Isocitric Acid

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Syntheses with a Chiral Building Block from the Citric Acid Cycle: (2*R*,3*S*)-Isocitric Acid by Fermentation of Sunflower Oil**

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The citric acid cycle constitutes a main metabolic process. Since its discovery in 1937 by H. A. Krebs, all of its intermediates have been prepared in multigramm amounts—with one exception: (2*R*,3*S*)-isocitric acid (**1**, D$_S$-*threo*-isocitric acid). As a new member of the chiral pool it would be an interesting starting material for organic synthesis. This chiral α-hydroxy tricarboxylic acid is mainly accompanied by its constitutional isomer, citric acid (**2**). However, attempts to separate **1** from **2** have so far been successful only on an analytical scale.

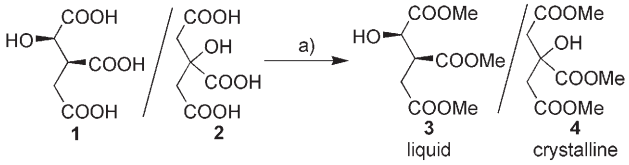
Though experiments have been carried out to achieve synthetic access to *ent*-isocitric acid (*ent*-**1**), again only milligram quantities were obtained.[1] As a result of the scarce availability of **1** there is virtually no application known for it in synthesis. In databases only (2*R*,3*S*)-isocitric acid trimethyl ester (**3**), (2*R*,3*S*)-isocitric acid lactone-2,3-dicarboxylic acid dimethyl ester (**5**), (2*R*,3*S*)-isocitric acid lactone-2,3-dicarboxylic acid (**6**), and (2*R*,3*S*)-isocitric acid lactone-2,3-dicarboxylic acid anhydride (**7**) are listed superficially.[2]

Surprisingly, no attempts have been made to obtain **1** by fermentation, although a large number of yeasts are known to produce and excrete citric acid and (2*R*,3*S*)-isocitric acid in varying ratios when grown on long-chain *n*-alkanes or glucose.[3] So far, these fermentations have been optimized for high levels of citric acid excretion.

Herein we describe a combination of ecologically desirable biotechnological and chemical methods yielding enantiopure (2*R*,3*S*)-isocitric acid (**1**) and its derivatives in kilogram amounts, thus representing an unadulterated application of the "white biotechnology for green chemistry" concept.[4] We discovered that the thiamine auxotrophic yeast *Yarrowia lipolytica* excretes organic acids in high percentage when it is grown on vegetable oils with an excess of thiamine under nitrogen-limited, aerobic conditions.[5] Our aim was to achieve the highest possible ratio of isocitric to citric acid and concomitant high isocitric acid concentration.

We succeeded in producing isocitric acid concentrations of 93 g L^{-1} and **1**/**2** ratios of 1.14:1 on the pilot-plant scale in the cultivation of wild-type *Y. lipolytica* EH59 on refined sun flower oil—a hitherto unrivalled achievement especially with regard to the use of renewable vegetable raw materials. After filtration of the biomass, electrodialysis was performed to convert the obtained trisodium salts into the free acids, before the removal of water was accomplished under reduced pressure. Then, it was time to search for an adequate process to separate the two isomers.

Esterification of the highly viscous concentrated solution yielded the corresponding triesters of both tricarboxylic acids. From this mixture citric acid trimethyl ester **4** crystallized as a colorless solid, while (2*R*,3*S*)-isocitric acid trimethyl ester **3** did not, as it is a liquid under standard conditions. Utilizing this formerly unknown fact, separation of the isomeric esters **3** and **4** could be carried out simply by filtration of **4** from **3** (Scheme 1). In view of the intended application of **1** in



Scheme 1. Esterification of the concentrated fermentation broth: a) MeOH, 2,2-dimethoxypropane, 10 mol% trimethylsilyl chloride, 3 days, RT, 80–88%.

stereoselective synthesis, a variety of different building blocks should become accessible by facile and efficient transformations. Therefore, we first searched for possible differentiation methods of the three carboxylic acid moieties. The formation of a five-membered lactone structure in **5** and **6** was easily accomplished starting from **3**. Both **5** and **6** could be converted into **1** as the parent compound (Scheme 2).

The anhydride **7** of **6** turned out to be the key element in most of the subsequent transformations. Selective ring opening yielded exclusively the monoester derivatives **8** and **9** with the ester groups at the C2 position (Scheme 3) and thus led to compounds with three differentiated carboxylic acid moieties. Crystalline (−)-menthyl ester **9** was also used to confirm the absolute stereochemistry of the fermentation product by X-ray crystallographic analysis (Figure 1).[6] Reduction of the

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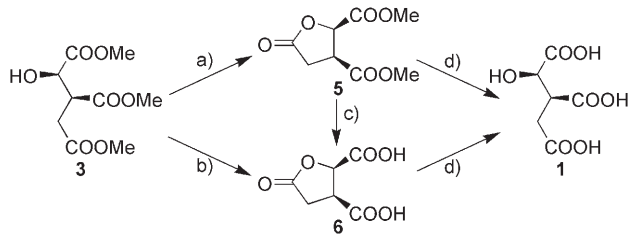
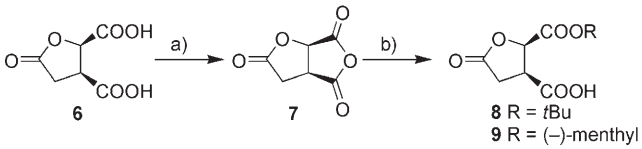


Scheme 2. Conversion of **3**: a) 10 mol% *para*-toluenesulfonic acid, toluene, reflux, 80%; b) 4.0 M HCl, reflux, 8 h, quant.; c) 1.0 M HCl, reflux, 4 h, quant.; d) 3 equiv NaOH, amberlite IR-120, quant.



Scheme 3. Formation of **7** and its regioselective opening: a) Ac_2O, 160°C, 15 min, 85%; b) for **8**: anhydrous *t*BuOH, reflux, 15 h, quant.; for **9**: (1*R*,2*S*,5*R*)-(−)-menthol, 100°C, 36 h, 27%.

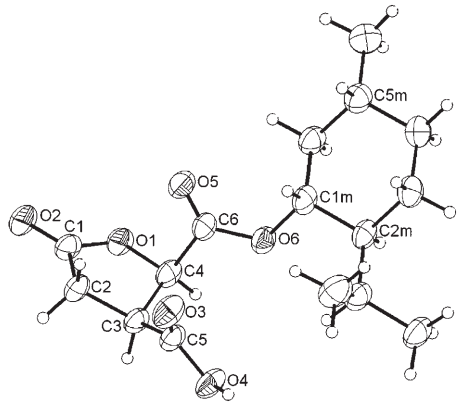
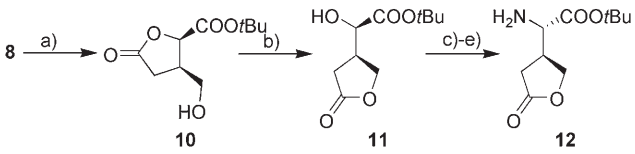


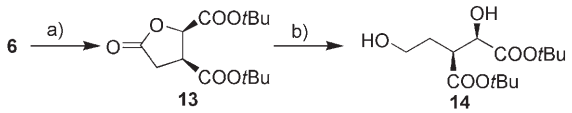
Figure 1. Crystal structure of **9**.[6]

mono-*tert*-butyl ester **8** yielded alcohol **10**, which rearranged to give **11**. An entry to the class of amino acids was opened with the non-natural lactone–amino acid **12**, which was synthesized via **11** from **10** (Scheme 4). Selective reduction of the lactone moiety was successful when starting from **13** and yielded the succinic acid derivative **14** (Scheme 5).



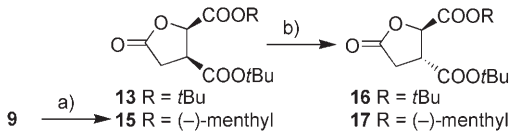
Scheme 4. Transformation and rearrangement of **8**: a) BH_3/THF, THF, 0°C→RT, 5 h, 88%; b) H^+, 78%; c) methanesulfonyl chloride, pyridine, CH_2Cl_2, 24 h; d) NaN_3, DMF, 47% over two steps; e) Pd/C, H_2, EtOAc, 8 h, 90%.

Finally, we searched for methods to synthesize other stereoisomers of isocitric acid. The unsymmetrically esterified



Scheme 5. Transformation of **6**: a) 2-methylpropene, cat. H_2SO_4, CH_2Cl_2, 5 d, 88%; b) $Ca(BH_4)_2$, THF/*i*PrOH, 84%.

compound **15** was used to find mild conditions to invert the conformation at the C3 stereocenter without concomitant loss of stereoinformation at C2 and hence complete racemization. These optimum conditions were applied to invert the readily available di-*tert*-butyl ester **13**, giving rise to derivatives of the non-naturally occurring (−)-*allo*-isocitric acid (Scheme 6).



Scheme 6. a) 2-Methylpropene, cat. H_2SO_4, CH_2Cl_2, 48 h, quant.; b) inversion of the conformation at C3 for **16**: DBU, CH_2Cl_2, 50°C, 30 min, 80% (based on recovered starting material); for **17**: DBU, CH_2Cl_2, 50°C, 1 h, 89% (based on recovered starting material). DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene.

In summary, we succeeded in the production and isolation of (2*R*,3*S*)-isocitric acid, a substance from the citric acid cycle that was of only analytical interest until now. For the first time, (2*R*,3*S*)-isocitric acid is now available in kilogram amounts from the fermentation of vegetable oils such as sunflower oil. Its further transformation to new chiral building blocks points at its promising applications in natural product synthesis and its use as a starting material in the pharmaceutical industry.

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[6] Crystal data of $C_{16}H_{24}O_6$: Stoe IPDS-2T diffractometer, $M_m = 312.35\ g\,mol^{-1}$, crystal size $0.1 \times 0.4 \times 0.9\ mm^3$, monoclinic, space group $P2_1$ (no. 4), $a = 821.95(9)$, $b = 561.50(4)$, $c = 1760.2(2)$ pm, $\beta = 97.141(9)°$, $V = 806.07(14) \times 10^6\ pm^3$, $Z = 2$, $T = 180(2)$ K, $\rho_{calcd} = 1.287\ g\,cm^{-3}$, $\mu = 0.098\ mm^{-1}$, $\lambda = 71.073$ pm ($Mo_{K\alpha}$), 10207 measured, 2805 independent reflections, $R_{int} = 0.073$, 2608 with $I > 2\sigma(I)$, 207 parameters, H atoms in idealized positions, R_1(observed reflections) = 0.051, wR_2(all data) = 0.15, max/min residual electron density peaks $0.21/-0.20\ e \times 10^6\ pm^3$; structure solution and refinement: SHELX-97 (G. M. Sheldrick, SHELXS-97, Program for the Solution of Crystal Structures, Universität Göttingen, **1997**). CCDC-665724 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

