Carbon-nitrogen bond formation to construct novel polyketide-indole hybrids from the indole-3-carbinol exposed culture of *Daldinia eschscholzii*

**Abstract**

A plenty of cytochrome P450s have been annotated in the *Daldinia eschscholzii* genome. Inspired by the fact that some P450s have been reported to catalyze the carbon-nitrogen (C-N) bond formation, we were curious about whether hybrids through C-N bond formation could be generated in the indole-3-carbinol (I3C) exposed culture of *D. eschscholzii*. As expected, two skeletally undescribed polyketide-indole hybrids, designated as indol-polyketides A and B (1 and 2), were isolated and assigned to be constructed through C-N bond formation. Their structures were elucidated by 1D and 2D NMR spectra. The absolute configurations of 1 and 2 were determined by comparing the recorded and calculated electronic circular dichroism (ECD) spectra. Furthermore, the plausible biosynthetic pathways for 1 and 2 were proposed. Compounds 1 and 2 exhibited significant antiviral activity against H1N1 with IC₅₀ values of 45.2 and 31.4 μM, respectively. In brief, compounds 1 and 2 were reported here for the first time and were the first example of polyketide-indole hybrids pieced together through C-N bond formation in the I3C-exposed culture of *D. eschscholzii*. Therefore, this study expands the knowledge about the chemical production of *D. eschscholzii* through precursor-directed biosynthesis (PDB).

1. Introduction

Microbes and plants produce diverse natural products (also called secondary metabolites), which remain an important source of medicines, pesticides and chemical tools [1,2]. In particular, the central significance of natural products as prime starting molecules for the drug discovery is reflected both by the fact that half of all the new chemical entity-based drugs introduced in the last three decades—540 out of 1073—are ‘nature-derived’ [3]. With the development of society, the demand for new compounds for drug discovery pipelines has become more and more urgent. To address the issue, several strategies have been developed, such as bioactivity-guided isolation [4], engineering strategies [5], and genome-focused approaches [4]. However, we are increasingly disturbed by the fact that more and more known natural products were re-isolation, which is incredibly time consuming [4]. To overcome such frustration, precursor-directed biosynthesis (PDB) approach was thus established to selectively and efficiently produce target new natural or semi-natural skeletons [6,7].

Scrutiny of the structures of marketed drugs, we found that both polyketides [8] and indole alkaloids are among the privileged scaffolds [9]. Polyketides represent a viable source of chemically diverse and biologically active natural compounds, of which many have become important clinical therapeutics [10]. Besides, indole alkaloids are one of the hot topics as an important source of lead compounds that have resulted in diverse clinical medicines [11]. Such information reminds us to ask whether the hybrids of polyketides and indoles could be another valuable source of lead compounds. Screening of our fungal library, we found that *D. eschscholzii*, a fungus residing originally in the *Tenodera aridifolia* gut generated a plenty of polyketides without detecting any alkaloids [12,13]. After supplementation of indole-3-carbinol (I3C) in the culture of *D. eschscholzii*, we obtained an antibacterial and anti-inflammatory polyketide-indole hybrid, dalestindole, which was stereoselectively biosynthesized by *D. eschscholzii* through class II aldolase catalyzed C-C bond formation between 3,3′-didiarylpropylene (DMP) and C-3 of fungal chromene [14]. Furthermore, two more skeletal polyketide-indole hybrids, named indolchromins A and B, were...
identified by piecing polyketide and indole together through C–C bond formation [15]. Notably, C–N bond formation is another key step toward the construction of novel skeletons [16] and many cytochrome P450s have been evidenced to be responsible for C–N bond formation [17,18]. Through the bioinformatics analysis, we found that a total of 119 cytochrome P450s have been annotated in the *D. eschscholzii* genome [19] (Fig. 1), encouraging us to hypothesize that the I3C exposed-culture of *D. eschscholzii* could generate new polyketide-indole hybrids through C–N bond formation. Gratifyingly, the endeavor led to the characterization of two new skeletal polyketide-indole hybrids, named indolopolyketone A and B (1 and 2), formed through C–N bond formation between polyketide and DIM, an oxidative dimer of I3C. We herein provide details of the structure identification, plausible biosynthetic pathway and biological activities of 1 and 2.

2. Materials and methods

2.1. General experimental procedures

Silica gel (200–300 mesh) for column chromatography (CC) and GF254 (10–20 mm) for TLC (thin layer chromatography) were produced by the Qingdao Marine Chemical Company, China. The ODS silica gel (50 μm) was purchased from YMC Co. Ltd, Japan, and Sephadex LH-20 from Pharmacia Biotech, Sweden. Reagents and solvents used in the study were of analytical grade. HPLC separations were carried out using an Agilent ODS column (5 μm, 250 × 10 mm), a 1260 infinity II preparative binary pump G7161A, and a UV detector G7114A. MS spectra were determined on an Agilent 6546 LC/TOF-MS spectrometer operating in a positive mode with direct infusion. NMR spectra were analyzed on a Bruker DRX400 and Varian Inova-600 NMR spectrometer with TMS as an internal standard. Electronic CD (ECD) spectra were acquired on a JASCO J-810 chirascan. Optical rotations were recorded on a Rudolph Research Analytical Autopol IV automatic polarimeter. IR spectra were acquired in KBr disks on a Nexus 870 FT-IR spectrometer.

2.2. Fungal cultivation and extraction

*Daldinia eschscholzii* IFB-TL01 was cultured on Petri dishes of potato dextrose agar (PDA) at 28 °C for 3 days. The fresh mycelium taken from the grown fungal colony was inoculated into the flasks (always 1-L sized), each containing 400 mL of malt-extract medium (20 g/L malt extract, 20 g/L sucrose, 1 g/L peptone). After a 2-day incubation at 28 °C with an agitation of 200 rpm, 20 mL of culture liquid was transferred as inoculating seed into each flask containing 400 mL of ME medium. I3C at the final concentration of 1.0 mM was added into the flasks at 72 h after inoculation. Cultivations were continued for the ensuing 10 days at 28 °C with agitation (200 rpm). The broth was collected and extracted with EtOAc.

2.3. Polyketide-indole hybrids from the I3C-exposed fungal cultivation

*In vacuo* evaporation of EtOAc from the above extract gave the residue (25.63 g), which was subjected to CC over silica gel with petroleum ether/acetone mixtures (v/v 100:2, 100:5, 10:1, 5:1, 3:1, 2:1, 1:1) to yield seven fractions (A–G). The CC fraction F (5.14 g) derived from the petroleum ether/acetone (2:1) was verified to be alkaloid-rich by the LC-HR-MS analysis, thereby being subjected to CCs over ODS with a gradient of MeOH/H2O (30:70 → 100:0, v/v) to give seven subfractions.
The subfraction F6 (0.32 g) was then subjected to the semi-preparative RP-HPLC equipped with a Xbridge C18 PN FWXB 12505-2510 (10 × 250 mm) using 75% MeOH in water for 30 min (flow rate: 2 mL/min) to yield 1 (5.7 mg, tR = 17.6 min) and 2 (4.2 mg, tR = 21.2 min).

2.4. Chiral HPLC separation of 1 and 2

The commercialized chiral column (CHIRALPAK® IA, Lot No. IA00CG-RE001, 10 × 250 mm) was used for the Chiral HPLC resolution of racemates 1 and 2 with n-hexane/ethanol (85:15, v/v) as the mobile phase.

2.5. Computational details

The corresponding excited-state calculations were performed at the ground-state optimized geometries. Time-dependent density functional theory (DFT) in combination with polarizable continuum model (PCM), dielectric constant ε = 32.64 for MeOH (TD-DFT/PCM) with the same basis set was carried out to calculate the spin-allowed excitation energy and rotational strength of the lowest 100 excited states. The ECD spectra were generated using the program SpecDis [20] by applying a Gaussian band shape with the width of 0.20 eV, from oscillator strengths and dipole-velocity rotational strengths, respectively.

2.6. Cells and viruses [21]

Vero cells were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in 5% CO2. Madin-Darby canine kidney (MDCK) cells were grown in DMEM medium supplemented with 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin. Influenza A virus H1N1 (A/Puerto Rico/8/34), and H1N1 (A/Virginia/ATCC/2009) were purchased from ATCC (USA), and propagated in 10-day-old embryonated eggs for three days at 36.5 °C.

2.7. Cytopathic effect (CPE) inhibition assay

The antiviral activity was evaluated by the CPE inhibition assay [21]. Briefly, MDCK or Vero cells in 96-well plates were infected with influenza A virus at a multiplicity of infection (MOI) of 0.1, respectively, and therefore, we were encouraged to address the fungal biosynthesis of DIM [19]. Therefore, compound 1 was hypothesized to be produced through the Michael addition of 3-methyleneindolium (3MI, an electrophile) and formaldehyde-releasing) pathways may explain the biosynthesis of DIM [22]. Therefore, compound 2 was generated through another round of Michael addition of DIM with PBE0, which may be catalyzed by an unknown P450 enzyme. Compound 2 was generated through another round of Michael addition of DIM with PBE0, which may be catalyzed by an unknown P450 enzyme.

3. Results

3.1. Phylogenetic analysis-based recognition of cytochrome P450 in D. eschscholzii

The genome of D. eschscholzii was deposited at https://www.ncbi.nlm.nih.gov. GenBank: GCA_000751375.2, GenBank: GCA_001951055.1, and GenBank: GCA_000261445.1, respectively. A total of 119 cytochrome P450s have been annotated in the D. eschscholzii genome [19] (Fig. 1).

3.2. Structure elucidation

Indolpolyketone A (1) was obtained as a yellow amorphous powder. Its molecular formula was determined to be C25H24N2O2 on the basis of high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) ion at m/z 431.1731 (431.1730 calcld for C27H24N2O4Na), implying 17° of unsaturation. The 1H NMR spectrum of 1 showed characteristic signals for the presence of a 2-methylchroman-5-ol moiety and a DM motif by comparing with those of indolchrmns A [15] and DIM [22], respectively (Table S1 and Fig. S4-9). However, in the case of 1, the 2-methylchroman-5-ol motif was found to anchor on the N-1 atom of DIM by the absence of NH-1 signal and the HMBC correlations of the H-4 double doublet at δ 5.88 with C-2’, C-10, C-5 and C-2 (Fig. 2). Chiral HPLC of 1 (Fig. S1) afforded (+)-(2R,4R)-1 (2.1 mg, tR = 10.556 min) and (−)-(2S,4S)-1 (1.8 mg, tR = 12.076 min), whose absolute configurations were determined by comparing the recorded and calculated ECD spectra (Fig. 3). In theory, 1 should have four enantiomers, but only two more stable trans-oriented enantiomers were obtained owing to be interchangeable via retro-Michael reaction.

Indolpolyketone B (2) was obtained as a yellow amorphous powder, which was evidenced to have a molecular formula of C36H31N3O2 (23° of unsaturation) from the Na+ liganded molecular ion at m/z 560.2307 (560.2308 requires C36H31N3O2Na) in its HR-ESI-MS. Its 1H and 13C NMR spectral data (Table S2 and Fig. S10-16) were well comparable to those of 1 except for a set of signals ascribable for an additional (3-indolyl)methyl motif, that was shown to attach to C-2’ resonating downfield at δ23.149. Such an indole trimere was demonstrated to anchor on C-4 via a C-N bond with the pentaketide moiety by the absence of NH-1 and 4-carbonyl resonances, but the presence of H-4 signal at δ23 8.4 (Fig. 2). This assumption was further determined by the HMBC correlations of H-4 with C-2’ and C-7’ a (Fig. 2).

Chiral HPLC of 2 (Fig. S2) gave (+)-(2R,4R)-2 (1.5 mg, tR = 7.374 min) and (−)-(2S,4S)-2 (1.6 mg, tR = 7.975 min), which were stereochemically assigned by comparing their ECD spectra with those of 1 (Fig. 4). These two enantiomers formed dominantly upon the addition reaction owing to the trans-orientation of the 2,4-substituents.

3.3. The putative biosynthetic pathways of 1 and 2

The isolation of 1 and 2 highlights the possibility of expanding the chemical skeleton through hybridizing indole vestiges and polyketides. Therefore, we were encouraged to address the fungal biosynthesis of 1 and 2. Previously, we identified DIM from the same I3C-exposed fungal culture [22,23], and we have addressed the formation of DIM through three pathways [24]. Due to the slight acidity in the I3C-exposed culture of D. eschscholzii (Fig. S3), at least two (CO2 –liberating and formaldehyde-releasing) pathways may explain the biosynthesis of DIM [24] (Fig. 5). In addition, 1-(2,6-dihydroxyphenyl)but-2-ene-1-one (PBE0) was recognized as a reactive polyketide intermediate and results from the orchestration of the polyketide synthase (ChrA) ketoreductase (KR) domain with that of the KR partner (ChrB) in D. eschscholzii [19]. Therefore, compound 1 was hypothesized to be produced through the Michael addition of DIM with PBE0, which may be catalyzed by an uncertain P450 enzyme. Compound 2 was generated through another round of Michael addition of DIM with PBE0, which may be catalyzed by an uncertain P450 enzyme.

3.4. Bioactivity tests

The obtained compounds 1 and 2 were assayed for their antiviral activity against H1N1. As a result, 1 and 2 exhibited significant antiviral activity against H1N1 with IC50 values of 45.2 and 31.4 μM, respectively.

4. Discussion and conclusion

Natural products are the major source of lead compounds for drug development and represent the majority of small-molecule drugs that were already on the market [25,26]. However, due to the repeated
discovery of known compounds, the hit rate of new skeletal compounds come down every year since the study of streptomyces metabolites reached its summit in the 1970s [27]. To further expand the chemical diversity, development of new strategies for the discovery of novel compounds is of great significance.

Recently, precursor-directed biosynthesis (PDB) has become an important approach for the biosynthesis of a variety of semi-natural chemicals. PDB exploits the native biosynthetic machinery of a producing organism in culture medium supplemented with non-native substrates that compete against native substrates in situ, thus greatly expands the pharmaceutical library of lead compounds with promising or even enhanced biological performance [28, 29]. Although the production of metabolites constructed by PDB are usually in low yield and need to be further purified from a complex mixture, however, through rational design of PDB, such access to target compounds remains economical, practical and environmental benign.

In fact, the key to the successful application of PDB is the selection of the proper platform (such as a fungus) and precursors. D. eschscholzii was rich in polyketides that was pieced together through promiscuous couplings of radicals derived from 1,3,6,8-tetrahydroxynaphthalene, 1,3,8-trihydroxynaphthalene and 1,8-dihydroxynaphthalene. Based on the genome of D. eschscholzii, pksTL gene was found to participate in the biosynthesis of these naphthol-derived polyketides [30, 31]. In addition, the combined application of the functional dimorphism of the polyketide synthase ChrA KR with ChrB allows the fungal generation of PBEO, which is inclined to cyclization spontaneously [19]. PBEO is an active intermediate, which facilitates the fungal production of expanded polyketide diversity [19]. Therefore, choosing D. eschscholzii as the platform of PDB seems reasonable. A body of work showed that cytochrome P450s involved in the formation of C–N bonds. For example, StaN in Streptomyces sp. TP-A0274 is responsible for the C-N bond formation between the nitrogen at N-12 of aglycone and the carbon at C-5' of deoxysugar [32]. TleB from Streptomyces blastmyceticus and its homolog HinD from Streptoalloteichus hindustanus, were characterized to catalyze unusual intramolecular C–N bond formation to generate indolactam V from the dipeptide N-methylvalyl-tryptophanol [33]. A total of 119
cytochrome P450s have been annotated in the *D. eschscholzii* genome [19] (Fig. 1), which endows a great opportunity to construct new compounds by forming C–N bonds. However, further experimental verification is desired to ascertain the exact role and mechanism of the P450 enzyme involved in the C–N bond formation in 1 and 2.

Additionally, indole-3-carbinol is abundant in cruciferous vegetable and shows cancer-preventive potency in diverse models [34,35]. Under acid environment, I3C dehydrate to form an active intermediate 3MI, which could further polymerize to form oligomeric products, such as DIM and 2-(indol-3-ylmethyl)-3,3′-dindolylmethane (LTr1) [24,36]. The I3C-exposed culture of *D. eschscholzii* was slightly acidic (Fig. S3), thus facilitating the transformation of I3C into 3MI, an acceptable precursor for the PDB-based generation of new chemicals.

In conclusion, 1 and 2 were characterized as skeletally undescribed
polyketide-indole hybrids with the two substructures pieced together through C–N bond formation in 13C-exposed culture of *D. eschscholzii*. This study expands the knowledge about the chemical production of *D. eschscholzii*, thereby increasing the possibility to afford new bioactive molecules that may invigorate the drug discovery pipelines.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.02.004.

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