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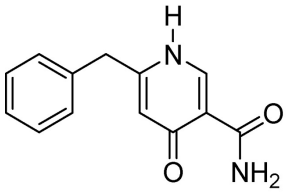


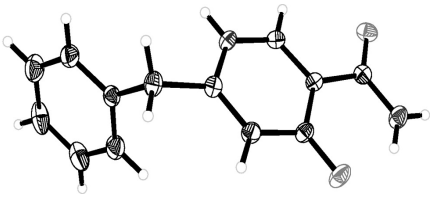

# Structural Revision of Aspernigrin A, Reisolated from *Cladosporium herbarum* IFB-E002

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Aspernigrin A was reisolated as a secondary metabolite of the *Cynodon dactylon*-associated endophytic fungus *Cladosporium herbarum* IFB-E002 coproducing rubrofusarin B, fonsecinone A, 7-hydroxy-4-methoxy-5-methylcoumarin, orlandin, kotanin, and 3$\beta$,5$\alpha$,6$\beta$-trihydroxyergosta-7,22-diene. The structure of aspernigrin A, previously elucidated to be 4-benzyl-6-oxo-1,6-dihydropyridine-3-carboxamide (**1**), was revised as 6-benzyl-4-oxo-1,4-dihydropyridine-3-carboxamide (**2**) on the basis of its additional NMR spectroscopic data and the X-ray crystallographic analysis.

In continuation of our characterization of structurally novel and/or biologically active metabolites from endophytes,[1,2] we found that the extract of *Cladosporium herbarum* IFB-E002 (an endophytic fungus residing in the normal leaves of *Cynodon dactylon*) was substantially inhibitory on xanthine oxidase (XO) and on the growth of the colon cancer cell line SW1116. The subsequent investigation of the extract was therefore performed under bioassay guidance, leading to the reisolation of aspernigrin A,[3] rubrofusarin B,[4,5] fonsecinone A,[5] 7-hydroxy-4-methoxy-5-methylcoumarin,[6] orlandin,[7] kotanin,[8] and 3$\beta$,5$\alpha$,6$\beta$-trihydroxyergosta-7,22-diene.[9] As we reported earlier,[1] rubrofusarin B and fonsecinone A were inhibitory on XO with $IC_{50}$ values of 16.8 and 19.5 $\mu$mol $L^{-1}$, respectively, and the former was cytotoxic to the SW1116 tumor cell line ($IC_{50}$: 15.7 $\mu$mol $L^{-1}$).

Aspernigrin A was recently characterized from the culture of a sponge-derived *Aspergillus niger* strain with its structure assigned mainly from its NMR and MS data as 4-benzyl-6-oxo-1,6-dihydropyridine-3-carboxamide (**1**).[3] However, an error could be discerned with the carbon resonance assignment of the conjugated amide carbonyl at $\delta$ 177.6, much more downfield than its usual value around $\delta$ 166.[10] Although the $^1$H and $^{13}$C NMR, EIMS, and HMBC data of aspernigrin A we reisolated were nearly identical to those reported,[3] an "alternative" structure (**2**) was proposed for aspernigrin A. This assumption was demonstrated to be more reasonable by its NOED spectrum, which displayed, upon irradiating the NH proton (H-1) signal at $\delta$ 12.17, the anticipated NOE enhancements of the H-2 singlet at $\delta$ 8.32 and the methylene (H-7) singlet at $\delta$ 3.90, suggesting the presence of a 4-pyridone (not 2-pyridone[3]) motif in the molecule of aspernigrin A. Moreover, the unambiguous reassignment of its $^1$H and $^{13}$C NMR data is given in Table 1, and its $^1$H−$^1$H COSY and NOED evidence illustrated in Figure 1. Furthermore, its melting point and UV and IR absorption bands, not mentioned earlier,[3] are reported in the Experimental Section.

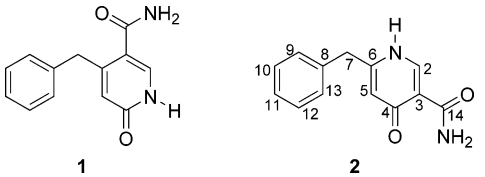


**Table 1.** $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR Data of **2** (DMSO-$d_6$)

| position | $\delta_H$ ($J$ in Hz) | $\delta_C$ (DEPT) | HMBC (H→C) |
|---|---|---|---|
| 1(N*H*) | 12.17 br s | | |
| 2 | 8.32 s | 142.2 d | 4, 6, 14 |
| 3 | | 118.0 s | |
| 4 | | 178.1 s | |
| 5 | 6.22 s | 118.9 d | 3, 4, 6, 7, 14 |
| 6 | | 151.2 s | |
| 7 | 3.90 s (2H) | 38.2 t | 5, 6, 8, 9/13 |
| 8 | | 137.4 s | |
| 9/13 | 7.28 m (2H) | 129.2 (d × 2) | 8, 11 |
| 10/12 | 7.34 m (2H) | 129.3 (d × 2) | 9/13, 11 |
| 11 | 7.27 m | 127.5 d | 9/13, 10/12 |
| 14 | | 165.9 s | |
| CON*H*$_2$ | 9.51 br s 7.40 br s | | |

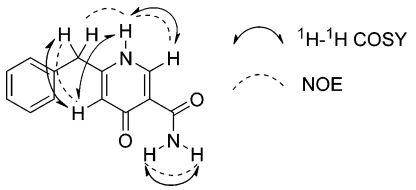


**Figure 1.** Key $^1$H−$^1$H COSY correlations and NOE difference spectral evidence for **2**.

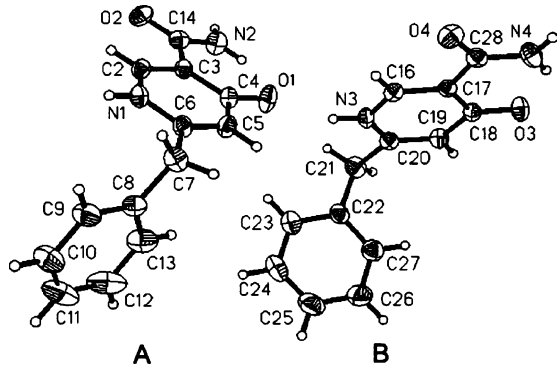


**Figure 2.** X-ray molecule structure of **2**, showing 30% probability displacement ellipsoids and the atom-numbering scheme.

The proposed structure of **2** was confirmed by an X-ray diffraction study with the result shown in Figure 2. Each of the asymmetric units of **2** contains two molecules. The bond lengths and angles (Table 2) of the two rings in each molecule are comparable with those observed in bis(4-maleimidophenyl)methane,[11] a compound containing diphenylmethane. The four rings in each unit are well-defined planes. The dihedral angles between the two aromatic rings

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**Table 2.** Selected Bond Lengths (Å) and Bond Angles (deg) of **2**

| | | | |
|---|---|---|---|
| O2−C14 | 1.236(2) | O4−C28 | 1.240(2) |
| O1−C4 | 1.267(2) | O3−C18 | 1.266(2) |
| N2−C14 | 1.331(2) | N4−C28 | 1.327(2) |
| N1−C2 | 1.341(2) | N3−C16 | 1.334(2) |
| N1−C6 | 1.355(2) | N3−C20 | 1.358(2) |
| C2−C3 | 1.362(3) | C16−C17 | 1.359(2) |
| C3−C4 | 1.436(3) | C17−C18 | 1.440(3) |
| C3−C14 | 1.492(3) | C17−C28 | 1.496(3) |
| C4−C5 | 1.427(3) | C18−C19 | 1.424(3) |
| C5−C6 | 1.346(3) | C19−C20 | 1.347(3) |
| C6−C7 | 1.503(3) | C20−C21 | 1.506(3) |
| C7−C8 | 1.510(3) | C21−C22 | 1.509(3) |
| C8−C9 | 1.372(3) | C22−C23 | 1.371(3) |
| C8−C13 | 1.384(3) | C22−C27 | 1.379(3) |
| C9−C10 | 1.376(3) | C23−C24 | 1.405(4) |
| C10−C11 | 1.369(4) | C24−C25 | 1.356(4) |
| C11−C12 | 1.368(4) | C25−C26 | 1.347(4) |
| C12−C13 | 1.383(3) | C26−C27 | 1.376(3) |
| C2−N1−C6 | 121.65(17) | C16−N3−C20 | 121.50(16) |
| N1−C2−C3 | 122.67(18) | N3−C16−C17 | 122.83(18) |
| C2−C3−C4 | 118.32(18) | C16−C17−C18 | 118.55(18) |
| C2−C3−C14 | 116.98(17) | C16−C17−C28 | 116.79(18) |
| C4−C3−C14 | 124.70(18) | C18−C17−C28 | 124.64(17) |
| O1−C4−C5 | 121.53(18) | O3−C18−C19 | 121.48(18) |
| O1−C4−C3 | 122.59(18) | O3−C18−C17 | 123.10(18) |
| C5−C4−C3 | 115.88(18) | C19−C18−C17 | 115.41(17) |
| C6−C5−C4 | 122.72(19) | C20−C19−C18 | 122.97(19) |
| C5−C6−N1 | 118.76(18) | C19−C20−N3 | 118.69(19) |
| C5−C6−C7 | 125.04(19) | C19−C20−C21 | 125.4(2) |
| N1−C6−C7 | 116.19(18) | N3−C20−C21 | 115.87(18) |
| C6−C7−C8 | 113.35(17) | C20−C21−C22 | 112.33(17) |
| C9−C8−C13 | 118.1(2) | C23−C22−C27 | 117.6(2) |
| C9−C8−C7 | 120.7(2) | C23−C22−C21 | 122.2(2) |
| C13−C8−C7 | 121.1(2) | C27−C22−C21 | 120.1(2) |
| C8−C9−C10 | 121.4(2) | C22−C23−C24 | 120.3(3) |
| C11−C10−C9 | 120.0(3) | C25−C24−C23 | 120.0(3) |
| C12−C11−C10 | 119.7(3) | C26−C25−C24 | 120.5(3) |
| C11−C12−C13 | 120.1(3) | C25−C26−C27 | 119.8(3) |
| C12−C13−C8 | 120.6(3) | C26−C27−C22 | 121.8(2) |
| O2−C14−N2 | 122.96(19) | O4−C28−N4 | 123.2(2) |
| O2−C14−C3 | 120.62(19) | O4−C28−C17 | 120.36(19) |
| N2−C14−C3 | 116.41(18) | N4−C28−C17 | 116.40(19) |

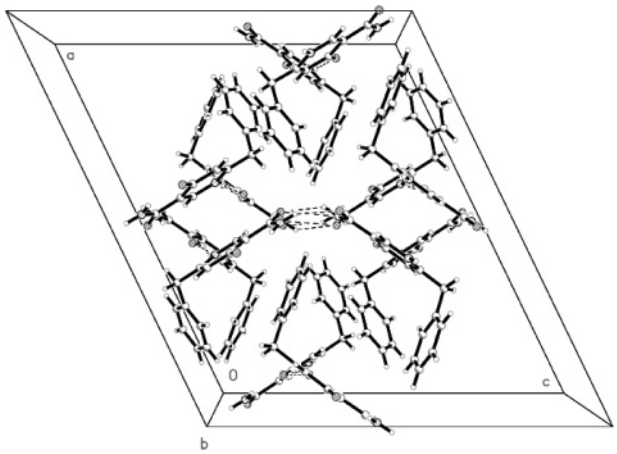


**Figure 3.** Perspective view of **2**, showing the hydrogen contacts.

in each molecule are 97.6(1)° for molecule A and 76.3(1)° for molecule B, being significantly different from each other. All the oxygen and nitrogen atoms contribute to the formation of H-bonds. Due to the strong intramolecular hydrogen bonds (N2−H···O1 and N4−H···O3), the acylamide group and the quinone oxygen atom constitute a plane, which is nearly parallel (the dihedral angles are 1.9(1)° and 2.9(1)° for molecule A and for molecule B, respectively) to the plane to which they attach. Every two adjacent molecules are linked together through the intermolecular hydrogen bonds between N atoms and the O atoms, which further join into a one-dimensional zigzag chain (as shown in Figure 3).

## Experimental Section

**General Experimental Procedures.** Melting points were determined on a Boetius micromelting point apparatus and are uncorrected. The UV spectra were recorded on a Hitachi U-3000 spectrophotometer and IR on a Nexus 870 FT-IR spectrometer. All NMR experiments were performed in DMSO-$d_6$ on a Bruker DRX500 NMR spectrometer with $^1$H and $^{13}$C nuclei observed at 500 and 125 MHz, respectively. EIMS experiments were run on a VG-ZAB-HS mass spectrometer at 70 eV. HRESIMS was recorded on a Mariner System 5304 instrument. Silica gel (200−300 mesh) for column chromatography and $GF_{254}$ (10−20 mm) for TLC were produced by Qingdao Marine Chemical Factory, Qingdao, China. Sephadex LH-20 was purchased from Pharmacia Biotech, Sweden. All other chemicals were of analytical grade.

**Biological Material.** *Cladosporium herbarum* IFB-E002 was an endophytic fungus isolated according to the protocol detailed elsewhere[12] from surface-sterilized fresh leaves of *Cynodon dactylon* collected in early November 2001 from Yancheng Biosphere Reserve, Jiangsu Province, China. The identification of the fungal strain was accomplished on the basis of its morphological characteristics[13] with an additional confirmation from its 18S rDNA sequence, which gave a 98% sequence similarity to those accessible at the BLASTN of *C. herbarum*.

The fresh mycelium of *C. herbarum* grown on PDA medium at 28 °C for 6 days was inoculated into 1000 mL flasks containing 400 mL of PD medium. After 4-day incubation at 28 °C on a rotary shaker at 150 rpm, 15 mL of culture liquid was transferred as seed into 250 mL flasks each preloaded with evenly mingled medium composed of water (15 mL), grain (7.5 g), bran (7.5 g), yeast extract (0.5 g), tartrate sodium (0.1 g), glutamine sodium (0.1 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), and pure corn oil (0.1 mL). The next cultivation was performed for 35 days at 28 ± 1 °C, and the relative humidity was 60−70% with initial water content of the medium at ca. 67%.

**Extraction and Isolation.** The crude solid fermentation product (3.5 kg, not completely dried) was collected and extracted thrice with MeOH (15 L) at room temperature. In vacuo evaporation of the solvent from extract yielded a brown residue (120 g), which immediately dissolved in MeOH (ca. 500 mL) on a $H_2O$ bath (ca. 50 °C) with occasional shaking. This MeOH solution was kept at −20 °C overnight after being cooled to room temperature. Filtration of the cold liquor gave a waxy substance and filtrate that was concentrated under reduced pressure to give a brown-black solid (40 g). This solid was redissolved in a MeOH/acetone (85/15) mixture, and the resulting solution was stored at −20 °C overnight. Filtration of the cold solution gave a salty precipitate and filtrate that was concentrated in vacuo to yield a black material (37 g), which was chromatographed over a silica gel column (400 g, 200−300 mesh) eluted with $CHCl_3$/MeOH mixtures (100:0 → 0:100, v/v) of increasing polarity. The eluates (500 mL each) were combined to give eight fractions according to TLC monitoring. The XO inhibitory fraction (fraction 2, 2.4 g) was rechromatographed on a silica gel column (80 g, 200−300 mesh) eluted with $CHCl_3$/MeOH mixtures (200:1 → 10:1, v/v) followed by repeated gel filtration over Sephadex LH-20 ($CHCl_3$/MeOH, 1:1, v/v) to give rubrofusarin B (15 mg) and fonsecinone A (6 mg). Fraction 3 (1.6 g) was further separated over a silica gel column (30 g, 200−300 mesh) with a $CHCl_3$/MeOH gradient to give 7-hydroxy-4-methoxy-5-methylcoumarin (12 mg). Repeated chromatography of fraction 4 (3.6 g) on a silica gel column (80 g, 200−300 mesh; $CHCl_3$/MeOH, 100:1 → 10:1, v/v) followed by gel filtration over Sephadex LH-20 ($CHCl_3$/MeOH, 1:1, v/v) gave orlandin (8 mg), kotanin (5 mg), 3$\beta$,5$\alpha$,6$\beta$-trihydroxyergosta-7,22-diene (4 mg), and compound **2** (15 mg). Compound **2** obtained as white amorphous powder was recrystallized as prisms from the mixture of $CHCl_3$/MeOH (1:1, v/v).

**Aspernigrin A (2):** colorless crystals; mp 197−199 °C; UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 217 (4.25), 257 (4.68) nm; IR (KBr) $\nu_{max}$ 3347, 3215, 3065, 2861, 2713, 2614, 1665, 1601, 1584, 1498, 1455, 1350, 1249, 863, 730, 698 $cm^{-1}$; $^1H$ and $^{13}C$ NMR, see Table 1; EIMS (70 eV) *m/z* 228 $[M]^+$ (65), 227 (90), 211 (44), 210 (100), 185 (22), 155 (36), 154 (48); HRESIMS *m/z* 251.0811 $[M + Na]^+$ (calcd for $C_{13}H_{12}N_2O_2Na$, 251.0796).

**X-ray Crystallographic Analysis of 2.** A colorless prism of **2** (0.39 × 0.26 × 0.15 mm) was selected for the data collection, and the structure was solved by the direct method using SHELXS-97.[14] Crystal data of **2**: $C_{13}H_{12}N_2O_2$, $M_r$ = 228.25 g $mol^{-1}$, monoclinic, space group $C_2/c$, $a$ = 21.944(2) Å, $b$ = 12.132(2) Å, $c$ = 19.326(2) Å, $\beta$ = 115.836(2)°, $V$ = 4630.4(7) $Å^3$, $Z$ = 16, $F(000)$ = 1920, $\lambda$(Mo Kα) = 0.71073 Å, $\mu$(Mo Kα) = 0.090 $cm^{-1}$, $D_x$ = 1.310 g $cm^{-3}$, $T_{min}/T_{max}$ = 0.9752/0.9857, reflections/parameters = 4988/307, goodness of fit on $F^2$ = 0.874, $R_1$, $wR_2$ [$I \geq 2\sigma(I)$] = 0.0479/0.1013, $R_1$, $wR_2$ (all data) = 0.0971/0.1315. The structure of **2** was refined by SHELXL-97. All non-hydrogen atoms were refined with anisotropic thermal parameters, and hydrogen atoms calculated on the ideal positions were refined with isotropic thermal parameters and included in the calculations of the structure factors.

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**Supporting Information Available:** This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre, as CCDC No. 262012. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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