determined their antimicrobial activities against selected Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE). The general structure of the urnucratins may be described as one substituted naphthalene ring system and one partially saturated substituted naphthalene ring system joined by spiroether and carbon−carbon linkages. The partially saturated naphthalene ring is fused with an epoxide ring.

RESULTS AND DISCUSSION

Compound **1** was obtained as a brown powder, and its molecular formula of $C_{20}H_{12}O_6$, which possessed 15 degrees of unsaturation, was determined from its HRESIMS. Combined analysis of the ^{1}H NMR, ^{13}C NMR, DEPT, and HMBC spectra of **1** (Table 1) revealed that there were 21 carbon signals, which included two carbonyl groups (δ_C 195.1 and 189.7), three oxygenated methines (δ_H/δ_C 3.66/53.7, 4.18/51.7, and 6.04/79.8), one quaternary oxygenated carbon (δ_C 86.5), and 14 aromatic or alkene carbons. Two sharp, hydrogen-bonded proton singlets at δ_H 9.28 and 12.35 were determined to be two hydroxyl groups substituted at the aromatic carbons C-9 (δ_C 159.3) and C-9′ (δ_C 162.4), respectively, based on the HMBC correlation of δ_H 9.28/δ_C 159.3 and δ_H 12.35/δ_C 162.4. According to the $^{1}H-^{1}H$ COSY spectrum of **1**, correlations of H-2 (δ_H 4.18)/H-3 (δ_H 3.66), H-3/H-4 (δ_H 6.04), H-2′ (δ_H 6.39)/H-3′ (δ_H 6.79), H-7 (δ_H 7.09)/H-8 (δ_H 6.93), H-6′ (δ_H 6.80)/H-7′ (δ_H 7.42), and H-7′/H-8′ (δ_H 6.97) were observed. Further, four substructure units could be established as $-C_2H-C_3H-C_4H-$, $-C_{2'}H=C_{3'}H-$, $-C_7H=C_8H-$, and $-C_{6'}H=C_{7'}H-C_{8'}H=$. From the HMBC spectrum of **1**, the correlations of H-7/C-9, H-7/C-5, H-8/C-10, H-2/C-1, H-2/C-10, H-3/C-4, H-3/C-5, H-4/C-5, and H-4/C-6 were found, and the first substructure of **1** was established as **1a** (Figure 2a). HMBC correlations of H-2′/C-10′, H-3′/C-4′, H-3′/C-5′, H-

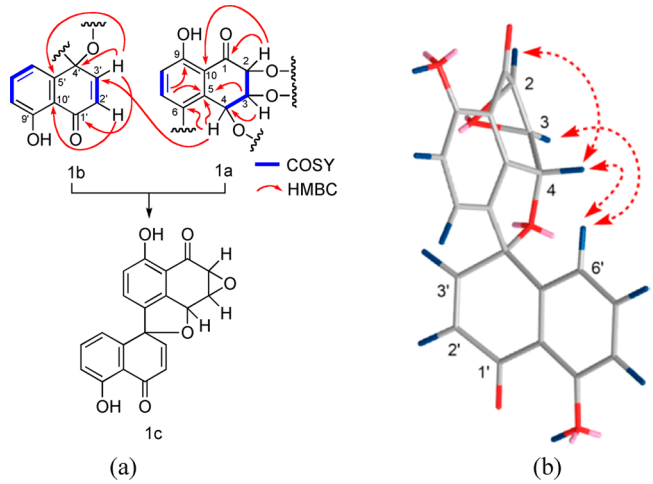

Figure 2. (a) Partial structures of key $^{1}H-^{1}H$ COSY and HMBC correlations of **1**. (b) Key NOESY correlations of **1**.

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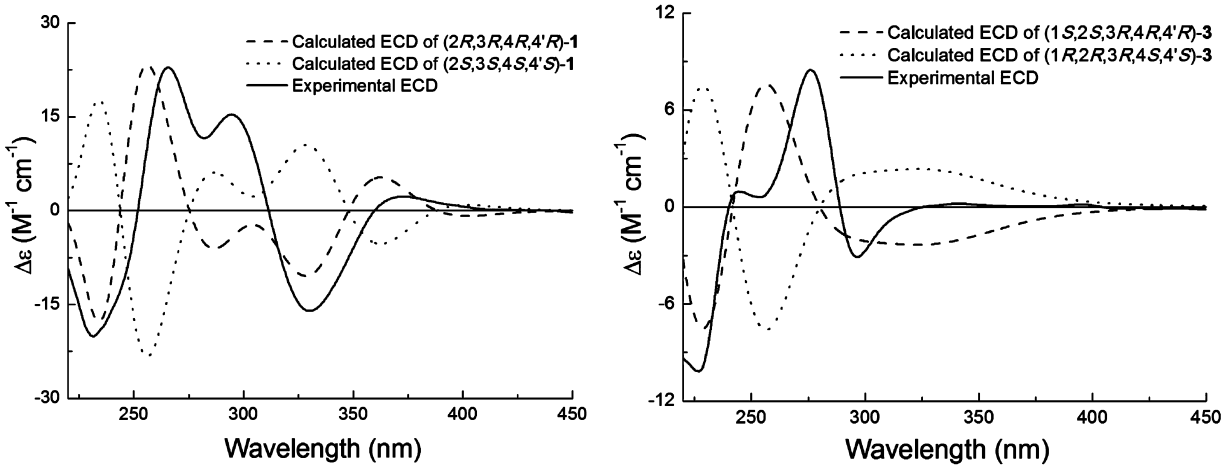

Figure 3. Calculated and experimental CD spectra of **1** and **3**.

6′/C-10′, H-6′/C-8′, H-8′/C-9′, H-8′/C-10′, H-8′/C-6′, H-7′/C-9′, and H-7′/C-5′ revealed that there was an aromatic ring connecting to the double bonds via a quaternary oxygenated carbon (C-4′).

There was a typical signal pair for the double bond [H-2′ (δ_H 6.39)/C-2′ (δ_C 127.6), H-3′ (δ_H 6.79)/C-3′ (δ_C 149.1)] signals conjugated to the carbonyl group C-1′ (δ_C 189.7). This could be further confirmed by the HMBC correlation of H-3′/C-1′. Thus, the second substructure of **1** was deduced as **1b** (Figure 2a). A weak $^4J_{C,H}$ correlation between H-4 and C-3′ was observed in the HMBC spectrum of **1**, and this revealed that substructures **1a** and **1b** were connected through an oxygen substituted on both C-4 and C-4′. As a consequence, there were two degrees of unsaturation remaining, which meant that two more rings needed to be formed to complete the structure of **1**. Since all of the atoms on **1** had been assigned to substructures **1a** and **1b**, a connection between these two substructures could be formed by a C−C bridge located at C-6 and C-4′. The last possible ring on **1** could be formed only between C-2 and C-3 as an epoxide. The existence of the epoxide functionality was also evident from one pair of coupled proton doublets resonating between 3.5 and 4.6 ppm and the two high-field oxygen-bearing carbon resonances between 51 and 54 ppm. Thus, the planar structure of **1** was established as **1c** (Figure 2a), and all of the signals in the 2D NMR spectra were assigned unambiguously (Table 1). Compound **1** contains an unusual bisnaphthalospiroether skeleton with one oxygen bridge and one C−C bridge and therefore represents a new subclass of bisnaphthalenes. Its structure is different from the most notable bisnaphthalenes, the spiroxins, which contain two oxygen bridges and one C−C bridge.[16]

The relative configuration of **1** was determined by the NOESY correlations of H-4/H-6′, H-3/H-6′, and H-2/H-4 (Figure 2b).

Compound **2** was obtained as a brown powder, and its molecular formula was deduced as $C_{21}H_{14}O_6$ from its HRESIMS. Reviewing the ^{1}H and ^{13}C NMR data revealed that these were very similar to those of **1**, except that there was one methoxyl group (δ_H/δ_C 3.99/56.5) in **2** replacing one of the hydroxyl groups of **1**. Thus, compound **2** was deduced to have the same spirobisnaphthalene skeleton as **1**. Further, HMBC analysis revealed that the methoxyl group was substituted on C-9′ based on the correlation between the methoxyl group (δ_H 3.99) and C-9′ (δ_C 160.5). All of the chemical shifts of **2** were assigned unambiguously in the ^{1}H NMR, ^{13}C NMR, HMQC, and HMBC experiments (Table 1). The relative configuration of **2** was determined by NOESY.

Compound **3** was isolated as a yellow powder, and its molecular formula was found to be $C_{21}H_{16}O_6$ on the basis of its HRESIMS. The ^{1}H and ^{13}C NMR of **3** revealed that **3** possessed the same spirobisnaphthalene skeleton as **2**, but the carbonyl group at C-1 in **2** was replaced with a hydroxyl group (δ_H/δ_C 5.52/69.2) at C-1 in **3**.

This observation was confirmed by the HMBC correlations of H-2 (δ_H 3.72)/C-1, H-1/C-3 (δ_C 52.0), H-1/C-5 (δ_C 134.5), H-1/C-9 (δ_C 156.0), H-1/C-10 (δ_C 114.4), H-2/C-10, and H-3 (δ_H 3.90)/C-5. As in **2**, the methoxyl group (δ_H/δ_C 3.96 (s)/56.2) of **3** was located at C-9′ according to the HMBC correlation between its signal at δ_H 3.96 and the signal at δ_C 160.2. In order to determine the relative stereochemical orientation of H-1, NOESY experiments were applied. The NOESY correlation for H-1/H-3 revealed that H-1 had the same orientation as H-3. Careful analysis of the NOESY spectrum of **3** led to the successful establishment of its relative stereochemistry.

To determine the absolute configurations of **1** and **3**, quantum chemical CD calculations were undertaken. The preliminary conformational distribution search was performed using the Sybyl 18 software package using the Tripos force field with default parameters. The corresponding minimum geometries were further fully optimized using DFT at the B3LYP/6-31G(d) level, as implemented in the Gaussian 09 program package. The stable conformers obtained were then submitted to CD calculation by means of time-dependent (TD) DFT calculations (B3LYP/6-31G(d)) with Gaussian 09. The overall predicted CD spectra of **1** and **3** by quantum chemical CD calculations were subsequently compared with the experimental ones. This comprasion revealed good agreement between the calculated and the measured CD curves (Figure 3), whereas the enantiomers of **1** and **3** showed the opposite results, respectively. Thus, the absolute configurations of the chiral carbons were determined to be 2*R*, 3*R*, 4*R*, and 4′*R* in **1** and 1*S*, 2*S*, 3*R*, 4*R*, and 4′*R* in **3** (Figure 1). The absolute configuration at C-1 of **3** was further confirmed on the basis of the circular dichroism of a complex formed in situ with $[Rh_2(OCOCF_3)_4]$, with the inherent contribution subtracted. The Rh complex of **3** displayed a positive E band (Figure 4), correlating with a 1*S* absolute configuration.[17,18]

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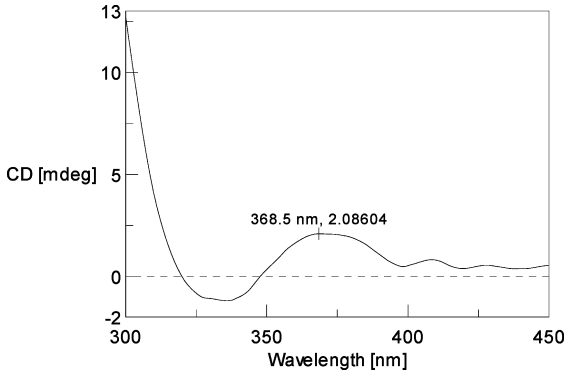

Figure 4. CD spectrum of the Rh complex of **3** with the inherent CD spectrum subtracted.

The absolute stereochemistry of **2** was found to be the same as **1** by the comparison of their optical rotations, as well as by their proposed biogenesis (Figure S23).[19]

The antimicrobial activities of urnucratins A–C against the Gram-positive bacteria *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *S. aureus* (MRSA, MW2), vancomycin-resistant *Enterococcus faecium* (VRE), *E. faecalis* (ATCC 29212), and *Streptococcus pyogenes* (UWL) were determined. The results of these standard minimum inhibitory concentration (MIC) assays are displayed in Table 2. As a unique class of spirobisnaphthalenes, urnucratins A (**1**) and B (**2**) showed the most promising antimicrobial potencies against the four pathogens tested.

Table 2. Antibacterial Activity of Urnucratins A–C (1–3)

	MIC value (μg/mL)				
compound	*S. aureus* (ATCC 29213)	*MRSA* (MW2)	*E. faecium* (VRE1)	*E. faecalis* (ATCC 29212)	*S. pyogenes* (UWL)
urnucratin A (**1**)	2	2	1	0.5	0.5
urnucratin B (**2**)	8	8	8	8	4
urnucratin C (**3**)	64	64	64	64	64
vancomycin[a]	1	1	64	2	1
tetracycline[a]	0.25	0.125	4	16	0.125

[a]Vancomycin and tetracycline: positive controls.

None of the urnucratins showed activity against the Gram-negative bacteria that were tested (*E. coli* and *P. aeruginosa*). The significantly higher potencies of urnucratins A and B, relative to urnucratin C, indicate that the presence of the carbonyl group in the structure of urnucratins A and B is most likely associated with their antibacterial activity. Finally, the cytotoxicities of **1–3** were assessed using the trypan blue exclusion method against J774A.1 murine monocyte cells, and all had IC_{50} values greater than 100 μM.[20]

More than 100 bisnaphthalene analogues have been studied, and this class of compounds is reported to possess a broad range of biological activities.[12] Among these bisnaphthalenes, xanalteric acids I and II, recently isolated from an *Aspergillus* species, were found to be antimicrobial but had relatively weak anti-MRSA activities.[21] The urnucratins, a new subclass of bisnaphthalenes reported here for the first time, may offer a promising entry point for the development of new anti-infective drugs of novel structure, due primarily to their observed low MIC values and relative lack of toxicity. Further investigation of the mechanism of action of this class of compounds is warranted, and additional studies are now under way in our laboratories.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with an Autopol IV automatic polarimeter (Rudolph Research Analytical, USA) in CH_2Cl_2 at 25 °C. UV spectra were recorded with a BioSpec-1601 spectrophtometer (Shimadzu, Japan). CD spectra were recorded on a JASCO J-815 spectropolarimeter. The ^1H and ^{13}C NMR, HMQC, HMBC, ^1H–^1H COSY, and NOESY experiments were carried out on Varian Inova 500 MHz and Bruker 600 MHz spectrometers in $CDCl_3$, and the chemical shifts (δ) are reported in parts per million (ppm) relative to Me_4Si (δ = 0) as an internal standard. The coupling constants (J values) are shown in Hz. HRESIMS was obtained using an Agilent LC/MSD TOF. Analytical and preparative TLCs were performed by using 250 and 500 μm precoated silica gel 60 Å F_{254} plates (Whatman Partisil), repectively. HPLC was carried out using an Alltech Econosphere C_{18} column (4.6 × 150 mm, 5 μm; 22 × 250 mm, 10 μm) with a Waters 600E pump and 996 photodiode array detector. Sephadex LH-20 was purchased from Merck Co., Ltd.

Isolation and Purification. The fruiting bodies of *Urnula craterium* were collected in La Crosse County, Wisconsin, USA, in May 2009. The specimens were identified by T.J.V., and a voucher (registration no. F110) was deposited in the herbarium at the Department of Biology, University of Wisconsin–La Crosse (UWL).

The dried, powdered fungal fruiting bodies ("mushrooms") of *U. craterium* (35.0 g) were extracted with 3% MeOH in CH_2Cl_2 (300 mL) by stirring overnight at room temperature to yield 1.1 g of extract after evaporation of the solvents under vacuum. A 100 mg aliquot of the *U. craterium* crude extract was separated on a preparative TLC plate (Whatman Partisil, silica gel 60 Å, size 20 × 20 cm, layer thickness 500 μm; CH_2Cl_2–MeOH (96:4)), which created five subfractions. The active subfraction 2 was further purified on a Sephadex LH-20 column (20 mm × 300 mm, CH_2Cl_2–MeOH (1:1)), leading to pure compound **1** (22.0 mg). Next, 330 mg of crude extract was placed on two preparative TLC plates (Whatman Partisil, silica gel 60 Å, size 20 × 20 cm, layer thickness 500 μm) and developed by a solvent system of CH_2Cl_2–MeOH (9:1). Two subfractions, UC06-1 (120 mg) and UC06-2 (230 mg), resulted. Active subfraction UC06-1 was further separated on a preparative TLC plate developed using CH_2Cl_2–MeOH (99:1), followed by purification on a Sephadex LH-20 column (20 mm × 300 mm; CH_2Cl_2–MeOH (1:1)), which identified pure compound **2** (15.0 mg). For identification of compound **3**, the crude extract of *U. craterium* (680 mg) was subjected to flash column silica gel chromatography (Φ 45 mm × 300 mm), eluting with a gradient mixture of CH_2Cl_2–EtOAc (9:1, 8.5:1.5, 8:2, 7:3, 1:1, 4:6, and 0:1, each 600 mL). Eleven fractions, UC28-1–11 were collected. The active fractions UC28-9 (38.3) and UC28-10 (11.6 mg) were combined and then partitioned on a preparative TLC plate (Whatman Partisil, silica gel 60 Å, size 20 × 20 cm, layer thickness 500 μm; CH_2Cl_2–MeOH (97:3)) forming eight subfractions, UC31-1–8. The most active subfraction, UC31-5 (18.6 mg), was purified on a Sephadex LH-20 column (20 mm × 300 mm), eluting with a mixture of CH_2Cl_2–MeOH (1:1) to give **3** (14.0 mg).

Urnucratin A (1): brown powder; $[\alpha]^{30}_D$ −4.1 (CH_2Cl_2; c 0.09); UV (MeOH) λ_{max} 225, 260, and 342 nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS m/z 349.0694 $[M + H]^+$, calcd 349.0712.

Urnucratin B (2): brown powder; $[\alpha]^{30}_D$ −4.0 (CH_2Cl_2; c 0.12); UV (MeOH) λ_{max} 229, 257, and 339 nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS m/z 385.0663 $[M + Na]^+$, calcd 385.0688.

Urnucratin C (3): brown powder; $[\alpha]^{30}_D$ +1.7 (CH_2Cl_2; c 0.16); UV (MeOH) λ_{max} 228, 279, and 338 nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS m/z 365.0982 $[M + H]^+$, calcd 365.1025.

Antimicrobial Activity. The MIC assays were performed according to Clinical and Laboratory Standards Institute guidelines, and all end points were recorded in μg/mL.[22] *Staphylococcus aureus*

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(ATCC 29213), methicillin-resistant *S. aureus* (MRSA, MW2), vancomycin-resistant *Enterococcus faecium* (VRE1), *E. faecalis* (ATCC 29212), and *Streptococcus pyogenes* (UWL) were used in these assays. Vancomycin (Sigma-Aldrich, St. Louis, MO; purity >90%) and tetracycline (Sigma-Aldrich, St. Louis, MO; purity 99%) served as the positive antibacterial controls.

Cytotoxicity Assays. Cytotoxicities of purified natural products **1**–**3** were assessed using the tryptan blue exclusion method against J774A.1 murine monocyte cells according to the method of Hathaway et al.[20] Puromycin (Sigma Chemical Co.; purity >98%) was used as positive control (IC_{50} = 10 μM).

■ ASSOCIATED CONTENT

Supporting Information

Detailed descriptions of the experimental procedures, a listing of HRMS and NMR spectra for compounds **1**–**3**, and the quantum chemical CD calculations and experimental CD spectra for compounds **1** and **3** are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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