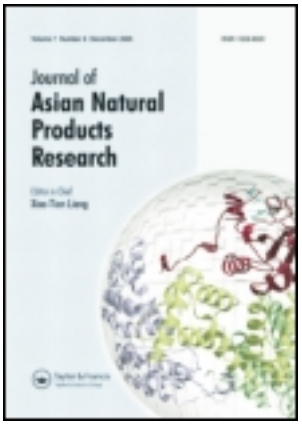

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Biotransformation of oleanolic acid by *Alternaria longipes* and *Penicillium adametzi*

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Microbial transformation of oleanolic acid (**1**) was carried out. Six transformed products (**2**–**7**) from **1** by *Alternaria longipes* and three transformed products (**8**–**10**) from **1** by *Penicillium adametzi* were isolated. Their structures were elucidated as 2α,3α,19α-trihydroxy-ursolic acid-28-*O*-β-D-glucopyranoside (**2**), 2α,3β,19α-trihydroxy-ursolic acid-28-*O*-β-D-glucopyranoside (**3**), oleanolic acid 28-*O*-β-D-glucopyranosyl ester (**4**), oleanolic acid-3-*O*-β-D-glucopyranoside (**5**), 3-*O*-(β-D-glucopyranosyl)-oleanolic acid-28-*O*-β-D-glucopyranoside (**6**), 2α,3β,19a-trihydroxy-oleanolic acid-28-*O*-β-D-glucopyranoside (**7**), 21β-hydroxyl oleanolic acid-28-*O*-β-D-glucopyranoside (**8**), 21β-hydroxyl oleanolic acid (**9**), and 7α,21β-dihydroxyl oleanolic acid (**10**) based on the extensive NMR studies. Among them, **10** was a new compound and compounds **5** and **8**–**10** had stronger cytotoxic activities against Hela cell lines than the substrate. At the same time, it was reported for the first time in this paper that the skeletons of compounds **2** and **3** were changed from oleanane to uranane and seven glycosidation products were obtained by biotransformation.

Keywords: biotransformation; oleanolic acid; *Alternaria longipes*; *Penicillium adametzi*

1. Introduction

Oleanolic acid, a natural pentacyclic triterpenoid found in many plant species, has attracted attention due to its important pharmacological properties, such as anti-inflammation, antitumor, and antidiabetes activities [1,2]. In recent years, to find safe, effective, stable, and an innovative new drug target molecules or lead compounds, the structural modification of oleanolic acid and the structure–activity relationship of its derivatives were reported [3,4], but the reaction sites were mainly at C-3 and C-28, and the reaction procedures including hydroxylation, oxidation, and acetylation focused on the introduction of heteroatom into structure were reviewed.

Microbial transformation is defined as an enzymatic reaction by micro-organisms with metabolic activities to modify the structures of bioactive substrates. It has such advantages as high stereo- or region-selectivities, as well as mild reaction conditions over chemical synthesis [5]. Some reactions fulfilled with difficulty in the process of chemical synthesis are facile by microbial transformation. Microbial transformation is a useful technique to modify the structures of biologically active compounds. But there have been a few reports on biotransformation of oleanolic acid in recent years [6–9]. In our previous study, we had screened the biotransformation of oleanolic acid by 25 kinds of fungi

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and determined the transformation efficiency [10].

In this paper, the biotransformation of oleanolic acid (**1**) by *Alternaria longipes* and *Penicillium adametzi* was performed to find out new chemical entities and with the aim of improving its activities and solubility. Nine products were obtained, and their structures were elucidated based on the extensive spectral data, including 2D NMR. Among them, compound **10** was new and compounds **5** and **8**–**10** had stronger cytotoxic activities *in vitro* against Hela cell lines than the substrate.

2. Results and discussion

The structures of unknown transformed products were elucidated on the basis of their ^{1}H, ^{13}C NMR, HSQC, and HMBC spectral data and the known compounds were identified by comparison of their NMR spectral data with those reported in literature [11–15]. Incubation of oleanolic acid (**1**) with *A. longipes* for 7 days yielded six compounds (**2**–**7**) and using the same methods of microbiologic culture, three transformed products (**8**–**10**) were obtained from *P. adametzi*. Their structures were con -firmed as 2α,3α,19α-trihydroxy-ursolic acid-28-*O*-β-D-glucopyranoside (**2**), 2α,3β, 19α-trihydroxy-ursolic acid-28-*O*-β-D-glucopyranoside (**3**), oleanolic acid 28-*O*-β-D-glucopyranosyl ester (**4**), oleanolic acid-3-*O*-β-D-glucopyranoside (**5**), 3-*O*-(β-D-glucopyranosyl)-oleanolic acid-28-*O*-β-D-glucopyranoside (**6**), 2α,3β,19a-trihydroxy-oleanolic acid-28-*O*-β-D-glucopyranoside (**7**), 21β-hydroxyl oleanolic acid-28-*O*-β-D-glucopyranoside (**8**), 21β-hydroxyl oleanolic acid (**9**), and 7α,21β-dihydroxyl oleanolic acid (**10**). Compound **10** was an unreported product. Skeleton-rearranged metabolites, compounds **2** and **3**, were obtained from this biotransformation involving a methyl migration, whose skeletons were changed from oleanane to ursane.

As one skeleton-rearranged metabolite, compound **2** was obtained as a white powder and its ESI-MS analysis gave a molecular formula of $C_{36}H_{58}O_{10}$. The ^{1}H NMR spectra showed six quaternary methyl groups and one tertiary methyl group at δ 1.05 (3H, d, H-29), which indicated that the skeleton of mother nucleus was changed from oleanane to ursane. Compared with the literature [14], the structure of compound **2** was determined as 2α,3α,19α-trihydroxy-ursolic acid-28-*O*β-D-glucopyranoside. ^{13}C NMR spectral data of compound **3** are similar to those of compound **2** except that an oxygenated signal at δ 83.8 (CH, by DEPT) was present instead of a signal at δ 79.3 due to C-3. On the same analysis methods with compound **2**, the structure of compound **3**, the other skeleton-rearranged metabolite, was determined as 2α,3β,19α-trihydroxyl ursolic acid-28-*O*-β-D-glucopyranoside.

Compound **10** was previously unreported and was obtained as a white power. It was optically active, with $[\alpha]_D^{17}$ +47.3 ($c = 0.15$, MeOH). The molecular formula $C_{30}H_{48}O_5$ was established by analysis of positive HR-EI-MS, which gave a quasi-molecular ion peak at *m/z* 511.3297 $[M + Na]^+$. The ^{13}C NMR spectrum displayed resonances for 30 carbons, which were assigned as seven methyls, eight methylenes, seven methines, and eight quaternary carbons by analysis of DEPT spectrum. The HMQC spectrum showed two new cross-peaks between the proton signals at δ 3.99 and 4.26 with the carbon signals at δ 72.5 and 72.9 in comparison with the parent compound, indicating that compound **10** contained two oxygenated atoms compared with **1**. In the HMBC spectrum, H-29 and H-30 exhibited cross-peaks with C-20 (δ 36.8), C-19 (δ 47.6), and C-21 (δ 72.5), and H-26 (δ 1.33) exhibited cross-peak with C-7 (δ 72.9). In the ^{1}H–^{1}H COSY spectrum, the signal at δ 3.99 exhibited cross-peaks with H-22 (δ 2.36) and the signal at δ 4.26 exhibited cross-peaks with H-6 (δ 1.82), indicating that the two oxygenated carbons at δ 72.5

and 72.9 were attributed to C-21 and C-7, respectively. Furthermore, the NOESY spectrum also showed cross-peaks of H-7 with H-27, and H-21 with H-29. Accordingly, the configuration of hydroxyl group was determined to be β. On the basis of the above evidence, the structure of **10** was determined to be 7α,21β-dihydroxyl oleanolic acid.

Based on the time course in the biotransformation of oleanolic acid (**1**) (Figures 1 and 2), the pathways shown in Figures 3 and 4 were proposed for the biotransformation of oleanolic acid cultured with *A. longipes* and *P. adametzi*, respectively. The glycoside products, compounds **4** and **5** were detected at 24 h of incubation, while other products **2**, **3**, **6**, and **7** were obtained at 48 h of incubation, which were shown in Figure 1. Biogenetically, the oleanane and ursane skeletons share a common intermediate, and the

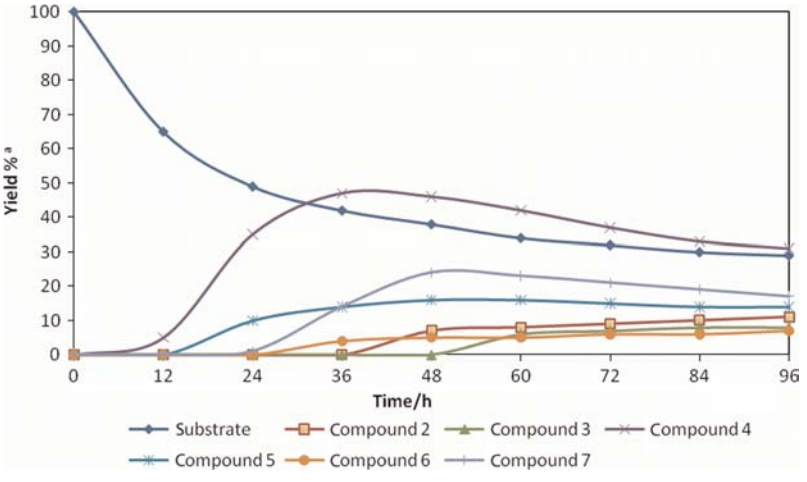

Figure 1. Time course of the biotransformation of oleanolic acid (**1**) by *A. longipes*.
Note: [a]Yield is expressed as a percentage relative to the total amount of reaction products on a molar basis.

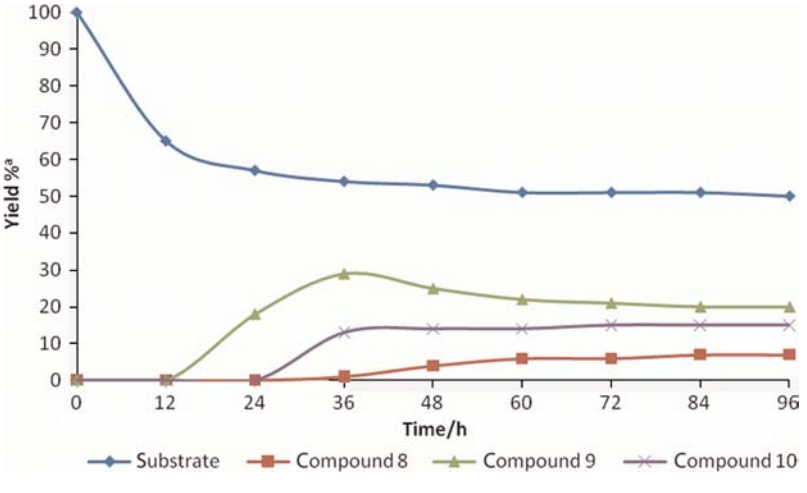

Figure 2. Time course of the biotransformation of oleanolic acid (**1**) by *P. adametzi*.
Note: [a]Yield is expressed as a percentage relative to the total amount of reaction products on a molar basis.

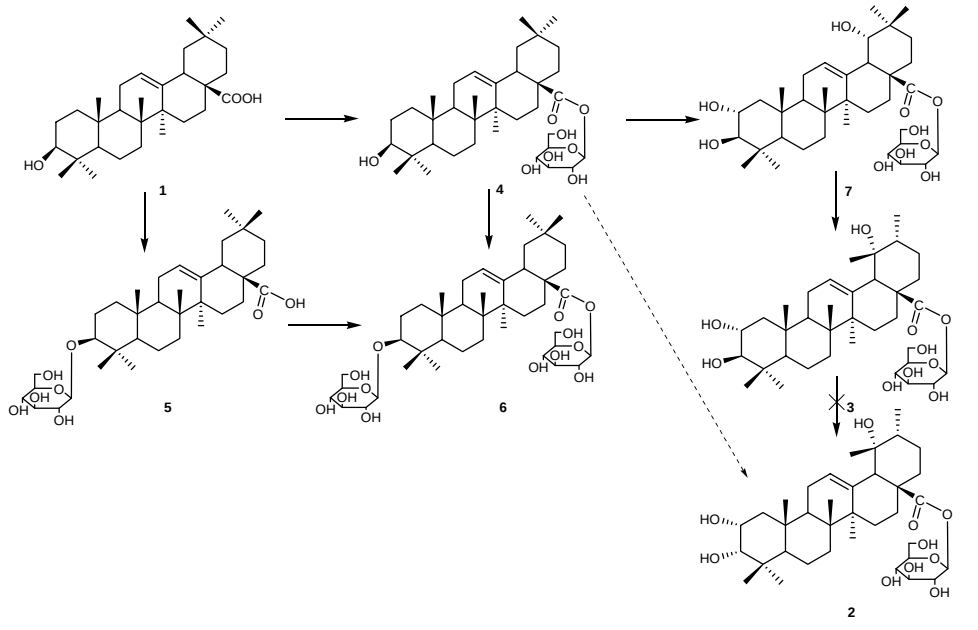

Figure 3. Possible biotransformation pathways of oleanolic acid (**1**) by *A. longipes*.

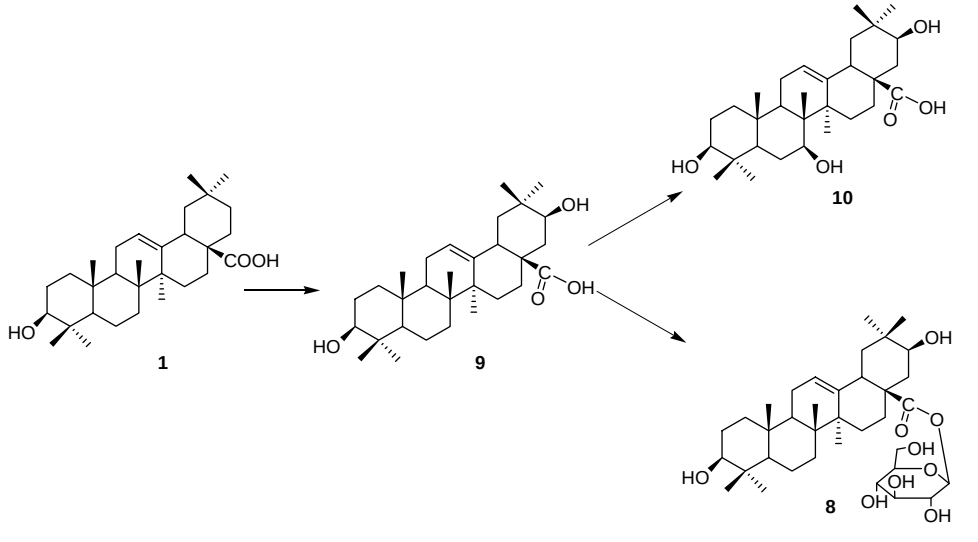

Figure 4. Possible biotransformation pathways of oleanolic acid (**1**) by *P. adametzi*.

ursane nucleus is biosynthesized from an oleanane precursor via a methyl migration from C-20 to C-19 [16]. To investigate the mechanism of this biotransformation and to study the biotransformation pathway, a conversion experiment was performed on utilizing compound **7** as substrate. Compound **7** was found to be converted to compound **3** when incubated with *A. longipes* under the same conditions for 2 days. On the other hand, no appreciable reaction could be detected for compound **2** after extended incubation for 4 days. This implies that oleanolic acid **1** is converted

stepwise to the final product compound **3** via one intermediate, compound **7**, which is further converted to compound **2** through extended incubation (Figures 3 and 4). Otherwise, it was reported for the first time in this paper that the skeletons of compounds **2** and **3** were changed from oleanane to uranane by biotransformation approach, which was formed by the participation of 'retro-biosynthetic' methyl migration from C-20 to C-19.

At the same time, seven glycosidation products are reported in this paper. Glycosylation is a characteristic biotransformation reaction in plant cells, because glycosyltransferases are widespread in plants [17]. But glycosidation is not common in the microbial transformation in aquatic conditions. Recently, the synthesis mechanism of oligosaccharides was reported by a chemoenzymatic method using cyclodextrin glucanotransferase [18]. Microbial transformation, which is also an enzymatic reaction with good region- and stereoselectivity, can overcome the limitation of chemical synthesis and satisfy the demands. So, the capacity of *A. longipes* and *P. adametzi* for the glycosylation of oleanolic acid should be useful for the preparation of even more highly water-soluble oleanolic glycosides. This procedure overcomes the time-consuming protection and deprotection steps necessary in chemical synthesis.

Thus, the ability and regioselectivity of *A. longipes* and *P. adametzi* for the glycosylation and methyl migration of oleanolic acid are elucidated for the first time in this paper.

The MTT bioassay showed that all the transformed products had cytotoxicities against Hela (human cervical carcinoma) cells. Compounds **5** and **8**–**10** had significant inhibitory effects against Hela cells with IC_{50} values of 7.4, 25.0, 7.6, and 0.78 μM, and the IC_{50} values of substrate and other products were all greater than 25.0 μM, respectively. Compounds **5** and **8**–**10** had more potent inhibitory effects than the substrate **1**. The pharmaco-experiment indicated that hydroxylation at C-21 (**9**), C-7 (**10**), and C-3 (**5**) glycosylation could increase the cytotoxicities, but the glycosylation at C-28 (**8**) would significantly reduce the activities.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a digital polarimeter in methanol on a Jasco P-1020 digital polarimeter (model AUTOPOL, Japan). The mass spectra were recorded on an ion trap mass spectrometer. The IR spectrum was obtained on an Avatar 360 FT-IR spectrophotometer. The ^{1}H NMR spectra were recorded on a Bruker ARX-400 spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) in pyridine-d_5 as solvent. The HR-ESI-MS were recorded on a Jeol HX 110 mass spectrometer. Chromatography was performed with Diaion HP20 (Mitsubishi Chemical Corporation, Tokyo, Japan), silica gel (200–300 mesh, Qingdao Haiyang Chemical Corporation, Qingdao, China). Preparative HPLC was performed in Thermo Scientific Spectra SYSTEM P1000 LC (Thermo Fisher Scientific, Waltham, MA, USA). Pump type: Spectra SYSTEM P1000, detector: Spectra SERIES UV100, column: RP-18 (5 μm, 250 × 10 mm) (YMC-Pack ODA-A, YMC Company Ltd., Kyoto, Japan, flow rate: 4.5 ml/min, elution with MeOH–H_2O). TLC plates (silica gel) were used to check the purity of compounds and all spots were detected by spraying with 10% H_2SO_4, followed by heating. All reagents used were of analytical grades. Oleanolic acid (**1**) was supplied by JF-natural (Tianjin Jianfeng Natural Product R&D Ltd. Co., Tianjin, China). The purity of **1** was above 98% determined by HPLC.

3.2 Fungi

A. longipes AS 3.2875 and *P. adametzi* AS 3.447 were purchased from the China General Microbiological Culture Collec-

tion Center in Beijing, China. All cultures were maintained on potato dextrose agar slants and stored in a refrigerator at 5°C prior to use.

3.3 Biotransformation and isolation

Stock cultures of fungi were stored on potato dextrose agar slants at 5°C. Seed cultures of each fungus were obtained by transferring fungi from stock cultures to a liquid medium containing ($g\,L^{-1}$) potato (20%) and glucose (2%). The experiments were conducted in conical flasks (500 ml), containing the same liquid medium (200 ml) inoculated with the fungi. The flasks were shaken at 28°C and 160 rpm for 48 h. The substrates (oleanolic acid, **1**), previously dissolved in a minimum volume ($15\,mg\,ml^{-1}$) of dehydrated alcohol, were evenly distributed among the flasks (15 mg per flask of substrate), except one that was kept as a control, and the reaction was allowed to proceed for 4 days. The mycelium was then removed by filtration, and the broth was dealt in an ultrasonic bath for 30 min and extracted with *n*-BuOH for three times (*n*-BuOH was used in the same volume with broth every time) and all extracts were combined and dried. After filtration, the solvents were evaporated under reduced pressure, and the crude extract was fractionated by chromatography. Controls were carried out in order to verify the action of the medium (without fungus) on the substrates and the presence of similar metabolites on the fungi cultures (without substrates).

3.3.1 *Biotransformation of oleanolic acid (**1**) by* A. longipes

The crude extract obtained from the biotransformation of oleanolic acid (**1**) (17.4 g) was chromatographed on Diaion HP20 (Φ 45 cm × 3.7 cm) eluted stepwise by H_2O, 30% EtOH, 50% EtOH, 70% EtOH, and 95% EtOH to give five corresponding fractions (Q-1–Q-5). Fraction Q-4 (4.6 g) was separated by a silica gel open column (Φ 35 cm × 3.7 cm) with $CHCl_3$–MeOH gradient elution to yield six subfractions (Fractions Q-41–Q-46). Crystallization of Fraction Q-44 yielded compound **4** (294 mg, 4.54% yield). The purification of Fraction Q-45 (395 mg) on PHPLC with MeOH–H_2O (3:2) afforded metabolites **2** (13 mg, 0.2% yield), **3** (15 mg, 0.23% yield), and **5** (20 mg, 0.31% yield). Using the same method, Fractions Q-46 (384 mg) were purified by PHPLC with MeOH–H_2O (1:1) to furnish **6** (6 mg, 0.10% yield) and **7** (65 mg, 1.03% yield).

3.3.2 *Biotransformation of oleanolic acid (**1**) by* P. adametzi

The crude extract obtained from the biotransformation of oleanolic acid (**1**) (14.6 g) was chromatographed on Diaion HP20 (Φ 45 cm × 3.7 cm) eluted stepwise by H_2O, 30% EtOH, 50% EtOH, 70% EtOH, and 95% EtOH to give five corresponding fractions (Qi-1–Qi-5). Fr. Qi-4 (1.8 g) was separated by a silica gel open column (Φ 25 cm × 2.8 cm) with $CHCl_3$–MeOH gradient elution to yield six subfractions (Qi-41–Qi-46) and crystallization of Fraction Qi-42 yielded compound **9** (203 mg, 3.13% yield). Fr. Qi-3 (1.7 g) was separated by an ODS open column with MeOH–H_2O gradient elution to yield six subfractions (Qi-31–Qi-36). The purification of Fraction Qi-33 (577 mg) on PHPLC with MeOH–H_2O (85:15) afforded metabolite **10** (185 mg, 2.90% yield). Using the same method, Fractions Qi-36 (237 mg) was purified by PHPLC with MeOH–H_2O (7:3) to furnish **8** (13 mg, 0.20% yield).

3.3.3 *7α,21β-dihydroxyl oleanolic acid (**10**)*

White powder; Liebermann–Burchard reaction was positive; $[\alpha]_D^{17}$ 47.3 ($c = 0.15$, MeOH). IR (KBr) ν_{max}: 3422, 3130, 2962,

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of **10** in pyridine-d_5 (δ in ppm, J in Hz).

No.	^{1}H	^{13}C	No.	^{1}H	^{13}C
1	1.62 m, 1.06 o	38.9 t	16	2.31 m, 2.22 m	25.7 t
2	1.85 m	28.2 t	17		48.8 s
3	3.47 m	77.9 d	18	3.55 m	42.6 d
4		39.1 s	19	2.15 m, 1.53 m	47.6 t
5	1.09 o	53.1 d	20		36.8 s
6	2.03 m, 1.82 m	30.5 t	21	3.99 dd, $J = 11.1, 5.2$ Hz	72.5 d
7	4.26 m	72.9 d	22	2.36 m	41.8 t
8		45.6 s	23	1.23 s	28.6 q
9	1.75 m	48.3 d	24	1.03 s	16.6 q
10		37.6 s	25	0.98 s	15.6 q
11	2.06 m	24.0 t	26	1.33 s	10.8 q
12	5.67 t, $J = 3.3$ Hz	123.1 d	27	1.52 s	26.0 q
13		144.0 s	28		179.4 s
14		43.9 s	29	1.29 s	29.8 q
15	2.70 td, $J = 14.5, 4.4$ Hz, 2.19 m	32.3 t	30	1.30 s	17.7 q

Notes: Overlapped signals are indicated by 'o'. The carbon and proton signals were unambiguously assigned through the HMQC spectrum and HMBC spectrum.

2873, 1696, 1399, 1073, and 860 cm^{-1}. For ^{1}H and ^{13}C NMR spectral data see Table 1. HR-ESI-MS *m/z*: 511.3357 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_5Na$, 511.3399).

3.4 Time course and HPLC analysis

Time course experiments were performed by a procedure similar to normal transformation experiments except that 5 mg/ml of substrate was administered to each of eight flasks (250 ml) containing 60 g of liquid medium. At a regular time interval (12 h), one of the flasks was tested to evaluate the conversion yields of the products by HPLC methods.

The HPLC analysis was carried out using an analytical Luna ODS C_{18} column (250 × 4.6 mm i.d., 5 μm; Phenomenex Inc., Guangzhou, China). The isocratic mobile phase was MeOH–1% glacial acetic acid water solution (90:10, v/v). The mobile phase was filtered through 0.45 μm Millipore filters before use. The injection volume was 20 μl and the flow rate was 1.0 ml/min. Peaks were detected at 208 nm, which is the maximum absorbance of these products. The system was operated at room temperature (25°C).

3.5 Bioassay

The cell line was maintained in an RPMI 1640 medium (GIBCO/BRL, MD, USA) supplemented with 10% (v/v) of fetal bovine serum, 100 IU/ml of penicillin and 100 μg/ml of streptomycin at 37°C, and 5% CO_2, and grown in 96-well microtiter plates for the assay. All compounds were dissolved in DMSO. After 48 h of incubation, compounds underwent serial dilution to give final concentrations of 10^{-7}–10^{-4} mol/L. The cell growth was evaluated by the MTT assay. The activities were measured as IC_{50} values, which are a concentration of the test compound (μM) that inhibited cell growth by 50%.

Acknowledgements

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