# Diketopiperazines from the *Cordyceps*-Colonizing Fungus *Epicoccum nigrum*

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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 antimicorbial, 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*; Clavicipataceae),[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 $C_{18}H_{18}N_2O_7S_2$ (11 degrees of unsaturation) on the basis of its HRESIMS (*m/z* 461.0448 $[M + Na]^+$; $\Delta$ +0.5 mmu). The $^1H$ and $^{13}C$ NMR data of **1** (Table 1) suggested the presence of three exchangeable protons ($\delta$ 5.56, 5.74, and 6.24, respectively), three methylenes, nine methines (five bonded to oxygen or nitrogen), two heteroatom-bonded $sp^3$ quaternary carbons, and four carbonyl carbons including two ketone ones. These data accounted for all of the $^1H$ and $^{13}C$ resonances for **1** and revealed structural similarity

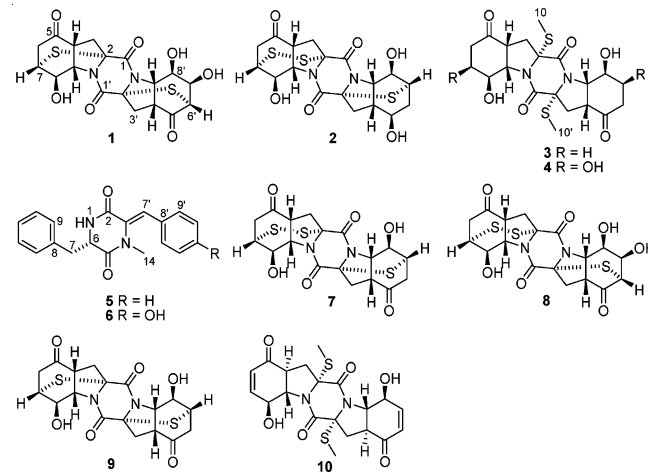


**Table 1.** NMR Spectroscopic Data (500 MHz, DMSO-$d_6$) for **1** and **2**

| | **1** | | **2** | |
|---|---|---|---|---|
| position | $\delta_C$, mult. | $\delta_H$ (*J* in Hz) | $\delta_C$, mult. | $\delta_H$ (*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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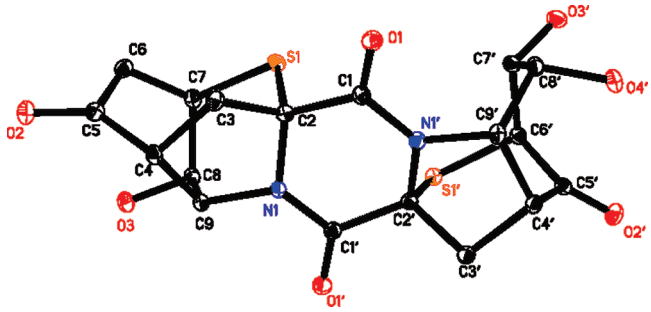



**Figure 1.** Thermal ellipsoid representation of **1**.

to those of both epicoccins B (**8**) and D (**9**).[11] Specifically, the $^1$H and $^{13}$C 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 $^1$H−$^1$H 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 $^1$H−$^1$H coupling constants and by comparison of its $^1$H 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 pseudoequatorially 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 $C_{18}H_{20}N_2O_6S_3$ (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 ($\delta_H/\delta_C$ 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 $^1$H−$^1$H 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 $^1$H−$^1$H 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 $C_{20}H_{26}N_2O_6S_2$ (nine degrees of unsaturation) by HRESIMS (*m/z* 477.1125 [M + Na]$^+$; Δ +0.5 mmu). Analysis of its $^1$H and $^{13}$C NMR data (Table 2) revealed one methyl group, three methylenes, three methines (one of which was oxygenated), and two carbonyl carbons ($\delta_C$ 165.6 and 207.7, respectively). These data accounted for only half of its elemental composition, suggesting its symmetrical nature. Interpretation of the $^1$H−$^1$H 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′ ($\delta_H/\delta_C$ 1.90/14.2) and the HMBC cross-peak from $H_3$-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**.

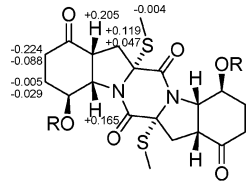
**Table 2.** NMR Spectroscopic Data (500 MHz, DMSO-$d_6$) for **3** and **4**

| | **3** | | **4** | |
|---|---|---|---|---|
| position | $\delta_C$, mult. | $\delta_H$ (*J* in Hz) | $\delta_C$, mult. | $\delta_H$ (*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) |

The relative configuration of **3** was also assigned by analysis of its $^1$H−$^1$H 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 pseudoequatorially oriented. NOESY correlations of $H_3$-10/10′ with H-3b/3′b and of H-3a/3′a with H-4/4′ indicated that $H_3$-10/10′ was opposite H-4/4′. Further comparison of the $^1$H NMR data of **3** with those of **10**[14] and rostratin B[5] 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 ($\Delta\delta = \delta_S - \delta_R$) 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 $C_{20}H_{26}N_2O_8S_2$ (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 ($\delta_H/\delta_C$ 1.88, 2.12/25.8) was replaced by an oxymethine ($\delta_H/\delta_C$ 4.13/65.7) in **4**. This was supported by $^1$H−$^1$H COSY correlations of OH-7/7′ ($\delta_H$ 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




**3a** R = (*R*)-MTPA
**3b** R = (*S*)-MTPA

**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**

| | **5** | | **6** | |
|---|---|---|---|---|
| position | $\delta_C$, mult. | $\delta_H$ (*J* in Hz) | $\delta_C$, mult. | $\delta_H$ (*J* 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 $C_{19}H_{18}N_2O_2$ (12 degrees of unsaturation) by HRESIMS (*m/z* 329.1265 $[M + Na]^+$; $\Delta$ +0.1 mmu). Analysis of its $^1H$ and $^{13}C$ NMR data (Table 3) revealed one amide proton ($\delta_H$ 8.55), one *N*-methyl group ($\delta_H/\delta_C$ 2.62/34.94), one methylene, one methine indicative of an amino acid α-CH group ($\delta_H/\delta_C$ 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 $\delta_H$ 7.02−7.34 suggested the presence of two monosubstituted aryl rings. HMBC correlations from $H_2$-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 $H_3$-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 ($H_3$-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 method[17] 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 ($\Delta$ +0.5 mmu) by HRESIMS, consistent with a molecular formula of $C_{19}H_{18}N_2O_3$ (12 degrees of unsaturation). Analysis of its NMR data (Table 3) revealed its structural similarity to **5**, except that the aromatic proton ($\delta_H$ 7.29) attached to C-11′ ($\delta_C$ 127.89) was replaced by a phenolic proton ($\delta_H$ 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** ($[\alpha]^{25}_D$ +334) with that of **5** ($[\alpha]^{25}_D$ +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 $EC_{50}$ values of 13.5, 42.2, and 27.9 $\mu$M, respectively (the $CC_{50}$ values for these compounds were all greater than 100 $\mu$M; the positive control indinavir showed an $EC_{50}$ 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 $^{13}C$ NMR data were acquired with Bruker Avance-400, 500, and 600 spectrometers using solvent signals (DMSO: $\delta_H$ 2.50/$\delta_C$ 39.5; pyridine-$d_5$: $\delta_H$ 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* (Clavicipataceae) 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 $MgSO_4 \cdot 7H_2O$, $CaCO_3$, $FeSO_4 \cdot 7H_2O$, and $ZnSO_4 \cdot 7H_2O$; 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 RPHPLC (Agilent ZORBAX SB-C18 column; 5 μm; 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; $[\alpha]^{25}_D$ +68.0 (*c* 0.17, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 224 (3.04) nm; IR (neat) $\nu_{max}$ 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 → C-2, 4, 5, 9; H-4 → C-2, 4, 8, 9; H-6a → C-5, 7, 8; H-7 → C-2, 5, 9; H-3′a → C-2′, 5′, 9′; H-4′ → C-2′, 3′, 5′; H-6′ → C-2′, 5′, 7′, 8′; OH-8 → C-7, 8, 9; OH-7′ → C-7′; OH-8′ → C-8′, 9′; HRESIMS *m/z* 461.0448 (calcd for $C_{18}H_{18}N_2O_7S_2Na$, 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 × 0.18 × 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 Kα radiation, $\lambda = 0.71073$ Å at 173(2) K. Crystal data: $C_{18}H_{20}N_2O_8S_2$, $M = 456.48$, space group monoclinic, $P2_1$; unit cell dimensions $a = 10.861(2)$ Å, $b = 6.3257(13)$ Å, $c = 13.029(3)$ Å, $V = 887.9(3)$ Å$^3$, $Z = 2$, $D_{calcd} = 1.707$ mg/m$^3$, $\mu = 0.356$ mm$^{-1}$, $F(000) = 476$. The structure was solved by direct methods using SHELXL-97[26] 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; $[\alpha]^{25}_D$ +52.0 (*c* 0.08, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 215 (3.34) nm; IR (neat) $\nu_{max}$ 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 → C-2, 5, 9; H-3b → C-4, 5, H-4 → C-2, 3, H-6a → C-5, 7, 8; H-9 → C-2, 4, 5, 7, 8; H-3′a → C-4′; H-3′b → C-2′, 5′; H-4′ → C-5′; H-6′a → C-5′, 7′, 9′; H-8′ → C-2′, 7′, 9′; H-9′ → C-2′, 5′, 7′, 8′; OH-8 → C-9; OH-8′ → C-8′, 9′; NOESY correlations (DMSO-$d_6$, 600 MHz) H-9′ ↔ OH-5′; HRESIMS *m/z* 479.0376 (calcd for $C_{18}H_{20}N_2O_6S_3Na$, 479.0381).

**Epicoccin G (3):** colorless powder; $[\alpha]^{25}_D$ +69.0 (*c* 0.17, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 224 (3.83) nm; IR (neat) $\nu_{max}$ 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 → C-1/1′, 2/2′, 4/4′, 5/5′; H-6a/6′a → C-8/8′; H-6b/6′b → C-3/3′, 5/5′, 7/7′, 8/8′; H-7b/7′b → C-6/6′; H-9/9′ → C-4/4′, 7/7′, 8/8′; $H_3$-10/10′ → C-2/2′; NOESY correlations (DMSO-$d_6$, 600 MHz) H-4/4′ ↔ H-3a/3′a, 9/9′; H-6a/6′a ↔ H-8/8′; $H_3$-10/10′ ↔ H-3b/3′b; HRESIMS *m/z* 477.1125 (calcd for $C_{20}H_{26}N_2O_6S_2Na$, 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/3b′), 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, $H_3$-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, $H_3$-10/10′).

**Epicoccin H (4):** colorless powder; $[\alpha]^{25}_D$ −175.0 (*c* 0.09, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 230 (3.83) nm; IR (neat) $\nu_{max}$ 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 → C-1/1′, 2/2′, 4/4′, 5/5′, 10/10′; H-3b/3′b → C-2/2′, 4/4′, 5/5′, 9/9′, 10/10′; H-4/4′ → C-2/2′, 3/3′, 5/5′, 9/9′; $H_2$-6/6′ → C-5/5′, 7/7′, 8/8′; OH-7/7′ → C-6/6′, 7/7′, 8/8′; OH-8/8′ → C-7/7′, 9/9′; H-9/9′ → C-4/4′, 5/5′, 7/7′, 8/8′; $H_3$-10/10′ → C-2/2′; key NOESY correlations (DMSO-$d_6$, 600 MHz) H-4/4′ ↔ H-3a/3′a, 9/9′; $H_3$-10/10′ ↔ H-3b/3′b; HRESIMS *m/z* 509.1023 (calcd for $C_{20}H_{26}N_2O_8S_2Na$, 509.1028).

**Diphenylalazine A (5):** white powder; $[\alpha]^{25}_D$ +332.0 (*c* 0.48, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 215 (3.78), 295 (3.72) nm; IR (neat) $\nu_{max}$ 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 → C-3, 5; H-6 → C-2, 5, 7, 8; H-7a → C-5, 6, 8, 9, 13; H-9 → C-7, 11, 13; H-10 → C-8, 12; H-11 → C-9, 13; H-12 → C-8, 10; H-13 → C-7, 9, 11; H-14 → C-3, 5; H-7′ → C-2, 9′; H-9′ → C-11′; H-10′ → C-8′, 12′; H-11′ → C-10′, 13′; H-12′ → C-8′, 10′; H-13′ → C-9′, 10′; NOESY correlations (DMSO-$d_6$, 400 MHz) H-1 ↔ H-7a, 7b, 9, 13; H-6 ↔ H-9, 13, 14; $H_2$-7 ↔ H-1, 9, 13; H-9 ↔ H-1, 6; H-14 ↔ H-6, 12′, 13′; H-7′ ↔ H-9′; HRESIMS *m/z* 329.1265 (calcd for $C_{19}H_{18}N_2O_2Na$, 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 $NaHCO_3$ (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 RPHPLC analysis (Kromasil $C_{18}$ 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 $(Et_3NH)_3PO_4$ 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; $[\alpha]^{25}_D$ +334.0 (*c* 0.213, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 205 (4.04), 228 (3.84), 310 (3.93) nm; IR (neat) $\nu_{max}$ 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 → C-2, 5; $H_2$-7 → C-5, 6, 8; H-10, 12 → C-8; H-7′ → C-2, 9′; H-9′ → C-11′; H-10′, 12′ → C-8′; $H_3$-14 → C-3, 5; HRESIMS *m/z* 345.1210 (calcd for $C_{19}H_{18}N_2O_3Na$, 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 ($3 \times 10^4$/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% $CO_2$. 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% ($CC_{50}$) 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 $3 \times 10^4$ 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 $EC_{50}$ based on p24 antigen expression level were calculated.

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**Supporting Information Available:** $^{1}H$ and $^{13}C$ 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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