Production of 2-Methylisocitric Acid from n-Paraffins by Mutants of *Candida lipolytica*

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Previously, we found that many kinds of yeasts produced large amounts of citric and (+)-isocitric acids from various carbon sources, including *n*-paraffins. Recently, Akiyama *et al.* succeeded in changing the ratio of accumulated amounts of citric acid to (+)-isocitric acid by using mutants of *Candida lipolytica* ATCC 20114. Fluoroacetate-sensitive mutants of *C. lipolytica* ATCC 20114, which show extremely low aconitase activity as compared with that of the parent strain, were found to produce citric acid in high yield but little (+)-isocitric acid. The finding suggested that fluoroacetate-resistant mutants, if present, might show high aconitase activity and produce (+)-isocitric acid in high yield and the attempt was made to obtain such mutants from *C. lipolytica* IFO 1659. The fluoroacetate-resistant mutants thus obtained were examined for their citric and (+)-isocitric acid productivities. Unexpectedly, no significant difference was found in the ratio of (+)-isocitric acid between the mutants and the parent strain. Some of the mutants, however, were found to produce an unknown acid in large amounts from *n*-paraffins.

By means of counter-current distribution, the unknown acid was isolated as its lactone in a yield of 21 g from the culture broth of mutant strain No. R-2 which was grown at 26°C for 10 days on a rotary shaker in fifty of 300-ml Erlenmeyer flasks, each containing 20 ml of a medium composed of *n*-paraffins (C_{12} to C_{15}) 8 ml/dl, NH_{4}Cl 0.3%, KH_{2}PO_{4} 0.05%, MgSO_{4}, 7H_{2}O 0.05%, thiamine hydrochloride 0.05 ppm, Span 80 0.05%, CaCO_{3} 8% and tap water. The lactone was recrystallized from ethyl acetate-chloroform (1:10) in long needles; mp 153°C (uncorr.), [α]^{20}_{D}-26.2° (c=1.0, H_{2}O), -24.4° (c=1.0, MeOH); NMR (60 Mcps, d-CH_{3}COCH_{3} δ(ppm); 1.78 (singlet, 3H, CH_{3}-group), 2.9 (octet, 2H, CH_{2}-groups), J_{ab} 23 cps, J_{ac} 10 cps and J_{bc} 9.6 cps. Anal. Found: C, 44.71; H, 4.13. Calcd. for C_{7}H_{8}O_{6}: C, 44.69; H, 4.29%. It was titrated as a dibasic acid with pK_{a1} of 2.77 and pK_{a2} of 4.74 in cold water. By treatment with excess alkali in hot water, the lactone ring was opened and one more acidic group became detectable by back titration. After drying with a calculated amount of NaOH solution, the lactone yielded trisodium salt; [α]^{25}_{D}+5° (c=1.8, 0.4 n HCl). The NMR spectrum of the lactone showed a singlet methyl peak, an octet methylene peak which coupled with a triplet methine group and a singlet peak due to two carboxyl protons. The geminal coupling constant of the methylene group was 23 cps, indicating that the group is adjacent to the carbonyl group of the lactone. From these results, the structure of the lactone was determined to be one of four isomers of 2-methylisocitric acid γ-lactone. Comparison of the ORD spectrum of the lactone with those of *threo*-D-γ-isocitric acid lactone ((−)-isocitric acid lactone) and *erythro*-L-α-isocitric acid lactone ((+)-alloisocitric acid lactone) indicated that the lactone is either *threo*-D-γ-2-methylisocitric acid lactone or *erythro*-D-α-2-methylocitic acid lactone (Table I). Intramolecular nuclear Overhauser effect on the lactone was further examined. Irradiation of the 2-methyl protons caused a profound, intensity increase (15% of the original intensity) of the 3-methine proton signal in the lactone NMR spectrum, indicating a spatial closeness of less than 2.97 Å between the methyl and methine protons. Since the internuclear distances between the two groups obtained from Dreiding models are 2.74 and 3.64 Å for *threo*-D-γ-2-methylocitic acid lactone and *erythro*-D-α-2-methylocitic acid lactone, respectively, the nuclear Overhauser effect data strongly suggest that the lactone is the former. Further
TABLE I. 

<table>
<thead>
<tr>
<th>Isomer</th>
<th>[α]_D</th>
<th>ORD</th>
<th>Lactone Acid</th>
<th>Lactone Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>erythro-D&lt;sub&gt;3&lt;/sub&gt;- Isocitric</td>
<td></td>
<td></td>
<td>+ Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>erythro-L&lt;sub&gt;3&lt;/sub&gt;- Isocitric&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td></td>
<td>C.e.</td>
<td>C.e.</td>
</tr>
<tr>
<td>three-D&lt;sub&gt;3&lt;/sub&gt;- Isocitric&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>three-L&lt;sub&gt;3&lt;/sub&gt;- Isocitric</td>
<td></td>
<td></td>
<td>d 1</td>
<td>d 1</td>
</tr>
<tr>
<td>Methyl-</td>
<td></td>
<td></td>
<td>Negative</td>
<td>C.e.</td>
</tr>
<tr>
<td>Isocitric</td>
<td></td>
<td></td>
<td>C.e.</td>
<td>C.e.</td>
</tr>
</tbody>
</table>

<sup>a</sup> ORD spectra were obtained on a JASCO ORD/UV 5 polarimeter.

<sup>b</sup> From Penicillium fermentation.

<sup>c</sup> From yeast fermentation.

<sup>d</sup> ORD curves of erythro-D<sub>3</sub>-isocitric and three-L<sub>3</sub>-isocitric acids are mirror images of those of erythro-L<sub>3</sub>-isocitric and three-D<sub>3</sub>-isocitric acids, respectively.

<sup>e</sup> C.e. means Cotton effect.

Support for the conclusion was obtained from a biological activity of the acid. The sodium salt of the acid was found to inhibit competitively NADP-linked isocitrate dehydrogenase. The Ki value for the enzyme was determined to be 1.0 x 10^{-7} M, indicating high affinity of the acid for the enzyme. On the other hand, three- and erythro-L<sub>3</sub>-isocitric acids were found to have little affinity for the enzyme. In view of these results, it is safely assumed that the acid is three-D<sub>3</sub>-2-methylisocitric acid.

Although 2-methylisocitric acid was originally synthesized by Rach in 1886 as a mixture of four isomers, its physiological significance has not yet been understood. Gawron and Mahajan<sup>11</sup> showed that horse heart aconitase catalyzes hydration of 2-methylaconitic acid to give methylcitric and 2-methylisocitric (probably three-D<sub>3</sub>-2-methylisocitric) acids. This is the first evidence indicating possible occurrence of three-D<sub>3</sub>-2-methylisocitric acid in the living organism. This acid itself has recently been shown to be active as a substrate for fungal and bacterial isocitrate lyases.<sup>3</sup>

In this connection, it is noticeable that methylcitrate, another hydration product from 2-methylaconitic acid by horse heart aconitase, is found as a major metabolite of propionate in some patients with propionate acidemia.<sup>9</sup>

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REFERENCES

1) M. Abe and T. Tabuchi, Agr. Biol. Chem., 32, 392 (1968);