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Novel biotransformation of pentacyclic triterpenoid acids by *Nocardia* sp. NRRL 5646

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Abstract—Six pentacyclic triterpene acids, ursolic acid, oleanolic acid, betulinic acid, 23-hydroxybetulinic acid, glycyrrhetinic acid, and senegenin, were metabolized by the microbe *Nocardia* sp. NRRL 5646 to selectively furnish their corresponding 28-methyl esters. Notably, ursolic acid (**1**) was converted to oleanolic acid methyl ester (**4**) via two intermediates, oleanolic acid (**2**), and ursolic acid methyl ester (**3**), which are formed by participation of 'retro-biosynthetic' methyl migration from C-19 to C-20. Senegenin (**11**) was selectively converted to a nortriterpene methyl ester, senegenic acid methyl ester (**12**), via an unprecedented C–C bond cleavage. The stereochemical assignments of compounds **11** and **12** were made unambiguously for the first time using 2D NMR spectroscopy.

Natural products (NPs) are an incredibly diverse group of small (usually molecular weight less than 1500 Da) organic compounds isolated from a variety of natural sources, principally plants. The reason that NPs capture the imagination of organic chemists and pharmaceutical scientists is because of their well documented and wide ranging biological activities and their skeletal diversity and intriguing functional group characteristics, which render them as indispensable leads for probing biological system status and for drug discovery with new bioassay systems. Recently, we demonstrated that microbial transformation could be a fruitful tool to enhance the structural diversity of natural triterpene glycosides.[1] As a continuation of these efforts to expand the structural diversity of natural products using microbial transformation, a group of pentacyclic triterpene acids (TAs) were chosen for biotransformation study. TAs are very widely distributed in the plant kingdom and have been shown to possess a wide range of biological activities. For example, ursolic acid (**1**) and betulinic acid (**5**) possess anti-cancer and anti-HIV activities.[2,3] Betulinic acid is in preclinical development. It is envisioned that biotransformation of these TAs may provide analogs, which could be utilized for screening for new activities or for studying SAR. A panel of microbes was utilized to screen for their ability to transform the TAs. *Nocardia* sp. NRRL 5646 was found to convert these substrates efficiently to less polar metabolites. This organism is widely used for transformation because of its broad spectrum of enzymatic capability, including carboxylic acid and aldehyde reduction, phenol methylation, and the noteworthy skeleton rearrangement of quinovic acid glycosides via methyl migration.[1] In this report, the biotransformation results of six triterpenoid acids, ursolic acid (**1**), oleanolic acid (**2**), betulinic acid (**5**), 23-hydroxybetulinic acid (**7**), glycyrrhetic acid (**9**), and senegenin (**11**), by *Nocardia* sp. are described. Each of the six triterpene acids was incubated on a preparative scale with the *Nocardia* sp. according to the standard, two-stage fermentation protocol.[1,4] The work-up and isolation of the metabolites were carried out as described previously.[1]

A 200 mg sample of **1** ($C_{30}H_{48}O_3$, $M_r = 456$) was used for the scale-up incubation and three metabolites, coded MT-1 (**2**), MT-2 (**3**), and MT-3 (**4**), were formed from the biotransformation, as evident from the HPTLC analysis. Silica gel chromatographic separation of the

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transformation residue resulted in the isolation of compounds **2** (21%), **3** (9%), and **4** (13% yield).

MT-1 (**2**) was obtained as a white powder. The positive ion ESI-MS showed a quasimolecular ion at *m/z* $[M+H]^+$ 457.2, and a molecular formula $C_{30}H_{48}O_3$was deduced from the HR-ESI-MS, indicating that **2** has the same molecular formula as that of the parent compound **1**. The structure of **2** was assigned as oleanolic acid by comparison of its ^1H and ^{13}C NMR spectra with literature data,[5] as well as co-TLC comparison with an authentic sample.

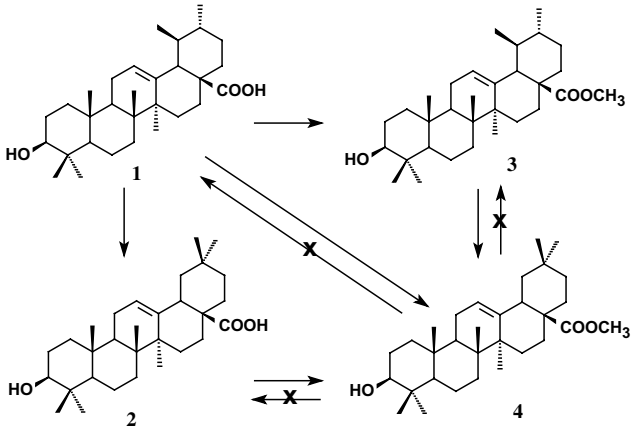
MT-2 (**3**) and MT-3 (**4**) were isolated as colorless powders, and their structures were readily assigned to ursolic acid methyl ester and oleanolic acid methyl ester, respectively, by comparison of their ^1H and ^{13}C NMR data with the literature.[6–9] Thus methylation at the C-28 carboxylic acid of ursolic acid (1) occurred through catalysis by the enzyme system of *Nocardia* sp. to form the corresponding ester **3**. It is also notable (Scheme 1) that two, skeleton-rearranged metabolites, oleanolic acid (**2**) and oleanolic acid methyl ester (**4**), were also obtained from this biotransformation involving a methyl migration. Biogenetically, the ursane and oleanane skeletons share a common intermediate, and the ursane nucleus is biosynthesized from an oleanane precursor via a methyl migration from C-20 to C-19.[10] The current observation confirms the unique ability of this *Nocardia* sp. to catalyze a 'retro-biosynthetic' conversion from the ursane to the oleanane skeleton. This is the second example of a 'retro-biosynthetic' transformation with this organism. A similar conversion was observed in the biotransformation utilizing quinovic acid glycosides as substrates.[1]

To investigate the mechanism of this biotransformation, several experiments were performed. Both oleanolic acid (**2**) and ursolic acid methyl ester (**3**) were found to be converted to oleanolic acid methyl ester (**4**) when incubated with *Nocardia* sp. under the same conditions. On the other hand, no appreciable reaction could be detected for substrate **4** after extended incubation for four days. This implies that ursolic acid (**1**) is converted stepwise to the final product oleanolic acid methyl ester (**4**) via two intermediates, namely, oleanolic acid (**2**), and ursolic acid methyl ester (**3**), which are further converted to oleanolic acid methyl ester (**4**) through extended incubation.

The source of the methyl group in the enzyme system of *Nocardia* sp. (inter- or intramolecular) has yet been defined. In subsequent experiments, simple alcohols such as ethanol, glycerol, isopropanol, and butanol showed no effect on the biotransformation, implying that methylation may not be catalyzed by the general lipases or esterases, which might be inhibited by exogenous alcohols. Thus, it would be suggested that methyl ester formation could most likely result from an S-adenosylmethionine-dependent methyltransferase, a member of a large family of enzymes with broad biological activity including transmethylation.[11]

Betulinic acid (**5**) and 23-hydroxybetulinic acid (**7**) (Fig. 1) are members of the so-called 'lupane-type' pentacyclic triterpenes, which were shown to exhibit potent apoptotic and anti-HIV activities.[12,13] Incubation of **5** and **7** with *Nocardia* sp. afforded their corresponding methyl esters, betulinic acid methyl ester (**6**) and 23-hydroxybetulinic acid methyl ester (anemosapogenin methyl ester, **8**), respectively. The structures were identified on the basis of their almost super-imposable NMR data (Table 1) with literature values for betulinic acid methyl ester,[9,14] and 23-hydroxybetulinic acid.[15]

Glycyrrhetinic acid (**9**), a potent and selective inhibitor of 11β-hydroxysteroid dehydrogenases,[16] is the aglycone of the saponins from the well-known Traditional Chinese Medicine licorice (*Glycyrrhiza glabra*). Similarly, **9** was converted to its methyl ester, glycyrrhetinic acid methyl ester (methyl glycyrrhetinate, **10**) by incubation



Scheme 1. Biotransformation of ursolic acid (**1**), oleanolic acid (**2**) and their corresponding methyl esters (**3** and **4**) by *Nocardia* sp.

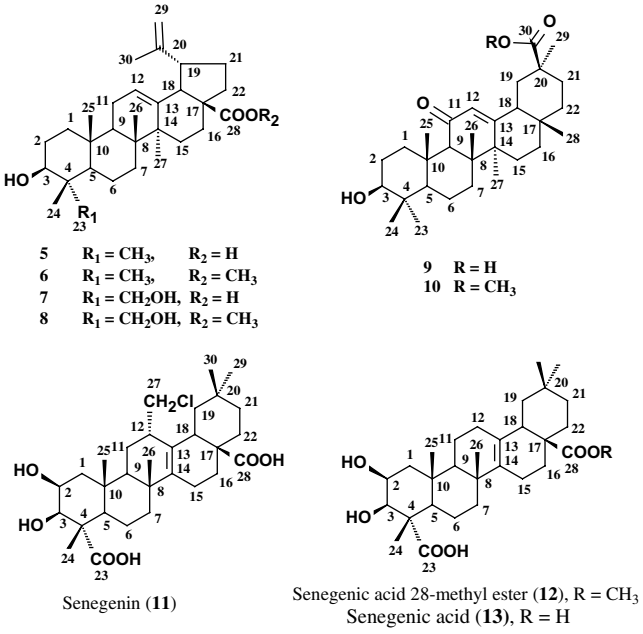


Figure 1. Structures of pentacyclic triterpene acids biotransformed by *Nocardia* sp.

Table 1. NMR spectral data of compounds **5–12** and senegenic acid[a]

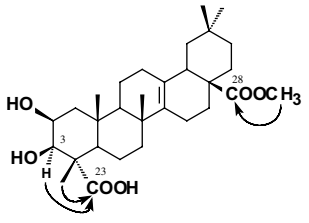
C	5	6	7	8	9	10	11	12	Senegenic acid (**15**)
1	39.3	39.3	39.2	39.1	39.5	39.8	43.7	44.4	44.9
2	28.3	28.3	27.9	27.9	26.7	27.0	70.5	71.2	71.8
3	78.2	78.1	73.6	73.4	77.8	78.0	74.8	75.5	76.0
4	38.5	39.5	42.9	42.7	37.6	37.8	52.5	53.4	54.0
5	55.9	55.9	48.9	48.8	55.6	55.5	51.7	51.5	52.0
6	18.8	18.8	18.6	18.6	17.0	17.0	19.4	18.0	18.5
7	34.9	34.8	34.6	34.5	32.1	32.7	37.8	38.8	39.5
8	41.2	41.1	41.2	41.0	44.1	44.1	38.6	37.8	38.4
9	50.2	50.9	50.5	50.9	61.0	61.1	56.7	56.8	57.3
10	37.4	37.1	37.6	37.4	36.1	37.5	35.8	36.6	37.2
11	21.3	21.1	21.3	21.2	199.1	199.2	20.4	20.7	21.1
12	26.2	26.0	26.2	26.0	124.4	124.5	44.8	23.8	24.0
13	38.7	38.6	38.7	38.6	165.4	165.7	129.7	130.0	130.7
14	42.9	42.7	42.9	43.0	45.1	45.2	143.3	136.5	137.0
15	30.3	30.1	30.3	30.1	28.0	28.2	20.5	21.4	21.9
16	32.9	32.4	32.9	32.3	28.0	28.8	20.7	31.0	31.9
17	56.7	56.8	56.7	56.8	35.6	35.8	46.0	44.9	45.3
18	47.8	47.6	47.8	47.6	40.4	40.7	38.8	39.2	39.9
19	49.7	49.8	49.7	49.8	36.0	36.5	41.3	41.0	41.9
20	151.4	150.9	151.4	150.9	42.9	42.8	30.0	30.3	30.9
21	31.3	31.0	31.8	31.0	34.1	34.0	32.9	33.7	34.6
22	37.6	37.5	37.6	37.4	39.9	39.8	30.4	31.7	32.2
23	28.7	28.7	68.2	67.9	29.0	29.4	180.0	180.3	180.8
24	16.4	16.4	12.9	12.9	20.8	21.2	11.0	13.2	13.4
25	16.4	16.3	16.5	16.2	16.5	16.7	16.5	17.6	18.1
26	19.2	18.8	19.5	18.6	17.8	18.1	17.7	20.3	20.8
27	15.2	14.9	14.9	14.9	18.7	18.8	46.8		
28	178.9	176.5	178.9	176.5	16.0	16.1	179.8	177.6	180.2
29	109.9	110.1	109.9	110.1	20.7	20.7	31.7	32.6	32.7
30	19.8	19.4	19.5	19.4	180.9	178.6	23.4	24.3	25.1
OCH_3		51.3		51.3		51.8		51.3	

[a] Recorded in pyridine-d_5 at 500 MHz (^{1}H) and 125 MHz (^{13}C).

with *Nocardia* sp. The structure of **10** was confirmed by comparison of its NMR data with those reported in the literature.[17,18]

Senegenin (**11**) is an artificial aglycone from the vigorous ethanolic hydrochloride hydrolysis of the saponins from *Polygala senega*.[19,20] When **11** was incubated with *Nocardia* sp., a less polar metabolite was detected by HPTLC from the incubation mixture, which was subjected to silica gel chromatography to afford **12** as a colorless powder. The molecular formula of **12** was established as $C_{30}H_{46}O_6$ by HR-ESI-FTMS in which a pseudo-molecular ion was detected at *m/z*: 503.3223 $[M+H]^+$ {calcd for $C_{30}H_{47}O_6$: 503.3372}, revealing that chloride has been eliminated during the biotransformation. The ^{13}C NMR spectra of **4** (Table 1) revealed its similarity with senegenic acid (**13**) based on the almost identical NMR data with the literature values for **13**,[21] except that one additional oxygenated methyl signal was observed at δ 51.3 and the ^{13}C signal of the C-28 carboxylic acid of **12** was shifted 2.6 ppm upfield compared with that of **11**.[21] This implied that **12** was a methyl ester of senegenic acid (**13**).[19] There are two possibilities for the methyl ester location due to the presence of two carboxylic acid residues. Conclusive evidence from the HMBC spectrum allows assignment of the methyl ester to the C-28 carboxylic acid, as the ^{1}H signal at δ 4.61 (d, J = 3.0 Hz), assignable to H-3α, was shown to be long range correlated to the ^{13}C signal at δ 180.3. This signal was then assigned to the carbonyl group at the C-23 position (Scheme 2). In addition, the small coupling constant (e/e coupling) of the H-3 doublet indicated that the vicinal hydroxyl groups at C-2 and C-3 should be β-oriented. Therefore, the structure of **12** was elucidated as senegenic acid 28-methyl ester, suggesting a highly selective methyl esterfication between the carboxylic acid functionalities at C-23 and C-28. Since there are no NMR data reported for **12**, a complete, unambiguous assignment was done through 2D NMR techniques (Table 1).

In conclusion, this is the first report that *Nocardia* sp. NRRL 5646 can catalyze specific methyl esterification at the C-28 position of pentacyclic triterpene acids. In addition, the ursane skeleton was isomerized to the oleanane skeleton, consistent with the previous transformation of quinovic acid glycosides, which also possess an aglycone with the ursane skeleton.[1] This suggests the presence of a unique enzyme system in *Nocardia* sp.



Scheme 2. Key H–C long-range correlations of **12** from HMBC.

catalyzing this unusual 'retro-biosynthetic' conversion. Strikingly, the 12-chloromethylene group of senegenin (**11**) was eliminated during the biotransformation with *Nocardia* sp. Although there are many reports on enzyme-catalyzed C–C bond cleavage reaction by lyases from a variety of substrates such as aromatic aldehydes,[22] indoles,[23] and lignins.[24] To our knowledge, this is the first report that a C–C bond cleavage was catalyzed by microbe *Nocardia* sp. In addition, the results reported here further exemplify the biocatalytic efficacy of *Nocardia* sp. toward complex natural products, allowing one-step transformations that are presently very difficult, if not impossible, to be performed using chemical methods, such as the conversion of **1** to **2**, and of **11** to **12**, in which skeleton rearrangements occur by methyl migration and the elimination of a chloromethylene group, respectively. Finally, the versatile catalytic capabilities of *Nocardia* sp. to generate structural diversity in already complex natural products are of pharmaceutical interest and await further exploration.

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Supplementary data

Experimental details for the incubation and separation of the metabolites from incubation mixture, and detailed ^{1}H and ^{13}C NMR assignments of compounds **1**–**13**. The supplementary data is available online with the paper in ScienceDirect. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2005.01.155.

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