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Regio- and stereo-selective hydroxylations of ingenane diterpenoids by *Mortierella ramanniana* and *Gibberella fujikuroi*

WU Yi-Qing, CAO Yue, LIU Xin, CHENG Zhi-Hong*

Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai 201203, China

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[ABSTRACT] The regio- and stereo-selective hydroxylations of two ingenane diterpenoids, 20-deoxyingenol (**1**) and 13-oxyingenol dodecanoat (**2**), by the filamentous fungi *Mortierella ramanniana* and *Gibberella fujikuroi* were investigated in the present study. Four undescribed metabolites (**3**–**6**) of substrate **1** and two undescribed metabolites (**7** and **8**) of substrate **2** were isolated. All the metabolites were identified as hydroxylated ingenane derivatives by extensive NMR and HR-ESI-MS data analyses. All the biotransformed compounds and the substrates were evaluated for their cytotoxicities against three human cancer cell lines, including human colon cancer Caco-2, breast cancer MCF-7, and adriamycin (ADM)-resistant MCF-7/ADM cell lines. All ingenane alcohols (**1**, and **3**–**6**) displayed no significant cytotoxic activities. The substrate 13-oxyingenol dodecanoat (**2**) showed moderate cytotoxicity with IC_{50} values being 35.59 ± 5.37 μmol·L^{-1} (Caco-2), 24.04 ± 4.70 μmol·L^{-1} (MCF-7), and 22.24 ± 5.19 μmol·L^{-1} (MCF-7/ADM). However, metabolites **7** and **8** displayed no significant cytotoxicity. These results indicated that the hydroxylation at the C-13 aliphatic acid ester of substrate **2** can significantly reduce the cytotoxic activity.

[KEY WORDS] Microbial transformation; *Mortierella ramanniana*; *Gibberella fujikuroi*; Diterpenoid; Ingenane; Hydroxylation

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Introduction

Ingenane diterpenoids with a 5/7/7/3-fused-ring skeleton mostly present as polyesters with long-chain aliphatic acids located at C-2, C-5 or C-13. They have received considerable attention, due to a wide variety of biological activities, including anti-proliferative [1] and antiviral effects [2] and modulatory effect on IFN-γ in NK92 cells [3]. Many ingenane derivatives were obtained through phytochemical or chemical synthesis [4-5].

Enzymes in fungi have been utilized for biotransformation of a wide range of natural products since 1960s [6-7]. Compared to the chemical approach, the greatest advantages of microbial transformation are environmental friendly, regio- and stereo-selective reactions under mild conditions [8]. Therefore, microbial transformation can be an alternative to enrich the library of ingenane derivatives, especially as a tool of selectively introducing hydroxyl groups into the ingenane skeleton at non-active carbon positions.

In the present work, the biocatalytic abilities of 14 microorganisms to convert 20-deoxyingenol (**1**) and 13-oxyingenol dodecanoat (**2**) were screened in order to obtain new ingenane derivatives with better bioactivities and solubility. Two fungi, *Mortierella ramanniana* CGMCC 3.03413 and *Gibberella fujikuroi* CICC 40272, were chosen as whole-cell catalysts for the biotransformations of **1** and **2,** respectively. Four undescribed ingenane derivatives (**3**–**6**) were isolated and identified after incubation of *M. ramanniana* with **1**, and two undescribed ingenane derivatives (**7**–**8**) were obtained after the incubation of *G. fujikuroi* with **2**. Moreover, the cytotoxicity of the substrates and all metabolites were also evaluated in three human cancer cell lines *in vitro*.

Results and Discussion

Biotransformation of 20-deoxyingenol (1)

Among 14 screened microorganisms, *Mortierella ramanniana* CGMCC 3.03413 was found to be able to metabolize 20-deoxyingenol (**1**) into several products based on TLC analysis. Preparative incubation of *M. ramanniana* with **1** for 5 days yielded four hydroxylated products (**3**–**6**) (Fig. 1).

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[*Corresponding author] Tel: 86-21-51980157; Fax: 86-21-5198-0017; Email: chengzhh@fudan.edu.cn

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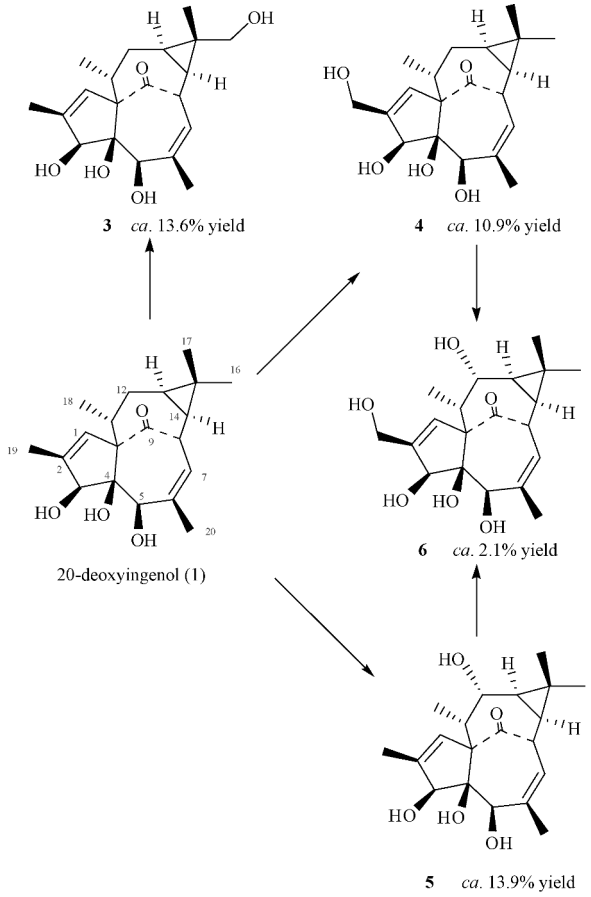

Fig. 1 Proposed biotransformation pathways of 20-deoxyingenol (1) by *Mortierella ramanniana* CGMCC 3.03413

Metabolite **3** was obtained in a 13.6% yield as a powder. Its molecular formula was determined as $C_{20}H_{28}O_5$ by positive-ion HR-ESI-MS (*m/z* 371.182 9 $[M + Na]^+$), indicating a monohydroxylated product of **1**. The ^1H and ^{13}C NMR spectra of **3** were similar to those of substrate **1** (Tables 1 and 2), except for the absence of the Me-16 signal (Table 1), which was replaced by a CH_2OH group (δ_C 72.6; δ_H 3.25, d, J = 11.2 Hz, and δ_H 3.27, d, J = 11.2 Hz) (Tables 1 and 2).

The HMBC correlations of H_2-16 with C-13 (δ_C 21.1), C-14 (δ_C 21.5) and C-15 (δ_C 31.4) indicated the presence of a hydroxyl group at C-16. Moreover, the NOE correlations of H_2-16 with α-orientated H-13 (δ_H 0.82) and H-14 (δ_H 0.91) also supported the hydroxylation at C-16. Thus, the structure of metabolite **3** was identified as 16-hydroxy-20-deoxyingenol.

Metabolite **4** was obtained as a powder in a 10.9% yield. The HR-ESI-MS ion at *m/z* 371.183 2 $[M + Na]^+$ established the molecular formula of **4** as $C_{20}H_{28}O_5$, suggesting another monohydroxylated product. The similarity between the NMR spectra of **1** and **4** for the 7/7/3-fused rings implied a hydroxyl addition in the 5-membered ring. This additional hydroxyl group was introduced in the methyl group converting it to a CH_2OH group (δ_C 60.7) which showed two doublets coupled each other at δ_H 4.24 (J = 14.5 Hz) and δ_H 4.20 (J = 14.6 Hz) in the HSQC spectrum. In the HMBC experiment, correlations between the alkenyl proton (δ_H 6.11) and C-10 (δ_C 73.5), C-3 (δ_C 77.6) and C-4 (δ_C 86.2) as well as correlations between H-1 and C-19 (δ_C 60.7) were observed, indicating that the new hydroxyl group was located at C-19. Thus, the structure of metabolite **4** was identified as 19-hydroxy-20- deoxyingenol.

Metabolite **5** (13.9% yield) had the molecular formula of $C_{20}H_{28}O_5$ on the basis of HR-ESI-MS (*m/z* 371.183 0 $[M + Na]^+$), indicating that a hydroxyl group was introduced into the substrate **1**. The ^1H NMR and ^{13}C NMR spectra of **5** showed a new oxygen-bearing methine signal appeared at δ_H 4.77 (1H, d, J = 5.4 Hz) and δ_C 70.7 (Tables 1 and 2). The ^{13}C NMR signals of C-11 and C-13 were shifted downfield by 7.4 and 5.4 ppm (Table 2), respectively, obviously suggesting the

Table 1 ^1H NMR spectroscopic data (600 MHz, CD_3OD) for compounds 1–8

Position	1	2	3	4	5	6	7 [a]	8 [a]
1	5.84, s	5.89, d, (1.4)	5.83, d, (1.3)	6.11, s	5.82, d, (1.0)	6.12, s	5.89, d, (1.1)	5.91, s
3	4.34, s	4.41, s	4.34, s	4.54, s	4.31, s	4.54, s	4.4, s	4.41, s
4	-	3.83, s	-	-	-	-	3.83, s	3.83, s
5	3.37, s	-	3.37, s	3.42, s	3.31, m	3.41, s	-	-
7	5.68, d, (3.3)	6.02, d, (3.9)	5.7, dd, (3.3, 1.5)	5.68, dd, (3.3, 1.5)	5.62, m	5.65, dd, (3.2, 1.4)	6.02, d, (4.4)	6.03, d, (4.4)
8	4.25, d, (10.3)	4.05, dd, (12.1, 4.1)	4.29, m	4.22, m	4.08, dd, (12.3, 3.0)	4.13, dd, (9.6, 1.6)	4.06, dd, (12.0, 4.3)	4.06, dd, (12.1, 4.3)
11	2.43, m	2.45, m	2.48, m	2.45, m	2.40, d, (5.4)	2.47, d, (3.7)	2.45, m	2.45, m
12	2.43, m	2.74, d, (3.2)	2.46, m	2.44, m	4.77, d, (5.4)	4.82, m	2.74, d, (3.0)	2.74, d, (3.0)
	73, dd, (10.7, 2.4)	2.71, d, (3.1)	1.74, m	1.75, m	,	,	2.71, d, (3.0)	2.71, d, (3.0)
13	0.68, dd, (15.3, 8.3)	-	0.82, m	0.69, m	0.57, t, (8.6)	0.61, t, (8.5)	-	-
14	0.82, dd, (11.8, 8.5)	1.08, m	0.91, m	0.83, dd, (11.9, 8.4)	0.95, m	0.95, m	1.55, m	1.56, m
16	1.07, s	1.07, s	3.25, d, (12.2)	1.07, s	1.1, s	1.13, s	1.07, s	1.07, s
			3.27, d, (11.9)					
17	1.14, s	1.22, s	1.20, s	1.14, s	1.19, s	1.23, s	1.12, s	1.22, s
18	0.96, d, (7.0)	0.96, d, (7.2)	0.97, m	0.98, dd, (7.1)	0.95, m	1.00, d, (7.1)	0.96, d, (7.2)	0.97, d, (7.2)
19	1.84, s	1.85, d, (1.3)	1.84, s	4.24, d, (14.5)	1.83, d, (1.1)	4.25, d, (14.6)	1.85, s	1.86, s