FULL PAPER

Two New Ring *A*-Cleaved Lanostane-Type Triterpenoids and Four Known Steroids Isolated from Endophytic Fungus *Glomerella* sp. F00244

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Two new ring *A*-cleaved lanostane-type triterpenoids, glometenoid A (**1**) and glometenoid B (**2**), together with four known steroids, (20*S*,22*E*,24*R*)-ergosta-7,22-dien-3*β*,5α,6*β*-triol (**3**), (3*β*,5α,8α,22*E*)-ergosta-6,22-diene-3,5,8-triol (**4**), 5α,8α-epidioxy-22*E*-ergosta-6,22-dien-3*β*-ol (**5**), and ergosterol (**6**), were isolated from the endophytic fungus *Glomerella* sp. F00244. Their structures were elucidated by comprehensive spectroscopic analyses of NMR and MS data. Their antimicrobial activities were evaluated against pathogenic bacteria *Bacillus subtilis* ATCC 9372, *Staphylococcus aureus* ATCC 25923, *Bacillus pumilus* CMCC (B) 63202, *Micrococcus luteus* CMCC28001, and pathogenic fungi *Candida albicans* AS2.538 and *Aspergillus niger* ACCC30005, but no inhibition was observed at a concentration of 20 μg/ml. Further cytotoxicity assessment revealed that compound **1** exhibited weak antiproliferative activity against ovarian cancer HeLa cell.

Keywords: Triterpenoid, Steroid, Endophytic fungus, Biogenesis, Mevalonic acid pathway.

Introduction

Endophytic fungi living in a plant's internal tissues or organs without causing any apparent or immediate symptoms or diseases in the host plant have been viewed as excellent sources of natural products with unique structures and interesting biological properties [1]. In previous research done by our group members for secondary metabolites from the endophytic fungi, a series of new compounds were isolated with antibacterial or antitumor activities [2 – 6].

Ring *A*-cleaved lanostane-type triterpenoids are a class of triterpenoids with interesting structure and diverse bioactivities. Several such compounds have been isolated recently. For instance, klainedoxalanostenone, with moderate xanthine oxidase inhibitory activity, has been isolated from the stem bark of *Klainedoxa gabonensis* [7]; two new ring *A*-cleaved triterpenes, poricoic acid AE and poricoic acid CE, have been isolated from the surface layer of the mushroom *Poria cocos* [8]; schisanlactone G, a new ring *A*-cleaved 3,4-seco-lanostane triterpenoid, from *Schisandra sphenanthera* has been isolated [9]; five new ring *A*-cleaved compounds with exomethylene *γ*-lactone ring, exhibiting broad cytotoxicity against tested cell lines, have been isolated from the apical buds of *Gardenia sootepensis.* [10]; and six ring *A*-cleaved cycloartane triterpenes have been isolated from the gum resin of *Gardenia gummifera* and *Gardenia lucida* [11]. Recently, we have embarked on the research of secondary metabolites from an endophytic strain F00244 with the observed cytotoxicities for its crude extract from primary screening. Six compounds were found, including two new ring *A*-cleaved lanostane-type triterpenoids (**1** and **2**), and one known *D*-homoannulated steroid (**3**), which was isolated for the first time from microbes [12], and three known tetracyclic steroids (**4** – **6**) [13 – 16] (*Fig. 1*). Here, we present the isolation, structure elucidation, and antimicrobial and cytotoxic activities of compounds **1** – **6**.

Results and Discussion

The strain F00244 was isolated from the stem of mason pine grown in Xiamen botanical garden, Fujian province, P. R. China, and identified as *Glomerella* sp. according to its sequence of rDNA (ITS1-5.8S-ITS2). The fermentation culture was extracted successively with AcOEt. The AcOEt extract was purified by repeated column chromatographies (*RP-18*, *Sephadex LH-20*, and silica gel) to afford compounds **1** – **6**.

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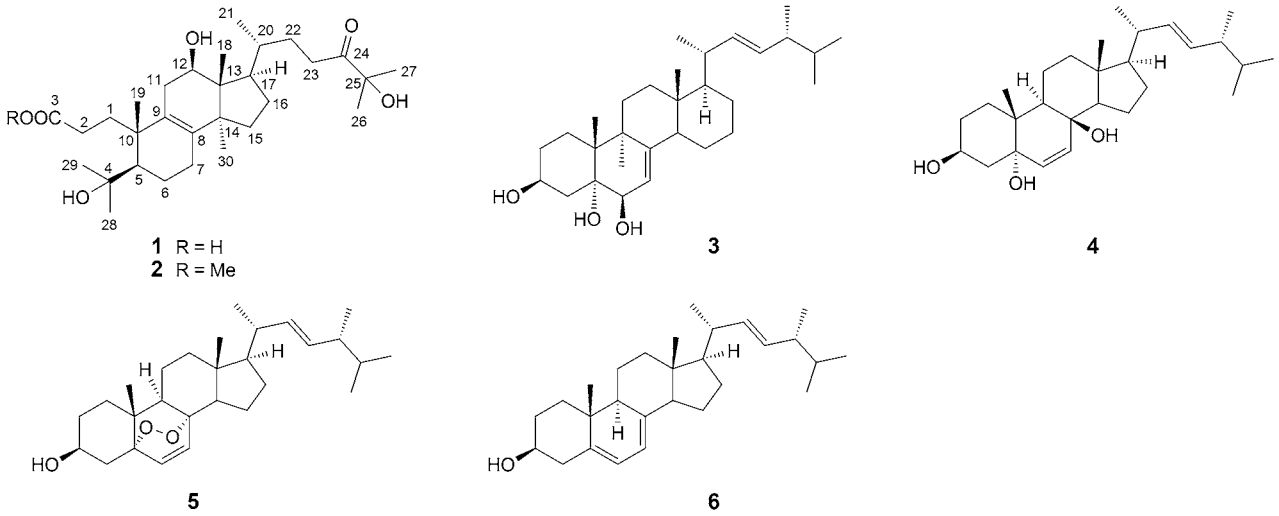

Fig. 1. Structures of compounds **1** – **6**.

Compound **1** was obtained as yellow amorphous powder, and its molecular formula, $C_{30}H_{50}O_6$, was established on the basis of the HR-ESI-MS (*m*/*z* 529.3607 $[M + Na]^+$). Analysis of the IR spectrum indicated the presence of a OH group with intramolecular H-bond as well as a COOH group with IR absorption at 2955, 2923, and 1727 cm^{-1}, respectively. The ^1H- and ^{13}C-NMR (DEPT) spectra of **1** (*Table 1*) displayed signals for nine quaternary C-atoms (one keto, one COOH, two olefinic, and two O-bearing), four CH groups (one O-bearing), nine CH_2 groups, and eight Me groups. According to the numbers of C-atoms and Me groups, **1** was speculated as triterpenoid initially. The structure of fragment **1a** was determined on the basis of the $^1H,^1H$-COSY correlations of H–C(1) ↔ H–C(2) and H–C(6) ↔ H–C(7), along with the HMBCs from the H-atoms of Me(19) to C(1), C(10), C(9), and C(5), H-atoms of Me(29) and Me(28) to C(4) and C(5), H–C(5) to C(4), C(10), H–C(6) to C(4), C(5), and C(10), and H–C(2) to C(1) and C(3) (*Fig. 2*). Similarly, fragments **1b** and **1c** were established as shown in *Fig. 2*. The HMBCs of Me(19) with C(9), H–C(11) with C(9) and C(8), and Me(30) with C(8) indicated that fragments **1a** and **1b** were linked *via* C(8) and C(9). Finally, the HMBCs from H–C(17) to C(20), C(21), and C(22), and to C(13), C(16), and C(18) indicated that fragments **1b** and **1c** were linked together *via* C(17), indicating a lanostane-type triterpenoid [17]. The relative configuration of **1** was established on the basis of NOSEY spectra. The observed strong NOESY cross-peaks at H–C(12)/H–C(17), H–C(12)/Me(30), H–C(17)/Me(21), and Me(18)/H–C(23) indicated that **1** had the same relative configuration as that of the structure of lanosterol (*Fig. 3*). Taken together, the structure of compound **1** was established as 4,12*β*,25-trihydroxy-24-oxo-3,4-secolanost-8-en-3-oic acid (glometenoid A).

Compound **2** was obtained as white amorphous powder, and its molecular formula, $C_{31}H_{52}O_6$, was established on the basis of the HR-ESI-MS (*m*/*z* 543.3662 $[M + Na]^+$ and 559.3448 $[M + K]^+$). The IR absorptions at 2958, 2923, and 1711 cm^{-1} suggested the presence of a OH group with intramolecular H-bond and a COOH group, respectively. The ^{13}C-NMR and DEPT spectra of **2** displayed 31 C-atoms, including nine quaternary C-atoms, four CH groups, nine CH_2 groups, and nine Me groups (one O-bearing). The ^1H- and ^{13}C-NMR spectra of **2** were similar to those of **1** with the exception of the additional MeO group at *δ*(H) 3.68 (*s*) and *δ*(C) 51.74 (*q*; *Table 2*), which suggested **2** was the methyl ester derivative of **1**. Thus, **2** was deduced as methyl 4,12*β*,25-trihydroxy-24-oxo-3,4-secolanost-8-en-3-oate (glometenoid B). The relative configuration of **2** was assigned the same as of **1** for biogenetic considerations.

By analysis and comparison of their NMR spectra, MS data, and specific rotations with those reported [12 – 16], compounds **3** – **6** were elucidated as known steroids.

Previously, compound **4** ((3*β*,5α,8α,22*E*)-ergosta-6,22-diene-3,5,8-triol) was reported to show inhibitory effects in the antineuroinflammatory test [13]. Compound **5** (5α,8α-epidioxy-22*E*-ergosta-6,22-dien-3*β*-ol) was an inhibitor of HL60 leukemia cell growth and apoptosis inducer [14]. Compound **6** (ergosterol) is the precursor of vitamin D2, which is very important for human health [16]. Here, all isolated compounds (**1** – **6**) were evaluated for their cytotoxicities against HeLa and HepG2 cancer cell lines. Only compound **1** exhibited weak antiproliferative activity against the ovarian cancer cell line (HeLa) with 21% growth inhibition at a concentration of 10 μM. In the antimicrobial assay, compounds **1** – **6** were tested against pathogenic bacteria *Bacillus subtilis* ATCC 9372, *Staphylococcus aureus* ATCC 25923, *Bacillus pumilus* CMCC (B) 63202, *Micrococcus luteus* CMCC28001, and pathogenic fungi *Candida albicans* AS2.538 and *Aspergillus niger* ACCC30005. No inhibition was observed at a

concentration of 20 μg/ml. Although compounds **1** – **6** were not active in our current available bioassays, a broader panel of bioassays for evaluation of these compounds could be conducted in the future.

Table 1. ^{1}H- and ^{13}C-NMR (600 and 150 MHz, resp., in $CDCl_3$), and HMBC Data of **1**. δ in ppm, J in Hz

Position	δ(H)	δ(C)	HMBC (H → C)
1	1.75 – 1.82 (*m*, H_α), 2.61 – 2.68 (*m*, H_β)	32.9	C(3), C(2), C(10), C(5), C(9), C(19)
2	2.10 – 2.16 (*m*, H_α), 2.31 – 2.38 (*m*, H_β)	29.1	C(1), C(3)
3	–	179.1	–
4	–	75.6	–
5	1.46 (*s*, 1 H)	48.0	C(6), C(28), C(4)
6	1.45 – 1.51 (*m*, H_α), 1.56 – 1.61 (*m*, H_β)	23.2	C(10), C(5), C(4)
7	1.96 – 2.03 (*m*, 2 H)	26.4	–
8	–	139.3	–
9	–	131.4	–
10	–	42.6	–
11	1.82 – 1.88 (*m*, H_α), 2.41 – 2.48 (*m*, H_β)	33.6	C(9), C(13), C(12)
12	4.16 (*dd*, *J* = 7.8, 7.8, 1 H)	72.9	C(18), C(17)
13	–	48.8	–
14	–	53.5	–
15	1.21 – 1.25 (*m*, H_α), 1.69 – 1.75 (*m*, H_β)	31.5	C(30), C(14)
16	1.55 – 1.62 (*m*, H_α), 1.89 – 1.95 (*m*, H_β)	25.2	C(20)
17	1.86 – 1.93 (*m*, 1 H)	50.7	C(18), C(21), C(16), C(20), C(13), C(22)
18	0.75 (*s*, 3 H)	10.3	C(13), C(17), C(14), C(12)
19	1.22 (*s*, 3 H)	21.9	C(1), C(10), C(5), C(9)
20	1.63 – 1.70 (*m*, 1 H)	34.0	C(23)
21	1.03 (*d*, *J* = 6.60, 3 H)	21.1	C(22), C(20), C(17)
22	1.92 – 1.99 (*m*, H_α), 2.10 – 2.17 (*m*, H_β)	29.2	C(21)
23	2.54 – 2.60 (*m*, 2 H)	33.8	C(22), C(24)
24	–	215.1	–
25	–	76.3	–
26	1.40 (*s*, 3 H)	26.6	C(27), C(25), C(24)
27	1.40 (*s*, 3 H)	26.5	C(26), C(25), C(24)
28	1.28 (*s*, 3 H)	27.4	C(29), C(5), C(4)
29	1.30 (*s*, 3 H)	33.5	C(28), C(5), C(4)
30	0.97 (*s*, 3 H)	24.8	C(15), C(13), C(14), C(8)

Comparing with the known closest analogs of ring *A*-cleaved lanostane-type triterpenoids, including seco-coccinic acids (F – K) [18] and ascotrichic acid B [19], our new compounds glometenoid A (**1**) and B (**2**) with OH group at C(4) and C(12), and ketone group at C(24) represented a higher oxidation status through more hydroxylation or oxidation steps. Additionally, to better understand the potential biosynthetic relationship between compounds (**1** – **6**), we proposed the biogenetic pathways (*Schemes 1 – 3*). The mevalonic acid (MVA) pathway was recognized as a main route to the biosynthesis of terpenes and steroids [20]. After cyclization of 2,3-oxidosqualene, the triterpenoid underwent various modifications to give two important intermediates lanosterol [21] and ergosterol (**6**) [16] (*Scheme 1*). It was presumed that ergosterol underwent the steps of hydroxylation and ring *D* homoannulation to afford cerevisterol (**3**), and ergosterol was peroxidized to give **5**, then the cleavage of endoperoxide bridge of **5** was required to produce **4** (*Scheme 2*). As for biogenesis of compounds **1** and **2**, we postulated that the H–C(3) group of lanosterol was oxidized to C(3)=O. Subsequent *Baeyer–Villiger* oxidation gave the seven-membered lactone ring, which, after hydrolysis and a series of oxidation reactions, lead to the product glometenoid A (**1**). Finally, esterification of **1** lead to glometenoid B (**2**; *Scheme 3*).

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Experimental Part

General

TLC: precoated silica-gel *GF254* plates (0.20 – 0.25 mm; *Qingdao Marine Chemical Factory*, Qingdao, P. R. China). Spots were visualized under UV light or by spraying with I_2, H_2SO_4, and *Dragendorff* reagent. Column chromatography (CC): silica gel (SiO_2, 200 – 300 mesh; *Qingdao Marine Chemical Factory*). Silica gel *GF 254* (10 – 40 μm, *Qingdao Marine Chemical Plant*, Qingdao, P. R.

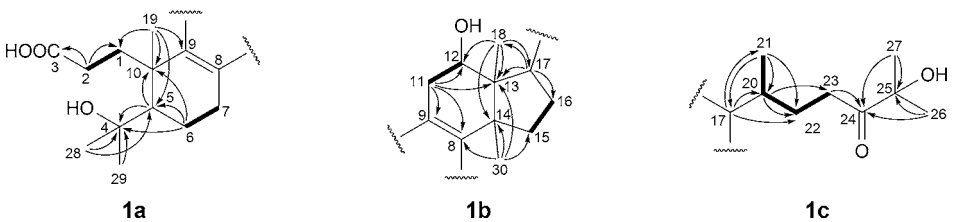


Fig. 2. The structures of fragments **1a** – **1c** in compound **1** and selected HMBCs (H → C) and ^{1}H,^{1}H-COSY (▬) correlations.

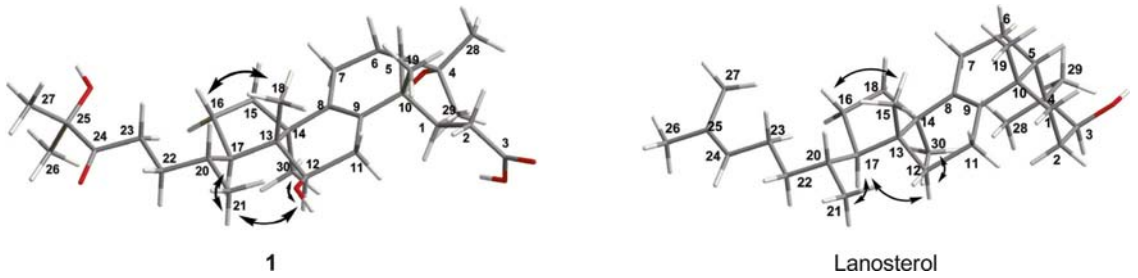


Fig. 3. Selected NOESY correlations (H ↔ H) and relative configuration of compound **1** and lanosterol.

Table 2. The comparison of the ^{1}H- and ^{13}C-NMR data (600 and 150 MHz, resp.; in $CDCl_3$) of **1** and **2**

Position	**1**		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
1	1.75 – 1.82 (*m*, H_α), 2.61 – 2.68 (*m*, H_β)	32.9	1.70 – 1.80 (*m*, H_α), 2.62 – 2.69 (*m*, H_β)	32.8
2	2.10 – 2.16 (*m*, H_α), 2.31 – 2.38 (*m*, H_β)	29.1	2.09 – 2.15 (*m*, H_α), 2.25 – 2.31 (*m*, H_β)	29.3
3	–	179.1	–	174.9
4	–	75.6	–	75.3
5	1.46 (*s*, 1 H)	48.0	1.40 (br., 1 H)	48.0
6	1.45 – 1.51 (*m*, H_α), 1.56 – 1.61 (*m*, H_β)	23.2	1.44 – 1.50 (*m*, H_α), 1.56 – 1.61 (*m*, H_β)	23.3
7	1.96 – 2.03 (*m*, 2 H)	26.4	1.96 – 2.03 (*m*, 2 H)	26.4
8	–	139.3	–	139.4
9	–	131.4	–	131.8
10	–	42.6	–	42.7
11	1.82 – 1.88 (*m*, H_α), 2.41 – 2.48 (*m*, H_β)	33.6	1.82 – 1.88 (*m*, H_α), 2.40 – 2.48 (*m*, H_β)	33.6
12	4.16 (*dd*, *J* = 7.8, 7.8, 1 H)	72.9	4.15 (*dd*, *J* = 7.7, 7.7, 1 H)	73.0
13	–	48.8	–	48.8
14	–	53.5	–	53.5
15	1.21 – 1.25 (*m*, H_α), 1.69 – 1.75 (*m*, H_β)	31.5	1.21 – 1.29 (*m*, H_α), 1.70 – 1.75 (*m*, H_β)	31.5
16	1.55 – 1.62 (*m*, H_α), 1.89 – 1.95 (*m*, H_β)	25.2	1.54 – 1.62 (*m*, H_α), 1.92 –1.98 (*m*, H_β)	25.3
17	1.86 – 1.93 (*m*, 1 H)	50.7	1.86 – 1.93 (*m*, 1 H)	50.7
18	0.75 (*s*, 3 H)	10.3	0.75 (*s*, 3 H)	10.3
19	1.22 (*s*, 3 H)	21.9	1.21 (*s*, 3 H)	21.8
20	1.63 – 1.70 (*m*, 1 H)	34.0	1.64 – 1.70 (*m*, 1 H)	34.0
21	1.03 (*d*, *J* = 6.6, 3 H)	21.1	1.04 (*d*, *J* = 6.7, 3 H)	21.1
22	1.92 – 1.99 (*m*, H_α), 2.10 – 2.17 (*m*, H_β)	29.2	1.92 – 1.98 (*m*, H_α), 2.08 – 2.16 (*m*, H_β)	29.2
23	2.54 – 2.60 (*m*, 2 H)	33.8	2.54 – 2.61 (*m*, 2 H)	33.9
24	–	215.1	–	215.8
25	–	76.3	–	76.2
26	1.40 (*s*, 3 H)	26.6	1.40 (*s*, 3 H)	26.6
27	1.40 (*s*, 3 H)	26.5	1.40 (*s*, 3 H)	26.6
28	1.28 (*s*, 3 H)	27.4	1.28 (*s*, 3 H)	27.3
29	1.30 (*s*, 3 H)	33.5	1.30 (*s*, 3 H)	33.4
30	0.97 (*s*, 3 H)	24.8	0.96 (*s*, 3 H)	24.8
31	–	–	3.68 (*s*, 3 H)	51.7

China). Medium-pressure reversed-phase *RP-18* (*Merck*, Darmstadt, Germany), and *Sephadex LH-20* (*Amersham Biosciences*, Sweden). Optical rotations: *PerkinElmer 341* polarimeter (*PerkinElmer Inc.*, Waltham, MA, USA), in $CHCl_3$. IR Spectra: *Nicolet FT-IR 360* spectrophotometer (*Thermo Electron Corp.*, Madison, WI, USA), in KBr; $\tilde{\nu}$ in cm^{-1}. ^{1}H- and ^{13}C-NMR spectra: *Bruker DRX-600* spectrometer (*Bruker Co.*, Bremen, Germany) at 600 and 150 MHz, resp., in $CDCl_3$ or (D_6)DMSO; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. HR-Q-TOF-MS: *Bruker Daltonics Bio* TOF-Q mass spectrometer (*Bruker Co.*, Bremen, Germany); in *m*/*z* (rel. %).

Isolation and Fermentation of the Fungal Strain

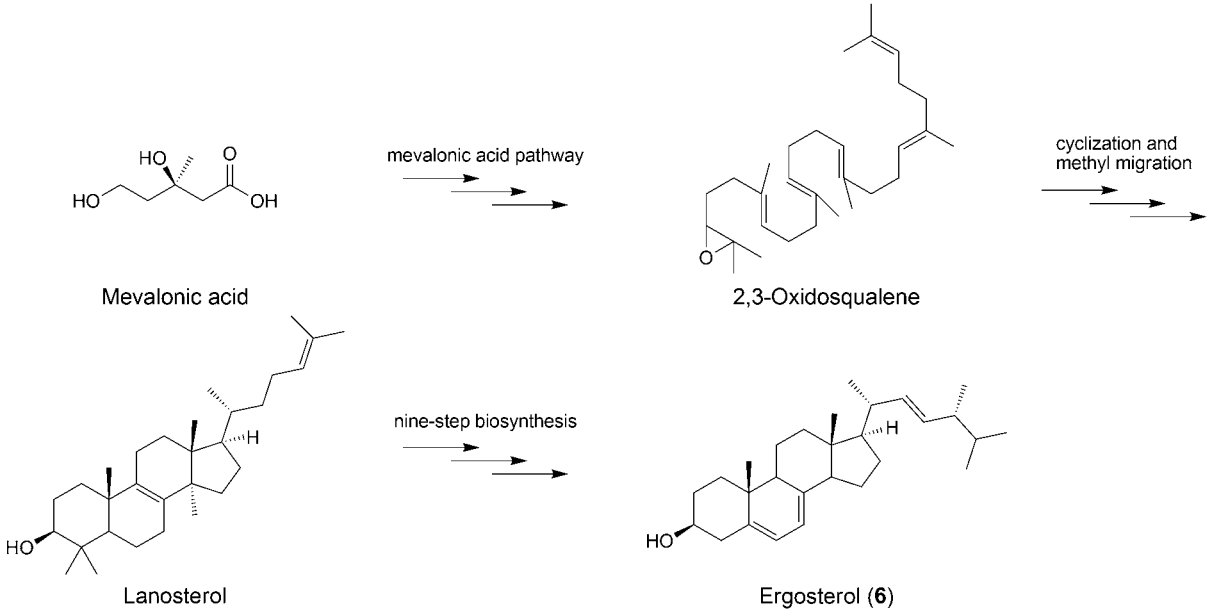
The fungus F00244 was isolated by potato dextrose agar (PDA) medium from plant stem of mason pine, which was collected from Xiamen botanical garden, Fujian province, P. R. China. It was identified as *Glomerella* sp. on the basis of internal transcribed spaces (ITS) sequencing with universal primers ITS1 and ITS4. The special amplified ITS1-5.8SrDNA-ITS4 fragment was 538 bp, which exhibited 99% sequence identity with *Glomerella septospora.* (GenBank accession number GU935908.1). We employed bioassay-guided strategy for the isolation of these metabolites. First, the fungus *Glomerella* sp. F00244 was fermented in small scale (500 ml) and the crude extract was screened for antimicrobial and antitumor activities. Having the antitumor bioactivity of the crude extract, the strain was statically cultured on PDA medium at 28 °C for 14 d. A total of 15 l of fungal solid culture was prepared.

Extraction and Isolation

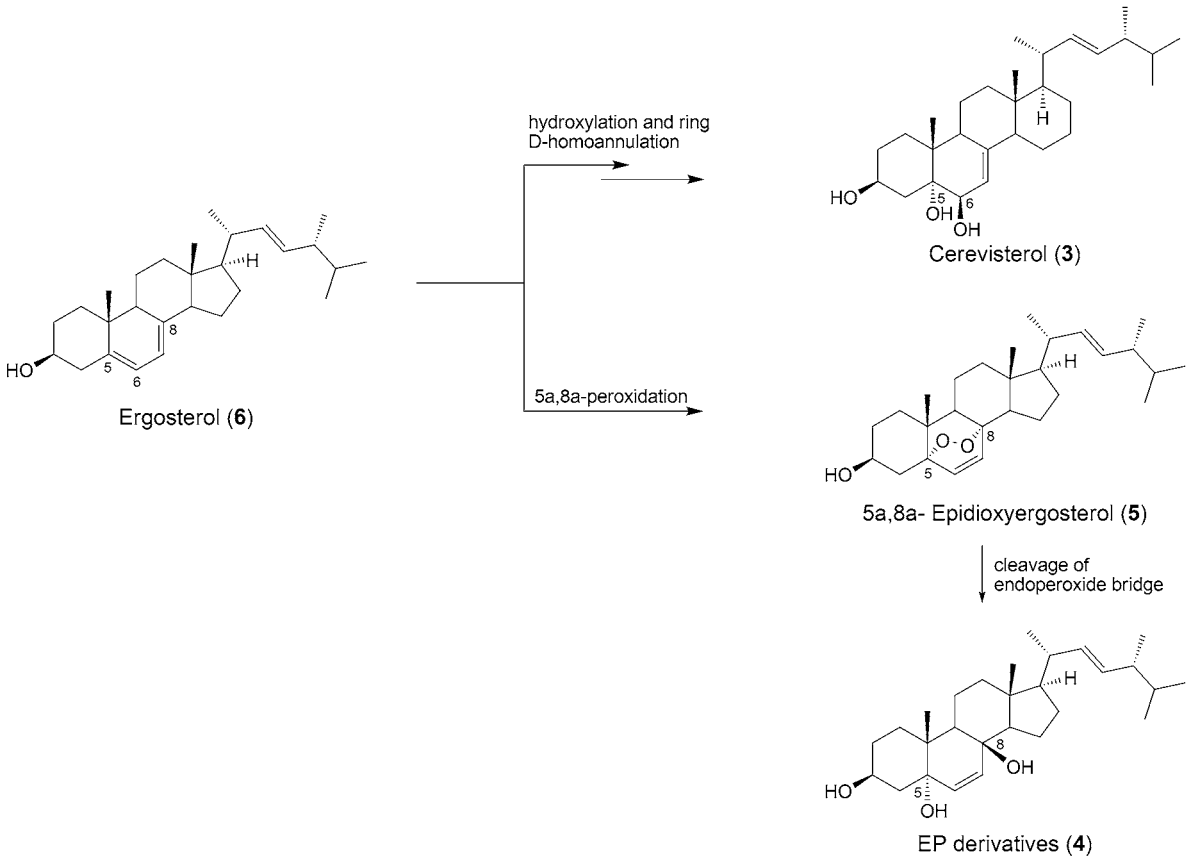
The fermented material was diced and extracted with mixed solvent of AcOEt/MeOH/AcOH (80:15:50) (5 × 3 l). The org. soln. was combined and concentrated in vacuum at 45° to yield crude syrup (4.0 g). The crude syrup was suspended in AcOEt and washed with H_2O, and then the AcOEt layer was concentrated and resuspended in MeOH. The MeOH soln. was filtered and concentrated to give the crude extract. Meanwhile, the insoluble residue was dissolved in $CHCl_3$ to give C-extract (400 mg). The crude extract was resuspended in MeOH and washed with petroleum ether (PE). Both the MeOH layer and the PE layer were concentrated to yield dark brown extract (M-extract, 1.9 g) and light brown extract (P-extract, 1.5 g), resp.

The M-extract (1.9 g) was subjected to medium-pressure liquid chromatography (MPLC; 170 g of *RP-18*;

Scheme 1. Proposed biosynthesis of lanosterol and ergosterol (**6**).

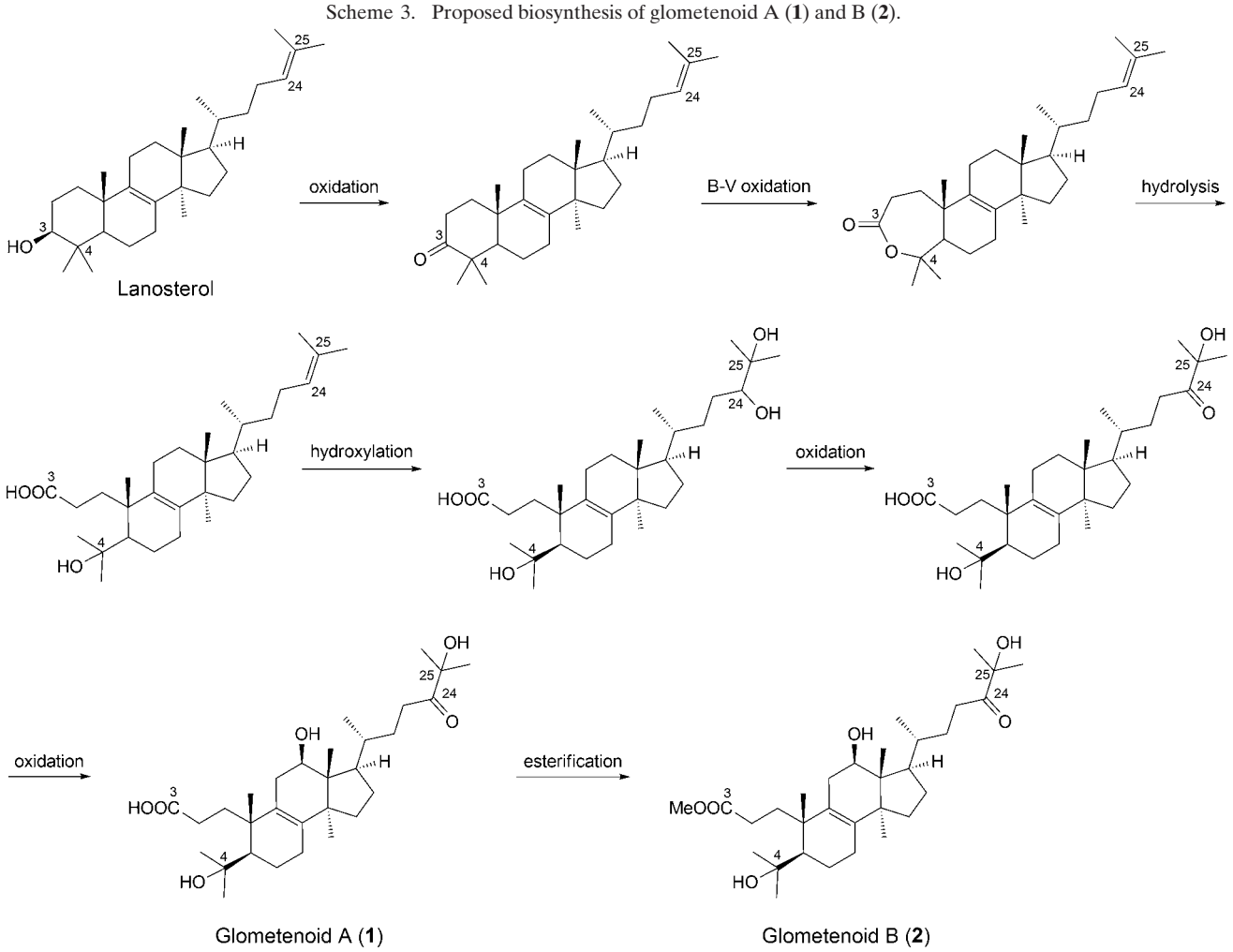


Scheme 2. Proposed biosynthesis of cerevisterol derivatives (**3**), EP derivatives (**4**), and 5α,8α-epidioxyergosterol (**5**).



MeOH/ddH_2O = 0%, 30%, 50%, 70% 100%, and acetone, 2 l for each gradient) to afford *Fr. C* (93.6 mg, 70% of MeOH) and *D* (900 mg, 100% of MeOH). *Fr. C* was subjected to MPLC (30 g of *RP-18*; MeOH/ddH_2O 7:3) to afford fractions *Fr. C-a* (25 mg) and *Fr. C-b* (10 mg). *Fr. C-a* was further purified by *Sephadex LH-20* in MeOH

Scheme 3. Proposed biosynthesis of glometenoid A (**1**) and B (**2**).



and silica CC eluted with PE/AcOEt (4:1) to yield compound **1** (16.4 mg). *Fr. C-b* was purified by silica CC using PE/AcOEt (4:1) as the eluent to yield compound **2** (2.1 mg). *Fr. D* (900 mg) was subjected to a *Sephadex LH-20* (160 g) using MeOH as the mobile phase to yield two portions, *Fr. D-f* (80 mg) and *Fr. D-g* (148 mg). *Fr. D-g* (148 mg) was sequentially subjected to a *Sephadex LH-20* (MeOH) twice and silica CC (PE/AcOEt 50:1) to yield compound **5** (1.3 mg). *Fr. D-f* (80 mg) was sequentially subjected to a *Sephadex LH-20* (acetone/MeOH 4:1) and silica CC (PE/AcOEt 3:1) to yield compound **3** (0.8 mg). The C-extract (400 mg) was purified on silica CC using PE/AcOEt (30:1) as the eluent to obtain compound **6** (85.5 mg). The P-extract (1.5 g) was sequentially subjected to silica CC (AcOEt), *Sephadex LH-20* (acetone/MeOH, *v*/*v*, 4:1), and MPLC (30 g of *RP-18*, acetone) to yield compound **4** (6.0 mg).

Bioassays

The cytotoxicities of compounds **1** – **6** were evaluated by means of the MIT the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay against human cancer cell lines HepG2 and HeLa, using cisplatin as positive control [22]. The antimicrobial activities of **1** – **6** were tested against pathogenic bacteria *Bacillus subtilis* ATCC 9372, *Staphylococcus aureus* ATCC 25923, *Bacillus pumilus* CMCC (B) 63202, *Micrococcus luteus* CMCC28001, and pathogenic fungi *Candida albicans* AS2.538 and *Aspergillus niger* ACCC30005 using disk diffusion method at 20 μg/ml [23].

4,12*β*,25-Trihydroxy-24-oxo-3,4-secolanost-8-en-3-oic Acid (= **3-[(3*R*,3a*R*,4*R*,6*S*,7*R*,9b*S*)-2,3,3a,4,5,6,7,8,9,9b-Decahydro-4-hydroxy-3-[(2*R*)-6-hydroxy-6-methyl-5-oxoheptan-2-yl]-7-(2-hydroxypropan-2-yl)-3a,6,9b-trimethyl-1*H*-cyclopenta[*a*]naphthalen-6-yl]propanoic Acid**; **1**). Yellow crystals. $[\alpha]_D^{25}$ = +50.0 (*c* = 0.12, $CHCl_3$). IR (KBr): 2957, 2926, 1727, 1711. ^{1}H- and ^{13}C-NMR: *Table 2*. HR-ESI-MS: 529.3607 ($[M + Na]^+$, $C_{30}H_{50}NaO_6^+$; calc. 529.3505).

Methyl 4,12*β*,25-Trihydroxy-24-oxo-3,4-secolanost-8-en-3-oate (= **Methyl 3-[(3*R*,3a*R*,4*R*,6*S*,7*R*,9b*S*)-2,3,3a,4,5,6,7,8,9,9b-Decahydro-4-hydroxy-3-[(2*R*)-6-hydroxy-6-methyl-5-oxoheptan-2-yl]-7-(2-hydroxypropan-2-yl)-3a,6,9b-trimethyl-1*H*-cyclopenta[*a*]naphthalen-6-yl]propanoate**; **2**) White crystals. $[\alpha]_D^{25}$ = +47 (*c* = 0.04, $CHCl_3$). IR (KBr): 2957,

2925, 1711, 1665. ^{1}H- and ^{13}C-NMR: *Table 2*. HR-ESI-MS: 543.3662 ([M + Na]$^{+}$, $C_{31}H_{52}NaO_6^{+}$; calc. 543.3662).

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