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Biosynthesis of ursolic acid derivatives by microbial metabolism of ursolic acid with *Nocardia* sp. strains—Proposal of new biosynthetic pathways

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ABSTRACT

Our studies of the microbial-metabolism of triterpenoid ursolic acid by various *Nocardia* sp. strains, have led to the proposal of two novel pathways to produce triterpenoid derivatives. *Nocardia* sp. NRRL 5646, *Nocardia* sp. 44822 and *Nocardia* sp. 44000 generated the following ursolic acid derivatives: ursolic acid methyl ester, ursonic acid, ursonic acid methyl ester, 3-oxoursa-1,12-dien-28-oic acid and 3-oxoursa-1,12-dien-28-oic acid methyl ester. *Nocardia* sp. 45077 synthesized ursonic acid and 3-oxoursa-1,12-dien-28-oic acid while *Nocardia* sp. 46002 produced only ursonic acid and *Nocardia* sp. 43069 showed no metabolism at all. The conversion of ursolic acid by *Nocardia* sp. NRRL 5646 was independent of the medium used for the fermentation. An increase in temperature from 28 °C to 36 °C doubled the reaction rate of the biotransformation. The analysis of ursane metabolites was done by HPLC, while their structures were established using HPLC–$APCI_{pos}$-MS/MS and HPLC–NMR spectroscopy. The pseudo molecular ion peaks were determined by HPLC–$APCI_{pos}$-MS and used to measure their molecular weight. The product ion spectra of the metabolites showed the characteristic fragments of Δ^{12}-oleanes and Δ^{12}-ursanes indicating that a substitution in ring A or B was responsible for the decrease in molecular weight.

Based on these results, two new biosynthetic pathways are proposed. These new pathways can presumably be used as strategic routes for the biotechnological production of triterpenoid derivatives. It is assumed that a 3β-hydroxysteroid dehydrogenase and a 3-ketosteroid-Δ^1-dehydrogenase are involved in the transformation of the steroid.

1. Introduction

Medicinal plants including sage, rosemary, thyme and lavender contain distinctive amounts of two pentacyclic triterpenoids (ursolic acid and oleanolic acid; Fig. 1). The spectrum of biological activities that have been attributed to these compounds have made them pharmaceutically interesting. Triterpenes have been reported to have antiviral [1], antibacterial [2], anti-tubercular [3], anti-HIV [4], anti-cancer [5], anti-diabetic [6], antioxidant [7], and anti-inflammatory [8] properties.

Both triterpene compounds are found in the raw plant material. Therefore, an efficient separation of the triterpenoids has to be accomplished prior to commercialization or drug use. A modification of the target compounds can simplify the purification protocol and could lead to identification of other compounds of pharmaceutical interest. Such a modification can be carried out by chemical or by microbial/enzymatic means. With respect to the complex nature of the triterpenes the latter is beneficial to achieve a modification with an optimal regio- and enantioselectivity without possible degradation of the carbon skeleton. Thus, microbial transformation is often the only rational way to obtain the desired product from a precursor [31]. In addition, the microbial biotransformation requires neither laborious protecting group chemistry nor any co-factor supplement. However, the realization of a desired biotransformation reaction and the screening of an appropriate microbial strain is still a great challenge. For example, Zhang and Zhi-Hong [9] describe a novel method of triterpene-modification, where both ursolic acid and oleanolic acid are transformed to oleanolic acid methyl ester by *Nocardia* sp. NRRL 5646. That kind of biotransformation results in a single component (oleanolic acid methyl ester) that could be subsequently hydrolyzed to the corresponding triterpene acid

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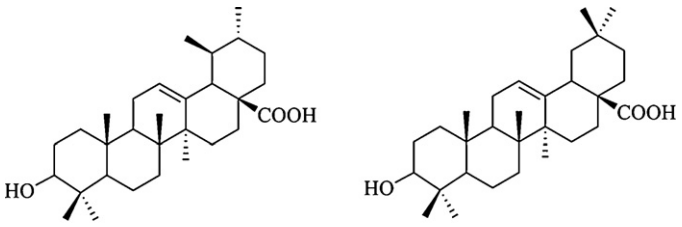

Ursolic acid Oleanolic acid

Fig. 1. Molecular structure of ursolic acid and oleanolic acid.

prior to commercialization of the compound. Special concern is related to hydroxylation reactions. Thus, Collins et al. reported the hydroxylation of ursolic acid methyl ester to yield methyl 3β,7β,21β-trihydroxyursa-9(11),12-dien-28-oate by incubation of the methyl ester with *Mucor plumbeus* (ATCC4740) [32]. A modification of the ursane skeleton was also shown by Cheng et al., who described the hydroxylation of a quinovic acid derivate at C-30 while incubating the substrate in the presence of *Streptomyces griseus* [33]. However, a very recently published review by Parra et al. covers the main literature published in the field of microbial transformations of triterpenoids and is recommended for further information concerning microbial strains and their related biotransformations on corresponding oleanane and ursane skeletons [34].

In this study, we investigate the microbial metabolism of ursolic acid by *Nocardia* sp. 5646 and other *Nocardia* strains to propose novel pathways that can be used for the production of bioactive ursolic acid derivatives.

2. Materials and methods

2.1. Substrates and organisms

Ursolic acid and oleanolic acid were obtained from Sigma–Aldrich (Germany); ursolic acid methyl ester and oleanolic acid methyl ester were obtained from LGC Standards (Germany).

Chemicals used were of analytical-grade. HPLC grade solvents and deuterated solvents were used for the HPLC and NMR analysis, respectively. *Nocardia* sp. NRRL 5646 was a gift from the ARS Culture Collection in Peoria, Illinois (USA). All other *Nocardia* strains were obtained from the German Collection of Microorganisms and Cell Cultures DSMZ (Germany).

2.2. Biomass production

Microbial cultures were grown according to a two-stage fermentation protocol.

Medium A for *Nocardia* sp. NRRL 5646 [10]: 20 g glucose, 5 g yeast extract, 5 g soya peptone, 5 g NaCl, 5 g K_2HPO_4, 0.75 g $MgSO_4$ per 1 L of distilled water, adjusted to pH 7.0 with 6 M HCl.

Medium B for *Nocardia* sp. NRRL 5646, *Nocardia* sp. 43069, *Nocardia* sp. 43572, *Nocardia* sp. 44000, *Nocardia* sp. 44822 and *Nocardia* sp. 45077 (German Collection of Microorganisms and Cell Cultures: Medium 65): 4 g glucose, 4 g yeast extract, 10 g malt extract per 1 L of distilled water, adjusted to pH 7.2 with 6 M HCl.

Medium C for *Nocardia* sp. 46002 (German Collection of Microorganisms and Cell Cultures: Medium 426): 10 g glucose, 10 g peptone, 2 g casein peptone, 2 g yeast extract, 6 g NaCl per 1 L of distilled water, adjusted to pH 7.8 with 6 M HCl.

The starter culture (500 mL baffled Erlenmeyer flasks filled with 150 mL medium) was inoculated and incubated at 100 rpm at 28 °C (Certomat BS-T, B. Braun Biotech International, Germany). After 72 h the starter culture was transferred to a 5 L baffled Erlenmeyer flask (filled with 1350 mL medium) which was incubated for 24 h at the same conditions.

The biomass was separated by centrifugation at 5 °C and 8000 × *g* (Hermle Z 383 K, Hermle Labortechnik GmbH, Germany). It was washed three times for 20 min with 200 mL 0.5% NaCl.

2.3. Biotransformation

In a 500-mL baffled Erlenmeyer flask, 10 g of moist biomass was suspended in 100 mL of phosphate buffer (5 g NaCl, 5 g K_2HPO_4, 0.75 g $MgSO_4$ per 1 L of distilled water adjusted to pH 7.0 with 6 M HCl) and 6 mL of substrate (5 mg/mL ursolic acid in ethanol) was added. Two controls (6 mL substrate added to 100 mL phosphate buffer; 6 mL ethanol added to 10 g of moist biomass suspended in 100 mL phosphate buffer) were run synchronously. The flasks were incubated as duplicates at 100 rpm at 28 °C (Certomat BS-T, B. Braun Biotech International, Germany) for at least 20 days. Samples of 8 mL each were drawn during the biotransformation for analysis after substrate addition. The reaction was stopped by freezing the samples. The first sample was taken immediately after the substrate addition, the second sample was taken after 2 or 3 days and thereafter the interval of the sampling varied from 4 to 8 days.

2.4. Sample preparation for HPLC

The samples were defrosted and extracted with 16 mL ethyl acetate. The organic phase was separated by centrifugation for 20 min at 20 °C and 8000 × g (Hermle Z 383K, Germany) and concentrated under reduced pressure (220 mbar) at 40 °C *in vacuo* (Laborota 4003-control, Heidolph Instruments GmbH & Co. KG, Germany). The dry residue was solubilized in 3 mL of the mobile phase and then filtered (13 mm syringe filter 0.45 μm PTFE, VWR International, Germany) and analyzed by HPLC. Standard deviation of ursolic acid determination was estimated at 0.3%.

2.5. Analytical instrumentation

HPLC analyses were carried out on a Waters alliance 2695 with Waters PDA detector 2998. Methanol with 0.1% formic acid in the ratio of 92:8 (0.4 mL/min; isocratic method) was used as the mobile phase. A Phenomenex Luna C18(2) 5 μm, 250 mm × 4.6 mm column was used. The UV spectra of the metabolites were obtained from a PDA detector using a detection wavelength of 212 nm.

For HPLC–$APCI_{pos}$-MS/MS analysis we used a SCIEX API 2000 MS/MS tandem mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface (Applied Biosystems, Germany) coupled to a HPLC system (PerkinElmer Series 200). HPLC conditions were as described above. Data was acquired and evaluated using Analyst Software 1.4.2 (Applied Biosystems, Germany). APCI settings were as follows: positive ionization mode, 2.0 μA needle current, nitrogen as curtain gas (350 at 25 psi), 30 psi GS1, 40 psi GS2, 21.0 V declustering potential, 290.0 V focusing potential, 5.0 V entrance potential and a 2.3 kV electron multiplier voltage. The mass spectrometer was operated in the full scan and product ion scan modes. The total scan duration was 0.5 s with a 2-ms pause between mass ranges. Fragmentations using nitrogen (5 units) as a collision gas were performed at a collision energy of 27 eV.

^{1}H NMR spectra were recorded by HPLC–NMR in the "stopped-flow" mode. We used an 1100 Series HPLC system (Agilent Technologies, CA, USA) with a 600 MHz Avance spectrometer (Bruker BioSpin, Germany) and a 3 mm LCSEI flow probe. The column mentioned above was used. The mobile phase used was acetronitril-d_3 with D_2O in the ratio of 92:8 (0.4 mL/min; isocratic method). The chromatographic conditions and stopped-flow NMR "peak parking" were controlled by the Bruker software. The samples were concentrated under reduced pressure (340 mbar) at 40 °C *in vacuo* (Laborota 4003-control, Heidolph Instruments GmbH & Co. KG, Germany). The dry residue was solubilized in 2 mL CH_3OH-d_4 and the HPLC results were measured at a wavelength of 212 nm.

3. Results

3.1. Generation of the metabolites

In our experiments oleanolic acid was converted by *Nocardia* sp. NRRL 5646 to oleanolic acid methyl ester within 2 days (data not shown). However, the biotransformation of ursolic acid showed an unexpected product spectrum. It did not metabolize into oleanolic acid methyl ester as reported by Zhang and Zhi-Hong [9]. Information concerning the structure elucidation is presented below. Additional *Nocardia* strains were screened for their ability to transform ursolic acid.

Biotransformation by *Nocardia* sp. NRRL 5646 is presented in Fig. 2. Half of the ursolic acid was converted by the third day and only 5% remained following 20 days. Ursolic acid methyl ester was detectable after the first day, maximal value was measured by the third day and it was completely transformed after 20 days. Ursonic acid methyl ester was detected on the third day and maximal value was detected by day 6. By day 20 the peak area decreased to 30% of the maximum value. Ursonic acid was generated on day 6 (maximum value) and decreased to approximately 4% of the maximal levels by day 20. 3-Oxoursa-1,12-dien-28-oic acid was detected on day 17 (maximum value) and decreased instantly, thus on day 20 the absorbance was 83% of the maximum value. 3-Oxoursa-1,12-dien-28-oic acid methyl ester appeared on day 17 and showed a maximum value on day 20.