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Biotransformation of triptolide by *Cunninghamella blakesleana*

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Abstract—Biotransformation of triptolide **1** by *Cunninghamella blakesleana* (AS 3.970) was carried out. Seven biotransformation products were obtained and four of them were characterized as new compounds. On the basis of their NMR and mass spectral data, their structures were characterized as 5α-hydroxytriptolide **2**, 1β-hydroxytriptolide **3**, triptodiolide **4**, 16-hydroxytriptolide **5**, triptolidenol **6**, 19α-hydroxytriptolide **7** and 19β-hydroxytriptolide **8**. All the new transformed products (**2**, **3**, **7** and **8**) were found to exhibit potent in vitro cytotoxicity against some human tumor cell lines.

1. Introduction

Tripterygium wilfordii Hook.f, Lei Gong Teng in Chinese, was used in traditional Chinese medicine for the treatment of various diseases including rheumatoid arthritis, nephritis, systemic lupus erythematous and skin disorders, as well as in male-fertility control.[1,2] Triptolide, a diterpenoid triepoxide, isolated from the Chinese herbal plant, *T. wilfordii* Hook.f by Kupcham in 1972[3] has been shown to be effective in the treatment of autoimmune diseases[4] and to have potent antileukemic and antitumor activities.[5,6]

As a biologically active agent, the application of triptolide was limited due to its strong toxicity. To find more effective compounds with less toxicity, structural modifications of triptolide and its analogues by chemical synthetic methods have been carried out extensively in recent years.[7–10] Biotransformation is now becoming a useful tool for structural modifications of bioactive natural products. However, structural modifications of triptolide by biological methods have not been performed. In the present paper, we report for the first time the successful biotransformation of triptolide by *Cunninghamella blakesleana* (AS 3.970) and structural elucidation of the biotransformation products.

2. Results and discussion

Triptolide was successfully bioconverted by *C. blakesleana* (AS 3.970). The substrate was added into a 2-day-old cultivation of the microorganisms and seven products were isolated by chromatographic methods after an additional 5 days' incubation. Their structures were characterized as 5α-hydroxytriptolide **2**, 1β-hydroxytriptolide **3**, triptodiolide **4**, 16-hydroxytriptolide **5**, triptolidenol **6**, 19α-hydroxytriptolide **7** and 19β-hydroxytriptolide **8**, respectively, on the basis of their IR, 1D NMR, 2D NMR and mass spectroscopic data. Among the products isolated, compounds **2**, **3**, **7** and **8** have not been reported before. The biotransformation reaction is illustrated in Scheme 1.

Product **2** was obtained as colorless crystals and had the molecular formula of $C_{20}H_{25}O_7$ (*m/z* 377.1595 $[M+H]^+$, calcd 377.1600) based on its HRMS, suggesting that a hydroxyl group has been introduced into the substrate molecule. The IR absorbance peaks at 3470, 1762, 1677, 1079, 1029 cm^{-1} indicated the presence of hydroxyl and ketone groups. ^1H and ^{13}C NMR spectra of **2** were similar to those of **1** except for the signals corresponding to C-5, C-10 and H-5. The signal of H-5 (δ 2.67, br.d) disappeared in the ^1H NMR spectrum of **2**. In the HMQC spectrum, a new signal (δ 70.12 ppm) of a quaternary carbon supports the fact that a tertiary carbon was hydroxylated. On the basis of $^1H-^1H$ COSY and HMBC analysis, the hydroxylated tertiary carbon was assigned to C5. Additionally, the proton signal at δ 5.33 ppm (5-OH) showed correlations with the signal at δ 1.75 ppm (H-1α), δ 2.17 ppm (H-6α) and δ 4.86 ppm (H-19) in the NOESY spectrum. Therefore, compound **2** was identified as 5α-hydroxytriptolide, which is a new compound. All the ^{13}C and ^1H NMR data of **2** were assigned using extensive NMR techniques (DEPT, ^1H-H COSY, HMQC, HMBC and NOESY). The 1D and 2D NMR spectral data of **2** were summarized in Table 1.

Product **3** was obtained as colorless crystals and its Time of Flying Mass Spectrum (TOFMS) gave the quasi-molecular

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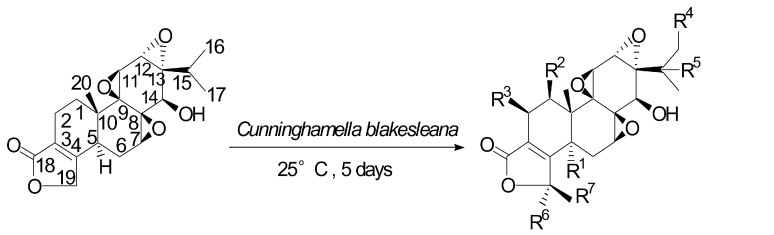


triptolide **1**

2 R^1=OH, R^2=R^3=R^4=R^5=R^6=R^7=H
3 R^2=OH, R^1=R^3=R^4=R^5=R^6=R^7=H
4 R^3=OH, R^1=R^2=R^4=R^5=R^6=R^7=H
5 R^4=OH, R^1=R^2=R^3=R^5=R^6=R^7=H
6 R^5=OH, R^1=R^2=R^3=R^4=R^6=R^7=H
7 R^6=OH, R^1=R^2=R^3=R^4=R^5=R^7=H
8 R^7=OH, R^1=R^2=R^3=R^4=R^5=R^6=H

Scheme 1. Biotransformation of triptolide by *Cunninghamella blakesleana*.

ion peak at *m/z* 394 $[M+NH_4]^+$ and *m/z* 399 $[M+Na]^+$. The IR spectrum of product **3** revealed the presence of hydroxyl and ketone groups. High resolution MS $[M+H]^+$ at 377.1598 was consistent with the formula $C_{20}H_{25}O_7$ (calcd 377.1600). By comparing its ^{13}C NMR spectrum with that of **1**, the signal of C-1 had shifted downfield from 29.76 to 71.09 ppm, which suggested **3** to be a hydroxylated product of **1** at C-1 position. This deduction was also supported by a long-range coupling between H-20 and C-1 on the basis of its HMBC spectrum. In the NOESY spectrum, the correlations of 1-OH with H-2β, H-11 and H-20 strongly supported the orientation of the 1-OH as β-configuration. This conclusion was supported by the correlations of H-1 with H-2α and H-5α in the NOESY spectrum. On the basis of the above analysis, the structure of **3** was elucidated to be the 1β-hydroxy derivative of compound **1**, which is also a new compound. All the ^{13}C and ^1H NMR data of **3** were assigned according to extensive NMR techniques (DEPT, ^1H-H COSY, HMQC, HMBC and NOESY). The 1D and 2D NMR spectral data of **3** were summarized in Table 2.

Product **4** was obtained as colorless crystals and its TOFMS gave the quasi-molecular ion peak *m/z* at 394 $[M+NH_4]^+$. After comparison of the spectroscopic data with those reported in the literature,[3] compound **4** was identified as triptodiolide, which is a known structure.

Product **5** was obtained as colorless crystals and its TOFMS gave the quasi-molecular ion peak *m/z* at 394 $[M+NH_4]^+$. Its NMR spectral and chemical data were in good agreement with those of 16-hydroxytriptolide reported by Ma et al.[11]

Product **6** was obtained as colorless crystals and its TOFMS gave the quasi-molecular peak at *m/z* 394 $[M+NH_4]^+$. It was a known compound identified as triptolidenol based on its spectral data, which are in good agreement with those reported in the literature.[12]

Table 1. NMR data and correlated relations in 2D NMR of **2** (DMSO-d_6, *J* in Hz)

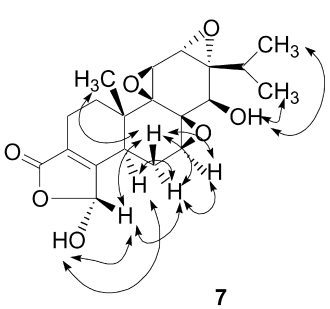
	^1H	^{13}C	HMBC (C→H)	NOESY
1	1.74 (ddd, 6, 5.5, 12) 1.03 (dd, 5.5, 12.5)	23.6	H-20	H-1β, 11, 5-OH H-1α, 2β, 11
2	2.07 (br.d) 1.94–2.00 (m)	17.3	H-1αβ	H-2β H-1β, 20
3		124.6	H-1β, 19	
4		163.3	H-19, 5-OH	
5		70.1	H-1β, 2α, 6αβ, 7, 20, 5-OH	
6	2.15–2.18 (m) 2.11 (dd, 13, 15)	30.8	H-7, 5-OH	H-7, 19, 20, 5-OH H-7, 19, 20,
7	3.33 (d, 5)	59.2	H-6α, 14	H-6αβ
8		61.9	H-6α, 7, 14	
9		63.1	H-7, 12, 14, 20	
10		39.4	H-1αβ, 2α, 6β, 20, 5-OH	
11	3.72 (d, 3)	56.4	H-12	H-1αβ, 12
12	3.52 (d, 3)	54.5	H-11, 14	H-11, 15, 16, 17
13		64.4	H-11, 14, 15, 16, 17, 14-OH	
14	3.36 (d, 7)	71.4	14-OH	H-15, 16, 17, 14-OH
15	2.15 (sept, 7)	27.8	H-14, 16, 17	H-12, 14, 15, 17
16	0.74 (d, 7)	17.1	H-15, 17	H-12, 14, 15, 17
17	0.88 (d, 7)	18.1	H-15, 16	H-15, 16
18		173.8		
19	4.86 (s)	69.1		H-6α, 5-OH
20	0.98 (s)	16.8		H-6αβ, 2β
5-OH	5.33 (s)			H-6α, 12, 19
14-OH	4.61 (d, 7)			H-7, 14, 15

Table 2. NMR data and correlated relations in 2D NMR of **3** (DMSO-d_6, J in Hz)

	^{1}H	^{13}C	HMBC (C→H)	NOESY
1	3.74 (quin, 4.5)	71.1	H-2α, 20, 1-OH	H-2α, 5, 11, 1-OH
2	2.21 (br.d)	26.2	1-OH	H-2α, 20
	1.80–1.84 (m)			H-2β, 12
3		121.9	H-2α	
4		161.5	H-2αβ, 19	
5	2.64 (br.d)	39.8	H-6αβ, 7, 20	H-1α, 5, 7
6	2.13–2.19 (m)	22.3	H-7	H-5, 6β, 7
	1.85 (dd, 14, 15)			H-6α, 20
7	3.34 (d, 5.5)	59.6	H-6αβ, 14	H-6α
8		61.1	H-6αβ, 7, 14	
9		64.7	H-11, 14-OH	
10		40.0	H-2αβ, 5, 6αβ, 20, 1-OH	
11	4.21 (d, 3)	54.9	H-12	H-1, 5, 12, 14, 1-OH
12	3.59 (d, 3)	54.0	H-11, 14	H-11, 15, 16, 17
13		66.1	H-11, 12, 14, 15, 16, 17, 14-OH	
14	3.32 (d, 8)	71.4	H-7, 14, 15, 16, 17	H-15, 16, 17, 14-OH
15	2.12 (sept, 7)	27.4	H-14, 16, 17	H-12, 14, 16, 17, 14-OH
16	0.76 (d, 7)	16.7	H-15, 17	H-12, 14, 15, 17
17	0.88 (d, 6.5)	17.6	H-15, 16	H-12, 14, 15, 16
18		172.6		
19	4.76 (q, 18)	70.2		H-5, 6αβ
20	1.01 (s)	8.8		H-2β, 6β, 1-OH
1-OH	4.01 (d, 4)			H-1, 2β, 11, 2-OH
14-OH	4.61 (d, 8)			H-14, 15

Products **7** and **8** were obtained as a pair of epimers at a ratio of about 3:1 on the basis of their ^{1}H NMR spectral data. We had tried to separate the epimers by a number of methods but finally failed. They were colorless crystals and their TOFMS showed the quasi-molecular peak at *m/z* 394 $[M+NH_4]^+$ and *m/z* 399 $[M+Na]^+$. Products **7** and **8** did not produce a mauve spot as products **3**–**5** did on TLC by spraying with the Kedde solution but produced a brown spot with 10% H_2SO_4. This result suggested the loss of metheylene group adjacent to α,β-unsaturated lactone which was necessary for the Kedde reaction. Hence one can deduce that the metheylene of C-19 might have been substituted in products **7** and **8**. IR spectra showed strong hydroxyl group absorption at 3432 and 3274 cm^{-1}. High-resolution mass measurement on the $[M+H^+]$ at *m/z* 377.1596 was consistent with the molecular formula $C_{20}H_{24}O_7$ (Calcd 377.1600), suggesting that a hydroxyl group had been introduced into the substrate molecule at C-19. The ^{13}C NMR spectra of **7** and **8** showed new peaks at 97.2 and 98.1 ppm, respectively. DEPT analyses showed that the number of secondary carbons changed from 4 to 3, and the number of tertiary carbons increased from 6 to 7. All these data suggested that **7** and **8** were hydroxylated products of triptolide at C-19. All the ^{1}H and ^{13}C NMR data were assigned according to ^{1}H–^{1}H COSY, HSQC and HMBC spectra. The relative stereochemistry was confirmed by NOESY experiment (Scheme 2). The strong correlation between 2.49 ppm (H-5) and 7.79 ppm (19-OH) of **7** suggested the α-configuration of 19-hydroxytriptolide in **7**. Therefore, 19-OH in **8** should be of β-configuration. The signal at 6.00 ppm (H-19) of **7** correlated with H-6α and H-6β supported the orientation of the 19-OH as α-configuration in **7**. The signal at 6.05 ppm (H-19) of **8** was not observed to have correlations with H-6α and H-6β. On the basis of the above analysis, the structures of **7** and **8** were identified as 19α-hydroxytriptolide and 19β-hydroxytriptolide, respectively, which are a pair of new compounds. The ^{1}H and ^{13}C NMR spectral data of compounds **7** and **8** were summarized in Table 3.

The new products obtained from current investigation were evaluated for their bioactivity against human tumor cell lines. By MTT assay, products **2**, **3**, and mixture of **7** and **8** were found to exhibit potent in vitro cytotoxic activities against several human tumor cell lines. Product **2** has IC_{50} values of 0.30, 1.50, 1.05, 0.34, 0.47 μM against KB,



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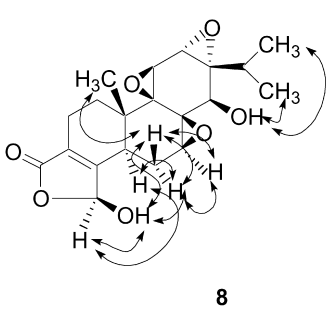

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Scheme 2. NOESY correlations of biotransformed products **7** and **8**.

Table 3. ^{1}H and ^{13}C NMR spectral data of **7** and **8** (DMSO-d_6, J in Hz)

Position	^{1}H	^{13}C	^{1}H	^{13}C
1	1.19 (ddd, 12, 12, 6) 1.29 (dd, 12, 6)	29.0	1.19 (ddd, 12, 12, 6) 1.29 (dd, 12, 6)	29.1
2	2.12 (br.d) 1.92–1.99 (m)	16.6	2.12 (br.d) 1.92–1.99 (m)	16.4
3		126.8		127.0
4		160.5		159.3
5	2.49 (br.d)	39.5	2.59 (br.d)	40.3
6	2.19–2.22 (m) 1.86 (dd, 15, 13)	22.3	2.21–2.23 (m) 1.92 (dd, 15, 13)	22.7
7	3.37 (d, 5.5)	59.7	3.33 (d, 5.5)	60.1
8		61.0		60.9
9		64.3		64.3
10		35.3		35.4
11	3.86 (d, 3)	55.2	3.86 (d, 3)	55.2
12	3.53 (d, 3)	54.3	3.53 (d, 3)	54.3
13		64.8		64.7
14	3.31 (d, 7.5)	71.3	3.31 (d, 7.5)	71.3
15	2.11 (sept, 7)	27.5	2.11 (sept, 7)	27.5
16	0.75 (d, 7)	16.8	0.75 (d, 7)	16.8
17	0.87 (d, 7)	17.7	0.87 (d, 7)	17.7
18		170.6		170.6
19	6.00 (d, 8)	97.3	6.05 (d, 8.5)	98.1
20	0.96 (s)	13.9	0.94 (s)	14.1
14-OH	4.72 (d, 7.5)		4.36 (d, 7.5)	
19-OH	7.79 (d, 8)		7.60 (d, 8.5)	

BGC_{823}, MCF-7, Hela and HL-60 cells, respectively. Product **3** showed significant cytotoxicity against KB, BGC_{823}, MCF-7, Hela and HL-60 cells with IC_{50} values of 0.12, 7.65, 0.87, 0.32, 0.07 μM, respectively. Mixture of products **7** and **8** had cytotoxic activities against KB and HL60 cells with IC_{50} values of 46.5 and 52.6 μM, respectively.

Biotransformation is now becoming an increasingly important tool available to synthetic chemists in the structural modifications of natural or synthetic organic compounds. The rich enzymes in microorganisms could be used for biotransform or synthesize some natural products. In this study, we report a powerful method for the preparation of a variety of derivatives from triptolide using *C. blakesleana* (AS 3.970) as a biocatalyst. All of the new products were found to exhibit potent in vitro cytotoxic activities against human tumor cell lines. Further studies on their bioactivity are under investigation.

3. Experimental

3.1. General procedures

Melting points were measured with an XT4A micro-melting point apparatus and uncorrected. Optical rotations were recorded on a Perkin–Elmer 243B polarimeter using MeOH as solvent with a 1 cm path length. IR spectra were recorded on an Avatar 360 FT-IR spectrophotometer in KBr pellets. 1D and 2D NMR spectra were run on a Bruker DRX-500 spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) in DMSO-d_6 with TMS as an internal standard. The Chemical shift values (δ) were given in parts per million (ppm), and the coupling constants were in hertz (Hz). High resolution positive SIMS were performed on a Bruker Apex II FI-ICR mass spectrometer. TOFMS were measured with a Perkin–Elmer QSTAR mass spectrometer. All the solvents used for extraction and isolation were of analytical grade. TLC was performed on silica gel G. (10–40 μM). Separation and purification were carried out by column chromatography on silica gel (200–300 mesh). Silica gel was purchased from Qingdao Marine Chemical Group Co., P. R. China. Triptolide was detected on TLC by spraying with the Kedde reagent or 10% H_2SO_4.

3.2. Substrate

Triptolide **1** was purchased from Institute of Medical Sciences of Fujian. Its structure was characterized by ^{1}H NMR, ^{13}C NMR and MS spectra. The purity of triptolide, determined by RP-HPLC evaluation using methanol–water (60:40) is 98%. The substrate was dissolved in acetone and diluted to 10 mg/mL before use.

3.3. Microorganisms

C. blakesleana (AS 3.970) were purchased from China General Microbiological Culture Collection Center.

3.4. Medium

All culture and biotransformation experiments were performed in potato medium. Potato medium was produced by the following procedure: 200 mg of minced husked potato were boiled in water for one hour, then the solution was filtered and the filtrate were added with water to 1 L after addition of 20 mg of glucose.

3.5. Biotransformation

The screening-scale run was performed in 250 mL Erlenmeyer flasks containing 60 mL of potato medium. Microorganisms were transferred into the flasks from the slants. The cultures were cultivated on a rotary shaker at 160 rpm at 25°C. For preparative transformation, 1 mL of substrate solution was added into a 1000 mL Erlenmeyer flasks containing 300 mL potato medium that had been precultured for 48 h. In total 500 mg of substrate were added. The incubation was continued for 5 days under the same conditions as described above. Culture controls consisted of fermentation blanks in which microorganisms were grown without substrate but with the same amount of acetone. Substrate controls were composed of sterile medium containing the same amount of substrate and incubated under the same conditions.

3.6. Extraction and isolation of biotransformed products

After 5 days of incubation, the culture was filtered and the filtrate was extracted with the same volume of ethyl acetate for three times. The organic phase was evaporated to dryness in vacuo. The residues were dissolved in acetone. The solution was spotted on silica gel TLC plate which was developed by petroleum ether (60–90°C)–ethyl acetate (1:5) and visualized by spraying with 10% H_2SO_4 solution. TLC chromatography showed that *C. blakesleana* had the ability to biotransform triptolide. Transformed products were more polar than the substrate. No transformation products were found in the controls.

The residue (2.5 g) was subjected to column chromatography over silica gel and eluted with petroleum ether–ethyl acetate gradient (4:1 to pure ethyl acetate). Biotransformation of **1** by *C. blakesleana* resulted in 90 mg of **2** (18% yield), 9 mg of **3** (1.8% yield), 15 mg of **4** (3% yield), 12 mg of **5** (2.4% yield), 10 mg of **6** (2% yield) and 100 mg of **7** and **8** (20% yield).

3.6.1. 5α-Hydroxytriptolide 2. Colorless crystals; mp 250–252°C; $[\alpha]_D^{25}=-132.9$ (*c* 0.12, MeOH); IR (KBr) ν_{max} 3470, 2962, 2932, 1762, 1677, 1079, 1029 cm^{-1}; TOFMS *m/z* 377 $[M+H]^+$, 394 $[M+NH_4]^+$, 399 $[M+Na]^+$, 775 $[2M+Na]^+$; HRMS: calcd for $C_{20}H_{25}O_7$ $[M+H]^+$, 377.1600, found 377.1594. The ^1H and ^{13}C NMR spectral data are summarized in Table 1.

3.6.2. 1β-Hydroxytriptolide 3. Colorless crystals; mp 226–228°C; $[\alpha]_D^{25}=-126.6$ (*c* 0.15, MeOH); IR (KBr) ν_{max} 3466, 2965, 2927, 1749, 1672, 1073, 1023 cm^{-1}; TOFMS *m/z* 377 $[M+H]^+$, 394 $[M+NH_4]^+$, 770 $[2M+NH_4]^+$; HRMS: calcd for $C_{20}H_{25}O_7$ $[M+H]^+$, 377.1600, found 377.1598. The ^1H and ^{13}C NMR spectral data are summarized in Table 2.

3.6.3. 19α-Hydroxytriptolide 7 and 19β-hydroxytriptolide 8. The title compounds were obtained as a mixture. Colorless crystals; TOFMS *m/z* 394 $[M+NH_4]^+$, 770 $[2M+NH_4]^+$, 775 $[2M+Na]^+$; HRMS: calcd for $C_{20}H_{25}O_7$ $[M+H]^+$, 377.1600, found 377.1598. The ^1H and ^{13}C NMR spectral data are summarized in Table 3.

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