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# Microbial oxidation and glucosidation of echinocystic acid by *Nocardia corallina*

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ABSTRACT

Echinocystic acid (3β,16α-dihydroxy-olean-12-en-28-oic acid **1**) is a complex compound with many bioactivities. Microbial transformation of echinocystic acid (**1**) by *Nocardia corallina* CGMCC4.1037 has been studied. Incubation of echinocystic acid with *N. corallina* CGMCC4.1037 afforded three metabolites: 3-oxo-16α-hydroxy-olean-12-en-28-oic acid (**2**), 3β,16α-dihydroxy-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (**3**), and 3-oxo-16α-hydroxy-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (**4**). Product (**4**) was a new product. Their structure elucidation was mainly based on the HRMS and NMR data. The possible mechanism of the regio-selective oxidation of the 3-OH and a probable sequence of these transformations were also discussed. This is the first report on the microbial transformation of echinocystic acid (**1**). This method may open a new route to the future modification of echinocystic acid (**1**).



## 1. Introduction

The pentacyclic triterpene echinocystic acid (**1**) is a very interesting compound. It was first isolated from *Echinocystis fabacea* in 1934 [1]. Because of its complex structure, it took more than 10 years to establish its structure [2–8]. Its absolute configuration was confirmed in 1974 [9]. Since its discovery, especially in recent years, echinocystic acid and its saponins have been found in many plants [10–14]. Echinocystic acid and its saponins have been reported to have cytotoxic effects against different cell lines, including the J774.A1, HEK-293, and WEHI-164 cell lines [15], the HepG2, HL-60 cells [16,17], the A375, Hela, and L929 cell lines [18] in vitro. Echinocystic acid and its saponins have many other bioactivities, including anti-HIV activities [19], antifungal activities [20], inhibitory activity toward pancreatic lipase [21], immunostimulatory effect [22,23], inhibition of mast cell degranulation [24], and the interleukin-18 inhibitory activities [25].

Although more and more bioactivities of echinocystic acid and its saponins have been reported, there is almost no chemical or enzymatic modification of echinocystic acid reported. Structural diversity is very important for the future research.

Enzymes have been used to catalyse the transformation of both macromolecular substrates and small molecules [26] in aqueous environments as well as in organic solvent [27]. Because of their high regio- and chemo-selectivity, enzymes have been widely used by chemists seeking selective catalytic agents [28–30]. The diversity of potentially useful enzymes and microbes at the chemist's disposal is now vast. The microbial transformation can be used as a powerful tool to create extremely unusual compounds [31–35]. We have showed that the microorganisms can transform the 7-epi-10-deacetylbaccatin III (7-epi-10-DAB III) to very important intermediate 10-deacetylbaccatin III (10-DAB III) [36]. We hope to utilize the power of microbes to transform echinocystic acid and to obtain some products that were difficult to obtain from chemical methods for further bioactivity assay or chemical modifications. A series of microbes were screened for the transformation of echinocystic acid (**1**). Herein, we reported in this article the selective oxidation and glucosidation of echinocystic acid (**1**) by *Nocardia corallina* CGMCC4.1037.

## 2. Experimental

### 2.1. General experimental procedures

NMR spectra were measured on a Bruker DRX-500 spectrometer (Rheinstetten, Germany) with $Me_4Si$ as internal standard (at 500 MHz for $^1H$ NMR and at 125 MHz for $^{13}C$ NMR). High-resolution electrospray ionization mass spectra (ESI-MS) were obtained with a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The reaction mixture and products were analyzed on Si gel GF254 plates (Qingdao Oceanic Chemicals, Qingdao, China). The mobile phase was petroleum ether/THF (2:1) and $CHCl_3$/MeOH (9:1). The visualization of TLC plates was performed by spraying with 10% $H_2SO_4$ in methanol followed by heating. The analytical HPLC was carried out on a Waters™ 600 Controller and Pump, with Waters™ 996 Photodiode Array Detector (Waters Cor-

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poration, Milford, MA, USA) and a GraceSmart RP 18 column (5 μm, 4.6 mm × 250 mm) (Grace Vydac, Hesperia, CA, USA). The mobile phase consisted of 60% methanol and 40% water. The flow rate was 1 ml/min. The column temperature was set at 25°C. The detection wave-length was 210 nm. The injection volume was 20 μl. General solvent and reagents were purchased from Beijing Chemical Industry Company (Beijing, China). The 3D model was generated using a Chem3D® ultra version 8 (Cambridgesoft Corporation, Cambridge, MA, USA).

### 2.2. Microorganism and the substrate echinocystic acid (**1**)

The microbe *N. corallina* CGMCC4.1037 was purchased from China General Microbiological Culture Collection center (CGMCC, Beijing, China).

The substrate echinocystic acid was purchased from Beijing Feiside(First) Bio-pharmaceutical Development Co., Ltd. (Beijing, China). Its structure was confirmed by comparison its $^{1}$H, $^{13}$C NMR spectra with those reported in the literature [22,37].

### 2.3. Microbial transformation of echinocystic acid (**1**)

The pH value of the beef-protein medium (peptone 10.0 g/l, beef extract 3.0 g/l, NaCl 5.0 g/l, Aoboxing Biotechnology Co., Ltd., Beijing, China) was adjusted to 7.0 before sterilized in autoclave for 20 min at 121 °C. The flasks were incubated at 27 °C and 170 rpm on an orbital shaker. Two control cultures with the same medium and with only substrate or with only fungus *N. corallina* CGMCC4.1037 were used in the same experimental conditions.

Cultures were grown according to the standard two-stage fermentation protocol. Stage I seeds were inoculated with microorganism obtained from freshly grown agar slants.

To eight 1000 ml Erlenmeyer flasks each containing 500 ml of sterile liquid beef-protein medium were added small part of *N. corallina* CGMCC4.1037 stage I seeds. The cultures were incubated for 48 h, after which time a total of 220 mg echinocystic acid (dissolved in 4.4 ml ethanol) was evenly distributed among eight flasks. The cultures were further incubated for 7 days, then harvested and extracted three times with equal volumes (500 ml) of $CH_2Cl_2$ and evaporated to dryness under reduced pressure to give a brown oil (300 mg).

### 2.4. Isolation of metabolites

The crude residue (300 mg) was first purified by column chromatography on silica gel (300–400 mesh, 20 g) eluted with a stepwise petroleum ether/acetone from 100:1 to 1:4 (1500 ml). Five fractions were obtained: fraction A (64.3 mg), B (87.5 mg), C (57.8 mg), D (19.6 mg), and E (69.1 mg). Fraction A was further recrystallized in petroleum ether/THF (3:1) to afford a metabolite (**2**) (50.1 mg, 22.8%). Fraction B was unreacted echinocystic acid (87.5 mg). Fraction D was further purified by column chromatography on silica gel (300–400 mesh, 15 g) eluted with a stepwise $CHCl_3$/MeOH from 19:1 to 9:1 (700 ml) to afford two products (**3**) (1.4 mg, 0.5%) and (**4**) (0.8 mg 0.4%).

### 2.5. Compound data

#### 2.5.1. Echinocystic acid (3β,16α-dihydroxy-olean-12-en-28-oic acid **1**)

$^{1}$H NMR (500 MHz, pyridine-$d_5$): 5.66 (1H, brs, H-12), 5.26 (1H, s, H-16), 3.63 (1H, dd, $J$ = 4.0, 9.0 Hz, H-18), 3.46 (1H, dd, $J$ = 5.0, 11.0 Hz, H-3), 1.84 (3H, s, H-27), 1.22 (3H, s, H-23), 1.18 (3H, s, H-30), 1.07 (3H, s, H-24), 1.05 (3H, s, H-29), 1.02 (3H, s, H-26), 0.93 (3H, s, H-25); $^{13}$C NMR (125 MHz, pyridine-$d_5$): 180.4 (C-28), 145.5 (C-13), 122.8 (C-12), 78.5 (C-3), 75.2 (C-16), 56.3 (C-5), 49.3 (C-17), 47.7 (C-9), 47.7 (C-19), 42.6 (C-14), 41.9 (C-18), 40.3 (C-8), 39.8 (C-4), 39.4 (C-1), 37.8 (C-10), 36.6 (C-21), 36.6 (C-15), 34.0 (C-7), 33.7 (C-29), 33.3 (C-22), 31.5 (C-20), 29.2 (C-23), 28.6 (C-2), 27.6 (C-27), 25.1 (C-30), 24.3 (C-11), 19.2 (C-6), 18.0 (C-26), 17.0 (C-24), 16.1 (C-25). The literatures [22,34] reported NMR data, we reported our data here for convenient purpose for readers.

#### 2.5.2. 3-Oxo-16α-hydroxy-olean-12-en-28-oic acid (**2**)

White solid, mp 320–322 °C, HR-ESI-MS: 469.3235 $[M-H]^-$ (calcd 469.3312), 939.6538 $[2M-H]^-$. $^{1}$H NMR (500 MHz, pyridine-$d_5$): 5.65 (1H, s, H-12), 5.25 (1H, s, H-16), 3.63 (dd, $J$ = 4.5, 15.0 Hz, H-18), 1.87 (s, H-27), 1.18 (s, H-23), 1.13 (s, H-30), 1.06 (s, H-24), 1.05 (s, H-29), 1.02 (s, H-26), 0.97 (s, H-25); $^{13}$C NMR (125 MHz, pyridine-$d_5$): 216.2 (C-3), 180.0 (C-28), 145.2 (C-13), 122.2 (C-12), 74.7 (C-16), 55.4 (C-5), 48.9 (C-17), 47.4 (C-4), 47.3 (C-19), 46.4 (C-9), 42.2 (C-14), 41.6 (C-18), 39.8 (C-8), 39.2 (C-1), 37.0 (C-10), 36.2 (C-21), 36.1 (C-15), 34.4 (C-2), 33.3 (C-29), 32.9 (C-7), 32.9 (C-22), 31.1 (C-20), 27.1 (C-23), 26.7 (C-27), 24.7 (C-30), 23.9 (C-11), 21.6 (C-24), 19.9 (C-6), 17.3 (C-26), 15.1 (C-25). The literature [35] reported the NMR data with DMSO-$d_6$ as solvent. We report the NMR data in pyridine for further references.

#### 2.5.3. 3β,16α-dihydroxy-olean-12-en-28-oic acid 28-O-β-D-glucopyranoside (**3**)

White solid, HR-ESI-MS: 657.4050 $[M+Na]^+$ (calcd 657.3973), 1291.8213 $[2M+Na]^+$, 495.3593 $[M+Na-C_6H_{10}O_5]^+$. $^{1}$H NMR (500 MHz, pyridine-$d_5$): 6.33 (1H, d, $J$ = 8.0 Hz, H-1 of Glc), 5.63 (1H, s, H-12), 5.32 (1H, s, H-16), 3.53 (1H, dd, $J$ = 4.5, 14.5 Hz, H-18), 3.45 (1H, dd, $J$ = 4.5, 11.5 Hz, H-3), 1.82 (s, H-27), 1.20 (s, H-23), 1.17 (s, H-30), 1.04 (s, H-24), 1.02 (s, H-29), 0.99 (s, H-26), 0.95 (s, H-25); $^{13}$C NMR (125 MHz, pyridine-$d_5$): 176.4 (C-28), 144.9 (C-13), 123.2 (C-12), 78.6 (C-3), 74.6 (C-16), 56.7 (C-5), 49.6 (C-17), 47.8 (C-9), 47.7 (C-19), 42.5 (C-14), 41.8 (C-18), 40.6 (C-8), 39.8 (C-4), 39.5 (C-1), 37.9 (C-10), 36.6 (C-21), 36.4 (C-15), 34.0 (C-7), 33.6 (C-29), 32.6 (C-22), 30.4 (C-20), 28.6 (C-23), 27.7 (C-2), 25.1 (C-27), 24.3 (C-30), 23.3 (C-11), 18.0 (C-6), 17.0 (C-26), 16.2 (C-24), 14.6 (C-25), 96.3 (C-1 of glucose), 79.7 (C-3 of glucose), 79.3 (C-5 of glucose), 74.6 (C-2 of glucose), 71.6 (C-4 of glucose), 62.7 (C-6 of glucose). The literature [34] reported only partially data of this compound.

#### 2.5.4. 3-Oxo-16α-hydroxy-olean-12-en-28-oic acid 28-O-β-D-glucopyranoside (**4**)

White solid, HR-ESI-MS: 655.3937 $[M+Na]^+$ (calcd 655.3814), 1287.7982 $[2M+Na]^+$, 493.3374 $[M+Na-C_6H_{10}O_5]^+$. $^{1}$H NMR and $^{13}$C NMR see Table 1.

### 2.6. Microbial metabolism of 3-oxo-16α-hydroxy-olean-12-en-28-oic acid (**2**) by N. corallina CGMCC4.1037

*N. corallina* CGMCC4.1037 was first grown in one 1000 ml culture flasks containing 500 ml of medium for 2 days. To this flask was added 10 mg of (**2**). The cultures were further incubated for another 7 days and then were extracted three times with EtOAc (3 × 500 ml). The combined extracts were dried over anhydrous $Na_2SO_4$ and evaporated to dryness at 50°C under reduced pressure to afford an orange brown residue. The residue was analyzed by TLC ($CHCl_3$/MeOH 9:1) and HPLC with compound (**4**) as reference. No compound (**4**) was detected.

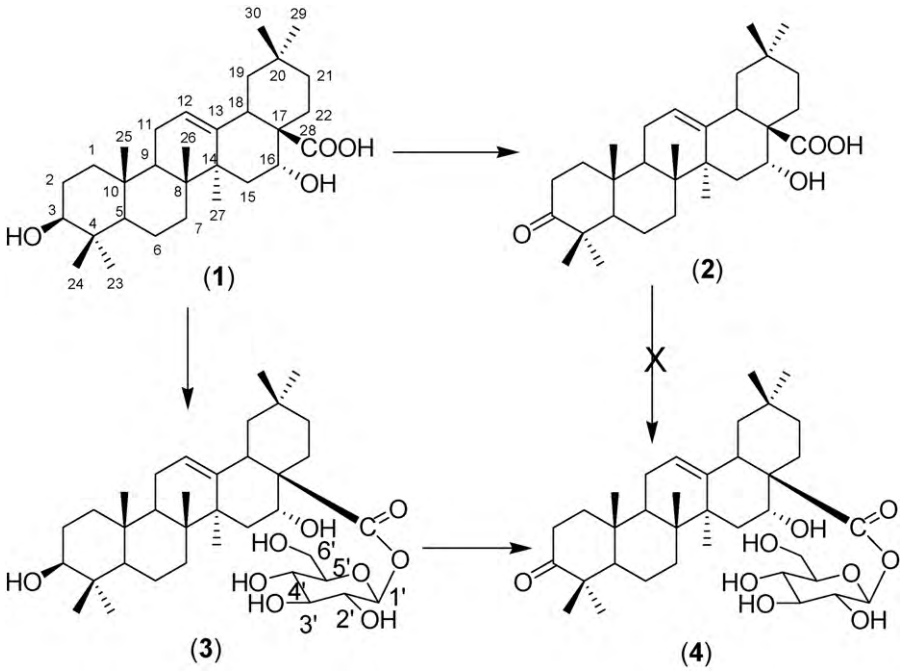
## 3. Results and discussion

The preliminary screenings with 43 collected microbes showed that *N. corallina* CGMCC4.1037 transformed echinocystic acid (**1**)

**Table 1**
NMR data of compound (**4**) (pyridine-$d_5$, 500 MHz for $^1$H NMR and 125 MHz for $^{13}$C NMR).

| No. | H | C | HMBC(H → C) |
|---|---|---|---|
| 1 | 1.37 (m) | 40.5 | |
| 2 | $\alpha$ 2.57 (m), $\beta$ 2.44 (m) | 35.6 | 10 |
| 3 | – | 217.6 | |
| 4 | – | 48.7 | |
| 5 | 1.35 (m) | 56.6 | |
| 6 | 1.44 (m) | 20.5 | |
| 7 | $\alpha$ 1.44 (m), $\beta$ 1.57 (m) | 34.1 | |
| 8 | – | 41.3 | |
| 9 | 1.83 (m) | 47.7 | |
| 10 | – | 38.2 | |
| 11 | 1.98 (m) | 25.2 | |
| 12 | 5.65 (s) | 123.8 | 11 |
| 13 | – | 145.8 | |
| 14 | – | 43.4 | |
| 15 | $\alpha$ 1.83 (m), $\beta$ 2.43 (m) | 37.3 | |
| 16 | 5.30 (s) | 75.6 | |
| 17 | – | 50.4 | |
| 18 | 3.54 (dd, 5.0, 14.0) | 42.7 | 17 |
| 19 | $\alpha$ 1.41 (m), $\beta$ 2.82 (t, 14.0) | 48.4 | 20 |
| 20 | – | 32.1 | |
| 21 | $\alpha$ 1.27 (m), $\beta$ 2.56 (m) | 37.2 | |
| 22 | $\alpha$ 2.15 (m), $\beta$ 2.45 (m) | 33.4 | |
| 23 | 1.14 (s) | 27.9 | 3, 4, 5, 24 |
| 24 | 1.04 (s) | 22.8 | 3, 4, 5, 23 |
| 25 | 0.98 (s) | 16.4 | 1, 5, 9, 10 |
| 26 | 1.16 (s) | 18.7 | 7, 8, 9, 14 |
| 27 | 1.81 (s) | 28.4 | 8, 13, 14, 15 |
| 28 | – | 177.2 | – |
| 29 | 1.06 (s) | 34.5 | 19, 20, 21 |
| 30 | 1.02 (s) | 25.8 | 19, 20, 21 |
| 28-*O*-Glc | | | |
| 1′ | 6.33 (d, 8.5) | 97.2 | 28 |
| 2′ | 4.18 (t, 7.0) | 75.5 | |
| 3′ | 4.07 (m) | 80.2 | |
| 4′ | 4.32 (t, 4.0) | 72.4 | 3′ |
| 5′ | 4.32 (t, 4.0) | 80.7 | 6′ |
| 6′ | 4.40 (dd, 4.0, 10.0); 4.48 (dd, 2.0, 10.0) | 63.5 | 4′ |

Mult., multiplicity, s, singlet; d, doublet; t, triplet; m, multiplet. The assignment of the data was deducted from the $^{13}$C NMR, H-HCOSY, HSQC and HMBC.




**Scheme 1.** Microbial oxidation and glucosidation of echinocystic acid by *N. corallina* CGMCC4.1037.

into three products (Scheme 1). From the silica gel TLC analysis, it was found that the major product was less polar than the substrate echinocystic acid (**1**), while the other two minor products were much more polar than the substrate echinocystic acid (**1**).

Compound (**2**) was obtained as a white solid. It was determined to have the molecular formula $C_{30}H_{46}O_4$ by its HR-ESI-MS data ($[M-H]^-$: 469.3235). It was two protons less than the substrate echinocystic acid (**1**). Comparing the $^1$H NMR spectrum of substrate (**1**) with product (**2**), the major difference was the disappearance of the signal at $\delta$ 3.46 ppm in product (**2**), while this signal was attributed to H-3 in echinocystic acid (**1**). Comparing the $^{13}$C NMR spectrum of product (**2**) to that of the substrate (**1**) the $^{13}$C NMR data of (**1**) are listed in the experimental section for comparative purposes. they were pretty similar except for the chemical shifts of C-2, C-3, and C-4. The most significant difference was carbon-3 in compounds (**1**) and (**2**): The hydroxyl group ($\delta$ 78.5 ppm) in substrate (**1**) was replaced with a ketone group ($\delta$ 216.2 ppm) in product (**2**). The chemical shifts of C-2 and C-4 in product (**2**) were down shifted (5.8 and 7.6 ppm respectively). From its polarity, its MW, its $^1$H NMR spectrum, and particularly its $^{13}$C NMR spectrum, the structure of product (**2**) was characterized as 3-oxo-16α-hydroxy-olean-12-en-28-oic acid. This ketone was previous isolated from natural resources [38].

Compound (**3**) was obtained as a white solid. Its structure was deducted from its HRMS data and NMR spectra. Its molecular formula was determined as $C_{36}H_{58}O_9$ on the basis of its HRMS data ($[M+Na]^+$: 657.4050). It has six carbons more than the substrate echinocystic acid (**1**). Comparing the $^1$H NMR spectrum of product (**3**) to that of the substrate echinocystic acid (**1**), product (**3**) has an anomeric proton at δ 6.33 (1H, d, $J$ = 8.0 Hz) and typical glucose moiety protons at $\delta$ 4.00–4.46 ppm. The other $^1$H NMR signals of these two compounds were very similar. The $^1$H NMR spectrum of product (**3**) suggested that it may have a glucose moiety in the molecular. In the $^{13}$C NMR spectrum, chemical shift of the C-28 carboxyl group in product (**3**) was observed at $\delta$ 176.4 ppm (4 ppm up-shifted from the substrate (**1**)), implying that there was a sugar linkage was formed at the C-28 carboxyl group. This was confirmed by an anomeric carbon observed at $\delta$ 96.3 ppm. The sugar was defined as glucose based on its HRMS, $^1$H NMR spectrum and $^{13}$C NMR spectrum. The coupling constant ($J$ = 8.0 Hz) of the anomeric hydrogen at $\delta$ 6.33 ppm indicated that the glucose had a β-configuration. The structure of product (**3**) was elucidated as 3β,16α-dihydroxy-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside. This compound was previously obtained as a hydrolysis product of a saponin [37].

Compound (**4**) was obtained as a white solid. Its molecular formula was determined as $C_{36}H_{56}O_9$ on the basis of its HRMS spectrum ($[M+Na]^+$: 655.3937). It is two protons less than the product (**3**). The $^1$H NMR spectrum of compound (**4**) was similar to that of compound (**3**) except for the disappearance of a signal at $\delta$ 3.45 ppm for the 3-β-H in compound (**3**). The $^{13}$C NMR data of compounds (**3**) and (**4**) are very similar except for the carbon-3 ($\delta$ 78.6 and $\delta$ 217.6 ppm respectively). From the above evidence, the carbon-3 of compound (**4**) was a ketone group instead of a hydroxyl group. This assignment of compound (**4**) could also be deducted from the comparison its HRMS and NMR data to that of compound (**2**). Thus the structure of (**4**) was elucidated as 3-oxo-16α-hydroxy-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside. It was a new compound.

The glycosidation of triterpenoids in microbial transformations was very rare [39] and the yield of the glucosidation product was very low [40] which was also true to our results. The glycosidase favorably catalyzed hydrolysis than glycosidation in aqueous condition while catalyzed reversed hydrolysis and transglycosidations in dry media [41]. The microbial transformations usually occur in aqueous condition which is hard to accumulate glycosidation products.

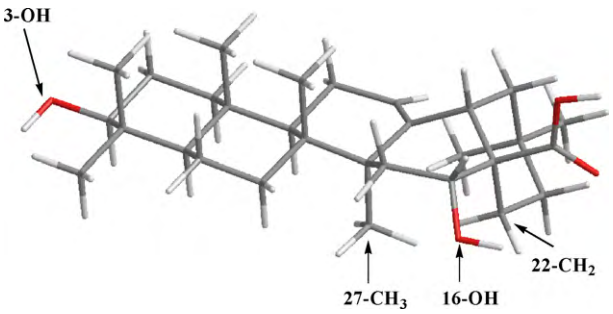



**Fig. 1.** 3D Model of echinocystic acid (**1**).

There are two hydroxyl groups in echinocystic acid (**1**) at C-3 and C-16 with only 3-OH transformed. The probable reason may be resulted from the spatial position of these two hydroxyl groups (Fig. 1). The 3-β-OH was at equatorial position and it was more accessible to enzymes. The 16-α-OH was at axial position and was more "crowded" with other groups like the 27-$CH_3$ and 22-$CH_2$. The spatial position of 16-α-OH may prevent enzymes accessing it. This microbial transformation provided a convenient way to differentiate the two hydroxyl groups of echinocystic acid (**1**) and thus opened a new route for future modification of this compound.

The possible sequence of these transformations was shown in Scheme 1. The product (**2**) had the highest yield and was directly transformed from the substrate (**1**). Product (**3**) should also be a direct glucosidation product from substrate (**1**) albeit in low yield. There are two routes leading to the product (**4**). The first one is the glucosidation of product (**2**). The second one is the oxidation of product (**3**). We have incubated product (**2**) with *N. corallina* CGMCC4.1037 at the same condition as that used in our experiments and found that the product (**2**) could not be transformed to product (**4**). Since this microorganism had the ability to oxidize 3-OH to 3-ketone, the oxidation of product (**3**) to afford product (**4**) is more likely. Unfortunately, we do not have enough product (**3**) to complete this experiment.

## 4. Conclusion

Echinocystic acid (**1**) was a complex compound with lot of bioactivities. There was no structure modification of this compound reported. Herein, we reported for the first time biotransformation of echinocystic acid (**1**) by *N. corallina* CGMCC4.1037. Incubation of echinocystic acid (**1**) with *N. corallina* CGMCC4.1037 afforded three metabolites: 3-oxo-16α-hydroxy-olean-12-en-28-oic acid (**2**), 3β,16α-dihydroxy-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (**3**), 3-oxo-16α-hydroxy-olean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (**4**). The glycosidation of echinocystic acid (**1**) was very rare in triterpenoid biotransformation. The selective oxidation of the 3-OH in echinocystic acid (**1**) that was not easily achievable by conventional synthetic methods provided a convenient way to differentiate the two hydroxyl groups in this compound. This may open a new route for future modification of echinocystic acid (**1**).

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