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Direct microbial-catalyzed asymmetric α-hydroxylation of betulonic acid by *Nocardia* sp. NRRL 5646

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ABSTRACT

Microbial transformation of betulonic acid by *Nocardia* sp. NRRL 5646 in preparative scale resulted in the isolation of one unexpected asymmetric α-hydroxylation product, methyl 2α-acetoxy-3-oxo-lup-20(29)-en-28-oate, and one known compound methyl 3-oxo-lup-20(29)-en-28-oate. The structures of metabolites were elucidated unambiguously by ESI-MS, 2D-NMR spectroscopy. This is the first successful microbial transformation of ketone α-hydroxylation of lupane-type pentacyclic triterpenes.

Since the discovery of betulinic acid (**1**) as a selective inhibitor of human melanoma,[1] lupane-type pentacyclic triterpenes have been found to involve more activities, such as anti-cancer, anti-HIV, and anti-inflammatory activities.[2–4] Due to its complex skeleton and few chemical active sites, the organic structural modifications were tethered on C-3, C-20, and C-28,[5–7] hereby enhancing the structural diversity is very essential for the research and development of these compounds. In recent studies, we demonstrated that microbial transformation could be a fruitful tool to enhance the structural diversity of pentacyclic triterpenes, some novel reactions such as the direct conversion of the ursane to the oleanane triterpene skeleton, regio-selective methyl hydroxylation, and methyl migration were carried out by different microorganisms.[8–10] As a continuation of these efforts, lupane-type triterpene betulonic acid (**2**) (Fig. 1) was chosen for biotransformation study. The microbe *Nocardia* sp. NRRL 5646 which has been used to catalyze numerous valuable reactions, including carboxylic acid and aldehyde reduction, phenol methylation, and flavone hydroxylation[11–13], was used for the biotransformation and the substrate was incubated on preparative scale according to the standard two-stage fermentation protocol.[14]

A 110 mg sample of **2** ($C_{30}H_{46}O_3$, M_r = 454) was used and two less polar metabolites labeled Met-**1** (**3**) and Met-**2** (**4**) were developed after the biotransformation. Silica gel chromatographic separation of the transformation residue resulted in the isolation of compounds **3** and **4** in 49.8% and 6.42% yield, respectively, based on weight relative to starting substrate.

Met-**1** (**3**) was obtained as colorless needles in methanol. The positive ion ESI-MS showed a quasimolecular ion at *m/z* $[M+Na]^+$ 491.4 and the formula was deduced as $C_{31}H_{48}O_3$, indicating a 14 amu mass increase in substrate **1**. Based on NMR data, a new proton singlet appeared at δ_H 3.67 (s, 3H) and one additional oxygenated methyl signal was observed at δ 51.3 in ^{13}C NMR spectrum. Comparing that with compound **2**, the ^{13}C signal of carboxyl group of C-28 was shifted up-field 5.6 ppm from 182.3 to 176.7 ppm, this implied that Met-1 (**3**) was a methyl ester of betulonic acid. Therefore, Met-**1** was identified as methyl 3-oxo-lup-20(29)-en-28-oate.

Met-**2** (**4**) was isolated as white powder. The molecular formula of **4** was established as $C_{33}H_{50}O_5$ by HR-ESI-TOF-MS in which a quasimolecular ion was detected at *m/z* 549.3548 $[M+Na]^+$ (calcd for $C_{33}H_{50}O_5Na$, 549.3550), suggesting a 72 amu mass increase in substrate **1**. Based on the NMR data, the same C-28 carboxylic acid methyl ester structure was observed by comparing that with Met-1. Additionally, in the ^1H NMR spectrum, two new proton signals displayed at δ_H 2.13 (s, 3H) and 5.59 (1H, dd, J = 13.25 Hz, J = 6.15 Hz), and the signal at δ_H 2.98 (2H, m) of Met-1 was disappeared. In the ^{13}C NMR spectrum, three new carbon signals, respectively, displayed at δ_C 170.3, 20.7 and 71.8 (Table 1), and the signal at δ_C 47.0 of Met-1 was disappeared. In the 2D NMR spectra, the proton signal at δ_H 2.13 (s, 3H) should be directly

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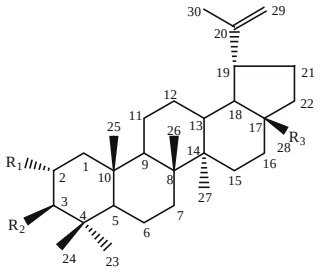

compound	R_1	R_2	R_3
1	H	OH	COOH
2	H	=O	COOH
3	H	=O	$COOCH_3$
4	AcO	=O	$COOCH_3$

Figure 1. The structures of compounds **1**–**4**.

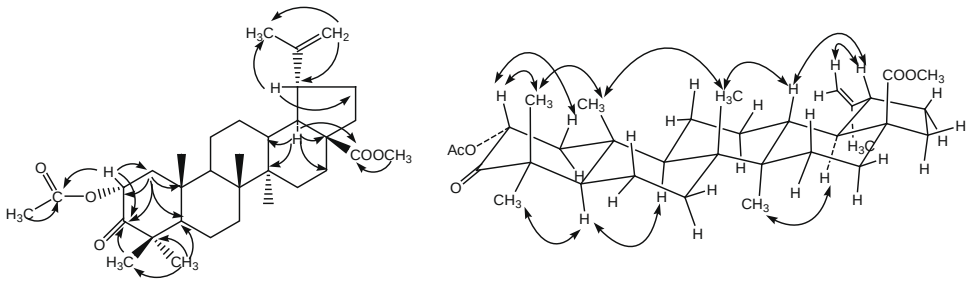

Figure 2. Key H–C HMBC and H–H NOESY spectra of compound **4**.

Table 1
^{13}C NMR spectral data of compound Met-2 (125 MHz, $CDCl_3$)

Carbon	Met-2	Carbon	Met-2	Carbon	Met-2
1	46.1	12	25.3	23	24.7
2	71.8	13	38.2	24	21.0
3	209.5	14	42.5	25	16.5
4	48.6	15	29.7	26	16.1
5	57.3	16	32.1	27	14.6
6	19.0	17	56.5	28	176.6
7	34.1	18	49.4	29	109.8
8	40.8	19	47.0	30	19.3
9	50.3	20	150.3	OCH_3	51.3
10	38.2	21	30.6	CH_3CO	170.3
11	21.2	22	36.9	CH_3CO	20.7

attached to the carbon signal at δ_C 20.7 according to the correlation analysis of HSQC spectrum, and which was also observed to correlate to the carbon signal at δ_C 170.3 from HMBC spectrum, indicating the presence of an acetoxyl group in the molecule. And the proton signal at δ_H 5.59 should be assigned to the new methine signal at δ_C 71.8 on the basis of the HSQC and DEPT spectral data analysis. Comparing the ^{13}C NMR and DEPT spectral of Met-2 with those of Met-1, C-1,C-3 signals shifted down-field by 6.5 ppm and up-field by 8.6 ppm, respectively, and the disappearance of a methylene signal at 34.2 ppm (C-2) suggested that the acetoxyl group should be substituted at C-2 and this was further confirmed by the two-bond and three-bond correlation in HMBC spectrum (Fig. 2). The relative stereochemistry of the acetoxyl group at C-2 was established as α (equatorial) based on NOESY spectrum. The NOESY spectrum showed key correlations between every two of H-2, H-$1_{(equa)}$, CH_3-24 and CH_3-25, suggesting that the H-2 should be β configuration. In view of all these observations, Met-2 was characterized as methyl 2α-acetoxy-3-oxo-lup-20(29)-en-28-oate.

Optically active α-hydroxy carbonyl moieties are commonly found in some important natural products. Extensive efforts have been exerted to generate new methodologies to enantioselectively synthesize these compounds[15] and our research provided an alternative method to achieve this. In the microbial transformation of betulonic acid, at least two activities were involved in ketone α-hydroxylation, that is, the introducing of asymmetric hydroxyl group at C-2 and the acetylation of the hydroxyl group. Hydroxylation and acetylation are two consecutive processes with the assumption that the two catalytic active domains are proximate, which prevent the intramolecular interconversion of active groups and the formation of isomers which frequently occurred during the ketone α-hydroxylation synthesis.[16,17] To our knowledge, unlike 3-OH triterpenoids that were derived from oxidosqualene or squalene, 2-OH was introduced after the formation of triterpenoid skeleton[18] and the *ortho*-hydroxyl groups are important functional groups of triterpenes for anti-inflammatory and antitumor activities,[19,20] so the research to explore whether the α-hydroxylation catalyzed by *Nocardia* sp. occurs on other types of pentacyclic triterpenes is ongoing. Finally the versatile catalytic capabilities of *Nocardia* sp. to generate structural diversity in already complex natural products are of pharmaceutical interest and await for further exploration.

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