Microbial transformations of diterpene acids

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The ability of *Rhodococcus* bacteria to transform dehydroabietic and isopimaric acids with a high degree of conversion (up to 95%) was detected.

The microbial oxidation of various compounds, including diterpenoids, is of considerable interest as a key stage in the synthesis of practically valuable compounds because of the high stereoselectivity of biotransformations and the ability of microorganisms to modify chemically non-activated molecular fragments.[1–3]

Dehydroabietic acid **1** and isopimaric acid **2** are naturally available compounds, and they were selected as the test compounds for two reasons. First, the hydroxylated derivatives of these acids are interesting as intermediates in the synthesis of antiviral agents.[4–6] Second, the microbial degradation of resin acids is an essential stage in waste treatment at pulp mills.[7] We decided on nocardiaform actinomycetes, in particular, *Rhodococcus* bacteria, as potential biocatalysts for the hydroxylation of terpenoids.

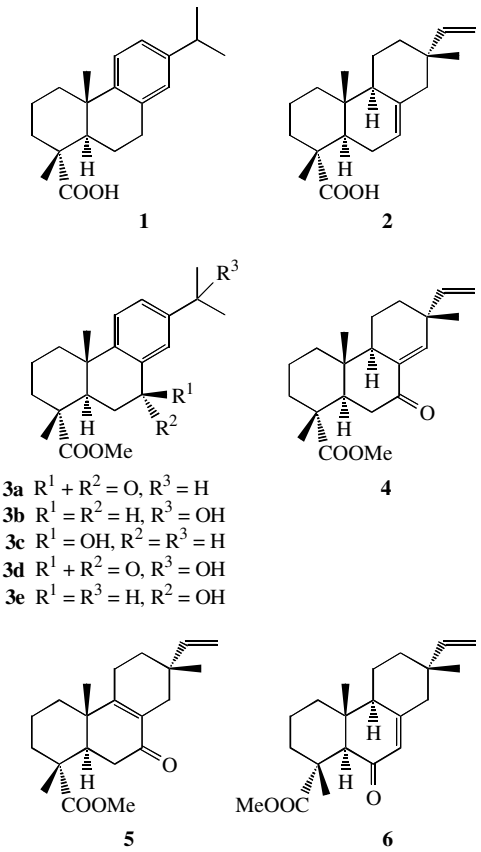
In this study, we tested 276 rhodococci strains [*R. erythropolis* (121†), *R. fascians* (2), *R. "longus"* (9), *R. opacus* (6), *R. rhodochrous* (15) and *R. ruber* (123) maintained in the Regional Specialised Collection of Alkanotrophic Microorganisms at IEGM[8]] and 39 freshly isolated strains of *Rhodococcus* sp. for the ability to transform diterpene acids. Microorganisms were grown in a mineral medium (pH 6.8–7.0) of the following composition (g dm^{-3}): KNO_3, 1.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; NaCl, 1.0; $MgSO_4$, 0.2; $CaCl_2$, 0.02; $FeCl_3$,[8] 0.001; yeast extract, 0.1; and dehydroabietic or isopimaric acid as a carbon source (dissolved in ethanol), 0.5. In some cases, *n*-hexadecane (0.5–1.0 vol.%) was used as a co-substrate. Cultivation was carried out at 28 °C in a rotary shaker (150 rpm) for 6 to 7 days.

The majority of the tested strains exhibited insignificant transforming activities (no higher than 5%), whereas some rhodococci were able to transform from 35.3 to 95.4% initial diterpenoids. Thus, *R. erythropolis* IEGM 267, *R. ruber* IEGM 472 and IEGM 474 selectively oxidised dehydroabietic acid, and *R. erythropolis* IEGM 192, *R. ruber* IEGM 457, IEGM 467 and IEGM 468 oxidised isopimaric acid.

It should be noted that the representatives of *R. ruber* and *R. rhodochrous* having an orange-red non-diffusing pigment were found most active when diterpenoids were used as sole growth substrates. *R. erythropolis* strains exhibited the highest diterpenoid-transforming activity when *n*-hexadecane was used as a growth substrate.

The biotransformations of dehydroabietic acid **1** by *R. ruber* IEGM 472 and IEGM 474 resulted in the appearance of a compound with $m/z = 328$ (12.4 and 14.6%, respectively) in the reaction mixture (samples were analysed by GC/MS). The conversion of dehydroabietic acid was 75.6–82.3% (in terms of a decrease in the concentration of the parent compound). The ^{1}H NMR and mass spectra of the methylated reaction product were consistent with the published data[9] for 7-oxomethyldehydroabietate **3a**.‡

According to our data, at 70% conversion of dehydroabietic acid by *R. erythropolis* IEGM 267, the methylated reaction products contained 15-hydroxymethyldehydroabietate **3b**‡ (23.6%) as the major constituent in a mixture with previously described minor compounds,[9–11] namely, 7-oxomethyldehydroabietate **3a**, 7β-hydroxymethyldehydroabietate **3c**‡ and 7-oxo-15-hydroxymethyldehydroabietate **3d**.‡ It should be noted that the autooxidation of methyldehydroabietate resulted in the formation of 7α-hydroxymethyldehydroabietate **3e**.[12]



Rhodococci were found to exhibit high stereoselectivity in the enzymatic oxidation of isopimaric acid **2**. In the transformation of isopimaric acid by *R. ruber* IEGM 457, a compound with $m/z = 330$ (14%) dominated in the reaction mixture; this compound was identified as methyl 7-oxoisopimara-8(14),15-dien-18-oate **4**‡ by IR, UV and ^{1}H NMR spectroscopy.[13] At the same time, *R. ruber* IEGM 467 and IEGM 468 converted

† Number of strains is indicated in the brackets.

‡ Selected data for **3a**: mp 64–66 °C, $[\alpha]_D^{23}$ +15.1° (*c* 3.3, $CHCl_3$); lit.,[9] mp 65–67 °C, $[\alpha]_D^{23}$ +7.8° (*c* 5.2, EtOH).
For **3b**: mp 79–81 °C, $[\alpha]_D^{22}$ +44.7° (*c* 2.3, $CHCl_3$); lit.,[10] mp 82–83 °C, $[\alpha]_D^{24}$ +54° ($CHCl_3$).
For **3c**: mp 95–96 °C, $[\alpha]_D^{21}$ +56° (*c* 1.5, $CHCl_3$); lit.,[11] mp 92–93 °C, $[\alpha]_D^{24}$ +56° (1% EtOH).
For **4**: mp 89–90 °C, $[\alpha]_D^{20}$ –11° (*c* 0.3, $CHCl_3$); lit.,[13] mp 90–92 °C, $[\alpha]_D^{20}$ –10° (*c* 0.2, $CHCl_3$).
For **5**: mp 60–61 °C, $[\alpha]_D^{20}$ +88° (*c* 3.4, $CHCl_3$); lit.,[13] mp 58–59 °C, $[\alpha]_D^{24}$ +88° (*c* 0.34, $CHCl_3$).
IR, UV and ^{1}H NMR spectra of **3a–d**, **4** and **5** were consistent with the corresponding published data.

acid **2** to an isomeric product (14%), which was identified after methylation as methyl 7-oxoisopimara-8(9),15-dien-18-oate **5**‡ by ^{1}H NMR spectroscopy and mass spectrometry, as well as by comparing with an authentic sample.[13]

Comparative studies of the diterpenoid-transforming activity of some procaryotic organisms, in particular, *Micrococcus* bacteria (which are abundant in natural rhodococci containing microbiocenoses[14]) showed that micrococci were able to accumulate isomeric ketones **4** and **5** during isopimaric acid transformations. Thus, the oxidation of isopimaric acid by *M. freudenreichii* IEGM 425 led to the formation of products identified as ketones **5** and **4** (38.2 and 5.7%, respectively) after methylation (isopimaric acid conversion was 82%). The GC/MS analysis of the mixture of methylated products revealed the presence of another isomeric ketone, which most likely corresponds to methyl 6-oxoisopimara-7,15-dien-18-oate **6**.

Isopimaric acid is more toxic and more stable to biodegradation under natural conditions than other diterpene resin acids.[7] However, under co-oxidation with *n*-hexadecane, isopimaric acid was almost completely degraded by *R. erythropolis* IEGM 192 used in this study without accumulation of intermediate products. This fact is important for the development of treatment techniques for the purification of resin acid-contaminated industrials wastes (*e.g.*, pulp mill effluents).

Thus, *Rhodococcus* bacteria were successfully used in the transformation of naturally available diterpene compounds. Among the bacterial strains tested, the Rhodococcus representatives were more active in the transformation of diterpene acids with high degrees of regioselectivity and stereoselectivity.

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