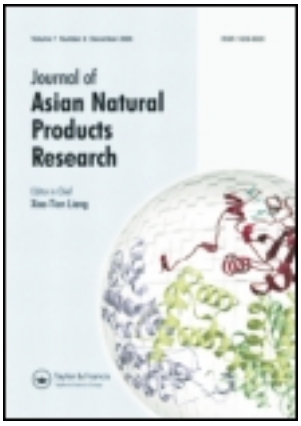

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Microbial transformation of diosgenin by filamentous fungus *Cunninghamella echinulata*

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Microbial transformation of diosgenin (**1**) by suspended-cell cultures of the filamentous fungus *Cunninghamella echinulata* CGMCC 3.2000 was investigated. Incubation of the substrate diosgenin (**1**) with this fungus led to the isolation of three products: two known compounds, (25*R*)-spirost-5-en-3β,7β,12β-triol (**2**) and (25*R*)-spirost-5-en-3β,7β,11α-triol (**3**), and a new compound (25*R*)-spirost-5-en-3β,7α,11α-triol (**4**). The structural elucidations of the three compounds were achieved mainly by the MS, 1D and 2D NMR spectroscopic methods and comparison with known compounds. *C. echinulata* CGMCC 3.2000 has not been used before in the biotransformation of diosgenin (**1**).

Keywords: microbial transformation; biotransformation; hydroxylation; diosgenin *Cunninghamella echinulata* CGMCC 3.2000

1. Introduction

Diosgenin is a steroidal sapogenin widely existing in a variety of plants species [1–6]. In recent years, many investigations have been reported on the important pharmacological attributes of diosgenin, such as anticancer [7–10], antiskin aging [11], and antagonistic effect on cardiovascular action [12]. In addition to its important pharmacological attributes, diosgenin has served as starting materials for the synthesis of steroid hormones [13].

Microbial transformations of steroidal sapogenins have provided an alternative method for obtaining new steroidal sapogenin derivatives that were hard to be synthesized by classic chemical methods [12,14,15]. Microbial transformation of diosgenin has been used in the preparation of pharmaceutically significant compounds. Some micro-organisms such as *Aspergillus nidulans* and *Rhizopus* sp. have been established to be capable of transforming diosgenin to key intermediates for the manufacture of sex hormones by the breaking of the carbon chain of diosgenin [16]. Several micro-organisms could convert diosgenin to diosgenone [16]. *Streptomyces virginiae IBL-14* could also transform diosgenin to diosgenone, and then to isonuatigenone by C-25 tertiary hydroxylation [17]. Previous research also showed that microbial hydroxylations of diosgenin were mainly on the carbons of the rings B, C, and D [18–20].

In this study, the ability of the fungus *Cunninghamella echinulata* CGMCC 3.2000 to transform diosgenin has been investigated for the first time. This fermentation led to the formation of three metabolites. The characterization of three metabolites, a new compound and two

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known compounds, was mainly based on the spectroscopic data.

2. Results and discussion

Biotransformation of diosgenin **1** with *C. echinulata* CGMCC 3.2000 led to the isolation of three transformation products **2**, **3**, and **4** (Figure 1).

Product **2** has a molecular weight of 446, i.e. 32 units more than that of the substrate. The ^{1}H NMR and ^{13}C NMR spectra showed that it had two more —OH than the substrate diosgenin **1**. By comparison of its NMR spectral data with those reported in the literature [18], its structure was confirmed as (25*R*)-spirost-5-en-3β,7β,12β-triol. This compound was also a microbial transformation product by micro-organisms *Cunninghamella elegans* [18] and *Cunninghamella blakesleeana* [19].

Product **3** also has a molecular formula of $C_{27}H_{42}O_5$ and a molecular weight of 446, which suggested that two oxygen atoms were incorporated into the substrate **1**. After the careful study of its ^{1}H NMR, ^{13}C NMR and ^{1}H–^{1}H COSY and comparison of its NMR spectral data with the reported similar compounds [18,21–24], compound **3** was identified as (25*R*)-spirost-5-en-3β,7β,11α-triol. Although compound **3** was a known compound identified by chemical methods [19–20], its structure was identified for the first time by 1D and 2D NMR spectra. We thus provide a detailed NMR spectral data for further reference (Table 1).

Compound **4** has the same MF and MW as those of compounds **2** and **3** from the HR-electrospray ionization (ESI)-mass spectra (MS). From the ^{1}H NMR and HSQC spectra, it was shown that it has two more hydroxyl groups than the substrate **1**. The NMR spectral data of compound **4** were very similar to those of compound **3** except for the signals at positions 5, 6, 7, 8, and 9 (Table 1). In the ^{1}H–^{1}H COSY spectrum, the signal at δ 3.84 (br s) had correlation with H-6 at δ 5.67 (dd, $J = 6.0$, 1.2 Hz; Figure 2). In the HMBC spectrum, H-6 has correlations with C-7 and C-8. The first —OH group must be at position 7, since the coupling constants of H-7 (br s) were different from H-7 (t, 7.8 Hz) in compound **3** (Table 1). The hydroxyl group in compound **4** may be at α-orientation. The α-orientation of 7-hydroxyl group could also be deducted from the chemical

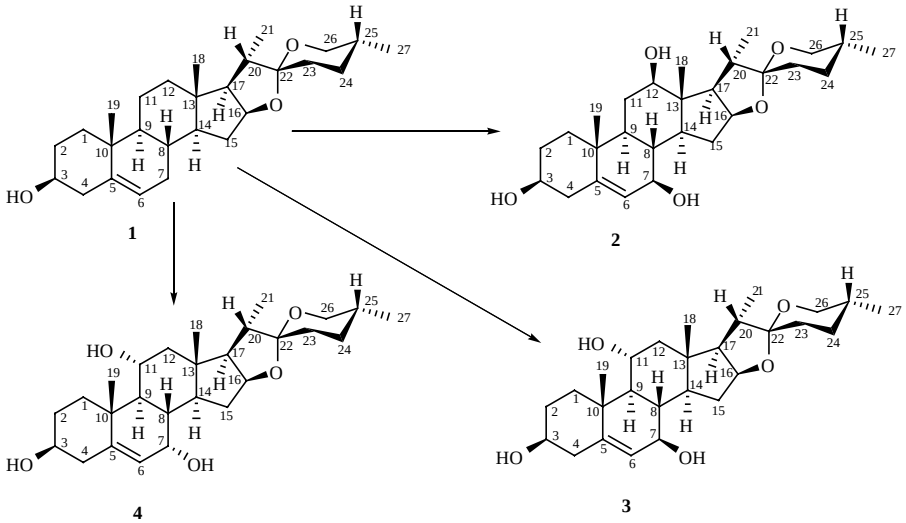

Figure 1. Structures of diosgenin **1** and compounds **2**–**4**.

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data (δ) of compounds **3** and **4** (in $CDCl_3$).

	3		**4**	
1	1.55 (m) 2.58 (dt, 3.6, 13.8)	39.0	1.19 (m) 2.59 (dt, 3.6, 13.8)	38.8
2	1.54 (m, o) 1.83 (m, o)	31.8	1.52 (m, o) 1.82 (m, o)	31.6
3	3.56 (m)	71.5	3.59 (m)	71.4
4	2.23 (t, 11.4, 13.8) 2.34 (m, o)	42.2	2.31 (t, 10.8, 13.8) 2.36 (ddd, 2.4, 7.8, 13.8)	42.5
5		143.6		146.8
6	5.32 (br s)	125.7	5.67 (dd, 6.0, 1.2)	123.6
7	3.84 (t, 7.8)	72.7	3.84 (br s)	65.0
8	1.49 (m, o)	40.7	1.62 (m, o)	37.2
9	1.10 (t, 10.8)	54.6	1.31 (m, o)	49.1
10		38.3		39.0
11	4.05 (ddd, 15.6, 10.8, 5.5)	68.8	4.08 (ddd, 15.3, 11.2, 5.8)	68.8
12	1.25 (m, o) 2.06 (dd, 4.8, 5.4, 11.7)	51.2	1.25 (m, o) 2.05 (dd, 4.8, 5.4, 11.7)	50.7
13		41.3		40.7
14	1.35 (m, o)	54.8	1.61 (m, o)	49.4
15	1.58 (m, o) 2.36 (m, o)	34.3	1.27 (m, o) 2.12 (m, o)	31.4
16	4.46 (q, 15.3, 7.8)	81.2	4.49 (q, 14.1, 7.8)	80.9
17	1.83 (m, o)	61.3	1.85 (m, o)	61.8
18	0.82 (s)	17.2	0.80 (s)	17.1
19	1.21 (s)	18.8	1.17 (s)	18.1
20	1.83 (m, o)	41.7	1.87 (m, o)	41.7
21	0.99 (d, 7.2)	14.5	0.98 (d, 6.6)	14.5
22		109.2		109.3
23	1.62 (m, o) 1.60 (m, o)	31.4	1.59 (m) 1.69 (m, o)	31.6
24	1.44 (m, o) 1.60 (m, o)	28.8	1.45 (m, o) 1.64 (m, o)	28.8
25	1.60 (m, o)	30.3	1.62 (m, o)	30.3
26	3.38 (t, 10.8) 3.47 (m)	66.9	3.38 (t, 10.8) 3.47 (m)	66.9
27	0.79 (d, 6.0)	17.1	0.79 (d, 6.6)	17.1

shift of C-5 (δ 146.6), C-6 (δ 123.6), and C-7 (δ 64.6), because the chemical shifts of its 7β-isomer would be C-5 (δ 143.7), C-6 (δ 125.5), and C-7 (δ 72.6), respectively [18,21,22]. The α-orientation of 7-hydroxyl was finally confirmed by the NOE between H-7 and H-8 in its NOESY spectrum (Figure 2). The carbon that has a chemical shift of δ 68.8, bearing a proton signal at δ 4.08 (ddd, 15.3, 11.2, 5.8 Hz), was assigned position 11, because the H-7 correlated to H-8, while H-8 has a correlation with H-9 in the $^{1}H-^{1}H$ COSY spectrum. In the meantime, H-9 was correlated to the proton at δ 4.08 which must be at position 11 (Figure 2). This hydroxyl group was put to 11-α-OH because of the NOE of H-11 (δ 4.08) with Me-18 and Me-19 (Figure 2). Therefore, the structure of compound **4** was fully confirmed to be (25*R*)-spirost-5-en-3β,7α,11α-triol. This compound was a new compound.

Cunninghamella echinulata CGMCC 3.2000 has been first investigated for the introduction of double hydroxyl groups into diosgenin **1**. Incubation of the substrate **1** with *C. echinulata* CGMCC 3.2000 resulted in the formation of three metabolites **2**, **3**, and **4**. Compound **2** was found before in the biotransformation of diosgenin **1** using *C. elegans* and *C. blakesleeana* [18–19]. Product **3** was also found in the microbial transformation of diosgenin **1** using *Helicostylum piriforme.* Since compound **3** was found in the early 1960s, its structure was determined mainly by chemical method. The detailed assign-

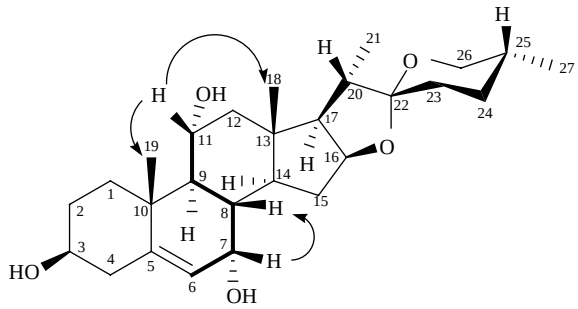

Figure 2. Key $^{1}H-^{1}H$ COSY (bold line) and NOE correlations (arrows) of product **4**.

ment of its NMR spectral data was very useful for future investigators; we thus provide the fully assigned NMR spectral data of compound **3**. It is very interesting to note that the same compound (25*R*)-spirost-5-en-3β,7β,12β-triol **2** was obtained through the microbial transformation of diosgenin **1** using fungi *C. elegans* and *C. blakesleeana* by previous researchers [18–19]. These results showed that the three fungi produced same or same functional enzyme(s) due to the common property that they come from the genus *Cunninghamella*. As a result, they all had the ability to hydroxylate at the C-7 and C-12 of diosgenin. *Cunninghamella echinulata* CGMCC 3.2000 can also selectively hydroxylate diosgenin **1** at positions 11 and 7 to afford compounds **3** and **4**. These two compounds were isomers at position 7.

In conclusion, biotransformation with *C. echinulata* provided an effective hydroxylation method of diosgenin (**1**) that was hard to be synthesized from classic chemical methods.

3. Experimental

3.1 General experimental procedures

Optional rotations were recorded on a Perkin-Elmer 341 polarimeter. Melting points were recorded on a Fisher-Johns micromelting apparatus and are uncorrected. The IR spectra were measured on a Perkin-Elmer 983G infrared spectrometer with KBr pellets. ^{1}H, ^{13}C, and 2D NMR spectra ($CDCl_3$ as solution) were recorded on a Bruker Avance-600. Chemical shifts (δ) are reported in parts per million (ppm) with TMS as an internal standard. Coupling constants (J) were given in Hertz (Hz). MS were measured in positive-ion mode by ESI technique on an Esquire 3000 mass spectrometer, using methanol as solvents. All chemical reagents used were of analytical grades. Sterilization was carried out in an YX-2800 autoclave. Aseptic operation was carried out in SW-CJ-ID laminar flow cabinet. Incubation was carried out on a HZQ-X100 constant temperature shake incubator. Thin layer chromatography (TLC) analyses were carried out on pre-coated silica gel GF254 plates (0.25 mm thick, Qingdao Marine Chemical Factory, Qingdao, China). Visualization of the TLC plates was performed by 10% H_2SO_4–EtOH reagent, followed by heating. Silica gel (ZCX-II, 200–300 mesh, Qingdao Marine Chemical Factory, Qingdao, China) was used for column chromatography.

3.2 Substrate, micro-organism, and culture medium

The substrate diosgenin was provided by Kunming Institute of Botany, Chinese Academy of Sciences. *Cunninghamella echinulata* CGMCC 3.2000 was purchased from China General Microbiological Culture Collection Center, Beijing, China. The strain was grown on potato dextrose

agar (PDA) at 25°C and stored at 4°C. The medium for *C. echinulata* CGMCC 3.2000 was prepared by the following procedure: To 200 g of mincing husked potato, 1000 ml of water was added. The mixture was boiled for half an hour before it was filtered. To the filtrate, 20 g glucose was added with 20 g glucose.

3.3 Fermentation of compound 1 and extraction of metabolites

The fungal medium was transferred into 1000 ml conical flasks (500 ml medium each) and was autoclaved at 121°C for 20 min. The mycelia of *C. echinulata* CGMCC 3.2000 were transferred from PDA slant to 34 flasks and allowed to grow in shake incubator at 28°C for 200 rpm. After 3 days, compound **1** (2000 mg, dissolved in 116 ml hot anhydrous ethanol) was evenly distributed among the 34 flasks. The incubation continued at the same conditions. Parallel control experiments were conducted which included an incubation of the fungus without compound **1** and an incubation of compound **1** in the medium without fungus. After 6 days, the contents of each flask were homogenized in a blender (15,000 rev/min) and extracted with two volumes of ethyl acetate for three times. The extract was evaporated under reduced pressure on a rotary evaporator (45°C). The resulting brown gum (14.89 g) was analyzed by TLC.

3.4 Isolation of the metabolites

The crude gum was dissolved in acetone, absorbed on silica gel (35 g), and applied to column chromatography. The elution system consisted of gradient mixtures of dichloromethane and acetone. Elution with dichloromethane/acetone (100:1, 4:1, 3:1, 1:1) gave substrates **1** (1566 mg), **2** (16 mg) **3** (88 mg) and **4** (14 mg), respectively.

3.4.1 (25R)-Spirost-5-en-3β,7α, 11α-triol (4)

White crystalline solid (methanol). Mp 270–272°C, $[\alpha]_D^{20} - 61.0$ ($c = 0.295$, $CHCl_3$). IR (KBr) ν_{max} (cm^{-1}): 3379, 3367, 3273, 2951, 2928, 2910, 2872, 1458, 1379, 1240, 1180, 1043, 979. For the ^1H NMR and ^{13}C NMR spectral data, see Table 1. HR-ESI-MS: *m/z* 447.3114 $[M + H]^+$ (calcd for $C_{27}H_{43}O_5$, 447.3105).

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