Isolation and Identification of (–)-Methylcitric Acid and 2-Methyl-*cis*-aconitic Acid from the Culture of *Candida lipolytica*

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Previously, we found that from a mixture of *n*-alkanes a fluoroacetate-tolerant mutant, No. R-2, derived from *Candida lipolytica* IFO 1659 produced an appreciable amount of *threo*-D₅-2-methylisocitric acid which competitively inhibited isocitrate dehydrogenase.¹⁾ These findings suggested the possible occurrence of unknown metabolic pathway of *n*-alkanes by *C. lipolytica* and hence, an attempt was made to isolate and identify related intermediates, if present, in the culture broth in order to elucidate the pathway.

Consequently, we have newly isolated and identified fumaric, *trans*-aconitic, 2-methyl-*cis*-aconitic and (–)-methylcitric acids from the culture of *C. lipolytica* No. R-2. The isolation of 2-methyl-*cis*-aconitic acid provides the first evidence for the occurrence of the acid in living organisms, although Gawron and Mahajan²⁾ chemically synthesized the acid and showed that horse heart aconitate hydratase catalyzed hydration of the acid, giving a mixture of methylcitric and 2-methylisocitric acids. Although Ando *et al.*³⁾ found methylcitric acid as a major metabolite of propionate in some patients with propionic acidemia, it is worth noting that, besides 2-methylisocitric and methyl-*cis*-aconitic acids, this acid is produced from *n*-alkanes by a fluoroacetate-tolerant mutant of *Candida*. This paper deals with the isolation and identification of these acids produced by *C. lipolytica* No. R-2.

The strain was grown at 26°C for 10 days on a rotary shaker (220 rpm) in two hundred and fifty 500-ml Erlenmeyer flasks each containing the medium described in the previous paper.¹⁾ The culture broth was collected and filtered through Kieselguhr-layer and the filtrate was concentrated under reduced pressure. The concentrate was heated and filtered at an elevated temperature to remove citric, isocitric and 2-methyl-

isocitric acids as calcium salt.*² The filtrate was diluted and then adjusted to pH 1.8 with 4 N sulfuric acid. After removal of calcium sulfate and thorough concentration of the filtrate, the residual sirup was extracted repeatedly with ethyl acetate. The combined extract was evaporated to a sirup, which was then heated until foaming ceased at 100°C under reduced pressure in order to dehydrate thoroughly. The residual sirup (ca. 80 g) was partitioned between 400 ml of ethyl acetate and 200 ml of water, fifteen transfers counter-current distribution being conducted. After each fraction had been checked by paper chromatographic analysis using ethyl acetate-acetic acid-water (50:12:10) as a solvent-mixture and bromphenol blue as a detecting reagent, ethyl acetate phases of fractions No. 1 and No. 2 containing an unknown acid, A (*R_f* 0.92), were collected and evaporated to dryness. The residue (13 g) was suspended in water and neutralized with 0.4 N barium hydroxide. By concentrating the solution, a precipitate of barium salt appeared. The precipitate collected by filtration was dissolved in 1 N hydrochloric acid and extracted with ether. After the ether extract had been dried over anhydrous sodium sulfate, ether was removed by evaporation. On storage for several days under reduced pressure over phosphorus pentoxide, the residual sirupy mass (4.5g) gradually crystallized. The crystalline mass was recrystallized from ethyl acetate-benzene in colorless prisms: mp 51~52°C; *Anal.* Found: C, 49.37; H, 3.46; Calcd. for C₇H₆O₅: C, 49.42; H, 3.56%; UV λ_{max}^{E₁O_H} 216 nm (ε=5,680); NMR δ (ppm, in tetrahydrofuran-*d*₆) 2.05 (3H, CH₃-C=C), 3.47 (2H, C=C-CH₂), 10.48 (1H, COOH). The acid thus obtained showed the maleic anhydride ring system (positive hydroquinone test²⁾). In aqueous solution, it was titrated as a tribasic acid with pK_{a1} of 2.8, pK_{a2} of 4.5 and pK_{a3} of 6.6. From these results, the isolated acid was inferred to be 2-methyl-*cis*-aconitic acid anhydride.

Subsequently, aqueous phases of the fractions No. 5 to No. 9 were combined and concentrated. Appropriate portions of the concentrate were chromatographed by the method of Bulen *et al.*⁴⁾ A column, 12 mm in diameter, containing 30 g of silicic acid dispersed in 9 ml of 0.5 N sulfuric acid was used and elution was carried out with solvent mixtures of varying proportions of chloroform: 1-butanol equilibrated with 0.5 N sulfuric acid: elution was started with 100 ml of 95:5 solvent mixture and followed by elutions with 100 ml each of mixtures with increasing contents of butanol at 10% intervals. Eluate was fractionated in 3-g portions, each of which was examined by PPC, GC and other tests. Composite fraction I (tube No. 35~41) contained fumaric acid (*R_f* 0.86); II (tube No. 43~65) 2-methylisocitric acid lactone (*R_f* 0.75); III (tube No. 66~74) *trans*-aconitic acid (*R_f* 0.72); IV (tube No. 100~111) isocitric acid lactone (*R_f* 0.53) and V

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*² Based on the insolubility of calcium salts of these three acids at higher temperatures.

FIG. 1. IR Spectra of Methylcitric Acid Prepared Enzymatically (a) and Product B Produced by *Candida lipolytica* No. R-2 (b) in KBr.

(tube No.117~124) another unknown acid, B(Rf0.44).^{*} In order to isolate the unknown acid, B, column chromatography was repeated several times and fraction V was collected. This fraction, after concentration, was rechromatographed by the same procedure and concentrated to a sirup under reduced pressure. The sirup gradually crystallized on prolonged standing. The crystalline mass (650 mg) was recrystallized from ethyl acetate-chloroform in colorless prisms: mp 116°C; $[\alpha]_D^{20} -8^\circ$ ($c=7$, in H_2O); *Anal.* Found: C, 40.25; H, 4.97; Calcd. for $C_7H_{10}O_7$: C, 40.78; H, 4.89; NMR δ (ppm, in D_2O) 1.17 (3H, doublet, $J=7.2$ Hz, CH_3-CH), 2.61 (1H, quartet, $J=7.2$, CH_3-CH), 2.73 (2H, quartet, $J=15.6$, $-CH_2-$). The IR spectrum is shown in Fig. 1. It was titrated potentiometrically as a tri-basic acid with pK_{a1} of 3.1, pK_{a2} of 4.8 and pK_{a3} of 6.7 (neutralization equivalent: Found 67, Calcd. 69). After the neutralized solution had been dried, the trisodium salt remained: $[\alpha]_D^{20} -17^\circ$ ($c=2.0$, in H_2O). Methylation of the free acid with diazomethane gave the methyl ester as a gas chromatographically pure colorless sirup: $[\alpha]_D^{20} +12^\circ$ ($c=1.5$, in MeOH). A solution of 300 mg of the methyl ester in 1 ml of methanol was saturated with dry ammonia. On cooling, a white precipitate (160 mg) separated. The precipitate was recrystallized from water: mp 212~

214°C (uncorr., decomp.); $[\alpha]_D^{16} -48^\circ$ ($c=0.2$, in H_2O). From these chemical and physical properties, this acid was inferred to be one of the four isomers of methylcitric acid.

For the purpose of preparing authentic samples of 2-methyl-*cis*-aconitate and methylcitrate, the trisodium salt of 2-methylisocitric acid isolated from a culture of the mutant¹⁾ was treated with aconitate hydratase by the method of Gawron and Mahajan²⁾ to give a mixture of methyl-*cis*-aconitate, methylcitrate and the residual substrate. From the reaction mixture, these acids were separated each other and isolated just as described above. The chemical and physical data of the enzymatically prepared samples were well coincident with those of the acids isolated from the culture broth, respectively.

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^{*} Approximate amounts of these acids in the culture broth and their gas chromatographic behaviors will be shown in a separate paper.