# 5-METHYL-4-OXO-OCTANE-1,8-DIOIC ACID AS A MICROBIOLOGICAL DEGRADATION PRODUCT OF CHOLIC ACID

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## 1. Introduction

In continuing our studies on the microbiological degradation of bile acids, we have recently found that *Streptomyces rubescens*, grown on 3-(7aβ-methyl-1,5-dioxo-3aα-perhydroindan-4α-yl)propionic acid ($C_{13}$ acid) as its sole carbon source, produces (+)-(5*R*)-methyl-4-oxo-octane-1,8-dioic acid ($C_9$ acid) as one of the metabolites [1]. We have also reported that this organism is able to convert directly 3α,7α,12α-trihydroxy-5β-cholanoic acid (cholic acid) into various nitrogen-containing compounds of $C_{18}$, $C_{16}$ and $C_{13}$ perhydroindane-carboxylic acids and that one of these is the nitrogeneous derivative of the above $C_{13}$ acid itself [2]. It is therefore conceivable that *S. rubescens* might be able to metabolize cholic acid to the $C_9$ acid via the $C_{13}$ acid. Our continued interest in defining the intermediates and reaction sequence, involved in the microbiological catabolism of cholic acid, has prompted us to prove a direct formation of the $C_9$ acid form cholic acid. From our results obtained so far, it has been considered that *Arthrobacter simplex* [3], that is capable of converting cholic acid into (4*R*)-4-[4α-(2-carboxyethyl)-7aβ-methyl-5-oxo-3aα-perhydroindan-1β-yl]valeric acid ($C_{18}$ acid) [4,5], has a more intensive ability for the cholic acid degradation than *S. rubescens*. Thus, a large scale incubation of cholic acid with *A. simplex* was undertaken to obtain the $C_9$ acid. The present paper describes a direct transformation of cholic acid into the $C_9$ acid by *A. simplex*.

Incubation of cholic acid with *A. simplex* was conducted on a kg scale with the use of jar fermentors, and a mixture of transformation products was treated as described in the experimental part. The expected $C_9$ acid was at first isolated as its dimethyl ester in a yield of about 0.1%. Then, the ester was saponified to yield the free acid, which is identical with (±)-5-methyl-4-oxo-octane-1,8-dioic acid [1].

In contrast to a previous work [1], the $C_9$ dicarboxylic acid isolated in the present experiment was optically inactive. This result could be explained on the basis of racemization of the $C_9$ acid which might be expected to occur in the isolation process, because of enolization of an oxo group at C-4 adjacent to the asymmetric carbon atom at C-5. Another question has been raised whether the $C_9$ acid isolated in the present experiment, that is not traced with radioisotope, is formed from cholic acid. Although this question is not yet resolved, we believe, from our previous results [1–5], that *A. simplex* probably is capable of converting cholic acid into the $C_9$ acid via the $C_{18}$ acid.

On the microbiological degradation pathway of cholic acid, we have already isolated a number of possible intermediates and proposed the following hypothetical pathways: cholic acid → several $C_{24}$ intermediates → $C_{18}$ acid in *A simplex* [4,5]; cholic acid → several $C_{24}$ intermediates → $C_{18}$ acid → $C_{16}$ intermediates → $C_{13}$ acid → $C_9$ acid in *S. rubescens* [1,2]; cholic acid → several $C_{24}$ intermediates → $C_{22}$ acids in *Streptomyces gelaticus* [6]. The present result demonstrates the possibility that cholic acid could be metabolized by micro-organisms to the $C_9$ acid via certain common intermediates. However, more definitive conclusions about the metabolic fate of cholic acid by micro-organisms must await the identification of more catabolites and the enzymic studies on the various catabolites isolated so far.



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## 2. Experimental

### 2.1. *Incubation*

*A. simplex* grown on a nutrient agar was transferred into a 500 ml Sakaguchi's incubation flask containing a seed medium (100 ml) consisting of (g/l): glucose, 5; peptone, 5; corn steep liquor, 5; sodium cholate, 1. The inoculated flask was incubated at 28° on a reciprocal shaker for 3 days to give seed cultures. The seed culture (700 ml) was transferred into a 30 jar fermentor containing a synthetic medium (20 l) consisting of (g/l): $(NH_4)_2SO_4$, 2; $KH_2PO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; yeast extract, 0.5; cholic acid (used as sodium salt), 1; defoamer (Nissan Uniol D-2000, Nippon Oils and Fats Co., Ltd., Tokyo, Japan), 4 ml. Both media for flask and jar cultures were sterilized at 120° for 20 min and the pH was adjusted to 7.0–7.4 with alkali before sterilization. The jar-fermentation was carried out for 3 days at 30° with air rate of 20 l/min at a pressure of 0.5–0.7 kg/cm$^2$ with an agitator speed of 250 rpm.

### 2.2. *Isolation of the* $C_9$ *acid*

The culture broth was concentrated *in vacuo* at around 40° to one-twentieth of its original volume and centrifuged at 5860 × *g* for 30 min to remove the cells. The supernatant was acidified to pH 2 with dil. HCl and extracted with ethyl acetate. The extracts were evaporated *in vacuo* to dryness at room temperature. In this experiment, the incubation of cholic acid (1.14 kg) resulted in the formation of a mixture of products (784 g). The mixture was esterified by heating with methanol (7 l) containing 0.3% (w/v) toluene-*p*-sulphonic acid for 2 hr under reflux. The mixture was treated in the usual manner to yield a mixture of methyl esters. A solution of the mixture in benzene–light petroleum (3:1, v/v; 5 l) was passed through alumina (Woelm neutral, grade I; M.Woelm Eschwege, Germany; 3 kg) to remove some coloured materials and defoamer, and the column was washed with the same solvents. The filtrate and washings were combined and evaporated *in vacuo* to yield an oily residue. The residue was recrystallized from ether–light petroleum to yield the dimethyl ester of the $C_{18}$ acid (m.p. 39–40°, 473 g). The mother liquor was distilled under reduced pressure and a distillate (4.09 g) at 130° (oil-bath temperature)/0.04 mm was collected. The coloured residue (56 g) was not further treated. The distillate gave two major spots ($R_f$ 0.52 and 0.82), as visualized by spraying with conc. $H_2SO_4$ followed by heating, on thin-layer chromatography with the use of silica gel $GF_{254}$ (E.Merck A.-G., Darmstadt, Germany) and cyclohexane–ethyl acetate–acetic acid (5:5:1, by volume). The slower moving spot ($R_f$ 0.52) was sandwiched in between two spots detectable by the ultraviolet scanner before spraying with conc. $H_2SO_4$. An aliquot (200 mg) of the distillate was subjected to preparative thin-layer chromatography with the use of four plates (20 cm × 20 cm, 0.75 mm thick) of silica gel $HF_{254}$ (see above) and a mixture of solvents as described above. An area, which was sandwiched in between two bands as detected by the ultraviolet scanner, was scraped from the plates and extracted with chloroform–methanol (1:1, v/v). The extracts were treated with ether to remove contaminated silica gel and gave a colourless oil (67 mg), which showed infrared (IR) bands (in chloroform) at 1732 (C=O and $CO \cdot O \cdot CH_3$) cm$^{-1}$. The nuclear magnetic resonance (NMR) spectrum (Varian A-60 spectrometer with tetramethylsilane as internal standard in $CDCl_3$) exhibited bands at 1.13 (3H; doublet; J 6.9 c/sec; $CH_3$ at C-5) and 3.68 (6H; singlet; 2 $CO \cdot O \cdot CH_3$ at C-2 and C-7) ppm. Both IR and NMR spectra of this ester and the dimethyl ester (oil), prepared with diazomethane from the $C_9$ acid [1], were identical. The remaining distillate (3.89 g) was chromatographed on alumina (120 g, Woelm neutral, grade II). Elutions with 20 and 30% benzene in light petroleum gave an oil (719 mg). The oil (620 mg) was hydrolyzed with 2% (w/v) methanolic alkali and the resulting products were recrystallized from ether–light petroleum to yield (±)-5-methyl-4-oxo-octate-1,8-dioic acid (305 mg), m.p. 55–57° and $[\alpha]_D^{27}$ 0° (*c* 1.016 in chloroform) (Found: C, 53.25; H, 7.04. Calc. for $C_9H_{14}O_5$: C, 53.46; H, 6.98%). Identity of this acid with a sample partially synthesized in a previous paper [1] was established by mixed m.p. and by comparison of the IR spectrum.

## References

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