A Xanthocillin-like Alkaloid from the Insect Pathogenic Fungus *Cordyceps brunnearubra* BCC 1395

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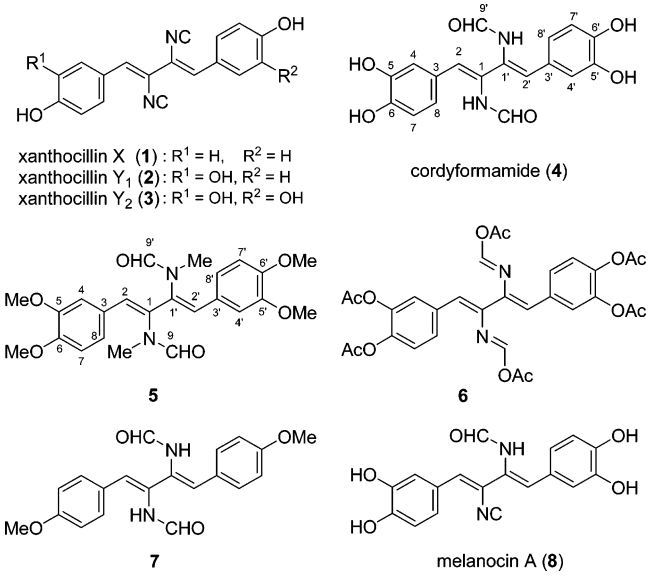
Cordyformamide (**4**), the plausible biogenetic precursor of xanthocillin Y_2, was isolated from a culture broth of the insect pathogenic fungus *Cordyceps brunnearubra* BCC 1395. Cordyformamide was found to exhibit activity against the malarial parasite *Plasmodium falciparum* K1 with an IC_{50} value of 18 μM, whereas it showed weak or no cytotoxicity.

Xanthocillins are naturally occurring isocyanides possessing antibiotic activity.[1] Xanthocillins X (**1**), Y_1 (**2**), and Y_2 (**3**) were first isolated from *Penicillium notatum*,[2] whereas xanthocillin X (**1**), its mono- and dimethyl ether, and methoxyxanthocillin X dimethyl ether, isolated from *Aspergillus* sp., were reported to exhibit antiviral activity.[3] To date, a number of related compounds have been isolated from various groups of fungi such as 6′,8′-dimethoxyxanthocillin X dimethyl ether from *Basipetospora* sp.,[4] NK372135A, -B, and -C from *Neosartoria fischeri*,[5] antibiotic MK4588 from *Leptosphaeria* sp.,[6] darlucins from *Sphaerellopsis filum*,[7] and melanocins from *Eupenicillium shearii*.[8] In our continuous research on novel secondary metabolites of insect pathogenic fungi,[9] we isolated a new alkaloid, cordyformamide (**4**), from *Cordyceps brunnearubra* BCC 1395. The structure of this compound is closely related to that of xanthocillin Y_2 (**3**), but it possesses two formamide groups instead of the isonitrile functionalities in **3**. Herein we report the structure elucidation and biological activity of **4**.



Cordyformamide (**4**) was isolated as a white powder. The molecular formula of **4** was determined by HRMS (ESI-TOF) as $C_{18}H_{16}N_2O_6$. The IR spectrum of **4** showed broad and intense absorption bands at ν_{max} 3505, 3259, and 1631 cm^{-1}. Due to the complexity of the ^1H and ^{13}C NMR resonance pattern as described below, **4** was converted to the hexamethylated derivative **5** (excess MeI, K_2CO_3, in DMF; rt, 20 h). The molecular formula of **5** was established by HRMS as $C_{24}H_{28}N_2O_6$; therefore, it is a hexamethylated product. The ^{13}C NMR spectrum of **5** showed 12 carbon resonances, indicating a symmetrical dimeric structure. Analysis of ^1H, ^{13}C, DEPT, and HMQC data revealed that one-half of this symmetric molecule, $C_{12}H_{14}NO_3$, possesses a formyl group (δ_C 163.8, δ_H 8.09), four downfield sp^2 methines at δ_C 126.9 (δ_H 6.53), 122.8 (δ_H 6.89), 111.5 (δ_H 6.84), and 111.2 (δ_H 6.85), two methoxy groups overlapped at δ_C 56.0 (δ_H 3.92, 3H, s; and 3.90, 3H, s), one amide *N*-methyl at δ_C 31.3 (δ_H 3.18, 3H, s), and four downfield quaternary sp^2 carbons resonating at δ_C 149.9, 149.2, 134.7, and 126.2. The presence of a 3,4-dimethoxyphenyl group was deduced from the HMBC data: correlations from the methoxy protons δ_H 3.90 and 3.92, respectively, to the downfield quaternary aromatic carbons C-5 (δ_C 149.2) and C-6 (δ_C 149.9); from H-4 (δ_H 6.84) to C-3 (δ_C 126.2), C-6, and C-8 (δ_C 122.8); from H-7 to C-3 and C-5; and from H-8 to C-3, C-4 (δ_C 111.5), and C-6. Both H-4 and H-8 exhibited additional long-range correlations to the olefinic methine carbon at δ_C 126.9 (C-2), while the adjacent olefinic proton H-2 (δ_H 6.53) showed cross-peaks with C-4, C-8, and a quaternary olefinic carbon at δ_C 134.7 (C-1). These data enabled the connection between C-2 and C-3. An *N*-methylformamide group was evident from the HMBC correlations from *N*-methyl protons to the δ_C 163.8 carbonyl (C-9) and from the formyl proton (H-9) to the *N*-methyl carbon (δ_C 31.3). The amide *N*-methyl protons were also correlated to the δ_C 134.7 quaternary carbon (C-1). On the basis of these data the structure of the $C_{18}H_{16}N_2O_6$ unit was assigned, and the identical units should be coupled at C-1/C-1′ as shown in **5**.

Likewise, acetylation of **4** (Ac_2O, pyridine) afforded the hexaacetate **6**, which was assigned the molecular formula $C_{30}H_{28}N_2O_{12}$ by HRMS. This compound also exhibited a simple NMR signal pattern in $CDCl_3$; therefore, its structure was elucidated by interpretation of NMR data in a similar manner as described above for **5**. On the basis of these structural assignments of the derivatives **5** and **6**, the structure of the parent compound (cordyformamide) was proposed as shown in **4**.

The complex ^1H and ^{13}C NMR signal pattern of cordyformamide (**4**) is due to the rotational hindrance of its *N*-formyl groups. The ^1H NMR spectrum of **4** in DMSO-d_6 showed four amide N*H* signals at δ_H 9.47 (br s), 9.34 (br s), 9.23 (d, J = 11.0 Hz), and 9.17 (d, J = 11.1 Hz), coupled respectively with formyl protons (H-9/H-9′) resonating at δ_H 8.18 (two broad singlets, overlapped; dihedral angle, H−N−C(9)−H, is close to 90°), 7.78 (d, J = 11.2 Hz; dihedral angle is close to 180°), and 7.84 (d, J = 11.2 Hz; dihedral angle is close to 180°). Therefore, the ^1H NMR spectrum of **4** should contain resonances of two symmetric conformers and a nonsymmetric conformer. It is consistent with the appearance of four H-2/H-2′ resonances (δ_H 6.37, 6.36, 6.34, and 6.30), four C-2/C-2′ resonances (δ_C 124.1, 123.0, 122.5, and 121.1), and four C-3/C-3

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Table 1. NMR Data for Cordyformamide (**4**) in DMSO-d_6

position	δ_C[a]	δ_H (mult., J in Hz)
1, 1′	130.3, 129.7, 131.2, 129.7	
2, 2′	124.1, 123.0, 121.1, 122.5	6.30 (s), 6.34 (s), 6.36 (s), 6.37 (s)
3, 3′	126.8, 127.0, 127.1, 127.4	
4, 4′	115.9, 115.9, 116.1, 116.1	6.68−6.72 (m)
5, 5′	145.3, 145.4, 145.5, 145.6	
6, 6′	145.6, 145.7, 145.8, 146.0	
7, 7′	121.7, 121.7, 121.9, 121.9	6.75−6.80 (m)
8, 8′	116.4, 116.5, 116.7, 116.7	7.01 (d, 7.6), 7.01 (d, 7.6), 6.92 (d, 8.8), 6.92 (d, 8.8)
9, 9′	160.8, 160.8, 165.1, 165.1	8.18 (s), 8.18 (s), 7.78 (d, 11.2), 7.84 (d, 11.2)
N*H*		9.47 (s), 9.34 (s), 9.17 (d, 11.1), 9.23 (d, 11.0)

[a] Assignment of carbons between C-5/C-5′ and C-6/C-6′ can be interchanged.

resonances (δ_C 127.4, 127.1, 127.0, and 126.8); however, resonances were partly overlapped in other positions (Table 1). Recently, Tatsuta and Yamaguchi reported the stereoselective synthesis of (*Z*,*Z*)-xanthocillin X dimethyl ether and its (*E*,*E*)-isomer via diformamide intermediates such as (*Z*,*Z*)-**7**.[10] It is described that (*Z*,*Z*)-**7** showed a complex ^{1}H NMR signal pattern.

The symmetrical ^{1}H and ^{13}C NMR data of **5** and **6** indicated that cordyformamide (**4**) should possess either (*Z*,*Z*)- or (*E*,*E*)-geometry. Although attempts to recrystallize **4**, **5**, and **6** for X-ray diffraction analysis met with failure, the NOESY data of **4** strongly suggested that it should be the (*Z*,*Z*)-isomer. Thus, the singlet amide N*H* protons at δ_H 9.47 (s) and 9.34 (s) showed correlation with H-8 (δ_H 7.01, d, J = 7.6 Hz, superimposed). Weak cross-peaks between N*H* and H-7 (δ_H 6.80 and 6.78, respectively) were also observed, while no correlation was found between N*H* and H-2 (δ_H 6.37 and 6.36) of the same unit. In addition, the overlapping singlet formyl protons at δ_H 8.18 showed a cross-peak with H-8. Similarly, the doublet N*H* protons at δ_H 9.17 (d, J = 11.1 Hz) and 9.23 (d, J = 11.0 Hz) exhibited intense cross-peaks with H-8 (δ_H 6.92, d, J = 8.8 Hz, superimposed) along with weak correlations with H-7 (δ_H 6.78 and 6.75, respectively). Again, no cross-peak between N*H* and H-2 (δ_H 6.34 and 6.30) was observed. It should be noted that naturally occurring xanthocillins have been shown to be (*Z*,*Z*)-isomers.

The structure of cordyformamide (**4**) is closely related to melanocin A (**8**),[8] which possesses one isocyanide and one formamide group. Both **4** and **8** are likely to be biogenetic precursors of xanthocillin Y_2 (**3**). In our fermentation study *C. brunnearubra* BCC 1395 proved to be a very slow-growing fungus. Neither **3** nor **8** was detected in the extract even after 116 days incubation of BCC 1395.

Cordyformamide (**4**) exhibited modest activity against the malarial parasite *Plasmodium falciparum* K1 with an IC_{50} value of 18 μM. This compound also showed weak cytotoxicity to BC cells (human breast cancer) at IC_{50} 39 μM, while it was inactive against KB cells (oral human epidermoid carcinoma; up to 56 μM), NCI-H187 cells (human small cell lung cancer; up to 56 μM), and noncancerous Vero cells (up to 140 μM). Compounds **5** and **6** did not show activity in these assays.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Varian CARY 1E UV/vis spectrophotometer. FT-IR spectra were recorded on a Bruker VECTOR 22 spectrometer. NMR spectra were recorded on a Bruker AV500D spectrometer. ESI-TOF mass spectra were measured with a Micromass LCT mass spectrometer.

Fungal Material. *C. brunnearubra* was isolated on an Isoptera termite collected in Sam Lan National Park, Saraburi Province, Thailand, and it was identified by Dr. Nigel L. Hywel-Jones, BIOTEC. This fungus was deposited at the BIOTEC Culture Collection (BCC) as BCC 1395 on July 26, 1997.

Fermentation and Isolation. *C. brunnearubra* BCC 1395 was cultured for 116 days in potato dextrose broth (PDB; potato starch 4.0 g, dextrose 20.0 g, per liter; 20 × 250 mL) at 25 °C under static conditions. The cultures were filtered, and the combined filtrate (5 L) was extracted with EtOAc (2 × 5 L). The EtOAc layer was concentrated under reduced pressure to obtain a pale brown solid (722 mg). This extract was triturated in MeOH (50 mL) at rt for 18 h and filtered by suction to leave a white powder (**4**, 366 mg).

Cordyformamide (4): white powder; UV (MeOH) λ_{max} (log ϵ) 204 (4.49), 348 (4.46) nm; IR (KBr) ν_{max} 3505, 3259, 1631, 1363, 1273, 1110, 910, 796 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 1; HRMS (ESI-TOF) *m*/*z* 379.0909 $[M + Na]^+$ (calcd for $C_{18}H_{16}N_2O_6Na$, 379.0906).

Methylation of 4. Compound **4** (50 mg) was treated with MeI (0.50 mL) and K_2CO_3(s) (200 mg) in DMF (2 mL) at rt for 20 h. The mixture was diluted with EtOAc and washed with H_2O. The EtOAc layer was concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel (2% MeOH in CH_2Cl_2) to afford **5** (60 mg) as a white solid: IR (KBr) ν_{max} 1665, 1597, 1513, 1269, 1149, 1019, 819, 637 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.09 (2H, s, 9-C*H*O and 9′-C*H*O), 6.89 (2H, dd, J = 8.2, 1.6 Hz, H-8 and H-8′), 6.85 (2H, d, J = 8.0 Hz, H-7 and H-7′), 6.84 (2H, d, J = 1.7 Hz, H-4 and H-4′), 6.53 (2H, s, H-2 and H-2′), 3.92 (6H, s, 6-OCH_3 and 6′-OCH_3), 3.90 (6H, s, 5-OCH_3 and 5′-OCH_3), 3.18 (6H, s, 1-NCH_3 and 1′-NCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 163.8 (d, C-9 and C-9′), 149.9 (s, C-6 and C-6′), 149.2 (s, C-5 and C-5′), 134.7 (s, C-1 and C-1′), 126.9 (d, C-2 and C-2′), 126.2 (s, C-3 and C-3′), 122.8 (d, C-8 and C-8′), 111.5 (d, C-4 and C-4′), 111.2 (d, C-7 and C-7′), 56.0 (q, 6-OCH_3 and 6′-OCH_3), 56.0 (q, 5-OCH_3 and 5′-OCH_3), 31.3 (q, 1-NCH_3 and 1′-NCH_3); HRMS (ESI-TOF) *m*/*z* 463.1848 $[M + Na]^+$ (calcd for $C_{24}H_{28}N_2O_6Na$, 463.1845).

Acetylation of 4. Compound **4** (5.0 mg) was treated with Ac_2O (50 μL) in pyridine (0.2 mL) at rt for 24 h. The mixture was diluted with EtOAc and washed with H_2O. The EtOAc layer was concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel (MeOH/CH_2Cl_2) to afford **6** (3.5 mg) as a white solid: IR (KBr) ν_{max} 1772, 1664, 1498, 1375, 1211, 1112, 1013, 910 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.69 (2H, s, H-9 and H-9′), 7.25 (2H, d, J = 8.4 Hz, H-7 and H-7′), 7.19 (2H, dd, J = 8.5, 2.1 Hz, H-8 and H-8′), 7.17 (2H, d, J = 2.0 Hz, H-4 and H-4′), 6.71 (2H, s, H-2 and H-2′), 2.32 (6H, s, 5-OCOCH_3 and 5′-OCOCH_3), 2.32 (6H, s, 6-OCOCH_3 and 6′-OCOCH_3), 2.25 (6H, s, 9-OCOCH_3 and 9′-OCOCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.8 (s, 9-O*C*OCH$_3$ and 9′-O*C*OCH$_3$), 167.8 (s, 6-O*C*OCH$_3$ and 6′-O*C*OCH$_3$), 167.8 (s, 5-O*C*OCH$_3$ and 5′-O*C*OCH$_3$), 162.3 (d, C-9 and C-9′), 131.0 (s, C-1 and C-1′), 130.9 (s, C-3 and C-3′), 128.0 (d, C-2 and C-2′), 126.7 (d, C-8 and C-8′), 124.3 (d, C-7 and C-7′), 123.9 (d, C-4 and C-4′), 22.9 (q, 9-OCO*C*H$_3$ and 9′-OCO*C*H$_3$), 20.7 (q, 6-OCO*C*H$_3$ and 6′-OCO*C*H$_3$), 20.7 (q, 5-OCO*C*H$_3$ and 5′-OCO*C*H$_3$); HRMS (ESI-TOF) *m*/*z* 631.1540 $[M + Na]^+$ (calcd for $C_{30}H_{28}N_2O_{12}Na$, 631.1540).

Biological Assays. The assay for activity against *P. falciparum* (K1, multidrug-resistant strain) was performed using the microculture radioisotope technique described by Desjardins.[11] A standard antimalarial compound, dihydroartemisinin, showed an IC_{50} value of 4.2 nM in the same assay system. Cytotoxicity of the purified compounds against human epidermoid carcinoma cells (KB), human breast cancer cells (BC), human lung cancer cells (NCI-H187), and African green monkey kidney fibroblast cells (Vero) was evaluated using the colorimetric method.[12]

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **4**−**6** and NOESY spectrum of **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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