

Diketopiperazines from the *Cordyceps*-Colonizing Fungus *Epicoccum nigrum*Huijuan Guo,[†] Bingda Sun,[†] Hao Gao,[‡] Xulin Chen,[§] Shuchun Liu,[†] Xinsheng Yao,[‡] Xingzhong Liu,[†] and Yongsheng Che^{*†}

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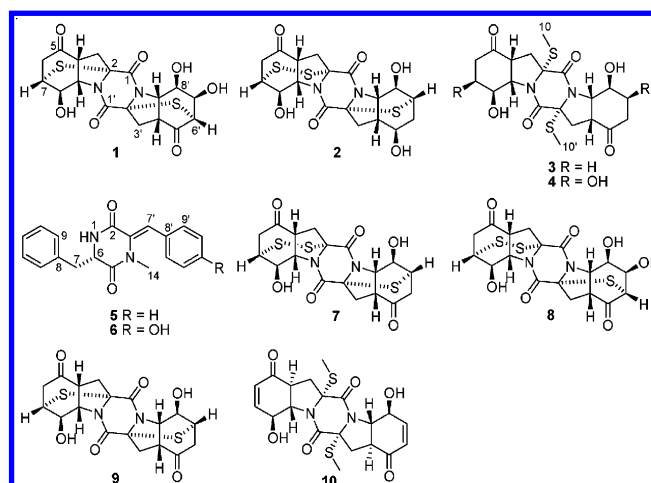
New diketopiperazines, epicoccins E–H (**1–4**) and diphenylalazines A (**5**) and B (**6**), have been isolated from the solid-substrate fermentation culture of the *Cordyceps*-colonizing fungus *Epicoccum nigrum*. The structures of **1–6** were determined primarily by NMR experiments, and the structure of **1** was confirmed by X-ray crystallography. The absolute configurations of **3** and **5** were assigned using the modified Mosher (**3**) and Marfey's (**5**) methods, respectively. Compounds **3–5** showed inhibitory effects on HIV-1 replication in C8166 cells.

Diketopiperazines (DKPs) are an important class of compounds displaying a variety of biological effects, such as antimicrobial, herbicidal, antiviral, immunosuppressive, and antitumor activities.^{1–3} Naturally occurring DKPs have frequently been isolated from fungal sources. Examples include golmaenone, a radical scavenger from a marine-derived *Aspergillus* sp.,⁴ the rostratins, cytotoxic DKP disulfides from a marine-derived *Exserohilum rostratum*,⁵ the epicorazines, antibacterial metabolites from a basidiomycete *Stereum hirsutum*,⁶ and the verticillins, dimeric DKPs from a marine-derived *Penicillium* sp.⁷

Cordyceps sinensis (Berk.) Sacc. (Anamorph: *Hirsutella sinensis*; Clavicipitaceae),⁸ known as Chinese caterpillar fungus or “Dong Chong Xia Cao” (caterpillar-in-winter, herb-in-summer), is the combination of the fungus and the dead caterpillar larva of the moth *Hepilus* spp. It has been known and used for centuries in traditional Chinese medicine (TCM).⁹ In nature, it is found only at high altitudes on the Himalayan Plateau and is therefore difficult to harvest. Chemical investigations of the fungal species that colonize the fruiting body of *C. sinensis* (*Cordyceps*-colonizing fungi) in our laboratory have led to the isolation of structurally diverse and biologically active metabolites.^{10–12} In a search for anti-HIV-1 agents from this unique fungal source, the fungus *Epicoccum nigrum* (XZC04-CS-302) isolated from a sample of *C. sinensis* collected in Linzhi, Tibet, People's Republic of China, was grown in a solid-substrate fermentation culture. Its organic solvent extract showed inhibitory effects on HIV-1 replication in C8166 cells. Fractionation of the extract afforded six new DKPs, named epicoccins E–H (**1–4**) and diphenylalazines A (**5**) and B (**6**), together with the known epicoccins A (**7**), B (**8**), and D (**9**), which were previously isolated by us from a different strain of *Cordyceps*-colonizing *E. nigrum*.¹¹ Details of the isolation, structure elucidation, and anti-HIV-1 activity of these metabolites are reported herein.

Results and Discussion

Epicoccin E (**1**) was assigned the molecular formula C₁₈H₁₈N₂O₇S₂ (11 degrees of unsaturation) on the basis of its HRESIMS (*m/z* 461.0448 [M + Na]⁺; Δ +0.5 mmu). The ¹H and ¹³C NMR data of **1** (Table 1) suggested the presence of three exchangeable protons (δ 5.56, 5.74, and 6.24, respectively), three methylenes, nine methines (five bonded to oxygen or nitrogen), two heteroatom-bonded sp³ quaternary carbons, and four carbonyl



carbons including two ketone ones. These data accounted for all of the ¹H and ¹³C resonances for **1** and revealed structural similarity

Table 1. NMR Spectroscopic Data (500 MHz, DMSO-*d*₆) for **1** and **2**

position	1		2	
	δ _C , mult.	δ _H (J in Hz)	δ _C , mult.	δ _H (J in Hz)
1	160.8, qC		162.9, qC	
2	70.5, qC		74.2, qC	
3a	43.2, CH ₂	2.76, br d (12)	45.7, CH ₂	2.54, d (12)
3b		2.94, dd (12, 8.0)		2.76, dd (12, 8.0)
4	44.9, CH	3.04, t (8.0)	43.4, CH	3.08, t (8.0)
5	207.2, qC		207.9, qC	
6a	41.5, CH ₂	2.88, br d (11)	38.0, CH ₂	2.44, br d (18)
6b		3.16, dd (11, 5.0)		3.05, d (18)
7	41.4, CH	3.73, t (5.0)	45.4, CH	3.72, br d (10)
8	65.3, CH	3.95, dd (5.0, 2.5)	64.8, CH	4.59, br d (3.0)
9	60.6, CH	4.67, d (8.0)	62.3, CH	4.46, br d (8.0)
1'	157.4, qC		159.9, qC	
2'	74.0, qC		69.7, qC	
3'a	51.9, CH ₂	2.87, br d (11)	41.6, CH ₂	2.55, d (12)
3'b		2.99, dd (11, 7.0)		2.78, dd (12, 8.0)
4'	45.1, CH	3.08, t (7.0)	38.8, CH	2.69, t (8.0)
5'	203.3, qC		61.0, CH	4.27, dd (8.0, 5.0)
6'a	53.7, CH	3.18, d (5.0)	36.5, CH ₂	1.93, dd (14, 8.0)
6'b				2.69, dd (14, 8.0)
7'	78.1, CH	4.39, t (5.0)	42.4, CH	3.35, d (8.0)
8'	63.3, CH	4.44, dd (7.0, 5.0)	66.7, CH	3.78, br d (3.0)
9'	60.6, CH	4.62, t (7.0)	57.5, CH	4.33, br d (8.0)
OH-8		6.24, d (2.5)		6.04, d (3.0)
OH-5'				4.80, d (5.0)
OH-7'		5.74, d (5.0)		
OH-8'		5.56, d (4.0)		5.80, d (3.0)

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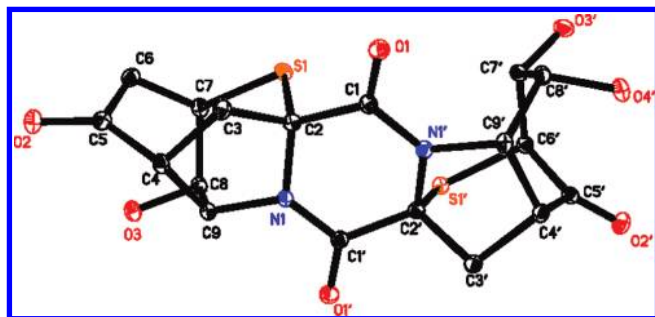


Figure 1. Thermal ellipsoid representation of **1**.

to those of both epicoccins B (**8**) and D (**9**).¹¹ Specifically, the ¹H and ¹³C NMR chemical shifts for the left portion of **1** were nearly identical to those of the symmetrical **9**, whereas those for the right portion of **1** closely matched the right half of **8**. These observations were supported by relevant ¹H–¹H COSY and HMBC correlations, leading to assignment of the gross structure of **1** as shown.

The relative configuration of **1** was assigned on the basis of its ¹H–¹H coupling constants and by comparison of its ¹H NMR data with those of the known precedents **8** and **9**. The same small coupling constant (8.0 Hz) observed between H-4 and H-9 as in **8** and **9** suggested their *cis* relationship, whereas a coupling constant of 5.0 Hz between H-7 and H-8 indicated that they were pseudo-equatorially oriented with respect to the cyclohexanone ring. Absence of coupling between H-8 and H-9 implied that they were nearly perpendicular to each other. Thus, the relative configuration for the left portion of **1** was assigned to be the same as that of **9**. Similar assignment was made for the right portion of **1**, which possesses the same relative configuration as the right portion of **8**.

The gross structure and the relative configuration of **1** were confirmed by single-crystal X-ray diffraction analysis, and a perspective ORTEP plot is shown in Figure 1. The presence of two sulfur atoms in **1** and the value of the Flack parameter (0.045)¹³ also allowed assignment of the absolute configurations of all the stereogenic centers in **1** as 2*R*,4*R*,7*R*,8*R*,9*S*,2'*R*,4'*R*,6'*S*,7'*R*,8'*R*,9'*S*.

The molecular formula of epicoccin F (**2**) was established as C₁₈H₂₀N₂O₆S₃ (10 degrees of unsaturation) by HRESIMS (*m/z* 479.0376 [M + Na]⁺; Δ +0.5 mmu). The presence of three sulfur atoms in this formula implied that **2** could possess the same sulfur linkages as in epicoccin A (**7**). Indeed, the NMR data of **2** (Table 1) revealed nearly identical structural features to those of **7**, except that one carbonyl carbon (δ 207.8) was replaced by an oxymethine (δ_H/δ_C 4.27/61.0) in **2**, suggesting that the C-10 ketone functionality was reduced. This observation was consistent with the fact that the molecular weight of **2** is two mass units higher than that of **7** and was confirmed by ¹H–¹H COSY correlations of H-5' with H-4', OH-5', and H-6', as well as HMBC cross-peaks from H-4' and H-6'a to C-5'. Therefore, the gross structure of epicoccin F was established as depicted in **2**.

The relative configuration of **2** was deduced from ¹H–¹H coupling constants and NOESY data. The coupling patterns for relevant protons on the left portion of **2** were identical to their counterparts on the left portion of **7**, indicating that the left halves of both metabolites possess the same relative configuration. The 8.0 Hz coupling constant observed for H-4' and H-9' revealed their *cis* relationship, whereas the lack of coupling between H-4' and H-5', H-7' and H-8', and H-8' and H-9' indicated that the vicinal angle between the protons in each pair was close to 90°. In addition, NOESY correlation of H-9' with OH-5' placed H-5' on the face opposite that of H-4' and H-9'. Considering the absolute configuration established for **1** by X-ray data, the additional stereogenic center C-5' in **2** was deduced to have the *R* configuration.

Epicoccin G (**3**) was obtained as a colorless powder. It was assigned the molecular formula C₂₀H₂₆N₂O₆S₂ (nine degrees of unsaturation) by HRESIMS (*m/z* 477.1125 [M + Na]⁺; Δ +0.5

Table 2. NMR Spectroscopic Data (500 MHz, DMSO-*d*₆) for **3** and **4**

position	3		4	
	δ _C , mult.	δ _H (J in Hz)	δ _C , mult.	δ _H (J in Hz)
1/1'	165.6, qC		165.5, qC	
2/2'	71.6, qC		71.4, qC	
3a/3'a	34.1, CH ₂	2.27, dd (13, 8.0)	34.1, CH ₂	2.32, dd (13, 8.5)
3b/3'b		2.76, brd (13)		2.74, br d (13)
4/4'	44.0, CH	2.93, t (8.0)	43.5, CH	2.91, t (8.5)
5/5'	207.7, qC		207.3, qC	
6a/6'a	33.8, CH ₂	2.20, dt (17, 4.0)	43.2, CH ₂	2.39, dd (17, 4.0)
6b/6'b		2.59, ddd (17, 12, 6.0)		2.59, dd (17, 10)
7a/7'a	25.8, CH ₂	1.88, dd (17, 12)	65.7, CH	4.13, ddd (10, 5.0, 4.0)
7b/7'b		2.12, dd (17, 4.0)		
8/8'	64.7, CH	4.30, dd (5.5, 3.0)	68.3, CH	4.27, m
9/9'	63.4, CH	4.28, d (8.0, 5.5)	63.2, CH	4.32, dd (8.5, 4.0)
10/10'	14.2, CH ₃	1.90, s	14.1, CH ₃	1.92, s
OH-7/7'				5.08, d (5.0)
OH-8/8'		5.30, d (3.0)		5.30, d (3.0)

mmu). Analysis of its ¹H and ¹³C NMR data (Table 2) revealed one methyl group, three methylenes, three methines (one of which was oxygenated), and two carbonyl carbons (δ_C 165.6 and 207.7, respectively). These data accounted for only half of its elemental composition, suggesting its symmetrical nature. Interpretation of the ¹H–¹H COSY NMR data identified an isolated proton spin system, which was C-6/6'–C-9/9'–C-4/4'–C-3/3' (including OH-8/8'). HMBC correlations from H-3a/3'a to C-1/1' and C-5/5' and from H-6b/6'b to C-5/5' established the C-3/3'–C-6/6' fragment, completing the cyclohexanone ring. Upon extensive analysis of its 2D NMR data, the symmetrical 6–5–6–5–6 DKP skeleton was established for **3**, but without the cross-ring bridges as found in **1** or **2**. The chemical shift of C-10/10' (δ_H/δ_C 1.90/14.2) and the HMBC cross-peak from H₃-10/10' to C-2/2' indicated that C-10/10' was connected to the sulfur atom attached to C-2/2', which is closely related to that of exserohilone (**10**),¹⁴ except that the olefin unit in **10** was reduced in **3**.

The relative configuration of **3** was also assigned by analysis of its ¹H–¹H coupling constants and NOESY data. The 8.0 Hz vicinal coupling constant for H-4/4' and H-9/9' revealed their *cis* relationship, compared to the *trans* configuration deduced from the large coupling constant of 12.6 Hz for corresponding protons in exserohilone (**10**).¹⁴ No coupling was observed between H-8/8' and H-9/9', indicating that both protons are pseudo-equatorially oriented. NOESY correlations of H₃-10/10' with H-3b/3'b and of H-3a/3'a with H-4/4' indicated that H₃-10/10' was opposite H-4/4'. Further comparison of the ¹H NMR data of **3** with those of **10**¹⁴ and rostratin B⁵ established its relative configuration as shown.

The absolute configuration of **3** was determined using the modified Mosher method.^{15,16} Treatment of **3** with (*S*)- and (*R*)-MTPA Cl afforded the (*R*)-MTPA (**3a**) and (*S*)-MTPA esters (**3b**), respectively. The difference in chemical shift values (Δδ = δ_S – δ_R) for the diastereomeric esters **3b** and **3a** was calculated to assign the 8*S* absolute configuration. Therefore, the 1*R*,4*S*,5*S*,8*S*,9*S*,1'*R*,4'*S*,5'*S*,8'*S*,9'*S* absolute configuration was proposed for **3** on the basis of the Δδ results summarized in Figure 2.

Epicoccin H (**4**) was also obtained as a colorless powder. It was assigned the molecular formula C₂₀H₂₆N₂O₈S₂ (nine degrees of unsaturation) on the basis of HRESIMS analysis (*m/z* 509.1023 [M + Na]⁺; Δ +0.5 mmu). The NMR data of **4** (Table 2) revealed structural features nearly identical to those of **3**, except that the C-7 methylene (δ_H/δ_C 1.88, 2.12/25.8) was replaced by an oxymethine (δ_H/δ_C 4.13/65.7) in **4**. This was supported by ¹H–¹H COSY correlations of OH-7/7' (δ_H 5.08) with H-7/7' and HMBC cross-peaks from OH-7/7' to C-6/6', C-7/7', and C-8/8'. On the basis

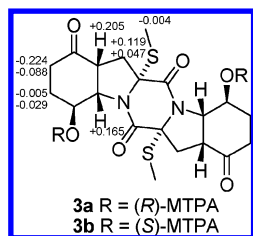


Figure 2. $\Delta\delta$ values (in ppm) = $\delta_S - \delta_R$ obtained for (*R*)- and (*S*)-MTPA esters **3a** and **3b**.

Table 3. NMR Spectroscopic Data (400 MHz, DMSO- d_6) for **5** and **6**

position	5		6	
	δ_C , mult.	δ_H (<i>J</i> in Hz)	δ_C , mult.	δ_H (<i>J</i> in Hz)
1		8.55, br s		8.40, br s
2	161.76, qC		162.5, qC	
3	131.44, qC		129.5, qC	
5	166.93, qC		167.1, qC	
6	56.35, CH	4.34, t (5.1)	63.5, CH	4.26, t (5.0)
7a	39.52, CH ₂	3.11, dd (14, 5.1)	30.5, CH ₂	3.16, dd (14, 5.0)
7b		3.09, dd (14, 5.1)		3.16, dd (14, 5.0)
8	135.61, qC		135.8, qC	
9	129.82, CH	7.18, d (7.6)	129.7, CH	7.17, d (7.4)
10	128.35, CH	7.26, t (7.6)	128.3, CH	7.25, t (7.4)
11	126.86, CH	7.14, t (7.6)	127.0, CH	7.17, t (7.4)
12	128.35, CH	7.27, t (7.6)	128.3, CH	7.25, t (7.4)
13	129.82, CH	7.16, d (7.6)	129.7, CH	7.17, d (7.4)
14	34.94, CH ₃	2.62, s	34.6, CH ₃	2.69, s
7'	117.47, CH	6.72, s	118.5, CH	6.64, s
8'	133.79, qC		124.0, qC	
9'	129.31, CH	7.02, d (7.4)	131.5, CH	6.91, d (8.4)
10'	127.99, CH	7.36, t (7.4)	115.0, CH	6.75, d (8.4)
11'	127.89, CH	7.29, t (7.4)	157.8, qC	
12'	127.99, CH	7.34, t (7.4)	115.0, CH	6.75, d (8.4)
13'	129.31, CH	7.02, d (7.4)	131.5, CH	6.91, d (8.4)
OH-11'				9.74, br s

of these data, the structure of **4** was elucidated as shown, and the absolute configuration of **4** was presumed to be the same as that of **3**.

The molecular formula of diphenylalazine A (**5**) was determined to be C₁₉H₁₈N₂O₂ (12 degrees of unsaturation) by HRESIMS (*m/z* 329.1265 [M + Na]⁺; Δ +0.1 mmu). Analysis of its ¹H and ¹³C NMR data (Table 3) revealed one amide proton (δ_H 8.55), one *N*-methyl group (δ_H/δ_C 2.62/34.94), one methylene, one methine indicative of an amino acid α -CH group (δ_H/δ_C 4.34/56.35), 14 aromatic/olefinic carbons (11 of which were protonated), and two carboxylic carbons. The ¹H NMR resonances for a cluster of 10 aromatic protons at δ_H 7.02–7.34 suggested the presence of two monosubstituted aryl rings. HMBC correlations from H₂-7 to C-9 and C-13, H-6 to C-5 and C-8, and H-1 (*N*-H) to C-5 established the phenylalanine (Phe) unit, whereas those from the olefinic proton H-7' to C-2 and C-9' and from H₃-14 to C-3 identified the *N*-methylated 3,7'-dehydrophenylalanine moiety. Key HMBC correlations from the α -proton of Phe (H-6) to C-2 and from the *N*-methyl proton (H₃-14) of 3,7'-dehydrophenylalanine to the carboxylic carbon of Phe (C-5) established the DKP core, thereby completing the gross structure of **5**.

Marfey's method¹⁷ was applied to assign the absolute configuration of the Phe residue resulting from acid hydrolysis of **5**. HPLC analysis of the 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) derivative of the acid hydrolysate of **5** gave the same retention time as that prepared from a sample of authentic L-Phe. Therefore, the Phe residue in **5** was assigned the L-configuration.

Diphenylalazine B (**6**) gave a pseudomolecular ion [M + Na]⁺ peak at *m/z* 345.1210 (Δ +0.5 mmu) by HRESIMS, consistent with a molecular formula of C₁₉H₁₈N₂O₃ (12 degrees of unsaturation). Analysis of its NMR data (Table 3) revealed its structural similarity

to **5**, except that the aromatic proton (δ_H 7.29) attached to C-11' (δ_C 127.89) was replaced by a phenolic proton (δ_H 9.74) in **5**. The ¹H–¹H coupling patterns observed for H-9' and H-10', and H-12' and H-13', were indicative of a *p*-substituted aryl ring, permitting assignment of the gross structure of **6** as shown. The Phe unit in **6** was assigned the L-configuration by comparison of the specific rotation of **6** ($[\alpha]_D^{25} +334$) with that of **5** ($[\alpha]_D^{25} +332$).

Compounds **1–6** were tested for in vitro anti-HIV-1 activity. Compounds **3–5** showed inhibitory effects on HIV-1 replication in C8166 cells, with EC₅₀ values of 13.5, 42.2, and 27.9 μ M, respectively (the CC₅₀ values for these compounds were all greater than 100 μ M; the positive control indinavir showed an EC₅₀ value of 8.2 nM).

Epicoccins E (**1**) and F (**2**) are new DKPs possessing the characteristic internal sulfur bridge(s). Although another class of DKPs, the epipolythiodioxopiperazines (ETPs), possess an internal disulfide bridge on the DKP ring,¹⁸ **1** and **4** incorporated the rare cross-ring sulfur bridges significantly different from those of the common ETPs, with epicoccins A–D as the only examples.¹¹ However, **1** differs from its most closely related known precedent **9** by virtue of the presence of a sulfur bridge linking C-2' and C-6' instead of C-2' and C-7' in **9**, whereas **2** differs from **7** by having a C-5'-OH instead of the C-5' ketone functionality. Epicoccins G (**3**) and H (**4**) are closely related to exserohilone (**10**),¹⁴ but differ in having a cyclohexenone moiety rather than a cyclohexanone ring. Diphenylalazines A (**5**) and B (**6**) are DKPs possessing a Phe and a dehydro *N*-Me-Phe unit, respectively, and are closely related to XR335 isolated from *Streptomyces* spp.,¹⁹ but differ in the identity of the substituents on the aryl ring of the dehydro *N*-Me-Phe unit.

It is generally accepted that the natural DKPs seem to be produced via a nonribosomal pathway, catalyzed by the nonribosomal peptide synthetases (NRPSs).^{20–22} Recently, a new family of enzymes named cyclodipeptide synthases (CDPSs), unrelated to the NRPSs, were reported to be involved in biosynthesis of the antibacterial agent albonoursin isolated from *Streptomyces noursei*.^{22,23} Even though some biosynthetic studies suggested that the sulfur atoms in the ETPs may be derived from methionine, cysteine, and sodium sulfate, the mechanism by which the sulfurs are incorporated remains to be fully investigated.¹⁸ Presumably, the biosynthesis of epicoccins E–H proceeds in a manner similar to that of other known precedents.²⁴

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on a Shimadzu Biospec-1601 spectrophotometer. IR data were recorded using a Nicolet Magna-IR 750 spectrophotometer. ¹H and ¹³C NMR data were acquired with Bruker Avance-400, 500, and 600 spectrometers using solvent signals (DMSO: δ_H 2.50/ δ_C 39.5; pyridine-*d*₅: δ_H 7.21, 7.58, 8.73) 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 data were obtained using a Bruker APEX III 7.0 T spectrometer.

Fungal Material. The culture of *E. nigrum* was isolated by Dr. Mu Wang from a sample of *C. sinensis* (Clavicipitaceae) collected in Linzhi, Tibet, in March 2004. The isolate was identified by one of the authors (B.S.) and assigned the accession no. XZC04-CS-302 in X.L.'s culture collection at the Institute of Microbiology, Chinese Academy of Sciences, Beijing. The isolate was cultured on slants of potato dextrose agar (PDA) at 25 °C for 15 days. 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. Twenty 500 mL Erlenmeyer flasks, each containing 150 mL of liquid media (2% maltose, 6% Glucidex 29, 0.7% peptone, 0.75% Pharmamedia, trace amounts of MgSO₄·7H₂O, CaCO₃, FeSO₄·7H₂O, and ZnSO₄·7H₂O; final pH 7.0) 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 (3 L) was freeze-dried and extracted with ethyl methyl ketone (MEK; 3×500 mL), and the organic solvent was evaporated to dryness under vacuum to afford the crude extract (6.0 g). The extract was fractionated by silica gel VLC using petroleum ether–EtOAc gradient elution. The fractions eluted with 25 (80 mg), 30 (100 mg), 60 (100 mg), 70 (100 mg), and 95% EtOAc (30 mg) were individually separated by Sephadex LH-20 column chromatography (CC) using MeOH as eluent. The resulting subfractions were combined into six fractions and subjected to further purification by semipreparative RPPLC (Agilent ZORBAX SB-C18 column; $5 \mu\text{m}$; 9.4×250 mm; 2 mL/min) using different gradients to afford epicoccins E (**1**; 5.0 mg; 60% MeOH in H_2O over 5 min, followed by 60–85% over 30 min), F (**2**; 5.0 mg; 60% MeOH in H_2O over 5 min, followed by 60–85% over 25 min), G (**3**; 7.0 mg; 35% MeOH in H_2O over 5 min, followed by 35–85% over 25 min), and H (**4**; 5.0 mg; same gradient as in purification of **3**); diphenylalazines A (**5**; 20 mg; 50% MeOH in water over 5 min, followed by 50–90% over 30 min) and B (**6**; 2.0 mg; 60% MeOH in H_2O over 5 min, followed by 60–100% over 30 min); and the known compounds epicoccins A (**7**; 3.2 mg; 40% MeOH in H_2O over 5 min, followed by 40–80% over 30 min), B (**8**; 10 mg; same gradient as in purification of **7**), and D (**9**); 10 mg; same gradient as in purification of **6**).

Epicoccin E (1): colorless needles (MeOH– H_2O); mp 273–275 °C; $[\alpha]_D^{25} +68.0$ (c 0.17, MeOH); UV (MeOH) λ_{max} (log ϵ) 224 (3.04) nm; IR (neat) ν_{max} 3438, 2923, 1727, 1666, 1422, 1323, 1287, 1133 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 1; HMBC data (DMSO- d_6 , 600 MHz) H-3a \rightarrow C-2, 4, 5, 9; H-4 \rightarrow C-2, 4, 8, 9; H-6a \rightarrow C-5, 7, 8; H-7 \rightarrow C-2, 5, 9; H-3'a \rightarrow C-2', 5', 9'; H-4' \rightarrow C-2', 3', 5'; H-6' \rightarrow C-2', 5', 7', 8'; OH-8 \rightarrow C-7, 8, 9; OH-7' \rightarrow C-7'; OH-8' \rightarrow C-8', 9'; HRESIMS m/z 461.0448 (calcd for $\text{C}_{18}\text{H}_{18}\text{N}_2\text{O}_7\text{S}_2\text{Na}$, 461.0453).

X-ray Crystallographic Analysis of 1.²⁵ Upon crystallization from MeOH– H_2O (10:1) using the vapor diffusion method, colorless crystals were obtained for **1**, and a crystal ($0.20 \times 0.18 \times 0.18$ mm) was separated from the sample and mounted on a glass fiber. Data were collected using a Bruker SMART 1000 CCD diffractometer with graphite-monochromated Mo K α radiation, $\lambda = 0.71073$ Å at 173(2) K. Crystal data: $\text{C}_{18}\text{H}_{18}\text{N}_2\text{O}_7\text{S}_2$, $M = 456.48$, space group monoclinic, $P2_1$; unit cell dimensions $a = 10.861(2)$ Å, $b = 6.3257(13)$ Å, $c = 13.029(3)$ Å, $V = 887.9(3)$ Å³, $Z = 2$, $D_{\text{calcd}} = 1.707$ mg/m³, $\mu = 0.356$ mm⁻¹, $F(000) = 476$. The structure was solved by direct methods using SHELXL-97²⁶ and refined using full-matrix least-squares difference Fourier techniques. All non-hydrogen atoms were refined with anisotropic displacement parameters, and all hydrogen atoms were placed in idealized positions and refined as riding atoms with the relative isotropic parameters. Absorption corrections were applied with the Siemens Area Detector Absorption Program (SADABS).²⁷ The 10 618 measurements yielded 3947 independent reflections after equivalent data were averaged, and Lorentz and polarization corrections were applied. The final refinement gave $R_1 = 0.0324$ and $wR_2 = 0.0814$ [$I > 2\sigma(I)$].

Epicoccin F (2): colorless powder; $[\alpha]_D^{25} +52.0$ (c 0.08, MeOH); UV (MeOH) λ_{max} (log ϵ) 215 (3.34) nm; IR (neat) ν_{max} 3418, 2931, 2856, 1664, 1417, 1320, 1051 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 1; HMBC data (DMSO- d_6 , 600 MHz) H-3a \rightarrow C-2, 5, 9; H-3b \rightarrow C-4, 5, H-4 \rightarrow C-2, 3, H-6a \rightarrow C-5, 7, 8; H-9 \rightarrow C-2, 4, 5, 7, 8; H-3'a \rightarrow C-2'; H-3'b \rightarrow C-2', 5'; H-4' \rightarrow C-5'; H-6'a \rightarrow C-5', 7', 9'; H-8' \rightarrow C-2', 7', 9'; H-9' \rightarrow C-2', 5', 7', 8'; OH-8 \rightarrow C-9; OH-8' \rightarrow C-8', 9'; NOESY correlations (DMSO- d_6 , 600 MHz) H-9' \leftrightarrow OH-5'; HRESIMS m/z 479.0376 (calcd for $\text{C}_{18}\text{H}_{20}\text{N}_2\text{O}_6\text{S}_3\text{Na}$, 479.0381).

Epicoccin G (3): colorless powder; $[\alpha]_D^{25} +69.0$ (c 0.17, MeOH); UV (MeOH) λ_{max} (log ϵ) 224 (3.83) nm; IR (neat) ν_{max} 3398, 2919, 2851, 1699, 1651, 1543, 1405, 1193 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 2; HMBC data (DMSO- d_6 , 600 MHz) H-3a/3'a \rightarrow C-1/1', 2/2', 4/4', 5/5'; H-6a/6'a \rightarrow C-8/8'; H-6b/6'b \rightarrow C-3/3', 5/5', 7/7', 8/8'; H-7b/7'b \rightarrow C-6/6'; H-9/9' \rightarrow C-4/4', 7/7', 8/8'; H₃-10/10' \rightarrow C-2/2'; NOESY correlations (DMSO- d_6 , 600 MHz) H-4/4' \leftrightarrow H-3a/3'a, 9/9'; H-6a/6'a \leftrightarrow H-8/8'; H₃-10/10' \leftrightarrow H-3b/3'b; HRESIMS m/z 477.1125 (calcd for $\text{C}_{20}\text{H}_{26}\text{N}_2\text{O}_6\text{S}_2\text{Na}$, 477.1130).

Preparation of (R)-MTPA (3a) and (S)-MTPA Esters (3b). A sample of **3** (1.0 mg, 0.002 mmol), (S)-MTPA Cl (10.0 μL , 0.052 mmol), and pyridine- d_5 (0.5 mL) were allowed to react in an NMR tube at ambient temperature for 6 days. The ^1H NMR data of the R-MTPA ester derivative (**3a**) were obtained directly on the reaction mixture: ^1H NMR (pyridine- d_5 , 500 MHz) δ 6.40 (2H, s, H-8/8'), 4.91 (2H, d, $J = 8.0$ Hz, H-9/9'), 3.33 (2H, d, $J = 18$ Hz, H-3b/3'b), 3.07 (2H, t, $J = 8.0$ Hz, H-4/4'), 2.67 (2H, m, H-7b/7'b), 2.61 (2H, dd, $J =$

14, 8.0 Hz, H-3a/3'a), 2.53 (2H, m, H-6b/6'b), 2.38 (2H, m, H-6a/6'a), 2.30 (2H, m, H-7a/7'a), 2.15 (6H, s, H₃-10/10').

Another sample of **3** (1.0 mg, 0.002 mmol), (R)-MTPA Cl (10.0 μL , 0.052 mmol), and pyridine- d_5 (0.5 mL) were processed as described above for **3a** to afford **3b**: ^1H NMR (pyridine- d_5 , 500 MHz) δ 6.46 (2H, s, H-8/8'), 4.74 (2H, d, $J = 8.0$ Hz, H-9/9'), 3.29 (2H, d, $J = 14$ Hz, H-3b/3'b), 2.86 (2H, t, $J = 8.0$ Hz, H-4/4'), 2.70 (2H, m, H-7b/7'b), 2.62 (2H, m, H-6b/6'b), 2.61 (2H, m, H-6a/6'a), 2.49 (2H, dd, $J = 14, 8.0$ Hz, H-3a/3'a), 2.30 (2H, m, H-7a/7'a), 2.15 (6H, s, H₃-10/10').

Epicoccin H (4): colorless powder; $[\alpha]_D^{25} -175.0$ (c 0.09, MeOH); UV (MeOH) λ_{max} (log ϵ) 230 (3.83) nm; IR (neat) ν_{max} 3416, 2922, 1707, 1645, 1418, 1246, 1076 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 2; HMBC data (DMSO- d_6 , 600 MHz) H-3a/3'a \rightarrow C-1/1', 2/2', 4/4', 5/5', 10/10'; H-3b/3'b \rightarrow C-2/2', 4/4', 5/5', 9/9', 10/10'; H-4/4' \rightarrow C-2/2', 3/3', 5/5', 9/9'; H₂-6/6' \rightarrow C-5/5', 7/7', 8/8'; OH-7/7' \rightarrow C-6/6', 7/7', 8/8'; OH-8/8' \rightarrow C-7/7', 9/9'; H-9/9' \rightarrow C-4/4', 5/5', 7/7', 8/8'; H₃-10/10' \rightarrow C-2/2'; key NOESY correlations (DMSO- d_6 , 600 MHz) H-4/4' \leftrightarrow H-3a/3'a, 9/9'; H₃-10/10' \leftrightarrow H-3b/3'b; HRESIMS m/z 509.1023 (calcd for $\text{C}_{20}\text{H}_{26}\text{N}_2\text{O}_8\text{S}_2\text{Na}$, 509.1028).

Diphenylalazine A (5): white powder; $[\alpha]_D^{25} +332.0$ (c 0.48, MeOH); UV (MeOH) λ_{max} (log ϵ) 215 (3.78), 295 (3.72) nm; IR (neat) ν_{max} 3242, 2953, 2920, 1686, 1628, 1542, 1493, 1076 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 3; HMBC data (DMSO- d_6 , 400 MHz) H-1 \rightarrow C-3, 5; H-6 \rightarrow C-2, 5, 7, 8; H-7a \rightarrow C-5, 6, 8, 9, 13; H-9 \rightarrow C-7, 11, 13; H-10 \rightarrow C-8, 12; H-11 \rightarrow C-9, 13; H-12 \rightarrow C-8, 10; H-13 \rightarrow C-7, 9, 11; H-14 \rightarrow C-3, 5; H-7' \rightarrow C-2, 9'; H-9' \rightarrow C-11'; H-10' \rightarrow C-8', 12'; H-11' \rightarrow C-10', 13'; H-12' \rightarrow C-8', 10'; H-13' \rightarrow C-9', 10'; NOESY correlations (DMSO- d_6 , 400 MHz) H-1 \leftrightarrow H-7a, 7b, 9, 13; H-6 \leftrightarrow H-9, 13, 14; H₂-7 \leftrightarrow H-1, 9, 13; H-9 \leftrightarrow H-1, 6; H-14 \leftrightarrow H-6, 12', 13'; H-7' \leftrightarrow H-9'; HRESIMS m/z 329.1265 (calcd for $\text{C}_{19}\text{H}_{18}\text{N}_3\text{O}_2\text{Na}$, 329.1266).

Absolute Configuration of Diphenylalazine A (5).¹⁷ A solution of **5** (1.5 mg) in 6 N HCl (1 mL) was heated at 100 °C for 16 h. Upon removal of excess HCl under vacuum, the hydrolysate was placed in a 1 mL reaction vial and treated with 1% solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA; 200 μL) in acetone followed by 1.0 M NaHCO₃ (40 μL). The reaction mixture was heated at 45 °C for 1.5 h, cooled to room temperature, and then acidified with 2.0 N HCl (20 μL). In a similar fashion, standard D- and L-Phe were derivatized separately. The derivatives of the hydrolysate and standard amino acids were subjected to RPPLC analysis (Kromasil C₁₈ column; 10 μm , 4.6×250 mm; 1.0 – 1.5 mL/min) at 25 °C using the following gradient program: solvent A, 50 mM (Et₃NH)₃PO₄ at pH 3.0; solvent B, acetonitrile; linear gradient, 10%–35% B in 60 min with UV detection at 340 nm. The retention times for the FDAA derivatives of **5** hydrolysate, standard L-Phe, and D-Phe were 44.75, 44.75, and 49.65 min, respectively.

Diphenylalazine B (6): white powder; $[\alpha]_D^{25} +334.0$ (c 0.213, MeOH); UV (MeOH) λ_{max} (log ϵ) 205 (4.04), 228 (3.84), 310 (3.93) nm; IR (neat) ν_{max} 3262, 2954, 2929, 1678, 1623, 1588, 1077 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 3; HMBC data (DMSO- d_6 , 400 MHz) H-6 \rightarrow C-2, 5; H₂-7 \rightarrow C-5, 6, 8; H-10, 12 \rightarrow C-8; H-7' \rightarrow C-2, 9'; H-9' \rightarrow C-11'; H-10', 12' \rightarrow C-8'; H₃-14 \rightarrow C-3, 5; HRESIMS m/z 345.1210 (calcd for $\text{C}_{19}\text{H}_{18}\text{N}_3\text{O}_3\text{Na}$, 345.1215).

Anti-HIV Bioassays.²⁸ Anti-HIV assays included cytotoxicity and HIV-1 replication inhibition evaluations. Cytotoxicity was measured by the MTT method as described in the literature. Cells (3×10^4 /well) were seeded into a 96-well microtiter plate in the absence or presence of various concentrations of test compounds in triplicate and incubated at 37 °C in a humid atmosphere of 5% CO₂. After a 4-day incubation, cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The concentration that caused the reduction of viable cells by 50% (CC₅₀) was determined. In parallel with the MTT assay, a HIV-1 replication inhibition assay was determined by p24 antigen capture ELISA. C8166 cells were exposed to HIV-1 (MOI = 0.058) at 37 °C for 1.5 h, washed with PBS to remove free viruses, and then seeded into a 96-well microtiter plate at 3×10^4 cells per well in the absence or presence of test compounds (indinavir sulfate was used as positive control). After 4 days, the supernatant was collected and inactivated by 0.5% Triton X-100. The supernatant was diluted three times, added to the plate coating with anti-p24 McAb (provided by Dr. Bin Yan, Wuhan Institute of Virology, Wuhan, People's Republic of China), and incubated at 37 °C for 1 h. After washing five times with PBST, the HRP-labeled anti-p24 antibody

(provided by Dr. Bin Yan) was added and incubated at 37 °C for 1 h. The plate was washed five times with PBST, followed by adding *o*-phenylenediamine (OPD) reaction mixture. The assay plate was read at 490 nm using a microplate reader within 30 min. The inhibition rate and the EC₅₀ based on p24 antigen expression level were calculated.

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Supporting Information Available: ¹H and ¹³C NMR spectra of epicoccins E–H (**1**–**4**) and diphenylalazines A (**5**) and B (**6**). This material is available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

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