Identification of (24E)-3α,7α-dihydroxy-5β-cholest-24-enoic acid and (24R,25S)-3α,7α,24-trihydroxy-5β-cholestanoic acid as intermediates in the conversion of 3α,7α-dihydroxy-5β-cholestanoic acid to chenodeoxycholic acid in rat liver homogenates

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Abstract Studies of chemical structure of the intermediates in the biosynthetic sequence between 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) and chenodeoxycholic acid have been undertaken. Radiolabeled DHCA was incubated with a rat liver preparation. The reaction products were converted to the *p*-bromophenacyl esters, and analyzed by reversed-phase high performance liquid chromatography. Under the conditions used, the radioactivity was found in (24E)-3α,7α-dihydroxy-5β-cholest-24-enoic acid (31%) and (24R,25S)-3α,7α,24-trihydroxy-5β-cholestanoic acid (7%) along with the starting material (62%). Neither the 24Z isomer of the α,β-unsaturated bile acid nor the other three isomers of the β-hydroxy bile acid were detected. The findings support the proposed pathway for the side chain cleavage in chenodeoxycholic acid biosynthesis, which is thought to be identical to that of cholic acid biosynthesis.—**Une, M., A. Inoue, T. Kurosawa, M. Tohma, and T. Hoshita.** Identification of (24E)-3α,7α-dihydroxy-5β-cholest-24-enoic acid and (24R,25S)-3α,7α,24-trihydroxy-5β-cholestanoic acid as intermediates in the conversion of 3α,7α-dihydroxy-5β-cholestanoic acid to chenodeoxycholic acid in rat liver homogenates. *J. Lipid Res.* 1994. **35:** 620–624.

Supplementary key words bile acid biosynthesis • α,β-unsaturated bile acid • β-hydroxy bile acid

The primary bile acids formed from cholesterol in mammalian liver are cholic acid and chenodeoxycholic acid. At the present time, it is believed that in the major pathway for the formation of the primary bile acids, elaboration of the cholesterol nucleus precedes the degradation of the side chain, forming 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α-diol as intermediates. The cleavage of the side chain of these C_{27} intermediates entails an ω-oxidation followed by a β-oxidation. It is well established that the end products of the ω-oxidation, 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) (1–3) and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) (4–6) are obligatory intermediates in the major pathway for the biosynthesis of cholic acid and chenodeoxycholic acid, respectively.

The chemical structure of intermediates in the pathway for the β-oxidation of THCA to form cholic acid has been studied by us (7, 8) and other investigators (9, 10). Thus, (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid (Δ^{24}-THCA), and (24R,25S)-3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid (24-OH-THCA) were identified as the intermediates in this biosynthetic route. However, not much is known about the mechanism of the conversion of DHCA to the corresponding C_{24} bile acid, chenodeoxycholic acid.

The present report describes the in vitro formation of 3α,7α-dihydroxy-5β-cholest-24-enoic acid and 3α,7α,24-trihydroxy-5β-cholestanoic acid from DHCA and the elucidation of the absolute configuration at C-24 and C-25 of these intermediates.

Abbreviations: TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; 24-OH-THCA, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid; Δ^{24}-THCA, 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid; DHCA, 3α,7α-dihydroxy-5β-cholestanoic acid; 24-OH-DHCA, 3α,7α,24-trihydroxy-5β-cholestanoic acid; Δ^{24}-DHCA, 3α,7α-dihydroxy-5β-cholest-24-enoic acid.

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MATERIALS AND METHODS

General

Melting points (mp) were determined with a Kofler hot-stage apparatus and are uncorrected.

Nuclear magnetic resonance (NMR) spectra were measured on a JEOL JMN-FX-400 spectrometer (400 MHz) using CD_3OD as a solvent and tetramethylsilane as internal standard.

Reference compounds

Chenodeoxycholic acid was a commercial product. (24E)- and (24Z)-3α,7α-Dihydroxy-5β-cholest-24-enoic acids ((24E)- and (24Z)-Δ^{24}-DHCA) (11) and (25RS)-3α,7α-dihydroxy-5β-cholestanoic acid ((25RS)-DHCA) (12) were chemically synthesized from chenodeoxycholic acid according to the method described previously.

Synthesis of 3α,7α,24-trihydroxy-5β-cholestanoic acid (Fig. 1)

*Methyl (24R,25S)-3α,7α,24-triacetoxy-12α-hydroxy-5β-cholestanoate (**III**).* (24R,25S)-3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid ((24R,25S)-24-OH-THCA) was prepared as described previously (13). The tetrahydroxycholestanoic acid (**I**) (450 mg) was methylated with ethereal diazomethane solution. To a solution of the methyl ester (**II**) (470 mg) in pyridine (7 ml) and benzene (20 ml) acetic anhydride (7 ml) was added. The reaction mixture was kept at room temperature for 15 h and diluted with ice-cold water. The reaction product was extracted with benzene (100 ml × 3) after acidification with dilute HCl. The extracts were washed with water, 5% $NaHCO_3$ and water, and dried over Na_2SO_4. Evaporation of the solvent left a residue (690 mg), which was recrystallized from methanol to give crystals of **III**; mp. 79–82°C. NMR, (δ ppm) 0.72 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 0.99 (3H, d, J=6.59 Hz, 21-CH_3), 1.13 (3H, d, J=7.14 Hz, 27-CH_3), 1.99, 2.02, 2.04 (9H, s, 3-,7-, and 24-OAc), 3.66 (3H, s,-$COOCH_3$), 3.98 (1H, m, 12-H), 4.55 (1H, m, 3-H), 4.88 (1H, m, 7-H), 5.02–5.12 (1H, m, 24-H).

*Methyl (24R,25S)-3α,7α,24-triacetoxy-12-oxo-5β-cholestanoate (**IV**).* To a solution of **III** (500 mg) in acetone was added 0.8 ml of Jone's reagent (chromium trioxide (2.2 g) dissolved in sulfuric acid (2.8 ml) and diluted with 10 ml of water). The reaction mixture was stirred at room temperature for 2 h, then poured into water (100 ml), and ex-

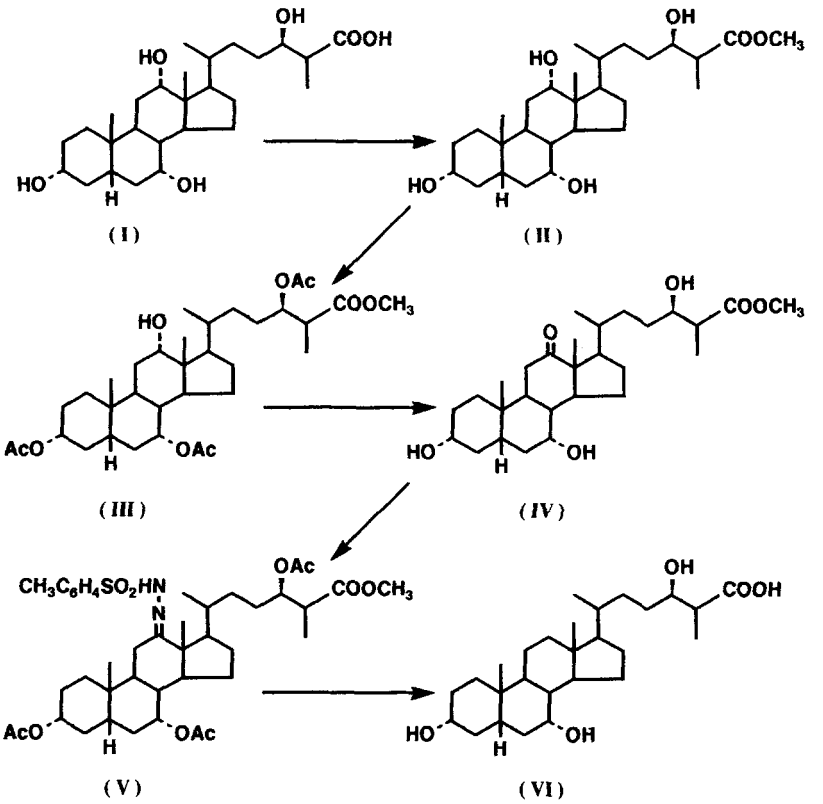

Fig. 1. Synthetic route of 3α,7α,24-trihydroxy-5β-cholestanoic acid from 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid. **I**, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid; **II**, methyl 3α,7α,12α,24-tetrahydroxy-5β-cholestanoate; **III**, methyl 3α,7α,24-triacetoxy-12α-hydroxy-5β-cholestanoate; **IV**, methyl 3α,7α,24-triacetoxy-12-oxo-5β-cholestanoate; **V**, methyl 3α,7α,24-triacetoxy-12-oxo-5β-cholestanoate 12-tosylhydrazone; **VI**, 3α,7α,24-trihydroxy-5β-cholestanoic acid.

tracted with ether (100 ml × 2). The ethereal extracts were washed with water, dried over Na_2SO_4, and evaporated into dryness. The residue was chromatographed on a silica gel (25 g) column using a mixture of benzene and ethyl acetate as an eluting solvent; the fractions containing a major products were collected and the solvent was evaporated to dryness to give a residue of **IV**, 421 mg. NMR, 0.83 (3H, d, J=6.59 Hz, 21-CH_3), 1.08 (3H, s, 18-CH_3), 1.09 (3H, s, 19-CH_3), 1.14 (3H, d, J=6.94 Hz, 27-CH_3), 2.00, 2.01, and 2.03 (9H, s, 3-,7-, and 24-OAc), 3.66 (3H, s,-$COOCH_3$), 4.52–4.60 (1H, m, 3-H), 4.98 (1H, m, 7-H), 5.03-5.12 (1H, m, 24-H).

*Methyl (24R,25S)-3α,7α,24-triacetoxy-12-oxo-5β-cholestanoate-12-tosylhydrazone (**V**).* To a solution of IV (400 mg) in acetic acid (10 ml) was added *p*-toluenesulfonyl hydrazide (400 mg). The reaction mixture was stirred at room temperature for 20 h, then poured into ice-cold water (100 ml) and extracted with ether (100 ml × 2). The ethereal extracts were washed with water, 3% Na_2CO_3, and water, and evaporated to dryness. The oily residue was chromatographed on a silica gel (25 g) column using a mixture of benzene and ethyl acetate as an eluting solvent. The fractions eluted with benzene–ethyl acetate 7:3 were collected and the solvent was evaporated to dryness to give a residue consisting mainly of **V**, 449 mg. NMR, 0.49 (3H, d, J=6.78 Hz, 21-CH_3), 0.85 (3H, s, 18-CH_3), 1.04 (3H, s, 19-CH_3), 1.15 (3H, d, J=7.15 Hz, 27-CH_3), 1.99, 2.02, and 2.05 (9H, s, 3-,7-, 24-OAc), 2.44 (3H, s, Ar-CH_3), 3.68 (3H, s,-$COOCH_3$), 4.52–4.60 (1H, m, 3-H), 4.95 (1H, m, 7-H), 5.00–5.08 (1H, m, 24-H), 7.38 and 7.81 (2H each, 2d, J=8.24 Hz, *p*-substituted phenyl).

*(24R,25S)-3α,7α,24-trihydroxy-5β-cholestanoic acid (**VI**).* To a solution of **V** (284 mg) in acetic acid (8 ml) at 50°C was gradually added $NaBH_4$ (300 mg) over a period of 15 min. The reaction mixture was allowed to stand at room temperature for 15 h. The reaction mixture was then poured into ice-cold water (100 ml) and extracted with ether (100 ml × 2). The ethereal extracts were washed with water, 2% $NaHCO_3$, and water, and the solvent was evaporated to dryness. The residue (187 mg) was chromatographed on a silica gel (25 g) column using ethyl acetate as an eluting solvent. The fractions containing a major product were collected. The solvent was evaporated in vacuo, and the resulting residue was hydrolyzed with 5% methanolic KOH (100 ml) under refluxing. The hydroxylate was extracted with ethyl acetate (100 ml × 2) after dilution with water (300 ml) and acidification with dilute HCl. The organic layer was washed with water and evaporated to dryness to give a residue consisting mainly of **VI** (116 mg). NMR, 0.70 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), 0.95 (3H, d, J=6.41 Hz, 21-CH_3), 1.12, (3H, d, J=6.96 Hz, 27-CH_3), 3.32–3.40 (1H, m, 3-H), 3.64–3.71 (1H, m, 24-H), 3.79 (1H, m, 7-H).

(24R,25R)-, (24S,25R)-, and (24S,25S)-3α,7α,24-trihydroxy-5β-cholestanoic acids were prepared from the corresponding 3α,7α,12α,24-trihydroxy-5β-cholestanoic acids exactly as described above for the (24R,25S)-isomer.

Labeled (25RS)-DHCA

To a solution of (25RS)-DHCA (50 mg) in acetic acid was added a solution of K_2CrO_4 (100 mg) in water (4 ml) with stirring at room temperature over a 5-min period. After 2 h the reaction mixture was poured into water (50 ml) and extracted with ether (50 ml × 2). The ethereal extracts were washed with water, and evaporation of the solvent gave a residue (47 mg) containing the 7-oxo derivative of DHCA. To a solution of the 7-oxo acid (10.9 mg) in methanol (1 ml) was added [^{3}H]sodium borohydride (31 mg, 50 mCi, NEN Research Products). The reaction mixture was allowed to stand at room temperature for 5 h and was then diluted with 20 ml of 0.01 N HCl. Extraction with ether and removal of the solvent by evaporation gave a residue which was chromatographed on a Lober column (RP-18, Merck) with 92% methanol as an eluting solvent. The column effluents were monitored by TLC (silica gel G plate, solvent system: isooctane–ethyl acetate–acetic acid 5:5:1) and also subjected to radioactivity measurements. The effluent fractions containing the radioactive DHCA were combined and the solvents were evaporated to dryness to yield 4.3 mg of [7β-^{3}H](25RS)-DHCA. The radiochemical purity of [7β-^{3}H]DHCA was greater than 99% by high performance liquid chromatography. The specific activity was 5.1 × 10^8 dpm/mg.

Incubation procedure

Male rats of the Wistar strain weighing about 200 g were used. The liver was minced and homogenized in 9 volumes (w/v) of 0.25 M sucrose by one stroke in a Potter-Elvehjem homogenizer. Cellular debris and nuclei were removed by centrifugation at 800 *g* for 10 min. The supernatant solution was used in the incubation experiments.

The incubation mixture contained the following in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0): 7.5 μmol ATP, 7.5 nmol FAD, 0.1 μmol CoASH, 10 μmol $MgCl_2$, and 1.0 mg protein. After preincubation at 37°C for 10 min, the reaction was started by the addition of [7β-^{3}H]DHCA (9 × 10^5 dpm) and the incubation was continued for 60 min. The reaction mixture was terminated by the addition of 6 N HCl (0.1 ml) and was extracted with a Sep-pak C_{18} cartridge (Waters, Milford, MA).

Analysis of incubation products

High performance liquid chromatography (HPLC) was carried out using a Waters (Milford, MA) M-45 solvent-delivery system equipped with a Shimadzu (Kyoto, Japan) SPD-1 UV detector; the wave length was 254 nm. A TSK GEL ODS-80-TM (4.6 mm i.d. × 15 cm, Tosoh, Tokyo, Japan) column was used. Samples were subjected

to HPLC together with the corresponding unlabeled authentic bile acid after *p*-bromophenasyl ester derivatization (7). A mixture of methanol–water 85:15 was used as the moving phase and the flow rate was 1 ml/min. The collected 0.5-ml fractions were counted in an Aloka LSC 3500 liquid scintillation spectrometer after addition of 7 ml of counting solution (Aquasol-2, NEN Research Products).

RESULTS

Four diastereoisomers at C-24 and C-25 of 24-OH-DHCA (**VI**) were prepared from the corresponding four isomers of 24-OH-THCA (**I**) via elimination of 12-hydroxyl group, respectively (Fig. 1). Methyl ester of **I** was partially acetylated to give **III** according to the method described previously (14). Chromic acid oxidation of **III** yielded the 12-oxo compound (**IV**), which was transformed to the 12-tosylhydrazone (**V**). The tosylhydrazone (**V**) was reduced with $NaBH_4$ to give 24-OH-DHCA (**VI**) followed by alkaline hydrolysis (15).

[7β-^{3}H](25RS)-DHCA was incubated with postnuclear supernatant in the presence of ATP, CoA, FAD, and Mg^{2+}. The incubation products were analyzed on HPLC after *p*-bromophenacyl ester detivatization. As shown in **Fig. 2,** the presence of two radioactive compounds other than the starting material was observed. The more rapidly eluted product was identical with (24R,25S)-24-OH-DHCA in its retention time on HPLC. The more slowly eluted peak was identical to that of (24E)-Δ^{24}-DHCA. No radioactivity was observed in the peaks corresponding to the other three isomers of 24-OH-DHCA and (24Z)-Δ^{24}-DHCA.

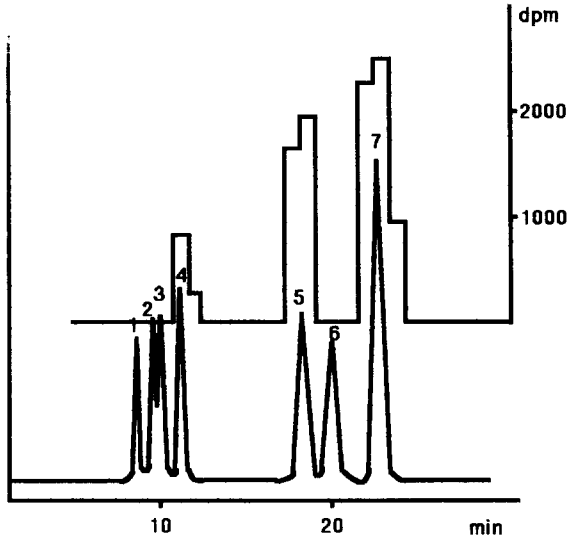

Fig. 2. Radio-HPLC of the incubation products of (25RS)-3α,7α-dihydroxy-5β-cholestanoic acid. Peak 1, (24S,25R)-3α,7α,24-trihydroxy-5β-cholestanoic acid; peak 2, (24S,25S)-3α,7α,24-trihydroxy-5β-cholestanoic acid; peak 3, (24R,25R)-3α,7α,24-trihydroxy-5β-cholestanoic acid; peak 4, (24R,25S)-3α,7α,24-trihydroxy-5β-cholestanoic acid; peak 5, (24E)-3α,7α-dihydroxy-5β-cholest-24-enoic acid; peak 6, (24Z)-3α,7α-dihydroxy-5β-cholest-24-enoic acid; peak 7, (25RS)-3α,7α-dihydroxy-5β-cholestanoic acid.

DISCUSSION

It is known that in the pathway for the biosynthesis of cholic acid the oxidative cleavage of THCA proceeds by a mechanism similar to the β-oxidation of fatty acids. Hence, the α,β-unsaturated bile acid, Δ^{24}-THCA, and the β-hydroxy bile acid, 24-OH-THCA, should be intermediates in this pathway (7–10, 16, 17). Farrants, Björkhem, and Pedersen (9) and Björkhem, Kase, and Pedersen (10) have detected Δ^{24}-THCA and 24-OH-THCA in the formation of cholic acid from THCA by rat liver peroxisomes. Further, we have reported the stereospecific formation of (24E)-Δ^{24}-THCA and (24R,25S)-24-OH-THCA from either (25R)- or (25S)-THCA in rat liver homogenates (7) and rat liver peroxisomes (8).

Another primary bile acid, chenodeoxycholic acid, is also biosynthesized from cholesterol in mammalian liver. By analogy to the biosynthesis of cholic acid, the major pathway from cholesterol to chenodeoxycholic acid involves DHCA as an obligatory intermediate, though alternative pathways may exist that do not involve the formation of DHCA (18). DHCA could be converted efficiently to chenodeoxycholic acid in human (4) and rat (5). In addition, it was also shown that chenodeoxycholic acid was formed from DHCA in human liver peroxisomes (16). That THCA competitively inhibited the formation of chenodeoxycholic acid from DHCA in hamster strongly suggests that THCA and DHCA share the same enzyme system for the oxidative cleavage of the side chain (19). Gustafsson (5) has reported the formation of 24-OH-DHCA in the incubation of DHCA with rat liver microsomes and the 100,000 *g* supernatant fluid. However, the possibility cannot be excluded that 24-OH-DHCA formed under the conditions used may be the direct hydroxylation product of DHCA at C-24. Thus, there is little evidence with regard to the existence of the possible intermediates, Δ^{24}-DHCA and 24-OH-DHCA, in the formation of chenodeoxycholic acid from DHCA.

The present study confirmed the formation of Δ^{24}-DHCA and 24-OH-DHCA as the incubation products of DHCA by rat liver homogenates. Though it is known that the peroxisomal fraction possesses the highest oxidative activity of the side chain of THCA (15, 16), postnuclear supernatant was used in the present study because the results in the experiments of THCA incubation with whole homogenates were identical with those with isolated peroxisomes (7, 8). By analogy to our previous experiment of THCA incubation, NAD^+ was omitted from

the incubation mixture. This may be the reason for the absence of chenodeoxycholic acid and increased formation of Δ^{24}-DHCA and 24-OH-DHCA. In the present study, the stereochemistries of the two products were deduced as (24E)-Δ^{24}-DHCA and (24R,25S)-24-OH-DHCA, based on the comparison of the retention times with authentic standards on HPLC. The absolute configuration in these compounds formed from DHCA is compatible with that in the products formed from the incubation of THCA with rat liver preparation in our previous study (7, 8). The present results indicate the involvement of the same enzyme system for the formation of cholic acid and chenodeoxycholic acid. Therefore, DHCA is oxidized to (24E)-Δ^{24}-DHCA, which in turn is hydrated to give (24R,25S)-24-OH-DHCA in the biosynthesis of chenodeoxycholic acid in rat liver as is known to be the case for cholic acid formation from THCA.

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