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# Substrate Stereochemistry of Isovaleryl-CoA Dehydrogenase

## II. Steric Course of C-3 Hydrogen Elimination[1]

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(3*S*)-[4-$^{13}$C]Isovaleryl-CoA was prepared and dehydrogenated to 3-methylcrotonyl-CoA by pure rat liver isovaleryl-CoA dehydrogenase. In its $^{13}$C NMR spectrum, the product showed a single enriched $^{13}$C resonance at δ27.73. Signal assignment of the methyls of 3-methylcrotonyl-CoA (3-pro-*Z*, δ21.93; 3-pro-*E*, δ27.73) was made by synthesizing reference samples of 3-[4-$^{13}$C]methylcrotonyl-CoA. In conjunction with our prior determination (D. J. Aberhart and C-H. Tann, 1981, *Bioorg. Chem.* **10**, 200–205) that isovaleryl-CoA dehydrogenase removes the 2-pro-*R* hydrogen of isovaleryl-CoA, the results show that the dehydrogenation proceeds with *anti* elimination of hydrogens from C-2 and C-3 of the substrate.


## INTRODUCTION

Tanaka and co-workers have firmly established that the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA, a step in the metabolism of leucine, is catalyzed by a specific enzyme, isovaleryl-CoA dehydrogenase (*1*). The existence of this enzyme was first indicated by the results of studies on the genetic defect of leucine metabolism: isovaleric acidemia (*2*). Patients having this disease excrete large amounts of isovaleric acid in their urine and are found to be deficient in isovaleryl-CoA dehydrogenating ability, whereas *n*-butyryl-CoA dehydrogenating activity remains normal (*3*). Recently, Tanaka and co-workers (*1*, *4*) have purified isovaleryl-CoA dehydrogenase to homogeneity. The pure enzyme does not dehydrogenate short, medium, or long straight-chain acyl-CoA, nor 2-methyl branched-chain acyl-CoA (i.e., isobutyryl- and 2-methylbutyryl-CoA, intermediates in valine and isoleucine metabolism, respectively, for which another specific dehydrogenase exists (*5*)).

In the absence of ETF[3] or other electron-transfer reagents, the enzyme cata-

[1] Part I, D. J. Aberhart and C-H. Tann (1981) *Bioorg. Chem.* **10**, 200–205.

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[3] Abbreviations used: ETF, electron-transfer flavoprotein; FAD, flavin adenine dinucleotide; PMS, phenazine methosulfate; SW, sweep width, PW, pulse width; Aq, acquisition time; LB, line broadening.



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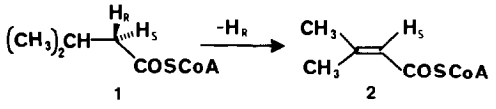
lyzes the exchange of a hydrogen atom from C-2 of isovaleryl-CoA, forming the basis of a simple assay (*6–9*). Five years ago, we reported (*10*) that the isovaleryl-CoA dehydrogenase reaction proceeds stereospecifically with loss of the 2-pro-*R* hydrogen of the substrate, **1** → **2**. This study was not conducted with the pure dehydrogenase, but instead was carried out by administering stereospecifically C-2 tritiated isovaleric acids to biotin-deficient rats. The main product, 3-hydroxyisovaleric acid, was produced after formation, *in vivo*, of isovaleryl-CoA, dehydrogenation to 3-methylcrotonyl CoA, hydration of the latter by crotonase, and hydrolysis of the hydration product. Since Ikeda *et al.* have shown that, in rats, other acyl-CoA dehydrogenases do not appreciably dehydrogenate isovaleryl-CoA (*11*), the results are relevant to the stereochemistry of the pure isovaleryl-CoA dehydrogenase. Unfortunately, we were unable to determine the C-3 stereochemistry of the dehydrogenation reaction using biotin-deficient rats on account of the unexpected nonstereospecific hydration of 3-methylcrotonyl-CoA in these rats (*12, 13*).[4] We therefore undertook, and now report, the determination of this C-3 stereochemistry using the pure isovaleryl-CoA dehydrogenase isolated from rat liver mitochondria.



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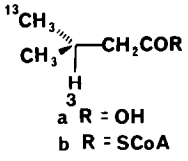
## RESULTS AND DISCUSSION

In principle, the hydrogen elimination from C-3 of isovaleryl-CoA could take place *syn* or *anti*, relative to the hydrogen abstracted from C-2 (or the reaction could be nonstereospecific). Our approach to the determination of this relative stereochemistry was to synthesize stereospecifically $^{13}C$-labeled isovaleryl-CoA, **3b**, convert it by using purified isovaleryl-CoA dehydrogenase to 3-methylcrotonyl-CoA, and determine by $^{13}C$ NMR the location of the $^{13}C$-enriched methyl group in the product.




(3*S*)-[4-$^{13}C$]Isovaleric acid, **3a**, was prepared from (2*RS*,3*S*)-[4-$^{13}C$]valine (97–99 at.% $^{13}C$) (*15*) by a modification of a previously described procedure (*14*). The product, **3a**, was converted to the required CoA ester, **3b**, by the mixed anhydride method (*6*), and the product was purified by preparative HPLC. (A nonstereospe-

[4] With purified crotonase, 3-methylcrotonyl-CoA undergoes stereospecific *syn* hydration on the 2-Re, 3-Re face of the double bond (*13*). We thank Professor Duilio Arigoni for informing us of this work.

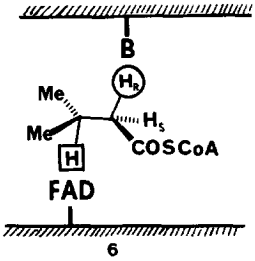
cifically labeled sample of (3*RS*)-[4-$^{13}$C]isovaleryl-CoA was also prepared—see Experimental for synthetic method. The two $^{13}$C-enriched methyl signals could not be resolved.)

Prior to carrying out the enzymatic dehydrogenation of **3b**, we wished to establish the $^{13}$C signal assignment of the C-3 methyls of 3-methylcrotonyl-CoA. For this purpose, 3-(*E*)-[4-$^{13}$C]methylcrotonic acid, **4a**, was synthesized by the method of Corey and Katzenellenbogen (*16, 17*). Nonstereospecifically labeled 3-[4-$^{13}$C]methylcrotonic acid (ca. 45 at.% $^{13}$C per methyl) was similarly prepared except that the reaction with $(^{13}CH_3)_2CuLi$ was run at 0°C instead of at −78°C. By mixing a portion of the nonstereospecifically labeled product with **4a**, a sample of 3-[4-$^{13}$C]methylcrotonic acid, **5a**, having ca. 67% (3-*E*) and ca. 33% (3-*Z*) labeling, was obtained. The latter and **4a** were each converted to the corresponding CoA esters, **4b** and **5b**, and the $^{13}$C NMR spectra were recorded. The stereospecifically labeled **4b** showed a single enriched-carbon resonance at δ27.78, whereas the sample labeled nonequally in both methyl groups showed two enriched-carbon signals at δ21.93 and δ27.76 in a ca. 1 : 2 ratio.


4 a R = OH 5
b R = SCoA

With the signal assignment secure, the substrate, **3b**, was subjected to dehydrogenation with pure rat liver isovaleryl-CoA dehydrogenase. After clean-up by Sephadex G-10 chromatography, the pure 3-methylcrotonyl-CoA was isolated by HPLC. The product showed a single resonance due to $^{13}$C-enriched carbon at δ27.73, showing that it was labeled as in **4b**.

This stereochemical information, in conjunction with our prior determination that the 2-pro-*R* hydrogen of isovaleryl-CoA is removed by the enzyme (*10*), shows that the two hydrogens are removed in an *anti* orientation. The steric course of this transformation is thus in accord with that established for all the other acyl-CoA dehydrogenase reactions whose steric courses have been investigated (for a summary, see (*9*)). The results strongly suggest that, in the active site of all of these acyl-CoA dehydrogenases, the C-2 proton-abstracting base (possibly a cysteine residue (*18*)) and C-3 hydride-accepting FAD unit are located in a similar antiperiplanar arrangement, as in **6**.

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## EXPERIMENTAL PROCEDURES

HPLC was performed using a Waters Associates liquid chromatograph with an M-6000A pump, a Model 450 variable-wavelength detector, and a U6K injector. Solvents were filtered through a 0.45-$\mu$m nylon filter and degassed by vacuum pumping. All injected samples were filtered through a Gelman Acro LC13 filter. $^{13}C$ NMR spectra were recorded on a Bruker WM-250 instrument (except in the case of the enzymatic dehydrogenation product **4b**, the spectrum of which was run on a Bruker WM-500 instrument) using 5-mm tubes.

### *Synthesis of (3S)-[4-$^{13}C$]Isovaleric Acid, **3a**, and (3RS)-[4-$^{13}C$]Isovaleric Acid*

(2*RS*, 3*S*)-[4-$^{13}C$]Valine (*15*) (480 mg, 4.1 mmol, 97–99 at.% $^{13}C$) in ice-cold 2.5 *N* $H_2SO_4$ (10 ml) was treated with KBr (1.6 g, 13.4 mmol) followed by $NaNO_2$ (0.45 g, 6.5 mmol) added in portions over 45 min while stirring at 0°C. Stirring was continued at 25°C for 1 h; then the solution was extracted with ether (3 × 50 ml). The extract was dried ($Na_2SO_4$) and evaporated to a viscous oil (800 mg). This was suspended in ice-cold $H_2O$ (12 ml) and treated with zinc (0.9 g, 30 mesh), followed by concentrated $H_2SO_4$ (0.25 ml) with vigorous stirring at 0°C for 5 min, then at 25°C for 30 min. The solution was separated from excess zinc, diluted with water (50 ml), and distilled to near dryness. The distillate was neutralized with diluted NaOH and then lyophilized to yield (3*S*)-[4-$^{13}C$]isovaleric acid sodium salt (207 mg). $^{13}C$ NMR δ23.12[5] (conditions: 62.896 MHz, SW 15151 Hz, PW 11 $\mu$s, RD 2 s, Aq 0.541 s, LB 1 Hz).

(3*RS*)-[4-$^{13}C$]Isovaleric acid was prepared by hydrogenation of 3-(3*EZ*)-[4-$^{13}C$]methylcrotonic acid (0.5 g) in ether (50 ml) with 5% Pd (C) (100 mg) at 200 psi in a Parr stirred pressure reactor for 1 h. After filtration, most of the ether was evaporated under reduced pressure, $H_2O$ (50 ml) was added, and the solution was neutralized with NaOH and lyophilized to yield (3*RS*)-[4-$^{13}C$]isovaleric acid (0.51 g). $^{13}C$ NMR δ23.03 (conditions: 62.896 MHz, SW 15151 Hz, PW 27 $\mu$s, RD 2 s, Aq 0.27 s, LB 3 Hz).

### *3-(3E)-[4-$^{13}C$]Methylcrotonic Acid, **4a**, and 3-(3EZ)-[4-$^{13}C$]Methylcrotonic Acid, **5a***

3-(3*E*)-[4-$^{13}C$]Methylcrotonic acid, **4a**, was synthesized as previously described (*17*). $^{13}C$ NMR ($CDCl_3$) δ27.64 (conditions: 62.896 MHz, SW 15151 Hz, PW 27 $\mu$s, RD 2 s, Aq 0.27 s, LB 3 Hz). (3*EZ*)-[4-$^{13}C$]Isovaleric acid, **5a**, was synthesized in a similar manner, except that the reaction of methyl tetrolate with $(^{13}CH_3)_2CuLi$ was conducted at 0°C. A mixture of **4a** + **5a** (ca. 1 : 1) had $^{13}C$ NMR ($CDCl_3$) δ20.45, 27.67 (ca. 1 : 2 integral ratio) (conditions as above).

[5] All $^{13}C$ NMR spectra reported in this paper were run on very dilute solutions and only the signals due to carbons enriched above natural abundance were observed.

*Synthesis and Purification of Coenzyme A Esters*

These were prepared, as previously described (*6*), by the mixed anhydride method. Typically, 80–100 mg of the sodium salt of the corresponding acid was used. In all cases, the crude products showed substantial impurities upon HPLC analysis, and were purified by preparative HPLC as follows.

HPLC analysis and purification of coenzyme A esters were performed by adaptation of the method of Corkey *et al.* (*19*). A μBondapak $C_{18}$ column, 3.9 mm × 30 cm (Waters Associates), was used. The elution solvent was 0.05 M $KPO_4$, pH 5.3 : MeOH (3 : 1) at 2.0 ml/min. The detector was set at 254 nm. Typically, injections of 10 μl of a 1 mg/ml solution were used for analytical purposes. The elution times for isovaleryl-CoA and 3-methylcrotonyl-CoA were ca. 14 min and ca. 11.5 min, respectively. Preparative separations were performed using the same conditions but with larger injections (100–150 μl) (the entire crude CoA ester product from ca. 80 mg free acid being dissolved in 1.5–2.0 ml $H_2O$).

The collected peaks from preparative runs were evaporated on a rotary evaporator at 25–30°C (vacuum pump) to remove most of the MeOH, and were then lyophilized. For desalting the product, the residue was dissolved in the minimum amount of $H_2O$ (2–3 ml) and rechromatographed by HPLC, using the same column but eluting with 0.05 M $NH_4OAc$, pH 5.3 : MeOH (3 : 1) at 2.0 ml/min (ca. 300- to 500-μl injections). The collected product solutions were evaporated as above to remove MeOH and then lyophilized to provide the product (ca. 10–15 mg). In all cases, the CoA esters purified in this manner showed extremely clean analytical HPLC chromatograms with no detectable impurities.

The purified $^{13}C$-enriched coenzyme A esters were examined by $^{13}C$ NMR ($D_2O$, 0.05 M $KPO_4$, pH 7.4, internal dioxane) (only enriched carbons detected): (3*S*)-[4-$^{13}C$]isovaleryl-CoA, **3b**, δ22.62; (3*RS*)-[4-$^{13}C$]isovaleryl-CoA, δ22.63; 3-(3*E*)-[4-$^{13}C$]methylcrotonyl-CoA, **4b**, δ27.78; 3-(3*EZ*)-[4-$^{13}C$]methylcrotonyl-CoA, **5b**, δ21.93 and 27.77 (ratio 1 : 2) (conditions: 62.896 MHz, SW 15151 Hz, PW 13 μs, RD 2 s, Aq 0.541 s, LB 1 Hz; S/N ca. 80–120 : 1).

*Conversion of (3S)-[4-$^{13}C$]Isovaleryl-CoA, **3b**, to 3-(E)-[4-$^{13}C$]Methylcrotonyl-CoA by Isovaleryl-CoA Dehydrogenase: Isolation of the Product*

A solution was prepared of pure rat liver isovaleryl-CoA dehydrogenase (*1*) (30 nM), FAD (100 μM), and PMS (3 mM) in $KPO_4$ (0.01 M)–EDTA (0.5 mM) buffer, pH 8.0. The final volume was 10 ml. Then **3b** (1.1 mg, 1.3 μmol) was added and the mixture was incubated at 30°C for 1 h, frozen, and lyophilized to a green paste which was stored at −70°C.

The paste was then redissolved in $H_2O$ (1.5 ml). The material could not be analyzed by HPLC at this point due to the high concentration of PMS. Although the retention time of PMS was considerably less than that of **3b** or **4b**, larger amounts of PMS (>ca. 1 μg) trailed badly and overlapped the coenzyme A ester peaks. Therefore, the redissolved incubation mixture was added to a 1.5 × 30-cm column of Sephadex G-10 (at 4°C). The column was eluted with $H_2O$ at ca. 0.4 ml/min, collecting 15-min fractions. The first three yellow fractions were combined

and lyophilized, yielding a few milligrams of yellow crystalline residue. The desired 3-methylcrotonyl-CoA could then be isolated from this material by preparative HPLC as described above (except that a Waters Associates NovaPak $C_{18}$ column was used with a flow rate of 1.2 ml/min. The retention times of isovaleryl-CoA and 3-methylcrotonyl-CoA were 7.5 and 5.5 min, respectively.) The chromatograms showed only traces of residual PMS (retention time 2.0 min). After desalting as described above, the pure 3-(*E*)-[4-$^{13}$C]methylcrotonyl-CoA, ca. 0.3 mg, was obtained as a white powder. $^{13}$C NMR ($D_2O$, 0.05 M $KPO_4$, pH 7.4, with internal dioxane reference) δ27.73 (conditions: 125.76 MHz, SW 29412 Hz, PW 4 μs, RD zero, Aq 0.557 s, LB 3 Hz; S/N ca. 50 : 1).

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