SYNTHESIS, ^{1}H-NMR and MASS SPECTRA OF 2-^{2}H-, 2,3-$^{2}H_2$- and 2,2-$^{2}H_2$-LABELLED SHORT CHAIN CARBOXYLIC ACIDS

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SUMMARY

2-^{2}H, 2,3-$^{2}H_2$ and 2,2-$^{2}H_2$ - short chain carboxylic acids were synthesized. Monodeuteration at the 2-position of the carboxylic acids was achieved via decarboxylation of the corresponding carboxyl-deuterated 2-alkylmalonic acids. 2,3-Dideuteration was achieved via catalytic deuteration of the corresponding 2,3- unsaturated carboxylic acids with sodium borodeuteride. 2,2-Dideuteration was achieved via repeated base catalyzed exchange reactions of the carboxylate sodium salts in sodium deuteroxide/deuterium oxide solution. Proton nuclear magnetic resonance and mass spectra of the obtained products are presented.

Key Words: Deuterium, Short Chain Carboxylic Acids, Mass Spectra, ^{1}H-NMR

INTRODUCTION

We required various short chain carboxylic acids labelled with deuterium at the C-2 and C-3 positions for the mechanistic studies of acyl-CoA dehydrogenases (1,2). Currently, five acyl-CoA dehydrogenases are known (3-7). Three of them are specific for straight chain acyl-CoA's with varying chain length (3,6), while two others act on branched chain acyl-CoA's: one is specific for 2-methyl-substituted acyl-CoA's (5) and the other for 3-methyl substituted substrate (4). In the presence of an appropriate electron acceptor(s), all of these dehydrogenases remove one hydrogen each from the C-2 and C-3 of suitable acyl-Coenzyme A esters, producing the corresponding trans-2-enoyl-Coenzyme A esters (complete reaction). In the absence of an electron acceptor, the enzyme and substrate form

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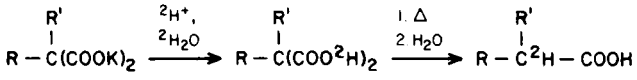
a complex but dehyrogenation of substrate does not occur (half reaction). We found, however, that when the half reaction was carried out in $^{2}H_{2}O$, deuterium was extensively incorporated into acyl-CoA substrate via a proton/deuteron exchange mechanism (1,2). In order to identify the deuterated products and to elucidate the enzyme-catalyzed exchange mechanism, we isolated the deuterated carboxylic acids from the enzyme reaction media and studied them using ^{1}H-NMR and gas chromatography-mass spectrometry (GC/MS). We synthesized several carboxylic acids labelled with deuterium at C-2 and C-3 position(s) as authentic standards for the identification of the enzymically deuterated products. In this report, we describe the syntheses and ^{1}H-NMR and mass spectra of these synthetically labelled compounds.

RESULTS AND DISCUSSION

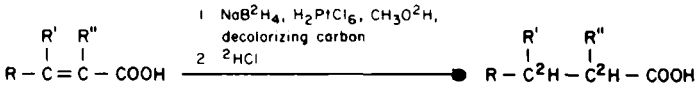
The synthetic schemes employed are summarized in the following reaction sequences:

I. Synthesis of alkyl-2-^{2}H carboxylic acids.



(R and R' vary depending on the desired product)

II. Synthesis of alkyl-2,3-$^{2}H_{2}$ carboxylic acids.



(R, R' and R" vary depending on the desired product)

III. Synthesis of alkyl-2,2-$^2H_2$ carboxylic acids.

$$R-CH_2-COONa \xrightarrow[2\ H^+,\ H_2O]{1\ NaO^2H,\ ^2H_2O,\ \Delta(5X)} R-C^2H_2-COOH$$

(R varies depending on the desired product)

Reaction sequence I was originally reported from our laboratory (8). We chose this method because the reaction is specific for monodeuteration, and is easily carried out in short times. We did, however, detect that some 2,2 dideuterated product is also formed. The formation of the dideuterated product was independent of the pyrolysis temperature and did not occur during the distillation purification process.

Reaction sequence II is an adaptation of the method originally described by Brown and Brown (9), in which sodium borodeuteride has been substituted for sodium borohydride. High deuterium enrichment percents were obtained for all compounds synthesized by this method. 2-Methyl-2-butenoic and 3-methyl-2-butenoic acid showed reduced reactivity under the conditions employed. This is in keeping with the previous finding that the substituted alkenes showed decreased reactivity in catalytic reduction . We explored other reduction methods such as the reaction with nickel-aluminum alloy and sodium hydroxide (10) and the reaction using hydrazine and sodium hydroxide (11), in which we utilized the deuterium analogues. These methods were found to be unsatisfactory because the use of highly alkaline conditions resulted in predominant 2,2,3-trideuteration.

Reaction sequence III is the method originally described by Atkinson, et al. (12). A very high deuterium enrichment can be obtained by this method. The method is time-consuming, however, requiring five separate exchange reactions of the carboxylate sodium salt in sodium deuteroxide solution.

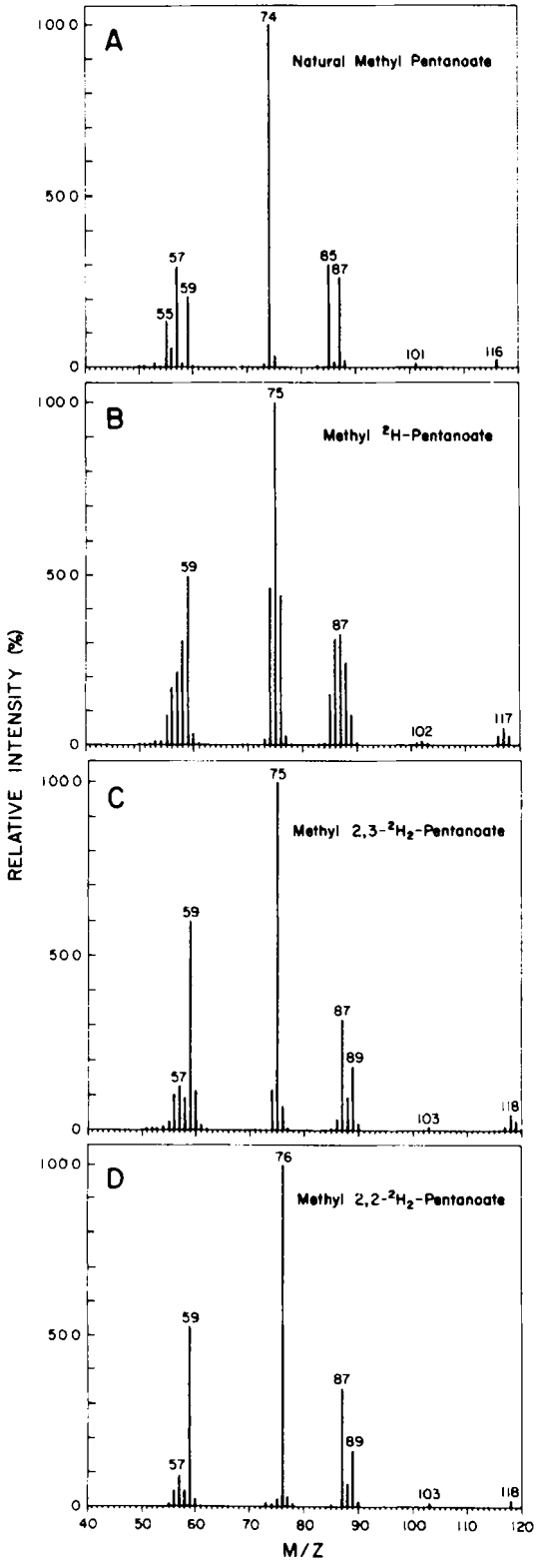


Fig. 1. Electron-impact mass spectra of the natural and deuterated pentanoic acids.

Mass spectra and ^{1}H-NMR were obtained for all the synthetic compounds to determine isotopic enrichment and position of labelling.

The mass spectra of the labelled pentanoic acids are described as a representative case for all the synthetic compounds (Fig. 1). The most characteristic features of the mass spectra for the three types of deuterium labelled compounds are found in the molecular and the McLafferty rearrangement ions. These are: 2-^{2}H compounds - the McLafferty rearrangement ion is shifted from m/z 74 to m/z 75 and the molecular ion is increased by one a.m.u. (Fig. 1B), 2,3-$^2H_2$ compounds - the McLafferty rearrangement ion is shifted from m/z 74 to m/z 75 and the molecular ion is increased by two a.m.u. (Fig. 1C) and 2,2-$^2H_2$ compounds - the McLafferty rearrangement ion is shifted from m/z 74 to m/z 76 and the molecular ion is increased by two a.m.u. (Fig. 1D). The ^{1}H-NMR results are described for all the synthetic compounds. ^{1}H-NMR spectra of the natural and deuterated pentanoic acids are presented in Fig. 2. Characteristic features of these spectra are summarized under EXPERIMENTAL. Changes in the ^{1}H-NMR of other carboxylic acids are essentially the same as those seen in the pentanoic acids. Spectral changes are limited to the signals of C-2, C-3, and C-4. In general, the major changes caused by deuterium labeling are as follows: in the signals of protons replaced with deuterium, the intensity of the signal decreases. The signal is slightly shifted upfield, and each peak of the signal is further split due to geminal ^{1}H-^{2}H coupling. In the signal of protons at the neighboring carbon, the number of peaks decrease if one of the two protons is completely replaced with deuteron, and each peak is slightly broadened due to vicinal ^{1}H-^{2}H coupling.

Based on the data obtained in this study, we were able to identify 2-monodeuterated acyl-CoA as the product of the acyl-CoA dehydrogenase-catalyzed proton/deuteron exchange (1,2). One of the prochiral C-2 protons was nearly 100% replaced with a deuteron by the stereospecific enzyme reaction while no C-3 protons were exchanged at all.

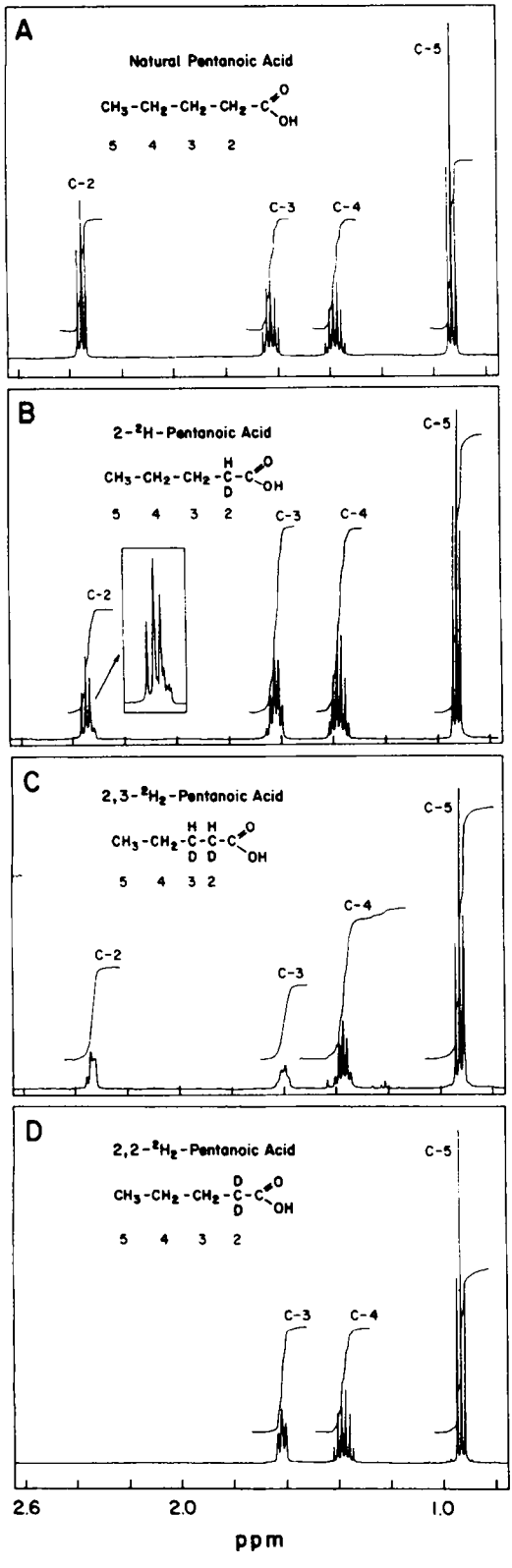


Fig. 2. ^{1}H-NMR spectra of the natrual and deuterated pentanoic acids.

EXPERIMENTAL

Materials. Water-$^2H_2$ (99.8 atom % ^{2}H), 20% deuterium chloride (99+ atom % ^{2}H) in ^2H_2O, sodium borodeuteride (98 atom % ^{2}H), 40% sodium deuteroxide (>99 atom % ^{2}H) in ^2H_2O, and methanol-O-^{2}H (>99.5 atom % ^{2}H) were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade and were purchased from appropriate vendors.

Analysis of Products. The chemical purity of all compounds was determined by gas chromatography of the methyl ester derivatives of the carboxylic acids using a packed 10% OV-17 column. The structures and isotopic enrichment of all products were verified by both gas chromatography-mass spectrometry (GC/MS) and proton nuclear magnetic resonance (NMR).

A Finnigan 4510 GC/MS/COM was used for mass spectral studies. The acids were analyzed as their methyl esters using a 10% OV-17 packed column as the inlet. The samples were chromatographed isothermally at temperatures ranging from 45°C for methyl butanoate to 90°C for methyl hexanoate. The injection port, separator, and transfer line were kept at 225°, 250° and 270°C, respectively. All samples were analyzed by both electron impact and chemical ionization. The ionizing voltage was 70eV for electron impact (EI) analysis and methane was used as reagent gas for chemical ionization analysis. The protonated molecular ions in chemical ionization were used to determine isotopic enrichment. Appropriate fragment ions obtained by electron impact were used to confirm the position(s) of deuterium incorporation.

Nuclear magnetic resonance was performed on the free acids in deuterated chloroform (3mg/ml) using a Bruker WM-500 spectrometer equipped with an Aspect 2000 computer. The structures and isotopic enrichment of the synthesized acids were determined by comparison of the obtained spectra to those of the corresponding natural abundance compounds and integration of the signals. There was good agreement between isotopic enrichments determined by NMR and GC/MS.

Hexanoic-2-^{2}H-acid (1). Diethyl 2-butylmalonate was synthesized and subsequently saponified according to the method described previously (8). 2-Butylmalonic acid was converted to the dipotassium salt. Five grams of the salt was dissolved in 10 ml of deuterium oxide and acidified to pH 1 with $^{2}H_2SO_4$. The solution was extracted with diethyl ether, dried over anhydrous sodium sulfate, filtered, and recrystallized by the addition of hexane. The recovered 2-butylmalonic acid (carboxyl-$^{2}H_2$) crystals were dried in a vacuum dessicator. The acid crystals were transferred to a 25 ml round-bottom flask equipped with a magnetic stirrer and a reflux condenser attached to a mineral oil bubbler. The flask was heated in an oil bath and the temperature was raised to 195° at which point decarboxylation started. After decarboxylation ceased, the flask was heated an additional hour at 180°. The resulting hexanoic-2-^{2}H-acid was purified by distillation under reduced pressure. The chemical purity and isotope enrichment were 99% and 49% with ^{2}H and 12% with $^{2}H_2$, respectively. The yield prior to the decarboxylation step was 50%. Although it was not determined accurately, the yield from the decarboxylation step was high as expected from the simple procedure.

Pentanoic-2-^{2}H-acid (2). This deuterated acid was prepared in the same manner as compound 1 from 2-propylmalonate. 1-Bromopropane was used as the alkylating agent. The chemical purity and yield were 99% and 59%, respectively. Isotope enrichment was 51% with ^{2}H and 15% with $^{2}H_2$.

3-Methylbutanoic-2-^{2}H-acid (3). This deuterated acid was prepared in the same manner as compound 1 from 2-isopropylmalonate. The chemical purity and yield were 99%, and 68%, respectively. Isotope enrichment was 52% with ^{2}H and 22% with $^{2}H_2$.

2-Methylbutanoic-2-^{2}H-acid (4). This deuterated acid was prepared in the same manner as compound 1 from 2-ethyl-2-methylmalonate. Dimethyl 2-Ethyl-2-methylmalonate was synthesized by alkylation of 2-methylmalonate with 1-bromoethane.

Saponification with potassium hydroxide was carried out for 12 hours. The chemical purity, isotope enrichment, and yield were 99%, 23%, and 47%, respectively.

Butanoic-2-^{2}H-acid (5). Commercially available 2-ethylmalonic acid (99%) was converted to the deuterium acid and decarboxylated as for compound 1. The chemical purity and isotopic enrichment were 99% and 51% with ^{2}H and 38% with $^2H_2$, respectively.

2-Methylpropanoic-2-^{2}H-acid (6). 2,2-Dimethylmalonic acid, which was previously synthesized in our laboratory (8), was converted to the deuterium acid and decarboxylated as for compound 1. The chemical purity and isotopic enrichment were 99% and 64%, respectively.

Hexanoic-2,3-$^2H_2$-acid (7). A 125 ml side arm erlenmeyer flask equipped with a magnetic stirring bar, a balloon, and an inlet glass tube fitted with a rubber serum cap, was immersed in a water bath at 25°. In the flask was placed 10.0 gram of decolorizing carbon, 40 ml of deuterated methanol(methanol-O-^{2}H), and 1 ml of a 0.2 M solution of hydrogen hexachloroplatinate (IV) in deuterated methanol. Five ml of a 1.0 M solution of sodium borodeuteride solution in deuterated methanol, stabilized with 0.004 mol of sodium deuteroxide, was injected into the flask through the rubber serum cap. After one minute, 4 ml of 6M deuterium chloride in deuterium oxide was likewise injected, causing the ballon to inflated with deuterium gas. 0.005 Mol of 2-hexenoic acid dissolved in 10 ml of deuterated methanol was injected to the vigorously stirred solution. As the reaction proceeded, the ballon became partially deflated, in which case additional amounts of the 1 M sodium borodeuteride solution were added so as to keep the balloon inflated. After the reaction had proceeded for one hour the pH was adjusted to >10 with sodium hydroxide and the solution was filtered and the deuterated methanol removed on a rotary evaporator. Ten ml of water was added and the

solution acidified to pH 1.0 with hydrochloric acid. The solution was extracted five times with equal volumes of diethyl ether. The extracts were dried over anhydrous sodium sulfate, filtered, and evaporated under a gentle stream of nitrogen to yield hexanoic-2,3-$^2H_2$-acid. The chemical purity and yield were 93% and 70%, respectively. Isotope enrichment was 73% with 2,3-$^2H_2$ and 11% with 2,2,3-$^2H_3$.

Pentanoic-2,3-$^2H_2$-acid (8). This deuterated acid was prepared in the same manner as compound 7 utilizing 2-pentenoic acid as the starting material. The chemical purity and yield were 98% and 61%, respectively. Isotope enrichment was 69% with 2,3-$^2H_2$ and 21% with 2,2,3-$^2H_3$.

3-Methylbutanoic-2,3-$^2H_2$-acid (9). This deuterated acid was prepared in the same manner as compound 7 utilizing 3-methyl-2-butenoic acid as the starting material. The chemical purity was 67%. Isotopic enrichment was 90% 2,3-$^2H_2$ and 2% 2,2,3-$^2H_3$. Significant amounts of the starting material remained unreacted under the conditions employed. The yield was 50%.

2-Methylbutanoic-2,3-$^2H_2$-acid (10). This deuterated acid was prepared in the same manner as compound 7 utilizing 2-methyl-2-butenoic acid as the starting material. The chemical purity was 42%. Isotopic enrichment was 62% 2,3-$^2H_2$ and 33% 2,2,3-$^2H_3$, respectively. Significant amounts of the starting material remained unreacted under the reaction conditions employed. The yield was 24%.

Butanoic-2,3-$^2H_2$-acid (11). This deuterated acid was prepared in the same manner as compound 7 utilizing 2-butenoic acid as the starting material. The chemical purity and yield were 99% and 68%, respectively. Isotopic enrichment was 73% with 2,3-$^2H_2$ and 11% with 2,2,3-$^2H_3$.

Hexanoic-2,2-$^2H_2$-acid (12). This deuterated acid was prepared by the method of Atkinson, et al (12). The chemical purity, isotopic enrichment and yield were 99%, 77% with 2,2-$^2H_2$ and 49%, respectively.

Pentanoic-2,2-$^2H_2$-acid (13). This deuterated acid was prepared in the same manner as compound 12. The chemical purity, isotopic enrichment and yield were 99%, 98% with 2,2-$^2H_2$ and 44%, respectively.

3-Methylbutanoic-2,2-$^2H_2$-acid (14). This deuterated acid was prepared in the same manner as compound 12. The chemical purity, isotopic enrichment and yield were 97%, 55% with 2,2-$^2H_2$ and 39%, respectively.

Butanoic-2,2-$^2H_2$-acid (15). This deuterated acid was prepared in the same manner as compound 12. The chemical purity, isotopic enrichment and yield were 99%, 98% with 2,2-$^2H_2$ and 44%, respectively.

^{1}H-NMR and MASS SPECTRA

The ^{1}H-NMR and mass spectra were described only for the deuterated pentanoic acids.

Unlabelled Pentanoic Acid. The ^{1}H-NMR and mass spectra of unlabelled and deuterium labelled pentanoic acids are shown in figure (2A). ^{1}H-NMR: H-2; a sharp triplet at 2.3549 ppm (2H) H-3; a quintet at 1.6256 ppm (2H) the three middle peaks have shoulders H-4; a sextet centered around 1.3778 ppm (2H) the four middle peaks have shoulders H-5; a sharp triplet at 0.9266 ppm (2H).

Pentanoic-2-^{2}H-acid. ^{1}H-NMR (Fig. 2B): H-2; there are two groups of signals (1.25H). One group is a sharp triplet at 2.3550 ppm, representing H from CH_2. The other group is a multiplet centered at 2.3346 ppm, representing H from CH^2H. H-3; a quartet centered around 1.6197 ppm (2H). Each peak is broadened due to vicinal H^2H coupling. H-4; a sharp sextet centered around 1.3776 ppm (2H). H-5; a sharp triplet at 0.9265 ppm (3H).

Mass Spectroscopy. Mass spectral data obtained by chemical ionization of the methyl ester of pentanoic-2-^{2}H-acid showed 51.0% monodeuteration and 15.1%

dideuteration at the 2-carbon. This same result is obtained also in the EI mass spectrum (Fig. 1B). The molecular ion peak is shifted up one a.m.u. to m/z 117, representing incorporation of one deuterium. Smaller amounts of m/z 116 and m/z 118 are also present, representing the unlabelled and dideuterated acids, respectively. The base peak at m/z 74 in the unlabelled compound (from McLafferty rearrangement with C2-C3 cleavage) is also predominantly shifted one a.m.u. higher to m/z 75 and to a lesser degree to m/z 76, representing mono- and dideuteration at the 2-carbon, respectively.

Pentanoic-2,2-^{2}H-acid. NMR (Fig. 2C): H-2: signal is absent. H-3: a triplet at 1.6216 ppm (2H). Each peak is broadened due to vicinal $H^{2}H$ coupling. H-4: a sextet centered around 1.3828 ppm (2H). The middle four peaks have shoulders. H-5: a sharp triplet at 0.9322 ppm (3H).

Mass Spectroscopy: Mass spectral data obtained by chemical ionization showed 98.4% dideuteration. This can be seen in the EI mass spectrum. The molecular ion is shifted up two a.m.u.'s to m/z 118. The base peak at m/z 74 in the unlabelled compound is also shifted up two a.m.u.'s to m/z 76.

Pentanoic-2,3-^{2}H-acid. NMR: H-2: a broadened, distorted doublet at 2.3414 ppm (1.1H). H-3: a broadened, distorted quartet centered around 1.6032 ppm (0.89H). H-4: a broadened quintet at 1.3722 ppm (1.7H). H-5: a sharp triplet at 0.9258 ppm (3H).

Mass Spectroscopy. Mass spectral data obtained by chemical ionization indicated 69.0% dideuteration and 20.9% trideuteration. The EI mass spectrum (Fig. 1C) shows the molecular ion predominantly shifted two a.m.u.'s higher to m/z 118, along with a lesser amount of m/z 119. The base peak is shifted up only one a.m.u. to m/z 75 because this fragment ion contains only carbons 1 and 2. The EI mass spectrum clearly indicates incorporation of two deuterium, one of which is at the 2-carbon. The NMR spectrum clearly shows that the other deuterium is incorporated into the 3-carbon.

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