Epicoccins A–D, Epipolythiodioxopiperazines from a *Cordyceps*-Colonizing Isolate of *Epicoccum nigrum*

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Epicoccins A–D (**1**–**4**), four unique epipolythiodioxopiperazines possessing unusual sulfur bridges, have been isolated from cultures of a *Cordyceps*-colonizing isolate of *Epicoccum nigrum*. The structures of these compounds were determined mainly by analysis of their NMR spectroscopic data. The relative and absolute configuration of epicoccin A (**1**) was established by single-crystal X-ray diffraction analysis. Epicoccin A (**1**) showed modest antimicrobial activity.

Natural products containing the diketoperazine moiety and sulfur bridge(s) have been isolated frequently from fungal sources. These compounds include the rostratins,[1] the epicorazines,[2–6] the leptosins,[7–10] sch52900,[11] gliotoxin,[12] the verticillins,[13,14] the chaetoseminudins,[15] gliocladine,[16] and the bionectins.[17] They displayed antiproliferative,[6] cytotoxic,[6,7,9,10,15] antitumor,[7] immunonodulatory,[15] antinematodal,[16] and antibacterial activities.[17] *Cordyceps sinensis* (Berk.) Sacc. (reclassfied as *Hirsutella sinensis* later),[18] also known as Chinese caterpillar fungus or "DongChongXiaCao" (summer-plant, winter-worm), has been widely used as a tonic and/or medicine for hundreds of years in the Orient. *Cordyceps* is a unique black, blade-shaped fungus found primarily at high altitude on the Qinghai-Tibetan plateau and endophytically parasitizes on dead caterpillars of the moth *Hepilus* spp., which lives 6 in. underground. In late autumn, chemicals on the skin of the caterpillars interact with the fungal spores and release the fungal mycelia, which then infect the caterpillar. By early summer of the following year, the fungal infestation has killed the caterpillar and the fruiting body can be seen protruding from the caterpillar's head. Chemical studies of *C. sinensis* have shown that the species can produce many different bioactive compounds, and the medicinal benefits of *C. sinensis* have been demonstrated extensively.[19]

During our ongoing investigations of unique fungal species as sources of new antibacterial natural products, a subculture of an isolate of *Epicoccum nigrum* (2203), a nonsporulating fungus colonizing *Cordyceps sinensis* (Berk.) Sacc., was grown in solid-substrate fermentation culture. Its organic solvent extract displayed antibacterial activity against *Bacilus subtilis* (ATCC 6633). Bioassay-guided fractionation of this extract led to the isolation of four new secondary metabolites, that have been named epicoccins A–D (**1**–**4**). Details of the isolation, structure elucidation, and biological activity of these compounds are reported here.

Epicoccin A (**1**) was obtained as a colorless powder. Its molecular formula was determined as $C_{18}H_{18}N_2O_6S_3$ (10 degrees of unsaturation) by HRCIMS analysis [*m/z* 455.0391 $(M + H)^+$; Δ −0.9 mmu], and was supported by ^{1}H and ^{13}C NMR data (Table 1). Analysis of ^{1}H, ^{13}C, and HMQC NMR data for epicoccin A (**1**) revealed the presence of four methylenes, eight methines (two oxymethines), two quaternaries, and four carbonyl carbons. These data accounted for all but two exchangeable protons and three sulfur atoms. Analysis of the COSY NMR data led to the identification of two isolated proton spin systems corresponding to the C-3–C-6 via C-9 and C-3′–C-6′ via C-9′ subunits of structure **1**. HMBC correlations of H_2-3, H-6a, and H-7 with the ketone carbon C-5 (δ_C 207.8) led to the completion of one cyclohexanone unit, while correlations of H_2-3′ and H-7′ with the other ketone carbon C-5′ (δ_C 207.4) revealed the presence of the second cyclohexanone unit in **1**. The chemical shifts of C-8 and C-8′ (both at δ_C 64.8) indicated that these two carbons were attached to hydroxy groups. HMBC correlations of H_2-3 with C-1, H-4 with C-2, H_2-3′ with C-1′, and H-4′ with C-2′ indicated that C-1 and C-3 were connected to C-2 and that C-1′ and C-3′ were connected to C-2′. Considering the downfield chemical shifts of C-2 (δ_C 74.2), C-2′ (δ_C 70.5), C-9 (δ_C 62.3), and C-9′ (δ_C 60.2), the presence of two nitrogen atoms in **1**, and the observation of HMBC correlations of H-9 with C-2 and H-9′ with C-2′, both C-2 and C-9 and C-2′ and C-9′ were attached to corresponding nitrogen atoms to complete the diketopiperazine unit. The chemical shifts of C-2, C-7 (δ_C 45.5), C-2′, and C-7′ (δ_C 41.8) indicated that these carbons were connected to sulfur atoms. By comparing the ^{1}H and ^{13}C NMR data of epicoccin A (**1**) with those reported for its closest known analogue rostratin D (**5**),[1] together with the lack of symmetry in **1**, it was clear that epicoccin A possesses significantly different sulfur linkages from those found in rostratins and other known compounds of this class. Since only three sulfur atoms are available, it was postulated that a disulfide bridge was present between C-2 and C-7 and a single sulfur bridge was present between C-2′ and C-7′ based on the observation that the chemical shifts of both C-2 (δ_C 74.2) and C-7

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Table 1. NMR Spectroscopic Data of Epicoccin A (**1**) in DMSO-d_6

position	δ_H[a] (J in Hz)	δ_C,[b] mult.	HMBC (H → C#)
1		162.0, qC	
2		74.2, qC	
3a	2.57, d (13)	45.6, CH_2	2, 4, 5, 9
3b	2.85, br, d (13)	45.6, CH_2	1, 2, 5, 9
4	3.10, br, d (8.5)	43.4, CH	2, 5, 8
5		207.8, qC	
6a	2.46, d (18)	38.0, CH_2	5, 8
6b	3.09, dd (18, 12)	38.0, CH_2	4, 5, 7, 8
7	3.74, dd (12, 5.1)	45.5, CH	5, 8
8	4.62, br, dd (5.1, 3.3)	64.8, CH	4, 6, 9
9	4.48, br, d (8.5)	62.3, CH	2, 3, 4, 5, 7, 8
8-OH	6.08, d (3.3)		7, 8, 9
1′		159.8, qC	
2′		70.5, qC	
3a′	2.76, d (13)	43.3, CH_2	1′, 2′, 5′, 9′
3b′	2.88, br, d (13)	43.3, CH_2	1′, 2′, 4′, 5′, 9′
4′	3.05, br, d (6.8)	45.0, CH	2′, 5′, 6′, 8′
5′		207.4, qC	
6a′	2.88, d (17)	41.3, CH_2	4′, 5′
6b′	3.11, dd (17, 11)	41.3, CH_2	7′, 8′
7′	3.74, br, d (11)	41.8, CH	2′, 5′, 6′, 8′, 9′
8′	3.98, br, s	64.8, CH	4′, 6′, 7′
9′	4.62, br, d (6.8)	60.2, CH	2′, 3′, 8′
8′-OH′	6.19, br, s		7′, 8′, 9′

[a] Recorded at 400 MHz. [b] Recorded at 100 MHz.

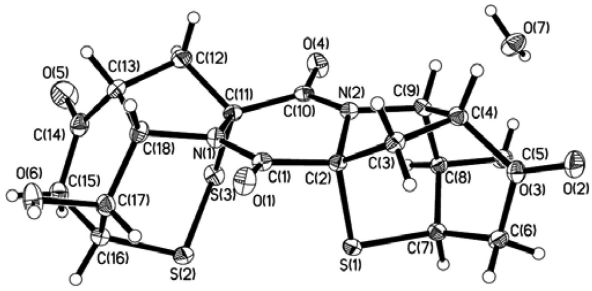

Figure 1. Thermal ellipsoid representation of **1**.

(δ_C 45.5) are 3.7 ppm downfield from those of C-2′ (δ_C 70.5) and C-7′ (δ_C 41.8) in the ^{13}C NMR spectrum of **1**.

Ultimately, the structure of epicoccin A (**1**) was confirmed by single-crystal X-ray crystallographic analysis, and a perspective ORTEP plot is shown in Figure 1. The X-ray data allowed determination of the relative configuration of epicoccin A, as depicted in **1**. In addition, the presence of sulfur atoms in compound **1** and the value of the Flack parameter $(-0.0357)^{20}$ determined by X-ray analysis also allowed assignment of the absolute configuration of all chiral centers in **1** as 2*R*, 4*R*, 7*R*, 8*R*, 9*S*, 2′*R*, 4′*R*, 7′*R*, 8′*R*, and 9′*S*.

The molecular formula of epicoccin B (**2**) was established to be $C_{18}H_{18}N_2O_7S_3$ (10 degrees of unsaturation) by analysis of its HRCIMS [*m*/*z* 471.0333 $(M + Na)^+$; Δ −1.6 mmu] and NMR data (Table 2), which is 16 mass units higher than that of compound **1**. Analysis of the ^1H and ^{13}C NMR data of **2** revealed the presence of structural features similar to those found in **1** (the disulfide portion of the molecule remained unchanged), except that one methylene unit (δ_H 2.88, 3.01; δ_C 41.3) on the right cyclohexanone moiety of the structure was replaced by an oxygenated methine unit (δ_H 4.36; δ_C 78.2) in the spectra of **2**. Analysis of ^1H–^1H COSY NMR data for **2** revealed the connectivity of this methine carbon to C-8′, indicating a change in the position of the sulfur linkage on the right half of compound **2**. In addition, key HMBC correlations of one exchangeable proton (δ_H 5.73; 7′-OH) with C-7′ and C-8′ and another exchangeable proton (δ_H 5.54; 8′-OH) with C-7′ and C-9′ were observed, indicating that C-7′ and C-8′ bear hydroxy groups. Although no COSY correlation (or discernible coupling) between H-6′ and H-7′ was observed, HMBC correlations of H-6′ with C-4′, C-5′, C-7′, and C-8′ positioned C-6′ between C-5′ and C-7′. These results required C-2′ and C-6′ to be connected to the same sulfur atom to form a sulfide linkage, and key HMBC correlation of H-6′ with C-2′ further supported this assignment. On the basis of these data, the planar structure of epicoccin B was established as shown in **2**.

Table 2. NMR Spectroscopic Data of Epicoccin B (**2**) in DMSO-d_6

position	δ_H[a] (J in Hz)	δ_C,[b] mult.	HMBC (H → C#)	NOESY[c]
1		161.8, qC		
2		73.7, qC		
3a	2.55, d (13)	45.6, CH_2	2, 4, 5, 9	
3b	2.85, br, d (13)	45.6, CH_2	1, 2, 4, 5	
4	3.10, br, d (8.5)	43.2, CH	2, 3, 5	
5		207.9, qC		
6a	2.46, d (19)	38.0, CH_2	5, 7, 8	
6b	3.09, br, d (19)	38.0, CH_2	5, 7	
7	3.74, br, s	45.4, CH	5, 8	
8	4.55, dd (7.0, 3.5)	64.8, CH	4, 6	
9	4.48, br, d (8.5)	62.4, CH	2, 3, 4, 5, 7, 8	
8-OH	6.09, d (3.5)		7, 8, 9	
1′		160.9, qC		
2′		73.2, qC		
3a′	2.85, br, d (13)	51.9, CH_2	2′, 5′, 9′	
3b′	3.01, br, d (13)	51.9, CH_2	2′, 4′, 5′	9′
4′	3.06, br, d (7.0)	45.2, CH	2′, 5′, 6′	6′
5′		203.6, qC		
6′	3.16, br, s	53.6, CH	2′, 4′, 5′, 7′, 8′	4′, 7′-OH
7′	4.36, br, d (6.0)	78.2, CH	5′, 6′, 9′	8′
8′	4.44, br, d (3.5)	63.2, CH	4′, 6′, 7′, 9′	7′
9′	4.58, br, d (7.0)	60.3, CH	2′, 3′, 4′, 5′, 7′, 8′	3b′
7′-OH	5.73, d (6.0)		7′, 8′, 6′	6′
8′-OH′	5.54, d (3.5)		7′, 8′, 9′	

[a] Recorded at 400 MHz. [b] Recorded at 100 MHz. [c] Recorded at 600 MHz.

The relative configuration of epicoccin B (**2**) was assigned by analysis of ^1H–^1H NMR coupling constants and NOESY data, and by comparison of its ^1H NMR data with those of epicoccin A (**1**). No coupling was observed between H-3b′ and H-4′, indicating that both protons must adopt a pseudoequatorial orientation, while a coupling constant of 7.0 Hz between H-4′ and H-9′ suggested a cis relationship between these two protons. The near-zero couplings observed between H-6′ and H-7′, H-7′, and H-8′ indicated that the vicinal angle between these protons was close to 90°. On the basis of these data, and considering the relative configuration established for **1**, the only possible conformation that could satisfy these requirements is depicted in Figure 2. NOESY correlations between H-3b′ and H-9′, H-4′ and H-6′, and H-6′ and H7′–OH further supported these assignments (Figure 2). The absolute configuration of epicoccin B (**2**) was proposed as shown by analogy to epicoccin A (**1**).

The elemental composition of epicoccin C (**3**) was determined to be $C_{18}H_{18}N_2O_6S_4$ (10 degrees of unsaturation) on the basis of HRESIMS [*m*/*z* 487.0131 $(M + H)^+$; Δ −1.1 mmu] and NMR data (Table 3). Detailed analysis of ^1H, ^{13}C, and HMQC NMR data for epicoccin C (**3**) revealed the presence of two methylene carbons, four methine carbons (one of which was oxygenated), one quaternary carbon (δ_C 73.7), and two carbonyl carbons (δ_C 162.8 and 207.8, respectively). These ^1H and ^{13}C NMR data accounted for only half of the carbon and hydrogen compositions given by the molecular formula, suggesting a symmetrical feature for epicoccin C (**3**). On the basis of these observations, and by comparison of its NMR data with those of epicoccin A (**1**), the structure of epicoccin C was proposed as shown in **3** and confirmed by analysis of its ^1H–^1H COSY NMR data. The configuration of epicoccin C (**3**) was again assigned by comparison of its NMR data with those of **1**.

Epicoccin D (**4**) was assigned the molecular formula of $C_{18}H_{18}N_2O_6S_2$ (10 degrees of unsaturation) on the basis of HRCIMS

Table 3. NMR Spectroscopic Data of Epicoccins C (**3**) and D (**4**) in DMSO-d_6

	epicoccin C (**3**)		epicoccin D (**4**)	
position	δ_H[a] (J in Hz)	δ_C,[b] mult.	δ_H[a] (J in Hz)	δ_C,[b] mult.
1/1′		162.8, qC		158.9, qC
2/2′		73.7, qC		71.1, qC
3a/3a′	2.55, d (13)	45.9, CH_2	2.76, d (13)	43.4, CH_2
3b/3b′	2.85, dd (13, 8.7)	45.9, CH_2	2.88, br, d (13, 8.7)	43.4, CH_2
4/4′	3.10, br, dd (8.7, 7.8)	43.2, CH	3.05, m	45.1, CH
5/5′		207.8, qC		207.4, qC
6a/6a′	2.43, br, d (19)	38.0, CH_2	2.88, br, d (19)	41.5, CH_2
6b/6b′	3.06, dd (19, 6.0)	38.0, CH_2	3.11, br, d (19)	41.5, CH_2
7/7′	3.70, br, s	45.3, CH	3.70, br, s	41.3, CH
8/8′	4.47, br, s	65.3, CH	4.00, br, s	65.3, CH
9/9′	4.46, br, dd (8.6, 7.8)	61.9, CH	4.66, br, d (8.2)	60.3, CH
8/8′-OH	6.06, d (3.3)		6.23, br, s	

[a] Recorded at 400 MHz. [b] Recorded at 100 MHz.

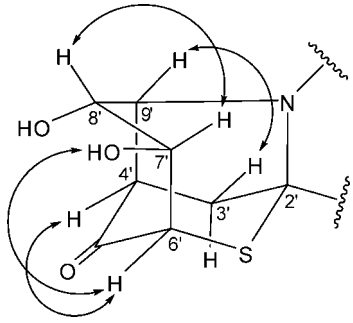

Figure 2. NOESY correlations used to establish the relative configuration for **2**.

analysis [m/z 423.0678 $(M + H)^+$; Δ –0.1 mmu] and NMR data (Table 3). Analysis of ^1H and ^{13}C NMR data of **4** revealed the presence of essentially the same structural features as those found in **3**. Considering the facts that the molecular formula of **4** is 64 mass units (two sulfur atoms) less than that of **3**, and the symmetry that was again evidenced by its ^1H and ^{13}C NMR data, the structure and configuration of epicoccin D were proposed as shown in **4** by comparison of its NMR data with those of **3**.

Epicoccin A (**1**) showed activity against *Bacilus subtilis* (ATCC 6633), affording a zone of inhibition of 12 mm at 100 μg/disk, where epicoccins B–D (**2**–**4**) were inactive at the same level (ciproflozaxin: 28 mm zone of inhibition at 100 μg/disk). However, none of these compounds showed antimicrobial activity against *Staphylococcus aureus* (ATCC 6538), *Streptococcus mutans* (ATCC 25175), *Enterococcus faecalis* (ATCC 19433), and *Sarcina lutea* (CMCC B28001) or antifungal activity against *Geotrichum candidum* (AS2.498), *Candida albicans* (ATCC 10231), and *Aspergillus fumigatus* (ATCC 10894) at 100 μg/disk. Epicoccins A–D (**1**–**4**) were also evaluated against two human cancer cell lines HeLa and HCT116, but none of them showed noticeable growth inhibitory effects.

Epicoccins A–D (**1**–**4**) are new members of the epipolythiodioxopiperazine class of compounds that possess unprecedented sulfur bridges. Presumably, the biosynthesis of epicoccins A–D (**1**–**4**) proceeds in a manner similar to that of other epidithiodioxopiperazines.[13] Rostratin D,[1] the most closely related known compound, could be a biosynthetic precursor for epicoccins A–D (**1**–**4**).[4] Although diketopiperazine-derived metabolites are encountered frequently in nature,[1,2,8,10] compounds containing sulfur bridges like those in **1**–**4** have not been previously reported from natural sources.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on an HP 8453 spectrophotometer. IR data were recorded using a Nicolet Magna-IR 750 spectrophotometer. ^1H and ^{13}C NMR data were acquired with Bruker Avance-400 and Bruker Avance-600 spectrometers using solvent signals (DMSO; δ_H 2.50/δ_C 39.5) as references. The HMQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. ESIMS data were recorded on a Bruker Esquire 3000^{plus} spectrometer. HRESIMS and HRCIMS data were obtained using a Bruker APEX III 7.0 T spectrometer.

Fungal Material. The culture of *E. nigrum* (2203) was isolated by Dr. Mu Wang from a sample of *Cordyceps sinensis* (Berk.) Sacc. that was collected in Linzhi, Tibet, on March 1, 2004. The isolate was identified by Mr. Bingda Sun and assigned the accession number 2203 in Professor X. Liu's culture collection at the Institute of Microbiology, Chinese Academy of Sciences, Beijing. The isolate was subcultured on PDA slants at 25 °C for 15 days. The agar plugs were used to inoculate 250 mL Erlenmeyer flasks, each containing 50 mL of media (0.4% glucose, 1% malt extract, and 0.4% yeast extract), and the final pH of the media was adjusted to 6.5 before sterilization. Flask cultures were incubated at 25 °C on a rotary shaker at 170 rpm for five days. Ten 500 mL Erlenmeyer flasks, each containing 150 mL of liquid media (3% maltose, 1% glucose, 0.08% yeast extract, 0.2% peptone, 0.05% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.001% $FeCl_3$, 0.0002% $ZnSO_4$, 0.0055% $CaCl_2$; final pH 6.0) and 30 g of vermiculite, were individually inoculated with 15 mL of the seed culture and incubated at 25 °C under static conditions for 40 days.

Extraction and Isolation. The fermented material (1.5 L) was freeze-dried and extracted with MEK (3 × 500 mL), and the organic solvent was evaporated to dryness under a vacuum to afford 0.79 g of crude extract. The extract was fractionated by Silica gel VLC using *n*-hexane–CH_2Cl_2–MeOH gradient elution. The fractions that were eluted with 100:5 CH_2Cl_2–MeOH (85 mg) were combined and further separated by semipreparative reversed-phase HPLC (Kramosil C_{18} column; 10 μm; 10 × 250 mm, 2 mL/min) to afford compounds **1**–**4** (**1**, 3.3 mg, t_R 15 min; **2**, 5.6 mg, t_R 18 min; **3**, 14 mg, t_R 21 min; **4**, 6.5 mg, t_R 14 min; 20% MeOH in H_2O over 5 min, 20–100% over 20 min).

Epicoccin A (1): colorless powder; $[\alpha]_D$ +365 (c 0.06, CH_3OH); UV (CH_3OH) λ_{max} 204 (ε 15 100); IR (neat) ν_{max} 3453, 2938, 1706, 1672, 1655, 1410, 994 cm^{-1}; ^1H NMR, ^{13}C NMR, and HMBC data, see Table 1; HRCIMS obsd m/z 455.0391 $[M + H]^+$, calcd for $C_{18}H_{19}N_2O_6S_3$, 455.0400.

X-ray Crystallographic Analysis of Epicoccin A (1).[19] Upon crystallization from MeOH–H_2O (10:1) using the vapor diffusion method, colorless crystals were obtained for **1**. A crystal (0.20 mm × 0.16 mm × 0.08 mm) was separated from the sample and mounted on a glass fiber, and data were collected using a Rigaku Saturn CCD area detector with a graphite monochromator and Mo Kα radiation at −160(1) °C. The cell dimensions were determined to be a = 7.5523(12) Å, b = 11.1218(14) Å, and c = 22.853(3) Å. The 17 832 measurements yielded 4557 independent reflections after equivalent data were averaged and Lorentz and polarization corrections were applied. The final refinement gave R_1 = 0.0375 and wR_2 = 0.0751.

Epicoccin B (2): amorphous powder; $[\alpha]_D$ +111 (c 0.04, CH_3OH); UV (CH_3OH) λ_{max} 212 (ε 14 600) nm; IR (neat) ν_{max} 3436, 2922, 2852, 1706, 1670, 1027 cm^{-1}; ^1H, ^{13}C NMR, and HMBC data, see Table 2; HRCIMS obsd m/z 471.0333 $[M + H]^+$, calcd for $C_{18}H_{18}N_2O_7S_3$, 471.0349.

Epicoccin C (3): amorphous powder; $[\alpha]_D$ +218 (*c* 0.12, CH_3OH); UV (CH_3OH) λ_{max} 204 (ε 13 100); IR (neat) ν_{max} 3457, 2926, 1704, 1677, 1650, 1409, 1051, 998 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; HRESIMS obsd *m*/*z* 487.0131 $[M + H]^+$, calcd for $C_{18}H_{19}N_2O_6S_4$, 487.0120.

Epicoccin D (4): amorphous powder; $[\alpha]_D$ +130 (*c* 0.01, CH_3OH); UV (CH_3OH) λ_{max} 206 (ε 14 500); IR (neat) ν_{max} 3355, 2953, 1710, 1700, 1682, 1615, 1450, 1068, 975 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; HRCIMS obsd *m*/*z* 423.0678 $[M + H]^+$, calcd for $C_{18}H_{19}N_2O_6S_2$, 423.0679.

Antimicrobial and Antifungal Bioassays. Antimicrobial and antifungal bioassays were conducted according to a literature procedure.[22] The bacterial strains were grown on Mueller-Hinton agar, the yeasts *Candida albicans* (ATCC 10231) and *Geotrichum candidum* (AS2.498) were grown on Sabouraud dextrose agar, and the fungus *Aspergillus fumigatus* (ATCC 10894) was grown on potato dextrose agar. Test compounds were absorbed onto individual paper disks (6 mm diameter) at 100 μg/disk and placed on the surface of agar. The assay plates were incubated at 25 °C for 48 h (at 37 °C for 24 h for antimicrobial activity) and examined for the presence of a zone of inhibition.

MTT Assay.[23] In 96-well plates, each well was plated with 10^4 cells. After cell attachment overnight, the medium was removed, and each well was treated with 50 μL of medium containing 0.2% DMSO or an appropriate concentration of test compounds (10 mg/mL as stock solution of a compound in DMSO and serial dilutions). Cells were treated at 37 °C for 4 h in a humidified incubator at 5% CO_2 first, and then, the medium was changed to fresh DMEM. MTT (Sigma) was dissolved in serum-free medium or PBS at 0.5 mg/mL and sonicated briefly. In the dark, 50 μL of MTT/medium was added into each well after the medium was removed from the wells and incubated at 37 °C for 3 h. Upon removal of the MTT/medium, 100 μL of DMSO was added to each well and shook at 60 rpm for 5 min to dissolve the precipitate. The assay plate was read at 540 nm using a microplate reader.

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Supporting Information Available: ^1H, ^{13}C, HMQC, and HMBC spectra of epicoccins A–D (**1**–**4**). This material is available free of charge via the Internet at http://pubs.acs.org.

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