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Microbial Transformations of Diosgenin by the White-Rot Basidiomycete *Coriolus versicolor*

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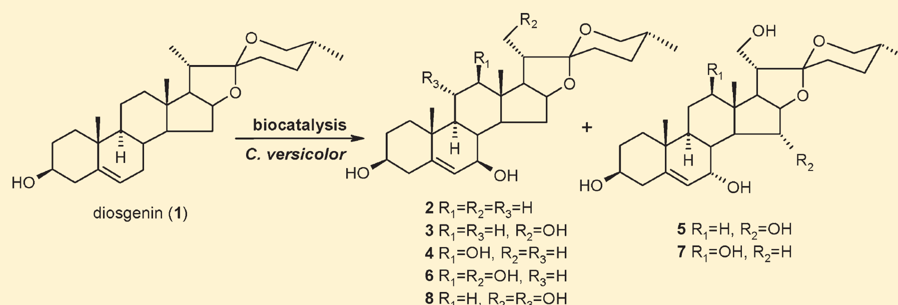
Supporting Information

ABSTRACT:



Microbial transformation of diosgenin (3β-hydroxy-5-spirostene) using white-rot fungus *Coriolus versicolor* afforded four previously unreported polyhydroxylated steroids, 25(*R*)-spirost-5-en-3β,7α,15α,21-tetraol (**5**), 25(*R*)-spirost-5-en-3β,7β,12β,21-tetrol (**6**), (25*R*)-spirost-5-en-3β,7α,12β,21-tetraol (**7**), and (25*R*)-spirost-5-en-3β,7β,11α,21-tetraol (**8**), along with three known congeners, 25(*R*)-spirost-5-en-3β,7β-diol (**2**), 25(*R*)-spirost-5-en-3β,7β,21-triol (**3**), and 25(*R*)-spirost-5-en-3β,7β,12β-triol (**4**). These structures were elucidated by 1D and 2D NMR as well as HR-ESIMS analysis. In addition, we provide evidence for two new microbial hydroxylations of diosgenin: C-21 primary carbon hydroxylation and C-15 hydroxylation. The 3β-hydroxyl group and double bond in the B-ring of diosgenin were found to be important structural determinants for their activity.

Since the research and development of steroid drugs in the 1950s, much importance has been attached to key intermediates produced by microbial biotransformations, including hydroxylation, dehydrogenation/reduction, esterification, methylation, and methoxylation, effected by either isolated enzymes or whole cells.[1−6] Compared to chemical synthesis, microbial transformations are generally carried out under mild conditions, following the principles of green chemistry, and have the advantage of high regio- and stereoselectivity.[5] In addition, microbial metabolic studies have been successfully used as model systems in predicting metabolic pathways in humans, as well as enhancing the efficacy of drugs by metabolic activation. Therefore, microorganisms have served as *in vitro* models for predicting mammalian drug metabolism.[7]

Diosgenin (**1**), namely, 25(*R*)-spirost-5-en-3β-ol, is a commercially available material used for producing steroid drugs and hormones since the last century. In recent years, a wide array of new biological activities of diosgenin have been disclosed, such as decreasing the risk of developing dementia in opiate abusers with HIV infection, anticancer and vasodilator activities, as a depigmenting agent, and antiaging of skin in mice.[8−14] These studies demonstrate the importance of diosgenin and its derivatives as potential lead compounds for the treatment of some diseases.

Diosgenin was previously subjected to several structural modification studies to secure new derivatives via microbial transformation.[15−18] Specifically, the hydroxylation reactions of diosgenin by several fungi and bacteria, such as *Cunninghamella blakesleeana*, *C. elegans*, *Helicostylum piriforme*, *Streptomyces virginiae*, and *Syncephalastrum racemosum*, were shown to occur at the C-7, C-9, C-11, C-12, and C-25 positions.[15−18] In addition, two side-chain cleavage intermediates of diosgenin were produced by *C. elegans* and *Aspergillus nidulans*.[19,20]

In an attempt to obtain new pharmacologically active derivatives of diosgenin, we carried out the biotransformation of diosgenin using the white-rot fungus *Coriolus versicolor*, selected after screening 14 fungi, which led to the isolation of seven metabolites, **2**−**8**. The structures were established by spectroscopic analysis. Furthermore, effects of some modification of rings A and B of diosgenin on the biotransformations were examined using diosgenin derivatives including diosgenone (**1a**), 3-acetyldiosgenin (**2a**), and tigogenin (5,6-dihydrodiosgenin, **2b**). Herein, we describe the isolation, structure elucidation, and preliminary antiglioma and NF-κB inhibition activities of biotransformed metabolites

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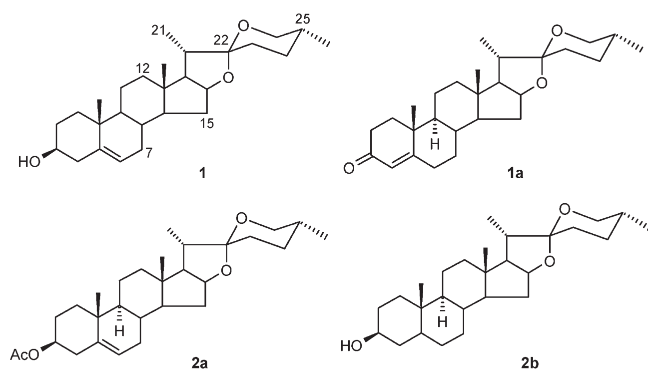
of diosgenin by *C. versicolor* and determinants for its biotransformation.



RESULTS AND DISCUSSION

An EtOAc extract (ca. 9.0 g), recovered from cultures of *C. versicolor* incubated with diosgenin, was subjected to column chromatography on silica gel, RP-18, and Sephadex L-20. This process afforded seven polyoxygenated compounds, **2** (13 mg), **3** (11 mg), **4** (10 mg), **5** (25 mg), **6** (3 mg), **7** (15 mg), and **8** (8 mg), four of which were new (**5**, **6**, **7**, and **8**). The structures (Figure 1) of these biotransformation products were characterized by IR, HR-ESIMS, and 1D and 2D NMR techniques and comparison of the data with literature values. Compound **2** was previously identified as 25(*R*)-spirost-5-en-3*β*,7*β*-diol (**2**) as a metabolite of **1** transformed by *Cunninghamella blakesleeana*[17] and *C. echinulata*.[4] Compound **3** was characterized as 25(*R*)-spirost-5-ene-3*β*,7*β*,21-triol.[6]

Metabolite **4** has a molecular formula of $C_{27}H_{42}O_5$ as deduced from its HR-ESIMS, suggesting a dihydroxylated derivative of **1**. Compared to **2**, the appearance of a new oxygenated methine carbon signal at C-12 (δ 80.1) was observed in addition to the allylic hydroxylated carbon C-7*β* (δ 73.5). Moreover, the large upfield shift ($\Delta\delta$ −5.4 ppm) of the angular C-18 methyl carbon (δ 16.4 to 11.0) suggested a *γ*-effect arising from 12*β*-hydroxyl substitution. The corresponding 12*α*-hydroxyl substitution produced a downfield effect ($\Delta\delta$ +1.1 ppm). All evidence suggested the presence of 12-OH with a *β*-configuration. It was thus assigned as 25(*R*)-spirost-5-en-3*β*,7*β*,12*β*-triol (Figure 1). This compound was obtained before as a diosgenin metabolite of *C. elegans*,[15] *C. blakesleeana*,[17] and *C. echinulata*.[4]

Metabolite **5** has a molecular formula of $C_{27}H_{42}O_6$ as determined by its HR-ESIMS as well as by its ^{13}C NMR spectrum, indicating a structure containing three more oxygen atoms than **1**. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) of **5** revealed that three hydroxyl groups were incorporated into **1**. Compared to compound **3**, the presence of the hydroxyl group at C-21 in **5** was observed from one methylene group (δ 62.9) in the ^{13}C NMR spectrum and proton signals at δ 3.66 and 3.51 in the ^{1}H NMR spectrum, which was further supported by the HMBC and COSY spectra (Figure 2). Furthermore, hydroxylation was disclosed by the appearance of two new methine carbinol protons at δ 4.15 and 4.02, which were correlated by the HSQC spectrum to the methine carbons C-7 and C-15 (δ 66.0 and 79.1), respectively. In the ^{1}H−^{1}H COSY spectrum, a cross-peak observed between the olefinic proton H-6 and methine proton H-7 showed that a hydroxyl group was introduced at C-7. In the HMBC spectrum, only a correlation of C-7 (δ 66.0) with H-6

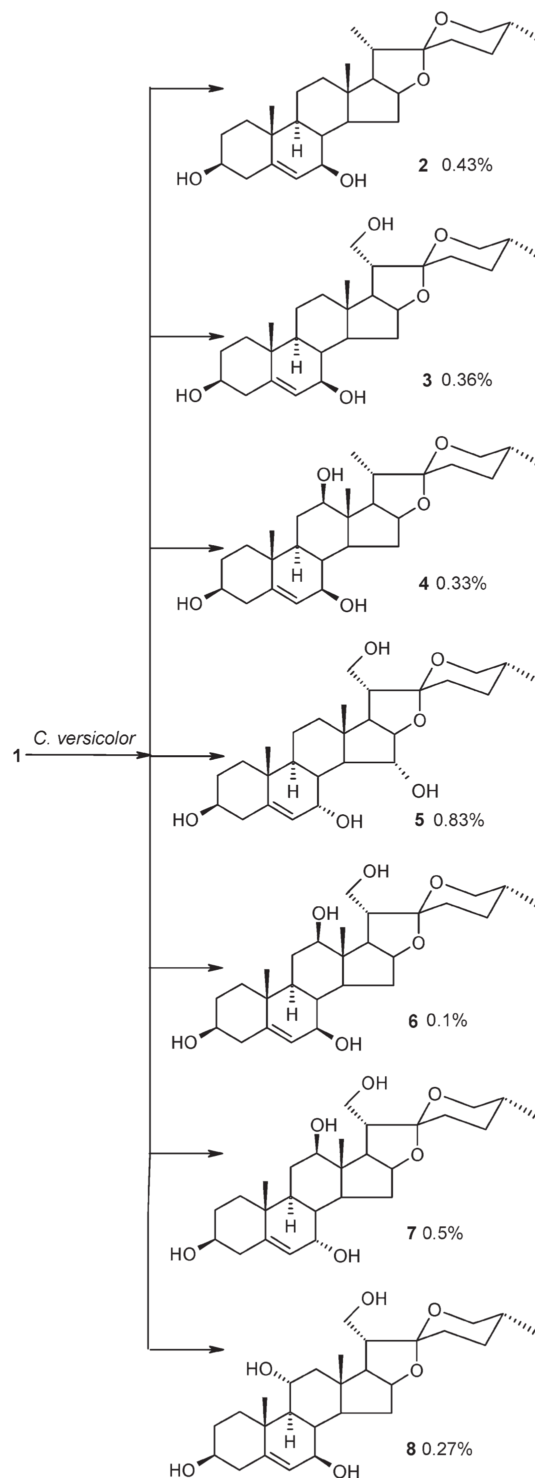


Figure 1. Biotransformations of diosgenin (**1**) by *Coriolus versicolor*.

(δ 5.51) provided the placement. On the other hand, the *α*-configuration of 7-OH was established by comparison of the C-7 chemical shift in the ^{13}C NMR spectrum of **5** with those reported for sterols with a 5-ene-3*β*,7-diol substructure (7*α*-OH, δ 65.3; 7*β*-OH, δ 73.3)[21] and 7*β*-oriented compounds **2**−**4**. This was further supported by the NOE effects of H-7 (δ 4.15) with H-8*β* (δ 1.85) in the NOESY spectrum as well as by comparing the singlet of H-7 in **5** with the doublet of H-7 in the

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Table 1. ^{13}C (125 MHz) NMR Data for Diosgenin (1) and Its Metabolites 2−8 (CD_3OD)[a]

	1	2	3	4	5	6	7	8
position	δ_C, mult.	δ_C, mult.	δ_C, mult.	δ_C, mult.	δ_C, mult.	δ_C, mult.	δ_C, mult.	δ_C, mult.
1	38.5 t	38.2 t	37.7 t	38.2 t	38.2 t	38.2 t	38.1 t	40.2 t
2	32.4 t	32.4 t	33.8 t	32.5 t	33.8 t	33.2 t	33.1 t	32.5 t
3	72.4 d	72.1 d	72.1 d	72.1 d	71.9 d	72.1 d	71.9 d	72.4 d
4	42.9 t	42.6 t	42.6 t	42.5 t	42.9 t	42.5 t	42.8 t	43.2 t
5	142.3 s	144.1 s	144.1 s	144.1 s	147.2 s	144.0 s	146.6 s	144.2 s
6	122.2 d	127.3 d	127.3 d	127.4 d	123.7 d	127.4 d	124.9 d	127.6 d
7	33.1 t	73.7 d	73.7 d	73.5 d	66.0 d	73.4 d	65.6 d	73.4 d
8	32.8 d	41.0 d	41.2 d	40.1 d	38.9 d	40.2 d	37.8 d	41.2 d
9	51.6 d	49.8 d	49.8 d	49.3 d	44.4 d	49.3 d	43.0 d	55.5 d
10	37.8 s	37.7 s	38.2 s	37.8 s	38.2 s	37.8 s	38.6 s	39.5 s
11	22.0 t	22.1 t	22.0 t	31.5 t	21.7 t	29.8 t	29.6 t	69.3 d
12	40.9 t	40.8 t	40.3 t	80.1 d	40.7 t	80.3 d	80.3 d	51.2 d
13	41.4 s	41.9 s	42.0 s	47.3 s	41.8 s	47.3 s	46.6 s	42.5 s
14	57.8 d	57.1 d	57.3 d	55.8 d	56.0 d	55.7 d	49.5 d	56.5 d
15	32.7 t	35.1 t	35.3 t	34.7 t	79.1 d	34.7 t	32.1 t	35.5 t
16	82.2 d	82.6 d	83.2 d	82.6 d	91.9 d	83.4 d	83.1 d	83.5 d
17	63.8 d	63.3 d	59.8 d	62.7 d	58.5 d	60.6 d	61.0 d	59.7 d
18	16.7 q	16.8 q	16.6 q	11.0 q	17.5 q	11.0 q	11.0 q	17.5 q
19	19.8 q	19.5 q	19.5 q	19.4 q	18.8 q	19.4 q	18.6 q	19.1 q
20	43.0 d	43.0 d	51.0 d	43.8 d	51.1 d	51.0 d	51.0 d	51.1 d
21	14.9 q	14.9 q	63.1 t	14.0 q	62.9 t	64.2 t	64.2 t	63.1 t
22	110.6 s	110.6 s	110.2 s	110.8 s	109.7 s	110.2 s	110.2 s	110.1 s
23	32.3 t	32.3 t	32.3 t	32.3 t	32.1 t	32.2 t	32.1 t	33.8 t
24	29.9 t	29.9 t	29.8 t	30.0 t	29.9 t	29.8 t	29.8 t	29.9 t
25	31.4 d	31.5 d	31.3 d	31.5 d	31.3 d	31.4 d	31.3 d	31.4 d
26	67.9 t	67.9 t	67.8 t	67.9 t	67.9 t	67.7 t	67.7 t	67.9 t
27	17.5 q	17.5 q	17.5 q	17.4 q	17.5 q	17.4 q	17.4 q	17.6 q

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

NMR spectra of **2**−**4**. In addition, in comparison to **3** (Table 1), the chemical shift of the methine carbon C-16 was shifted downfield by 8.7 ppm, suggesting hydroxylation of carbon C-15 (δ 79.1), as deduced from the COSY correlations between H-14 (δ 1.73) and H-15 (δ 4.02), and H-15 (δ 4.02) and H-16 (δ 4.23) (Figure 2). The configuration of the hydroxyl group at C-15 was shown to be α-oriented from the NOESY correlations between H-15 (δ 4.02) and H-7 (δ 4.15) and H-8 (δ 1.85) (Figure 2). Thus, the structure of **5** was determined to be (25*R*)-spirost-5-ene-3β,7α,15α,21-tetraol. It is worth noting that 15α-hydroxylation was previously not reported in the microbiological transformation of C_{27} steroids.[15]

Metabolite **6** has a molecular formula of $C_{27}H_{42}O_6$ as determined from its HR-ESIMS as well as from its ^{13}C NMR spectrum (Table 1), indicating a metabolite containing three more oxygen atoms than **1**. Comparison of ^{13}C NMR data of **6** with that of **4** and **5** suggested that **6** was a monohydroxylation derivative of **4**. It had a hydroxyl group substitution at the C-21 primary carbon atom (δ 64.2), as evidenced by COSY correlations between H-21 [δ 3.68 (1H) and 3.61 (1H)] and H-20 (δ 2.10) (Figure 2) and by HMBC correlations of H-21 (δ 3.68 and 3.61) with C-17 (δ 60.6), C-20 (δ 51.0), and C-22 (δ 110.2) (Figure 2). The location and β-configuration of the hydroxyl group at C-7 (δ 73.4) were established by the HMBC and NOESY spectra (Figure 2). The HMBC correlations between C-12 (δ 80.3) and H-11 (δ 1.66) and H-18 (δ 0.82) indicated that the remaining hydroxyl group was located at C-12. In addition, the NOE enhancements of H-12 (δ 3.41) with H-9 (δ 1.22) and H-14 (δ 1.29) on the α-side confirmed the β-configuration for 12-OH. The structure of **6** was thus established as 25(*R*)-spirost-5-ene-3β,7β,12β,21-tetraol (Figure 1).

Metabolite **7** has a molecular formula of $C_{27}H_{42}O_6$ as determined from its positive ion HR-ESIMS as well as from its ^{13}C NMR spectrum, indicating that **7** has the same molecular formula as **6**. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **7** were similar to those of **6**. The only difference was that the chemical shift of C-7 was δ 65.6 in **7** instead of δ 73.4 in **6**, suggesting that **7** is an epimer at C-7 of **6**. The location of the hydroxyl group was further determined by the HMBC data, which showed correlations of H-7 (δ 3.81) with C-5 (δ 146.6), C-6 (δ 124.9), and C-9 (δ 43.0), indicating the presence of a hydroxyl group at C-7 in **7**. The α-configuration of the 7-OH was deduced from the cross-peaks of H-7 with H-8 (δ 1.61, m) in the NOESY experiment (Figure 2). Assignment of the NMR data for **4** was achieved by COSY, HSQC, HMBC, and NOESY experiments. Thus, it was established as (25*R*)-spirost-5-en-3β,7α,12β,21-tetraol (Figure 1).

Metabolite **8** had a molecular formula of $C_{27}H_{42}O_6$ as determined from its positive ion ESIMS as well as from its ^{13}C NMR spectrum, indicating **8** contained one additional hydroxyl

Table 2. ^{1}H (500 MHz) NMR Data for Metabolites 5−8 (CD_3OD)[a]

	5	6	7	8
position	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1	1.86, m	1.83, m	1.85, m	2.64, dt (3.4, 3.1, 13.7)
	1.12, m	1.07, m	1.17, m	1.10, m
2	1.85, m	1.80, m	1.79, m	1.75, m
	1.66, m	1.67, m	1.68, m	1.56, m
3	3.45, m	3.41, m	3.48, m	3.43, m
4	2.32, m	2.27, m	2.27, m	2.25, m
	2.26, m			
6	5.51, br d (1.8)	5.26, br s	5.60, dd (5.2, 1.4)	5.30, br s
7	4.15, br d (8.0)	3.71, m	3.81, br s	3.73, br d (8.5)
8	1.85, m	1.63, m	1.61, m	1.54, m
9	1.27, m	1.22, m	1.46, m	1.10, m
11	1.58, m	1.66, m	1.65, m	3.96, ddd (5.2, 10.7, 10.7)
	1.46, m		1.42, m	
12	1.71, m	3.41, m	3.42, dd (11.0, 4.7)	1.18, m
	1.20, m			2.05, m
14	1.73, m	1.29, m	1.61, m	1.36, m
15	4.02, dd (3.0, 11.0)	2.36, m	2.21, m	2.38, m
		1.68, m	1.52, m	1.60, m
16	4.23, dd (3.0, 8.0)	4.53, m	4.55, dd (14.0, 7.7)	4.60, m
17	2.11, m	2.11, m	2.15, m	1.88, m
18	0.85, s	0.82, s	0.82, s	0.84, s
19	1.01, s	1.09, s	1.03, s	1.20, s
20	2.07, m	2.10, m	2.12, m	2.05, m
21	3.66, dd (11.0, 6.0)	3.68, overlapped	3.70, dd (10.0, 4.0)	3.68, dd (11.0, 6.5)
	3.51, dd (11.0, 7.0)	3.61, overlapped	3.64, t (10.1)	3.52, dd (11.0, 7.5)
23	1.80, m	1.64, m	1.81, m	1.87, m
	1.49, m	1.51, m	1.42, m	1.65, m
24	1.61, m	1.61, m	1.64, m	1.60, m
	1.45, m	1.41, m	1.40, m	1.43, m
25	1.65, m	1.65, m	1.59, m	1.67, m
26	3.45, m	3.43, m	3.40, m	3.43, m
	3.38, m	3.30, m	3.31, m	3.31, m
27	0.78, d (6.0)	0.77, d (6.0)	0.78, d (6.4)	0.78, d (6.5)

[a] Assignments were done by ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, and NOESY experiments.

group compared with **3**. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **8** were similar to those of **3**. A noticeable difference was that a methylene group (δ 22.0) at C-11 of **3** was oxidized to a hydroxyl methine (δ 69.3) in **8**. The position of the hydroxyl group was determined by the HMBC correlation of H-11 (δ 3.96) with C-10 (δ 39.5), indicating the presence of a hydroxyl group at C-11 in **8**. The α-orientation of this alcohol was verified by the cross-peaks of H-11β (δ 3.96) with Hβ-18 (δ 0.84) in the NOESY spectrum (Figure 2). Assignment of the NMR data for **4** was achieved by COSY, HSQC, HMBC, and NOESY experiments. On the basis of the above evidence, **8** was established as (25*R*)-spirost-5-en-3β,7β,11α,21-tetraol (Figure 1).

To explore the effects of modifications of rings A and B of diosgenin on the biotransformation, incubations of the four diosgenin analogues, diosgenone (**1a**), 3β-acetyldiosgenin (**2a**), 7β-hydroxyldiosgenin (**2**), and tigogenin (**2b**), were separately performed with *C. versicolor* under the same conditions as for diosgenin. However, no biotransformed products were detected by HPLC. Evidently, rings A, B, C, and D and the C-21 position of these substrates could not be attacked by the *C. versicolor* enzymes. The subtle structural differences of these compounds could explain their nonreactivity. Tigogenin has no double bond, and diosgenone (**1a**) includes a 3-ketone group instead of a 3-hydroxyl group and a double bond at C-4, while 3β-acetyldiosgenin (**2a**) contains a 3-acetyl group instead of a hydroxyl group. We postulate that the free 3β-hydroxyl group of the A-ring and a double bond at C-5 of the B-ring play a crucial role in diosgenin metabolism by *C. versicolor* and that they are required to act as substrate. It was previously shown that selective modifications occurring at C-7β, C-7α, C-12β, C-15α, and C-21 positions likely contribute to substrate conformation changes.[18,22]

C. versicolor is able to degrade lignin and phenolic compounds by an extracellular fungal oxygenase system called lacasses. Numerous cytochrome P450 monooxygenases (P450s) have been known to play a key role in the biosynthesis and metabolism of steroids by fungi and plants due to their ability to catalyze regio- and stereospecific hydroxylation reactions.[23] In the present study, to clarify if the hydroxylations were mediated by

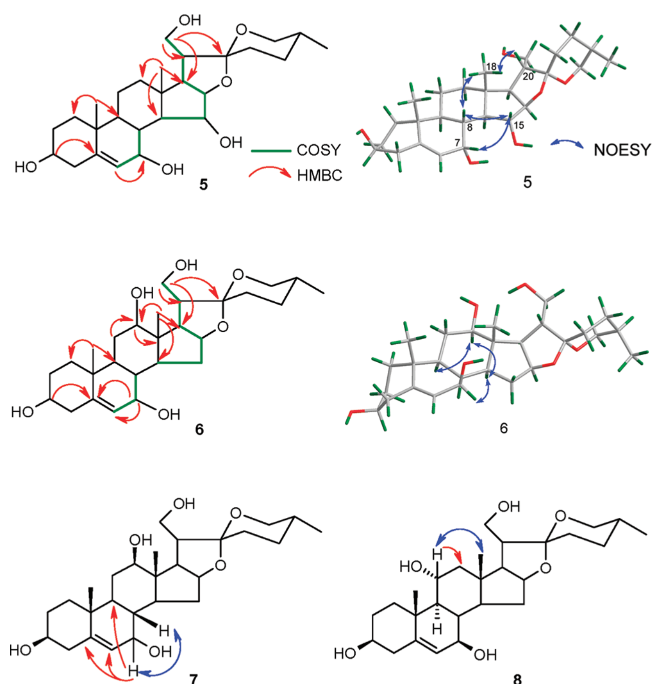

Figure 2. Key COSY, HMBC, and NOESY correlations of metabolites **5**−**8**.

P450-type enzymes, we conducted inhibitor experiments. Inhibitors of hydroxylase (P450), 2,2′-bipyridyl and 8-hydroxyquinoline (0.3−0.6 mM), were added to separate cultures at 3 h before addition of **1**. No hydroxylation products were detected, suggesting the involvement of P450-catalyzed oxygenations in the biotransformation of diosgenin by *C. versicolor*.

Interestingly, the allylic hydroxylation observed in the transformation of diosgenin with *C. versicolor* suggests that metabolite **2** could be an intermediate in the biotransformation of **1** to **8**. However, when metabolite **2** was added to the culture, no transformation products were detected. To the best of our knowledge, this is the first report on this type of spirostanol compounds with a hydroxyl group at C-21.

The *in vitro* cytotoxic activities of diosgenin (**1**) and its five biotransformed metabolites (**2**−**6**) and of compounds **1a** and **2a** were tested against the U87 glioma cell line by using the XTT method.[24] Compound **3** (100 μM) showed very weak inhibition (28.2%), but the remaining compounds were inactive. The compounds were inactive against the human tumor necrosis factor-induced activation of NF-κB (nuclear factor-kappaB).[25]

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured on a X-4 micromelting point apparatus and are uncorrected. Optical rotations were measured with a Perkin Elemer model 314. IR spectra was determined using a Bruker Tensor 27 spectrometer with KBr pellets. 1D and 2D NMR spectra (^{1}H NMR, ^{13}C NMR, DEPT, HSQC, HMBC, COSY, NOESY) were recorded using a Bruker Avance-500 or an AvanceIII-500 instrument with tetramethylsilane as internal standard in CD_3OD. ESIMS and HR-ESIMS were obtained on a Esquire6000 and an API QSTAR Pulsar 1 spectrometer.

HPLC was performed on a Shimadzu LC-6AD with a diode array detector (Shimadzu, Japan) using an analytical Hypersil ODS2 C_{18} column (4.6 × 250 mm, 5 μm; Dalian Elite Analytical Instruments Co., Ltd.). The HPLC column was isocratically eluted with MeOH−H_2O (78:22) in 15 min at a flow rate of 1.0 mL·min^{-1}, at λ 210 and 230 nm. Preparative HPLC was carried out on a Waters 600E (Waters, USA) with a UV200 detector (Dalian Elite Analytical Instruments Co., Ltd., China) and a Hypersil ODS2 C_{18} column (20 × 250 mm, 5 μm, Dalian Elite Analytical Instruments Co., Ltd.). The preparative HPLC column was isocratically eluted with MeOH−H_2O (78:22) in 15 min with a flow rate 7.0 mL·min^{-1} and with UV detection at 210 nm.

Column chromatography was performed on silica gel (200−300 mesh, Qingdao Marine Co., Ltd., China), RP-18 (40−63 μM; Merck, Darmstadt, Germany), and Sephadex LH-20 (20−100 μm; Amersham Biosciences), respectively. Thin-layer chromatography (TLC) was carried out on silica gel GF_{254} precoated plates (0.20−0.25 mm; Qingdao Marine Co., Ltd.). Fractions were monitored by TLC, and spots were visualized by spraying the plate with 5% H_2SO_4 solution in EtOH followed by heating at 110 °C until the colors appeared. All reagents and solvents were of analytical grade.

The substrates diosgenin and tigogenin were donated by National Engineering Research Center for Phytochemistry in West China. Other substrates, diosgenone (**1a**), 25(*R*)-spirost-5-en-3β,7β-diol (**2**), and 3β-acetyldiosgenin (**2a**), were prepared by our group.

Microorganism and Culture Medium. The microbes used in this study were *Sphaceloma* de Bary LN14 (an endophytic fungus) isolated from *Melia azedarach* L., *Thelephora japonica*, *Coriolus versicolor*, *Polyporus picipes*, *Cunninghamella blakesleeana*, *Cunninghamella elegans*, *Cunninghamella echinulata*, *Absidia orchidis*, *Apergillus ocharceus*, *Penicillium citrinum*, *Rhizopus chinensis*, *Mucor mucedo*, and *Mucor wutungchiao*. These organisms were maintained on potato-dextrose agar (PDA) medium slants and stored at 4 °C.

All of the microorganisms were freshly subcultured prior to the biotransformation experiment. All preliminary screening experiments were performed by a two-stage fermentation procedure in a medium consisting of distilled water (1000 mL), potato (200 g), glucose (20 g), KH_2PO_4 (3 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), and vitamin B_1 (10 mg). The media were autoclaved at 121 °C for 30 min before use. These media were also supplemented with 2,2′-bipyridyl and 8-hydroxyquinoline as an ethanolic solution (20 mM) to prevent oxidation of the steroid nucleus.[20]

Culture Conditions and Biotransformation Procedures. Microorganisms were inoculated in Erlenemeyer flasks (250 mL) containing 60 mL of the above fermentation medium. The flasks were incubated at 26 °C on a rotary shaker at 80 rpm for 48 h. The substrate diosgenin (3.0 g) dissolved in acetone (5 mg/mL) was added to each flask. Final concentration of the substrate in this medium was 0.1 mg/mL, and incubation was continued for 5 days. Parallel controls were conducted as follows: an incubation of the fungus under identical conditions but without substrate and an incubation of substrate without the fungus under the same conditions. The results of the biotransformation were analyzed periodically by HPLC.

Biotransformation was performed in 500 mL Erlenemeyer flasks, each containing 200 mL of this fermentation PDA liquid medium. All the operations were the same as the procedures described above.

Extraction and Isolation of Biotransformation Products. At the end of fermentation, the mycelium was filtered off and washed in EtOAc. The filtrate was ultrasonically extracted three times with an equal volume of the same solvent. The organic phase was combined and dried over anhydrous Na_2SO_4 followed by filtration. The EtOAc was evaporated under reduced pressure to provide the crude extract.

The EtOAc extract (ca. 9.0 g) was fractionated by column chromatography on silica gel and eluted with a gradient of $CHCl_3$−MeOH (100:0 to 0:100) to provide fractions A−K. The remaining diosgenin was recycled in about 50% yield from fractions A−C. Fraction D was subjected to column chromatography on silica gel and eluted with $CHCl_3$−acetone (85:15) to yield subfraction D2. Subfraction D2 was rechromatographed on a RP-18 (MeOH−H_2O, 70:30), followed by

purification with a Sephadex LH-20 column ($CHCl_3$−MeOH, 1:1), to give compounds **2** (13 mg) and **3** (11 mg).

Fraction G was separated by column chromatography over silica gel and eluted with $CHCl_3$−acetone (80:20) to obtain subfraction G4, which was separated by column chromatography over silica gel eluting with $CHCl_3$−MeOH (97:3) and further purified by a Sephadex LH-20 column (MeOH) to furnish compound **4** (10 mg). Fraction H was subjected to a Sephadex LH-20 column, eluting with MeOH to give subfraction H3. Subfraction H3 was applied to silica gel column chromatography using a mixture of $CHCl_3$−acetone (70:30) to yield crude product **5**, which was purified by column chromatography over silica gel, eluted by $CHCl_3$−MeOH (97:3), yielding pure product **5** (25 mg). Fraction I was purified by a preparative HPLC column isocratically eluted with MeOH−H_2O (78:22) at a retention time of 6.52 min to afford compound **6** (3 mg).

Fraction J was chromatographed by a Sephadex LH-20 column (MeOH) to obtain three subfractions, J1−J3. Subfraction J3 was subjected to a Sephadex LH-20 column ($CHCl_3$−MeOH, 1:1) and then purified by a RP-18 column (MeOH−H_2O, 40:60 to 55:45), further followed by recrystallization, to afford **7** (15 mg). Fraction K was chromatographed by a Sephadex LH-20 column (MeOH) to obtain three subfractions, K1−K3. The subfraction K2 was separated by a Sephadex LH-20 column ($CHCl_3$−MeOH, 1:1) and by a RP-18 column (MeOH−H_2O, 40:60 to 55:45) and furher purified by silica gel column chromatography ($CHCl_3$−MeOH, 10:1) to yield **8** (8 mg).

*25(R)-Spirost-5-en-3β,7β-diol (=7β-hydroxyldiosgenin, **2**):* colorless needle crystal (MeOH); mp 215−216 °C, lit. mp 216−219 °C;[17] $[\alpha]^{20}_{D}$ −55 (*c* 0.45, MeOH), lit. $[\alpha]^{15}_{D}$ −75 (*c* 1.0, $CHCl_3$);[17] IR (KBr) ν_{max} 3422, 1638, 1054, 982, 922, 900, 865 cm^{-1}; ^{13}C NMR data see Table 1; ESIMS (positive ion mode) *m/z* (rel int) 431.3 $[M + H]^+$, 413.3 $[M + H - H_2O]^+$, 395.3 $[M + H - 2H_2O]^+$. All NMR and IR data of **2** were identical to the literature data.[4]

*25(R)-Spirost-5-en-3β,7β,21-triol (**3**):* colorless needle crystal (MeOH); mp 226−227 °C; $[\alpha]^{20}_{D}$ −43 (*c* 0.2, MeOH); IR (KBr) ν_{max} 3422, 1618, 981, 920, 900 cm^{-1}; ^{13}C NMR data see Table 1; ESIMS (positive ion mode) *m/z* 469.2 $[M + Na]^+$ HR-ESIMS (positive ion mode) *m/z* 447.3112 $[M + H]^+$ (calcd 447.3105 for $C_{27}H_{43}O_5$). All NMR data of **3** were in accordance with the literature data.[6]

*25(R)-Spirost-5-en-3β,7β,12β-triol (**4**):* colorless needle crystal (MeOH); mp 183−185 °C, lit; mp 182−184 °C;[15] $[\alpha]^{20}_{D}$ +19 (*c* 0.49, MeOH); IR (KBr) ν_{max} 3415, 1632, 1052, 981, 920, 898 cm^{-1}; ^{13}C NMR data see Table 1; HR-ESIMS (positive ion mode) *m/z* 447.3109 $[M + H]^+$ (calcd 447.3105 for $C_{27}H_{43}O_5$). All NMR data of **4** were in accordance with the literature data.[4,15]

*25(R)-Spirost-5-en-3β,7β,15α,21-tetraol (**5**):* colorless needle crystal (MeOH); mp 186−187 °C; $[\alpha]^{20}_{D}$−117 (*c* 0.21, MeOH); IR (KBr) ν_{max} 3422, 1636, 1054, 980, 922, 900, 865 cm^{-1}; ^{13}C and ^1H NMR data see Tables 1 and 2; ESIMS (positive ion mode) *m/z* (rel int) 485.2 $[M + Na]^+$, 462.3 $[M]^+$, 445.2 $[M - OH]^+$, 427.2 $[M - H_2O]^+$; HR-ESIMS (positive ion mode) *m/z* 485.2872 $[M + Na]^+$ (calcd 485.2879 for $C_{27}H_{42}O_6Na$).

*(25R)-Spirost-5-en-3β,7β,12β,21-tetraol (**6**):* colorless needle crystal (MeOH); $[\alpha]^{19}_{D}$−23 (*c* 0.15, MeOH); ^{13}C and ^1H NMR data see Table 1 and Table 2; ESIMS *m/z* 485 $[M + Na]^+$, 948 $[2M + Na + H]^+$; HR-ESIMS (positive ion mode) *m/z* 485.2877 $[M + Na]^+$ (calcd 485.2879 for $C_{27}H_{42}O_6Na$).

*(25R)-Spirost-5-en-3β,7α,12β,21-tetraol (**7**):* colorless needle crystal (MeOH); mp 249−250 °C; $[\alpha]^{16}_{D}$ −50.2 (*c* 0.08, MeOH); IR(KBr) ν_{max} 3402, 1630, 978, 922, 902 cm^{-1}; ^{13}C and ^1H NMR data see Table 1 and Table 2; ESIMS *m/z* 485.2 $[M + Na]^+$; HR-TOF-ESIMS (negative ion mode) *m/z* 497.2656 $[M + Cl]^-$ (calcd 497.2669 for $C_{27}H_{42}O_6Cl$).

*(25R)-Spirost-5-en-3β,7β,11α,21-tetraol (**8**):* Ccolorless needle crystal (MeOH); mp 250−252 °C; $[\alpha]^{16}_{D}$ −15.3 (*c* 0.15, MeOH); IR(KBr) ν_{max} 3386, 1644, 995, 960, 899 cm^{-1}; ^{13}C and ^1H NMR data see Table 1 and Table 2; ESIMS *m/z* 463.4 $[M + H]^+$; HR-TOF-ESIMS (negative ion mode) *m/z* 497.2656 $[M + Cl]^-$ (calcd 497.2665 for $C_{27}H_{42}O_6Cl$).

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 a 5% CO_2 humidified atmosphere. The cytotoxicity assay was performed according to the XTT method. Briefly, U87 cells were seeded onto 96-well microtiter plates at a density of 1000/well and treated with the tested compounds for 48 h, following which 10 μL of XTT was added. The plates were incubated for another 4 h at 37 °C. After treatment, cell viability was determined by the XTT colorimetric assay according to the manufacturer's protocol.[24]

NF-κB Luciferase Assay. Human embryonic kidney cells 293 were used to monitor alteration of the NFκB (nuclear factor-kappaB) pathway. This cell line contains chromosomal integration of a luciferase reporter construct regulated by the NFκB response element. Transcription factors can bind to the response element when stimulated by certain agents, allowing transcription of the luciferase gene. The gene product, luciferase, reacts with the substrate, emitting light that was detected using a LUMIstar Galaxy BMG luminometer. After incubating treated cells, they were lysed in Reporter lysis buffer, and the assay was performed using the Luc assay system from Promega.[25]

ASSOCIATED CONTENT

Supporting Information. NMR spectra for new compounds **5**−**8** are available free of charge via the Internet at http://pubs.acs.org.

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