

## A Metabolic Variation in *Penicillium spiculisporum* Lehman

### I. Production of (+)- and (—)-Decylcitric Acids

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(+)-Decylcitric acid and (—)-decylcitric acid, two new fungal metabolites, have been found in a strain of *Penicillium spiculisporum* Lehman. The pathway of their biosynthesis has been enzymically demonstrated.

*Penicillium spiculisporum* Lehman is known to produce spiculisporic acid.<sup>1</sup>

The enzymic synthesis of spiculisporic acid from lauryl coenzyme A and  $\alpha$ -ketoglutarate has been described in a preliminary report.<sup>2</sup>

During the work on the biosynthesis of spiculisporic acid it appeared that the ability of the mould to produce the enzyme performing the condensation of lauryl coenzyme A with  $\alpha$ -ketoglutarate slowly declined and after about three years of handling in the laboratory the culture had entirely lost the activity of the enzyme. This kind of degeneration of an organism is not an unusual matter when dealing with the production of secondary metabolites.

When trying to restore the enzyme activity, however, it was found that a cell-free preparation from the "degenerated" culture contained an enzyme that catalyzed the condensation of lauryl coenzyme A and oxalacetate. The product formed was presumably decylcitrate (Fig. 1). A fresh spiculisporic acid-producing culture from Centraalbureau voor Schimmelcultures, Baarn,

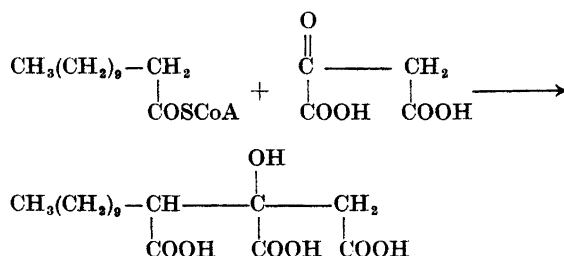


Fig. 1. Biosynthesis of decylcitric acid.

Table 1.  $R_F$  values of the tricarboxylic acids. Kieselgel G nach Stahl. Solvent: Benzene-dioxane-acetic acid 90:25:4. Spots were detected by spraying with 2',7'-dichlorofluorescein.

Spiculisporic acid	0.63
(-)-Decyleitric acid	0.44
(+)-Decyleitric acid	0.25

Netherland, did not show any tendency to produce the oxalacetate-condensing enzyme, but the presence of the spiculisporic acid-forming enzyme was easily demonstrated. The "degenerated" culture was confirmed by the Centraalbureau voor Schimmelfcultures to be identical with *P. spiculisporum* Lehman.

Hesitating to define the process that has taken place in the organism, we draw attention to the rare phenomenon involved in this case. Here the loss of the capacity to produce a secondary metabolite is compensated for by the ability to produce a new structurally related compound and, furthermore, the change in metabolism is confined to a defined enzyme reaction.

A thorough investigation of the culture filtrate from "degenerated" *P. spiculisporum*, using thin-layer chromatography, showed that the compound formed in the enzymic synthesis from lauryl coenzyme A and oxalacetate (compound E) occurred in substantial amounts as an excretion product. Thin-layer chromatography also indicated that the main product in the ether extract of the culture filtrate was another substance (compound X) belonging to the same group of compounds as spiculisporic acid and the enzymically formed product ( $R_F$ -values are given in Table 1).

The two compounds could be obtained as a crude mixture, either as a precipitate after acidification and chilling of the filtrate or as an ether extract from the acidified filtrate. Counter current distribution in a system of benzene-ether-water gave after nine transfers half the amount of compound X in almost pure form. Compound E was purified by column chromatography on Celite with chloroform-butanol as solvent. Recrystallisations of compound X and compound E from acetone-ligroin and water, respectively, gave the pure products.

The results of the elementary analysis of compound E corresponded to the composition of decyleitric acid. For some unaccountable reason the elementary analysis of compound X did not give reproducible figures. It appeared, however, that compounds E and X yielded practically identical IR-spectra, with strong absorption bands in the carbonyl region (1680—1700  $\text{cm}^{-1}$ ). Furthermore, the two compounds also showed practically identical mass spectra, with the molecular peak at 314 mass units, corresponding to decyleitric acid minus water. Both compounds also titrated as tribasic acids with the molecular weight of decyleitric acid. Alkali fusion of compound X resulted in the formation of lauric acid, identified by the melting point and paper chromatography.

The described properties of the compounds indicate that they are isomers of decyleitric acid. As decyleitric acid is carrying two asymmetric carbon atoms, four optical isomers are possible. Compound X, as well as compound E, as expected gave plain rotary dispersion curves, the former showing dextro

Table 2. Specific rotation of the decylcitric acids. Values for  $[\alpha]^{20}$ . 18.0 mg substance in 1.0 ml acetone solution.

Wavelength, Å	Compd. X	Compd. E	Compd. E transformed from X
3640	+0.241	-0.463	-0.468
4360	+0.113	-0.342	-0.344
5460	+0.039	-0.226	-0.220
5780	+0.043	-0.197	-0.197

rotation and the latter laevo rotation, over the wavelengths investigated (Table 2).

The compound obtained in the enzymic reaction is thus (–)-decylcitric acid, but the main product in the culture filtrate is (+)-decylcitric acid. One would expect the presence of an isomerase in the organism carrying out the inversion of (–)-decylcitrate to (+)-decylcitrate. It was found that (+)-decylcitric acid was almost quantitatively converted to (–)-decylcitric acid when treated with hot pyridine. Evidently the equilibrium favours the formation of (–)-decylcitrate; however, by heating slightly above the melting point it has been possible partially to convert the (–) form into the (+) form.

The presence of an isomerase in the organism was demonstrated by incubating a crude cell-free preparation, or a partially purified condensing enzyme, with (+)-decylcitrate- $^{14}\text{C}$  and (–)-decylcitrate- $^{14}\text{C}$  separately, both isomers being obtained by biological labelling from acetate- $^{14}\text{C}$ . Instead of effecting a conversion of the (–) form into the (+) form, as the enzymic formation of (–)-decylcitrate and the relative proportion of the substances in the culture medium indicated, the isomerase transformed the (+)-decylcitrate into (–)-decylcitrate, in accordance with the conversion in pyridine. The electrophoretically homogeneous enzyme preparation produces exclusively (–)-decylcitrate; thus, this compound has to be considered to be the primary product. Although the equilibrium in the isomerase reaction favours the formation of (–)-decylcitrate, the predominance of the (+)-decylcitrate in the culture filtrate could be a consequence of its faster excretion due to its higher water solubility as compared to the (–) form. Another circumstance that might favour the presence of the (+) form in the culture medium is that the (–) form can be reutilized. When the  $^{14}\text{C}$ -labelled acids were administered separately to replacement cultures, the (–)-decylcitrate was rapidly metabolized, on the other hand, the (+) form was very slowly or not at all taken up by the organism.

The purification procedure and the characteristics of the condensing enzyme will be published.

#### EXPERIMENTAL

*Culture conditions.* *Penicillium spiculisporum* Lehman was grown on a shake table (1" stroke, 250 rpm) at 28°C in 500 ml conical flasks. Each flask contained 250 ml Czapek-Dox medium ( $\text{NaNO}_3$  2.0 g,  $\text{KH}_2\text{PO}_4$  1.0 g,  $\text{KCl}$  0.5 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.1 g, Difco yeast extract 1.0 g, Glucose 40 g, distilled water 1 l). When the mycelia

were going to be used for enzyme studies the cultures were harvested after 3 days. The decylcitric acids were isolated with best yields 4–5 days after incubation.

*Isolation of (+)- and (-)-decylcitric acids.* The culture filtrates from 4 flasks were acidified with conc. HCl and the solution was left in the refrigerator for 16 h. The precipitate was filtered off and dried at room temperature, yield 1.5–2 g. The mixture was dissolved in the first tube (200 ml) of a counter current distribution apparatus using benzene-ether (1:1) as the mobile phase and water as the stationary phase. After 9 transfers the contents of tube 2 to 5 were pooled and (+)-decylcitric acid extracted into the organic phase after acidification of the mixture. Thin-layer chromatography (Kieselgel G nach Stahl suspended in 0.1 M oxalic acid; solvent: benzene-dioxane-acetic acid (90:25:4) of the evaporated extract showed very little contamination with (-)-decylcitric acid. Recrystallization from acetone-ligroin gave the pure product, m.p. 133–34°C.

(-)-Decylcitric acid was isolated from the crude precipitate by using column chromatography. 1 g was dissolved in 100 ml of 2 % butanol in chloroform and adsorbed on a column of 200 g Celite equilibrated with phosphate buffer, pH 3.5.<sup>3</sup> The column was washed with 500 ml chloroform followed by 300 ml 5 % butanol in chloroform. The (-)-decylcitric acid was eluted with 800 ml of 20 % butanol in chloroform. The solution was evaporated to dryness at reduced pressure and the decylcitric acid recrystallized from water. Yield 81 mg. (Found: C 56.99; H 8.51. Calc. for C<sub>18</sub>H<sub>28</sub>O<sub>7</sub>: C 57.9; H 8.45).

*Alkali fusion.* 200 mg of (+)-decylcitric acid was heated with 1.5 g of KOH and a few drops of water at 300° for 45 min. After cooling the mixture was dissolved in 10 ml of water and then acidified by the addition of conc. HCl. The acid solution was extracted with petroleum ether (b.p. 40–60°). Recrystallization from methanol-water after removal of the petroleum ether yielded 18.0 mg of lauric acid, m.p. 41° (lit. 44°). The identity of the lauric acid was further established by paper chromatography on Whatman Ac 82 using 60 % ethanol as solvent.

*Conversion of (+)-decylcitric acid to (-)-decylcitric acid.* 100 mg of (+)-decylcitric acid was heated with an excess of pyridine on the steam bath overnight, and the solution was then acidified and extracted with ether. After treatment with active carbon and recrystallization from water the product was pure (-)-decylcitric acid as judged from TLC and optical rotation.

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