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Stereospecific Multiple Isotopic Labeling of Benzyl Alcohol

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Abstract

Isotopically labeled enzymatic substrates and biological metabolites are useful for many mechanistic analyses, particularly the study of kinetic and equilibrium isotope effects, determining the stereospecificity of enzymes, and resolving metabolic pathways. Here we present the 1-pot synthesis, purification, and kinetic analysis of 7*R*-[²H]-phenyl-[¹⁴C]-benzyl alcohol. The procedure involves a chemoenzymatic synthesis that couples formate dehydrogenase to alcohol dehydrogenase with a catalytic amount of nicotinamide cofactor. The reaction goes to completion overnight, and the measurement of a competitive kinetic isotope effect on the enzymatic oxidation of the purified product identified no ¹H contamination. This measurement is very sensitive to such isotopic contamination and verified the high level of isotopic and enantiomeric purity yielded by the new synthetic procedure.

Keywords

Benzyl alcohol; isotopic labeling; alcohol dehydrogenase; kinetic isotope effects

Isotopically labeled compounds can be profoundly useful in determining the stereospecificity of enzymes and resolving metabolic pathways, where the isotope is followed in downstream products and secreted metabolites. Additionally, such compounds can help to elucidate the chemical mechanism of enzymes and other organic reactions, as well as to decipher the physical processes involved in the activation of specific bonds. A very common technique in enzymology, for example, is the measurement of kinetic isotope effects (KIEs), which assess the ratio of rates for reactions that differ only in isotopic composition (i.e., isotopologues):

$$KIE = \frac{k_L}{k_H} \quad [1]$$

where k_L and k_H are the rates with the light and heavy isotopes, respectively. KIEs can report on the extent to which a particular step in a reaction mechanism is rate limiting, the structures of transition states, the role of nuclear quantum mechanical effects on a reactive trajectory, and even the effects of enzyme dynamics on the catalyzed chemistry.^{1–4}

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Applications of specific relevance to the currently reported compound are studies of the enzyme alcohol dehydrogenase (ADH) using KIEs.^{1, 3, 5} The reaction of ADH entails the oxidation of an alcohol using nicotinamide adenine dinucleotide (NAD⁺) as a cofactor. Study of this system has led to tremendous advances in the understanding of hydrogen transfer processes by enzymes, but has been slowed since 1999 by the unavailability of stereospecifically-labeled benzyl alcohols, which are necessary to test certain theoretical predictions.⁶⁻⁸

Measuring hydrogen KIEs with tritium (i.e., $k_{\text{hydrogen}}/k_{\text{tritium}}$) leads to the largest—and thus least ambiguous—values of all KIEs. To use manageable levels of radioactivity, such measurements require a competitive method, where the two isotopologues are mixed together with only a trace amount of tritium, and they compete for the enzyme's active site.⁹ The most common way to analyze the results of a competitive experiment is to track the fractionation of the two isotopologues using different radioactive labels. For hydrogen KIEs, the heavy isotopologue is often the ³H-labeled substrate so only the light substrate, which has either ¹H or ²H at the position of interest, requires a remote ¹⁴C label. Most competitive KIE experiments, therefore, require doubly labeled (¹⁴C and ²H) substrates. Many KIE experiments in ADH, for example, have taken advantage of this kind of complex labeling scheme.^{7, 10-13} Those experiments, however, were limited by the lack of stereospecific labeling, which prohibited directly testing primary (1°) or secondary (2°) isotope effects without the interference of the hydrogen isotope at the geminal position. Recently, for example, it became apparent that two theoretical explanations for 1°–2° coupled motion⁶ could be resolved by measuring the effect of isotopic substitution at the 1° position on the 2° KIE in yeast ADH (yADH); that is, measurement of the 2° H/T KIE with either H or D as the transferred isotope.¹⁴ The D-transfer reaction requires that the ¹⁴C-labeled material be stereospecifically labeled with ²H at the 1° position (pro-*R*) and ¹H at the 2° position (pro-*S*). In particular, the measurement requires 7*R*-[²H]-phenyl-[¹⁴C]-benzyl alcohol with an isotopic- and enantio-purity greater than 99.5%.

Figure 1A shows the overall synthetic approach we report here. The synthesis coupled two enzymatic reactions in the same reaction mixture. The first reaction, catalyzed by formate dehydrogenase (FDH) was the transfer of ²H[−] from formate-d to NAD⁺, producing 4*R*-NADD. The second reaction, catalyzed by horse ADH (hADH), was the transfer of the *R*-²H[−] from NADD to the *re*-face of phenyl[¹⁴C]-benzaldehyde, yielding the desired 7*R*-[²H]-phenyl-[¹⁴C]-benzyl alcohol. The NAD⁺ cofactor was only present in catalytic amounts as it was recycled by the two enzymes, and the only source of reducing hydride equivalents was the formate-d (99.8 %D). The 500 μl reaction contained 100 mM phosphate (pH 7.5), 140 mM DCO₂Na, 10 mM phenyl[¹⁴C]-Benzaldehyde (2 mCi/mmol), and 0.1 mM NAD⁺. All materials were from Sigma, except the phenyl[¹⁴C]-Benzaldehyde, which was from ViTrax. The reaction was initiated by the simultaneous addition of pre-mixed FDH from *Candida boidinii* and hADH such that the final concentration of both enzymes was approximately 1 U/ml. The reaction was incubated at room temperature and reached complete conversion to products (i.e. quantitative conversion of benzaldehyde to benzyl alcohol) overnight, as evidenced by HPLC analysis (Figure 1, B and C).

The ^2H -content of the only source of reducing hydrides (formate-d) was 99.8% (actual lot analysis) and the enzymatic reactions are expected to be highly stereospecific. To be sure, though, we tested the isotopic and enantiomeric purity of the synthesized material by using it in a competitive KIE experiment, which is very sensitive to isotopic contamination (even 0.5% of ^1H -contamination at the pro-*R* position would lead to a decreasing trend in KIE as a function of fractional conversion to products).⁹ We co-purified the material with the other isotopologue in our experiment (racemic 7[^2H , ^3H]-benzyl alcohol) by HPLC on a C-18 column (5×250 mm) using 88:12 H_2O :MeCN at 1 ml/min. We then added this copurified material to a final concentration of 1 mM in a reaction containing 80 mM glycine (pH 8.5), 300 mM semicarbazide, 10 mM NAD^+ , and 1 mg/ml yADH isozyme 2 at 25° C. These conditions reflect the conditions used by previous competitive KIE experiments on yADH.^{7, 10, 13} At a range of fractional conversions, we quenched 100 μl aliquots of the reaction by the addition of 10 μl saturated HgCl_2 . We then separated the reactants from products by HPLC using 76:12:12 H_2O :MeOH:MeCN at 1 ml/min and analyzed the ^3H and ^{14}C contents of the fractions by liquid scintillation counting. From this, we calculated the KIE as a function of fractional conversion to products as¹⁵

$$KIE = \frac{\log(1-f)}{\log(1-f * \frac{R_p}{R_\infty})} \quad [2]$$

where R_p and R_∞ are the ratio of ^3H : ^{14}C (heavy to light isotope) in products at a fractional conversion, f , and when the reaction reaches completion, respectively.

If the ^{14}C -labeled material had more than 0.5 % isotopic contamination (due either to overall isotopic impurity or due to enantiomerically mislabeled material), a trend in the observed KIE as a function of fractional conversion would have been detectable within experimental error.⁹ For example, if instead of pure ^2H at the 7 pro-*R* position, the material contained more than 0.5 % ^1H , that contaminant would react much faster than the properly ^2H -labeled material due to a 1° KIE of around 3.5.¹⁶ Thus, the observed KIE at low fractional conversion would be significantly inflated, but would approach the “real” KIE at high fractional conversion, after the light (or enantiomerically mislabeled) contaminant has been consumed. Our reaction shows no decreasing trend (within experimental error) in the KIE as a function of fractional conversion and the magnitude of the KIE is within the expected range for this reaction^{7, 8} (Figure 1D), providing strong evidence that the synthesized material is isotopically and enantiomerically pure. We note as a word of caution that initial attempts to synthesize the material using the same procedure, but with yADH instead of hADH, led to significant ^1H -contamination at the 7 pro-*R* position. While the reason for this ^1H -contamination is not clear, as the only reducing equivalent in the system is the ^2H from DCO_2Na , the analysis of the contaminated product confirmed the usefulness of the above analysis.

In summary, we present here a synthetic route to 7*R*-[^2H]-phenyl-[^{14}C]-benzyl alcohol, which may have a number of applications in studying the mechanism of ADH, and could find use in synthetic routes to other materials that are useful for a variety of biochemical

applications. Additionally, the coupled synthetic system, which uses a NAD⁺ cofactor in catalytic amounts, could easily be adapted to other synthetic procedures.

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Figure 1.

(A) Synthesis of 7R-[²H]-phenyl-[¹⁴C]-benzyl alcohol. (B) HPLC chromatogram of the starting material for the synthesis, measured in radioactivity counts per minutes (CPM). (C) HPLC chromatogram of the synthetic reaction mixture after overnight incubation. The HPLC method was 75:25 H₂O:MeCN at 1 ml/min on a C-18 column. Commercially available standards (panel D) showed that the larger peak in panel B is benzaldehyde and the larger peak in panel C is benzyl alcohol. The smaller peak in both chromatograms, which does not change during the course of the reaction, is likely to be benzoic acid that formed from the oxidation of the benzaldehyde starting material prior to using it in this synthesis. The starting material, purchased from ViTrax, normally comes packaged in toluene, which apparently provides stability against oxidation. In order to use the material in this enzymatic synthesis, we requested that it be packaged in water, which the manufacturer had not tested for stability. Regardless, this contaminant did not affect the synthesis and was easily removed from the product by HPLC purification. (D) HPLC chromatogram of commercial standards of benzyl alcohol and benzaldehyde mixed in approximately 1:1 ratio, measured in

UV absorbance at $\lambda=263$ nm. A small peak of acid is also visible, which is common to commercial benzaldehyde. Note that apparent retention times differ slightly between the peaks here and those in panels B and C because the UV sensor is attached ahead of the radioactivity sensor. (E) KIE as a function of fractional conversion (f) for $0.2 < f < 0.8$, where the reactants and products both have sufficient quantities of ^3H and ^{14}C to accurately assess the KIE. The lack of a declining trend provides strong evidence of the isotopic and enantiomeric purity of the synthesized materials.