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Synthesis of linoleic acids combinatorially labeled at the vinylic positions as substrates for lipoxygenases

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Abstract

Mammalian lipoxygenases have been implicated in a number of inflammation-related human diseases. Soybean lipoxygenase-1 is the archetypical example of known lipoxygenases. Here we report the synthesis of linoleic acid and (11,11)-d2-linoleic acid which are combinatorially labeled at the vinylic positions (9, 10, 12, and 13). Combinatorial labeling schemes allow for the simultaneous determination of KIEs in enzymatic reactions using NMR. Substrates are, thus, available as probes of detailed mechanism in kinetic isotope effect (KIE) studies of lipoxygenases.

Human lipoxygenases which catalyze the oxidation of arachidonic acid have been implicated in a number of diseases, including asthma,[1a] atherosclerosis,[1b] colorectal cancer,[1c] prostate cancer,[1d] and arthritis.[1a] Because these enzymes catalyze the same basic reaction as plant lipoxygenases, mechanistic studies of the easily obtainable soybean lipoxygenase-1 (SLO-1) are of importance to the design of mechanistic inhibitors.[2] Soybean Lipoxygenase-1 (SLO-1) catalyzes the oxidation of linoleic acid (**1**) to 13-(*S*)-hydroperoxy-9,11-(*Z*,*E*)-octadecadienoic acid (13-(*S*)-HPOD) (Fig. 1). This conversion proceeds by the initial rate-determining abstraction of the *pro-S* hydrogen atom from the methylene at C11. Following this, in an ordered fashion, is the combination of molecular oxygen with the intermediate radical.[3] Transfer of an electron and proton from the active site to the resulting peroxyl radical (**3**) regenerates the active Fe(III) form of the enzyme and results in product (**4**). The initial rate-determining step leads to the formation of a radical which may be in one of three configurations. Energetics favor the formation of

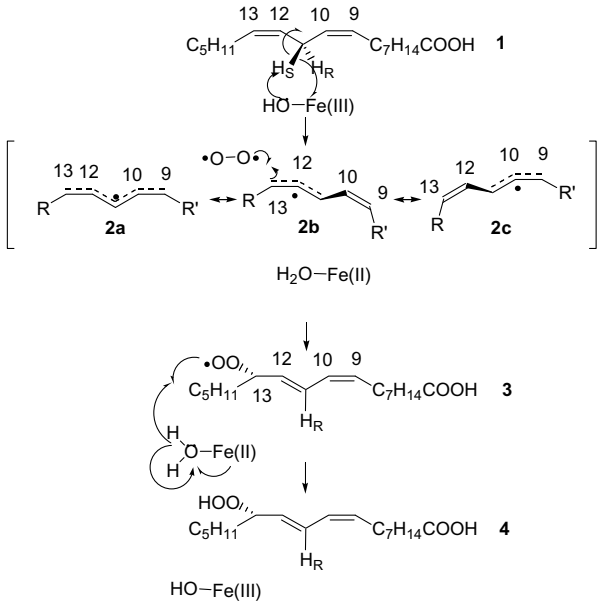

Fig. 1. Consensus mechanism for oxidation of linoleic acid catalyzed by SLO-1.

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the fully delocalized pentadienyl radical (**2a**). The regiospecificity of the oxygen combination with the radical has led to the proposal of an allyl radical delocalized over positions 11–13 (**2b**). Finally, data collected from EPR experiments suggest a radical delocalized over positions 9–11 (**2c**).[4] Work by Knapp et al. indicates that the protein controls the regio- and stereospecificity of O_2 insertion, suggesting that any of the three proposed intermediates are possible.[5]

Secondary deuterium kinetic isotope effects (KIEs) can be potent probes of structural changes that occur in isotope-sensitive reaction steps.[6] However, given the generally small magnitude of these isotope effects, it is useful to perform the experiments competitively. Competitive isotope effects can be carried out in three basic ways. The remote label method has been widely used in determining secondary KIEs in enzymatic systems.[7] This method requires the synthesis of molecules where tritium is specifically incorporated into the remote position at tracer levels and reports the presence of a deuterium in the position of interest. While this approach is very accurate, impurities containing the tritium label can adversely affect the result of the measurement. Isotope ratio mass spectrometry can be used to accurately measure isotope effects if the reactant or product being analyzed can be selectively converted into a volatile molecule, where the isotopic site of interest in the original analyte is present.[8] This technique is usually applied to investigations of heavy atom isotope effects (C, O, and N). A third possibility involves the use of NMR-active nuclei as isotopic probes.[9] Although NMR is not as quantitatively accurate as scintillation counting or isotope ratio mass spectrometry, several isotope effects can be determined simultaneously. One reason for this approach to have only seen a wide application in small molecule organic reactions is the large amounts of sample needed for accurate analysis of isotopomers at natural abundance. This constraint can be circumvented through combinatorial labeling schemes, where only positions of interest in the substrate are labeled (Fig. 2).

The synthetic approach to combinatorially labeled linoleic acids taken here is optimally suited to the measurement of multiple secondary deuterium: the incorporation of <5% deuterium at positions 8, 9 and 11, 12 allows optimization of NMR signals while minimizing any double labeling in these positions.[9] Double labeling is minimized in order to avoid spurious contributions to the measured isotope effects from breakdowns in the rule of the geometric mean.[10] Because the oxidation of linoleic acid catalyzed by lipoxygenases is thought to occur largely by a tunneling mechanism, it is instructive to measure secondary deuterium isotope effects as a function of the tunneling entity. For this reason, combinatorially labeled linoleic acids were synthesized separately with protium (**1a**) and deuterium (**1b**) at the methylene central to the pentadiene moiety.

The tail fragments (**7a** and **7b**) of these targets were synthesized in high yield from the commercially available methyl 2-octynoate (Scheme 1). The reduction[11] step typically resulted in crude yields in excess of 95%; whereas, the bromination[12] step was essentially quantitative. The reduction of propargylic alcohols with $LiAlH_4$ is known to give a (*Z*)-allylic alcohol.[13] Some of this undesired product (typically less than 1% for $LiAlD_4$ and 2–10% for $LiAlH_4$) was formed from over-reduction and was removed by reacting the crude product with *m*-chloroperoxybenzoic acid in chloroform.[14] The resulting epoxide formed from the allylic alcohol was then separated from the propargylic alcohol using flash chromatography (see Supplementary data). The presence of the undesired (*Z*)-allylic alcohol can be minimized from the outset by using a smaller excess of $LiAlL_4$ and by adding the ester starting material slowly to the $LiAlL_4$/diethyl ether slurry.

Because no labeling is performed on the head fragment (**11**) of the target molecules prior to the alkyne coupling step, the synthesis of these fragments utilized inexpensive starting materials and lower-yield reactions that were performed on a larger scale (Scheme 2). Synthesis of 9-decyn-1-ol (**10**) can be performed on technical grade 9-decen-1-ol since contaminating isomers with internal double bonds will not yield terminal alkynes at the end of the synthesis and will not undergo coupling. The efficiency of this scheme lies in the purification steps. The 9-decyn-1-ol (**10**) is distilled away from brominated contaminants. No purification step is undertaken immediately following bromination. The Jones oxidation results in the desired 9-decynoic acid with an expected ester condensation product, 9′-decynyl 9-decynoate.[15] The methylation

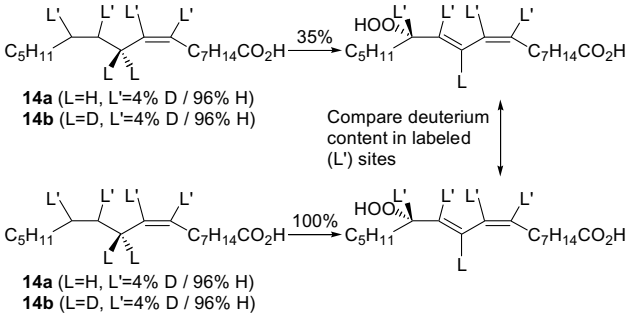
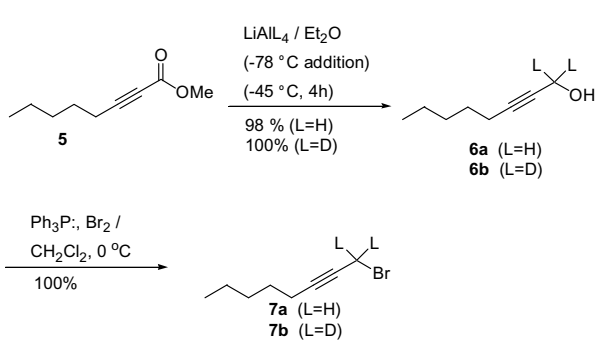
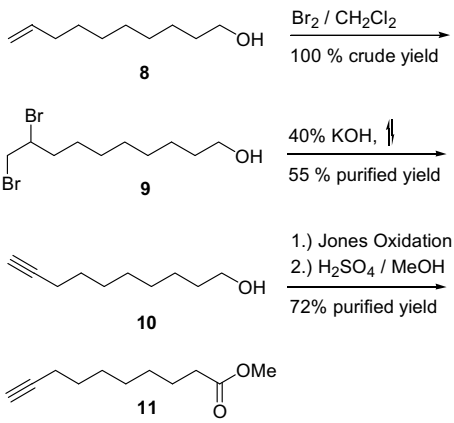


Fig. 2. Conceptual depiction of secondary kinetic isotope effect experiments for which **1a** and **1b** were synthesized.



Scheme 1. Synthesis of fragment **7**.



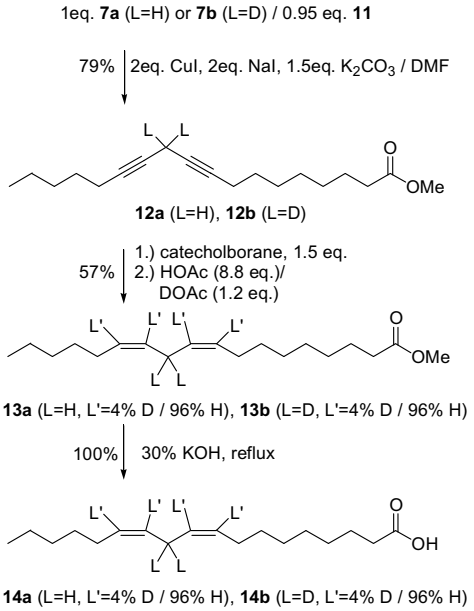
Scheme 2. Synthesis of fragment **11**.

step following oxidation allows one to reclaim this byproduct as the methyl ester via transesterification. The product, 9-decynoate (**11**), is then purified by flash chromatography over silica (see Supplementary data).

The coupling[16,17] and reduction steps[18] (Scheme 3) which conclude this synthesis illustrate its advantages over other similar syntheses. The most likely alternative to a simple Cu(I) coupling is a Grignard-mediated Cu(I) coupling.[19–21] The primary advantage of the coupling reaction used here is the tolerance of groups that are labile in the presence of Grignard reagents. While this method has been particularly useful for some couplings, an attempt to couple 9-decynoic acid with **7a** led to very small yields of the desired product.

The reduction step, using catecholborane, offers a number of benefits in this synthesis. Reduction of alkynes to (*Z*)-alkenes is most commonly performed using Lindlar's catalyst. If one is using a reduction method to label vinylic positions, this method poses two obstacles. First, adsorbed hydrogen on the platinum surface must be displaced by the label or mixture of label and carrier if the degree of labeling is to be controlled. Second, metal-mediated reductions tend to exchange hydrogens at allylic positions.[22] If this technique were used to make substrates for isotope effect measurements at the vinylic positions, it could lead to two situations whereby isotope effects would be incorrectly measured. If label is mistakenly incorporated at a site that has an intrinsically larger normal isotope effect (i.e., the *pro-S*-11-position), the isotope effect on the vinylic positions would be overestimated. Likewise, mistaken incorporation of label at positions with inherently lower normal or inverse isotope effects would lead to underestimation of the isotope effect at the vinylic positions. This problem is particularly troublesome in isotope effects measured using scintillation counting and mass spectrometry.

Additional advantages of using catecholborane follow. First, one can ensure full reduction of the alkyne to the alkene by using 1.5 or more equivalents of catecholborane.[18] Second, over-reduction does not occur since the resulting alkenylborane is electron poor and sterically hindered. These aspects of the catecholborane reduction are quite critical, since separation of compounds that differ only in one element of unsaturation would require an HPLC separation step, which severely limits the amount of material that can be purified. For the combinatorial labeling undertaken in this synthesis, the label was incorporated using a mixture of acetic acid and readily available deuterated acetic acid for acetolysis of the alkenylborane. In order to achieve approximately 4% labeling, the deuterated acetic acid had to be present at 20% in the acetolysis mixture, implying a primary isotope effect of about 5 for acetolysis of internal alkenylcatecholboranes.



Scheme 3. Coupling of fragments **7** and **11**; subsequent reduction of diyne.

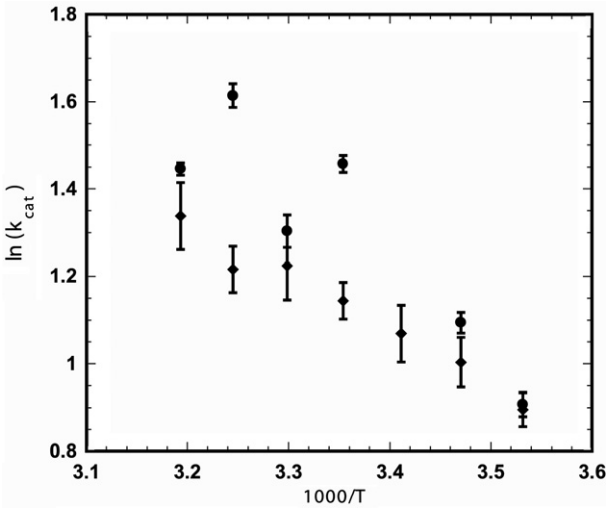


Fig. 3. Comparison of the temperature dependence for k_{cat} for SLO-1 catalyzed oxidation of d31-linoleic acid (filled circles) and **14b** (filled diamonds).

To confirm the viability of the newly synthesized combinatorially labeled linoleic acid, **14b**, the temperature dependence of the unimolecular rate constant, k_{cat}, was tested against data obtained with perdeuteriolabeled d31-linoleic acid for SLO-1. Figure 3 shows that the unimolecular rate constant has a similar temperature dependence for both substrates, an indication that synthesis-dependent impurities are not influencing the enzyme-catalyzed oxidation of the synthesized substrate. If any differences are to be noted for the substrates, it would be that the d31-labeled substrate has a higher rate constant on average. This may be due to incomplete deuterium incorporation, since this substrate was isolated from a perdeuterated set of fatty acids isolated from plants grown on deuterated sources.

The synthesis included in this letter provides a means by which alkenes may be combinatorially labeled for use in the simultaneous determination of isotope effects. Aside from the utility of the target molecules in enzyme studies, the synthesis of molecules containing (*Z*,*Z*)-pentadienyl moieties has been improved upon.

Acknowledgments

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Supplementary data

Experimental procedures and spectral characterization (^{1}H NMR) of all synthetic intermediates and end products. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.04.023.

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