Tetrahedron 67 (2011) 4206–4211

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

New approaches to the structural modification of olean-type pentacylic triterpenes via microbial oxidation and glycosylation

Yu-Yao Zhu [a], Li-Wu Qian [a,c], Jian Zhang [a,*], Ji-Hua Liu [b], Bo-Yang Yu [b,*]

[a] Department of Complex Prescription of Traditional Chinese Medicine, China Pharmaceutical University, 639# Long Mian Avenue, Nanjing 211198, People's Republic of China
[b] Key Laboratory of Modern Chinese Medicines, China Pharmaceutical University, Ministry of Education, 24# Tongjia Xiang Road, Nanjing, 211198, People's Republic of China
[c] Department of Natural Products, Anhui Institute of Materia Medica, Hefei 230022, People's Republic of China

ARTICLE INFO

Article history:
Received 6 January 2011
Received in revised form 30 March 2011
Accepted 15 April 2011
Available online 21 April 2011

Keywords:
Biotransformation
Pentacyclic triterpene
Glycosylation
Oxidation
Streptomyces griseus ATCC 13273
Aspergillus ochraceus CICC 40330

ABSTRACT

Microbial transformation of 4 olean-type pentacyclic triterpenes (OPTs), 3-oxo oleanolic acid (**1**), 3-acetyl oleanolic acid (**2**), oleanolic acid (**3**), and esculentoside A (**4**) was studied. After the screening of 12 strains of microbes, preparative biotransformation by two strains of *Streptomyces griseus* ATCC 13273 and *Aspergillus ochraceus* CICC 40330 resulted in the isolation of 10 metabolites. The microbial catalyzed high efficient regio-selective methyl oxidation and glycosylation were discovered, which could be provided as an alternative method to expand the structural diversity of OPTs. All the structures of the metabolites were elucidated unambiguously by ESI-MS, ^{1}H NMR, ^{13}C NMR, and 2D-NMR spectroscopy.

1. Introduction

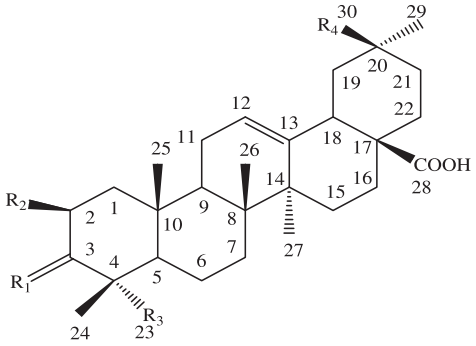
Olean-type pentacyclic triterpenes (OPTs) are of the bioactive natural products, which have been shown to possess a wide range of pharmacological applications, such as anti-tumor,[1] anti-HIV,[2] anti-microbial,[3] anti-diabetic,[4,5] and anti-inflammatory activities.[6] The structural diversity of the natural originated OPTs mainly attributed to the substitution of hydroxyl or carbonyl groups to the methyl or methenyl carbons of the skeleton and the formation of corresponding glycosides.[7] The presence of such functional groups, especially at C-3, C-28, and C-30, was also essential for the biological activities of OPTs.[8–10] Hereby, the researches on the structural modification of OPTs were focused on how to introduce the functional groups to the skeleton with the aim to enrich the structural diversities and explore more effective and/or less toxicity derivatives.[11,12] In view of the complex skeleton and less active position, microbial transformation was employed as an efficient method to conduct the modification of OPTs nowadays.[13] And in our previous studies, some novel microbial transformation of pentacyclic triterpenoids such as methyl migration, asymmetric ketone α-hydroxylation, and methyl hydroxylation[14–17] have been reported individually. As a continuation work to explore the microbial transformation of OPTs, 3-oxo oleanolic acid (**1**) was used as the substrate, by the screening of 12 strains of microbes, we found that there were two more polar metabolites in the cultures of *Streptomyces griseus* ATCC 13273 and one more polar metabolite in the cultures of *Aspergillus ochraceus* CICC 40330 with high yield. To further investigate the biotransformation capability of the two microbes and explore the influence of the substitutions on C-3 and C-20 to the reactions, another three substrates 3-acetyl oleanolic acid (**2**), oleanolic acid (**3**), and esculentoside A (**4**, Fig. 1) were screened, the other seven metabolites were detected. In this paper, we described the isolation and structural elucidation of all the 10 metabolites prepared in our research. It is envisioned that microbial transformation could play a more important role in the structural modification to expand the structural diversities of OPTs.

2. Results and discussion

3-Oxo oleanolic acid (**1**) (200 mg) was added into a 2-day-old cultivation of *S. griseus* ATCC 13273 and two more polar metabolites (**5** and **6**) were detected by HPTLC. Compound **5** was the main metabolite (84.3 mg) during the initial 48 h' incubation, while to the end of the 120 h incubation, compound **6** reached the highest amount (125.1 mg). Furthermore, when the same amount of compound **1** incubated with *A. ochraceus* CICC 40330, another more polar metabolite **7** (146.6 mg) was isolated.

* Corresponding authors. Tel.: +86 25 86185157; fax: +86 25 86185158 (J.Z.); tel.: +86 25 83271321; fax: +86 25 83313080 (B.-Y.Y.); e-mail addresses: wilson1978@163.com (J. Zhang), boyangyu59@163.com (B.-Y. Yu).

0040-4020/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2011.04.055



Compound	R_1	R_2	R_3	R_4
3-oxo oleanolic acid (**1**)	O	H	CH_3	CH_3
3-acetyl oleanolic acid (**2**)	$CH_3COO(\alpha)$, H(β)	H	CH_3	CH_3
Oleanolic acid (**3**)	OH(α), H(β)	H	CH_3	CH_3
Esculentoside A (**4**)	Glc-Xyl-O(α), H(β)	OH	CH_2OH	$COOCH_3$

Fig. 1. The structure of substrates **1**–**4**.

Compound **5** was isolated as colorless powder, the HR-ESI-MS of compound **5** showed an $[M-H]^-$ ion at *m*/*z* 483.3088 (calcd for $C_{30}H_{43}O_5$, 483.3115), indicating a 30 amu mass increase to that of compound **1**. The ^{13}C NMR spectrum exhibited a new signal at *δ* 180.9 ppm and only six characteristic methyl signals appeared at *δ* 26.6, 26.0, 21.6, 20.0, 17.2, and 15.0 ppm (Table 1) with the absence of one methyl signal in the substrate. This evidence suggested that one methyl group might be oxidized to a carboxyl group. Furthermore, by comparing the ^{13}C NMR spectrum of **1**, the carbon signal at *δ* 23.5 ppm attribute to C-30 shifted up-field 3.5 ppm to *δ* 20 ppm in the metabolite, indicating that the oxidization was on C-29. In addition, the three-bond correlation between *δ* 1.59 (s, 3H, H-30) ppm and 180.9 ppm was also observed in the HMBC spectrum. The relative stereochemistry of the carboxyl group was established as equatorial based on NOESY experiments. The NOESY spectrum of **5** showed NOE enhancements between *δ* 1.59 (s, 3H, H-30) and 3.45 (dd, 1H, J_1=14.0 Hz, J_2=4.0 Hz, H-18) ppm (Fig. 2), this further evidenced that the methyl group of C-29 was oxidized to a carboxyl group in the biotransformation. Based on all the spectroscopic data and comparison with that of serratagenic acid,[18] compound **5** was identified as 3-oxo-olean-12-en-28,29-dioic acid.

Table 1
^{13}C NMR spectral data of **5**, **6**, **8**, **9** (recorded in C_5D_5N, 125 MHz)

Carbon	**5**	**6**	**8**	**9**
1	39.1	39.0	39.4	38.7
2	34.4	34.5	28.1	28.4
3	216.1	213.4	78.1	80.2
4	47.4	53.8	39.8	43.2
5	55.3	56.7	55.8	56.4
6	19.8	18.9	18.8	19.1
7	32.6	32.2	33.3	33.6
8	39.7	38.6	39.0	39.8
9	47.2	46.4	48.1	48.2
10	37.0	36.1	37.4	37.2
11	23.9	23.0	23.9	23.9
12	123.0	121.7	123.3	123.0
13	144.3	143.3	144.3	144.4
14	42.3	41.0	42.2	42.1
15	28.3	27.2	28.3	28.3
16	23.9	22.7	23.9	24.1
17	46.7	45.5	46.7	46.6
18	41.0	40.0	41.1	41.1
19	41.2	39.9	41.1	41.1
20	42.6	41.4	42.6	42.6
21	30.0	28.1	30.0	29.3
22	32.4	31.2	32.4	32.4
23	26.6	19.8	28.8	23.5
24	21.6	64.1	16.5	64.6
25	15.0	14.4	15.6	16.0
26	17.2	16.2	17.5	17.3
27	26.0	24.9	26.1	26.0
28	179.9	178.7	179.9	179.9
29	180.9	179.8	181.0	180.5
30	20.0	18.9	20.0	20.0

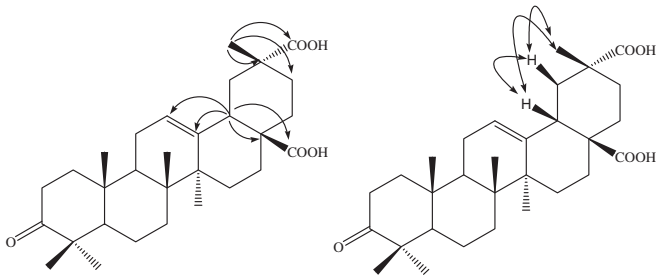


Fig. 2. Key HMBC and NOESY correlations of compound **5**.

Compound **6** was isolated as colorless powder, the HR-ESI-MS of compound **6** showed an $[M-H]^-$ ion at *m*/*z* 499.3060 (calcd for $C_{30}H_{43}O_6$, 499.3065), which indicated an increment of 16 amu as compared to compound **5**. The ^{13}C NMR spectrum of **6** exhibited a new signal at *δ* 64.1 (Table 1), while the ^1H NMR spectrum of **6** showed two new proton signals at *δ* 4.29 (d, 1H, *J*=11.0 Hz) and 3.83 (d, 1H, *J*=11.0 Hz) ppm. The DEPT experiment showed the presence of a new CH_2 signal at *δ* 75.5 ppm and the disappearance of a CH_3 signal, confirming that **6** was a monohydroxylated metabolite of **5**. In the HSQC experiment, the new proton signals showed correlations with the new carbon signal at *δ* 64.1 ppm, and in the HMBC experiment, the new CH_2 signals show long-range (two- and three-bond) $^1H-^{13}C$ correlations with C-3 and C-4, confirming C-23 or C-24 as the site of hydroxylation. Furthermore, the relative stereochemistry of the hydroxyl group was established as axial based on NOESY data. The NOESY spectrum of **6** showed NOE enhancements between the new proton signals with *δ* 1.10 ppm (s, 3H, H-25) and 2.77 ppm (td, 1H, J_1=14.5 Hz, J_2=6.0 Hz, H-2β) (Fig. 3), indicating that hydroxyl group was substituted at C-24. Based on all the evidences, metabolite **6** was characterized as 24-hydroxy-3-oxo-olean-12-en-28,29-dioic acid.

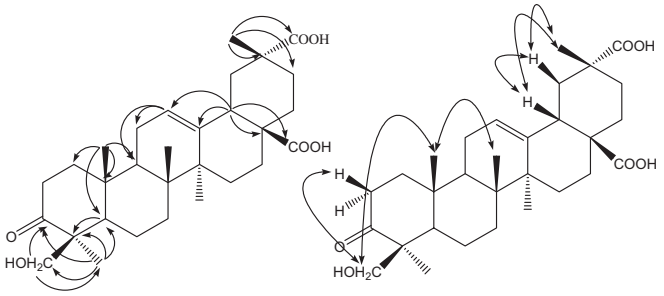


Fig. 3. Key HMBC and NOESY correlations of compound **6**.

Compound **7** was isolated as colorless powder, the ESI-MS of compound **7** showed an $[M+Na]^+$ ion at *m*/*z* 639.8 corresponding to the molecular formula of $C_{36}H_{56}O_8Na$, indicating a 162 amu mass increase of **1**. It was suggested that a hexose moiety was introduced. Whereas, the ^{13}C NMR spectrum showed the presence of six new signals at *δ* 95.8, 79.4, 78.9, 74.1, 71.2, and 62.2 ppm (Table 2), which are characteristic of β-D-glucose.[19] The anomeric proton H-1′ of the glucose moiety in **7** resonated as a doublet at *δ* 6.32 ppm. A coupling constant of 8.5 Hz for H-1′ indicated that the stereochemistry of the glycosidic linkage at C-1′ of D-glucose is β. The anomeric carbon at *δ* 95.8 was typical for an ester glycosidic linked sugar at C-28 carboxy group. The location of the glucose moiety was further confirmed by

Table 2
^{13}C NMR spectral data of **7**, **10**–**14** (recorded in C_5D_5N, 125 MHz)

Carbon	7	10	11	12[a]	13	14
1	39.2	39.0	44.0	43.2	44.8	44.9
2	34.7	28.1	70.8	70.3	71.6	71.6
3	216.2	78.2	82.7	73.6	73.1	73.0
4	47.4	39.4	42.6	40.8	42.4	42.4
5	55.4	55.9	47.5	47.5	48.2	48.1
6	19.8	18.9	18.0	17.4	18.2	18.3
7	32.5	33.1	33.0	32.0	33.0	32.9
8	39.8	40.0	39.6	38.9	39.8	40.0
9	47.2	48.2	48.3	47.9	48.5	48.6
10	36.9	37.4	36.8	36.1	37.2	37.2
11	23.8	23.5	23.6	22.9	23.9	23.9
12	122.7	122.9	123.3	122.3	123.4	123.4
13	144.2	144.2	144.2	143.1	144.6	143.8
14	42.2	42.2	42.2	40.5	42.2	42.2
15	28.2	28.3	28.1	27.2	28.3	28.2
16	23.6	23.7	23.7	22.7	23.9	23.5
17	46.2	47.1	45.9	45.0	46.7	46.5
18	41.8	41.8	43.9	41.8	42.8	43.2
19	47.0	46.3	43.2	41.3	26.3	42.4
20	30.8	30.8	44.1	42.0	51.3	43.9
21	34.0	34.1	30.8	29.9	38.0	30.5
22	32.5	32.6	34.3	33.1	34.1	34.0
23	26.6	28.7	65.1	69.0	67.8	67.7
24	21.6	16.5	14.7	13.1	14.5	14.5
25	15.0	15.7	17.0	16.6	17.4	17.5
26	17.3	17.6	17.2	16.3	17.2	17.3
27	26.0	26.1	26.0	25.5	26.2	26.1
28	176.4	176.4	179.5	178.7	179.9	176.1
29	33.1	33.2	28.2	28.0	71.1	28.2
30	23.4	23.9	176.9	176.4	175.8	177.0
OCH_3			51.4	51.4	51.5	51.6
Glc 1	95.8	95.8				95.8
2	74.1	74.2				74.1
3	79.4	79.3				79.3
4	71.2	71.2				71.0
5	78.9	79.0				78.9
6	62.2	62.3				61.9
Xyl 1			106.5			
2			75.1			
3			78.3			
4			70.7			
5			67.0			

[a] Recorded in CD_3OD.

the chemical shift of C-28, which shifted up-field from δ 182.8 to 176.4 by 6.4 ppm. By comparison of the spectroscopic data with those reported in the literature,[20] compound **7** was established as 28-*O*-β-D-glucopyranosyl 3-oxo-olean-12-en-28-oate.

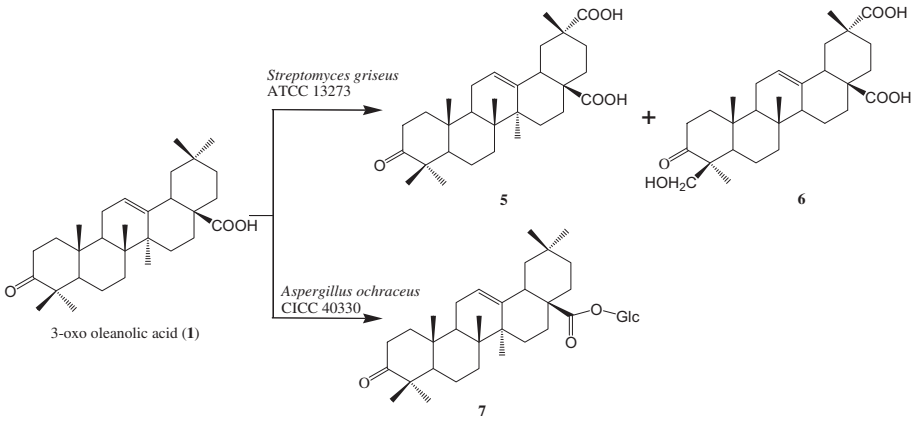
In the biotransformation of compound **1** with the two microorganisms, two reactions were observed: the regio-selective oxidation of the C-29 methyl group and the glycosylation of the C-28 carboxyl group (Scheme 1). While 3-acetyl oleanolic acid (**2**) was subjected to the cultures, to our surprise, no metabolite was detected. The subsequent biotransformation of oleanolic acid (**3**) was conducted; 200 mg of compound **3** was added into the 2-day-old fermentation broth of the two microorganisms, respectively. As we expected, two more polar products, compounds **8** (98.6 mg) and **9** (118.7 mg) were isolated from the cultures of *S. griseus* ATCC 13273 and one more polar metabolite compound **10** (137.6 mg) was isolated from the culture of *A. ochraceus* CICC 40330.

The molecular formula of **8**, $C_{30}H_{46}O_5$, was established by the $[M-H]^-$ ion at *m*/*z* 485.5 in negative ion mode of ESI-MS, indicating a 30 amu mass increase following the transformation. The ^{13}C NMR spectrum showed the presence of a new carbon signal at 181.0 ppm (Table 1) and the disappearance of a methyl group carbon signal at 33.1 ppm of C-29. Based on the similar ^1H and ^{13}C NMR data of **5** and comparison of the spectroscopic data in the literature,[18] **8** was identified as serratagenic acid.

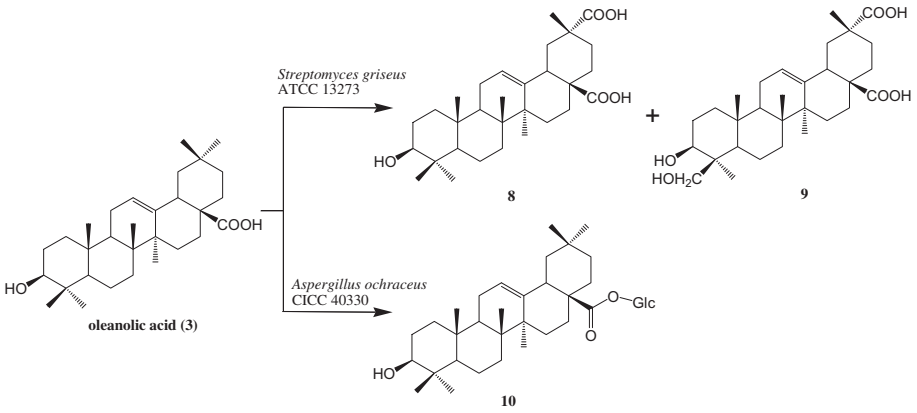
The molecular formula of **9** was established as $C_{30}H_{46}O_6$ by HR-ESI-MS in which a quasimolecular ion was detected at *m*/*z* 501.3183 $[M-H]^-$ (calcd for $C_{30}H_{45}O_6$, 501.3221), indicating a 16 amu mass increase to that of compound **8** following the transformation. The DEPT spectrum showed the presence of a new methylene carbon signal at δ 64.6 ppm (Table 1), with the disappearance of one methyl group carbon signals attribute to C-24, this indicated that the C-24 methyl group might be oxidized to the corresponding hydroxymethyl group, which was similar to that of compound **6**, the HMBC and NOESY experiments further confirmed the structure of the compound **9** as 3β,24-dihydroxy-olean-12-en-28,29-dioic acid.

The molecular formula of **10**, $C_{36}H_{58}O_8Na$, was established by the $[M+Na]^+$ ion at 641.2 in positive ion mode of ESI-MS, indicating a 162 amu mass increase in **3**. The ^{13}C NMR spectrum showed the presence of six new signals at δ 95.8, 79.3, 79.0, 74.2, 71.2, and 62.3 ppm (Table 2), indicating the presence of a glucose moiety. Based on all the observations and comparison of the spectroscopic data with those reported in the literature,[20] **10** was established as 28-*O*-β-D-glucopyranosyl,3β-hydroxy-olean-12-en-28-oate.

To the substrate of compound **3**, the same reactions were observed (Scheme 2), this indicated that the substituent group on C-3 was very crucial to the biotransformation. Another substrate, esculentoside A (**4**), which contained sugar moiety on C-3 hydroxyl group, methyl carboxyl group of C-30, and free hydroxyl group on C-23 was used to test whether these substitutions would affect the biotransformation. Compound **4** (200 mg) was added to the two cultures, respectively, two main less polar products, **11** (24.6 mg) and **12** (56.0 mg), were isolated from the culture of *S. griseus* ATCC



Scheme 1. Microbial transformation of **1**.



Scheme 2. Microbial transformation of **3**.

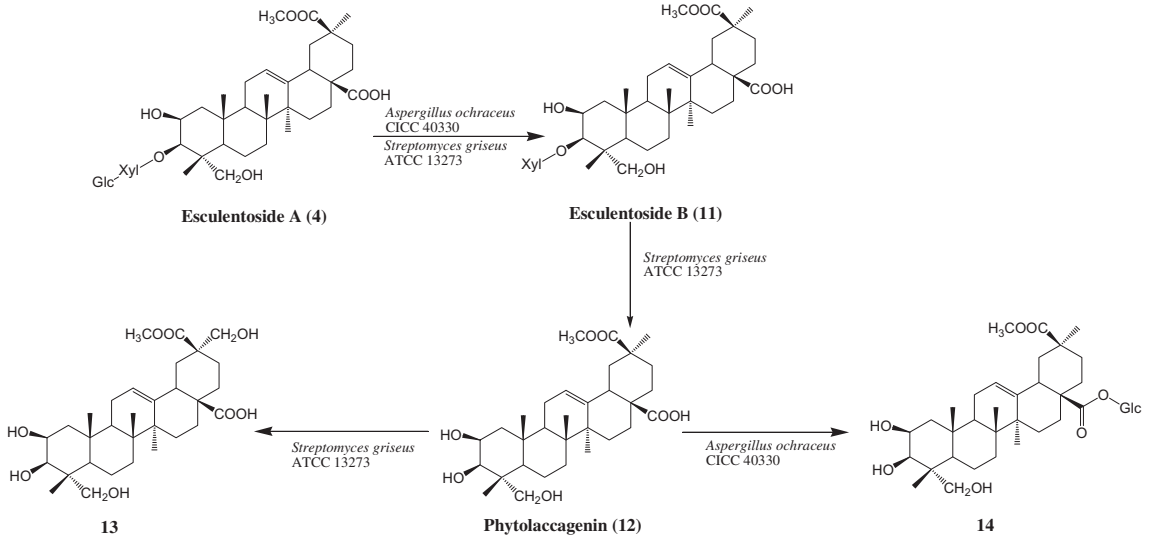
13273 and one same product **11** (112.4 mg) was detected as the only metabolite in the cultures of *A. ochraceus* CICC 40330.

The molecular formula of compound **11**, $C_{36}H_{56}O_{11}$, was established by the $[M-H]^-$ ion at *m*/*z* 663.2 in negative ion mode of ESI-MS, indicating a 162 amu mass loss in the transformation. This indicated that one hexose moiety might be hydrolyzed, this was further confirmed by the comparison of the spectroscopic data with those reported in the literature,[21] and compound **11** was identified as esculentoside B. The ESI-MS of compound **12** showed the $[M-H]^-$ ion at *m*/*z* 531.5 in negative ion mode, indicating another 132 amu mass loss in the following transformation. This suggested that another pentose moiety might be hydrolyzed, this was further confirmed by the comparison of the spectroscopic data with those reported in the literature[21] and the TLC analysis of the acidic hydrolysis experiment, **12** was identified as phytolaccagenin.

While 200 mg of compound **12** was subsequently used as the substrate for the further biotransformation, one more polar metabolite **13** (128.8 mg) was isolated from the culture of *S. griseus* ATCC 13273 and another more polar metabolite **14** (167.8 mg) was isolated from the culture of *A. ochraceus* CICC 40330.

Compound **13** was isolated as colorless power, the molecular formula was established as $C_{31}H_{48}O_8$ by HR-ESI-MS in which a quasimolecular ion was detected at *m*/*z* 547.3252 $[M-H]^-$ (calcd for $C_{31}H_{47}O_8$, 547.3276), suggesting a 16 amu mass increase compared to **12**. After comparison of the NMR spectroscopic data with those reported in the literature,[17] **13** was characterized as 2β,3β,23,29-tetrahydroxy-olean-12-ene-28,30-dioic acid 30-methyl ester. The molecular formula of **14** was established as $C_{37}H_{58}O_{12}$ by HRMS in which a quasimolecular ion was detected at *m*/*z* 693.3822 $[M-H]^-$ (calcd for $C_{37}H_{57}O_{12}$, 693.3855), indicating a 162 amu mass increase of **12**. The ^{13}C NMR spectrum of **14** showed the presence of six new signals at δ 95.8, 79.3, 78.9, 74.1, 71.0, and 61.9 ppm (Table 2), indicating the presence of a glucose moiety. The anomeric carbon at δ 95.79 was typical for an ester glycosidic linked sugar at C-28 carboxy group. Based on the ^1H and ^{13}C NMR data and similar evidence of **7** and **10**, compound **14** was established as 28-*O*-β-D-glucopyranosyl phytolaccagenin.

To the biotransformation of **4** (Scheme 3), the two microorganisms showed different capability to the hydrolysis of the sugar moiety, the glycosidase in the *S. griseus* ATCC 13273 had no selectivity to the glucoside and the xyloside, while the selectivity of the glycosidase in *A. ochraceus* CICC 40330 was much higher, which could provide an alternative way to identify the sugar chain composition of glycosides. Compound **12** is the aglycone of **4**, the main structural differences between **12** and **1**, **2**, **3** were the different substituent of the oxygen-bearing group to the skeleton, and this greatly influenced the biotransformation of *S. griseus* ATCC 13273, the oxidation of the C-29 methyl group was blocked at the



Scheme 3. Microbial transformation of **4**.

hydroxymethyl step, and no C-24 oxidized product was detected, the further zymologic studies and the applications are in progress.

In conclusion, two main reactions, the regio-selective oxidation of the methyl group on C-4 and C-20 and the formation of glycosyl ester of C-28 carboxyl group were observed; both of these two reactions could provide a versatile strategy to expand the structural diversities of OPTs. It is particularly noteworthy that the microbial catalyzed oxygenation of unactivated sp^3 C–H bonds of the methyl group in one step could be achieved by the unique microbe *S. griseus* ATCC 13273, which is rich in P450 enzymes.[22,23] Compared with the synthetic chemical methods,[24] such attempts always involve the heavy metals and tedious steps, to develop the green catalytic methods for the oxidation of unactivated sp^3 C–H bonds will have widespread application in synthetic chemistry and the novel oxidation capability and the enzyme system of *S. griseus* deserves further exploration. To the natural OPTs, another feature of the structural diversities relies on the sugar moiety linked to hydroxyl or carboxyl groups; the sugars attached to skeletons often dictate key pharmacological properties and/or molecular mechanism of action, although there were a number of reports on the microbial glycosylation research,[25] the high efficient and the regio-selective glucosylation of the C-28 carboxyl group of *A. ochraceus* CICC 40330 will take great advantages over other methods.

The OPTs represent a promising and expanding platform for biologically active natural compounds whose potential is currently only partly exploited, our work underlines the value of microbial transformations as a powerful biocatalytic means to expand the structural diversities and prepare novel derivatives of OPTs. Reactions observed were highly regio-specific and functional group specific, and they occurred under the mildest of reaction conditions without the need for blocking groups typically required in synthetic chemistry. The facile biocatalytic oxidation of the methyl groups by whole cells illustrates the advantages and properties of microbial transformations very well in a reaction that is difficult to achieve using synthetic chemical methods.

3. Experimental

3.1. General procedures

NMR spectra were recorded on a Bruker AV-500 spectrometer in C_5D_5N or CD_3OD solution with TMS as the internal standard and chemical shifts were expressed in δ (parts per million). ESI-MS and HR-ESI-TOF-MS experiments were performed on an Agilent 1100 Series MSD Trap mass spectrometer and an Agilent 6210 ESI-TOF spectrometer, respectively. All the solvents used for extraction and isolation were of analytical grade. HPTLC was performed on precoated silica gel GF_{254} plates. Separation and purification were carried out by column chromatography on silica gel (200–300 mesh). Silica gel was purchased from Qingdao Marine Chemical Group Co., PR China.

3.2. Substrate

Compound **1** was prepared by the oxidation of **3** with Jones reagent and purified by crystallization in methanol. The ^1H NMR and ^{13}C NMR spectral data were identical to previous reports.[26]

Compound **2** was synthesized by stirring **3** with acetic anhydride in dry pyridine with the presence of 4-dimethylaminopyridine and purified by crystallization in methanol. The ^1H NMR and ^{13}C NMR spectral data were identical to previous reports.[27]

Compound **3** was purchased from Qingze Pharmacy Co. Ltd., Nanjing China.

Compound **4** was isolated from the roots of *Phytolacca esculenta*. Its structure was characterized by ^1H NMR, ^{13}C NMR, and MS spectra.[21]

The purity of all the substrates was above 98% by the HPLC-ELSD analysis.

3.3. Microorganisms

S. griseus ATCC 13273, *Gliocladium deliquescens* NRRL 1086, *Aspergillus* sp., *Mucor* sp., *Beauveria* sp., *Rhizopus* sp., and *Gibberella* sp. et al. were obtained from a courtesy of Prof. John P.N. Rosazza of University of Iowa, USA. *A. ochraceus* CICC 40330, *Absidia coerulea*, *Saccharomyces cerevisiae*, *Rhizopus chinensis*, and *Penicillium raistrickii* were purchased from China Center of Industrial Culture Collection.

3.4. Analytical-scale biotransformation

3.4.1. Analytical-scale biotransformation of S. griseus ATCC 13273[28]. Cultures were grown by a two-stage procedure in 25 ml of soybean meal glucose medium held in 125 ml culture flasks. The soybean meal glucose medium contained (in g/L) 20 glucose, 5 yeast extract, 5 soybean meal, 5 NaCl, and 5 K_2HPO_4 in distilled water and was adjusted to pH 7.0 with 6 N HCl before being autoclaved at 121 °C for 15 min. Cultures were incubated with shaking at 180 rpm at 28 °C. A 10% inoculum derived from 48-h-old stage I cultures was used to initiate stage II cultures, which were incubated for 24 h before receiving 10 mg of substrates in 1 mL of acetone or methanol and incubations were conducted as before. Substrate controls consisted of sterile medium and substrates incubated under the same conditions but without microorganism. Substrate-containing cultures were generally sampled by removing 3 mL of the entire culture at 24, 48, 72, and 120 h after addition of substrate. Cultures were extracted with equal volume of EtOAc. The organic phase was concentrated and spotted on silica gel HPTLC plate, which was developed by chloroform/methanol (10:1, v/v). The results were visualized by spraying with H_2SO_4 (10% in ethanol) and heating at 120 °C for 1–2 min.

3.4.2. Analytical-scale biotransformations of A. ochraceus CICC 40330[16]. Microbial cultures were grown according to the standard two-stage fermentation protocol. Screening experiments were done, using 125 mL DeLong culture flasks. The culture flasks held one fifth of their volume of the following medium: potato 200 g, glucose 20 g, KH_2PO_4 3 g, $MgSO_4$ 0.75 g. The pH value of the medium was adjusted to 7.0 using 6 N HCl before being autoclaved for 20 min at 121 °C and 15 psi. The flasks were incubated at 200 rpm at 28 °C. A 10% inoculum derived from 72-h-old stage I cultures was used to initiate stage II cultures, which were incubated for 24 h before receiving 10 mg of substrate in 1 mL ethanol, and the incubations were conducted as before. Two kinds of controls were run synchronously with the fermentation and worked-up in the same way. One was the blank culture in order to define and exclude the indigenous secondary metabolites generated by microorganisms. The other was the blank substrate control, i.e., **1** was added to the sterile broth without microorganisms, to test whether **1** would be chemically decomposed or spontaneously transformed under broth and fermentation conditions. Substrate-containing cultures were generally sampled by removing 3 mL of the entire culture at 24, 72, and 144 h after addition of substrate. The mycelium was filtrated and the broth was extracted with EtOAc (125 mL×3). The extracts were concentrated in vacuum and analyzed by HPTLC developed with $CHCl_3$/MeOH, 10:1 and 5:1 (v/v), respectively, and visualized by spraying with 10% sulfanilic acid in ethanol.

3.5. Preparative scale biotransformation, isolation, and identification of biotransformation product[16]

Using 24-h-old stage II cultures, substrate (200 mg) was distributed evenly among thirty 125 mL culture flasks. Substrate-

containing cultures were incubated for 5 days and then extracted with equal EtOAc for three times. The organic solvent layer was evaporated to dryness. The extract was subjected to silica gel column chromatography eluted with solvent system of chloroform/methanol to afford the products. The structure was identified based on its MS and NMR.

*3.5.1. 3-Oxo-olean-12-en-28,29-dioic acid (**5**).* Colorless powders (methanol); HR-ESI-TOF-MS *m/z* 483.3088 $[M-H]^-$ (calcd for $C_{30}H_{43}O_5$, 483.3115); ^1H NMR (C_5D_5N) δ 5.56 (t, 1H, J=3.5 Hz, H-12), 3.45 (dd, 1H, J_1=14.0 Hz, J_2=4.0 Hz, H-18), 1.59 (s, 3H, H-30), 1.26 (s, 3H, H-27), 1.14 (s, 3H, H-23), 1.02 (s, 3H, H-25), 1.01 (s, 3H, H-24), 0.91 (s, 3H, H-26); the ^{13}C NMR spectral data are summarized in Table 1.

*3.5.2. 3-Oxo-24-hydroxy-olean-12-en-28,29-dioic acid (**6**).* Colorless powders (methanol); HR-ESI-TOF-MS *m/z* 499.3060 $[M-H]^-$ (calcd for $C_{30}H_{43}O_6$, 499.3065); ^1H NMR (C_5D_5N) δ 5.54 (t, 1H, J=3.5 Hz, H-12), 4.30 (d, 1H, J=11.0 Hz, H-24α), 3.83 (d, 1H, J=11.0 Hz, H-24β), 3.44 (dd, 1H, J_1=14.0 Hz, J_2=4.0 Hz, H-18), 1.59 (s, 3H, H-30), 1.47 (s, 3H, H-23), 1.25 (s, 3H, H-27), 1.10 (s, 3H, H-25), 1.03 (s, 3H, H-26); the ^{13}C NMR spectral data are summarized in Table 1.

*3.5.3. 28-O-β-D-Glucopyranosyl 3-oxo-olean-12-en-28-oate (**7**).* Colorless powders (methanol); ESI-MS *m/z* 639.8 $[M+Na]^+$; ^1H NMR (C_5D_5N) δ 6.32 (d, 1H, J=8.5 Hz, H-1′), 5.45 (t, 1H, J=3.5 Hz, H-12), 4.48–4.02 (m, 6H), 3.21 (dd, 1H, J_1=14.0 Hz, J_2=4.0 Hz, H-18), 1.21 (s, 3H, H-27), 1.13 (s, 3H, H-23), 1.12 (s, 3H, H-25), 1.01 (s, 3H, H-24), 0.93 (s, 3H, H-30), 0.92 (s, 3H, H-29), 0.89 (s, 3H, H-26); the ^{13}C NMR spectral data are summarized in Table 2.

*3.5.4. 3β-Hydroxy-olean-12-en-28,29-dioic acid (**8**).* Colorless powders (methanol); ESI-MS *m/z* 485.5 $[M-H]^-$; ^1H NMR (C_5D_5N) δ 5.54 (t, 1H, J=3.5 Hz, H-12), 3.43 (dd, 1H, J_1=11.0 Hz, J_2=3.5 Hz, H-18), 1.56 (s, 3H, H-30), 1.27 (s, 3H, H-27), 1.20 (s, 3H, H-23), 1.02 (s, 3H, H-25), 1.00 (s, 3H, H-24), 0.88 (s, 3H, H-26); the ^{13}C NMR spectral data are summarized in Table 1.

*3.5.5. 3β,24-Dihydroxy-olean-12-en-28,29-dioic acid (**9**).* Colorless powders (methanol); HR-ESI-TOF-MS *m/z* 501.3183 $[M-H]^-$ (calcd for $C_{30}H_{45}O_6$, 501.3221); ^1H NMR (C_5D_5N) δ 5.55 (t, 1H, J=3.5 Hz, H-12), 4.47 (d, 1H, J=11.0 Hz, H-24α), 3.65 (d, 1H, J=11.0 Hz, H-24β), 3.43 (dd, 1H, J_1=14.0 Hz, J_2=4.0 Hz, H-18), 1.59 (s, 3H, H-30), 1.53 (s, 3H, H-23), 1.27 (s, 3H, H-27), 0.99 (s, 3H, H-26), 0.87 (s, 3H, H-25); the ^{13}C NMR spectral data are summarized in Table 1.

*3.5.6. 28-O-β-D-Glucopyranosyl 3β-hydroxy-olean-12-en-28-oate (**10**).* Colorless powders (methanol); ESI-MS *m/z* 641.2 $[M+Na]^+$; ^1H NMR (C_5D_5N) δ 6.32 (d, 1H, J=8.1 Hz, H-1′), 5.45 (t, 1H, J=3.4 Hz, H-12), 4.47–4.02 (m, 6H), 3.43 (dd, 1H, J_1=10.9 Hz, J_2=5.1 Hz, H-3); 3.21 (dd, 1H, J_1=14.1 Hz, J_2=4.4 Hz, H-18), 1.24 (s, 3H, H-27), 1.22 (s, 3H, H-23), 1.13 (s, 3H, H-25), 1.02 (s, 3H, H-24), 0.93 (s, 3H, H-30), 0.90 (s, 3H, H-29), 0.89 (s, 3H, H-26); the ^{13}C NMR spectral data are summarized in Table 2.

*3.5.7. Esculentoside B (**11**).* White powder; ESI-MS *m/z* 663.6 $[M-H]^-$; ^1H NMR (C_5D_5N) δ 5.59 (t, 1H, J=3.5 Hz, H-12), 5.05 (d, 1H, J=9.4 Hz, H-1′), 3.62 (s, 3H, $-OCH_3$), 1.55 (s, 3H, H-25), 1.32 (s, 3H, H-24), 1.24 (s, 3H, H-27), 1.18 (s, 3H, H-26), 1.04 (s, 3H, H-29); the ^{13}C NMR spectral data are summarized in Table 2.

*3.5.8. Phytolaccagenin (**12**).* Colorless powders (methanol); ESI-MS *m/z* 531.5 $[M-H]^-$; ^1H NMR (C_5D_5N) δ 5.32 (s, 1H, H-12), 4.29 (br s, 1H, H-2), 4.14 (d, 1H, J=9.5 Hz, H-3), 3.67 (s, 3H, $-OCH_3$), 1.25 (s, 3H, H-25), 1.13 (s, 3H, H-24), 1.13 (s, 3H, H-27), 0.96 (s, 3H, H-26), 0.78 (s, 3H, H-29); the ^{13}C NMR spectral data are summarized in Table 2.

*3.5.9. 2β,3β,23,29-Tetrahydroxy-olean-12-ene-28,30-dioic acid 30-methyl ester (**13**).* White powder; HR-ESI-TOF-MS *m/z* 547.3252 $[M-H]^-$ (calcd for $C_{31}H_{47}O_8$, 547.3276); ^1H NMR (C_5D_5N) δ 5.68 (t, 3H, J=3.5 Hz, H-12), 4.52 (d, 1H, J=3 Hz, H-2), 4.25 (d, 1H, J=4.5 Hz, H-3), 4.15 (d, 1H, J=10.5 Hz, H-23), 3.96 (dd, 2H, J_1=15.9 Hz, J_2=10.5 Hz, H-29), 3.71 (d, 1H, J=10.5 Hz, H-23), 3.67 (s, 3H, $-OCH_3$), 1.59 (s, 3H, H-25), 1.36 (s, 3H, H-24), 1.27 (s, 3H, H-27), 1.11 (s, 3H, H-26); the ^{13}C NMR spectral data are summarized in Table 2.

*3.5.10. 28-O-β-D-Glucopyranosyl phytolaccagenin (**14**).* Colorless powders (methanol); HR-ESI-TOF-MS *m/z* 693.3822 $[M-H]^-$ (calcd for $C_{37}H_{57}O_{12}$, 693.3855); ^1H NMR (C_5D_5N) δ 6.30 (d, 1H, J=8.1 Hz, H-1′), 5.60 (t, 1H, J=3.3 Hz, H-12), 4.52 (br s, 1H, H-2), 4.38–4.13 (m, 7H), 3.98 (d, 1H, J=9.5 Hz, H-3), 3.69 (d, 1H, J=10.5 Hz, H-23), 3.59 (s, 3H, $-OCH_3$), 3.19 (dd, 1H, J_1=14.0 Hz, J_2=4.0 Hz, H-18), 1.61 (s, H-25), 1.35 (s, H-24), 1.22 (s, H-27), 1.19 (s, H-26) and 1.17 (s, H-29); the ^{13}C NMR spectral data are summarized in Table 2.

Acknowledgements

This work was supported by the National Nature Science Foundation of China (NSFC No. 20602040, 30672603). Thanks also given to the financial support from the State Administration of Foreign Expert Affairs of China (No. 111-2-07) and the '111 Project' from the Ministry of Education of China.

References and notes

1. Huang, D.; Ding, Y.; Li, Y.; Zhang, W.; Fang, W.; Chen, X. *Cancer Lett.* **2006**, *233*, 289–296.
2. Konoshma, T.; Yasuda, I.; Kashiwada, Y.; Cosentino, L. M.; Lee, K. H. *J. Nat. Prod.* **1995**, *58*, 1372–1377.
3. Sashi, B. M.; Sucharita, S. *Phytochemistry* **1997**, *44*, 1185–1236.
4. Jung, S.; Ha, Y. *Biochem. J.* **2007**, *403*, 243–250.
5. Cheng, K.; Liu, J.; Liu, X.; Li, H.; Sun, H.; Xie, J. *Carbohydr. Res.* **2009**, *344*, 841–850.
6. Giner-Larza, E. M.; Manez, S.; Recio, M. C.; Giner, R. M.; Prieto, J. M.; Cerda-Nicolas, M.; Rios, J. L. *Eur. J. Pharmacol.* **2001**, *428*, 137–143.
7. Joseph, D. C.; Robert, A. H. *Nat. Prod. Rep.* **2010**, *27*, 79–132.
8. Nick, A.; Wright, A. D.; Rali, T.; Sticher, O. *Phytochemistry* **1995**, *40*, 1691–1695.
9. Recio, M. C.; Giner, R. M.; Manez, S.; Rios, J. L. *Planta Med.* **1995**, *61*, 182–185.
10. Sakurawi, K.; Yasuda, F.; Tozyo, T.; Nakamura, M.; Sato, T.; Kikuchi, J.; Terui, Y.; Ikenishi, Y.; Iwata, T.; Takahashi, K.; Konoike, T.; Mihara, S.; Fujimoto, M. *Chem. Pharm. Bull.* **1996**, *44*, 343–351.
11. Honda, T.; Rounds, B. V.; Bore, L.; Finlay, H. J.; Favaloro, F. G., Jr.; Suh, N.; Wang, Y.; Sporn, M. B.; Gribbl, G. W. *J. Med. Chem.* **2000**, *43*, 4233–4246.
12. Wen, X.; Sun, H.; Liu, J.; Cheng, K.; Zhang, P.; Zhang, L.; Hao, J.; Zhang, L.; Ni, P.; Zographos, S. E.; Leonidas, D. D.; Alexacou, K. M.; Gimisis, T.; Hayes, J. M.; Oikonomakos, N. G. *J. Med. Chem.* **2008**, *51*, 3540–3554.
13. Andres, P.; Francisco, R.; Andres, G.; Antonio, M. *Mini-Rev. Org. Chem.* **2009**, *6*, 307–320.
14. Cheng, Z. H.; Yu, B. Y.; Cordell, G. A.; Qiu, S. X. *Org. Lett.* **2004**, *6*, 3163–3165.
15. Zhang, J.; Cheng, Z. H.; Yu, B. Y.; Cordell, G. A.; Qiu, S. X. *Tetrahedron Lett.* **2005**, *46*, 2337–2340.
16. Qian, L. W.; Zhang, J.; Liu, J. H.; Yu, B. Y. *Tetrahedron Lett.* **2009**, *50*, 2193–2195.
17. Qian, L. W.; Zhang, J.; Liu, J. H.; Yu, B. Y. *Chin. J. Chem.* **2009**, *27*, 1434–1436.
18. Yu, S. S.; Yu, D. Q.; Liang, X. T. *Phytochemistry* **1995**, *38*, 695–698.
19. Strauss, A.; Spengel, S. M.; Schaffner, W. *Phytochemistry* **1995**, *38*, 861–865.
20. Li, J. F.; Chen, S. J.; Zhao, Y.; Li, J. X. *Carbohydr. Res.* **2009**, *344*, 599–605.
21. Woo, W. S.; Kang, S. S.; Yamasaki, K.; Tanaka, O. *Arch. Pharm. Res.* **1978**, *1*, 21–25.
22. Hosny, M.; Dhar, K.; Rosazza, J. P. N. *J. Nat. Prod.* **2001**, *64*, 462–465.
23. Gopishetty, S. R.; Heinemann, J.; Deshpande, M.; Rosazza, J. P. N. *Enzyme Microb. Technol.* **2007**, *40*, 1622–1626.
24. Lothar, B.; Tadashi, H.; Gordon, W. G. *J. Org. Chem.* **2000**, *65*, 6278–6282.
25. Feng, X.; Zou, Z. M.; Fu, S. B.; Sun, L. Z.; Su, Z. H.; Sun, D. A. *J. Mol. Catal. B: Enzym.* **2010**, *66*, 219–223.
26. Toshiro, K.; Kazuhiro, T.; Yoshitaka, A.; Isao, H. *J. Org. Chem.* **1997**, *62*, 960–966.
27. Zhang, Y.; Li, J. X.; Zhao, J.; Wang, S. Z.; Pan, Y.; Tanaka, K.; Kadota, S. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1629–1632.
28. Betts, R. E.; Walters, D. E.; Rosazza, J. P. N. *J. Med. Chem.* **1974**, *17*, 599–602.