[Chem. Pharm. Bull. 36(1) 142—145 (1988)]

Non-stereoselective Conversion of the Four Diastereoisomers at the C-24 and C-25 Positions of 3α,7α,12α,24-Tetrahydroxy-5β-cholestan-26-oic Acid into Cholic Acid

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(Received July 7, 1987)

The four diastereoisomers at the C-24 and C-25 positions of the title compound (varanic acid) were incubated with rat liver mitochondrial fraction supplemented with adenosine triphosphate, coenzyme A, nicotinamide adenine dinucleotide, and $MgCl_2$. All of these isomers were converted into cholic acid. Thus, the stereochemical configuration at the C-24 and C-25 positions has no significant effect on the efficiency of side chain cleavage of the tetrahydroxy acid into cholic acid.

Keywords——3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid; varanic acid; cholic acid; bile acid; rat liver homogenate; mitochondorial fraction; β-oxidation

In the biosynthesis of cholic acid in animals, cholesterol is first converted into 3α,7α,12α-trihydroxy-5β-cholestane and then the latter compound is oxidized to 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA). The acid is presumed to be transformed into cholic acid by a sequence of reactions analogous to that involved in the β-oxidation of fatty acids (Chart 1). 3α,7α,12α,24-Tetrahydroxy-5β-cholestan-26-oic acid (TeHCA) has been postulated as an intermediate of this biologically important C–C bond cleavage reaction, and in fact its formation[2-4)] from THCA and its conversion[2-4)] into cholic acid have been demonstrated in rat liver homogenates. *In vivo* studies in bile fistula patients also revealed the conversion of TeHCA into cholic acid.[5)] Further, this acid was found in the bile of the frog, *Bombina orientalis*, and its stereochemistry was determined as (24*R*,25*R*).[6)] However, little is known about the stereochemical configuration at the C-24 and C-25 positions of the intermediate TeHCA in mammalian cholic acid biosynthesis. Recently Hoshita and co-workers have prepared four stereoisomers of TeHCA and assigned their stereochemistry at the C-24 and C-

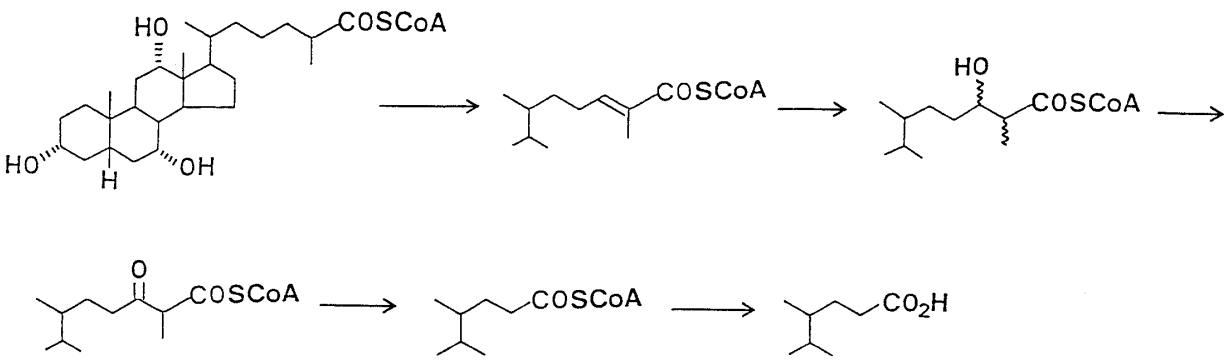

Chart 1. Postulated Biosynthetic Pathway to Cholic Acid from 3α,7α,12α-Trihydroxy-5β-cholestan-26-oic Acid CoA

25 positions.[7] More importantly, they reported the formation of the (24*R*,25*R*)-isomer as the sole TeHCA on incubation of (25*R*)- or (25*S*)-THCA with rat liver homogenate.[8] We have independently prepared the four stereoisomers of TeHCA and revised the stereochemical assignment of the above group.[9] To answer the question of which stereoisomer of TeHCA is the true intermediate in biosynthesis, it is essential to know the stereochemical specificity of the four isomers in the C–C bond cleavage reaction. The present paper describes the results of the incubation of TeHCA isomers with rat liver mitochondrial homogenate, demonstrating that all four stereoisomers are converted into cholic acid.

Results and Discussion

The four isomers were incubated with rat liver mitochondrial fraction in the presence of adenosine triphosphate (ATP), coenzyme A (CoA), nicotinamide adenine dinucleotide (NAD), and $MgCl_2$.[3,5] Non-labeled compounds was used as the substrate and the conversion yield was determined by gas chromatography-mass spectrometry (GC-MS) analysis without interference by endogenous cholic acid.

Figure 1 shows a typical gas liquid chromatography (GLC) result (analyzed in the form of trimethylsilyl (TMS) ether/Me ester) for the fraction corresponding to cholic acid/THCA (see Experimental for details). The THCA was added prior to extraction and this compound was used as an internal standard for quantification of the cholic acid produced by incubation. The peaks A and B correspond to cholic acid (*m/z*: 638 M^+, 623 M − Me, 548 M − TMSOH, 458 M − 2 × TMSOH, 368 M − 3 × TMSOH) and THCA (*m/z*: 665 M − Me, 590

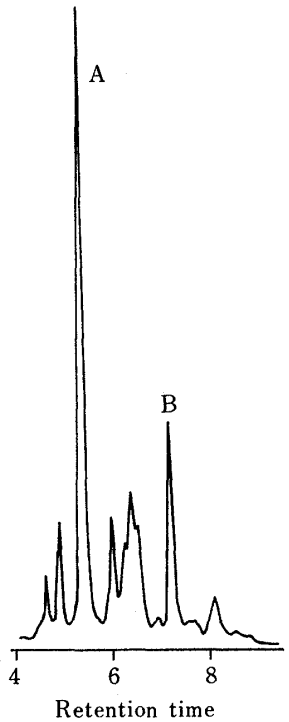

Fig. 1. GLC Analysis of the Incubation Product of (24*S*,25*R*)-TeHCA

A, cholic acid; B, THCA
Analyzed as TMS ether/Me ester derivatives. GC-MS conditions: 15 m OV-1 capillary column (Shimadzu CBP-1), oven temperature 240→280 °C (initial 2 min hold and then increased at 8 °C/min), injection temperature 280 °C.

Table I. Conversion of TeHCA into Cholic Acid by Incubation with Rat Liver Mitochondrial Fraction[a]

Substrate[b]/ condition	Cholic acid found (μg)	Conversion (%)
24*S*,25*R*	13.0	9.8
24*S*,25*R* (-CoA)	10.8	7.3
24*S*,25*S*	12.1	8.8
24*R*,25*S*	8.4	4.6
24*R*,25*R*	9.9	6.3
No substrate	4.4	0
THCA	17.9	15.4

a) The average value of two independent experiments is shown. *b*) The amount of each substrate was 100 μg.

M – TMSOH, 500 M – 2 × TMSOH), respectively. The results of the incubation are presented in Table I. When a boiled enzyme preparation was employed, the level of cholic acid found was the same as the endogenous level, confirming that the conversion was enzymatic.

It is evident that the four stereoisomers were converted into cholic acid regardless of the stereochemical configuration at the C-24 and C-25 positions. A slight preference (not significant) was seen for the (24*S*,25*R*)-isomer. In the absence of CoA, the formation of cholic acid was slightly decreased. The non-stereoselective conversion suggests that the oxidizing enzyme involved in this transformation does not require strict stereospecificity around the C-24 and C-25 stereochemical centers, although the possibility of the presence of an isomerase can not be neglected. However, it seems unlikely that four discrete enzymes transforming the respective isomers exist.

There has been some question as to whether or not TeHCA is an obligatory intermediate of cholic acid synthesis, since the conversion of TeHCA into cholic acid has been reported to be somewhat less effective than that of THCA.[3,5] Therefore THCA (as a C-25 epimeric mixture) was also incubated in the present work. Although some preference for THCA over any of the four stereoisomers of TeHCA was found (Table I), as reported previously,[3,5] the difference was not significant in relation to the above problem. A detailed comparison using stereochemically pure isomers of THCA will be required.

Hoshita *et al.*[8] demonstrated that the (24*R*,25*R*)-isomer is the sole TeHCA produced on incubation of THCA, while our present data indicate all the four isomers of TeHCA are equally well transformed into cholic acid. Therefore, the biologically important isomer of TeHCA may be the (24*R*,25*R*)-compound. It should be noted, however, that the orientation of the 24-hydroxyl group of the (24*R*,25*R*)-isomer is the opposite to that of (3*S*)-hydroxyfatty acid CoA ester, which was established as an intermediate of β-oxidation of long chain fatty acids.[10] The cause of this diversity is unclear at present, but the presence of the extra methyl group (27-Me) of TeHCA could influence the stereochemistry of hydration of α,β-unsaturated carboxyl CoA. Alternatively, the (24*R*,25*R*)-TeHCA may not be on the pathway of β-oxidation. In this connection, metabolic studies of 27-nor compounds, *e.g.*, 27-nor-3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (27-nor-THCA) and 27-nor-3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid (27-nor-TeHCA), would be interesting.

Experimental

Compounds——Four diastereoisomers of 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid were prepared as in the preceding paper[9] and their purity was proved to be more than 98%. 3α,7α,12α-Trihydroxy-5β-cholestan-26-oic acid was synthesized from Δ^{24}-THCA by catalytic hydrogenation.[8] NAD and ATP were obtained from Boehringer Co. (Mannheim, Germany). CoA was obtained from Sigma Chemical Co. (St. Louis, MO).

Incubation Procedure and Analysis of Incubation Product——Male rats of the Wistar strain weighing *ca.* 220 g were used. Rat liver homogenate, 20% (w/v), was prepared in 0.25 M sucrose solution with a loose-fitting glass homogenizer. The homogenate was centrifuged at 600 × *g* for 15 min. The supernatant fraction was centrifuged at 10000 × *g* for 20 min. The supernatant layer was discarded and the residual mitochondrial fraction was washed with 0.25 M sucrose. The washed residue was suspended in 0.1 M Tris–HCl buffer (pH 8.5) (1 ml of mitochondrial suspension corresponds to 0.8 g of rat liver; *ca.* 9 mg of protein/ml) and 4 ml of the suspension was distributed into individual 50-ml Erlenmeyer flasks. Further, 0.3 ml of a Tris–HCl buffer (pH 8.5) solution containing NAD (3 μmol), ATP (7 μmol), CoA (2.6 μmol) and $MgCl_2$ (30 μmol) was added to each flask. A substrate (100 μg) solution in MeOH (0.2 ml)–Tris–HCl (0.5 ml) was added and incubations were aerobically conducted for 1 h at 37 °C.

Incubations were terminated by the addition of 15 ml of 10% aqueous NaOH–ethanol (1 : 1, v/v). The whole mixture was heated at reflux for 6 h and cooled. The mixture, after addition of THCA (5 μg) as an internal standard, was acidified with 2 N HCl (15 ml), and extracted with AcOEt (30 ml × 2). The extract was dried over Na_2SO_4 and concentrated. The residue was treated with an excess of ethereal diazomethane. After the removal of the reagent and solvent by flushing with nitrogen, the residue was submitted to TLC (Merck, Silica gel F_{254} pre-coated plate), developed with $CHCl_3$–MeOH (8 : 1). The band corresponding to the position of cholic acid and THCA (*Rf* 0.3—0.4) was scraped off and the compounds were eluted from the silica gel with $CHCl_3$–MeOH (4 : 1). The concentrate was

derivatized with trimethylsilylimidazole (20 μl, warmed with a drier for 5 min) and analyzed by GC-MS (Shimadzu GC-MS DF 9020).

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