Stereochemistry in Dehydrogenation of 3α, 7α, 12α-Trihydroxy-5β-cholestan-26-oyl CoA during Cholic Acid Biosynthesis

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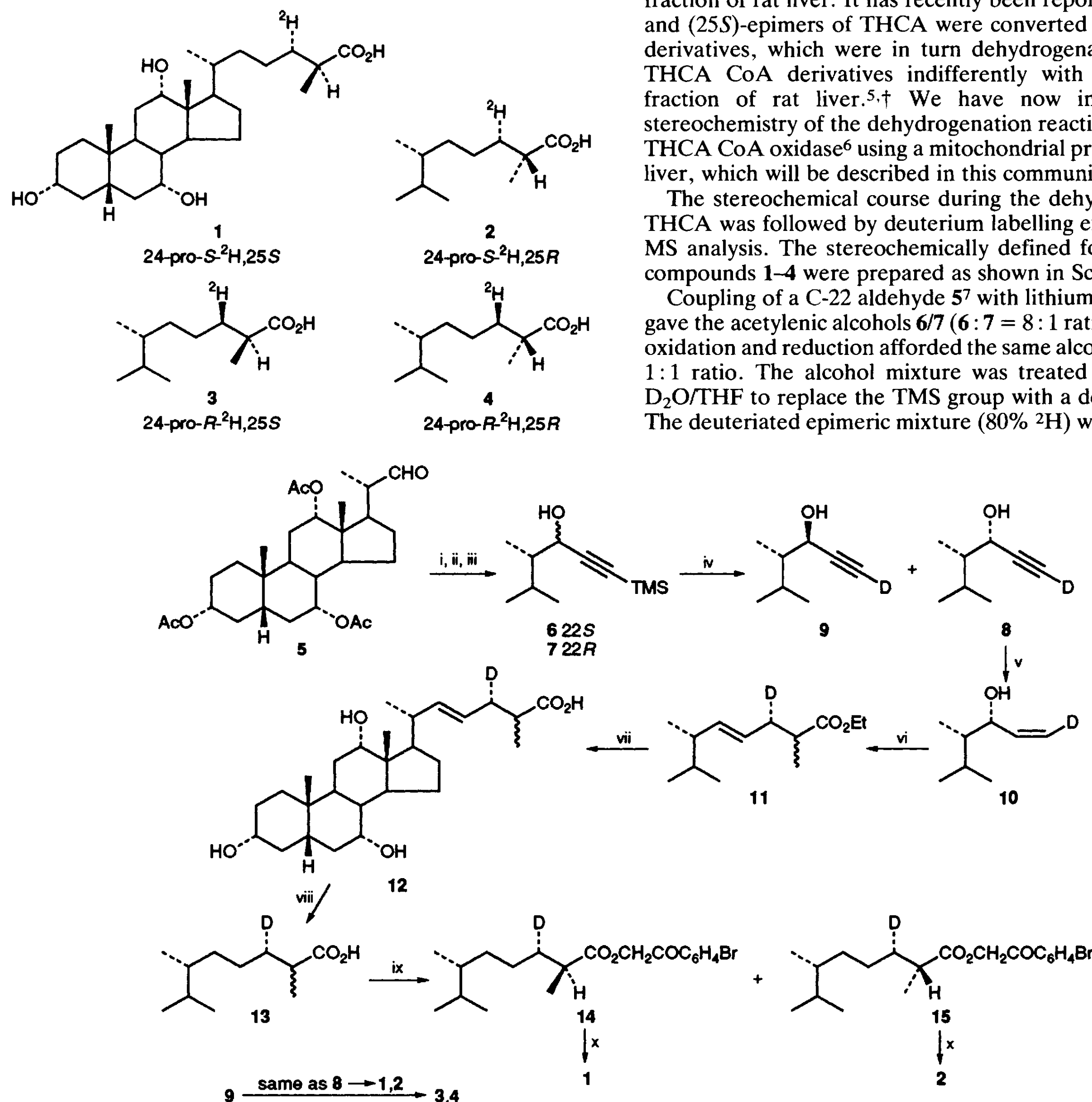
The dehydrogenation reaction, catalysed by 3α, 7α, 12α-trihydroxy-5β-cholestan-26-oyl CoA oxidase, is elucidated to proceed *via syn*-elimination of [24-pro-*R*]-hydrogen and 25-hydrogen.

The β-oxidation of fatty acid proceeds *via* α,β-unsaturated-, β-hydroxy-, and β-oxo-acyl CoA intermediates. Stereochemical courses established for the β-oxidation are: (*a*) the dehydrogenation of acyl CoA catalysed by acyl CoA oxidase yields (*E*)-enoyl CoA *via anti*-elimination of the pro-*R* hydrogen at C-3 and the pro-*R* hydrogen at C-2; (*b*) the addition of water catalysed by enoyl CoA hydratase takes place stereospecifically from the *si*-face at C-3 to form (3*S*)-hydroxyacyl CoA; and (*c*) the oxidation reaction catalysed by 3-hydroxyacyl CoA dehydrogenase is absolutely stereospecific for (3*S*)-hydroxyacyl substrate.[1] Although a similar β-oxidation pathway has been proposed for cholic acid biosynthesis from 3α, 7α, 12α-trihydroxy-5β-cholestan-26-oic acid (THCA),[2] little is known about its stereochemical course. We have reported the non-stereospecific formation of the four C-24/C-25 diastereoisomers of 3α, 7α, 12α, 24-tetrahydroxy-5β-cholestan-26-oic acid[3] and the conversion of the four isomers into cholic acid[4] in experiments using a mitochondrial fraction of rat liver. It has recently been reported that (25*R*)- and (25*S*)-epimers of THCA were converted into their CoA derivatives, which were in turn dehydrogenated into Δ^{24E}-THCA CoA derivatives indifferently with a peroxisomal fraction of rat liver.[5,†] We have now investigated the stereochemistry of the dehydrogenation reaction catalysed by THCA CoA oxidase[6] using a mitochondrial preparation of rat liver, which will be described in this communication.

The stereochemical course during the dehydrogenation of THCA was followed by deuterium labelling experiments and MS analysis. The stereochemically defined four deuteriated compounds **1–4** were prepared as shown in Scheme 1.

Coupling of a C-22 aldehyde **5**[7] with lithium TMS acetylide gave the acetylenic alcohols **6**/**7** (**6** : **7** = 8 : 1 ratio), which upon oxidation and reduction afforded the same alcohol **6**/**7**, but in a 1 : 1 ratio. The alcohol mixture was treated with TBAF in D_2O/THF to replace the TMS group with a deuterium atom. The deuteriated epimeric mixture (80% ^2H) was separated by



Scheme 1 *Reagents and conditions*: i, TMS —≡, *n*-BuLi, THF, −78 °C, **5**, 86% (**6** + **7**); ii, PCC, MS4A, CH_2Cl_2, 25 °C, 81%; iii, $NaBH_4$, MeOH, 0 °C, 72% (**6** + **7**); iv, $Bu_4N^+F^-$ (1.0 equiv.), THF–D_2O (5:1), 60 °C, 35% **8** and 38% **9**; v, H_2–Lindlar catalyst, quinoline, EtOAc, 25 °C, quant.; vi, $EtC(OEt)_3$, $EtCO_2H$, xylene, reflux, 83%; vii, LiOH, DME–H_2O, reflux, quant.; viii, H_2–10% Pd/C, EtOAc–EtOH, 25 °C, 76%; ix, *p*-$BrC_6H_4COCH_2Br$, Pr^i_2EtN, MeCN–MeOH, 25 °C, then HPLC separation, 53% **14** and 35% **15**; x, KOH, MeOH–H_2O, 0 → 25 °C, quant

a silica gel Lobar column, to give the less polar (22*S*)-**8** and the more polar (22*R*)-**9** acetylenic alcohols.

Hydrogenation of the alcohol **8** over Lindlar catalyst gave the (22*R*)-allylic alcohol **10**.‡ Orthoester Claisen rearrangement of **10** using triethyl orthopropionate and propionic acid gave the (25*RS*)-ester **11**. It is presumed that compound **11** has deuterium atom exclusively at the position of pro-*S* hydrogen on the basis of preferred transition state model of the rearrangement reaction.[8] The ester **11** was hydrolysed (leading to **12**), hydrogenated (leading to **13**) and derivatized to give the *p*-bromophenacyl ester **14/15**. At this stage the C-25 epimers were separated into a more mobile (25*S*)-**14** and a less mobile (25*R*)-**15** isomers by reversed-phase (ODS)-HPLC.[9] Alkaline hydrolysis of the esters **14** and **15** furnished [24-pro-*S*-^{2}H]-(25*S*)-**1** and [24-pro-*S*-^{2}H]-(25*R*)-**2** THCAs, respectively. The (22*R*)-alcohol **9** was converted into [24-pro-*R*-^{2}H]-(25*S*)-**3** and [24-pro-*R*-^{2}H]-(25*R*)-**4** isomers in the manner described for **8** (Scheme 1). The ^{2}H content of the final compounds **1–4** were estimated to be *ca.* 80% based on the FAB-MS spectral data. The stereochemical purity at C-25 of **1–4** was greater than 99%.§ Compounds **3** and **4** were presumed to be deuteriated exclusively at the position of [24-pro-*R*]-hydrogen.[8]

Compounds **1–4** (100 μg each) were incubated separately with the cell free system (10,000 g precipitate, 5 ml Tris-HCl buffer, pH 8.5, containing CoA, ATP and $MgCl_2$) prepared from rat liver, and the product, Δ^{24E}-THCA, was separated by HPLC as described previously.[3] Approximately 15 μg of Δ^{24E}-THCA was obtained from each incubations. The FAB-MS spectra of the Δ^{24E}-compounds (in the form of *p*-bromophenacyl ester) are shown in Fig. 1. Fig. 1(*a*) and (*b*) clearly indicate that most of deuterium was retained in the Δ^{24E}-THCAs derived from [24-pro-*S*-^{2}H]-substrates **1** and **2**. In contrast, Fig. 1(*c*) and (*d*) shows that deuterium was lost during the conversion from [24-pro-*R*-^{2}H]-substrates **3** and **4**. Hence, it is concluded that 24-pro-*R* hydrogen is eliminated in the dehydrogenation catalysed by THCA CoA oxidase. The observed MS results, taken together with the 24*E*-stereochemistry of the product, may lead to an idea that the dehydrogenation of (25*S*)-THCA (**1** and **3**) occurs *via anti*-elimination, whereas that of (25*R*)-THCA (**2** and **4**) proceeds *via syn*-elimination, although the idea seems highly unlikely in the light of enzyme reaction mechanism. The possibility of *syn*-elimination of **1** and **3** could be ruled out, since the formation of Δ^{24Z}-THCA was not observed.

HPLC analysis of the substrate (in the form of *p*-bromophenacyl ester) recovered from the incubation of **1** and **3** revealed that THCA was a *ca.* 1 : 1 mixture of (25*S*) and (25*R*)-isomers, whereas the recovered **2** and **4** retained the original (25*R*)-stereochemistry. This behaviour is consistent with our recent findings that the mitochondrial fraction of rat liver homogenate has an epimerase activity which catalyses the conversion of (25*S*)-THCA into (25*R*)-isomer probably in the form of CoA ester derivatives.[10] Thus, it is highly likely that **1** and **3** would isomerise into **2** and **4**, respectively, prior to the dehydrogenation. Based on these data, we are inclined to propose that the dehydrogenation occurs in *syn*-mode, wherein (25*R*)-THCA CoA, not (25*S*)-epimer, is a substrate for the oxidase. Further studies using a more purified enzyme system are apparently required to prove our proposal. *Syn*-elimination of H-24 and H-25 in the cholic acid biosynthesis is in marked contrast with *anti*-elimination in the fatty acid β-oxidation. This provides further evidence supporting the findings that the oxidase involved in bile acid synthesis is distinct from the fatty acid oxidase.[6]

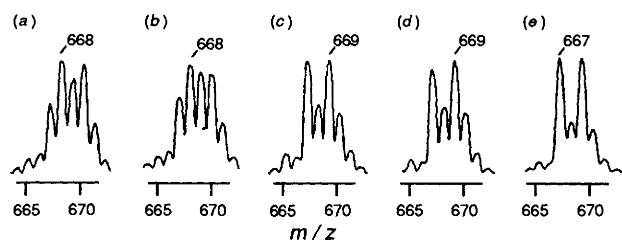


Fig. 1 [M + Na]$^+$ region of FAB–MS of Δ^{24E}-THCA derived from (*a*) [24-pro-*S*-^{2}H]-(25*S*)-THCA-**1**, (*b*) [24-pro-*S*-^{2}H]-(25*R*)-THCA-**2**, (*c*) [24-pro-*R*-^{2}H]-(25*S*)-THCA-**3**, (*d*) [24-pro-*R*-^{2}H]-(25*R*)-THCA-**4**, and (*e*) non-labelled Δ^{24E}-THCA

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Footnotes

† To our knowledge, the stereochemistry at C-25 [(25*R*) and/or (25*S*)] of THCA biosynthesised in mammalian liver has still remained obscure (I. Björkhem, *J. Lipid Res.*, 1992, **33**, 455), although THCA isolated from human bile has the (25*R*)-configuration (A. K. Batta, G. Salen, S. Shefer, B. Dayal and G. S. Tint, *J. Lipid Res.*, 1983, **24**, 94].

‡ The C-22 stereochemistry of **6–10** and the 22-epimer of **10** were assigned on the basis of their ^{1}H NMR data in comparison with those reported for the related compounds (*e.g.*, T. Eguchi, M. Yoshida and N. Ikekawa, *Bioorg. Chem.*, 1989, **17**, 294), and confirmed by applying a CD method [N. Harada, J. Iwabuchi, Y. Yokota, H. Uda and K. Nakanishi, *J. Am. Chem. Soc.*, 1981, **103**, 5590] to the 22-benzoates of **10** (CD: Δε +3.3 at 224 nm, MeOH) and 22-epimer of **10** (CD: Δε −1.3 at 224 nm, MeOH).

§ This was confirmed by preparing *p*-bromophenacyl ester of the acids followed by HPLC analysis.

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