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Synthesis of Site-Specifically ^{13}C Labeled Linoleic Acids

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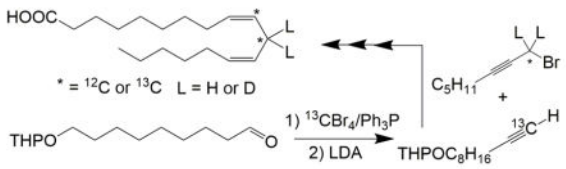
Abstract

Soybean lipoxygenase-1 (SLO-1) catalyzes the C-H abstraction from the reactive carbon (C-11) in linoleic acid as the first and rate-determining step in the formation of alkylhydroperoxides. While previous labeling strategies have focused on deuterium labeling to ascertain the primary and secondary kinetic isotope effects for this reaction, there is an emerging interest and need for selectively enriched ^{13}C isotopologues. In this report, we present synthetic strategies for site-specific ^{13}C labeled linoleic acid substrates. We take advantage of a Corey-Fuchs formyl to terminal ^{13}C-labeled alkyne conversion, using $^{13}CBr_4$ as the labeling source, to reduce the number of steps from a previous fatty acid ^{13}C synthetic labeling approach. The labeled linoleic acid substrates are useful as nuclear tunneling markers and for extracting active site geometries of the enzyme-substrate complex in lipoxygenase.

Graphical Abstract



Keywords

Secondary isotope effects; hydrogen tunneling; lipoxygenase; linoleic acid

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Supporting Information available. Experimental procedures and NMR characterization of synthetic compounds.

Lipoxygenases, widely represented in animals, plants, fungi and prokaryotes, have gained much attention in the field due to observations of enormous, non-classical primary deuterium kinetic isotope effects (KIEs) in the range of 20–120 for the native enzymes.[1] These enzymes catalyze the C-H abstraction from long chain polyunsaturated fatty acids in the process of preparing diverse (per)oxidized metabolites, important for growth, development and pathogenic defense in plants and various cell signaling and inflammatory response pathways in animals.[2] Importantly, soybean lipoxygenase-1 (SLO-1) has emerged as one of the paradigmatic enzymes in hydrogen tunneling, because the rate-determining hydrogen atom abstraction in the native form is associated with a very large primary KIEs at room temperature (*ca.* 80), in a nearly temperature independent manner (ΔE_a = 0.9 kcal/mol).[3] As shown in Figure 1, for the SLO-1 reaction, a mononuclear, nonheme ferric hydroxide accepts the transferred electron and proton, respectively, generating a ferrous water cofactor and a pentadienyl radical. Molecular oxygen is then inserted into the backbone, selectively at position 13 for wild-type SLO-1.[1a, 4] A second round of proton-coupled electron transfer (PCET) regenerates the active ferric-hydroxide cofactor and yields the product, *13S*-hydroperoxyoctadecadienoic acid. Also shown in Figure 1 are two amino acid sidechains, Leu546 and Leu754, which position the reactive carbon in proximity to the ferric-hydroxide reactive center and are integral for efficient hydrogen wave function overlap in SLO-1.[5]

Inflated, non-classical primary deuterium isotope effects (k_H/k_D > 7), together with deviations in Swain-Schaad relationships (especially for secondary D/T KIEs) and temperature independent KIEs, are considered as kinetic signatures for full tunneling in enzymatic reactions occurring at room temperature.[6] In the case of lipoxygenases, many synthetic strategies have been reported for site-specific deuterium labeled substrates, but there has also been an emerging interest in ^{13}C labels for these compounds. While the tunneling properties have been ascribed to the transferred hydrogen, recent evidence in SLO-1 reaction points towards the tunneling of heavy atoms in the backbone of the substrate.[7] In that study, the ^{13}C KIEs were measured from natural abundance ^{13}C using an NMR approach designed for small molecules by Singleton.[8] To overcome the relatively large errors associated with these measurements for enzymatic reactions, a high precision ^{1}H-detected ^{13}C heteronuclear single quantum coherence (HSQC) 2 dimensional (2D) NMR approach has been previously reported for the determination of such ^{13}C KIEs. [9] This technique requires the site-specific labeling of the carbon atom of interest and a carbon atom, adjacent to the target carbon atom so that they are spin-coupled, as a reporter.[10] While 1 dimensional (1D) ^{13}C NMR techniques are typically easier and faster and can be utilized to test these competitive isotope effects,[10] the HSQC approach[9] may offer enhanced signal-to-noise, which is particularly advantageous in the lipoxygenase reaction that is limited to low (micromolar) substrate concentrations. In addition, site-specific ^{13}C labeling of arachidonic acid has previously served to assign the magnetic resonance spectral contributions of the delocalized radicals, generated upon reaction with prostaglandin H synthase.[11] While arachidonic acid is the preferred substrate of mammalian lipoxygenases[2a] and exhibits rate constants and deuterium KIE for the reaction with SLO-1 that is comparable to linoleic acid, the latter is considered the physiological substrate for the plant enzyme.[12] This manuscript describes synthetic strategies for generating site-specific ^{13}C

enriched linoleic acids. These labeled substrates can be applied to evaluate the extent of tunneling in the substrate backbone from ^{13}C KIEs by 1D ^{13}C or 2D HSQC NMR techniques, to assign magnetic resonance signatures of the substrate radicals in the lipoxygenase reaction, and/or to resolve the elusive donor-acceptor distances in various lipoxygenase enzymes using magnetic resonance techniques sensitive to electron-nuclear couplings.[13]

Our first target is the isotopologue with ^{13}C enriched at the reactive carbon, C-11, of linoleic acid (11-[^{13}C]-**1**), which, as shown in Scheme 1, was synthesized in two parts with an unlabeled fragment, 2-(dec-9-yn-1-yloxy)tetrahydro-2*H*-pyran (**3**) and a ^{13}C labeled fragment, 1-bromo-2-octyne (1-[^{13}C]-**4**). As shown for example in Scheme 1, the two fragments **3** and **4** were joined via a Cu(I)-assisted Grignard coupling reaction. A similar strategy was previously used in our laboratory with 1,1-[$^{2}H_2$]-**4** and 9-decynoic acid that produced low yields of the desired, stable intermediate in the synthesis of monodeuterated or dideuterated linoleic acids.[14] To overcome these previous pitfalls, we started with commercially available 9-decyn-1-ol (**2**), which we protected through the overnight reaction of 3,4-dihydro-2*H*-pyran (DHP) to generate **3**. In this manner, only 1 *eq.* of the ethylmagnesium bromide (EtMgBr) is required to deprotonate the terminal alkyne, **3**. This strategy produced reasonable yields (*ca.* 70 %) of the desired 11-[^{13}C]-2-(octadeca-9,12-diyn-1-yloxy)tetrahydro-2*H*-pyran, 11-[^{13}C]-**5**. In the course of arachidonic acid synthesis for prostaglandin H synthase substrates, Peng *et al.* followed a similar approach that led to high yields (86%).[11] One caveat is that a fraction of the starting material **3** is virtually inseparable from product 11-[^{13}C]-**5** with silica gel purifications. We maintained the contaminating starting material from the coupling step to the end, because it did not interfere with the final steps. In our final purification step, linoleic acid could be separated cleanly and easily from this impurity using a reverse phase C18 column.

After the coupling that generates 11-[^{13}C]-**3**, the linoleic acid substrate could be completed (Scheme 1) by deprotection of 11-[^{13}C]-**5**, liberating the corresponding alcohol, 11-[^{13}C]-**6,** followed by oxidation to the acid, 11-[^{13}C]-**7**, with Jones reagent and hydrogenation to linoleic acid, 11-[^{13}C]-**1**. The hydrogenation reaction was performed with standard Lindlar catalyst and H_2. Further, our hydrogenation reactions were carried out in presence of quinolones which enhances selectivity for 9*Z*, 12*Z* alkene production.[15] HPLC analyses of the purified final products indicated only linoleic acid (C18:2 $\Delta^{9,12}$) with no detectable amounts of oleic (C18:1 Δ^{9}) or stearic (C18:0) acids, consistent with no over-reduction. ^{1}H NMR spectra were consistent with *cis*,*cis*-9,12 isomer. An alternative approach, described by our group previously,[16] catecholborane can also be used to achieve reduction selectivity.[15]

The ^{13}C labeled 1-bromo-2-octyne fragment, 1-[^{13}C]-**4**, was synthesized as described in Scheme 2. First, 2-octyn-1-ol (**10**) was generated from the deprotonation of the commercially available 1-heptyne **8** with EtMgBr that was subsequently reacted with ^{13}C-paraformaldehyde ([^{13}C]-**9**). This route has been described previously for the synthesis of 1-[^{2}H]-**10** and 1,1-[$^{2}H_2$]-**10**.[14, 17] Bromination of 1-[^{13}C]-**10** compounds to the corresponding desired 1-[^{13}C]-**4** was carried out cleanly with Ph_3P/Br_2.

As depicted in Scheme 3, the linoleic acids, 10-[^{13}C]-11,11-[$^{2}H_2$]-**1** and 10,11-[$^{13}C_2$]-11,11-[$^{2}H_2$]-**1** were also synthesized in two parts, with a common ^{13}C labeled C-1 to C-10 fragment (**2**). The label (^{12}C or ^{13}C) at reactive carbon in the final product, **1**, was controlled by the nature of the carbon isotope in the 1-bromo-2-octyne (**3**) fragment. In addition, deuterium was also included at the reactive carbon (C-11) for both linoleic acids in Scheme 3. Our lab has previously shown that for the WT SLO-1 enzyme chemistry is partially rate limiting with the protium substrate under certain conditions, but becomes fully rate limiting with deuterated substrate.[1a] Therefore, dideuterated substrates would be required to isolate the intrinsic KIE of the reactive carbon from NMR competitive measurements.

The protection of the 1,9-nonanediol (**11**) resulted in a mixture of starting material **11**, desired single protected 9-((tetrahydro-2*H*-pyran-2-yl)oxy)nonan-1-ol (**12**) and fully protected species. Because the starting material **11** was inexpensive and the desired compound **12** was easily separable with silica gel purification, we could prepare this compound on a relatively large scale. As illustrated in Scheme 4, oxidation of the alcohol **12** to the corresponding 9-((tetrahydro-2*H*-pyran-2-yl)oxy)nonan-1-ol (**13**) was carried out with pyridinium chlorochromate (PCC) and sodium acetate to maintain the THP protecting group. The highlight within this synthesis is the preparation of 2-(10-[^{13}C]-dec-9-yn-1-yloxy)tetrahydro-2*H*-pyran (10-[^{13}C]-**3**). This step was accomplished by a formyl-to-ethynyl conversion, first described by Corey and Fuchs,[18] using the aldehyde **13** and triphenylphosine-carbon tetrabromide-^{13}C. The inclusion of 1 *eq.* of anhydrous triethylamine[19] during the addition of the aldehyde **13** to the stirring triphenylphosphine-carbon tetrabromide solution in CH_2Cl_2 was essential to produce the desired 2-((10-[^{13}C]-10,10-dibromodec-9-en-1-yl)oxy)tetrahydro-2*H*-pyran (10-[^{13}C]-**14**). Due to the cost of the ^{13}C isotopologue, we reduced the starting equivalents of ^{13}C-carbontetrabromide from the recommended 2 *eq.*[18–19] to 1 *eq.* in this report. Even with only 1 *eq.* of $^{13}CBr_4$ (and 2 *eq.* Ph_3P), we were able to cleanly produce the desired 10-[^{13}C]-**14** in very satisfactory yields (*ca.* 90%).

It has been suggested that alkyne lithium salts couple with appropriate electrophiles in a two-step, one pot synthesis.[19] It could be envisioned that addition of 2 *eq.* of a strong base such as lithium diisopropylamide (LDA) would sequentially transform 10-[^{13}C]-**14** to a lithium alkyne salt. Subsequent addition of **4** would, in principle, act as a proper electrophile and undergo carbon-carbon bond formation, thus generating the desired 9,12-diyne. However, attempts to *directly* couple the lithium alkyne salt, generated *in situ*, with natural abundance 1-bromo-2-octyne (**4**) were not successful and upon work up gave only the alkyne 10-[^{13}C]-**3**. We therefore reacted the dibromoalkene, 10-[^{13}C]-**14**, with excess LDA at −70°C to ensure conversion to the lithium alkyne salt, which was warmed to room temperature and quenched to isolate the desired 2-(10-[^{13}C]-dec-9-yn-1-yloxy)tetrahydro-2*H*-pyran (10-[^{13}C]-**3**) in good yield (90%; Scheme 4).

For the synthesis of 1,1-[$^{2}H_2$]-**10** and 1-[^{13}C]-1,1-[$^{2}H_2$]-**10**, the ^{2}H and/or ^{13}C isotopologues were introduced onto 1-heptyne **8** using paraformaldehyde-d_2 and paraformaldehyde-^{13}C-d_2, respectively, forming the corresponding labeled 2-octyn-1-ol (1,1-[$^{2}H_2$]-**10** and 1-[^{13}C]-1,1-[$^{2}H_2$]-**10**). The route is comparable to that described for the synthesis of 1-[^{13}C]-**10** in

Scheme 2. Paraformaldehyde allows us to add not only ^{13}C at the reactive carbon, but also deuterium. The compound 1,1-[$^{2}H_2$]-**10** has also been prepared previously via reduction of the commercially available methyl 2-octynoate with $LiAlD_4$.[16] Reduction of 2-octynoate with $LiAlD_4$ produces slightly better yields of the 1,1-[$^{2}H_2$]-2-octyn-1-ol, but requires either additional purification or reaction steps to remove unwanted side products. For the synthesis of the deuterium and ^{13}C labeled 2-octyn-1-ol, the most direct route is through the use of paraformaldehyde-d_2-^{13}C, as outlined here. As illustrated in Scheme 2, bromination of these 2-octyn-1-ol (**10**) labels generated the respective labeled 1-bromo-2-octyne (**4**). The coupling and final steps for these labeled compounds are identical to those described above in Scheme 1 for 11-[^{13}C]-**1** and gave similar yields and purities for final products.

Unimolecular rate (k_{cat}) and Michaelis (K_M) constants were measured from the reaction of the linoleic acids synthesized here with WT SLO-1 at 30°C (see Table 1). Michaelis-Menton saturation curves were plotted from measuring the rate of product formation, monitored spectrophotometrically from the absorbance at 234 nm,[20] as a function of substrate concentration (2–80 μM). The kinetic parameters of 10-[^{13}C]-11,11-[$^{2}H_2$]-**1** and 10,11-[$^{13}C_2$]-11,11-[$^{2}H_2$]-**1** are compared to the commercially available perdeuterated substrate, extracted from alagal cells.[21] It is important to note that the k_{cat} for the perdeuterated substrate (k_{cat} = 5.7 s^{-1}) is taken from a recent report[22] and the apparent slight elevation of this value in reference to the k_{cat} magnitudes for the deuterated, carbon-13 labeled substrates, reported here (Table 1), is well within the typical variation from different enzyme preparations. Importantly, the K_M values for all substrates are identical. These data are validations to the synthesized compounds, 10-[^{13}C]-11,11-[$^{2}H_2$]-**1** and 10,11-[$^{13}C_2$]-11,11-[$^{2}H_2$]-**1**, as *bona fide* lipoxygenase substrates.

While there are several reports that describe routes to synthesize lipoxygenase substrates (linoleic and arachidonic acids) that are enriched with deuterons at various positions along the backbone (see for examples, refs [11, 14, 16–17, 23]), this report describes the strategy to label backbone carbons site-selectively in linoleic acid. An alternative approach has been reported for the site-specific ^{13}C labeling of arachidonic acids for substrates of PGHS and lipoxygenase. [11] In that report, Peng *et al.* used potassium ^{13}C-cyanide to add the ^{13}C label, primarily due to its comparatively low cost. Here, we chose to use the Corey-Fuchs aldehyde-alkyne conversion with ^{13}C-carbon tetrabromide as one source for the ^{13}C label, because this approach, although using a slightly more expensive starting material, led to a shorter route (11 steps vs 17 steps in ref [11]) for the final product.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- Strategies for site-specific ^{13}C isotope labeling of fatty acids
- Cu(I)-mediated cross coupling reactions
- Application of Corey-Fuchs aldehyde-alkyne conversion for selective ^{13}C insertion

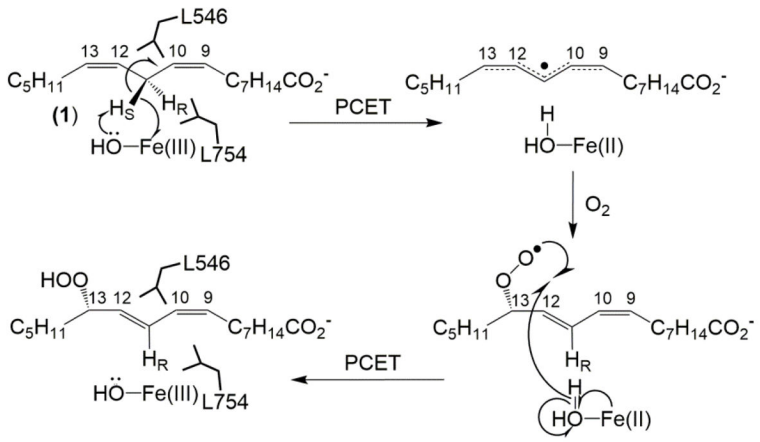
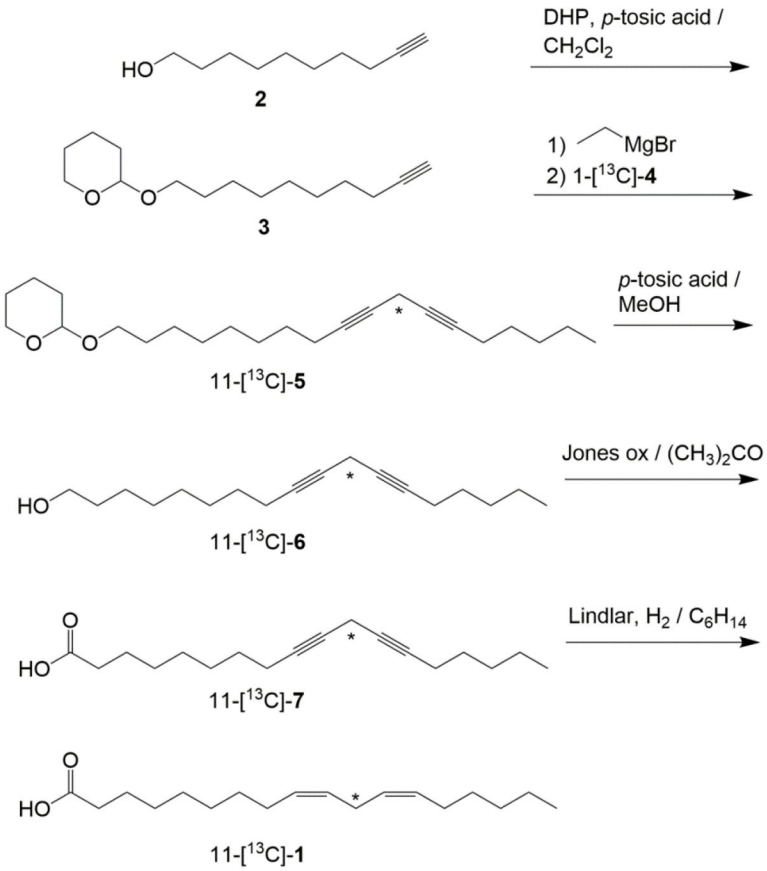


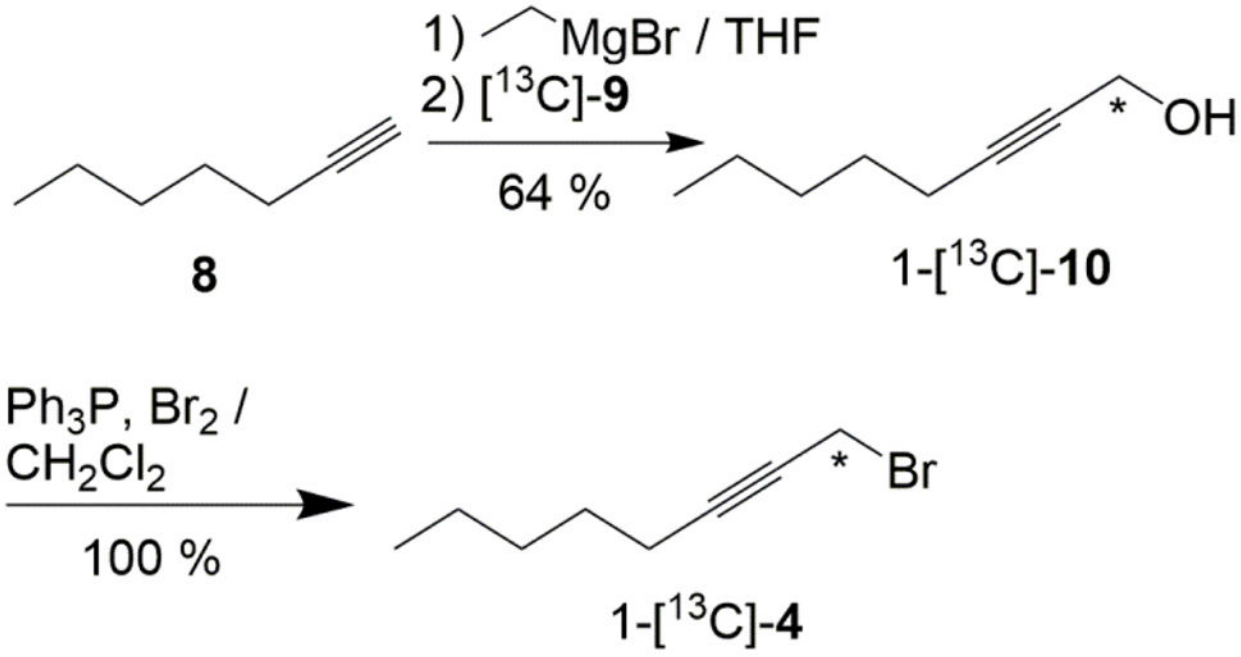
Figure 1.

Mechanism of linoleic acid peroxidation by soybean lipoxygenase-1. The first and final steps of the reaction are associated with proton-coupled electron transfer (PCET) processes. The important, conserved aliphatic residues (Leu546 and Leu754) that position the reactive carbon (C-11 of linoleic acid) against the active site ferric hydroxide cofactor in SLO-1 are modeled for reference.



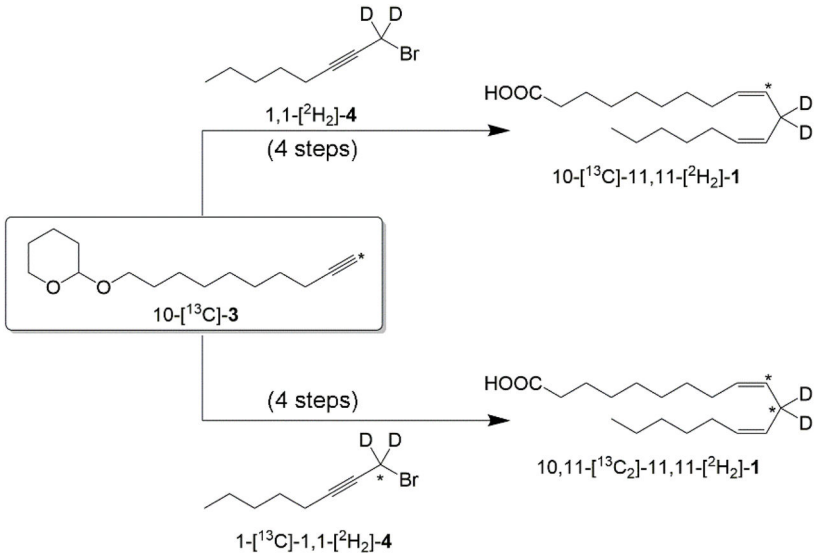
Scheme 1.

Synthesis of 11-[^{13}C] -**1**. The asterisks denote the position of the ^{13}C label.



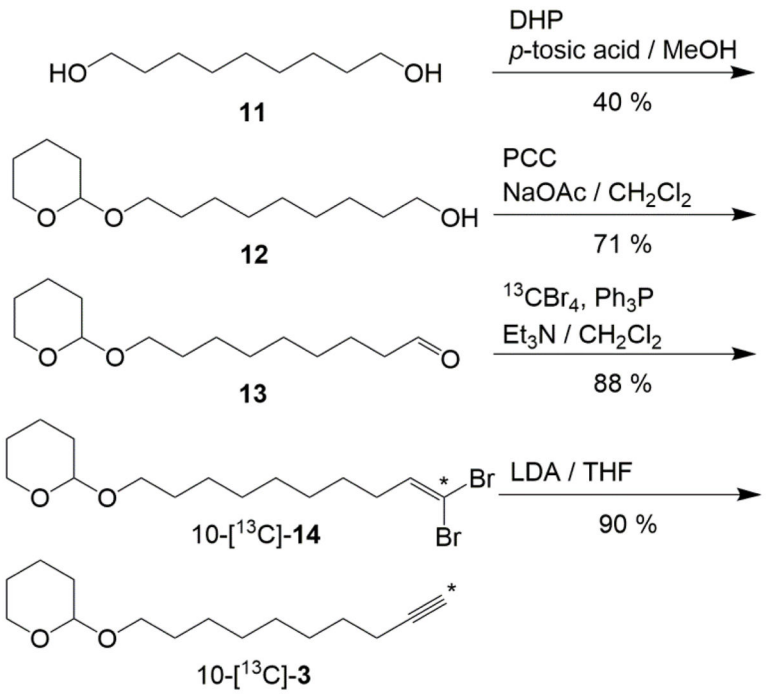
Scheme 2.

Synthesis of fragment 1-[^{13}C]-**4** using ^{13}C-paraformaldehyde, [^{13}C]-**9**. The asterisks denote the position of the ^{13}C label.



Scheme 3.

General strategy outline for ^{2}H, ^{13}C isotope labeling of linoleic acid substrates. The asterisks denote the position of the ^{13}C label. For details about the synthetic steps, refer to Scheme 1.



Scheme 4.
Synthesis of common fragment 10-[^{13}C]-**3**.

Table 1

Kinetic analysis of the ^{2}H, ^{13}C labeled substrates with WT SLO-1.[a]

Linoleic acid	k_{cat} (s^{-1})	K_M (μM)
[$^{2}H_{31}$]-**1**[b]	5.7 (0.1)	7.8 (0.5)
11,11-[$^{2}H_{2}$]-**1**[c]	4.5 (0.2)	7.7 (1.3)
10-[^{13}C]-11,11-[$^{2}H_{2}$]-**1**[d]	4.7 (0.2)	7.8 (1.0)
10,11-$^{13}C_{2}$-11,11-[$^{2}H_{2}$]-**1**[d]	4.5 (0.2)	7.6 (1.0)

[a]Reactions were conducted with 3.5 nM WT SLO, prepared as previously described,[5] at 30°C in 0.1 M borate, pH 9.0 buffer. Substrates were prepared at 1 mM working stocks in 0.1 M sodium borate (pH 9.0) buffer. Prior to use, the concentration of the effective substrate was confirmed enzymatically. Each kinetic data set was conducted with 7 substrate concentrations. For each substrate analyzed, the resulting kinetic parameters are averages from 3 individual data sets.

[b]Substrate is commercially available from Cambridge Isotopes. The first order rate (k_{cat}) is from ref [22]

[c]Synthesized from our lab previously; k_{cat} is consistent with previous report[20].

[d]Synthesized here.