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Microbiological transformation of diosgenin by resting cells of filamentous fungus, *Cunninghamella echinulata* CGMCC 3.2716

Ting Dong[a], Guang-Wei Wu[a], Xiao-Ning Wang[a], Jin-Ming Gao[a,*], Jian-Guang Chen[b], Shoei-Sheng Lee[b]

[a] *Natural Medicinal Chemistry Research Centre, College of Science, Northwest A & F University, Yangling, Shaanxi 712100, China*
[b] *School of Pharmacy, College of Medicine, National Taiwan University, Taipei 10051, Taiwan, ROC*

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ABSTRACT

Microbial transformation of the steroidal sapogenin diosgenin (**1**) by resting cells of the filamentous fungus, *Cunninghamella echinulata* CGMCC 3.2716 was studied. Four metabolites were isolated and unambiguously characterized as (25*R*)-spirost-5-ene-3β,7β-diol-11-one (**2**), (25*R*)-spirost-5-ene-3β,7β-diol (**3**), (25*R*)-spirost-5-ene-3β,7β,11α-triol (**4**), and (25*R*)-spirost-5-ene-3β,7β,12β-triol (**5**), by various spectroscopic methods (^{1}H, ^{13}C NMR, DEPT, ^{1}H–^{1}H COSY, HMBC, HSQC and NOESY). Compound **2** is a new metabolite. The NMR data and full assignment for the known metabolites (25*R*)-spirost-5-ene-3β,7β-diol (**3**) and (25*R*)-spirost-5-ene-3β,7β,11α-triol (**4**) are described here for the first time. The biotransformation characteristics observed included were C-7β, C-11α and C-12β hydroxylations. Compounds **1–5** exhibited no significant cytotoxic activity to human glioma cell line U87.

1. Introduction

Microbial transformations of compounds play vital roles in the preparation of new oxygenated derivatives with biological activities [1,2]. In particular, they have found widespread use in structural modification of steroids with high regio- and stereoselectivity [3], which are able to be accomplished via a variety of transformations on steroidal skeleton such as hydroxylation, Baeyer–Villiger oxidation, ketone reduction, methoxylation oxidation, reduction, isomerization, Michael addition, and reverse aldol reaction [4–6]. It has been proved that hydroxylation of steroidal compounds was catalyzed by cytochrome P450 monooxygenase systems almost existing in all eucaryotic microorganisms [7]. *Cunninghamella* genus has been widely applied in incorporating hydroxyl group of non-activated centers of steroids [8] and has frequently been used as microbial models of mammalian steroid drug metabolism [9–11].

Diosgenin (**1**), namely, (25*R*)-spirost-5-ene-3β-ol, has showed many biological activities, such as antioxidative [12,13], anti-aging of skin in mice [14], hypolipidemic [12,15], antiproliferative [16,17], proapopotic [18,19], anti-hyperpigmentation [20] as well as cancer chemopreventive [21,22]. Thus, the tendency of finding diosgenin derivatives by fungi which possessed the ability to retain the carbon skeleton of diosgenin has been noted. In previous studies, it had been reported that some microorganisms exhibited the ability to metabolize diosgenin [23–28]. The microbial transformations of diosgenin occurred at C-7, C-9, C-11, and C-12 positions, which were involved in hydroxylation, ketonization, methoxylation by various microorganisms.

In our continuing work on biotransformation of bioactive compounds [29], the ability to transform diosgenin (**1**) by the filamentous fungus *C. echinulata* resting cells has been investigated for the first time. From the fermentation broth of **1**, four metabolites were isolated and unambiguously characterized as (25*R*)-spirost-5-ene-3β,7β-diol-11-one (**2**), (25*R*)-spirost-5-ene-3β,7β-diol (**3**), (25*R*)-spirost-5-ene-3β,7β,11α-triol (**4**), and (25*R*)-spirost-5-ene-3β,7β,12β-triol (**5**) by spectroscopic methods. Additionally, the conversion pathway of metabolism of compounds **2–5** was explored preliminarily. To the best of our knowledge, this is the first report of the 11-oxo-diosgenin (**1**) by using fungal cell culture.

2. Experimental

2.1. General

Diosgenin, tigogenin, and acetyldiosgenin were provided from National Engineering Research Center for Phytochemistry in West China. Melting points were obtained with an X-4 micro-melting

* Corresponding author at: College of Science, Northwest A & F University, 3 Taicheng Road, Yangling, Shaanxi 712100, China.
Tel.: +86 29 87092335; fax: +86 29 87092335.
E-mail address: jinminggao@nwsuaf.edu.cn (J.-M. Gao).

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point apparatus and are uncorrected. Optical rotations were measured in methanol using a JASCO DIP-370 polarimeter. IR spectra were obtained using a JASCO IR Report-100 Infrared Spectrometer. 1D and 2D NMR spectra were recorded on Bruker AV400 and Bruker AV III-600 spectrometers (CD_3OD, δ_H 3.30 and δ_C 49.0 ppm). ESI (negative ion mode) mass spectra were measured on an Eaquire 2000 instrument. Silica gel (200–300 mesh) and pre-coated silica gel (0.25 mm) which were purchased from Qingdao Marine Chemical Company of China were used for column chromatography (CC) and TLC, respectively. TLC layers were activated at 105 °C for 1 h before use. Spots on TLC were visualized under UV light and by spraying with 5% H_2SO_4 in ethanol reagent followed by heating. All reagents used were of analytical grades.

2.2. Microorganisms and culture medium

The microbes *C. echinulata* CGMCC 3.2716, *Absidia orchidis* CGMCC 3.2458, *Aspergillus ochraceus* CGMCC 3.3876, *Penicillium citrinum* CGMCC 3.2788, *Rhizopus chinensis* CGMCC 3.1165, *Mucor mucedo* No.3.15, *Mucor wutungchiao* No.3.39 were purchased from Shaanxi Institute of Microbiology. The endogenetic fungus *Sphaceloma* de Bary LN-15 was isolated from *Melia azedarach* L. by our laboratory. *Thelephora japonica* S0230 and *Polyporus picipes* S0318 were purchased from Kunming Institute of Botany, the Chinese Academy of Sciences.

All the microorganisms were maintained on potato dextrose agar plates (mincing husked fresh potato 200 g l^{-1}, glucose 20 g l^{-1}, agar gel 15 g l^{-1}) at 4 °C and activated before use. The liquid growth medium was composed of distilled water (1000 ml), mincing husked fresh potato (200 g), glucose (20 g), $MgSO_4{\cdot}7H_2O$ (0.5 g), KH_2PO_4 (1.5 g), vitamin B_1 (10 mg). The medium was used after sterilized at 121 °C for 30 min.

2.3. Preparation of resting cells of microorganism

Microorganism was grown in 500-ml Erlenemeyer flasks containing 100 ml of liquid growth medium at 28 °C in a rotary shaker at 180 rpm, and harvested after 48 h. The cells was obtained by centrifugation and washed three times with 0.8% NaCl solution.

The cells were inoculated in 500-ml Erlenemeyer flasks containing 100 ml of phosphate buffer (0.45 g Na_2HPO_4, 0.34 g KH_2PO_4 in 100 ml of distilled water) for biotransformation. 0.8% NaCl and phosphate buffer were autoclaved at 121 °C prior to use.

2.4. Screening microorganism and microbial transformation

The screening-scale experiments for biotransformation of diosgenin were carried out with resting cell method to contain 60 ml phosphate buffer in 250-ml Erlenemeyer flasks. The cells were inoculated into each flask and cultured at 28 °C for 72 h in a rotary shaker at 180 rpm after adding the substrate. Diosgenin was dissolved in acetone with concentration of 5 mg ml^{-1}. Four milliliters of acetone solution was added to each flask. Control experiments were designed under the same conditions. The results of biotransformation of diosgenin were monitored by TLC. All the experiments were repeated three times.

The preparative scale biotransformation of diosgenin was carried out in 500-ml Erlenemeyer flasks containing 100 ml of phosphate buffer. The resting cells were reused three times for transformation without new preparation. The following procedures were the same to the screening scale biotransformation.

2.5. Extraction, isolation and identification of metabolites

The fermentations were filtered and extracted with ethyl acetate in three portions. The resulting mycelium was ultrasonically extracted with ethyl acetate. The organic phases were combined and dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to give 2.2 g of crude extract. The crude extract was subjected to silica gel column and eluted with chloroform–methanol (100:0–60:40) to provide three fractions A–C. Not transformed **1** (700 mg, 54%) was recycled from fraction A. Fraction B was separated by CC on silica gel (200–300 mesh) eluting with chloroform/acetone (93:7) to yield subfraction B2. Subfraction B2 was further purified by reversed phase RP18 column chromatography (methanol/H_2O, 70:30) to yield compounds **2** (20 mg, 1.5%) and **3** (12 mg, 0.9%). Fraction C was passed through a silica gel column with petroleum ether–acetone (75:25) as eluent to give subfraction C2. Subfraction C2 was submitted to a RP-18 column eluted with methanol–H_2O (60:40) and purified by Sephadex LH-20 column with methanol to afford compounds **4** (80 mg, 6.2%) and **5** (100 mg, 7.7%).

2.5.1. (25R)-spirost-5-ene-3β,7β-diol-11-one (*2*)

White crystalline solid. mp: 259–260 °C. $[\alpha]^{23}_D$ −10° (c = 0.30, MeOH). IR(KBr) ν_{max} 3405, 1691, 1678, 1057, 983, 922, 900, 864 cm^{-1}. ^1H NMR see Table 1; ^{13}C NMR see Table 2; ESI-MS (negative ion mode): m/z 443.2[M−H]$^-$, 425.2 [M−H-H_2O]$^-$. HR-ESI-MS (negative ion mode): m/z 443.2803 [M−H]$^-$ (calc. for $C_{27}H_{39}O_5$, 443.2799).

2.5.2. (25R)-spirost-5-ene-3β,7β-diol (*3*)

White crystalline solid. mp: 215–216 °C (lit. mp. 216–219 °C) [23]. $[\alpha]^{23}_D$ −45° (c = 0.31, MeOH) [lit.−75° ($CHCl_3$)] [23]. IR(KBr) ν_{max} 3458, 1671, 1054, 981, 921, 900, 864 cm^{-1}. ^1H NMR see Table 1; ^{13}C NMR see Table 2; ESI-MS (negative ion mode): m/z 429.1 [M−H]$^-$, 411.1[M−H-H_2O]$^-$.

2.5.3. (25R)-spirost-5-ene-3β,7β,11α-triol (*4*)

White crystalline solid. mp: 265–267 °C (lit. mp. 263–266 °C) [24]. $[\alpha]^{23}_D$ −47° (c = 0.30, MeOH) [lit.−47° (EtOH)] [24]. IR(KBr) ν_{max} 3384, 1635, 1046, 981, 922, 897, 864 cm^{-1}. ^1H NMR see Table 1. ^{13}C NMR see Table 2. ESI-MS (negative ion mode): m/z 445.2[M−H]$^-$, 427.1[M−H-H_2O]$^-$. HR-ESI-MS (negative ion mode): 445.2959[M−H]$^-$ (calc. for $C_{27}H_{41}O_5$, 445.2948).

2.5.4. (25R)-spirost-5-ene-3β,7β,12β-triol (*5*)

White crystalline solid. mp: 183–185 °C (lit. 182–184 °C) [25]. $[\alpha]^{23}_D$ −46° (c = 0.35, MeOH). IR(KBr) ν_{max} 3420, 1647, 1055, 980, 922, 897, 863 cm^{-1}. ESI-MS (negative ion mode): m/z 445.1[M−H]$^-$, 425.1[M-3H-H_2O]$^-$.

2.6. Biotransformation of metabolites *2–5*, tigogenin and acetyldiosgenin by C. echinulata resting cells

To explore a biotransformation pathway of diosgenin (**1**) and to determine the influence of the substrate structure on the reaction route, microbial transformations of compounds **2–5**, tigogenin and acetyldiosgenin with *C. echinulata* resting cells were carried out by the method described in Section 2.4. The results of fermentation were analyzed by TLC.

2.7. Cytotoxicity bioassay

Human glioma cell line U87 was obtained from the American Type Culture Collection (ATCC) and maintained in ATCC's recommended growth medium supplemented with 10% fetal bovine serum (Gibco; Invitrogen) at 37 °C in 5% CO_2 humidified atmosphere. The cytotoxicity assay was performed according to the XTT method. Briefly, U87 cells were seeded onto 96-well microtiter

Table 1
^{1}H NMR spectra of metabolites **2–5** (MeOH-d_4, TMS, δ in ppm, J in Hz)[a].

Position	2	3	4
1	2.62(1H-α,dt, J = 3.6,3.5,13.7) 0.95(1H-β, m)	1.86 (1H,dt, J = 3.5, 3.5, 13.4) 1.05 (1H, m)	2.62(1H-α,dt, J = 3.4, 3.1, 14.0)1.11(1H-β, m)
2	1.75(1H, m) 1.51(1H, m)	1.80 (1H, m), 1.61(1H, m)	1.73(1H, m) 1.52(1H, m)
3	3.40(1H, m)	3.42 (1H, m)	3.41(1H, m)
4	2.23(2H, m)	2.23(2H, m)	2.25(2H, m)
5			
6	5.25(1H, t, J = 1.8)	5.25(1H, t, J = 1.8)	5.29 (1H, brs)
7	3.97(1H, dt, J = 2.0, 2.0, 8.3)	3.71(1H, dt, J = 2.1, 2.1, 8.3)	3.72(1H, brd, J = 8.4)
8	1.90(1H, m)	1.61(1H, m)	1.54(1H, m)
9	2.07(1H, d J = 11.4)	1.08(1H, m)	1.11(1H, m)
10			
11		1.61(1H, m) 1.50(1H, m)	3.97(1H-β, ddd, J = 4.8, 10.6, 10.6)
12	2.35(1H-α, d J = 13.2) 2.25(1H-β, d J = 13.2)	1.75 (1H, m), 1.18(1H, td, J = 4.3, 12.8, 12.8)	1.99(1H-β, dd J = 6, 12), 1.23 (1H-β, m)
13			
14	1.98(1H, m)	1.26(1H, m)	1.34 (1H, m)
15	2.49(1H-α,m) 1.57(1H-β,m)	2.29 (1H-α, m), 1.61 (1H-β, m)	2.35(1H-α, m) 1.49(1H-β, m)
16	4.50(1H, dd, J = 7.5 15)	4.40 (1H, dd, J = 7.5 15)	4.4(1H, dd, J = 7.2 15)
17	1.95(1H, m)	1.73(1H, m)	1.73(1H, m)
18	0.72(3H, s)	0.81(3H, s)	0.82(3H, s)
19	1.25(3H, s	1.08(3H, s)	1.20(3H, s)
20	1.85(1H, m)	1.89(1H, dd, J = 7.2, 13.8)	1.89(1H, m)
21	0.95(3H, d, J = 7.0)	0.96(3H, d, J = 7.2)	0.97(3H, d, J = 7.2)
22			
23	1.70(1H, m), 1.58(1H, m)	1.69(1H, m), 1.48(1H, m)	1.68(1H,dd, J = 4.2 13.2) 1.56(1H, m)
24	1.62(1H, m) 1.43(1H, m)	1.61(1H, m) 1.43(1H, m)	1.60(1H, m) 1.43(1H, m)
25	1.60(1H, m)	1.61(1H, m)	1.60(1H, m)
26	3.45(1H, m), 3.33(1H, m)	3.45(1H, m), 3.33(1H, m)	3.45(1H, m), 3.33(1H, m)
27	0.79(3H, d, J = 6.4)	0.79(3H, d, J = 6.0)	0.78 (3H, d, J = 6.0)

[a] Assignments were based on DEPT, COSY, NOESY, HSQC, and HMBC experiments.

plates at a density of 1000/well and treated with the tested compounds for the 48 h, following which 10 μl XTT was added and incubated for another 4 h at 37 °C. After treatment, cell viability was determined by XTT colorimetric assay according to the manufacturer's protocol [30].

3. Results and discussion

Screening scale experiments showed that *C. echinulata* has an ability to transform compound **1** into its polar derivatives as determined by TLC, so a large scale experiment was carried out. Incubation of diosgenin (**1**) with the fungus *C. echinulata* resting cells produced four metabolites **2–5** (Fig. 1) in combination with purification by multiple column chromatography on silica gel, RP-18 and Sephadex LH-20. Metabolite **3**, **4** and **5** had already been isolated as biotransformed products of **1** with some fungi [23–25], but no NMR data for **3** and **4** were reported. The NMR data of **5** were identical with those reported in the literature [25]. This compound was reported previously from biotransformation of **1** by *Cunninghamella elegans* [25].

Compound **2** gave a pseudo-molecular ion peak at *m/z* 443.2803 in HR-ESI-MS (negative ion mode) spectrum coupled with ^{13}C NMR, corresponding to the molecular formula of $C_{27}H_{40}O_5$ ([M−H]$^-$ calc. 443.2799, 4.0 ppm error), with 8 unsaturated degree, indicating that **2** contained more two oxygen atoms than **1**. The IR spectrum showed the presence of hydroxyl and carbonyl functionalities (3405 and 1691 cm^{-1}), and the latter supported by carbon signal at 211.8 ppm in the ^{13}C NMR. The ^{13}C NMR spectrum of **2** disclosed 27 carbon signals which were recognized as 4 methyls, 8 methylenes, 10 methines, 5 quaternary carbon atoms including one carbonyl carbon. In the ^{1}H NMR spectrum, an additional hydroxy-bearing methine proton signal was exhibited at δ 3.97 (1H, dt, J = 2.0, 2.0, 8.3 Hz), while this OH-bearing methine carbon signal at δ 73.8 was seen, and the disappearance of C-7 methylene signal (δ 33.1) compared to **1** in the ^{13}C NMR spectrum, suggesting one hydroxyl group at C-7. Moreover, the ^{1}H–^{1}H COSY spectrum showed the correlations between H-7 (δ 3.97) and olefinic proton H-6 (δ 5.25), a

Table 2
^{13}C NMR data of diosgenin (**1**) and its metabolites **2–5** (MeOH-d_4, TMS, δ in ppm)[a].

Position	1	2	3	4
1	38.5	37.0	38.2	40.2
2	32.4	32.3	32.4	32.4
3	72.4	72.0	72.1	72.3
4	42.9	42.2	42.6	43.1
5	142.3	144.4	144.1	144.2
6	122.2	126.7	127.3	127.5
7	33.1	73.8	73.6	73.3
8	32.8	42.5	41.0	41.0
9	51.6	60.1	49.8	55.5
10	37.8	38.0	37.7	39.4
11	22.0	211.8	22.1	69.2
12	40.9	58.1	40.8	51.7
13	41.4	45.0	41.9	42.2
14	57.8	55.2	57.1	56.2
15	32.7	34.9	35.1	35.2
16	82.2	82.7	82.6	82.8
17	63.8	61.7	63.2	63.0
18	16.7	17.5	16.8	17.5
19	19.8	18.6	19.5	19.0
20	43.0	43.2	43.0	42.9
21	14.9	14.6	14.9	14.9
22	110.6	110.5	110.6	110.5
23	32.3	32.0	32.3	32.4
24	29.9	29.8	29.9	29.8
25	31.4	31.4	31.4	31.4
26	67.9	67.9	67.8	67.8
27	17.5	17.5	17.5	17.6

[a] Assignments were based on DEPT, COSY, NOESY, HSQC, and HMBC experiments.

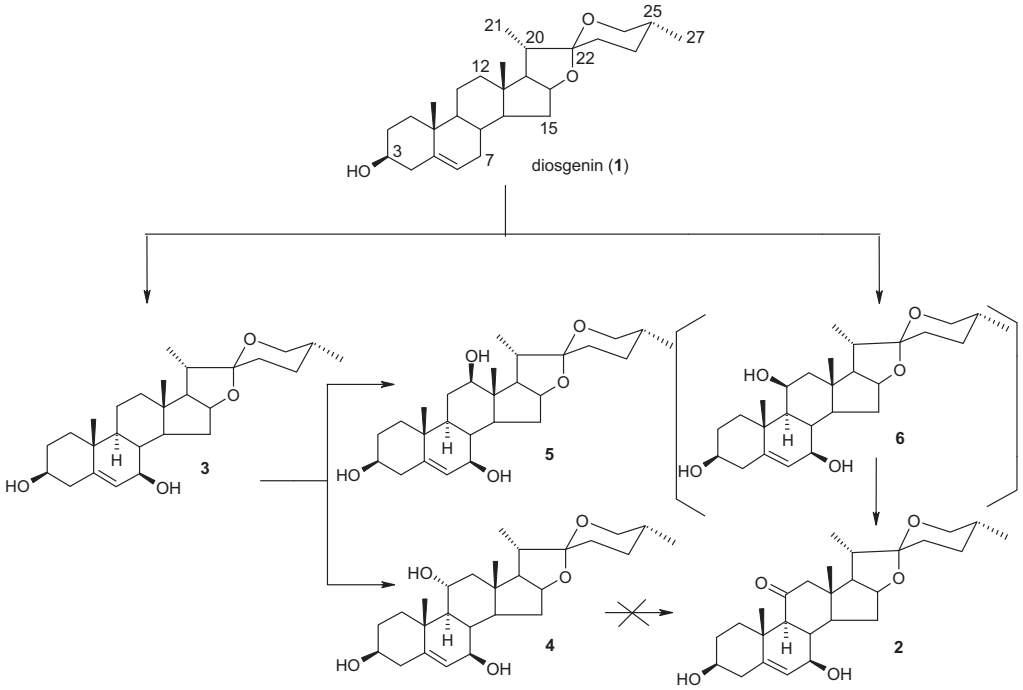

Fig. 1. Proposed biotransformation of diosgenin (**1**) by *C. echinulata* resting cells.

methine proton H-8 (δ 1.90) (Fig. 2). The HMBC spectrum showed the correlations from H-7 (δ 3.97) to C-5 (δ 144.4), C-6 (δ 126.7) and C-8 (δ 42.5) (Fig. 2), suggesting that the hydroxylation occurred at C-7. The β-configuration for 7-OH was assigned by NOESY correlations between Hα-7 (δ 3.97) and Hα-9 (δ 2.07), Hα-14 (δ 1.98) (Fig. 2). Furthermore, a C-11 methylene carbon signal at δ 22.0 in **1** was replaced by a carbonyl carbon at δ 211.8 in **2**. The $^2J_{H-C-H}$ spin system (δ 2.35, 1H, d, J = 13.2 Hz and δ 2.25, 1H, d, J = 13.2 Hz) was noted by the HSQC experiment, suggesting C-12 methylene carbon at δ 58.1 were adjacent to two quaternary carbon atoms. The HMBC spectrum showed the correlations of H-12 (δ 2.25, 2.35) and H-9 (δ 2.07) with C-11 (δ 211.8) (Fig. 2), suggesting the presence of this carbonyl group at C-11. Moreover, the downfield shifts of C-8 (δ 42.5), C-9 (δ 60.1), C-12 (δ 58.1), and C-13 (δ 45.0), in comparison to **1**, and a series of moderate γ-gauche effects which involved the carbon signals at C-1 (δ 37.0), C-19 (δ 18.6), C-14 (δ 55.2) and C-17 (δ 61.7) were observed, which also supported the placement of the carbonyl group at C-11 [4]. Finally, the structure of **2** was elucidated as (25*R*)-spirost-5-ene-3β, 7β-diol-11-one, which is a new metabolite.

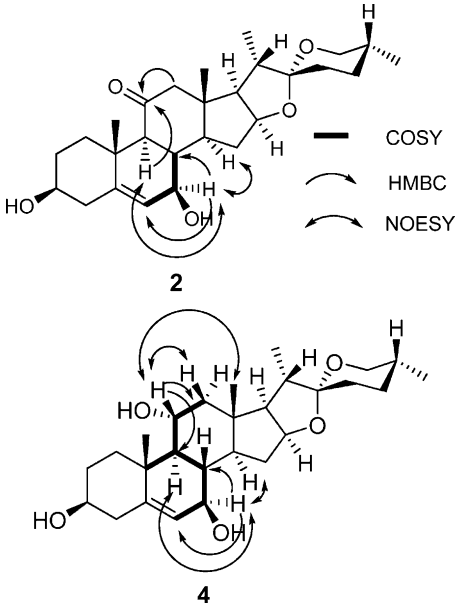

Fig. 2. Selected 2D NMR correlations of compounds **2** and **4**.

Compound **3** exhibited a molecular ion peak at *m/z* 429.1[M−H]$^-$ in negative ESI-MS spectrum coupled with ^{13}C NMR data, corresponding to the chemical formula $C_{27}H_{42}O_4$, suggesting that an oxygen atom was introduced into the molecule **1**. The IR spectrum indicated the presence of hydroxyl functionality (3458 cm^{-1}). The ^{1}H NMR data displayed a new downfield OH-bearing methine proton at δ 3.71 (1H, dt J = 2.1, 2.1, 8.3 Hz), while an OH-bearing methine carbon was observed at δ 73.6 along with C-7 methylene carbon at δ 33.1 disappearing in the ^{13}C NMR spectrum compared to compound **1**, suggesting the hydroxylation occurred at C-7, as supported by the downfield shifts of C-6 (δ 127.3) and C-8 (δ 41.0). Moreover, the COSY correlation of H-7 (δ 3.71) with olefinic proton H-6 (δ 5.25) also confirmed the hydroxyl group at C-7. The β-configuration at C-7 was determined by NOE effects between H-7 (δ 3.71) and H-14α (δ 1.26). Moreover, the physical–chemical properties of **3** were similar to that reported for 7β-hydroxyldiosgenin [23]. The structure of metabolite **3** was therefore elucidated as (25*R*)-spirost-5-ene-3β, 7β-diol. This compound was identified before as one of the biotransformed metabolites of diosgenin with *Cunninghamella blakesleeana* [23]. The ^{1}H and ^{13}C NMR data and full assignment of **3** are accomplished here for the first time by ^{1}H–^{1}H COSY, HMBC, HSQC and NOESY experiments.

The molecular formula of **4** was deduced as $C_{27}H_{42}O_5$ at *m/z* 445.2959 ([M−H]$^-$ calc. 445.2948, 1.1 ppm error) in negative HR-ESI-MS, 32 amu. higher than **1**, suggesting the incorporation of two oxygen atoms. The IR spectrum showed the hydroxyl absorption at 3384 cm^{-1}. The ^{1}H NMR spectrum exhibited two additional oxygen-bearing methine proton signals at δ 3.72 (1H, brd, J = 8.4 Hz) and 3.97 (1H, ddd, J = 4.8, 10.6, 10.6 Hz), while the ^{13}C NMR spectrum and DEPT experiments showed two additional downfield

methine carbon signals at δ 73.3 and 69.2, corresponding to two methylene signals of C-7 and C-11 missing, in comparison to compound **1** which indicated that two hydroxyl groups at both C-7 and C-11 were incorporated into compound **4**. The HMBC spectrum (Fig. 2) displayed the correlations from H-7 (δ 3.72) to C-5 (δ 144.2), C-6 (δ 127.5), C-8 (δ 41.0), and C-9 (δ 55.5). Moreover, the ^{1}H–^{1}H COSY spectrum (Fig. 2) showed correlations of H-7 (δ 3.72) with H-6 (δ 5.29) and H-8 (δ 1.54), which further confirmed one hydroxyl group at C-7. On the other hand, the HMBC correlations of C-11 methine proton signal at δ 3.97 with C-9 (δ 55.5), C-10 (δ 39.4), and C-12 (δ 51.7) were shown; the ^{1}H–^{1}H COSY spectrum showed correlations of H-11 (δ 3.97) with H-9 (δ 1.11) and H_2-12 (δ 1.99, 1.23), which further supported a hydroxylation at C-11. The NOESY correlations between Hα-7 (δ 3.72) and Hα-9 (δ 1.11), Hα-14 (δ 1.34), suggesting that an OH at C-7 in a β-orientation. In addition, the NOE correlations observed between Hβ-11 (δ 3.97) and Hβ-18 (δ 0.82) indicating an OH at C-11 to be β-oriented (Fig. 2). Thus, the structure of **4** was established as (25*R*)-spirost-5-ene-3β, 7β, 11α-triol. The ^{1}H and ^{13}C NMR data and full assignment of **4** are achieved here for the first time by COSY, HSQC, HMBC and NOESY experiments. This compound was identified before as one of the biotransformed metabolites of diosgenin with the fungus *Helicostylum piriforme* [24].

The anti-glioma activities of compounds **1–5** were tested using human glioma cell line U87. It was found that compounds **1–5** showed no marked cytotoxicity against human glioma cell line U87 with inhibition rates of 10–30% at a concentration of 100 μM.

The fungus *C. echinulata* has been utilized for the incorporation of various functionalities into steroidal skeleton. In the present study, the transformation products **2–5** derived from the stereoselective oxidation at C-7, C-11, and C-12 of **1** with *C. echinulata* reveal that this mold appears to possess different fungal cytochrome P450 mono-oxygenases. One enzyme effected 7β-hydroxylation at the allylic C-7 position, while there is probably another hydroxylase directed attack to C-7β/12β to afford **5**. Furthermore, a third 7β/11α-hydroxylase could be involved in the reaction occurring, with **4** being formed. Subsequent dehydrogenation of the hydroxyl group at C-11 to a keto group was catalyzed by dehydrogenase enzyme(s), leading to formation of **2**, presumably via a tentative 11β-intermediate **6**. In addition, cytochrome P450 enzymes present in *C. echinulata* can bind both on front and rear sides of the steroidal skeleton, resulting in the formation of the corresponding oxidized products.

On the other hand, we showed that 7β-hydroxylation occurred during the early stages of incubation to produce metabolite **3**, while 11α- and 12β-hydroxylation happened at the second day to afford products **4** and **5**. After 72 h, product **2** was produced by 11-oxidation (dehydrogenation). Incubation of metabolite **4** did not result in the production of metabolite **2**, which indicated that the oxidation of 11α-OH of **4** could not take place to C-11 keto functionality of **2** in the transformation. These suggest that the 11-oxo group of **2** could be produced via the oxidation of the above presumed 11β-hydroxy intermediate **6** (Fig. 1). However the intermediate **6** has not been isolated or detected, perhaps due to its swift oxidization. Therefore, we could make some kinetic assumptions that it is well known that an axial 11β-hydroxy group of **6** is much more active than an equatorial 11α-hydroxy group of **4** (namely, **6** has much higher energy than **4**), and as a result, oxidation rate of the former is quite faster than that of the latter under the same conditions. Once this reactive intermediate **6** could be formed, it will be fast converted into the ketone **2**. This process is catalyzed by the dehydrogenase present in this fungus. In addition, both compounds **4** and **5** were confirmed as final products since no detectable metabolites were accumulated after incubation of **4** and **5**, respectively. A proposed biotransformation pathway for **1** with *C. echinulata* is shown in Fig. 1.

In order to determine the structural requirements of biotransformation of diosgenin (**1**), feeding experiments done with diosgenin derivatives: tigogenin (5,6-dihydrodiosgenin) and acetyldiosgenin, respectively, were carried out to *C. echinulata*. Several attempts at hydroxylation of tigogenin or acetyldiosgenin resulted in the recovery of starting material. The results showed that no biotransformed products were detected, and that introduction of an acetyl group at C-3 or a 5α-dihydro moiety of B-ring in the substrates suppresses hydroxylation because the small structural modification could block binding of the substrate to an active-site of cytochrome P450 mono-oxygenases. Accordingly, it was disclosed that the double bond and free 3-OH may be crucial for biotransformation of **1**.

In conclusion, biotransformation of diosgenin (**1**) with the fungus *C. echinulata* provided four polar metabolites **2–4** and an effective procedure for the C-7β, 11α, and C-12β hydroxylations of **1**. It is of interest to find out whether Δ^5-unsaturation or hydroxyl group at C-3 in steroidal sapogenins had a significant impact on metabolism of disogenin by *C. echinulata*. This is the first report of the 11-oxo derivative of diosgenin (**1**) by using fungal resting cell culture. Moreover, the low yields of the isolated metabolites may be due to low solubility of the substrate and by inhibition effects [5]. Therefore, it is expected that more suitable fermentation conditions of the regioselective hydroxylation of the transformed products by microbial means will be optimized in terms of yields.

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References

[1] G.T. Maatooq, A.M. Marzouk, A.I. Gray, J.P. Rosazza, Phytochemistry 71 (2010) 262–270.
[2] T. Matsuda, R. Yamanaka, K. Nakamura, Tetrahedron: Asymmetry 20 (2009) 513–557.
[3] P. Fernandes, A. Cruz, B. Angelova, H.M. Pinheiro, J.M.S. Cabral, Enzyme Microb. Technol. 32 (2003) 688–705.
[4] M.I. Choudhary, N.T. Khan, S.G. Musharraf, S. Anjum, A. Rahman, Steroids 72 (2007) 923–929.
[5] S.B. Mahato, S. Garai, Steroids 62 (1997) 332–345.
[6] W.Y. Tong, X. Dong, Recent Pat. Biotechnol. 3 (2009) 141–153.
[7] T. Ohnishi, T. Yokota, M. Mizutani, Phytochemistry 70 (2009) 1918–1929.
[8] L.R. Lehman, J.D. Stewart, Curr. Org. Chem. 5 (2001) 439–470.
[9] D.E. Torres Pazmiño, M. Winkler, A. Glieder, M.W. Fraaije, J. Biotechnol. 146 (2010) 9–24.
[10] A.M. Clark, C.D. Hufford, Med. Res. Rev. 11 (1991) 473–501.
[11] S. Asha, M. Vidyavathi, Biotechnol. Adv. 27 (2009) 16–29.
[12] I.S. Son, J.H. Kim, H.Y. Sohn, K.H. Son, J.S. Kim, C.S. Kwon, Biosci. Biotechnol. Biochem. 71 (2007) 3063–3071.
[13] M. Kondeva-Burdina, U.L. Merce, M. Mitcheva, S. Nikolov, Toxicol. Lett. 172 (2007) S68.
[14] Y. Tada, N. Kanda, A. Haratake, M. Tobiishi, H. Uchiwa, S. Watanabe, Steroids 74 (2009) 504–511.
[15] G.H. Gong, Y. Qin, W. Huang, S. Zhou, X.H. Wu, X.H. Yang, Chem. Biol. Interact. 184 (2010) 366–375.
[16] M.J. Liu, Z. Wang, Y. Ju, R.N.-S. Wong, Q.Y. Wu, Cancer Chemother. Pharmacol. 55 (2005) 79–90.
[17] F. Li, P.P. Fernandez, P. Rajendran, K.M. Hui, G. Sethi, Cancer Lett. 292 (2010) 197–207.
[18] J. Bertrand, B. Liagre, G. Bégaud-Grimaud, M.O. Jauberteau, P. Cardot, J.L. Beneytout, S. Battu, J. Chromatogr. B 869 (2008) 75–83.
[19] J. Raju, R.P. Bird, Cancer Lett. 255 (2007) 194–204.
[20] J.S. Lee, K. Jung, Y.S. Kim, D. Park, Life Sci. 81 (2007) 249–254.
[21] C.T. Chiang, T.D. Way, S.J. Tsai, J.K. Lin, FEBS Lett. 581 (2007) 5735–5742.
[22] J. Raju, R. Mehta, Nutr. Cancer 61 (2009) 27–35.
[23] K. Kaneko, H. Mitsuhashi, K. Hirayama, Chem. Pharm. Bull. 17 (1969) 2031–2035.

[24] S. Hayakawa, Y. Sato, J. Org. Chem. 28 (1963) 2742–2743.
[25] G. Blunden, A.V. Patel, T.A. Crabb, Phytochemistry 29 (1990) 1771–1780.
[26] R. Saunders, P.S.J. Cheethamt, R. Hardman, Enzyme Microb. Technol. 9 (1986) 549–555.
[27] F.Q. Wang, C.G. Zhang, B. Li, W. Wang, D.Z. Wei, W.Y. Tong, Environ. Sci. Technol. 43 (2009) 5967–5974.
[28] Y. Zhao, L.M. Sun, X.N. Wang, T. Shen, M. Ji, X. Li, H.X. Lou, Chin. Chem. Lett. 21 (2010) 76–80.
[29] G.W. Wu, X.J. Li, Y. Gao, Y. Jia, J.M. Gao, Chin. Chem. Lett. 21 (2010) 446–448.
[30] N.W. Roehm, G.H. Rodgers, S.M. Hatfield, A.L. Glasebrook, J. Immunol. Methods 142 (1991) 257–265.