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Studies on the Substrate Stereochemistry of Enoyl-CoA Hydratase (Crotonase): Nonstereospecific Hydration of β-Methylcrotonate in Biotin-Deficient Rats

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Nuclear magnetic resonance methods have been developed for assignment of the absolute configuration of (4,4,4-d_3)-β-hydroxyisovalerate and (2-d_1)-β-hydroxyisovalerate. (*E*) and (*Z*)-(4,4,4-d_3)-β-methylcrotonate and (2-*d*)-β-methylcrotonate have been administered to biotin-deficient rats, and the resultant β-hydroxyisovalerate was isolated from their urine. The NMR spectra of derivatives of the biosynthetic products established that the hydration of β-methylcrotonate had proceeded nonstereospecifically.

INTRODUCTION

We recently reported on the stereochemistry of hydrogen elimination from C-2 in the conversion of isovalerate to β-hydroxyisovalerate in biotin-deficient rats (*1*). The overall process involves (at least) four enzymatic steps: formation of the CoA ester, dehydrogenation by isovaleryl-CoA dehydrogenase (or the general acyl-CoA dehydrogenase), hydration by enoyl-CoA hydratase (crotonase), and deacylation. The CoA acylation and deacylation steps would not be expected to involve stereochemical change in the acyl group. Thus, *in principle,* provided that the stereochemistry of the hydration step were known, the stereochemistry of the dehydrogenation step could be elucidated by determining the stereochemistry of the overall process. We now report the results of an investigation of the stereochemistry of the crotonase-dependent hydration of β-methylcrotonyl CoA in biotin-deficient rats. The results showed that, contrary to expectations, this hydration proceeds completely nonstereospecifically in this organism.[2]

RESULTS

Our approach to the determination of the stereochemistry of this hydration was to administer (*E*)- and (*Z*)-[4,4,4-d_3]-β-methylcrotonate, (**1a**) and (**1b**), and (2-*d*)-β-methylcrotonate, (**1c**), to biotin-deficient rats, and to determine by nmr methods the stereochemistry of the labeling in the resultant β-hydroxyisovalerate (**2a**).

[1] Address inquires and reprint requests to this author.
[2] A preliminary report of some aspects of this study has been published (*2*).

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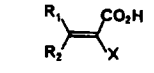
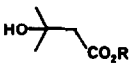


1a R_1=Me, R_2=CD_3, X=H
b R_1=CD_3, R_2=Me, X=H
c R_1=Me, R_2=Me, X=D
d R_1=Me, R_2=Me, X=H
e R_1=Me, R_2=Me, X=Br

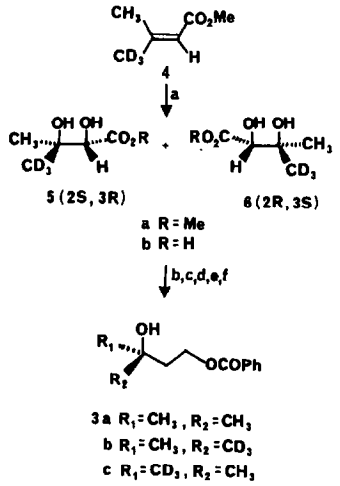


2a R=H
b R=Me

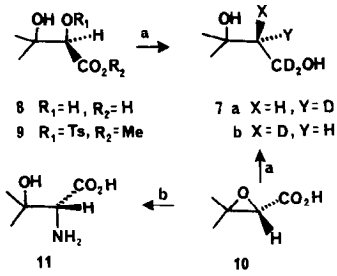
Before carrying out the administration of the labeled β-methylcrotonates to biotin-deficient rats, we first developed methods of assigning absolute configurations at C-3 and C-2 in the anticipated stereospecificially labeled metabolites.

Stereochemical Analysis of Deuterated Metabolites: Synthesis of Labeled Reference Compounds

Stereochemical analysis at C-3 could be performed by examination of the ^1H nmr spectrum of the derivative 3-methyl-1,3-butanediol-1-benzoate (**3**) (*3*). In the presence of the chiral lanthanide shift reagent, Eu(hfbc)$_3$, the methyl signals of (**3a**) became well separated after shifting to the region of δ 4–6. For signal assignment, a *partially* stereospecifically labeled reference sample was prepared (Scheme 1). Methyl (*E*)-[4,4,4-d_3]-3-methylcrotonate (**4**) was converted with OsO_4/$NaClO_3$ (*4*) to the (2*S*, 3*R*)- and (2*R*, 3*S*)-diols (**5a**) and (**6a**), which were saponified under mild conditions to the dihydroxy acids (**5b**) and (**6b**). Partial resolution of the mixture (*5*) gave a mixture enriched in (**6b**) (ca. 50% e.e.). The mixture was then reduced via the methyl ester monotosylates with $LiAlH_4$ to the mixture of diols which was converted to the corresponding monobenzoates (**3b**) + (**3c**) [ca. 50% e.e. (**3c**)]. The nmr spectrum of this product in the presence of Eu(hfbc)$_3$ showed two methyl singlets in a ratio of ca. 2:1, the downfield signal being the more intense.



SCHEME 1. Synthesis of stereospecifically labeled [4,4,4-d_3]-3-methyl-1,3-butanediol-1-benzoate. Reagents: (1) OsO_4/$NaClO_3$; (b) quinine; (c) CH_2N_2; (d) TsCl/pyridine; (e) $LiAlH_4$; (f) BzCl/pyridine.



SCHEME 2. Synthesis of stereospecifically labeled (2-d_1)-3-methyl-1,3-butanediol. Reagents: (a) $LiAlD_4$; (b) NH_3/H_2O.

A similar method was developed for stereochemical analysis of C-2 monodeuterated β-hydroxyisovalerate. Unfortunately the C-2 proton signals of neither (**3a**) nor of (**2b**) could be resolved in the presence of Eu(hfbc)$_3$. However, racemic [1,1,2-d_3]-3-methyl-1,3-butanediol, (**7a**) + (**7b**), in the presence of Eu(hfbc)$_3$, gave partially separated signals for the C-2 protons, adequately resolved for our purposes (Fig. 3a). Stereospecifically labeled samples (**7a**) and (**7b**) were then prepared (Scheme 2).

Optically pure (2*R*)-2,3-dihydroxy-3-methylbutyric acid (**8**) was converted to the methyl ester monotosylate (**9**), which was reduced with $LiAlD_4$ to (2*R*)-[1,1,2-d_3]-3-methyl-1,3-butanediol (**7a**). The enantiomer (**7b**) was prepared by a different route. Racemic 2,3-epoxy-3-methylbutyric acid was converted into the brucine salt, which, after recrystallization, was separated to yield (2*S*)-2,3-epoxy-3-methylbutyric acid (**10**). The absolute configuration of this previously unresolved acid was established by treatment of a portion of (**10**) with ammonia, resulting in the formation of D(−)-β-hydroxyvaline (**11**) (*6*). Since the epoxide opening by ammonia is a *trans* process (*7*), the epoxy acid must have had the (2*S*) configuration. Treatment of (**10**) with CH_2N_2 followed by $LiAlD_4$ gave (2*S*)-[1,1,2-d_3]-3-methyl-1,3-butanediol (**7b**).

The ^{1}H nmr spectra of (**7a**) and (**7b**) were then taken in the presence of adequate Eu(hfbc)$_3$ to shift the C-2 proton signal to a broadened singlet at ca. δ 12. Addition of a small amount of the racemic mixture, (**7a**) + (**7b**), to these samples then allowed assignment of the 2-*pro S* hydrogen of (**7**) as the downfield and the 2-*pro R* hydrogen of (**7**) as the upfield signal (Figs. 3b,c).

Biosynthetic Results: Stereochemistry of Hydroxyl Addition at C-3

(*E*)- and (*Z*)-[4,4,4-d_3]-3-methylcrotonic acids were administered to biotin-deficient rats following the procedure previously used for labeled isovalerates (*1*). The collected urine was then subjected to ultrafiltration, acidified, and extracted with CH_2Cl_2. A portion of the extract was treated with *p*-phenylphenacyl bromide to yield the *p*-phenylphenacyl esters of β-hydroxyisovalerate and of the recovered β-methylcrotonate. The mass spectra of both esters, from either (**1a**) or (**1b**), gave prominent molecular ions indicating the presence of only d_3 (ca. 65–70%) and d_0

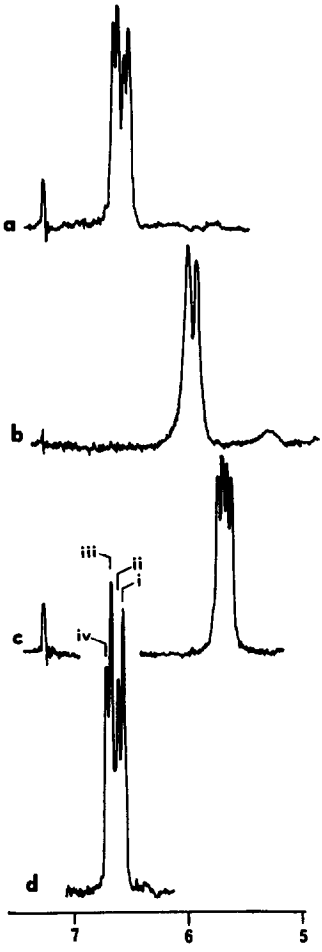


FIG. 1. Methyl group signals of Eu(hfbc)$_3$-shifted ^{1}H nmr spectra of 3-methyl-1,3-butanediol-1-benzoate. (a) Biosynthetic product from (**1a**); (b) unlabeled sample (**3a**); (c), synthetic mixture of (**3a**) + racemic [(**3b**) + (**3c**)], ratio (**3a**): [(**3b**) + (**3c**)] = 1:2; (d) as in (c) with additional (**3a**) added to the mixture.

(ca. 30–35%) species. The remainder of the extracts were methylated (CH_2N_2) and reduced with $LiAlH_4$ to 3-methyl-1,3-butanediol, which was converted to the monobenzoate, (*3*). These products, originating from (**1a**) or (**1b**), were then examined by nmr in the presence of Eu(hfbc)$_3$. Figure la shows the methyl region of this spectrum of the product derived from (**1a**). A similar spectrum was obtained from the product derived from (**1b**). Whereas the unlabeled (**3a**) as well as isotopically pure (d_3) synthetic, *racemic* [(**3b**) + (**3c**)] material gave *two* singlets under these conditions (Fig. 1b), the biosynthetic products unexpectedly gave *four* singlets. A mixture of racemic [(**3b**) + (**3c**)] plus unlabeled (**3a**) [ratio d_3: d_0, ca. 2:1] showed a similar pattern (Fig. 1c). The singlets due to the unlabeled component (**3a**) (i and iii, Fig. 1d) appeared to high field of peaks ii and iv due to the d_3 component [(**3b**) + (**3c**)], as shown by addition of further quantities of (**3a**) to

the mixture. Similar examples of the differential shifting of signals of CD_3 vs CH_3-containing compounds have been previously reported (*8, 9*); for other examples, see (*10*). In any case, the fact that the biosynthetic products, which are ca. 2:1 mixtures of $d_3 : d_0$ species, show *four* methyl singlets rather than *three* indicates that the d_3 species are racemic at C-3.

Examination of the ^1H nmr spectra of the recovered β-methylcrotonate *p*-phenylphenacyl esters from both precursors (**1a**) and (**1b**) suggested that the observed nonstereospecific labeling of (**3**) was not the result of isomerization of the starting materials prior to hydroxylation. Thus, the ^1H spectra (Figs. 2a,b) show nonequal peak heights for the vinylic methyl groups roughly consistent with the ratio of $d_3 : d_0$ species indicated by mass spectrometry. However, due to the presence of the endogenous d_0 species, an accurate evaluation of the possible involvement of partial isomerization could not be made by ^1H nmr. This problem was neatly solved by examination of the ^2H nmr spectra (Figs. 2c,d). Since the ^2H resonances were expected to be broadened relative to ^1H methyl signals, and since we wished to avoid overlapping peaks, the ^2H spectra were run in the presence of $Eu(fod)_3$. These spectra show that, in fact, a small amount of *cis/trans* isomerization had occurred, since both samples showed two deuterated methyl

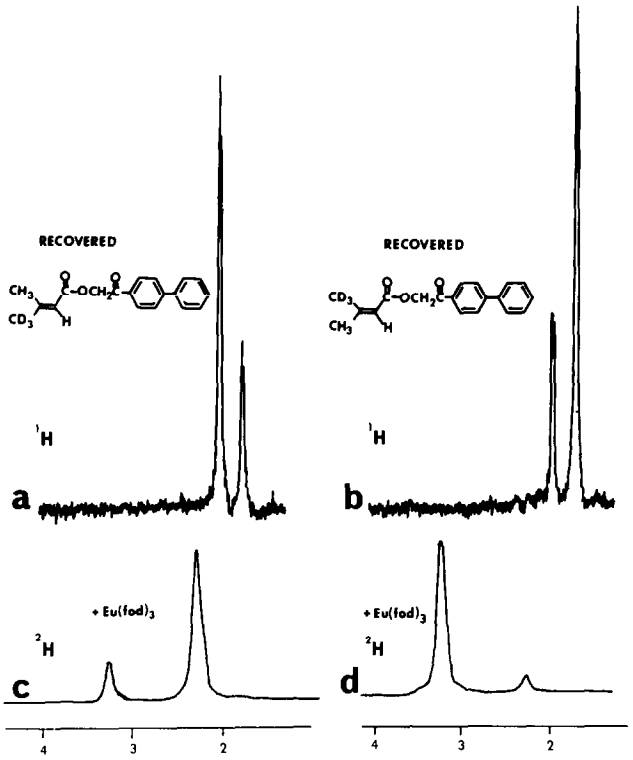


FIG. 2. Methyl group signals of β-methylcrotonic acid *p*-phenylphenacyl esters recovered from administration of (**1a**) and (**1b**) to biotin-deficient rats: (a) ^1H nmr, product recovered from (**1a**); (b), ^1H nmr, product recovered from (**1b**); (c) ^2H nmr with added $Eu(fod)_3$, product recovered from (**1a**); (d) ^2H nmr with added $Eu(fod)_3$, product recovered from (**1b**).

resonances but of nonequal intensities. However, this extent of isomerization would not be adequate to explain the complete nonstereospecificity of the labeling in the hydroxylated product.

Stereochemistry of Hydrogen Addition at C-2 of β-Methylcrotonate

As in the previous section, (2-*d*)-β-methylcrotonate (**1c**) was administered to biotin-deficient rats, and the urine was extracted. A portion of the extract was treated with *p*-phenylphenacyl bromide to form the *p*-phenylphenacyl ester of (**2a**) (the unmetabolized (**1c**) was not recovered in this experiment). The mass spectrum of the ester revealed, as before, the presence of exogenous (d_1) and endogenous material (d_0) in a ratio of ca. 2 : 1. The remainder of the extract was methylated (CH_2N_2) and reduced with $LiAlD_4$ to form (**7**). The ^1H nmr spectrum of this product was then examined in the presence of Eu(hfbc)$_3$. The spectrum (Fig. 3d) revealed the presence of both the 2-*pro R* and 2-*pro S* hydrogens in equal proportions, indicating that the product was racemic at C-2. Thus, both hydroxyl and hydrogen addition to β-methylcrotonate *apparently* proceeded nonstereospecifically in these rats.

Test of Nonenzymatic Hydration of β-Methylcrotonate and β-Methylcrotonyl CoA

The observation of nonstereospecificity in a biosynthetic process is often taken as evidence for the involvement of a non-enzyme-directed step in the process (*11, 12*). Indeed, methacrylyl CoA apparently can undergo spontaneous hydration in addition to crotonase-dependent hydration (*13*), although we found that stereospecifically labeled methacrylate was hydrated stereospecifically in

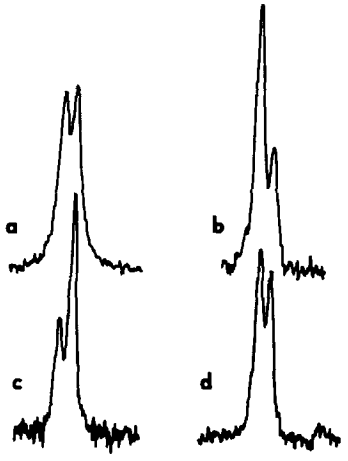


FIG. 3. Partial ^1H nmr spectra (C-2 proton signals) of (2-d_1)-3-methyl-1, 3-butanediol samples, in presence of Eu(hfbc)$_3$. (a) Racemic synthetic (**7a**) + (**7b**); (b) (**7a**) with additional racemic (**7a**) + (**7b**); (c) (**7b**) with additional racemic (**7a**) + (**7b**); (d) biosynthetic product obtained from biotin-deficient rats treated with (**1c**). Chemical shifts are ca. 11–12 (dependent on amount of Eu(hfbc)$_3$ added).

Pseudomonas putida (*14*). We therefore attempted to determine whether the substrate as free acid, or the presumed essential intermediate, β-methylcrotonyl CoA, was capable of undergoing spontaneous hydration at a significant rate. Since we could not conduct this study in the peritoneal cavity of a rat, the conditions therein were simulated by incubation of the test compounds in 0.2 *M* potassium phosphate buffer, pH 7.4, at 37°C. As expected, unlabeled (**1d**) under such conditions showed no trace of hydration over 5 weeks, and the d_3 analog (**1a**) did not exhibit *cis/trans* isomerization. When synthetic β-methylcrotonyl CoA was treated under the same conditions, no change was noted in the concentration of the unsaturated ester over 39 hr, as assayed with bovine liver crotonase. The results appear to indicate that spontaneous nonenzymatic hydration is not a significant process.

DISCUSSION

The results of this stereochemical investigation indicate that the overall hydration of β-methylcrotonate to β-hydroxyisovalerate in biotin-deficient rats proceeds with complete lack of stereospecificity. These results contrast sharply with our observation of stereospecific hydration of methacrylate in *P. putida* (*14*). Eggerer *et al.* (*15*) have also reported the stereospecific hydration of crotonyl CoA, although their studies were conducted with the pure crystalline crotonase, rather than using an intact organism.

In such an organism, it is difficult to evaluate conclusively the reason(s) for the observed lack of stereospecificity. The results of a control experiment suggest that the substrate does not hydrate under nonenzymatic conditions. The recovered starting material from (**1a**) and (**1b**) exhibited only a small degree (10–20%) of *cis/trans* isomerization, although it is conceivable that the coenzyme A ester might be subject to more extensive isomerization. The partially isomerized β-methylcrotonates isolated may be the result of hydrolysis of completely isomerized β-methylcrotonyl CoA into a pool of the stereospecifically labeled free acid. In fact, crotonase is known to possess the ability to catalyze certain *cis/trans* isomerizations of 2-ethylenic CoA derivatives (*16*). The significance of this process can probably best be evaluated using the isolated enzyme. Since only d_3-labeled and no d_2-labeled products were obtained from (**1a**) and (**1b**), the involvement of β-methylvinyl-CoA isomerase (*17*) to any significant extent is unlikely, unless the deuterium removed from the methyl group were reversibly transferred (intra- or intermolecularly) to the α- position. Another possibility might be that the observed nonstereospecific hydroxylation at C-3 might be due to the operation of β-hydroxybutyryl-CoA racemase or to the racemase activity of crotonase itself (*16*). It is not known whether β-hydroxyisovaleryl CoA is a substrate for the former enzyme. However, such racemase activity would not account for the nonstereospecificity of the labeling observed at C-2 of the product, nor would *cis/trans* isomerization of the unsaturated CoA ester. Thus, the exact reason for the observed nonstereospecificity in rats remains an open question, which can best be examined with the use of pure enzymes. Obviously the nonstereospecific hydration precludes the use of biotin-deficient rats for determining the relative

stereochemistry of hydrogen elimination from C-3 of isovalerate (although the stereochemistry of hydrogen loss from C-2 could still be determined in this way (*1*)).

EXPERIMENTAL

^{1}H nmr spectra were recorded on Varian HA-100 or EM-360 spectrometers. Chemical shifts are expressed in ppm (δ) from TMS = O. ^{2}H nmr spectra were recorded on a Varian HX-270 instrument at 41.44 MHz with proton decoupling. Samples were dissolved in alcohol-free $CHCl_3$ with internal $CDCl_3$ (δ 7.27) as reference. Mass spectra were recorded on a Nuclide 12-90-G mass spectrometer equipped with a Nuclide DA/CS 1.2 data acquisition system. Merck silica gel (HF 254 + 366) was used for thin-layer chromatography. Gas–liquid chromatography was performed using a Varian Model 920 instrument. Melting points were taken on a hot stage apparatus and are corrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tennessee.

(*E*) and (*Z*)-[4,4,4-d_3]-β-methylcrotonic acids, (**1a**) and (**1b**), were synthesized by published methods (*18–20*).

Synthesis of [2-d]-β-Methylcrotonic Acid (1c)

Sodium amalgam (9%, 180 g, 0.64 g atom Na) was added in portions over 10 min to a solution of 2-bromo-3-methylcrotonic acid (*21*), (**1e**), (19 g, 0.106 mol) in D_2O (150 ml) at 0°C with vigorous stirring. After stirring at 0°C for 2 hr, the solution was decanted, filtered, and acidified with concentrated HCl. The product was filtered, washed with a little cold H_2O, and recrystallized from H_2O, giving [2-*d*]-3-methylcrotonic acid (**1c**), 4.5 g, mp 68–69°C; nmr ($CDCl_3$) δ 1.93 (3H, s), 2.20 (3H, s), 11.79 (1H, br s) (complete disappearance of the vinyl H signal of (**1d**) at δ 5.7).

Synthesis of Stereospecifically Labeled [4,4,4-d_3]-3-Hydroxy-3-methyl-1-butyl Benzoates (3b) and (3c)

Methyl (*E*)-[4,4,4-d_3]-3-methylcrotonate, (**4**) (1.40 g, 12 mmol), was dissolved in a mixture of THF (12 ml) and H_2O (12 ml), and treated with $NaClO_3$ (2 g) and OsO_4 (61 mg) stirring at 25°C for 20 hr. The solution was extracted with $CHCl_3$ (4 × 25 ml), and the extract was dried (Na_2SO_4), and evaporated to an oil. Kugelröhr distillation gave *methyl (2S, 3R + 2R, 3S)-2,3-dihydroxy-3-methylbutyrate,* (**5a**) + (**6a**), bp_{200} 150–170°C; nmr ($CDCl_3$) δ 1.21 (3H, s), (3.2, br s, D_2O exchangeable), 3.84 (3H, s), 4.00 (1H, s).

The product (864 mg, 5.72 mmol) was stirred with 0.56 *N* NaOH/H_2O (10.2 ml, 1 eq) at 25°C for 48 hr. The solution was saturated with NaCl, acidified to pH 2 with HCl, diluted to 100 ml with saturated NaCl, and extracted continuously with ether for 22 hr. The extract was evaporated to yield the racemic acid, (**5b**) + (**6b**), as a viscous oil (418 mg, 3.54 mmol). This was dissolved in absolute EtOH (8.5 ml), and added to anhydrous quinine (1.16 g, 3.57 mmol, dried at 140°C *in vacuo* for 4 days), and the solution was kept at 4°C for 24 hr. The resultant crystals were recrystallized once from absolute EtOH (5 ml) to give the quinine salt of (**5b**) +

(**6b**) enriched in (**6b**), 266 mg, mp 200–201°C, $[\alpha]_D^{25}$ −139° (c, 2, H_2O); lit. (*5*) mp 208–209°C, $[\alpha]_D^{25}$ −141.8°. Although not optically pure, the quinine salt was not further recrystallized. The salt was dissolved in H_2O (5 ml), and added to a 1 × 8-cm column of Dowex 50W-X8, 50–100 mesh, H^+ form. Elution with H_2O and evaporation gave a mixture of (**5b**) (minor) + (**6b**) (major), which was methylated (CH_2N_2) to give the corresponding mixture of (**5a**) (minor) + (**6a**) (major), 84 mg, $[\alpha]_D^{25}$ −13° (c, 2, $CHCl_3$); lit. for optically pure (**6a**), $[\alpha]_D^{25}$ −28° ($CHCl_3$) (*5*).

The entire product in dry pyridine (2 ml) was treated with *p*-toluenesulfonyl chloride (152 mg; recrystallized from hexane) at 25°C for 24 hr. The solution was diluted with H_2O (25 ml), acidified (HCl), and extracted with ether. The extract was washed with dilute Na_2CO_3 and saturated NaCl, dried (Na_2SO_4), and evaporated to yield crude monotosylate, 165 mg, as a glass (single spot on TLC, R_f 0.3, solvent 25% EtOAc–hexane). The product was dissolved in dry THF (5 ml) and treated with $LiAlH_4$ (200 mg) refluxing for 38 hr. The cooled solution was treated dropwise with saturated Na_2SO_4, and the mixture was evaporated to yield crude *[4,4,4-d_3]-3-methyl-1,3-butanediol* (enriched in the (3*S*) enantiomer), 50 mg. The product, in dry pyridine (1 ml), was treated with benzoyl chloride (0.05 ml) at 25°C for 24 hr. After workup in the usual way, the product was purified by preparative TLC (R_f 0.4, solvent 25% EtOAc–hexane) to yield *[4,4,4-d_3]-3-hydroxy-3-methyl-1-butyl benzoate* (**3b**) + (**3c**) (enriched in (**3c**)), 20 mg as an oil; nmr ($CDCl_3$) δ 1.32 (3H, s) 1.65 (1H, br s, D_2O exchangeable), 1.98 (2H, t, J = 7 Hz), 4.52 (2H, t, J = 7 Hz), 7.45 (3H, m), 8.05 (2H, m).

Synthesis of (2R)-[1,1,2-d_3]-3-Methyl-1,3-butanediol (**7a**)

A solution of β-methylcrotonic acid (**1d**) (40 g, 0.4 mol) and $Na_2WO_4 \cdot 2H_2O$ (600 mg) in H_2O (40 ml) at 70°C was treated over 16 hr with 30% H_2O_2 (50 ml) added in small portions. Stirring was continued for an additional 14 hr at 70°C. The mixture was then cooled, acidified (HCl), saturated with NaCl, filtered, and extracted continuously with ether for 24 hr. Evaporation of the ether gave (2*RS*)-3-methyl-2,3-dihydroxybutyric acid, 28 g. A portion of this was resolved essentially as published (*5*) to yield (2*R*)-3-methyl-2,3-dihydroxybutyric acid (**8**), $[\alpha]_D^{25}$ −15° (*c* 2, 0.1 *N* HCl). The product was converted, as described for (**5a**) + (**6a**), into the methyl ester monotosylate (**9**), purified by preparative TLC (R_f 0.5, 45% EtOAc–hexane), glass, $[\alpha]_D^{25}$ +34.9° (*c* 4.4, $CHCl_3$); nmr ($CDCl_3$) δ 1.24 (6H, s), 2.45 (3H, s), 3.64 (3H, s), 5.66 (1H, s), 7.33 and 7.71 (4H, AB, J_{AB} = 8 Hz). The tosylate (2 g) in ether (50 ml) was added dropwise over 15 min to a refluxing solution of $LiAlD_4$ (1 g) in ether (30 ml). The mixture was then stirred at 25°C for 10 hr, and treated dropwise with saturated Na_2SO_4. The mixture was filtered and evaporated under reduced pressure to yield *(2R)-[1,1,2-d_3]-3-methyl-1,3-butanediol*, (**7a**), 640 mg, as an oil, nmr ($CDCl_3$) δ 1.3 (6H, s), 1.72 (1H, br s, $W_{1/2}$ = 5 Hz), 3.55 (2H, br m, $W_{1/2}$ = 11 Hz, D_2O exchangeable).

(2S)-2,3-Epoxy-3-methylbutyric acid (**10**)

A solution of β-methylcrotonic acid (**1d**) (30 g, 0.3 mol) and $Na_2WO_4 \cdot 2H_2O$ (*22*) (14 g, 0.042 mol) in water was warmed to 60°C and adjusted to pH 5.5 with

concentrated $NaOH_2$. To the stirred solution was added 30% H_2O_2 over 30 min, while maintaining the temperature at 60–65°C, and pH at 5.5 with additions of concentrated NaOH. Stirring at 60–65°C was continued for an additional 30 min, then the solution was cooled, saturated with $(NH_4)_2SO_4$, acidified with cold 5 *M* H_2SO_4, and extracted with ether (5 × 100 ml). The extract was dried (Na_2SO_4) and evaporated to give (±) 2,3-epoxy-3-methylbutyric acid as a viscous liquid, which could not be crystallized. The product polymerized if allowed to stand at 25°C for a few hours.

The crude epoxy acid (16 g, 0.14 mol) and brucine (54 g, 0.14 mol) were dissolved in hot MeOH (80 ml). The solution was filtered and boiled down to 50 ml. Hot acetone (100 ml) was added and again the solution was boiled down to 50 ml. This procedure was repeated once, boiling down to ca. 50 ml until the solution became cloudy. After standing at 25°C overnight, the mixture was filtered, and the crystals were washed with cold acetone. The product was recrystallized as above three times to yield the optically pure brucine salt of (*10*), 12 g, prisms, mp 173–175°C, $[\alpha]_D^{25}$ − 31.8° (*c* 4.9, H_2O). The product was dissolved in H_2O (60 ml) and treated with 1 *N* NaOH (30 ml). The precipitated brucine was separated by filtration, and the filtrate extracted with $CHCl_3$. The aqueous extract was then saturated with NaCl, acidified to pH 2.5 (HCl), and extracted with ether (5 × 40 ml). The extract was dried (Na_2SO_4), and evaporated to yield *(2S)-2,3-epoxy-3-methylbutyric acid,* (**10**), $[\alpha]_D^{25}$ − 16° (*c* 2.4, $CHCl_3$), viscous liquid.

A portion of (**10**) (900 mg) was mixed with concentrated NH_4OH, and heated in a sealed glass tube at 80°C for 16 hr. After cooling, the solution was evaporated under reduced pressure to a gum. The product was dissolved in 2 *N* NaOH (15 ml) and treated with benzoyl chloride (2 ml) with stirring at 25°C for 2 hr. The reaction mixture was acidified (HCl) and filtered. The filtrate was extracted with EtOAc and the solvent evaporated to yield a glass which was treated with CH_2N_2. The resultant product, *N-benzoyl-β-hydroxyvaline methyl ester,* was isolated by preparative TLC (solvent 80% EtOAc–hexane) as a viscous glass, 240 mg; nmr ($CDCl_3$) δ 1.35 (6H, s), 3.2 (1H, br s), 3.75 (3H, s), 7.45 (3H, m), 7.8 (2H, m). This product was then refluxed with 6 *N* HCl (20 ml) for 12 hr. After cooling, the solution was evaporated under reduced pressure. The residue was dissolved in H_2O (10 ml), and added to a 1 × 10-cm column of Dowex 50W-X8, 50–100 mesh, H^+ form, which was washed with H_2O until the washings were neutral. Elution with 2 *N* NH_4OH, followed by evaporation, reduced pressure gave *(2R)-3-hydroxyvaline,* (**11**), prisms from EtOH–H_2O, mp 199–200°C, $[\alpha]_D^{25}$ − 18.5° (*c* 2, 5 *N* HCl) (lit. (*6*) $[\alpha]_D^{25}$ − 13.5°).

(2S)-[1,1,2-d_3]-3-Methyl-1,3-butanediol (*7b*)

(2*S*)-2,3-Epoxy-3-methylbutyric acid (**10**) (500 mg) was treated with excess CH_2N_2/ether, and the solvent was evaporated. The product in ether (5 ml) was treated with $LiAlD_4$ (250 mg) at reflux for 2 hr, followed by stirring at 25°C for 16 hr. The mixture was treated with saturated Na_2SO_4 and filtered. The filtrate was evaporated under reduced pressure to yield (**7b**), 200 mg. A sample purified by

preparative GLC (15% SE-30, 110°C) had nmr ($CDCl_3$) δ 1.25 (6H, s), 1.70 (1H, br s, $W_{1/2}$ = 6 Hz), 2.80 (2H, br s, $W_{1/2}$ = 20 Hz, D_2O exchangeable).

Conversion of β-Methylcrotonic Acids to β-Hydroxyisovalerate in Biotin-Deficient Rats: Isolation of Metabolic Products and Derivatives

Labeled β-methylcrotonic acids (**1a**), (**1b**), or (**1c**) were administered to biotin-deficient rats exactly as previously described (*1*), except that each precursor was administered to two rats (50 mg/day/rat) for a total of 5–6 days. Urine was collected daily and kept frozen for eventual processing. The pooled urine was then thawed and filtered in an Amicon filtration cell through a PM 10 filter. This filtration step eliminated the formation of the emulsions which were encountered in our earlier work (*1*). The urine was then saturated with NaCl and acidified to pH 2 (HCl). The solution was then extracted with CH_2Cl_2 (4 × 50 ml), and the extract was dried (Na_2SO_4) and evaporated to a viscous gum (300–500 mg). This was dissolved in a little ether, and divided into two equal portions.

One portion was treated with excess CH_2N_2/ether (alcohol-free), and the solution was evaporated under reduced pressure to an oil (150–250 mg). This was examined by GLC (5% Carbowax 20 M, 80°C), which indicated the presence of methyl β-hydroxyisovalerate (**2b**) as the major component. The remainder of the methylated product was then dissolved in absolute ether (15 ml) and treated at reflux for 24 hr with excess $LiAlH_4$ (or $LiAlD_4$ in the case of metabolite obtained from (**1c**)). The mixture was then treated with saturated Na_2SO_4, and filtered. The filtrate was evaporated to an oil (200–300 mg) which was distilled in a Kugelröhr apparatus, bp 80–100°C (0.2 mm). GLC analysis (15% SE-30, 80°C) of the distillate (60–100 mg) indicated the presence of mainly 3-methyl-1,3-butanediol (70%), plus one major unidentified peak of longer retention time, and traces of other compounds.

The distillate, when obtained from administered (**1a**) or (**1b**), was dissolved in anhydrous pyridine (1 ml), and treated with benzoyl chloride (0.05 ml) at 25°C for 24 hr. Workup in the usual manner, followed by preparative TLC (R_f 0.4, solvent 25% EtOAc–hexane), yielded *3-hydroxy-3-methyl-1-butyl benzoate* (**3**), ca. 30–60 mg. When obtained from administered (**1c**), the $LiAlD_4$ reduction product was purified by preparative GLC to give the pure *(1,1,2-d_3)-3-methyl-1,3-butanediol* (**7**).

The *unmethylated* portion of the acidified urine extract was dissolved in 1 ml MeOH, and treated with 0.2 *M* KOH/MeOH added in portions (ca. 5–7 ml total) until a slight alkaline reaction (green color) was visible on moistened wide-range pH paper. Then α-bromo-*p*-phenylacetophenone (1 eq) was added, and the solution was refluxed for 3 hr. After a standard workup, the crude mixture of *p*-phenylphenacyl esters was separated by preparative TLC (20% EtOAc–hexane), and the two major bands corresponding to the esters of β-methylcrotonic acid (R_f 0.8) and β-hydroxyisovaleric acid (R_f 0.2) were extracted with ether. After rechromatography of the former (10% EtoAc–hexane, R_f 0.4), *β-methylcrotonic acid p-phenylphenacyl ester* (ca. 100 mg) was isolated, and was recrystallized twice from MeOH; mp 138–141°C; nmr identical with unlabeled sample except for

intensities of methyl signals (see text). An unlabeled sample of this compound prepared in a similar manner had mp 138–141°C; nmr ($CDCl_3$) δ 1.95 (3H, d, J = 1.5 Hz), 2.19 (3H, d, J = 1.5 Hz), 5.35 (2H, s) 5.86 (1H, qu, J = 1.5 Hz), 7.5 (5H, m), 7.64 and 7.97 (4H, AB, J_{AB} = 8 Hz).

Anal. Calcd for $C_{19}H_{18}O_3$: C, 77.53; H, 6.16. Found: C, 77.55; H, 6.24.

After rechromatography (solvent 35% EtOAc–hexane, R_f 0.3) and recrystallization from ether, the labeled *3-hydroxy-3-methylbutanoic acid p-phenylphenacyl esters* were obtained, mp 82–83°C, nmr identical with unlabeled analog (*1*) except for signal intensities at labeled positions.

Test of Spontaneous Hydration of β-Methylcrotonyl CoA

β-Methylcrotonyl CoA (Sigma) 0.880 mg, 1.01 μmol) was dissolved in 0.2 *M* potassium phosphate buffer, pH 7.4 (0.88 ml, 1.14×10^{-3} *M*). A sample (0.100 ml) assayed immediately by the procedure of Stern (*23*) with bovine liver crotonase (BLC) showed a total decrease in absorbance at 263 nm of 0.30. (The BLC was extracted from fresh beef liver and purified through the acid-heat step according to Stern (*22*). It had a specific activity of 1200 IU/mg protein. Although not homogeneous, this BLC preparation showed $\Delta OD_{263} = 0$ with isovaleryl CoA.) After incubation at 37°C for 39 hr, the β-methylcrotonyl CoA was reassayed as before ($\Delta OD_{263} = -0.29$).

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