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Design, Synthesis and Evaluation of Pentacyclic Triterpenoids Similar to Glycyrrhetinic Acid Via Combination of Chemical and Microbial Modification as Glycogen Phosphorylases Inhibitor

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A series of pentacyclic triterpenoids similar to glycyrrhetinic acid were designed and synthesized via the combination of chemical modification and microbial catalysis. All products were screened for the glycogen phosphorylases inhibitory activities in vitro. Within this series of derivatives, compound **5** displayed good inhibitory activities with IC_{50} value of 27.7 μM, which is better than that of the other derivatives and glycyrrhetinic acid. Structure–activity relationship (SAR) analysis of these inhibitors was also discussed.

Keywords: Biocatalysis, glycyrrhetinic acid, oleanolic acid, *Streptomyces griseus* ATCC 13273, *Aspergillus ochraceus* CICC 40330

Introduction

It is well established that licorice is still appreciated as a medicinal root. And the history of this plant as a pharmacological remedy dates back far into the past [1]. Glycyrrhizic acid (GL), the major bioactive component of licorice root extract, has a variety of pharmacological properties including anti-inflammatory, antioxidant and immune-modulating activities [2–5]. Furthermore, it also has been exploited in the treatment of hepatic injury [6]. Pharmacokinetic studies have shown that GL exhibits its pharmacological functions through its biologically active metabolite, 18β-glycyrrhetinic acid (GA), which is formed by presystemic hydrolysis (Fig. 1).

With the aim of enriching the structural diversities and exploring more effective and/or less toxicity derivatives, a lot of research has been focused on the modification of GA. Due to its complex skeleton and few chemical active sites, modifications were tethered on C-3, C-11, and C-30. Currently, biocatalysis is in the spotlight. It is increasingly being used to assist in synthetic routes to complex and chiral molecules of pharmaceutical interest [7–9]. Nowadays,

microbial transformation is being employed as an efficient method to conduct the modification of pentacyclic triterpenoids [10]. And in our previous studies, several novel modifications via microbial transformation have been reported individually [11, 12]. The oxygenation of unactivated sp^3 C-H bonds of the C-29 methyl group to a carboxyl group is a very special modification catalyzed by *Streptomyces griseus* ATCC 13273. It prompted us to design a number of compounds which are similar to GA synthesized from oleanolic acid (OA) by the combination of chemical and biocatalysis modification.

Glycogen phosphorylases (GP) play an important role in glucose metabolism, especially in glycogenolytic pathway. It is well known that liver GP is the major enzyme for controlling hepatic glucose output, and inhibition of this enzyme may afford a useful therapeutic approach for type II diabetes [13]. And pentacyclic triterpenoids such as maslinic acid and corosolic acid had been developed as a new class of natural GP inhibitors [14]. Herein, we report the design, synthesis and biological evaluation of several derivatives similar to GA as GP inhibitors.
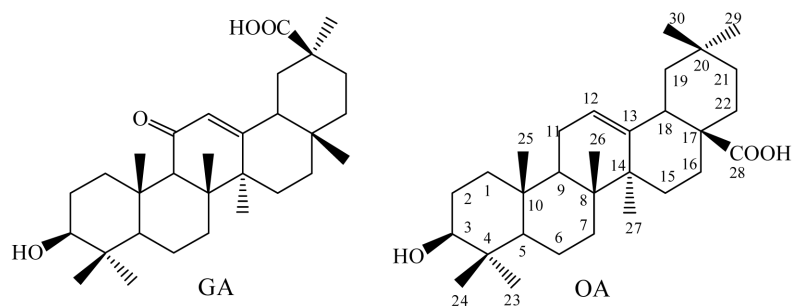
Fig. 1. Structure of GA and OA.

Materials and Methods

General Procedures

NMR spectra were recorded on a Bruker AV-500 spectrometer in CD_5N or $CDCl_3$ solution with TMS as the internal standard and chemical shifts were expressed in δ (parts per million). ESI-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. TLC was performed on precoated silica gel GF254 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., P. R. China.

Substrate

Compound **2** was purchased from Qingze Pharmacy Co. Ltd., Nanjing China.

Compound **3** was synthesized by stirring **2** with acetic anhydride in dry pyridine with the presence of 4-dimethylaminopyridine and purified by crystallization in methanol.

Preparation of compound **4** was carried out by the following steps: 70% t-BuOOH (2.0 ml, 14.0 mmol) was added to CH_2Cl_2 (20 ml) containing CrO_3 (10 mg, 0.1 mmol) and stirred for 15 min. Next, a solution of **3** (1.0 g, 2.0 mmol) was introduced. Stirring was maintained at 65°C for 6 h. The deprotection of the hydroxyl group was carried out in methanol with 4.0 mol/l NaOH at 60°C overnight.

Compound **7** was prepared by the oxidation of **4** with Jones Reagent and purified by crystallization in methanol.

Microorganisms

S. griseus ATCC 13273 was obtained courtesy of Prof. John P.N. Rosazza of University of Iowa, USA. *A. ochraceus* CICC 40330 was purchased from China Center of Industrial Culture Collection.

Preparative Scale Biotransformation, Isolation, and Identification of Biotransformation Product

Using 24-h-old stage II cultures, substrates (200.0 mg) were distributed evenly among thirty 150 ml culture flasks and incubated for 120 h. The cultures were collected and centrifuged at 5,500 g

for 10 min while the fermation was finished. Supernatants were extracted with EtOAc for 3 times. The organic solvent layer was evaporated to dryness. The extract was subjected to silica gel column chromatography eluted with different gradients of $CHCl_3$-MeOH to afford the products.

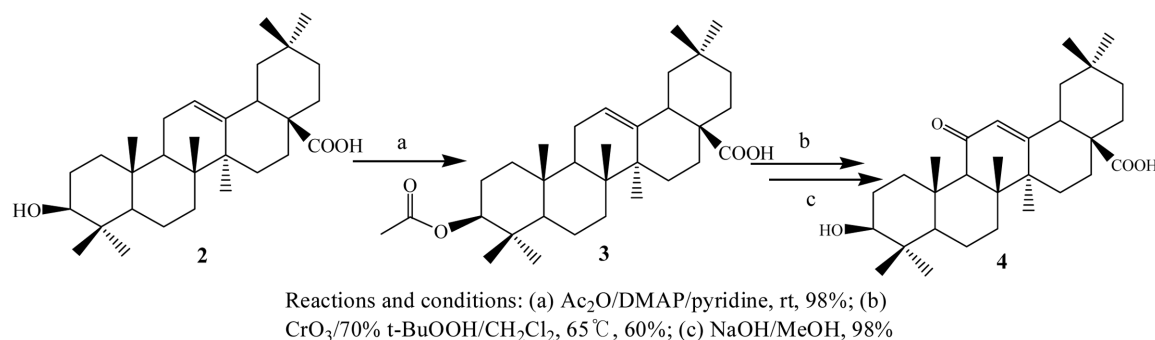### Biological Evaluations of These Products as GP Inhibitors

The inhibitory activity of the test compounds against rabbit muscle glycogen phosphorylase a (GPa) was monitored using microplate reader (Thermo) based on a published method [14, 15]. GPa activity was measured in the direction of glycogen synthesis by the release of phosphate from glucose 1-phosphate. Samples were dissolved in DMSO and diluted at different concentrations for IC_{50} determination, respectively. The enzyme was added to 100 μl of buffer containing 50 mM Hepes (pH 7.2), 100 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM glucose 1-phosphate, 1 mg/ml glycogen, and the test compound in 96-well microplates. After the addition of 150 μl of 1 M HCl containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green, the mixture was incubated at 22°C for 25 min, and then the phosphate absorbance was measured at 655 nm. The IC_{50} values were estimated by fitting the inhibition data to a dose dependent curve.

Results and Discussion

Identification of the Products Obtained by the Combination of Chemical Modification and Biocatalysis

Preparation of 11-keto oleanolic acid (**4**) was outlined in Fig. 2. Compound **3** was synthesized by stirring **2** with acetic anhydride in dry pyridine with the presence of 4-dimethylaminopyridine (DMAP) and purified by crystallization in methanol. The chromium-mediated allylic oxidation of **3** using 70% tert-butylhydroperoxide was carried out following the literature [16]. 4 mol/l NaOH was applied on deprotection of C-3 hydroxyl group in methanol at 60°C overnight with a three-step total yield of 63.2%. The ^{1}H-NMR and ^{13}C-NMR spectral data of **4** were identical to previous reports [17].

Compound **4** (200.0 mg) was added into a 2-day-old

Reactions and conditions: (a) Ac₂O/DMAP/pyridine, rt, 98%; (b)
CrO₃/70% t-BuOOH/CH₂Cl₂, 65 ℃, 60%; (c) NaOH/MeOH, 98%

Fig. 2. Preparation of 11-keto oleanolic acid (**4**).

cultivation of *S. griseus* ATCC 13273, and one metabolite (**5**) was detected by TLC at the end of 96 h incubation. Based on the isolation of silica gel column chromatography and purification in methanol, **5** (113.4 mg) was obtained from the fermentation broth with the yield of 53.4% (Fig. 3).

The ESI-MS of compound 6 showed an [M+Cl]⁻ ion at m/z 667.4 corresponding to the molecular formula of $C_{36}H_{55}O_9Cl$, indicating a 162 amu mass increase of **4**.

Compound **5** was isolated as colorless powder. The ESI-MS of **5** showed an [M-H]⁻ ion at m/z 499.4 corresponding to the molecular formula of $C_{30}H_{44}O_6$, indicating a 30 amu mass increase to that of compound **4** with the addition of two oxygen and absence of two hydrogen. The ¹³C NMR spectrum showed a new signal at δ 180.9 ppm and only six characteristic methyl signals appeared at δ 28.2, 23.5, 19.8, 19.5, 16.7, and 16.6 ppm with the absence of one methyl signal in the substrate (Table 1). It suggested that one

methyl group might be oxidized to a carboxyl group, and by comparing the signals of **4**, the carbon signal at δ 23.6 ppm attributed to C-30 of substrate shifted up-field 3.7 ppm to 19.8 ppm in the metabolite, indicating that the substitution carried out at C-29. In addition, the three-bond correlation between δ 1.49 (s, 3H, H-30) ppm and δ 180.5 ppm was observed in the HMBC spectrum indicating that the methyl group of C-29 was oxidized to a carboxyl group. The relative stereochemistry of the carboxyl group was further established as equatorial based on NOESY experiments. The NOESY spectrum of **5** showed NOE correlation 1.49 (s, 3H, H-30) and 3.48 (dd, 1H, J_1=11.5 Hz, J_2=4.5 Hz, H-18) ppm (Fig. 4), which can also indicate that the methyl of C-30 was without change and the additional carboxyl group attributed to C-29. Based on all the evidences, compound **5** was identified as 3β-hydroxy-11-oxo-olean-12-en-28, 29-dioic acid.
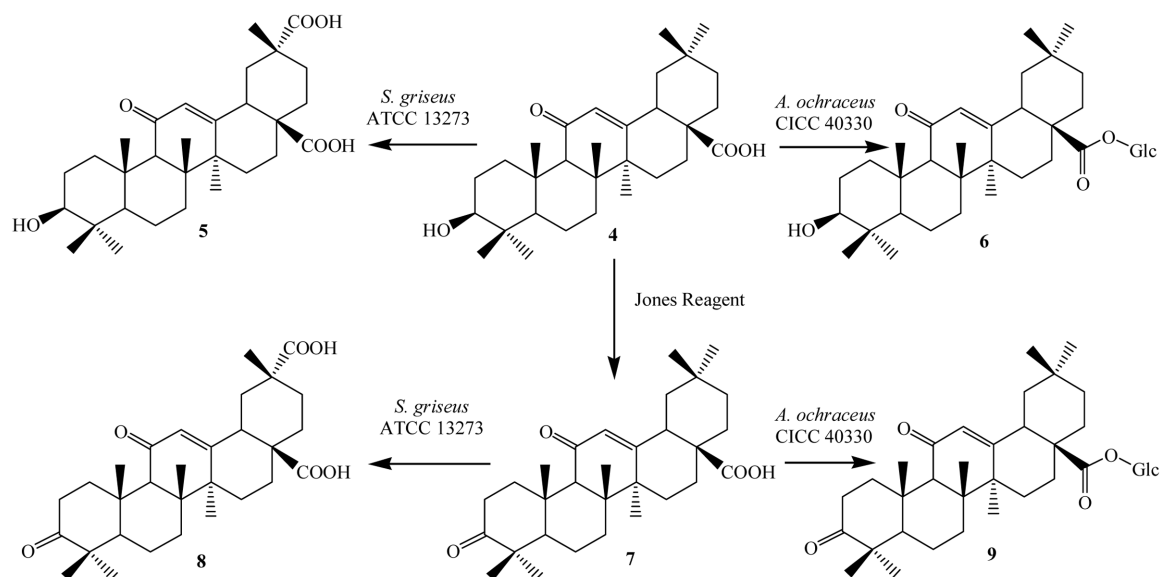
Fig. 3. Microbial transformation of *S. griseus* ATCC 13273 and *A. ochraceus* CICC 40330.

Table 1. ^{13}C-NMR spectral data of **4-9** (recorded in C$_5$D$_5$N, 125 MHz).

Carbon	4*	5	6	7#	8	9
1	39.2	39.8	39.9	41.6	39.3	39.3
2	27.3	28.8	28.8	36.0	34.4	34.2
3	78.8	77.9	78.0	219.0	215.9	216.3
4	39.2	39.8	39.8	49.6	47.7	47.6
5	55.0	55.5	55.5	57.2	55.1	55.2
6	17.4	17.9	18.0	20.9	19.0	18.8
7	31.7	31.5	31.6	34.1	31.4	31.6
8	45.1	45.4	45.6	46.8	45.2	45.4
9	61.8	62.3	62.4	62.9	61.4	61.6
10	37.4	37.9	37.9	38.8	37.2	37.2
11	200.4	200.0	200.0	201.4	199.3	199.6
12	128.1	128.6	128.4	129.9	128.4	128.2
13	168.3	168.9	168.7	170.6	169.5	168.9
14	43.5	44.1	44.0	45.5	44.2	44.1
15	27.8	28.4	28.2	29.7	28.4	28.2
16	22.7	23.6	23.6	24.5	23.6	23.5
17	46.0	46.2	46.5	47.9	46.2	46.6
18	41.4	41.5	42.2	43.4	41.5	42.3
19	44.2	39.4	44.4	46.1	40.0	44.2
20	30.7	42.3	30.7	32.6	42.3	30.6
21	33.7	33.4	33.8	35.3	32.5	33.5
22	32.8	29.2	32.8	33.5	29.2	32.1
23	28.2	28.2	28.2	28.4	26.7	26.7
24	16.3	16.6	16.6	23.2	21.5	21.3
25	15.6	16.7	16.8	17.5	15.8	15.5
26	19.4	19.5	19.5	20.6	19.1	18.9
27	23.4	23.5	23.4	25.3	23.4	23.3
28	182.4	179.5	176.1	184.2	179.5	176.2
29	32.9	180.5	33.3	34.7	180.4	33.1
30	23.6	19.8	23.2	25.4	19.8	23.5
Glc-1			96.0			95.9
2			79.5			79.5
3			78.9			78.8
4			74.2			74.3
5			71.2			71.2
6			62.2			62.5

*Recorded in CDCl$_3$.

#The signal of each carbon shifted down-field 2.0 ppm when compared with the literature [18].

While the broth of *A. ochraceus* CICC 40330 was used as the biocatalysis system, 11-keto oleanolic acid (**4**, 200.0 mg) was added, then metabolites **6** (130.6 mg, 46.0%) were isolated after 96h respectively (Fig. 3).

Compound **6** was isolated as colorless powder, the ESI-MS of compound **6** showed an [M+Cl]$^-$ ion at *m/z* 667.4 corresponding to the molecular formula of C$_{36}$H$_{56}$O$_9$, indicating a 162 amu mass increase of **4**. It was suggested that a hexose moiety was introduced. Whereas, the ^{13}C-NMR spectrum showed the presence of six new signals at δ 96.0, 79.5, 78.9, 74.2, 71.2, and 62.2 ppm, that are characteristic of β-D-glucose. The anomeric carbon at δ 96.0 was typical for an ester glycosidic linked sugar at C-28 carboxy group. By comparison of the spectroscopic data with those reported in the literature [18], compound **6** was established as 28-*O*-β-D-glucopyranosyl 3β-hydroxy-11-oxo olean-12-en-28-oate.

Furthermore, compound **7** was prepared by the oxidation of **4** with Jones Reagent and purified by crystallization in methanol with a yield of 95.8%. By comparision of the spectroscopic data with the literature, **7** was identified as 3, 11-dioxoolean-12-en-28-oic acid [19].

The same reaction was carried out while using compound **7** (200.0 mg) as the substrate in the procedure of biocatalysis, and the metabolites **8** (126.2 mg, 59.4%) and **9** (122.8 mg, 43.2%) were isolated respectively (Fig. 3). **8** was identified as 3, 11-dioxoolean-12-en-28, 29-dioic acid, and **9** was established as 28-*O*-β-D-glucopyranosyl-3, 11-dioxoolean - 12-en-28-oate.

Spectral data of compounds **4-9** were listed as follows.

3β-hydroxy-11-oxo-olean-12-en-28-oic acid (**4**). Colorless powders; ESI-MS *m/z* 470.6 [M+Cl]$^-$; ^{1}H-NMR (CDCl$_3$) δ 5.62 (S, 1H, H-12), 3.22 (dd, 1H, J_1=10.5 Hz, J_2=5.5 Hz, H-18), 1.36 (s, 3H, H-27), 1.11 (s, 3H, H-25), 0.99 (s, 3H, H-23), 0.94 (s, 3H, H-26), 0.93 (s, 3H, H-24), 0.92 (s, 3H, H-29), 0.78 (s, 3H, H-30); the ^{13}C-NMR spectral data is summarized in Table 1.

3β-hydroxy-11-oxo-olean-12-en-28, 29-dioic acid (**5**). Colorless powders; ESI-MS *m/z* 499.4 [M-H]$^-$; ^{1}H-NMR (C$_5$D$_5$N) δ 6.10 (S, 1H, H-12), 3.48 (m, 1H, H-18), 3.48(m, 1H, H-3), 3.24 (dt, 1H, J_1=13.0 Hz, J_2=3.5 Hz, H-1β), 1.50 (s, 3H, H-30), 1.42 (s, 3H, H-27), 1.30 (s, 3H, H-25), 1.24 (s, 3H, H-23), 1.21 (s, 3H, H-26), 1.05 (s, 3H, H-24); the ^{13}C-NMR spectral data is summarized in Table 1.

28-*O*-β-D-glucopyranosyl 3β-hydroxy-11-oxo-olean-12-en-28-oate (**6**). Colorless powders; ESI-MS *m/z* 667.4 [M+Cl]$^-$; ^{1}H-NMR (C$_5$D$_5$N) δ 6.31 (d, 1H, *J*=8.0 Hz, H-1') 5.97 (S, 1H, H-12), 4.47-4.03 (m, 6H), 3.48 (dd, 1H, J_1=11.5 Hz, J_2=4.5 Hz, H-18), 1.39 (s, 3H, H-27), 1.32 (s, 3H, H-25), 1.31 (s, 3H, H-23), 1.24 (s, 3H, H-26), 1.05 (s, 3H, H-24), 0.89 (s, 3H, H-29), 0.82 (s, 3H, H-30); the ^{13}C-NMR spectral data is summarized in Table 1.

3, 11-dioxo-olean-12-en-28-oic acid (**7**). Colorless powders;
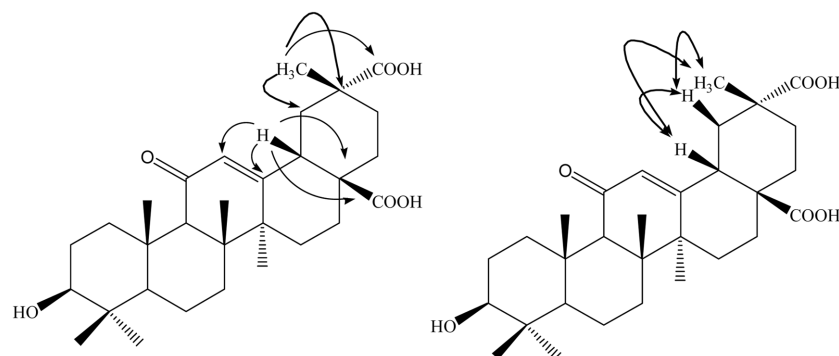
Fig. 4. Key HMBC and NOESY correlations of **5**.

ESI-MS m/z 467.3 [M-H]$^-$; ^{1}H-NMR (C$_5$D$_5$N) δ 5.66 (S, 1H, H-12), 3.00 (m, 1H, H-18), 1.37 (s, 3H, H-27), 1.22 (s, 3H, H-25), 1.09 (s, 3H, H-23), 1.03 (s, 3H, H-24), 0.96 (s, 3H, H-26), 0.94 (s, 3H, H-30), 0.93 (s, 3H, H-29); the ^{13}C-NMR spectral data is summarized in Table 1.

3, 11-dioxo-olean-12-en-28, 29-dioic acid (**8**). Colorless powders; ESI-MS m/z 497.3[M-H]$^-$; ^{1}H-NMR (C$_5$D$_5$N) δ 6.06 (S, 1H, H-12), 3.48 (dd, 1H, J_1=10.0 Hz, J_2=3.0 Hz, H-18), 1.46 (s, 3H, H-30), 1.35 (s, 3H, H-27), 1.24 (s, 3H, H-25), 1.14 (s, 3H, H-23), 1.11 (s, 3H, H-24), 1.00 (s, 3H, H-26); the ^{13}C-NMR spectral data is summarized in Table 1.

28-*O*-β-D-glucopyranosyl 3, 11-dioxo-olean-12-en-28-oate (**9**). Colorless powders; ESI-MS m/z 629.2 [M-Hl]$^-$; ^{1}H-NMR (C$_5$D$_5$N) δ 6.28 (d, 1H, J=8.0 Hz, H-1′) 5.67 (S, 1H, H-12), 4.51-4.02 (m, 6H), 3.24 (m, 1H, H-18), 1.35 (s, 3H, H-27), 1.32 (s, 3H, H-25) , 1.22 (s, 3H, H-26), 1.16 (s, 3H, H-23), 1.08 (s, 3H, H-24), 0.91 (s, 3H, H-29), 0.80 (s, 3H, H-30); the 13C-NMR spectral data is summarized in Table 1.

Biological Evaluation

The above-synthesized derivatives were evaluated in an enzyme inhibition assay against rabbit muscle glycogen phosphorylase a (GPa), an enzyme that shares considerable sequence similarity with human liver GPa. Caffeine was employed as the positive control. The IC$_{50}$ results are summarized in (Table 2). As illustrated in Table 2, three derivatives (**4**, **5**, **8**) exhibited better inhibitory activity than GA (**1**). Unfortunately there is no derivative better than OA (**2**).

Discussion

The structural diversity of natural originated pentacyclic triterpenes mainly attributed to the substitution of hydroxyl or carbonyl groups for the methyl or methenyl carbons of the skeleton and the formation of corresponding glycosides [20]. Hereby, the research on the structural modification of pentacyclic triterpenes was focused on how to introduce the functional groups to the skeleton with the aim of enriching the structural diversities and exploring more effective and/or less toxicity derivatives [15, 21].

Oleanolic acid is a common pentacyclic triterpenoids compound. But due to its complex skeleton and few chemical active sites, modifications were tethered on C-3 and C-29. In view of the complex skeleton and less active position, microbial transformation is being employed as an efficient method for modification nowadays [22]. In our previous research, the oxygenation of unactivated sp^3 C-H

Table 2. Result of rabbit muscle GPa inhibition assay.

Compound	X	Y	Z	R	IC$_{50}$ (μM)
caffeine	-	-	-	-	128.4 ± 15.8
1	-	-	-	-	69.7 ± 7.2
2	OH(β), H(α)	H$_2$	CH$_3$	H	18.2 ± 2.7
3	AcO(β), H(α)	H$_2$	CH$_3$	H	NA*
4	OH(β), H(α)	O	CH$_3$	H	65.7 ± 5.3
5	OH(β), H(α)	O	COOH	H	27.7 ± 2.9
6	OH(β), H(α)	O	CH$_3$	Glc	NA
7	O	O	CH$_3$	H	88.2 ± 11.4
8	O	O	COOH	H	35.4 ± 6.4
9	O	O	CH$_3$	Glc	NA

*No activity.

bonds of the C-29 methyl group to a carboxyl group by microbial transformation had been reported [12]. Furthermore, the chemo-selectivity and stereo-selectivity research of this modification were also investigated. Oxidation of the C-29 methyl group was proved to be a stable regio-selective reaction; the C-30 methyl group cannot be oxidized. The active –OH of C-3 and C-28 also influenced the microbial conventions seriously. If they were modified by acetylation or esterification, the reactions were blocked. Additionally, only one major metabolite could be detected in the biocatalysis system of *S. griseus* ATCC 13273 while using **4** as the substrate, which surprised us very much. The oxygenation of unactivated sp³ C-H bonds, catalyzed by *S. griseus* ATCC 13273, could form only one final metabolite of **5,** without a hydroxymethyl group at C-24. Accordingly, we concluded that introduction of carbonyl group at C-11 would block the oxygenation at C-24. Besides, the glucosylation also had good selectivity and efficiency, which conformed to the other pentacyclic triterpenoids substrates catalyzed by *A. ochraceus* CICC 40330.

Hence, we designed the synthesis of GA analogues using oleanolic acid as a substrate for the first time. These two biocatalysis systems in the modification of olean-type pentacyclic triterpenes were applied with definite purpose. In this research, an oleanolic acid derivative (**5**) similar with glycyrrhetinic acid was designed and synthesized by the combination of chemical modification and biocatalysis. And based on this, we have been working on the synthesis of glycyrrhetinic acid C-20 isomer.

Furthermore, a series of oleanolic acid derivatives have been designed, synthesized and evaluated as GP inhibitors. The results of GP inhibition assay indicated that compound **5** exhibited a better potency in derivatives. Preliminary sructure–activity relationships can be briefly discussed. Comparing the inhibitory activities of compounds **2** and **4**, modification at C-11 with a ketone group led to a little decrease of activity. The presence of a sugar moiety in triterpene saponins resulted in markedly decreased activity (**4** vs **6**, **7** vs **9**). It indicates the C-28 carboxyl group of pentacyclic triterpenoids seems to contribute to the GPa enzyme inhibitory activity. But it is worth mentioning that pentacyclic triterpenoids have very poor water solubility. The incorporation of sugar can improve water solubility, and the sugar ester bond can be easily hydrolyzed in vivo. Furthermore, comparing the inhibitory activities of compounds **4**, **5**, **7**, and **8** revealed the structure-activity relationship at the C-3 and C-29 positions. C-29 carboxyl group led to a better GPa inhibitory activity (**4** vs **7**, **5** vs **8**). On the other hand, oxidation of C-3 hydroxyl to a ketone group led to little decrease (**4** vs **5**, **7** vs **8**).

In conclusion, our work underlines the value of microbial transformations as a powerful biocatalytic means to design and synthesize novel derivatives of pentacyclic triterpenoids. The facile biocatalytic modification of the methyl groups without the need for blocking groups illustrates the advantages and properties of microbial transformations very well while also showing that it is difficult to achieve using synthetic chemical methods.

Acknowledgment

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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