IDENTIFICATION OF 27-NOR-3α, 7α, 12α-TRIHYDROXYCOPROSTAN-24-ONE APPARENTLY DERIVED FROM 3α, 7α, 12α-TRIHYDROXY-24-OXOCOPROSTANOIC ACID, A POSTULATED INTERMEDIATE OF BILE ACID BIOSYNTHESIS

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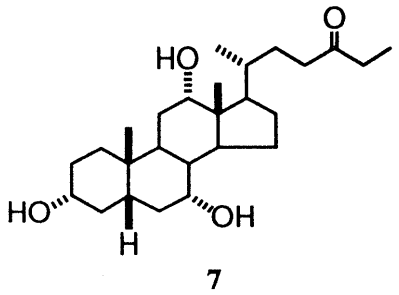
Incubation of [27-^{13}C]-3α,7α,12α-trihydroxycoprost-24-en-26-oic acid with rat liver homogenate followed by ^{13}C-NMR analysis of the incubation product has resulted in the identification of [26-^{13}C]-27-nor-3α,7α,12α-trihydroxycoprostan-24-one, supporting the idea that the substrate has been metabolized into 3α,7α,12α-trihydroxy-24-oxocoprostan-26-oic acid CoA derivative.

KEYWORDS cholic acid; 3α,7α,12α-trihydroxycoprost-24-en-26-oic acid; 27-nor-3α,7α,12α-trihydroxycoprostan-24-one; ^{13}C-label; β-oxidation

Cholic acid biosynthesis from cholesterol involves C-24/C-25 bond cleavage. A precursor of this carbon-carbon bond cleavage reaction is 3α,7α,12α-trihydroxycoprostan-26-oic acid (THCA) (**1**) and this acid is thought to be metabolized into cholic acid (**5**) by a mechanism (Chart 1) similar to that of the β-oxidation of fatty acids.[1] Among the intermediates shown in Chart 1, experimental evidence for the intermediary role of 24-ene-THCA[2,3] (**2**) and 24-hydroxy-THCA (TeHCA)[2,4] (**3**) has been accumulated. In contrast, little has been known on the formation and metabolism of 24-oxo-THCA (**4**) or its CoA derivative. We have recently reported the nonselective conversion of four diastereoisomers of TeHCA into cholic acid using rat liver homogenate.[5]

In our further studies on the mechanism of the C-C bond cleavage reaction, a ^{13}C-labeled compound, [27-^{13}C]-24-ene-THCA (**6**), has been prepared and the metabolic fate of this stable isotope labeled compound has been investigated with rat liver homogenate. These studies have led to the finding that 27-nor-3α,7α,12α-trihydroxycoprostan-24-one (**7**) is formed from 24-ene-THCA (**2**).



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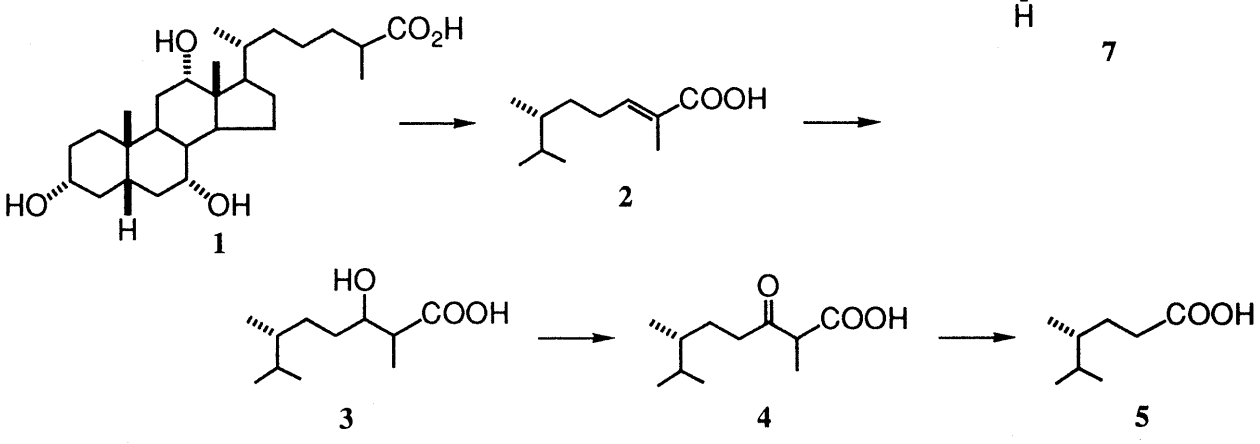

1 **2** **3** **4** **5**

Chart 1. Postulated Mechanism of Cholic Acid Biosynthesis
The acid residue could be CoA form in a biological system.

The ^{13}C-labeled acid **6** was synthesized according to Chart 2. The Wittig reaction of the protected aldehyde **8** with the ylide[6] generated from the phosphonium salt **9** by the action of *n*-BuLi afforded the unsaturated ester **10**. Alkaline

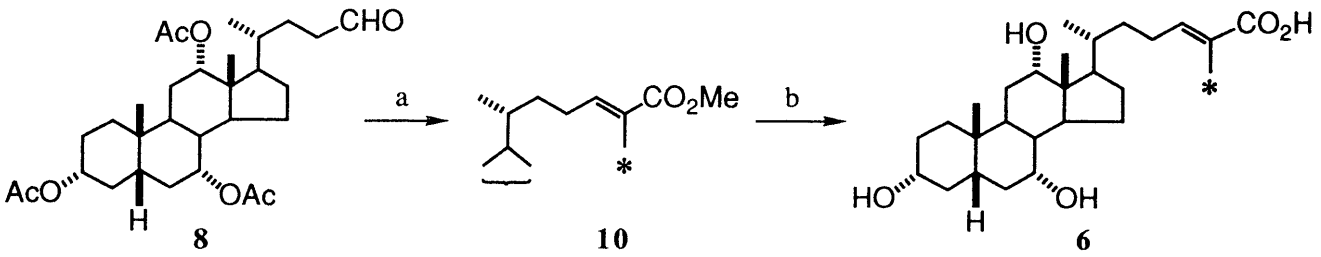

Chart 2. Synthesis of [27-^{13}C]-24-ene-THCA (**6**)
Reagents: a, [$Ph_3PCH(^{13}CH_3)CO_2Me]^+I^-$ (**9**), *n*-BuLi; b, KOH-MeOH.

hydrolysis of **10** furnished **6**. The ^{13}C-NMR and HPLC analysis of **6** indicated that **6** consists of 95% (24*E*)- and 5% (24*Z*)-isomer.[7]

The ^{13}C-labeled acid (**6**) (1.0 mg) was incubated with a rat liver homogenate (10,000g supernatant, 2 ml, Tris buffer pH 8.5; for experimental conditions, see ref. 5). The reaction was terminated by the addition of 3N-NaOH and ethanol. The mixture was heated at reflux for 5 h, acidified by 2N-HCl, and then extracted with ethyl acetate. The ^{13}C-NMR spectrum (Fig. 1) of the concentrated extract showed a new signal at δ8.2. The chemical shift appeared to be ascribable to a terminal methyl carbon (C-26) of the ethylketone moiety of 27-nor-3α,7α,12α-trihydroxycoprostan-24-one (**7**), apparently derived from 24-oxo-THCA (**4**) (amount of **7** formed was calculated as 100 μg by GC-MS analysis).

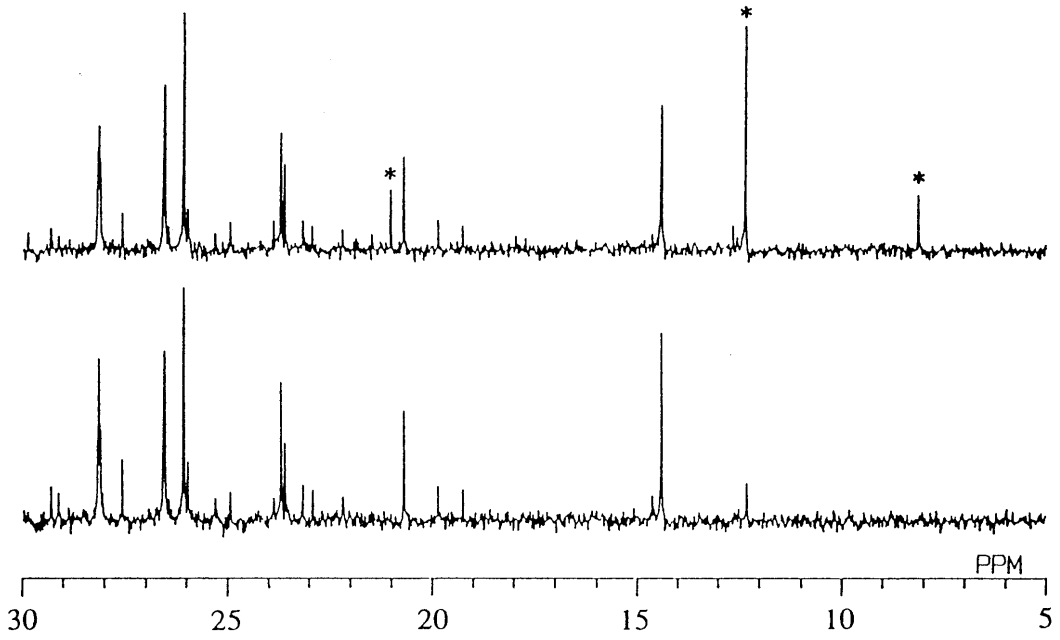

Fig. 1. ^{13}C-NMR Spectra (in Part) of the Incubation Product
Upper: from [^{13}C]-24-ene-THCA (**6**); lower: incubation medium background. Asterisked signal at δ8.2 is due to a new metabolite, whereas the other two asterisked signals are due to the unreacted substrate.

The structure and formation of **7** were firmly established as follows. First, the chemically synthesized authentic sample of **7**[8] exhibited its C-26 signal at δ8.2, which is completely identical to that found in the incubation mixture. Second, the extract was separated by p-tlc to give a fraction which has the same Rf value as that of the authentic **7**. This fraction was analyzed as the trimethylsilyl (TMS) ether derivative by GC-MS using a capillary column. A strong peak was observed at the retention time expected for the authentic **7**. Importantly, MS fragment ions of this peak

were shifted by one mass unit from the authentic sample; *e.g.*, *m/z*: 622 [M-Me], 547 [M-TMSOH], 532 [M-TMSOH-Me], 457 [M-2xTMSOH], and 367 [M-3xTMSOH]. It is apparent from these data that ethylketone **7** was derived from the ^{13}C-labeled substrate **6**.

Labile property of 24-oxo-THCA (**4**) was demonstrated by the following experiments. Hydrolysis of ethyl 3α,7α,12α-triformyloxy-24-oxocoprostan-26-oate[9] under alkaline conditions afforded the salt of 24-oxo-THCA.[10] However, attempted extractive isolation of the corresponding free acid failed, since neutralization (2N-HCl or sat. NH_4Cl) of the salt caused decomposition into ethylketone **7**. It is reasonably assumed that 24-oxo-THCA, if produced, would be decomposed to **7** during the work-up stage in the present work.

Hence, the identification of 7 in the incubation mixture strongly suggests the enzymatic formation of 24-oxo-THCA (**4**), presumably in the form of CoA derivative, from 24-ene-THCA (**2**). Ethylketone **7** has been previously identified in the bile alcohol composition of gallbladders of bullfrogs.[11]

Finally, it should be emphasized that successful characterization of **7** is facilitated by the use of ^{13}C label, which allowed us to predict a possible structure of the metabolite.

In relation to the ketone formation from β-keto-acid, it is interesting to note the following examples. Incubation of 3-oxostigmast-4-ene-26-oic acid with microbial cell-free system afforded a small amount of an ethylketone (27-norcholest-4-ene-3,24-dione),[12] and 9α-hydroxy-27-norcholest-4-ene-3,24-dione was isolated from the fermentation of sitosterol with a mutant of *Mycobacterium fortuitum*.[13] These ketones could be derived from intermediary keto-acids (or their CoA derivative) during a β-oxidation type reaction.

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7) In this text, all ^{13}C-NMR (125 MHz) spectra were recorded in CD_3OD, and the chemical shifts are expressed in reference to CD_3OD (δ 49.0). The chemical shifts of the labeled carbons are: δ 12.4 [(Z)-methyl] and 21.0 [(E)-methyl].
8) Ethylketone **7** was prepared starting with 3α,7α,12α-(tritetrahydropyranyloxy)-5β-cholan-24-al in three steps, *i.e.*, addition of ethylmagnesium bromide, PCC oxidation, and deprotection of THP group under an acidic condition. (**7**): mp 159-160°C, ^{13}C-NMR δ 36.8 (C-20), 17.8 (C-21), 31.2 (C-22), 40.0 (C-23), 215.1 (C-24), 36.6 (C-25), 8.2 (C-26).
9) The ester was prepared by the condensation of 3α,7α,12α-triformyloxy-5β-cholan-24-oic acid with magnesium salt of methylmalonic acid half ethyl ester [D. W. Brooks, L. D. -L. Lu, and S. Masamune, *Angew. Chem. Int. Ed. Engl.*, **18**, 72 (1979)]. ^1H-NMR ($CDCl_3$) δ 1.27 (t, J=7.1 Hz, $CH_2\underline{CH_3}$), 1.37 (d, J=7.2 Hz, 27-H_3), 3.51 (q, J=7.2 Hz, 25-H), 4.22 (q, J=7.1 Hz, $C\underline{H_2}CH_3$).
10) Rf value (developed on Merck HPTLC RP-18 plate with MeOH-water 9:1): (**4**, the basic methanol solution was directly applied on TLC plate) 0.58; (**5**) 0.50; (**7**) 0.38. Upon acidification of the KOH/MeOH reaction mixture the spot at 0.58 gradually decreased with concomitant appearance of the spot at 0.38.
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