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Synthesis of mono- and di-deuterated (2*S*,3*S*)-3-methylaspartic acids to facilitate measurement of intrinsic kinetic isotope effects in enzymes☆

Hyang-Yeol Lee, Miri Yoon and E. Neil G. Marsh*

Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-1055, USA

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Abstract—Kinetic isotope effects provide a powerful method to investigate the mechanisms of enzyme-catalyzed reactions, but often other slow steps in the reaction such as substrate binding or product release suppress the isotopically sensitive step. For reactions at methyl groups, this limitation may be overcomed by measuring the isotope effect by an *intra*-molecular competition experiment. This requires the synthesis of substrates containing regio-specifically mono- or di-deuterated methyl groups. To facilitate the mechanistic investigations of the adenosylcobalamin-dependent enzyme, glutamate mutase, we have developed a synthesis of mono- and di-deuterated (2*S*,3*S*)-3-methylaspartic acids. Key intermediates are the correspondingly labeled mesaconic acids and their dimethyl esters that potentially provide starting materials for a variety of isotopically labeled molecules.

1. Introduction

Isotope effects provide an extremely powerful tool to probe the mechanisms of chemical reactions and have proved particularly useful for investigating enzyme mechanisms.[1–4] Most isotope effect measurements rely on *inter*-molecular competition between labeled and unlabeled molecules. However, for an isotope effect to be measured in this way, it must be associated with the rate-determining step in reaction, or, in the case of isotope effects on V_{max}/K_m, occur either at or before the rate-determining step. In many enzyme reactions slow steps that are not isotopically sensitive, such as substrate binding, product release, or protein conformational changes, completely mask the intrinsic isotope effects, limiting our ability to learn about the chemical steps.

However, for chemical reactions that occur at methyl groups it is possible to measure *intrinsic* deuterium isotope effects in enzymes by specifically labeling the methyl carbon with one or two deuterium atoms. The isotope effect can be measured, even when the isotopically sensitive step is *not* rate determining, because it is manifested through *intra*-molecular competition between protium and deuterium atoms, which remain chemically equivalent even in the enzyme active site due to the rapid rotation of the methyl group. The principle of this experiment is illustrated in Figure 1.

This method of measuring isotope effects has proved especially useful for investigating enzyme reactions at unactivated methyl groups, for example, oxygenation reactions catalyzed by cytochrome p450 enzymes.[5] As part of our efforts to understand the mechanism of hydrogen atom transfer in the coenzyme B_{12}-dependent enzyme, glutamate mutase,[6–10] we sought to synthesize the substrate (2*S*,3*S*)-3-methylaspartate that was regio-specifically mono- or di-deuterated in the methyl group. These substrates allow us to measure the intrinsic kinetic isotope effects on hydrogen transfer between the substrate methyl group and the 5′-carbon of coenzyme B_{12} under single turnover conditions by setting up an *intra*-molecular competition between protium and deuterium atoms in the methyl group.

Our synthesis is based on the regio-specific deuteration of mesaconic acid (methylfumaric acid), which is an intermediate in the fermentation of glutamate by many anaerobic bacteria. Mesaconate is a versatile intermediate that can be readily converted to 3-methylaspartate through the action of β-methylaspartase (methylaspartate ammonialyase), an enzyme that has been used to synthesize a variety of aspartic acid analogs.[11,12] The enzyme-catalyzed reaction stereospecifically introduces an asymmetric center adjacent to the labeled methyl group. Mesaconate is also amenable to

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* Corresponding author. Tel.: +1 734 763 6096; fax: +1 734 615 3790; e-mail: nmarsh@umich.edu

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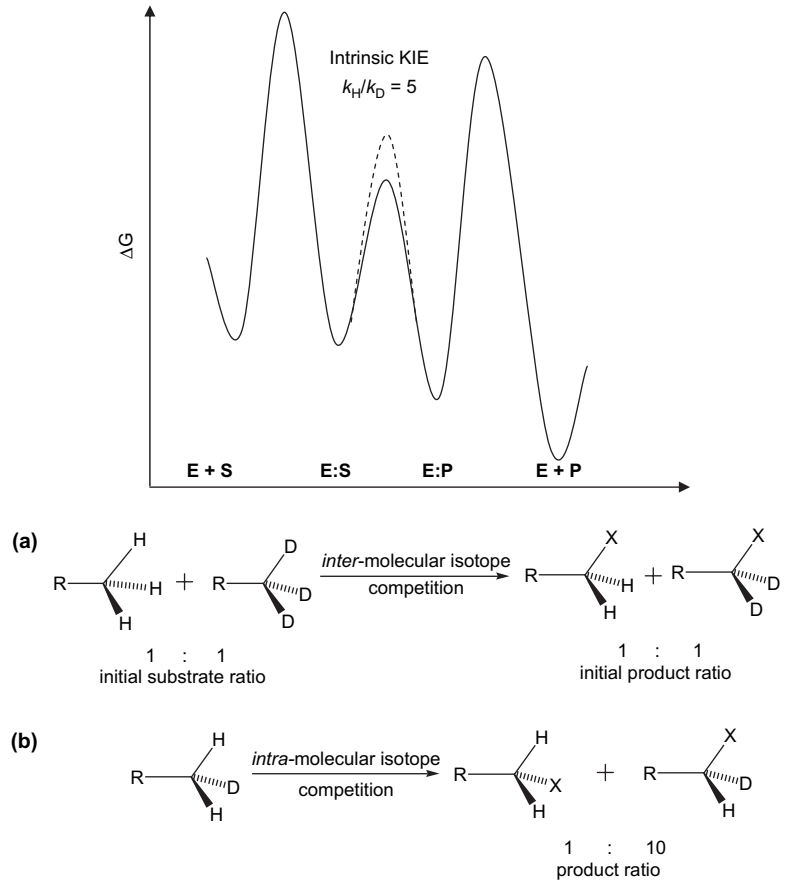

Figure 1. Illustration of the different outcomes obtained when measuring isotope effects in an enzyme-catalyzed reaction using *inter*-molecular versus *intra*-molecular competition experiments. Top: hypothetical free energy profile for an enzyme catalyzing the substitution of a hydrogen atom of a methyl group with functional group, X, in which substrate binding and product release mask the intrinsic deuterium kinetic isotope effect of 5 on the chemical step. Bottom: (a) the relative distribution of isotopically labeled products formed in an *inter*-molecular competition experiment; the ratio of deuterated to undeuterated products is the same as the starting materials, i.e., no isotope effect is expressed. (b) The relative distribution of isotopically labeled products formed in an *intra*-molecular competition experiment; here the full isotope effect is expressed (the ratio of deuterium-containing products is 10:1, and not 5:1, because there are twice as many protons as deuterons in the methyl group).

numerous chemical transformations, potentially allowing a wide range of labeled, branched-chain compounds to be synthesized.

2. Results and discussion

The strategy for the synthesis of the regio-specifically mono-deuterated methylaspartic acids is shown in Figure 2. Mesaconic acid **1** was first protected as its dimethyl ester **2** in 80% yield by refluxing overnight in methanol in the presence of 1.5% (v/v) sulfuric acid. As described previously,[13] these conditions yielded predominantly the *cis*-dimethyl ester **2a** with a small amount of the trans-isomer **2b**, the relative proportions varying somewhat from reaction to reaction. Both stereoisomers of **2** were converted, without separation, to 3-(bromomethyl)-fumarate dimethyl ester, **3**, by reaction with 1.5 equiv of *N*-bromosuccinimide and a catalytic amount (10%) of AIBN as a radical initiator. The reaction proceeded smoothly overnight to produce **3** in good yields (72%), with only the mono-brominated product being detected. During the reaction, the cis-stereoisomer is converted to the trans-form. This points to the formation of an allylic radical intermediate during the reaction, which would permit rotation around the double bond.

Compound **3** was carefully purified from unreacted **2** and other by-products by chromatography on silica gel. It is, of course, most important to remove any traces of **2** at this point, otherwise the isotopic purity of the final product will be diluted with unlabeled material. Introduction of deuterium was accomplished by reductive debromination using tributyltin deuteride in dry benzene at 55 °C with 10% AIBN as a radical initiator. This gave the mono-deuterated dimethyl methylfumarate **4** in 50% yield. Lastly, the ester was hydrolyzed using lithium hydroxide in aqueous tetrahydrofuran, room temperature, 30 h, to yield after acidification mono-deuterated mesaconic acid in 64% yield.

Mesaconic acid incorporating two deuterium atoms in the methyl group was synthesized by an analogous strategy starting with itaconic acid (Fig. 3). $^2H_4$-Mesaconic acid was synthesized by dissolving itaconic acid (methylenesuccinic acid) in 40% NaOD/D_2O and heating at 120 °C for 90 min.[14] This resulted in the isomerization of itaconate to mesaconate and the complete exchange of all four protons. After neutralization, the resulting d_4-mesaconic acid was converted to a mixture of *cis*- and *trans*-dimethyl ester (**7a** and **7b**), and then to deuterated 3-(bromomethyl)-fumarate dimethyl ester (**8**) as described above. The bromination reaction proceeds noticeably slower with the deuterated material,

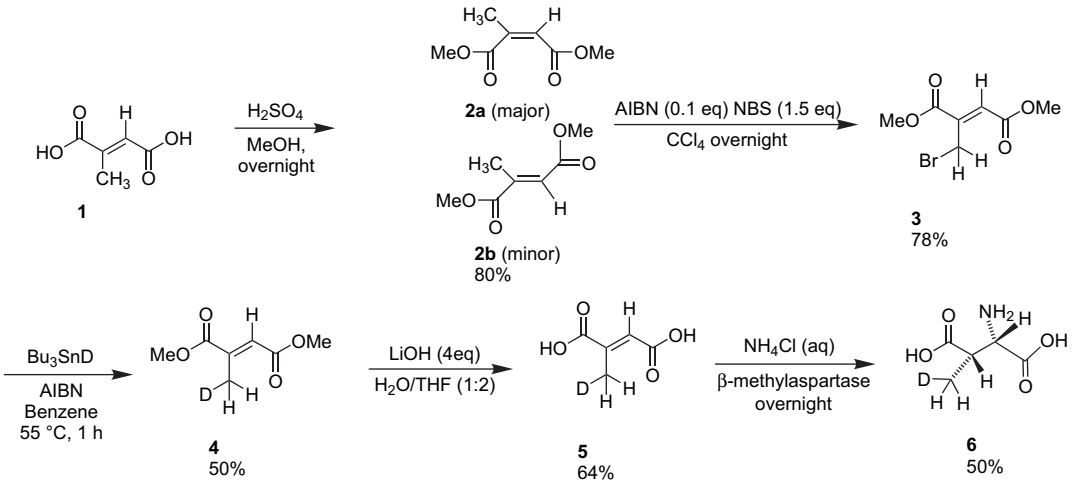

Figure 2. Strategy for the stereospecific synthesis of regio-specifically mono-deuterated (2*S*,3*S*)-3-methylaspartic acid.

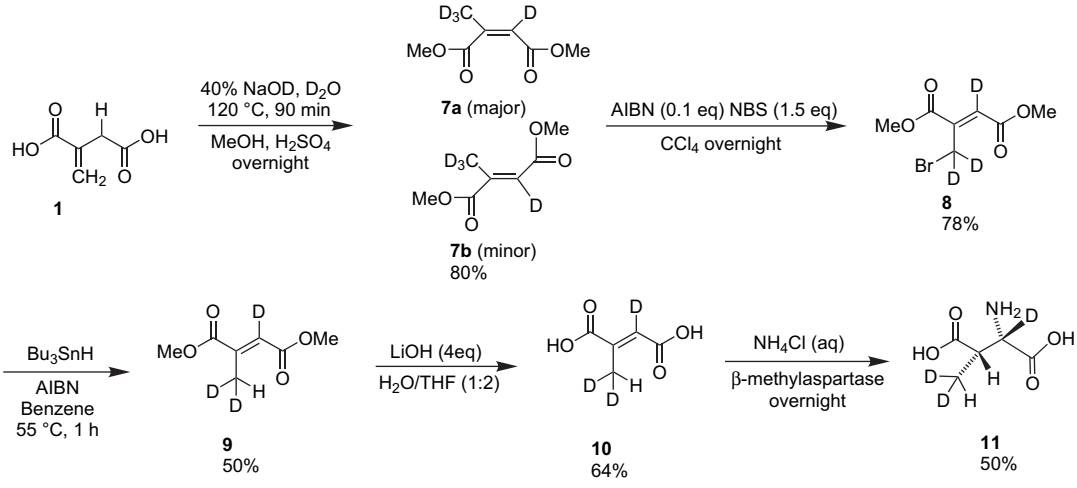

Figure 3. Strategy for the stereospecific synthesis of (2*S*,3*S*)-3-methylaspartic acid that is regio-specifically di-deuterated in the methyl group.

pointing to a significant deuterium kinetic isotope effect for this step. Reduction with tributyl-tin hydride yielded the dimethyl ester of mesaconate, **9**, containing two deuterium atoms in the methyl group, which was then hydrolyzed to give (2-$^2H_1$,methyl-$^2H_2$)-mesaconic acid, **10**.

The isotopic composition of the mesaconic acids was determined from the proton NMR spectrum, by taking advantage of the fact that incorporation of deuterium introduces an up-field shift in the protons of the methyl group by about 0.03 ppm (Fig. 4). The methyl protons of the non-deuterated compound appear as a doublet, ^4J=2 Hz, due to long-range coupling with the vinylic proton. The methyl group of the mono-deuterated material appears as an overlapping doublet of triplets that arises from the coupling of the protons to the spin 1 deuterium nucleus, $^4J_{HH}=^2J_{HD}$=2 Hz. Integration of the peak areas for these two signals in a sample of mono-deuterated mesaconic acid indicated that the deuterium content was at least 98 mol %. The proton decoupled ^{13}C NMR spectrum showed the expected triplet signal, $^1J_{CD}$=20 Hz, for the ^{13}C–deuterium coupling (Fig. 4).

The proton NMR spectrum of **10** showed a multiplet at 2.16 ppm exhibiting the expected quintet coupling pattern for two deuterium atoms coupling to the single proton $^2J_{HD}$= 2 Hz (Fig. 4). A weak signal is also present arising from the mono-deuterated methyl group. After accounting for the fact that the relative intensity of this signal is twice than that of the di-deuterated methyl group, the mono-deuterated material represents less than 2 mol % of the total. A signal from the mono-deuterated material is also evident in the ^{13}C NMR spectrum (Fig. 4), where its intensity is enhanced by relaxation through the proton spin. It was also evident from the proton spectrum of **9** that a small amount of protium, ~5%, was present at C-2 (data not shown), which appears to be exchanged during bromination. For our purposes, however, this does not present a problem since the α-hydrogen of amino acids can readily be exchanged with water enzymatically, as discussed below.

The isotopically labeled mesaconic acids were readily converted to the corresponding (2*S*,3*S*)-3-methylaspartic acids through the action of the enzyme methylaspartase in the

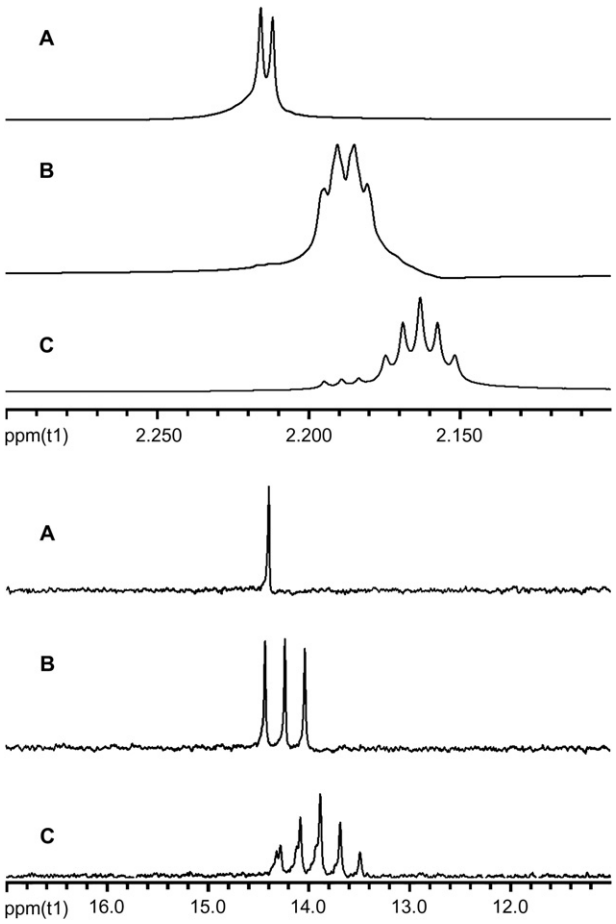

Figure 4. Analysis of the deuterium content of mesaconic acids by NMR. Top: proton NMR (400 MHz) showing the deuterium-induced changes in chemical shift and coupling pattern for the methyl resonance of the mono- and di-deuterated compounds. Bottom: ^{13}C NMR (100 MHz) showing the deuterium-induced changes in chemical shift and coupling pattern of the same compounds. Spectrum A: unlabeled material; spectrum B: mono-deuterated at the methyl group; spectrum C: di-deuterated at the methyl group.

presence of 0.5 M ammonium chloride. The deuterated amino acids were produced in ~50% yield and were shown to be active as substrates for glutamate mutase.[15] At this point deuterium present at C-2 of the methylaspartate can easily be removed by exchange with H_2O through the action of glutamate/aspartate aminotransferase.[10]

3. Conclusion

We have developed a procedure for the synthesis of mesaconic acids that are specifically mono- or di-deuterated in the methyl group. These compounds could be then enzymatically converted to the correspondingly deuterated (2*S*,3*S*)-3-methylaspartic acids, in this case to facilitate mechanistic experiments on glutamate mutase. More generally, mesaconic acid, or its dimethyl ester, provides a versatile 5-carbon fragment that may readily be elaborated to more complex molecules through a variety of synthetic transformations. Compounds containing methyl groups that are specifically mono- or di-deuterated should prove as useful mechanistic probes for enzymes that catalyze reactions at methyl groups.

4. Experimental

4.1. General

Bu_3SnH and Bu_3SnD were purchased from Acros Co., 40% sodium deuteroxide in D_2O was purchased from Cambridge Isotope Laboratories, Inc., mesaconic acid was purchased from Sigma, itaconic acid and *N*-bromosuccinimide were purchase from Aldrich. Recombinant β-methylaspartase was a gift from Professor David Gani (St. Andrews University) and was purified from *Escherichia coli* as described by Goda et al.[16]

4.2. Dimethyl methylmaleate (2a)

Compound **2a** was prepared from mesaconic acid by refluxing in methanol in the presence of H_2SO_4 using a previously described literature procedure.[17] The procedure yielded a colorless liquid comprising mainly **2a** with a small amount of the trans-isomer (**2b**). Yield of **2a**+**2b** was 80%.

4.3. Dimethyl bromomethylfumarate (3)

Compound **3** was prepared from the mixture of **2a** and **2b** that are obtained above using a previously described literature procedure.[17] Briefly, the mixture of **2a** and **2b** (3.16 g, 20 mmol) was reacted with *N*-bromosuccinimide (5.34 g, 30 mmol) and a catalytic amount of AIBN (0.33 g, 2 mmol) in carbon tetrachloride (45 mL) under reflux for 24 h. After workup and silica gel chromatography pure **3** was obtained in 78% yield.

4.4. Dimethyl $^2H_1$-methylfumarate (4)

The conversion of **3** into **4** was achieved by reduction with tributyltin deuteride and AIBN, based on a literature procedure.[18] Briefly, **3** (0.59 g, 2.5 mmol), Bu_3SnD (810 μL, 3 mmol), and 10 mol % AIBN (45 mg, 0.25 mmol) were stirred in 10 mL of dried benzene at 55 °C for 1 h. The mixture was cooled to room temperature and KF/Celite was added. After stirring the mixture overnight, it was concentrated and purified by silica gel column chromatography to give pure **4** in 50% yield. The ^1H and ^{13}C NMR spectra of **4** were identical to an authentic standard of the unlabeled compound **2a**, differing only as italicized due to the incorporation of deuterium at the methyl carbon: ^1H NMR ($CDCl_3$, 300 MHz) δ 6.68 (s, 1H), 3.71 (s, 3H), 3.67 (s, 3H), *2.17 (quartet, J=2 Hz, 2H)*; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 167.1, 165.8, 143.3, 126.1, 52.1, 51.2, 13.6 (triplet, *J*=20 Hz); ES-MS (positive ion mode) calcd for $C_7H_{10}DO_4$ $[M+H]^+$=160.1, *m/z* found=160.1; HRMS calcd for $C_6H_6DO_3$ $[M-OCH_3]^+$=128.0458, *m/z* found=128.0454.

4.5. $^2H_1$-Mesaconic acid (5)

A solution of **4** (200 mg, 1.26 mmol) in 3 mL of THF was added to a solution of LiOH hydrate (196 mg, 4.66 mmol) in 1.5 mL of water and stirred for 30 h. The reaction mixture was concentrated to remove THF and the pH was adjusted to ~1.0 by adding 3 M aqueous HCl. The solution was extracted with ethyl acetate three times, dried over sodium sulfate, and the solvent was removed by rotary evaporation. The solid residue was triturated with warm hexanes three

times to give **5** in 64% yield. The ^{1}H and ^{13}C NMR spectra of **5** were identical to an authentic standard of the unlabeled compound **1**, differing only as italicized due to the incorporation of deuterium at the methyl carbon: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.75 (br s, 1H), *2.19 (quartet, J=2 Hz, 2H)*; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.3, 169.2, 144.8, 128.2, *14.2 (triplet J=20 Hz)*; HRMS calcd for $C_5H_3DO_3^+$[M−H_2O]$^+$=113.0223, *m/z* found=113.0225.

4.6. $^2H_1$-Methyl-(2*S*,3*S*)-3-methylaspartic acid (6)

Compound **5** was converted to (2*S*,3*S*)-methylaspartic acid, **6**, through the action of β-methylaspartase. Deuterated mesaconic acid of 17.1 mg (130 μmol) was dissolved in 300 μL of 250 mM potassium phosphate buffer, pH 8.0, containing 20 mM potassium chloride, 2 mM magnesium chloride, and approximately 1.1 M ammonium hydroxide and converted to the diammonium salt. β-Methylaspartase of 60 units (360 μL of enzyme solution stored in 25 mM potassium phosphate buffer, pH 7.0, 50% glycerol) was added and the reaction mixture was incubated at 37 °C for 6 h. An additional 27 units (160 μL) of β-methylaspartase and 80 μL of 2 M ammonium chloride were added and the incubation was continued at 37 °C for a further 12 h. The decrease in 240 nm absorbance of the reaction mixture indicated that 105 μmol of mesaconate had been consumed. 50 μL of 12 N HCl was added to quench the reaction and the mixture, and heated at 94 °C for 5 min to precipitate the β-methylaspartase. The precipitated protein was removed by centrifugation of the suspension (12,000 rpm× 3 min). The supernatant solution was extracted five times with a mixture of 4 mL water and 24 mL ethyl ether to remove unreacted mesaconate. The solution was then adjusted to neutral pH and the concentration of **6** was determined to be 14 mM by assay with β-methylaspartase.[19] The final volume of the solution was 4.8 mL corresponding to a yield of 50%. The enzymatic properties of methylaspartate prepared by this method were identical to authentic material.

4.7. Dimethyl $^2H_4$-methylmaleate (7a)

Deuterated mesaconic acid was prepared based on the procedure described by Eagar et al.[14] A solution of itaconic acid (3.00 g, 23.1 mmol) in 30 mL of NaOD/D_2O was placed in the Parr reactor and heated to 120 °C for 1.5 h. After cooling to room temperature, the reaction mixture was poured into a 500 mL Erlenmeyer flask in an ice bath. Aqueous HCl of 6 M was slowly added to the reaction mixture to bring the pH to ∼1. Water was removed by freeze-drying in a lyophilizer. To the solid residue 30 mL of dilute HCl was added and the mixture was extracted with ethyl acetate (3×30 mL), the combined extracts were dried over sodium sulfate, and the solvent was then removed by rotary evaporation. The resulting white solid was converted to the dimethyl ester as described above to give a mixture of **7a** (major) and the trans-isomer **7b** with the total yield of 87%. The ^{1}H and ^{13}C NMR spectra of **7a** were identical to an authentic standard of the unlabeled compound **2a**, differing only as italicized due to the incorporation of deuterium: NMR ($CDCl_3$, 300 MHz) δ *6.68 (absent)*, 3.76 (s, 3H), 3.72 (s, 3H), *2.17 (absent)*; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 167.2, 165.9, 143.4, *126.0 (triplet, J=25 Hz)*, 52.3, 51.4, *13.4 (multiplet, J=20 Hz)*; ES-MS (positive ion mode), calcd for $C_7H_7D_4O_4$ [M+H]$^+$=163.1, *m/z* found=163.1.

4.8. Dimethyl bromo-$^2H_2$-methyl-$^1H_1$-fumarate (8)

The mixture of **7a** and **7b** was converted to **8** using the procedure described above for the conversion of **2a** and **2b** to **3**. Pure **8** was obtained in 72% yield. The ^{1}H and ^{13}C NMR spectra of **8** were identical to an authentic standard of the unlabeled compound **3**, differing only as italicized due to the incorporation of deuterium: ^{1}H NMR ($CDCl_3$, 400 MHz) *δ 6.79 (absent)*, *4.68 (absent)*, 3.84 (s, 3H), 3.78 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.1, 164.9, 142.5, *127.9 (triplet, J=26 Hz)*, 52.9, 52.2, *22.2 (multiplet, J=24 Hz)*; EIMS (positive), calcd for $C_6H_3D_3BrO_3$ [M−OCH_3]$^+$= 208.0, *m/z* found=209.0 and 207.0.

4.9. Dimethyl $^2H_2$-methyl-$^2H_1$-fumarate (9)

The conversion of **8** to **9** (1.00 g, 4.18 mmol) was carried out with tributyltin hydride (1.35 mL, 5.02 mmol) and 10 mol % AIBN (69 mg, 0.42 mmol) in 20 mL of dried benzene as described above for the conversion of **3** to **4**. Pure **9** was obtained in 53% yield. The ^{1}H and ^{13}C NMR spectra of **9** were identical to an authentic standard of the unlabeled compound **2a**, differing only as italicized due to the incorporation of deuterium: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.65 (s, 3H), 3.61 (s, 3H), 2.16 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 176.2, 165.9, 143.4, *125.9 (triplet, J=25 Hz)*, 52.3, 51.3, *13.4 (quintet, J=20 Hz)*; EIMS (positive), calcd for $C_6H_4D_3O_3$ [M−OCH_3]$^+$=130.1, *m/z* found=130.1.

4.10. (2-$^2H_1$,$^2H_2$-Methyl)-mesaconic acid (10)

The conversion of **9** to **10** was accomplished as described above for the conversion of **4** to **5** to give pure **10** in 78% yield. The ^{1}H and ^{13}C NMR spectra of **10** were identical to an authentic standard of the unlabeled compound **1**, differing only as italicized due to the incorporation of deuterium at the methyl carbon (Fig. 4): ^{1}H NMR ($CDCl_3$, 400 MHz) *δ 2.16 (quintet, J=2 Hz, 1H)*; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.4, 169.3, 144.6, *127.8 (triplet, J=25 Hz)*, *13.9 (quintet, J=20 Hz)*; HRMS calcd for $C_5HD_3O_3^+$[M−H_2O]=115.0349, *m/z* found=115.0347.

4.11. (2-$^2H_1$,$^2H_2$-Methyl)-(2*S*,3*S*)-3-methylaspartic acid (11)

Compound **10** was converted to (2*S*,3*S*)-methylaspartic acid, **11**, in ∼50% yield through the action of β-methylaspartase as described above.

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