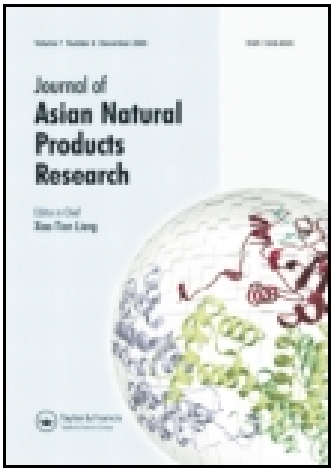

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Microbial transformation of Norkurarinone by *Cunninghamella blakesleana* AS 3.970

Yan-Qiu Shi[a], Xiu-Lan Xin[b], Hu-Cheng Zhang[b], Bao-Jing Zhang[c], Chang-Yuan Wang[c], Jie Hou[c], Qi-Peng Yuan[a]*, Sa Deng[c], Yan Tian[c] and Xiao-Chi Ma[c]*

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In this paper, microbial transformation of norkurarinone (**1**) by *Cunninghamella blakesleana* AS 3.970 was investigated and seven transformed products were isolated and characterized as kurarinone (**2**), 4″,5″-dihydroxykurarinone (**3**), 6″-hydroxyl-2′-methoxyl-norkurarinone 7-*O*-β-D-glucoside (**4**), 6″-hydroxyl-norkurarinone 4′-*O*-β-D-glucoside (**5**), 4″,5″-dihydroxynorkurarinone (**6**), 7-methoxyl-norkurarinone (**7**), and 7-methoxyl-4″,5″-dihydroxynorkurarinone (**8**), respectively. Among them, **3**–**5** are new compounds, and the glycosylation reaction in microbial transformation process was reported rarely. In addition, the cytotoxicities of transformed products (**1**–**8**) were also investigated.

Keywords: biotransformation; norkurarinone; *Cunninghamella blakesleana*; lavandulated flavonoid

1. Introduction

The Chinese crude drug 'Kushen', derived from the dry roots of *Sophora flavescens*, is a commonly used traditional Chinese medicine with gastric disturbance, antifebrile, and anthelmintic activities [1]. A large amount of lavandulated flavonoids were isolated and purified from this plant by phytochemical methods in recent years [2–4]. These flavonoids such as kurarinone and norkurarinone are the major constituents of *S. flavescens* with widely various biological activities including cytotoxicity [5] and glycosidase inhibition [6]. So more and more pharmacological researches of the lavandulyl flavonoids from *S. flavescens* were reported for the future development of new drug. However, the poor water solubility would limit its wide medicine use.

Microbial transformation is an useful biotechnique to modify chemical structures of natural products. And a large number of new natural products by microbial transformation have been reported for improving the bioactivities or studying the relationships of structures and activities in our previous works [7–11]. Meantime, microbial transformation could be used as the *in vitro* model to simulate the metabolism of natural products *in vivo* [12,13].

In this work, microbial transformation of norkurarinone (**1**) by *Cunninghamella blakesleana* AS 3.970 was studied to obtain the novel bioactive flavonoid derivatives with the lavandulyl groups. Totally seven transformed products of **1** were isolated and identified by widely spectral methods including NMR and MS. Among them, products **3**–**5** are new. And compounds **4** and

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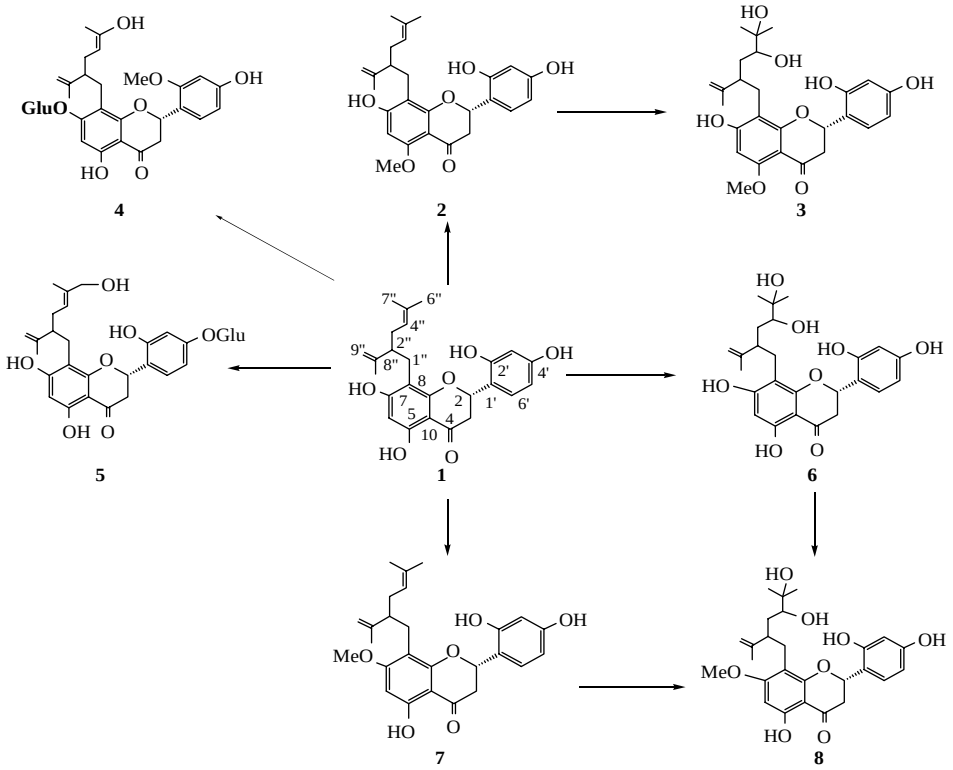

Figure 1. A possible biotransformation pathway of **1** by *C. blakesleana* AS 3.970.

5 were the glycoside derivatives of **1**, which was the rare microtransformation reaction. The biotransformation reactions of **1** by *C. blakesleana* AS 3.970 were hydroxylation, methylation, and glycosylation. In addition, the cytotoxicities of transformed products (**2**–**8**) against Hela and A375 cells were also investigated (Figure 1).

2. Results and discussion

In this study, compound **1** was added into the 36-h-old cultures of *C. blakesleana* AS 3.970 and continued to incubate for 5 days. And a blank culture control and substrate control were performed as described above. A total amount of 1.0 g of substrate was added to the culture liquid in the preparative-scale biotransformation. After 5 days of incubation, seven products were isolated by the preparative chromatographic methods. Based on the extensive spectra of ^{1}H NMR, ^{13}C NMR, and 2D NMR techniques, their structures were elucidated as kurarinone (**2**), 4″,5″-dihydroxyl-kurarinone (**3**), 6″-hydroxyl-2′-methoxyl-norkurarinone 7-*O*-β-D-glucoside (**4**), 6″-hydroxyl-norkurarinone 4′-*O*-β-D-glucoside (**5**), 4″,5″-dihydroxy-norkurarinone (**6**), 7-methoxyl-norkurarinone (**7**), and 7-methoxyl-4″,5″-dihydroxynorkurarinone (**8**), respectively. Among them, **3**–**5** are new products. The possible biotransformation pathway of **1** by *C. blakesleana* AS 3.970 was proposed. The biotransformation reactions such as demethylation, glycosylation, and hydroxylation were observed. And glycosylation was a rare reaction in the microbial transformation process.

Compounds **2** and **6**–**8** were identified as kurarinone, 4″,5″-dihydroxy-norkurarinone, 7-methoxyl-norkurarinone, and 7-methoxyl-4″,5″-dihydroxy-nor-kurarinone, respectively. Their ^{1}H NMR and ^{13}C NMR

spectral data were in agreement with those reported in literatures [14–16].

Compound **3** was obtained as yellow powder (MeOH). It was optically active, with $[\alpha]_D^{22}$ − 9.2 (*c* 0.3, MeOH). Its HR-ESI-MS provided a *pseudo*-molecular ion at *m/z* 471.1958 $[M-H]^-$, suggesting a molecular formula of $C_{26}H_{32}O_8$. Comparing with compound **2**, two oxygenated carbon signals at δ 80.0 and 81.5 were observed in the ^{13}C NMR spectrum of **3**, while the C=C double bond carbons at δ 124.4 and 131.6 in compound **2** disappeared [14]. The HMBC correlations of the carbon signal at δ 81.5 with the protons at δ 1.06 (Me-6″ and 7″) and 3.61 (H-4″) were observed, and the $^1H-^1H$-COSY correlations of H-3″ with H-2″ and H-4″ implied that two hydroxyl groups were introduced at C-4″ and C-5″ of the lavandulyl group, respectively. In addition, the circular dichroism (CD) spectrum of **3** showed a positive absorption at 315 nm and a negative absorption at 285 nm. Thus, the absolute configuration of C-2 was confirmed as *S* [14]. On the basis of the above analysis, compound **3** was elucidated as 4″,5″-dihydroxykurarinone. All the ^1H and ^{13}C NMR spectral data were unambiguously assigned by 2D NMR spectra (Table 1).

Compound **4** was obtained as yellow powder (MeOH). Its HR-ESI-MS provided a *pseudo*-molecular ion at *m/z* 615.2418 $[M-H]^-$, suggesting a molecular formula of $C_{32}H_{39}O_{12}$. The methoxyl group (δ 3.81) had the HMBC correlation with C-2′ (δ 159.8). And at the same time, the methoxyl group had the NOESY correlation with H-3′ (δ 6.64). These evidences proved that this methoxyl group should be located at C-2′. In addition, the carbon signals of δ 101.8, 75.4, 78.4, 71.8, 79.0 and 63.0 indicated that a sugar moiety was introduced in the structure of **4**. The sugar moiety was identified as D-glucose by acid hydrolysis. The anomeric proton coupling constant of 7.5 Hz and the anomeric carbon signal at δ 101.8 suggested that the configuration of glucopyranosyl should be β. The anomeric proton had the long-range correlation with the carbon signal at δ 165.4 (C-7), indicating that the glucopyranosyl moiety should be at C-7 position. The proton signal at δ 3.85 had HMBC correlations with C-7″ and C-4″, suggesting that the hydroxyl group should be located at C-6″. A positive absorption at 315 nm and a negative absorption at 285 nm in the CD spectrum of **4** indicated the absolute configuration of C-2 as *S*. On the basis of above analysis, compound **4** was characterized as 6″-hydroxyl-2′-methoxyl-norkurarinone 7-*O*-β-D-glucoside. All the ^1H NMR and ^{13}C NMR spectral data were unambiguously assigned by 2D NMR spectra (Table 1).

Compound **5** was afforded as yellow powder (MeOH). Its HR-FAB-MS provided a *quasi*-molecular ion at *m/z* 601.2298 $[M-H]^-$, suggesting the molecular formula of $C_{31}H_{38}O_{12}$. Similar with compound **4**, the carbon signals at δ 102.8, 74.2, 77.5, 71.0, 77.5 and 62.4 indicated that a sugar moiety was introduced in the molecular formula of **5**. Acid hydrolysis and thin-layer column-chromatography with the reference sample exhibited that the sugar was D-glucose. The anomeric carbon signal at δ 102.8 and the anomeric proton coupling constant (7.0 Hz) suggested that the configuration of glucopyranosyl should be β. In HMBC spectrum, the anomeric proton had long-range correlation with the carbon signal at δ 155.9, suggesting that glucopyranosyl moiety should be at C-4′ position. In addition, the methyl group at δ 1.45 had HMBC correlations with the carbon signals at δ 124.2 and 68.4. The proton signal at δ 3.80 had long-range correlations with C-5″ at δ 136.3 and C-7″ at δ 13.6. In NOESY spectrum, H-6″ (δ 3.80) had correlation with H-4″ (δ 5.00). All of the evidences suggested that the hydroxyl group should be located at C-6″. Similar to the CD spectrum of **1**, a positive absorption at 320 nm and a negative absorption at 290 nm implied that the absolute configuration of C-2 was *S*. On the basis of the above analysis, compound **5** was determined as 6″-hydroxyl-norkurarinone 4′-*O*-β-D-glucoside. All the ^1H NMR

Table 1. The ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectral data of **3**–**5** (DMSO-d_6).

	3		**4**		**5**	
No.	δ_H (J = Hz)	δ_C	δ_H (J = Hz)	δ_C	δ_H (J = Hz)	δ_C
2	5.62 (dd, 2.5, 13.0)	74.9	5.51 (dd, 2.5, 13.0)	76.3	5.85 (dd, 2.5, 13.0)	75.0
3	2.71 m	45.4	2.78 m	43.9	2.81 m	43.2
	3.03 m		3.04 m		2.92 m	
4	–	189.6	–	200.1	–	197.7
5	–	162.8	12.56 s	163.8	–	161.8
6	6.17 s	93.8	6.29 s	96.7	6.00 s	96.0
7	–	163.8	–	165.4	–	165.3
8	–	107.2	–	111.2	–	107.5
9	–	161.9	–	163.8	–	165.3
10	–	108.3	–	105.0	–	105.8
1′	–	118.4	–	119.8	–	120.8
2′	–	156.0	–	159.8	–	158.8
3′	6.45 (d, 2.5)	103.5	6.64 (d, 2.5)	100.4	6.49 (d, 2.5)	104.3
4′	–	159.1	–	160.8	–	155.9
5′	6.41 (dd, 8.5, 2.5)	107.8	6.43 (dd, 8.5, 2.5)	108.6	6.76 (dd, 8.5, 2.5)	110.1
6′	7.36 (d, 8.5)	128.3	7.34 (d, 8.5)	129.3	7.50 (d, 8.5)	127.7
1″	2.65 m	27.8	2.72 m	28.7	2.72 m	27.6
	2.63 m		2.58 m		2.61 m	
2″	2.52 m	44.8	2.51 m	48.6	2.54 m	47.4
3″	1.32 m	34.4	2.10 m	32.4	2.13 m	31.1
	1.58 m		2.04 m			
4″	3.61 m	80.0	5.30 (brt, 6.0, 6.5)	126.6	5.00 (brt, 6.0, 6.5)	124.2
5″	–	81.5	–	136.4	–	136.3
6″	1.06 s	25.9	3.85 brs	69.5	3.80 brs	68.4
7″	1.06 s	24.6	1.54 s	14.4	1.45 s	13.6
8″	–	148.5	–	150.1	–	148.9
9″	4.66 brs	111.0	4.56 brs	111.9	4.45 brs	111.1
10″	1.72 s	20.3	1.62 s	19.9	1.66 s	19.1
5-OMe	3.85 s	55.8				
2′-OMe			3.81 s	55.5		

Table 1 – *continued*

	3		4		5	
No.	δ_H (J = Hz)	δ_C	δ_H (J = Hz)	δ_C	δ_H (J = Hz)	δ_C
Glu-1			5.01 (d, 7.5)	101.8	4.82 (d, 7.0)	102.8
2			3.51 m	75.4	3.52 m	74.2
3			3.40 m	78.4	3.56 m	77.5
4			3.35 m	71.8	3.45 m	71.0
5			–	79.0	–	77.5
6			3.92 m	63.0	3.89 m	62.4
			3.71 m		3.71 m	

Table 2. *In vitro* cytoxic activities of transformed products.

	Tumor cell lines and IC_{50} (uM)			Tumor cell lines and IC_{50} (uM)	
No.	Hela	A375	No.	Hela	A375
1	30.6	49.8	**5**	23.6	68.9
2	35.8	61.5	**6**	>100	>100
3	>100	>100	**7**	55.7	>100
4	5.3	21.4	**8**	>100	>100

and ^{13}C NMR spectral data were unambiguously assigned by 2D NMR spectra (Table 1).

The cytotoxic activities of compounds **1**–**8** against Hela and A375 cells were tested. Our results indicated that the cytotoxicities of transformed products of **1** against Hela cells were more sensitive than A375 cells. The glycosylation in the skeleton of lavandulyl flavonoid would increase the cytotoxicities of products **4** and **5**. And the glycosylated derivative of C-7 showed better bioactivity than that of C-4′. While the methoxylation at C-7, hydroxylation at C-6″, and dihydroxylation at the double bond of C-4″ and C-5″ would decrease the cytotoxicities significantly. In addition, the methoxylation of C-5 did not have the significant influence on the cytotoxicities of **1** (Table 2).

3. Experimental

3.1 General experimental procedures

UV spectra were measured by Cary 300 spectrophotometer (Beijing Tongyong LLC, Beijing, China). IR spectra were recorded on a NEXUS-470 FT-IR spectrophotometer in KBr pellets (Thermo Fisher Scientific, Dubuque, IA, USA). Melting points were measured by XT_{4A} micromelting point apparatus and were uncorrected (Beijing Keyi Company, Beijing, China). CD spectra were performed on JASCO J-810 CD instrument (Jasco, Tokyo, Japan). ^{1}H NMR and ^{13}C NMR spectra were measured with an INOVA-500 spectrometer

(500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) in Dimethyl Sulfoxide (DMSO)-d_6 with TMS (tetramethylsilane) as internal standard (Varian, Las Vegas, NV, USA). HR-MS were obtained on a Bruker APEXII Fourier transform ion cyclotron resonance mass spectrometer (Bruke, Ettlingen, Germany). Thin Layer Chromalography (TLC) analyses were performed on silica gel G. All chemicals were purchased from Beijing Chemical Factory (Beijing, China). Silica gels were supplied by Qingdao Haiyang Chemical Group Co. (Qingdao, China). Norkurarinone (**1**) was isolated from *S. flavescens* by Y.-Q. Shi. The purity was above 97% which was determined by HPLC analysis.

3.2 Microorganism

Mucor subtilissimus AS 3.2454, *Mucor spinosus* AS 3.2450, *M. spinosus* AS 3.3447, *M. subtilissimus* AS 3.2456, *Alternaria alternata* AS 3.577, *A. alternata* AS 3.4578, *Alternaria longipes* AS 3.2875, *C. blakesleana lender* AS 3.970, *Cunninghamella echinulata* AS 3.3400, *Cunninghamella elegans* AS 3.2028, *Curvularia lunata* AS 3.4381, *Penicillium melinii* AS 3.4474, *Penicillium janthinellum* AS 3.510, *Rhizopus stolonifer* AS 3.3463, *R. stolonifer* AS 3.2050, *Syncephalastrum racemosum* AS 3.264, *Trichoderma viride* AS 3.2942, and *Rhizopus arrhizus* AS 3.2897 were purchased from China General Microbiological Culture Collection Center in Beijing, China.

3.3 Culture medium

All cultures of filamentous fungi in biotransformation experiments were performed in potato medium, which was made by the following compositions (liters): 200 g potato and 20 g glucose [17].

3.4 Culture and biotransformation

Preparative-scale transformation of **1** by *C. blakesleana* AS 3.970 was performed in a 1000-ml Erlenmeyer flask. The flasks were placed on the rotary shakers, operating at 180 rpm at 28°C. After 36 h of pre-culture, the substrate (10 mg) in 1 ml acetone was added to 350 ml medium. In total, 1000 mg of substrate was used for biotransformation. The incubation was continued under the above conditions for five additional days. The culture was filtered and the filtrate was extracted with same volume of EtOAc for four times. The organic phase was collected and concentrated to dryness *in vacuo*.

The crude extract (3.5 g) was subjected to silica gel column and eluted with chloroform–acetone [in a gradient manner from 100:3 (v/v) to 1:1, at a flow rate of 1.5 ml/min] to yield six fractions (I–VI). Fraction II (0.2 g) was subjected to preparative TLC and eluted with chloroform–acetone (5:1) to yield **2** (7 mg). Fraction IV (0.11 g) was separated by preparative HPLC and eluted with MeOH–H_2O (85:15, v/v) to give **7** (12 mg, Rt = 19.0 min). Fraction V (415 mg) was subjected to preparative HPLC and eluted with MeOH–H_2O (60:40) to yield **3** (12 mg, Rt = 15.5 min), **6** (16 mg, Rt = 25.0 min), and **8** (7 mg, Rt = 28.5 min). Fraction VI (460 mg) was isolated by HPLC with MeOH–H_2O (55:45) to obtain products **4** (7 mg, Rt = 16.5 min) and **5** (9 mg, Rt = 22.4 min). Purities of all the transformed products were above 95% by Rp-HPLC analysis, and the detected wavelength and the flow rate were set at 285 nm and 1.5 ml/min, respectively.

3.4.1 4″,5″-Dihydroxykurarinone (3)

Yellow powder (MeOH). m.p. 185–186°C. UV (MeOH) λ_{max}: 285, 325 nm; CD (MeOH): $\Delta\varepsilon_{315.1\,nm}$ +4.860, $\Delta\varepsilon_{285.5}$ −12.570. IR (KBr) ν_{max} (cm^{-1}): 3380, 2820, 1700, and 990; for ^{1}H NMR and ^{13}C NMR spectral data, see Table 1; HR-ESIMS: *m/z* 471.1958 [M–H]$^-$ (calcd for $C_{26}H_{31}O_8$, 471.2019).

3.4.2 6″-Hydroxyl-2′-methoxyl-norkurarinone 7-O-β-D-glucoside (4)

Yellow powder (MeOH). m.p. 287–288°C. UV (MeOH) λ_{max} 288, 320 nm; CD

(MeOH): $\Delta\varepsilon_{315} + 5.360$, $\Delta\varepsilon_{286.2} - 10.190$. IR (KBr) ν_{max} (cm^{-1}): 3290, 2850, 1760, and 1025; for ^1H NMR and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS: *m/z* 615.2418 $[M-H]^-$ (calcd for $C_{32}H_{38}O_{12}$, 615.2441).

*3.4.3 6″-Hydroxyl-norkurarinone 4′-O-β-D-glucoside (**5**)*

Yellow powder (MeOH). m.p. 256–257°C. UV (MeOH) λ_{max} 290, 320 nm; CD (MeOH): $\Delta\varepsilon_{320} + 3.290$, $\Delta\varepsilon_{290} - 16.810$. IR (KBr) ν_{max} (cm^{-1}): 3410, 2780, 1820, and 1150; for ^1H NMR and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS: *m/z* 601.2298 $[M-H]^-$ (calcd for $C_{31}H_{37}O_{12}$, 601.2285).

3.5 Bioassay

The cyctoxicities of transformed products (**2**–**8**) were evaluated by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [14]. Hela and A375 cells were maintained in RPMI1640 medium with 10% (v/v) fetal bovine serum and cultured in 96-well microtiter plates. Appropriate dilutions of compounds **2**–**8** were added to the culture. And then cells were continued to culture at 37°C, 5% CO_2 for 72 h. The concentration of test compound to give 50% inhibition of cell growth was expressed as IC_{50} value.

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