ORGANIC LETTERS

2012
Vol. 14, No. 8
2006–2009

Aromatic Polyketide Production in *Cordyceps indigotica*, an Entomopathogenic Fungus, Induced by Exposure to a Histone Deacetylase Inhibitor

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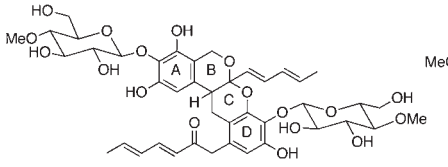
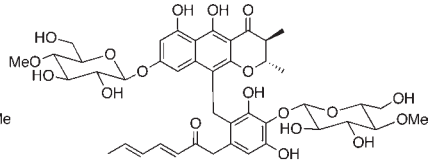
Received February 28, 2012

ABSTRACT



Indigotides C and D (BC ring junction: *cis*)



Indigotide E

Cultivation of *Cordyceps indigotica*, an entomopathogenic fungus, in the presence of suberoyl bis-hydroxamic acid (an HDAC inhibitor) greatly activated its polyketide synthesis apparatus to afford six novel aromatic polyketides, indigotides C−F (1−4), 13-hydroxyindigotide A (5), and 8-*O*-methylindigotide B (6). The structures of these compounds were determined by NMR spectroscopic analyses. Among the compounds, indigotides C−E (1−3) possessed unprecedented dimeric polyketide frameworks possibly generated via a [4 + 2] cycloaddition or Michael type reaction.

Filamentous fungi are well-known producers of a large number of diverse natural products, many of which have fascinating structures and important biological activities that have attracted the attention of natural product chemists, organic chemists, and pharmacologists. As fungal genomes have been sequenced, it has become clear that there are far more secondary metabolite-encoding biosynthetic gene clusters than were evident in previous chemical studies.[1] This suggests that fungi have dozens of unique gene cluster coding for uncharacterized polyketides and nonribosomal peptides. Many of these gene clusters are said to be transcriptionally suppressed under standard laboratory culture conditions.[2] Recently, it was demonstrated that epigenetic modification compounds, such as histone deacetylase (HDAC) inhibitors or DNA methyltransferase inhibitors, could induce transcriptional up-regulation of many PKS and NRPS-encoding gene clusters in a model fungus, *Aspergillus niger*.[3] A handful of new polyketides and nonribosomal peptides were isolated from fungi of the *Aspergillus* and *Penicillium* genera cultivated in the presence of HDAC inhibitors.[4,5] In an effort to obtain novel bioactive polyketides, we applied these chemicals to cultures of entomopathogenic fungi. Cultivation in the presence of 5-azacytidine (a DNA methyltransferase inhibitor), followed by extraction, resulted in the isolation of novel polyketides, including indigotides A (**7**) and B (**8**), from *Cordyceps indigotica*, while in the absence of an inhibitor, this species produces cyclic depsipeptides, destruxins A, B, and E, and a highly conjugated polyketide,

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10.1021/ol3005062
Published on Web 04/05/2012

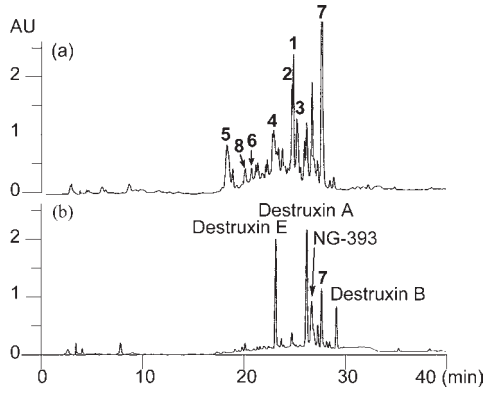

Figure 1. HPLC profiles of the EtOAc extracts of *C. indigotica* cultivated in the presence of SBHA 1 mM (a) and control (b) as detected by UV absorption at 215 nm.

NG-393, as the major constituents of the culture medium (Figure 1).[6] In our continuing search for diverse secondary metabolites produced by *C. indigotica* during cultivation in the presence of epigenetic modification compounds, we found that the addition of suberoyl bis-hydroxamic acid (SBHA) to the culture medium significantly enhanced the polyketide production and led to the isolation of six novel aromatic polyketides, indigotides C−F (**1**−**4**), 13-hydroxyindigotide A (**5**), and 8-*O*-methylindigotide B (**6**), together with **7** and **8**. In this paper, we discussed the isolation and structural elucidation of **1**−**6** and proposed a plausible pathway to account for their biosynthetic relationship (Scheme 1).

C. indigotica was cultivated in a PDB liquid medium containing 1 mM SBHA for 8 days under shaking conditions at 25 °C. The culture medium (10.8 L) was extracted with ethyl acetate, and the extract (1.4 g) was separated by Sephadex LH-20, silica gel column chromatography, and reversed-phase HPLC to afford eight aromatic polyketides, indigotides A (**7**, 157.0 mg), B (**8**, 4.8 mg), C (**1**, 5.6 mg), D (**2**, 5.3 mg), E (**3**, 18.4 mg), and F (**4**, 9.2 mg), 13-hydroxyindigotide A (**5**, 11.4 mg), and 8-*O*-methylindigotide B (**6**, 9.6 mg) (Figure 2).

The molecular formula of indigotide C (**1**) was determined to be $C_{42}H_{52}O_{18}$, as deduced from a negative HRFABMS at *m*/*z* 843.3068 $[M-H]^-$ (calcd 843.3075), requiring 17 degrees of unsaturation. The molecular formula was twice that of **7** ($C_{21}H_{26}O_9$).[6] The IR spectrum (1634 cm^{-1}) indicated the presence of an α,β-unsaturated carbonyl group. The ^1H NMR spectrum, with the assistance of the $^1H-^1H$ COSY spectrum, suggested the presence of two sets of 4-*O*-methyl-β-D-glucose (C-1″−C-6″, C-1‴−C-6‴) and (1*E*,3*E*)-1,3-pentadienyl moieties (C-9−C-13, C-9′−C-13′) (Table S1). The ^{13}C NMR spectrum revealed carbon signals due to one keto carbonyl carbon, ten quaternary sp^2 carbons, ten tertiary sp^2 carbons, one acetal carbon, one methine, one oxymethylene, two methylenes, two methyls, and signals corresponding to two

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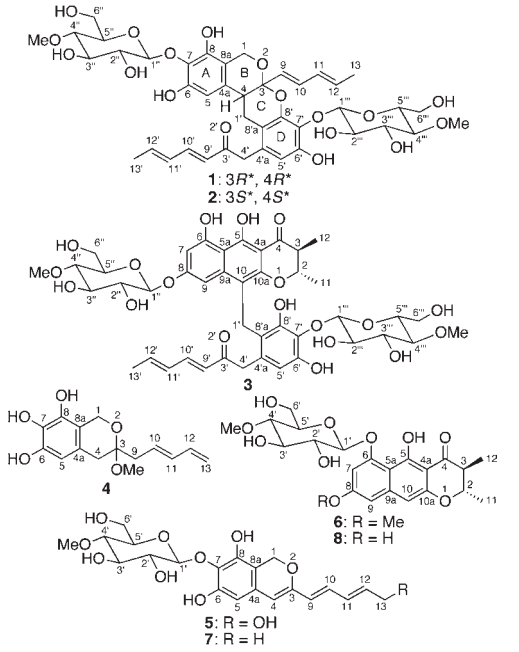

Figure 2. Structures of **1**−**8**.

4-*O*-methyl-β-D-glucoses (Table S1). Moreover, the NMR signals assignable to C-1−C-13 in **1** appeared at field strengths similar to the signals of the aglycone portion of **7**, except for the C-4 carbon at δ 37.6, the hydrogen at δ 3.06, and the C-3 carbon at δ 99.6. The HMBC correlations of H_2-1/C-3, C-4a, C-8, C-8a; H-4/C-3, C-4a, C-5, C-8a; H-5/C-6, C-7, C-8a indicated the presence of a 3,3,4,6,7,8-hexasubstituted 1*H*-isochroman core. The correlations of H-1″/C-7; H-9/C-3; H-10/C-3 indicated that a sugar moiety and a 1,3-pentadienyl moiety were present at C-7 and C-3, respectively. The partial structure A was thus determined, as shown in Figure 3. The long-range correlations from H-4′, H-9′, and H-10′ to C-3′ supported the 3,5-heptadien-2-one structure (C-4′−C-13′) (Figure 3). Furthermore, the partial structure B bearing a pentasubstituted benzene ring was suggested by the correlations of H_2-1′/C-4′a, C-8′, C-8′a; H_2-4′/C-4′a, C-5′, C-8′a; H-5′/C-6′, C-7′, C-8′a; H-1‴/C-7′ (Figure 3). The $^1H-^1H$ COSY correlations of H-4/Ha-1′, Hb-1′ allowed the connectivity between C-4 and C-1′ (Figure 3). The above functionality accounted for 16 of the 17 degrees of unsaturation, suggesting the presence of another ring. In accordance with the molecular formula, two of the five oxygenated carbons (C-3, C-6, C-8, C-6′, C-8′) were thought to be linked through an ether bond, and the other three possessed hydroxyl groups. The linkage was determined by converting **1** to the corresponding methyl ether **1a** (Scheme S1). The molecular weight of **1a** (HRFABMS: *m*/*z* 887.3724 $[M+H]^+$) increased by 42 mass units. The NMR spectrum of **1a** displayed three aromatic methoxy methyls newly produced (δ 3.86, 3.83, and 3.78), which showed NOEs with H_2-1, H-5, and H-5′, respectively. These results indicated that the three methoxy groups were located at C-8, C-6, and C-6′,

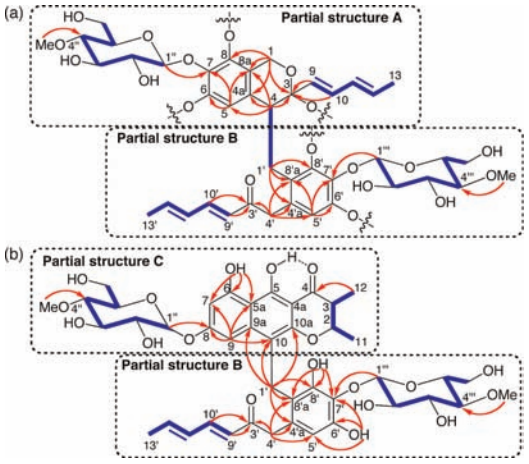

Figure 3. Key $^{1}H-^{1}H$ COSY (blue bold line) and HMBC (red arrow) correlations of **1** (a) and **3** (b).

and C-3 was linked with C-8′ through an ether bond, elucidating the planar structure of **1** as shown in Figure 2.

The relative configuration of **1** was determined on the basis of 1D-ROESY experiments and coupling constant analysis (Figure 3). The NOE of H-4/H-9 revealed a *cis*-fused BC ring. Furthermore, the NOEs of H-4/H-5, H-5/Ha-1′, and H-9/Hb-1′ indicated that the B-ring was in a pseudochair conformation, and C-1′ and C-9 were positioned equatorially (Figure 4). The dihedral angles of H-4/Ha-1′ and H-4/Hb-1′, shown in Figure 4, were characterized from the same coupling constants (5.2 Hz). The coexistence of **7** with **1** occurs with the supposed D-form of the sugar moieties in **1**.[6]

The molecular formula of indigotide D (**2**) was the same as that of **1**, as deduced from the HRFABMS.The spectral data of **2**, including the ^{1}H and ^{13}C NMR (Table S1), UV, and IR spectra (see the Supporting Information), agreed well with those of **1**. The correlation patterns in the $^{1}H-^{1}H$ COSY, HMBC, and 1D-ROESY experiments clearly indicated that the planar structure and the relative configuration of **2** were identical to those of **1**. Major differences were observed in the optical rotatory values [**2**: $[\alpha]^{25}_{D} = -25.9$ (*c* 0.29, EtOH); **1**: $[\alpha]^{25}_{D} = 0.0$ (*c* 0.15, EtOH)] and in the CD spectra (Figure 5), demonstrating that the aglycones of **1** and **2** were enantiomerically related.

Indigotide E (**3**) revealed a pseudomolecular ion peak at *m*/*z* 871.3046 $[M-H]^{-}$ in the negative HRFABMS, which suggested a molecular formula of $C_{43}H_{52}O_{19}$. Like compound **1**, the ^{1}H and ^{13}C NMR spectra of **3** indicated the presence of two 4-*O*-methyl-*β*-D-glucoses (Table S2). The ^{13}C NMR spectrum exhibited 43 signals, among which 21 signals (C-1′−C-13′, C-1‴−C-6‴, 4‴-OMe) were unambiguously assigned to the partial structure B, present in **1**, based on the HMBC correlations (Figure 3). In addition, 15 signals assignable to C-2−C-12 agreed well with those of the aglycone in **8** (Table S2). The only exception to this agreement was the observation of another quaternary sp^2 carbon (C-10) in **3**. These results indicated that **3** possessed

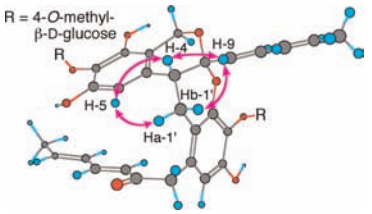

Figure 4. Key NOE correlations of **1**.

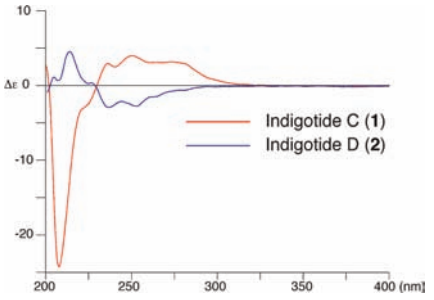

Figure 5. CD spectra of **1** and **2**.

the same 2,3-dimethyl-2,3-dihydro-4*H*-naphtho[2,3-b]-pyrane-4-one framework found in **8**. The ^{1}H NMR spectrum revealed a downfield shifted exchangeable proton at *δ* 15.79 (5-OH), which suggested the presence of the C-5 phenolic hydroxyl group that has a hydrogen bond with the C-4 carbonyl. In addition, the HMBC correlations of H-1″/C-8; 6-OH/C-5a, C-6, C-7; H-9/C-5a, C-7, C-8, C-10, along with the other correlations described in Figure 3, clearly revealed the partial structure C. The linkage between the two partial structures through C-10 and C-1′ was determined by the long-range correlations of H_2-1′/C-9a, C-10, C-10a. The large coupling constant between H-2 and H-3 (9.4 Hz) indicated a *trans*-diaxial relationship between H-2 and H-3. The negative Cotton effect around 335 nm in the CD spectrum showed that the absolute stereochemistry at C-2 was *S*.[6−8] Therefore, the structure of **3** was confirmed as shown in Figure 2.

The ^{13}C NMR spectrum of indigotide F (**4**), $C_{15}H_{18}O_5$ (HRFABMS: *m*/*z* 277.1069 $[M-H]^{-}$ (calcd 277.1076)), exhibited signals corresponding to five quaternary sp^2 carbons, four tertiary sp^2 carbons, one secondary sp^2 carbon, one acetal carbon, three methylenes, and one methoxy methyl (Table S3). The ^{1}H NMR spectrum displayed one singlet aromatic proton [*δ* 6.13 (s, H-5)], corresponding to a pentasubstituted benzene ring, five olefinic signals [*δ* 5.71 (dt, *J* = 15.4, 7.5 Hz), 6.20 (dd, *J* = 15.4, 10.7 Hz, H-11), 6.36 (dt, *J* = 17.3, 10.7 Hz,

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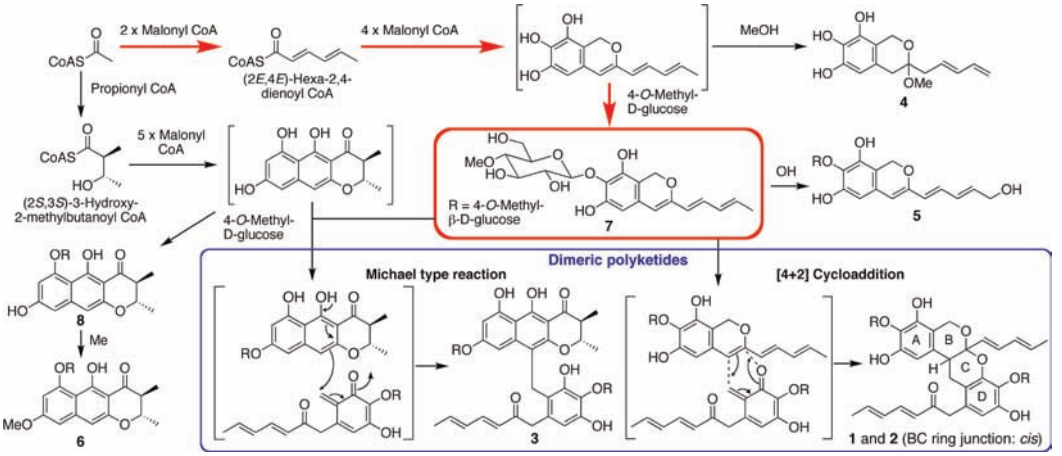
Scheme 1. Plausible Biosynthetic Relationship among the Isolated Polyketides



H-12), 5.13 (d, J = 17.3 Hz, Ha-13), 4.98 (d, J = 10.7 Hz, Hb-13)], and a methoxy methyl signal [δ 3.25 (s, OMe)], as well as signals corresponding to three methylenes (Table S3). The ^{1}H−^{1}H COSY correlations revealed the connectivity of C-9 to C-13, and the HMBC correlations of H_2-1/C-3, C-4a, C-8, C-8a; H_2-4/C-3, C-4a, C-5, C-8a; H-5/C-4, C-6, C-7 C-8a indicated the presence of a 3,3,6,7,8-pentasubstituted 1*H*-isochroman core (C-1−C-8a) (Figure S1). In addition, the long-range correlations from H-9, H-10, and OMe to C-3 revealed the position of the OMe group and the linkage between the acetal C-3 and C-9. The 6,7,8-trihydroxy substitution was deduced from the molecular formula. Thus, the structure of **4** was found to be as shown in Figure 2. The stereocenter at C-3 was thought to be racemic because the optical rotation of **4** was minimal ($[\alpha]^{25}_D$ = 0.0 (c = 0.38, EtOH)), and no remarkable Cotton effects were observed in the CD spectrum.

The molecular formula of **5** was determined to be $C_{21}H_{26}O_{10}$ by HRFABMS, which was one oxygen atom larger than that of **7**. The ^{1}H and ^{13}C NMR spectra permitted determination of **5** as the 13-hydroxy derivative of **7** (Table S4). Thus, the structure of **5** was found to be 13-hydroxyindigotide A (Figure 2).

The molecular formula ($C_{23}H_{28}O_{10}$) and the ^{1}H and ^{13}C NMR spectra of **6** (Table S2) suggested that the compound was the *O*-methylated derivative of **8**. The methylated position was determined based on the HMBC correlation between OMe and C-8; thus, the structure of **8** was 8-*O*-methylindigotide B (Figure 2).

In this study, we demonstrated that the addition of SBHA (an HDAC inhibitor) to the culture medium of *C. indigotica* significantly enhanced polyketide production, particularly through the biosynthetic pathway associated with the biosynthesis of indigotide A (**7**) (Figure 1 and Scheme 1). Activation of the polyketide biosynthetic pathway yielded many novel polyketides related to **7** (Scheme 1). Among these, indigotides C (**1**), D (**2**), and E (**3**) possessed unprecedented dimeric structures that may be generated via, respectively, an endo selective [4 + 2] cycloaddition of two indigotide A (**7**) molecules, or a Michael type reaction between an indigotide B analog and indigotide A (Scheme 1). This study suggested that epigenetic modification compounds can increase the biosynthesis of diverse fungal polyketides via activation of silent biosynthetic pathways.

Acknowledgment. This work was supported in part by a Grant-in-Aid for Scientific Research (Nos. 23710248 and 23590582) from the Ministry of Education, Science, Sports and Culture of Japan; A-STEP (FS-stage) (No. AS231Z01347G) from Japan Science and Technology Agency (JST); the Uehara Memorial Foundation; Japan Association for Chemical Innovation.

Supporting Information Available. Experimental procedure, figure, scheme, full spectroscopic data and NMR spectra of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.