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Genetic dereplication of *Trichoderma hypoxylon* reveals two novel polycyclic lactones

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ABSTRACT

Previous study demonstrated large scale production of trichochecenes which limited the discovery of novel metabolites in *Trichoderma hypoxylon*. By genetic deletion of trichothecene synthase encoding gene *thtri5*, we created the dereplication mutant which eliminated the production of trichothecenes. Through chemical isolation, we characterized a couple of rare new polycyclic lactones tricholactones A and B from the *thtri5* deletion strain. The structures of these two compounds were well determined by NMR, HR-ESI-MS and IECD analysis.

1. Introduction

New natural products (NPs) supply pioneering drug therapies and biological insight, which have played an important role in drug discovery because of their complexity and diversity of structures [1]. Fungi produce large number of bioactive secondary metabolites (SMs), which makes them meaningful to discover new NPs and applies in pharmaceutical industry [2]. Filamentous fungi are well known for manufacturing multiple new SMs [3,4]. The genus Trichoderma is famous among filamentous ascomycetes due to their high adaptability to variety of ecological conditions and various lifestyles [5]. Up to present, large number of structurally novel SMs was discovered from the genus *Trichoderma*. Four unprecedented polyketides trichodermatides A-D have been reported from marine-derived fungus *T. reesei* [6]. A battery of cytochalasins trichoderpyrone [7], trichoderones A and B [8], trichalasins A-F were discovered from *T. gamsii* [9–11], Trichothecenes were reported from *Trichoderma* sp. [12,13]. But, traditional fermentation and separation of SMs is time-consuming and laborious. It is an urgent need to find an efficient way to achieve novel SMs. Genetic dereplication is a new and efficient strategy to reduce the SMs background in fungi, and minimize the rediscovery of known compounds [14]. We have discovered a series of new SMs by genetic manipulations of either epigenetic regulators or global regulator from different fungi efficiently [15–18]. Some fungal species produce high amount of major metabolites which hinder the discovery of other novel compounds. Therefore, we attempted to reduce the SMs background to obtain more novel compounds based on genetic manipulation technology. One strain *Trichoderma hypoxylon* was characterized and reported to produce high amount of trichothecenes including harzianum B with 15.2% in the crude extracts [19]. In this study, we deleted the trichothecene biosynthetic gene *thtri5* [20] and led to the discovery of two novel polycyclic lactones tricholactones A (**1**) and B (**2**) (Fig. 1). The structures of **1** and **2** were determined by detailed analysis of NMR data, IECD and HR-ESI-MS analysis, together with comparison of chemical shift values of similar fragments.

2. Experimental section

2.1. General experimental procedures

UV spectra were measured using a Shimadzu UV-2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). IR spectra were obtained on a Thermo Nicolet Nexus 470 FT-IR spectrometer (Thermo Fisher Scientific, Massachusetts, USA). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance-500 spectrometer (Bruker Corporation, Karlsruhe, Germany). Chemical shifts were calibrated residual solvent signals (MeOH-d_4: δ_H 3.31 and 4.87, δ_C 49.0). High resolution electrospray ionization mass spectroscopy (HR-ESI-MS)

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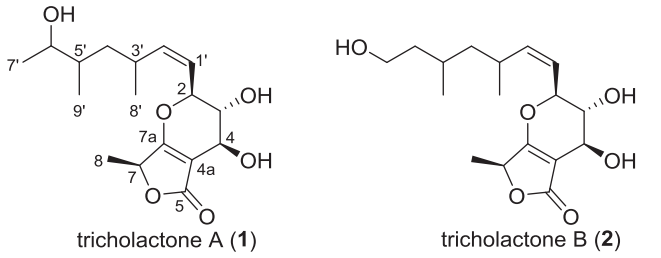

Fig. 1. Compounds **1** and **2** isolated from *T. hypoxylon thtri5* strain.

measurements were received on an Agilent Accurate-Mass-QTOF LC/MS 6520 instrument (Agilent Technologies Inc., California, USA). HPLC (high-performance liquid chromatography) analysis was carried out on a Waters HPLC system (Waters Corporation, Massachusetts, USA) equipped with a Waters 2998 photodiode array detector (HPLC column: C18, 250 × 4.6 mm, YMC Pack, S9 5 μm). Semi-preparative HPLC was performed on SSI HPLC system (Scientific Systems Inc., Pennsylvania, USA), using an ODS column (C18, 10 × 250 mm, YMC Pack, 5 μm).

2.2. Strains, media and growth conditions

By using genetic system creation, strain *T. hypoxylon* was deposited in the China General Microbiological Culture Collection Center (CGMCC 3.17906) [19]. *T. hypoxylon* and its transformants were kept at 25 °C on PDA-medium (potato 20%, dextrose 2%, agar 2%, natural pH) or PDB-medium (potato 20%, dextrose 2%, natural pH) which contained suitable antibiotics. *Escherichia coli* DH5a was used for plasmid DNA isolation, cultivated in Luria-Bertani (LB) medium.

2.3. Gene cloning, plasmid construction and genetic manipulations

To confirming the sensitivity for G418, the strain was cultivated on PDA medium in gradient concentration (FG401-01, TransGen Biotech) (100, 200, 300, 400, 500, 600 and 700 μg/ml). Using the same way to confirming hygromycin B sensitivity (K547, Amresco) (20, 30, 40, 50, 60 and 70 μg/ml). The control had no any antibiotics. The tested mycelia were inoculated on each plate and cultured for 7 days at 25 °C. The plasmids, PCR primer sets and PCR identification of *thtri5* deletion mutants used in this study are listed in Tables S1, S2 and Fig. S1 respectively. Deletion cassette construction and genetic manipulation were carried out as displayed by Liu [20]. Fungal protoplast preparation and transformation were presented as described by Bok & Keller [21].

2.4. Chemical analysis and characterization of compounds

The fungal strain was cultured on PDA medium at 25 °C for 7 days. Agar plugs were used to inoculate Fernbach flasks (500 ml), each containing 80 g of rice (a total of 2.5 kg) and 115 ml of water, and incubated at 25 °C for 30 days. The fermented material was soaked overnight by ethyl acetate for three times (6 L each time). The solution was concentrated to dryness under vacuum to afford a crude extract (12.0 g, HPLC trace, see Fig. S2), the crude residue was applied on a C-18 ODS column using a stepped gradient elution of MeOH-H_2O (20%, 40%, 60%, 80%, 100%) to yield 6 subfractions (fractions A1-A10). The fraction A3 (2.5 g) was separated by Sephadex LH-20 (MeOH) column chromatography to afford four fractions (A3-1 ~ A3-4). Fraction A3-1 (60.0 mg) was purified by RP-HPLC (SSI; YMC-Pack ODS-A column; 10 μm; 250 × 10 mm; 2 ml min^{-1}, 65% MeCN in H_2O for 30 min) to afford tricholactone A (**1**, 2.0 mg, t_R 25.2 min) and. Fraction A3-3 (40.0 mg) was purified by RP-HPLC (SSI; YMC-Pack ODS-A column; 10 μm; 250 × 10 mm; 2 ml min^{-1}, 55% MeOH in H_2O for 30 min) to afford tricholactone B (**2**, 2.0 mg, t_R 27.6 min).

Compound **1**: A colorless oil; $[\alpha]_D^{25}$ −1.5 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 230 nm; IR (MeOH) ν_{max}: 3404, 2963, 1743, 1663, 1328, 1087 cm^{-1} (Fig. S9); NMR spectral data, see Figs. S3–S8; HR-ESI-MS *m/z*: 327.18089 $[M+H]^+$ (Fig. S10) (calcd for $C_{17}H_{27}O_6$, 327.18076).

Compound **2**: A colorless oil; $[\alpha]_D^{25}$ −12.5 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 230 nm; IR (MeOH) ν_{max}: 3419, 2948, 1652, 1620, 1418, 1113 cm^{-1} (Fig. S17); NMR spectral data, see Figs. S11-S16; HR-ESI-MS *m/z*: 327.18121 $[M+H]^+$ (Fig. S18) (calcd for $C_{17}H_{27}O_6$, 327.18076).

Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data for **1** and **2** in methanol-d_4.

NO.	**1**		**2**	
	δ_H (*J* Hz)	δ_C	δ_H (*J* Hz)	δ_C
1	–	–	–	–
2	5.22–5.28, m	81.5, CH	5.22–5.28, m	81.5, CH
3	3.80–3.82, m	72.7, CH	3.81, t (4.0)	72.7, CH
4	4.32, d (3.0)	64.2, CH	4.31, d (4.0)	64.1, CH
4a	–	100.9, C	–	100.9, C
5	–	174.1, C	–	174.1, C
6	–	–	–	–
7	4.92–4.98, m	75.7, CH	4.92–4.98, m	75.7, CH
7a	–	179.2, C	–	179.2, C
8	1.44, d (7.0)	17.8, CH_3	1.44, d (6.5)	17.8, CH_3
1′	5.70, dt (10.0, 10.5)	123.7, CH	5.68, dt (10.0, 10.5)	123.5, CH
2′	5.46, t (10.5)	143.7, CH	5.48, t (10.5)	143.8, CH
3′	2.68–2.77, m	31.1, CH	2.72–2.76, m	31.1, CH
4′	1.21–1.23, m 1.42–1.44, m	41.3, CH_2	1.19–1.21, m 1.31–1.33, m	46.1, CH_2
5′	1.43–1.48, m	38.7, CH	1.52–1.62, m	28.5, CH
6′	3.58–3.64, m	72.4, CH	1.38–1.40, m 1.53–1.55, m	41.4, CH_2
7′	1.11, d (6.5)	19.9, CH_3	3.55–3.61, m	60.9, CH_2
8′	1.05, d (7.0)	22.4, CH_3	1.03, d (6.5)	22.2, CH_3
9′	0.91, d (6.5)	14.8, CH_3	0.93, d (6.5)	19.8, CH_3

3. Results and discussion

Tricholactone A (**1**) was obtained as colorless oil with the molecular formula $C_{17}H_{26}O_6$ as determined by HR-ESI-MS 327.18089 $[M+H]^+$ (Calcd for $C_{17}H_{27}O_6$, 327.18076), indicating five degrees of unsaturation. Analysis of the ^1H, ^{13}C, and HSQC NMR data (Table 1) of **1** revealed the presence of four methyl groups, one methylene unit, nine methines (five oxygenated), two oxygenated quaternary carbons, four olefinic carbons and one carbonyl groups. These data accounted for all the 1H and13C NMR resonances, implying that there might be two rings in structure **1**. The *Z* configuration of the double bond can be deduced from the coupling constant of 10.5 Hz from the two olefinic protons at δ_H 5.46 and 5.70 ppm. The $^1H-^1H$ COSY correlations (Fig. 2) established two isolated proton spin-systems corresponding to −C-4−C-3−C-2−C-1′− C-2′−C-3′ (8′–CH_3)−C-4′−C-5′ (9′–CH_3) −C-6′−C-7′− and −C-7−C-8−. The remaining connectivities were determined by detailed analysis of HMBC correlations. The correlations from H-2 to C-7a, H-3 to C-4a, H-4 to 7a constructed the hexatomic ether ring with

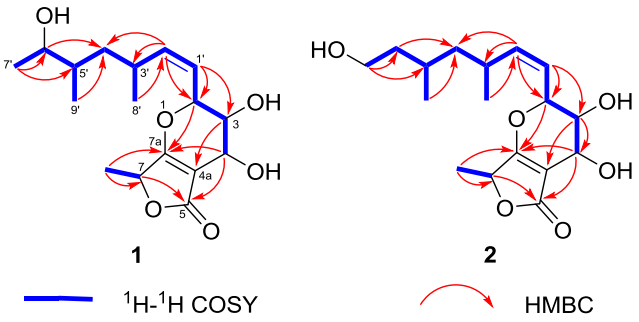

Fig. 2. $^1H-^1H$ COSY and key HMBC correlations of **1** and **2**.

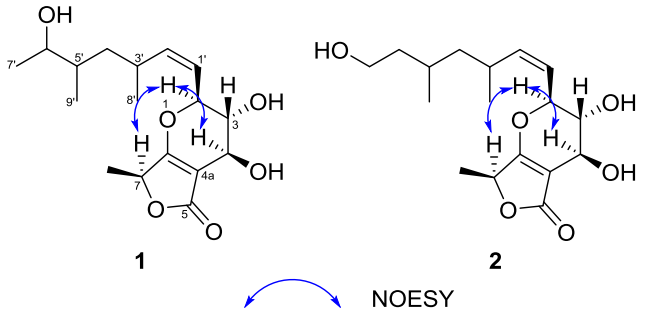

Fig. 3. Key ROESY correlations of **1** and **2**.

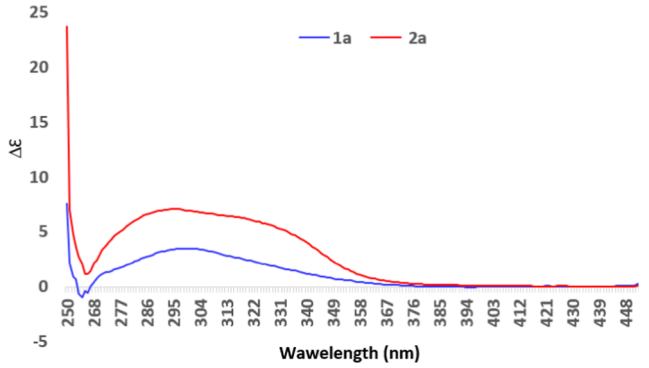

Fig. 4. $Mo_2(OAc)_4$-induced ECD spectra of compounds 1a and 2a in DMSO solution.

α, β-olefinic carbons. Those correlations between H-7 and C-5 (carbonyl carbon), 8-CH_3 and C-7a formed a α, β-unsaturated furanone ring with a methyl located at C-7. Then the structure of **1** was characterized. The relative configuration of **1** was determined by ROESY experiment (Fig. 3). The cross-peaks from H-2 to H-4 and H-7 revealed that those protons were close to each other in space and assigned as α-orientation. Because of the flexibility of carbon chain, the relative configuration of the units (C-3′~7′) couldn't be confirmed. Thus, the relative configuration of **1** was determined.

Tricholactone B (**2**) was obtained as colorless oil with the molecular formula $C_{17}H_{26}O_6$ as determined by HR-ESI-MS 327.18121 $[M+H]^+$ (Calcd for $C_{17}H_{27}O_6$, 327.18076). Analysis of the ^1H and ^{13}C NMR spectral data (Table 1) for **2** revealed its structural similarity, with tricholactone A (**1**). **2** was almost identical to **1** through the comparison of the ^{13}C NMR spectra of two compounds, except the 5 carbons alphatic side Chain [δ_C 41.2 (C-4′), 38.7 (C-5′), 72.4 (C-6′), 19.9 (C-7′), 22.4 (C-8′), 14.8 (C-9′)] in **1** is changed to be [δ_C 46.1 (C-4′), 28.5 (C-5′), 41.4 (C-6′), 60.9 (C-7′), 22.1 (C-8′), 19.8 (C-9′)], implying that the hydroxyl unit in **1** (C-6′) transfers to C-7′ in **2**. This hypothesis was finally confirmed by HMBC correlations from 7′-H to C-5′ and C-6′. Thus, the planar structure of **2** was established. The relative stereochemistry of **2** was determined on the basis of analysis of ROESY spectrum. Similar to compound **1**, the relative configuration of **2** was confirmed.

Due to the existence of a vic-diols unit on the pyran ring, a dimolybdenum tetraacetate $[Mo_2(OAc)_4]$-induced electronic circular dichroism (IECD) experiment was carried out to constructed the absolute configuration of C-4 and C-5 in tricholactones A (**1**) and B (**2**). The $Mo_2(OAc)_4$-induced ECD spectrum of the ester 1a and 1b exhibited positive Cotton effect at 315 nm (Fig. 4) [22] suggesting the *S* configuration of C-4 and C-5, respectively.

4. Conclusions

So far, approximately 100,000 fungal species are reported, but it is only 10% of the forecast [23]. Fungal secondary metabolites (SMs) are a remarkably source of new natural products with pharmaceutical value [24]. As we know, traditional isolation of new SMs is time-consuming and laborious for fungi. Several strategies have been developed by the utilization of fungal genome data. Manipulations of fungal regulators have made big progresses for the findings of novel fungal natural products. Deletions of epigenetic regulators (histone methyltransferase and deacetylase) led to the isolation of 15 new polyketides in a plant endophytic fungus *Pestalotiopsis fici* [17]; 11 new SMs were obtained by knocking out a histone acetyltransferase (HAT) gene Hat1 in *Metarhizium robertsii* [15]; Deletion of a global regulator *LaeB* led to the discovery of 4 novel polyketides in *Aspergillus nidulans* [16]. In addition, genetic manipulation of the COP9 signalosome subunit PfCsnE led to the discovery of 5 new compounds in *Pestalotiopsis fici* [18]. Through deletion of eight of the most highly expressed secondary metabolite gene clusters in *Aspergillus nidulans*, a "genetic dereplication" strain was created, and a novel compound was identified. This is demonstrated that genetic dereplication has been a good way to discover new fungal SMs. The strategy is eliminating the major known SM biosynthetic pathways [14]. *Trichoderma hypoxylon* was characterized and reported to produce high amount of trichothecenes including harzianum B with 15.2% in the crude extracts [19]. Based on our mature genetic manipulation, through the genome analysis, we found the biosynthetic gene (*thtri5*) of trichothecene, then deletion of *thtri5* was carried out and led to the discovery of two novel polycyclic lactones tricholactones A (**1**) and B (**2**), which displayed that it is an efficient strategy to achieve novel SMs through genetic manipulation of fungi.

In summary, we present a genetic approach for the dereplication of large amount of trichothecene which limits the finding of novel compounds in *T. hypoxylon*. The creation of deprelication mutant leads to the identification of two new polycyclic lactones tricholactones A and B. In the post-genomic era, it is very easy to define the biosynthetic genes with the relative metabolites. Therefore, this work provides an efficient way to engineer the desire fungal strains for new compound mining. Ultimately, the strategy of genetic dereplication could accelerate the process of pharmaceutical applications.

Declaration of Competing Interest

None.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103185.

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