A microbial model of mammalian metabolism: biotransformation of 4,5-dimethoxyl-canthin-6-one using Cunninghamella blakesleeana CGMCC 3.970

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Abstract

1. A filamentous fungus, Cunninghamella blakesleeana CGMCC 3.970, was applied as a microbial system to mimic mammalian metabolism of 4,5-dimethoxyl-canthin-6-one (1). Compound 1 belongs to canthin-6-one type alkaloids, which is a major bioactive constituent of a traditional Chinese medicine (the stems of Picrasma quassioides).
2. After 72 h of incubation in potato dextrose broth, 1 was metabolized to seven metabolites as follows: 4-methoxyl-5-hydroxyl-canthin-6-one (M1), 4-hydroxyl-5-methoxyl-canthin-6-one (M2), canthin-6-one (M3), canthin-6-one N-oxide (M4), 10-hydroxyl-4,5-dimethoxyl-canthin-6-one (M5), 1-methoxycarbonl-β-carboline (M6), and 4-methoxyl-5-O-β-D-glucopyranosyl-canthin-6-one (M7).
3. The structures of metabolites were determined using spectroscopic analyses, chemical methods, and comparison of NMR data with those of known compounds. Among them, M7 was a new compound.
4. The metabolic pathways of 1 were proposed, and the metabolic processes involved phase I (O-demethylation, dehydroxylation, demethoxylation, N-oxidation, hydroxylation, and oxidative ring cleavage) and phase II (glycosylation) reactions.
5. This was the first research on microbial transformation of canthin-6-one alkaloid, which could be a useful microbial model for producing the mammalian phase I and phase II metabolites of canthin-6-one alkaloids.
6. 1, M1−M5, and M7 are canthin-6-one alkaloids, whereas M6 belongs to β-carboline type alkaloids. The strain of Cunninghamella blakesleeana can supply an approach to transform canthin-6-one type alkaloids into β-carboline type alkaloids.

Keywords
4,5-Dimethoxyl-canthin-6-one, canthin-6-one alkaloids, Cunninghamella blakesleeana, microbial model of mammalian metabolism, microbial transformation

Introduction

Picrasma quassioides, a plant belonging to the family of simaroubaceae, has been used as a traditional Chinese medicine (TCM) for the treatment of gastroenteritis, eczema and snakebite (Jiao et al., 2010a). During our previous phytochemical investigations, canthin-6-one, β-carboline, and bis-β-carboline alkaloids were characterized in this species (Jiao et al., 2010a, b, 2011, 2015). Among the isolated alkaloids, canthin-6-one alkaloids, especially 4,5-dimethoxyl-canthin-6-one (1) and 4-methoxyl-5-hydroxyl-canthin-6-one were the major bioactive constituents (Shi et al., 2015; Xiao et al., 2012). A metabolic approach was applied to investigate the therapeutic mechanisms of TCM (Wang et al., 2014; Zhang et al., 2014). Up until now, mammalian metabolism of β-carboline alkaloids has already been investigated by spectroscopic analyses (Li et al., 2014; Zhao et al., 2012). However, there is no report about mammalian metabolism of canthin-6-one alkaloids. Microbial systems have been used as models for mammalian metabolism since many microbial metabolites formed from xenobiotics are similar to those formed in mammals (Ashe & Vidyavathi, 2009; Moody et al., 1999; Zhang et al., 1996). Furthermore, the fungal strains of Cunninghamella have been proposed as microbial systems to mimic mammalian metabolism of xenobiotics (Ashe & Vidyavathi, 2009; Piska et al., 2016; Quinn et al., 2015; Xie et al., 2005; Zhang et al., 1996).

Twenty-four strains were used for the preliminary screening of microbial transformation of 1. The screening result
showed that a strain of Cunninghamella blakesleeana can convert 1 to produce various metabolites. Then, scale-up fermentation of 1 with C. blakesleeana was carried out, and seven metabolites (Figure 1), including 4-methoxyl-5-hydroxyl-canthin-6-one (M1), 4-hydroxyl-5-methoxyl-canthin-6-one (M2), canthin-6-one (M3), canthin-6-one N-oxide (M4), 10-hydroxy-4,5-dimethoxyl-canthin-6-one (M5), 1-methoxycarbonyl-β-carboline (M6), and 4-methoxyl-5-O-β-d-glucopyranosyl-canthin-6-one (M7), were isolated from the fermented broths of 1. Among them, metabolite M7 was a new compound. The metabolic pathways of 1 were proposed, which involved O-demethylation, dehydroxylation, demethoxylation, N-oxidation, hydroxylation, oxidative ring cleavage (phase I), and glycosylation (phase II) (Figure 1). Details of the isolation and structural elucidation of metabolites are reported herein.

### Materials and methods

#### Materials

4,5-Dimethoxyl-canthin-6-one (1) was isolated and identified from the stems of P. quassioides by one of the authors (Wei-Hua Jiao) (1H and 13C NMR data see Table S1). Methanol (MeOH) was purchased from Yuwang Industrial Co. Ltd (Yucheng, China). Acetonitrile (CH3CN) was purchased from Oceanpak Alexative Chemical Co. Ltd (Gothenburg, Sweden). L-Cysteine methyl ester hydrochloride, D-glucose (D-Glc), L-glucose (L-Glc), L-cysteine methyl ester isothiocyanate, D-glucose (D-Glc), L-glucose (L-Glc), DMSO-d6, and CDCl3 were purchased from Sigma-Aldrich (Saint Louis, MO). Other organic solvents were analytical grade from Fine Chemical Co. Ltd (Tianjin, China).

#### General experimental procedures

UV data were recorded using a JASCO V-550 UV/vis spectrometer (Jasco International Co. Ltd, Tokyo, Japan). IR data were recorded on a JASCO FT/IR-480 plus spectrometer (Jasco International Co. Ltd). Optical rotations were measured on a JASCO P1020 digital polarimeter (Jasco International Co. Ltd). The ESIMS spectra were recorded on a Finnigan LCQ Advantage MAX mass spectrometer (Finnigan MAT GmbH, Bremen, Germany). The HRESIMS spectra were recorded on a Micromass Q-TOF mass spectrometer (Waters Corporation, Milford, CT). 1D and 2D NMR spectra were acquired with Bruker AV 400 and Bruker AV 600 spectrometers (Bruker BioSpin Group, Faellanden, Switzerland) using the solvent signals (DMSO-d6: δH 2.50/δC 39.5; CDCl3: δH 7.26/δC 77.0) as internal standards. Analytical HPLC was performed on a Dionex HPLC system equipped with an Ultimate 3000 pump, an Ultimate 3000 DAD, an Ultimate 3000 column compartment, an Ultimate 3000 autosampler (Thermo Fisher Scientific Inc., Sunnyvale, CA), and an Alltech (Grace) 2000ES ELSD (Alltech Co. Ltd, Portland, OR) using a Welch XB-C18 column (4.6 × 250 mm2; 5 µm) (Welch Materials Inc., Shanghai, China). Semi-preparative HPLC was performed on a Shimadzu LC-6-AD liquid chromatography system (Shimadzu Inc., Kyoto, Japan) with an SPD-20A detector using a YMC semipreparative column (10.0 × 250 mm2; 5 µm) (YMC Co. Ltd, Tokyo, Japan). Analytical thin-layer chromatography (TLC) was performed on silica gel GF254 plates (Yantai Chemical Industry Research Institute, Yantai, China). Column chromatography (CC) was performed on ODS (2.5 × 35 cm2; 50 µm) (YMC Co. Ltd).

#### Microorganisms and culture medium

All 24 microorganism strains were presented from College of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang, China. These strains were used for the preliminary screening of the biotransformation of 1 as follows: Absidia coerulea CGMCC 3.3389, Alternaria alternata CGMCC 3.4578, Alternaria longipes CGMCC 3.2875, Aspergillus carbonarius CICC 2087, Aspergillus flavus CGMCC 3.3554, Aspergillus fumigatus

![Figure 1. Proposed metabolic pathways of 4,5-dimethoxy-canthin-6-one (1).](image-url)
CICC 2168, Aspergillus niger CGMCC 3.1858, Aspergillus niger CGMCC 3.739, Aspergillus niger CGMCC 3.795, C. blakesleeana CGMCC 3.970, Cunninghamamella elegans CGMCC 3.1207, Fusarium avenaceum CGMCC 3.4594, Mucor polymorphosporus CGMCC 3.3443, Mucor spinosus CICC 40243, Mucor subtilissimus CGMCC 3.2454, Paecilomyces variotii CICC 4024, Penicillium admetezii CGMCC 3.4470, Penicillium citrinum CICC 4013, Penicillium decumbens CGMCC 3.5255, Penicillium janthinellum CGMCC 3.514, Penicillium melinii CGMCC 3.4474, Penicillium urticae CICC 4015, Sporotrichum sp. CGMCC 3.2882, and Syncephalastrum racemosum CGMCC 3.264.

Stock cultures were maintained at 4°C on agar slants containing PDA in an Eyela LTI-700 conventional incubator (Tokyo Rikakikai Co. Ltd, Tokyo, Japan). All initial screening experiments were performed in conical flasks (500 mL) containing 100 mL of PDB, which was sterilized by autoclaving at 121°C for 30 min using a MLS3750 autoclave (Sanyo Electric Co. Ltd, Moriguchi, Japan).

Fermentation procedures

The preliminary screening strains were cultured on slants of PDA at 25°C for days 2–5. Then, the cultures were incubated in the above-described flasks with shaking on a HZQ-8 rotator shaker (HDL Apparatus Co. Ltd, Beijing, China) operating at 220 ppm and 28°C. After 48 h, 5 mg of 1 was added to each flask and maintained for another 72 h under the same conditions. It is worth mentioning that culture controls and substrate controls were carried out to easily discover the positive strains. Culture controls consisted of culture medium, in which microorganisms were grown under identical conditions without 1. Substrate controls consisted of culture medium and the same amount of 1 incubated under the same conditions without microorganisms (Hsu et al., 2002).

Scale-up fermentation of 1 followed the above-described procedures except the number of flasks.

 Extraction and isolation

The fermented broths were extracted three times with ethyl acetate (EtOAc), and the organic solvent was removed under vacuum to yield a crude extract using an Eyela N-1001 rotary evaporator (Tokyo Rikakikai Co. Ltd, Tokyo, Japan). The crude extract was separated using ODS CC, which was eluted with MeOH:H2O to yield fractions. Isolation conditions of crude extracts was separated using ODS CC, successively eluted with evaporator (Tokyo Rikakikai Co. Ltd, Tokyo, Japan). The yields of metabolites were 1.1% (M1), 0.9% (M2), 0.5% (M3), 0.4% (M4), 1.5% (M5), 0.3% (M6), and 3.4% (M7), respectively.

Acid hydrolysis

M7 (1.0 mg) was hydrolyzed with 2 N of HCl for 1 h at 90°C. After extracting with EtOAc twice, the H2O layer was evaporated in vacuo to furnish a monosaccharide residue. The residue was dissolved in pyridine (1.0 mL) containing L-cysteine methyl ester hydrochloride (1.0 mg) and heated at 60°C. After 1 h, 10 µL of o-tolyl isothiocyanate was added to the reaction mixture and further reacted at 60°C for 1 h. Then, the reaction mixture was directly analyzed by the Dionex HPLC system and detected by an UV detector (at 250 nm).

Analytical HPLC was performed on the Welch XB-C18 column with isocratic elution of CH3CN:H2O:HCOOH (25:75:0.01, v/v/v) for 40 min at a flow rate of 0.8 mL/min. The standard monosaccharides of D-Glc and L-Glc were subjected to the same method (Chen et al., 2013).

Results and Discussion

Screening of strains

To search for the strains which can catalyze the biotransformation of 1, 24 strains (including two strains of Cunninghamamella) were screened. Analytical TLC of the crude extracts was carried out to screen the positive strains. For visualization of the alkaloidal spots on the TLC plates, modified Dragendorff’s reagent was used. The TLC results suggested that only one crude extract of the fermented broths of 1 with C. blakesleeana displayed distinct Dragendorff-positive spots rather than 1 compared with culture controls and substrate controls. Therefore, this strain was selected for scale-up fermentation.

Isolation of metabolites

A crude extract (1.86 g) was obtained after 0.28 g of 1 was evenly distributed among 56 flasks. Then, the extract was separated using ODS CC, successively eluted with MeOH:H2O (15:85, 30:70, 50:50, 70:30, 85:15, and 100:0, v/v) to yield 18 fractions (F1–F18), respectively. F10 was purified using semi-preparative HPLC with CH3CN:H2O (30:70, v/v) at a flow rate of 3 mL/min to yield M4 (1.1 mg). F12 was purified using semi-preparative HPLC with CH3CN–H2O (22:78, v/v) at a flow rate of 3 mL/min to yield M7 (9.6 mg). F13 was isolated using semi-preparative HPLC with CH3CN–H2O (30:70, v/v) at a flow rate of 3 mL/min to yield M2 (2.5 mg) and M5 (4.2 mg). F14 was isolated using semi-preparative HPLC with CH3CN–H2O (28:72, v/v) at a flow rate of 3 mL/min to yield M1 (3.1 mg), M3 (1.5 mg), and M6 (0.9 mg). The yields of metabolites were 1.1% (M1), 0.9% (M2), 0.5% (M3), 0.4% (M4), 1.5% (M5), 0.3% (M6), and 3.4% (M7), respectively.

Structural elucidation of metabolites

The known metabolites, 4-methoxyl-5-hydroxyl-canthin-6-one (M1) (Ohmoto & Koike, 1984) (Table S2), 4-hydroxyl-5-methoxyl-canthin-6-one (M2) (Li et al., 1993) (Table S3), canthin-6-one (M3) (Soriano-Agaton et al., 2005) (Table S4), canthin-6-one N-oxide (M4) (Soriano-Agaton et al., 2005) (Table S5), 10-hydroxy-4.5-dimethoxyl-canthin-6-one (M5) (Jiang & Zhou, 2008) (Table S6), and 1-methoxycarbonl-β-carboline (M6) (Ohmoto & Koike, 1982) (Table S7), were identified by spectroscopic analyses and comparison of their reported data.

Metabolite M7 was obtained as a yellowish amorphous powder and gave a positive reaction to modified Dragendorff’s reagent. UV spectrum of M7 displayed absorption bands at 209, 246, 264, 292, 301, 355, and 371 nm, which were similar to those of canthin-6-one skeleton. The quasi-molecular ion at m/z 429.1303 [M + H]+ by HRESIMS indicated that the molecular formula of M7 was C21H20N2O8 (13 degrees
of unsaturation). The $^{13}$C NMR spectrum of M7 showed 21 carbons. The non-exchangeable proton resonances were associated with the directly attached carbon atoms in the HSQC experiment. The analysis of the $^1$H—$^1$H COSY experiment and the coupling values of the protons indicated the presence of these subunits [C-5—C-6, C-9—C-10—C-11—C-12, and C-1’—C-2’—C-3’—C-4’—C-5’—C-6’(OH-6’)]. Combined with molecular formula, degrees of unsaturation, chemical shifts, and these deduced subunits, the key HMBC correlations (Figure 2) from H-1’ to C-15/C-5’ and from OCH$_3$-14 to C-14 built up the planar structure of M7. The assignments of all proton and carbon resonances are provided in Table S8. In the planar structure, there was a pyranohexose ring at C-2’-O-2’-D-glucopyranosyl-canthin-6-one (M7). The absolute configuration of the glucopyranosyl was established as $\beta$ from the coupling constant of the anomeric proton (H-1’) located at $\delta_\text{H}$ 5.32 (1H, d, $J = 7.3$ Hz). The absolute configuration of the glucopyranosyl was determined by HPLC analysis of products obtained from acid hydrolysis and derivatization reactions by L-cysteine methyl ester hydrochloride and o-tolyloisothiocyanate. Analytical HPLC was performed with an isocratic elution of CH$_3$CN:H$_2$O:HCOOH (25:75:0.01, v/v/v, 0.8 mL/min) for 40 min, and the peaks of the standard monosaccharide and sample derivatives were recorded at $\lambda$$_\text{UV}$ 287 (0-Glc), 153.5 (L-Glc), and 167 (M7) min, respectively. The evidence revealed that the glucopyranosyl of M7 was p-Glc. The key ROSY correlations between H-1’ and H-3’/H-5’/OCH$_3$-14 confirmed the above deduction. Therefore, metabolite M7 was identified as 4-methoxyl-5-O-$\beta$-d-glucopyranosyl-canthin-6-one, and is a new compound.

4-Methoxyl-5-O-$\beta$-d-glucopyranosyl-canthin-6-one (M7): yellowish amorphous powder; $\alpha$$_\text{D}$$^\text{19}$$^\text{c}$ = -57.4 (c 0.30, MeOH); UV (MeOH) $\lambda_{\text{max}}$ (log e) 209 (4.36), 246 (4.15), 264 (3.92), 292 (3.85), 301 (3.83), 355 (3.99), 371 (3.90) nm; IR (KBr) $\nu_{\text{max}}$ 3362, 2924, 2852, 1649, 1565, 1472, 1442, 1380, 1077, 1054 cm$^{-1}$; ESIMS (positive) m/z 451.2 [M + Na]$^+$; HRESIMS (positive) m/z 429.1303 [M + H]$^+$ (calculated for C$_{21}$H$_{21}$N$_2$O$_8$, 429.1292); $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$$_\text{H}$ 8.81 (1H, d, $J = 5.0$ Hz, H-5), 8.44 (1H, br d, $J = 8.2$ Hz, H-12), 8.30 (1H, br d, $J = 7.6$ Hz, H-9), 8.23 (1H, d, $J = 4.8$ Hz, H-6), 7.72 (1H, br t, $J = 7.6$ Hz, H-11), 7.54 (1H, br t, $J = 7.5$ Hz, H-10), 5.40 (1H, br s, OMe-2’/3’/4’), 5.32 (1H, d, $J = 7.3$ Hz, H-1’), 5.14 (1H, br s, OMe-2’/3’/4’), 5.05 (1H, br s, OH-2’/3’/4’), 4.43 (1H, br s, OH-6’), 4.38 (3H, s, OCH$_3$-14), 3.65 (1H, br d, $J = 11.5$ Hz, Ha-6’), 3.44 (1H, Hb-6’), 3.34 (1H, H-2’), 3.32 (1H, H-3’), 3.18 (1H, H-4’), 3.16 (1H, H-5’); $^{13}$C NMR (100 MHz, DMSO-d$_6$): $\delta$C 157.5 (C-16), 153.6 (C-14), 145.2 (C-5), 138.3 (C-13), 136.5 (C-15), 133.0 (C-3), 130.8 (C-11), 129.3 (C-7), 128.1 (C-2), 125.5 (C-10), 124.5 (C-8), 123.5 (C-9), 116.5 (C-6), 116.1 (C-12), 101.7 (C-1’), 77.6 (C-5’), 76.5 (C-3’), 74.2 (C-2’), 69.9 (C-4’), 61.7 (OCH$_3$-14), 60.9 (C-6’).

**Conclusion**

This was the first research on microbial transformation of 4,5-dimethoxy-canthin-6-one (1), and this study was also the first biotransformation case of canthin-6-one type alkaloids. After a series of procedures, seven metabolites were isolated and identified as follows: 4-methoxyl-5-hydroxyl-canthin-6-one (M1), 4-hydroxyl-5-methoxyl-canthin-6-one (M2), canthin-6-one (M3), canthin-6-one N-oxide (M4), 10-hydroxyl-4,5-dimethoxyl-canthin-6-one (M5), 1-methoxy-carbonyl-$\beta$-carboline (M6), and 4-methoxyl-5-O-$\beta$-d-glucopyranosyl-canthin-6-one (M7). Among them, M7 was identified as a new canthin-6-one glycoside.

The metabolic pathways of 1 were proposed, and the metabolic processes involved O-demethylation, dehydroxylation, demethoxylation, N-oxidation, hydroxylation, oxidative ring cleavage (Phase I), and glycosylation (Phase II) (Figure 1). Although there is still no report about mammalian metabolism of canthin-6-one alkaloids and there are only few reports on the pharmacokinetics of canthin-6-one alkaloids (Chen et al., 2016; Shi et al., 2015), most of the microbial metabolites of 1 in this research are the same as the expected mammalian metabolites according to the general rules of mammalian metabolism. Mammalian metabolism is mainly represented by "detoxification" processes, classified as phase I (functionalization) and phase II (conjugation) reactions. Phase I reactions consist of oxidation (O-dealkylation, N-dealkylation, N-oxidation, hydroxylation, and so on), reduction, and hydrolysis. Phase II reactions are synthetic reactions involving the conjugation of substrates or metabolites with common endogenous substances, including glycosylation, sulfonation, and glutathionylation (Azera, 1999; Srisilam & Veeresham, 2003). **Cunninghamella blakesleeanea** is an eukaryotic organism that possesses metabolizing enzyme systems similar to those of mammalian systems, and this strain has been proposed as a microbial model of mammalian metabolism in the past (Abourashed et al., 1999; Asha & Vidyavathi, 2009; Piska et al., 2016; Quinn et al., 2015; Xie et al., 2005; Zhang et al., 1996). Therefore, **C. blakesleeanea** could be a potential microbial system for producing the mammalian phase I and phase II metabolites of canthin-6-one alkaloids.

Additionally, metabolite M6, a $\beta$-carboline type alkaloid, was also produced by biotransformation of 1 in this study. Although the current biotransformation yield is low (0.3%) in this research, this strain of **C. blakesleeanea** may contribute a valuable enzyme or directly serve as an approach to transform canthin-6-one type alkaloids into $\beta$-carboline type alkaloids if the efficiency of biotransformation is improved by optimization of the fermentation conditions (Gong et al., 2011;...
Haldar et al., 2015; Shen et al., 2014). A hypothetical mechanism of oxidative ring cleavage from M1 to M6 is proposed (Figure 3).

Declaration of interest

This work was financially supported by grants from the National Natural Science Foundation of China (81422054), the Guangdong Natural Science Funds for Distinguished Young Scholar (2013050014287), Guangdong Special Support Program (2014TQ01R420), and Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme (Hao Gao, 2014). The authors declare no competing financial interest.

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**Supplementary material available online**