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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☆

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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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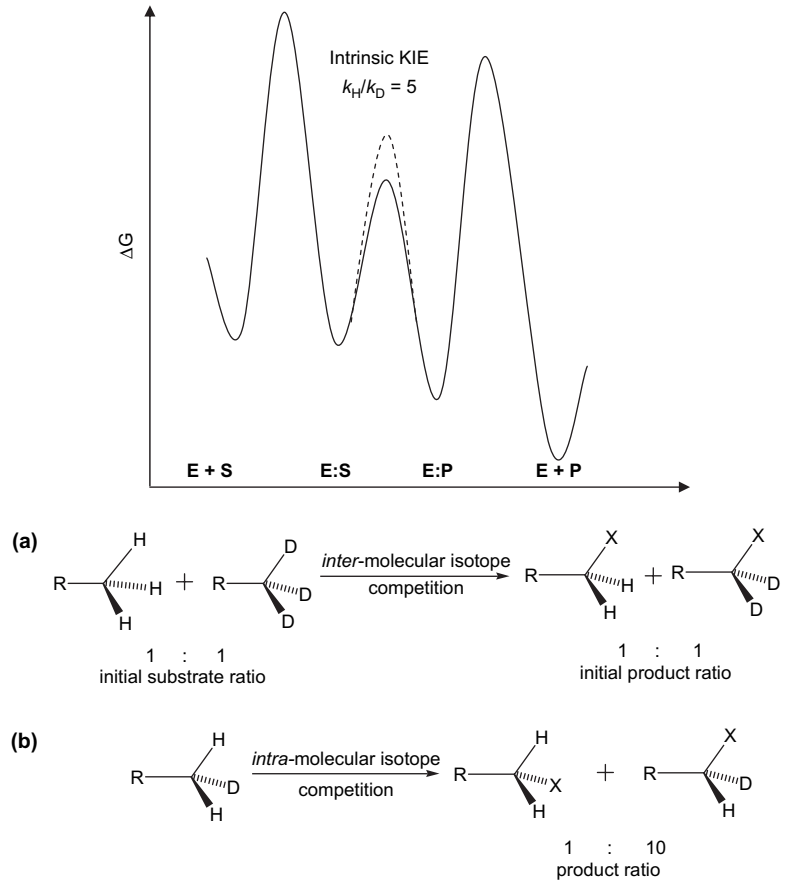


**Figure 1**. Illustration of the different outcomes obtained when measuring isotope effects in an enzyme-catalyzed reaction using *inter*-molecular versus *intra*-molecular competition experiments. Top: hypothetical free energy profile for an enzyme catalyzing the substitution of a hydrogen atom of 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,