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Synthesis of Isotopically Labeled Arachidonic Acids To Probe the Reaction Mechanism of Prostaglandin H Synthase

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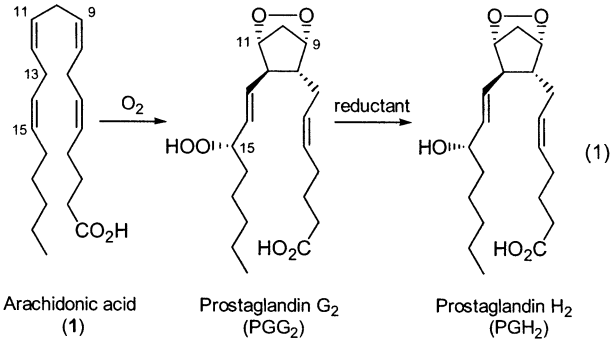
Abstract: Prostaglandin H synthase (PGHS) catalyzes the conversion of arachidonic acid to prostaglandin G_2 in the cyclooxygenase reaction. The first step of the mechanism has been proposed to involve abstraction of the *pro-S* hydrogen atom from C13 to generate a pentadienyl radical spanning C11−C15. We report here the synthesis of six site-specifically deuterated arachidonic acids to investigate the structure of the radical intermediate. The preparation of these compounds was achieved using a divergent scheme that involved one advanced intermediate for all targets. The synthetic design introduced the label late in the routes and allowed the utilization of common synthetic intermediates in the preparation of various targets. Both 13(*R*)- and 13(*S*)-deuterium-labeled arachidonic acids were synthesized in high enantiomeric purity as deduced from soybean lipoxygenase assays and mass spectrometric analysis of the resulting enzymatic products. Each synthetic compound was reacted under anaerobic conditions with the wide singlet tyrosyl radical of PGHS-2 to generate a radical intermediate that was analyzed by EPR. Deuterium substitution at positions 11, 13(*S*), and 15 resulted in the loss of one hyperfine interaction, indicating that the protons at these positions interact with the unpaired electron. Simulation of the spectra was achieved with one set of parameters that are consistent with the assignment of a pentadienyl radical. Use of 16-[$^2H_2$]-arachidonic acid indicated that only one of the protons at C16 gives rise to a strong hyperfine interaction. The findings are discussed in the context of two proposed mechanisms for the cyclooxygenase reaction.

The cyclooxygenase (COX) activity of prostaglandin H synthase (PGHS) catalyzes the first committed step in the biosynthesis of all prostaglandins and thromboxanes, the conversion of arachidonic acid (**1**) into prostaglandin G_2 (PGG_2; eq 1).[1] The peroxidase activity of PGHS catalyzes the subsequent reduction of PGG_2 to PGH_2. Many lipid signaling agents derived from PGH_2 are important modulators of cardiovascular, gastrointestinal, renal, and reproductive function, as well as crucial mediators of inflammation, fever, and allergy. The discovery that two PGHS isozymes exist in mammalian cells, PGHS-1 and PGHS-2,[2] has led to important improvements in nonsteroidal antiinflammatory drugs (NSAIDs).[3] PGHS-1 is a constitutively expressed protein in many tissues, whereas PGHS-2 expression is induced in specific tissues in response to mitogens, cytokines, and growth factors.[4] Most NSAIDs indiscriminately inactivate both COX-1 and COX-2 activities,



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[†] University of Illinois at Urbana-Champaign.
[‡] University of Texas Health Science Center at Houston.

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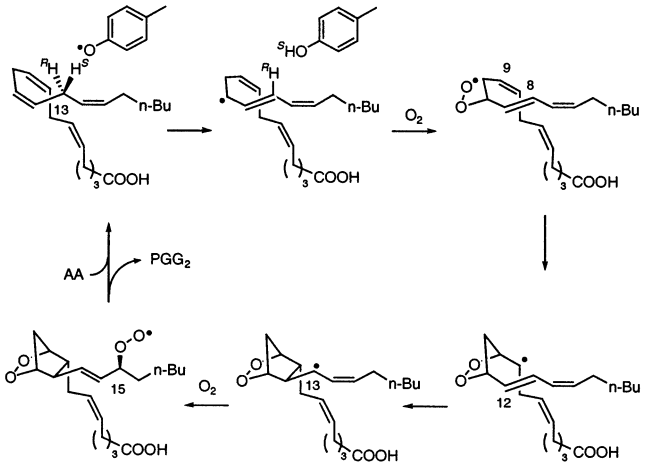
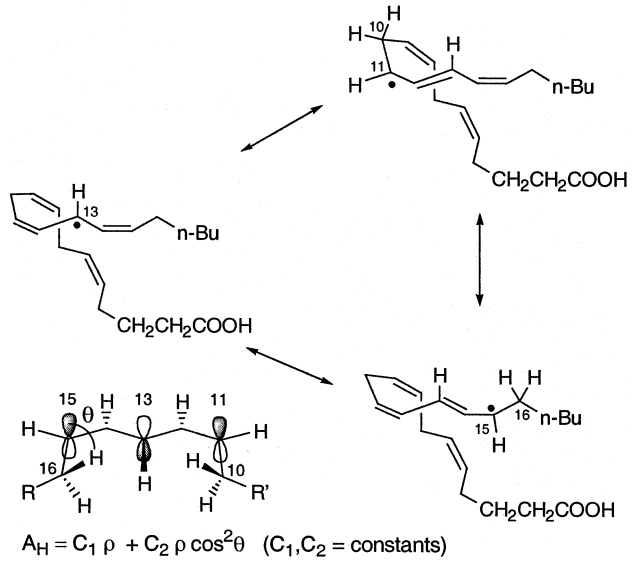
Scheme 1



Scheme 2



but selective inhibition of either isozyme may have important benefits. COX-2 inhibition reduces pain and fever, making the enzyme of great interest to the pharmaceutical industry.[5] Recently developed COX-2 selective inhibitors indeed lack many of the toxic side effects observed with nonselective inhibitors.[6] PGHS-1 is abundant in platelets and endothelium cells, and the product of the COX-1 reaction is a precursor to thromboxane A_2, a potent platelet aggregation factor. It is therefore believed that covalent inhibition of COX-1 via acetylation of an active site serine residue by aspirin contributes to the reduced risk of mortality from cardiovascular disease associated with regular administration of the drug.[7]

The initial mechanistic proposal for the cyclooxygenase reaction by Hamberg and Samuelsson[8] is shown in Scheme 1. Although direct support for many of the intermediates is lacking, a number of important findings since its original formulation in 1967 have supported the basic tenets of the overall mechanism. These include the detection of substrate-based radicals using spin trapping,[9,10] the identification of a tyrosyl radical as the initiator of catalysis,[11] the determination of X-ray structures that contain either substrate or product bound in the active site of the protein,[12,13] and the detection of a substrate-based radical by EPR spectroscopy.[14,15] Tsai and co-workers proposed that this latter radical signal is derived from a pentadienyl radical spanning positions C11−C15 of arachidonic acid.[15] Pentadienyl radicals have an odd-alternate spin distribution, and therefore the proposed radical in PGHS is expected to have spin density mainly at C11, C13, and C15 (Scheme 2). Consequently, the protons at these positions should give rise to large, anisotropic hyperfine splittings. Furthermore, the protons at positions 10 and 16 would also be expected to engage in a magnetic interaction with the unpaired electron, the size of which (A_H) would be dependent on the dihedral angle θ of the C−H bonds and the extended π-system that harbors the electron (Scheme 2). Site specifically deuterated arachidonic acids (**1**) can confirm the assignment of the structure of the radical and can aid in the determination of its conformation, since the presence of deuterium atom(s) should lead to predictable changes in the hyperfine pattern observed in the EPR spectra. In a preliminary report, we have used this strategy to confirm that the observed radical corresponds to a pentadienyl radical.[16] Here we describe the details of the synthesis of 11-[^{2}H]-**1**, 13(*R*)-[^{2}H]-**1**, 15-[^{2}H]-**1**, and 13(*R*),15-[$^2H_2$]-**1** to probe the spin density of the radical, and of 16-[$^2H_2$]-**1** to determine the dihedral angles of the C−H bonds at this position with respect to the singly occupied

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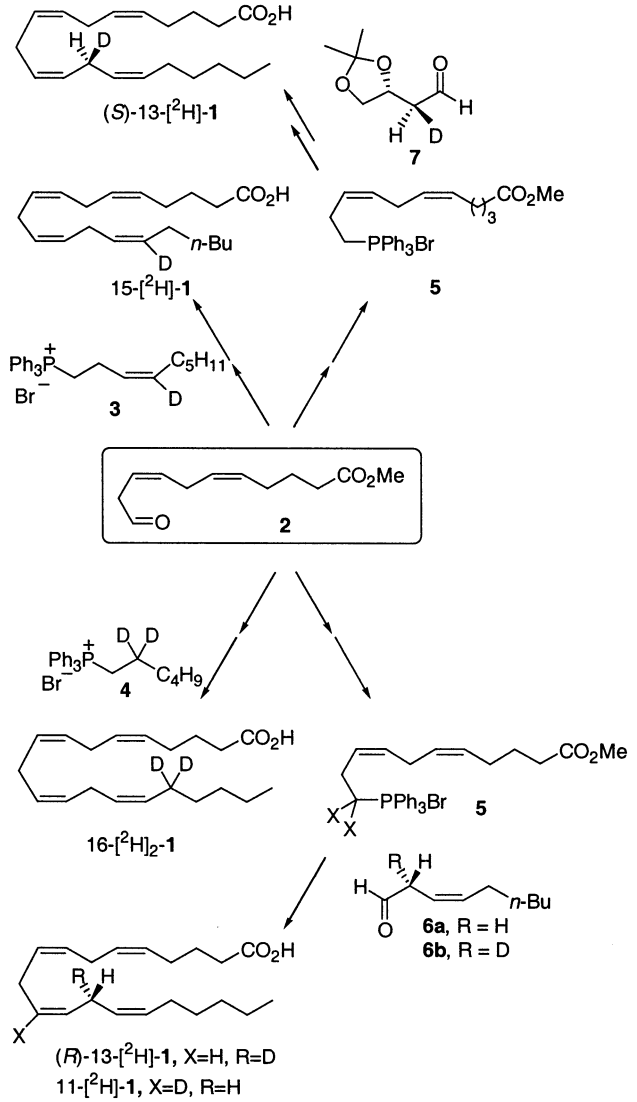
molecular orbital (SOMO). Also described is the preparation of 13(*S*)-[^{2}H]-**1**, a compound that has great value for the study of kinetic isotope effects in both PGHS and lipoxygenases.

Results

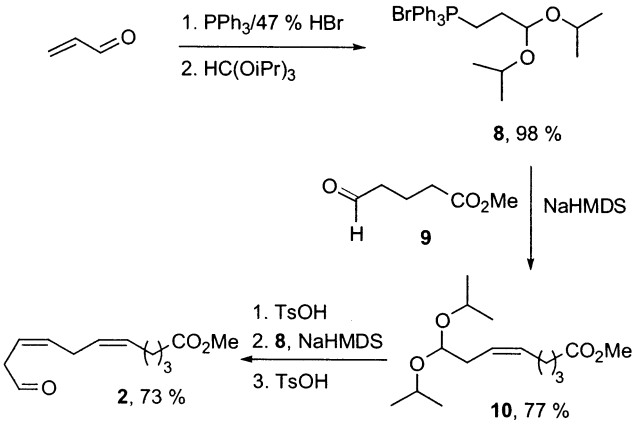
Synthetic Strategy. A number of different synthetic strategies have been reported for the preparation of arachidonic acid, most of which are not easily adaptable to the synthesis of all of the desired isotopically labeled targets.[17,18] Hamberg[19] and more recently Brash and co-workers[20] used in vivo conversion of tritium-labeled stearic acids to arachidonic acid by the fungus *Saprolegnia parasitica*. Whereas this approach is very successful for the preparation of radiolabeled products, it is not amenable to the preparation of deuterium-labeled products in high isotopic purity as a result of significant isotope dilution during the in vivo conversion.[20] Our synthetic routes were designed to introduce the labels at a late stage to reduce cost and minimize the risk of a decrease in isotopic purity. Furthermore, a divergent strategy that allowed preparation of all target compounds from one common advanced intermediate was preferred. We envisioned that aldehyde **2** could be converted into each of the target molecules (Scheme 3). Our routes to 15-[^{2}H]-**1** and 16-[^{2}H$_2$]-**1** required the Wittig reagents **3** and **4**, respectively. For the preparation of 13(*R*)-[^{2}H]-**1**, aldehyde **2** would be converted into phosphonium salt **5** and reacted with stereospecifically labeled aldehyde **6b**. A similar approach with the dideuterated analogue, 1-[^{2}H$_2$]-**5**, and unlabeled **6a** was expected to provide access to 11-[^{2}H]-**1**. Finally, reaction of **5** with stereospecifically deuterated aldehyde **7**, followed by further elaboration of the resulting triene, would give access to 13(*S*)-[^{2}H]-**1**.

The preparation of the common intermediate **2** was accomplished using the three-carbon homologation reagent **8**[21,22] for the construction of both olefinic bonds starting from aldehyde **9**, prepared in two steps from δ-valerolactone (Scheme 4). The Wittig reaction was carried out under salt-free conditions,[23,24] which provided the *Z*-isomer **10** exclusively. Deprotection of the acetal and repeated Wittig homologation produced **2** in 73% yield for three steps. This short synthesis of the advanced intermediate could be performed on a relatively large scale (2.5 g) to provide the common precursor to the target compounds.

Scheme 3



Scheme 4



Synthesis of 15-[^{2}H]- and 16-[^{2}H$_2$]-Arachidonic Acid. The synthetic route to transform **2** into the first two target molecules, 15-[^{2}H]- and 16-[^{2}H$_2$]-**1**, is illustrated in Scheme 5. Methylhexanoate was reduced with lithium aluminum deuteride,[25] followed by oxidation with chromium trioxide in pyridine to

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(22) Reagent **8** has been used previously for the synthesis of **1** (ref 17g), but in that study arachidonic acid was prepared in tail (C20) to head (C1) fashion, which does not allow straightforward introduction of deuterium label in the positions that are important for this study.

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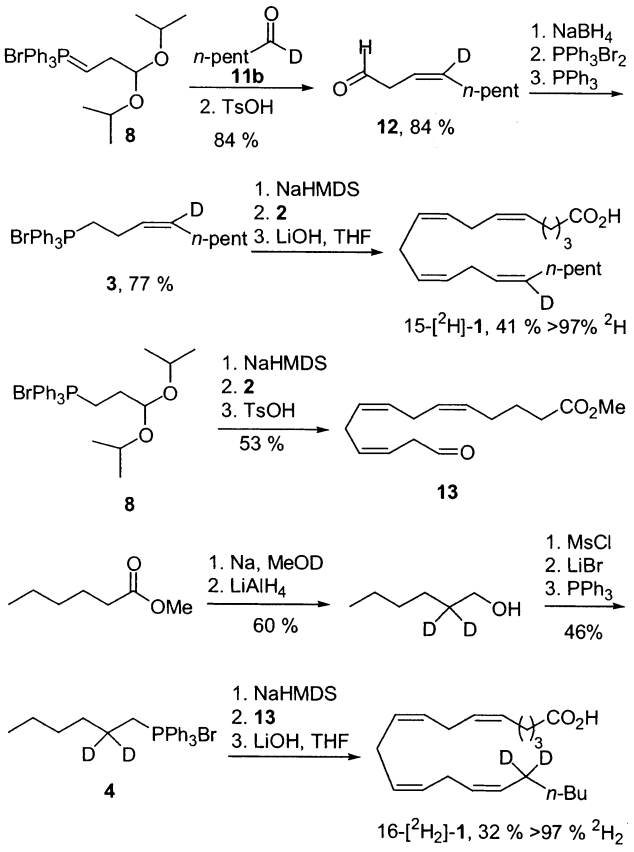
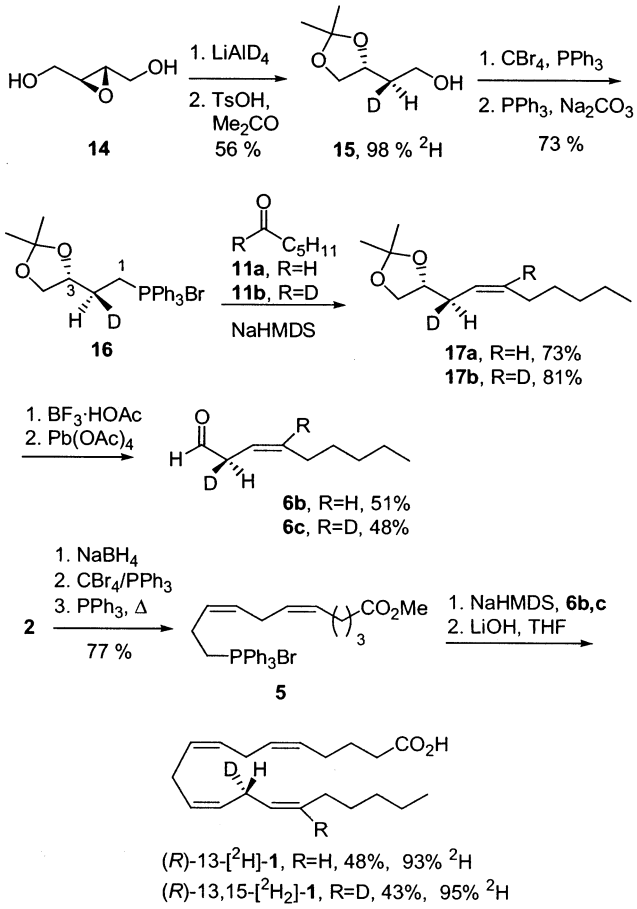
Scheme 5



Scheme 6



provide 1-[^{2}H]-hexanal **11b**.[26] The deuterated aldehyde was converted to alkene **12** employing once more the homologating reagent **8** in the presence of sodium bis(trimethylsilyl)amide (NaHMDS) followed by deprotection of the resulting olefination product. Compound **12** was reduced, brominated, and reacted with triphenylphosphine to provide the desired deuterium-labeled Wittig salt **3** in 77% yield. The final product was obtained by the Wittig reaction of aldehyde **2** and phosphonium salt **3** in the presence of NaHMDS, followed by hydrolysis of the resulting methyl arachidonate. The isotopic purity of 15-[^{2}H]-**1** was assessed by negative ion mode electrospray mass spectrometry, indicating >97% deuterium incorporation. 16-[$^2H_2$]-Arachidonic acid was accessed from aldehyde **13**, obtained by Wittig reaction of **8** and **2**. Phosphonium salt **4** was prepared from deuterated methylhexanoate, generated via solvent exchange in NaOMe/MeOD (Scheme 5). The final Wittig coupling of **13** and the ylide of **4** provided the desired product in moderate yield but with a high isotopic purity (>97%).

Preparation of 13(*R*)-[^{2}H]-Arachidonic Acid and Assay with Soybean Lipoxygenase. According to the design in Scheme 3, the synthesis of 13(*R*)-[^{2}H]-**1** from aldehyde **2** required conversion of the latter into Wittig synthon **5**, and the preparation of stereospecifically labeled aldehyde **6b**. The labeled fragment was obtained from the optically pure epoxy diol **14** (Scheme 6), prepared in four steps from L-dimethyl tartrate.[18,27] Opening of the epoxide ring with $LiAlD_4$ followed by in situ protection of the vicinal diol of the crude product provided **15** in moderate yield and high isotopic purity as determined by field ionization mass spectrometry.[28] Alcohol **15** was subsequently converted to the corresponding bromide, which was reacted with triphenylphosphine to give phosphonium salt **16**.[18] Wittig reaction with hexanal (**11a**) or 1-[^{2}H]-hexanal (**11b**), followed by deprotection and lead tetraacetate cleavage of the diol, provided compound **6b** to **6c**, respectively, which were used immediately, without purification, in the Wittig reaction with phosphonium salt **5**. This reagent was obtained in three steps in good yield from the key aldehyde **2**.

An obvious concern in the Wittig olefination of **6b** and **6c** involves the possibility of enolization, which could compromise the isotopic and stereochemical purity of the arachidonic acid product. However, a survey of the literature indicated that several other aldehydes containing a stereogenic center at the α-carbon have been olefinated with retention of the stereochemical integrity.[18,29] In an initial pilot study with unlabeled material, it was found that the desired arachidonic acid product was in fact formed, and hence the synthesis of 13(*R*)-[^{2}H]-**1** and 13(*R*),15-[$^2H_2$]-**1** was pursued. The final products were

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(28) All mass spectrometric determinations of isotopic purity were carried out by computer comparison of the spectra obtained for unlabeled and labeled synthetic material. The isotopic purity of the labeled compounds was adjusted to account for the intensity of the M + 1 peak observed for the unlabeled compounds.
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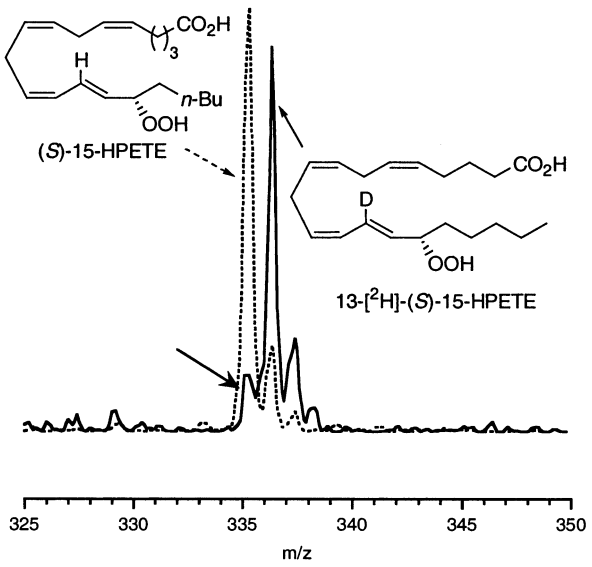


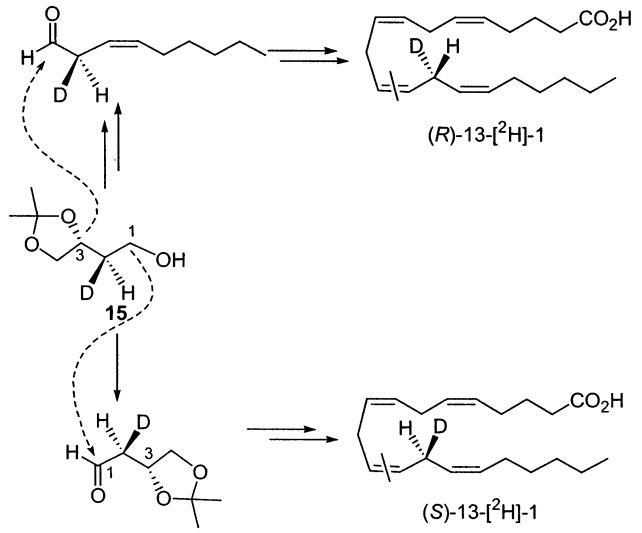
Figure 1. Negative ion mode ESI-MS of 15-HPETE produced by reaction of SLO-1 with unlabeled (dashed line) and 13(*R*)-deuterium-labeled arachidonic acid (solid line).

obtained by hydrolysis of the methyl esters with lithium hydroxide in aqueous THF. The isotopic purity of the products was assessed by electrospray mass spectrometry and revealed 93% deuterium incorporation for 13(*R*)-[^{2}H]-**1** and 95% bis-deuteration of 13(*R*),15-[$^2H_2$]-**1**.

Standard methods employed in asymmetric synthesis were unsuitable to determine the enantiomeric purity of the final product. Therefore, the optical purity of the target compound was determined by incubation with soybean lipoxygenase isozyme 1 (SLO-1). Under most conditions,[30] this enzyme converts arachidonic acid to 15(*S*)-hydroperoxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid (15-HPETE) by stereospecific abstraction of the *pro-S* hydrogen atom from position 13 of the substrate.[31] Hence, if the two synthetic 13(*R*)-deuterium-labeled arachidonic acids were enantiomerically pure, the enzymatic 15-HPETE product would contain the same levels of deuterium as the arachidonic acid substrate. Figure 1 shows the M − 1 ions observed in the negative ion mode electrospray mass spectrum of the products of the enzymatic reactions with 13(*R*)-[^{2}H]-**1** and unlabeled substrate.[32] The M − 1 ions differed by one mass unit, consistent with high enantioenrichment of 13(*R*)-[^{2}H]-**1**. The small amount of nondeuterated HPETE produced from 13-(*R*)-[^{2}H]-**1** (arrow, Figure 1) is predominantly due to the 7% unlabeled arachidonic acid present in the final product (Scheme 6). Taking the isotopic purity of the starting compound into account, computer simulation of the two spectra in Figure 1 allowed estimation of a lower limit of 95:5 for the enantiomeric ratio for the synthetic 13(*R*)-[^{2}H]-**1**. Similarly, the enantiomeric purity of 13(*R*),15-[$^2H_2$]-**1** was estimated as 96:4 (Supporting Information, Figure S1).

Preparation of 13(*S*)-[^{2}H]-Arachidonic Acid and Implications for Stereospecificity of SLO-1. Arachidonic acid labeled in the 13(*R*) position with deuterium is an important probe for

Scheme 7

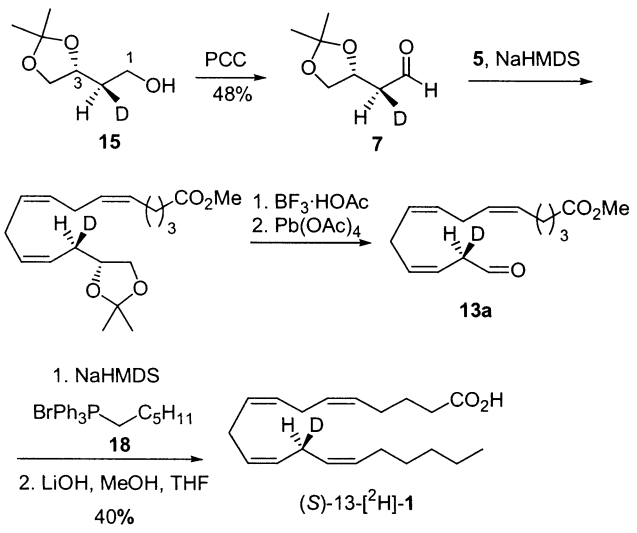


the radical formed upon abstraction of the 13(*S*) hydrogen atom by the tyrosyl radical. Its enantiomer, 13(*S*)-[^{2}H]-**1**, is equally useful to determine the deuterium kinetic isotope effect on both steady-state turnover and radical generation. Early studies by Hamberg and Samuelsson with 13(*S*)-[^{3}H]-**1** and PGHS-1 showed that recovered substrate exhibited increased specific radioactivity, thereby establishing that the first step in Scheme 1 is at least partially rate limiting.[8] The exact kinetic isotope effect (KIE) was not reported in this study, but from the results the KIE can be estimated to be approximately ∼2, although it is difficult to deduce a precise value. To provide a more accurate picture of the KIE displayed by both isozymes, we initiated the synthesis of 13(*S*)-[^{2}H]-arachidonic acid. Our initial approach is shown in Scheme 7. The isotopically labeled center in alcohol **15** served also as the precursor for the stereocenter in 13(*S*)-[^{2}H]-**1**. Instead of converting C3 of **15** into C12 of 13(*R*)-[^{2}H]-arachidonic acid (Scheme 6), we reasoned that the enantiomeric product should be accessible by elaboration of C1 of **15** into C12 of the product (Scheme 7). This strategy would allow both isomeric targets to be prepared from one advanced intermediate, **15**, which would be more economic than preparing the enantiomer of **15** requiring six steps from D-dimethyl tartrate. Hence, **15** was oxidized to the corresponding aldehyde **7** and reacted with phosphonium salt **5** (Scheme 8). Deprotection of the ketal, and lead tetraacetate cleavage of the resulting diol, provided **13a**. This aldehyde was subsequently converted into methyl arachidonate by olefination with phosphonium salt **18**, and the final product was obtained by hydrolysis of the methyl ester with lithium hydroxide in aqueous THF. The isotopic and optical purities were assessed as described above using the SLO-1 assay. If the final product was enantiomerically pure, the 15(*S*)-HPETE product from 13(*S*)-[^{2}H]-**1** should not contain any deuterium. However, the electrospray mass spectrum showed significant amounts of deuterated 15(*S*)-HPETE (Supporting Information, Figure S2), suggesting that the synthetic 13(*S*)-[^{2}H]-**1** consisted of a 80:20 mixture of enantiomers.

An alternative explanation for the relatively low enantiomeric purity of 13(*S*)-[^{2}H]-**1** involves a decreased stereofidelity of the hydrogen atom abstraction by SLO-1. Several studies have reported unusually large kinetic isotope effects on both k_{cat} and k_{cat}/K_m in the reaction of deuterated linoleic acids with SLO-1

(30) Kuhn, H.; Schewe, T.; Rapoport, S. M. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1986**, *58*, 273−311.
(31) Hamberg, M.; Samuelsson, B. *J. Biol. Chem.* **1967**, *242*, 5329−5335.
(32) Reduction of the hydroperoxide with $NaBH_4$ and MS analysis of the resulting alcohol provided isotopic compositions identical to those shown in Figures 1 and 2.

Scheme 8



and human 15-lipoxygenase.[33−36] These isotope effects of 35−80 well exceed the semiclassical limit and have been attributed to quantum mechanical tunneling.[35,37,38] Importantly, the significantly slower rate for deuterium atom abstraction from 11-(*S*)-[^{2}H]-*linoleic acid* has been shown to induce to some extent aberrant enzymatic abstraction of the hydrogen rather than the deuterium atom at position 11.[35] If deuterium substitution in the 13(*S*) position of *arachidonic acid* results in a similar isotope effect on the stereochemistry of hydrogen atom abstraction, it would mean that the ratio of protonated and deuterated HPETE in Figure 1 does not accurately reflect the enantiomeric purities of the labeled arachidonic acids. More specifically, the enantiomeric purity deduced from Figure 1 would be overestimated, whereas the purity of 13(*S*)-[^{2}H]-**1** would be underestimated.

One potential experiment to probe whether the stereoselectivity of hydrogen atom abstraction from **1** by SLO-1 indeed decreases significantly upon deuteration in the *pro-S* position at carbon 13 involves converting racemic 13-[^{2}H]-**1** into 15-(*S*)-HPETE and analyzing the product after completion of the reaction. If no significant erosion of stereospecificity occurs, one would expect a 1:1 ratio of deuterated and nonlabeled 13-(*S*)-HPETE product. On the other hand, if deuteration in the *pro-S* position results in abstraction of the *pro-R* hydrogen atom to a significant extent, the 15(*S*)-HPETE product would be enriched in deuterium. Unfortunately, our 13(*R*)- and 13(*S*)-deuterium-labeled arachidonic acids were prepared by different routes, and hence they could have different enantiomeric purities as suggested by the SLO assays. Therefore, mixing equal quantities of both synthetic materials would not necessarily provide racemic compound. Hence, we set out to prepare both 13(*R*)- and 13(*S*)-[^{2}H]-**1** in parallel using the route described in Scheme 6, starting with L-dimethyl tartrate for 13(*R*)-[^{2}H]-**1** and D-dimethyl tartrate for 13(*S*)-[^{2}H]-**1**. Analysis of the former compound by reaction with SLO-1 and subsequent negative ion electrospray mass spectrometry provided very similar results

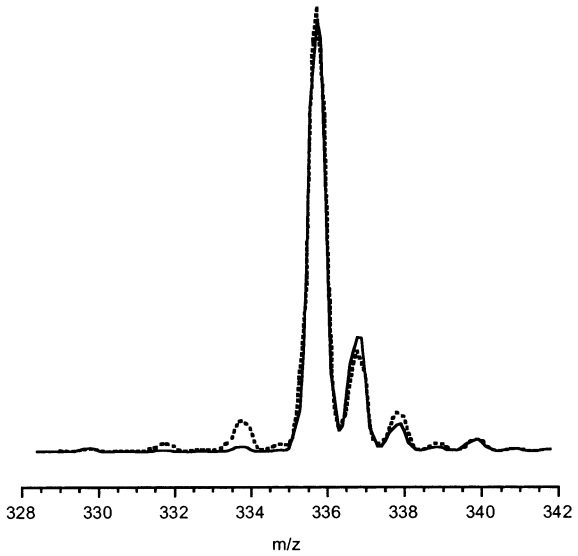
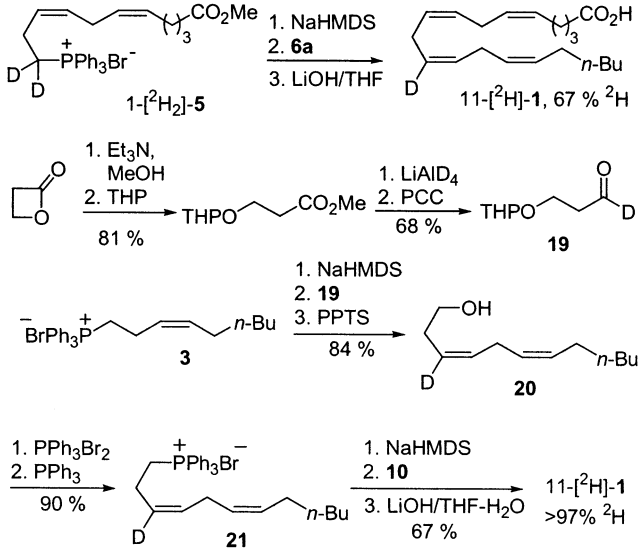


Figure 2. Negative ion mode ESI-MS of 15-HPETE produced by reaction of SLO-1 with unlabeled (dashed line) and 13(*S*)-deuterium-labeled arachidonic acid (solid line) prepared from D-dimethyl tartrate via the route in Scheme 6.

Scheme 9



as depicted in Figure 1. However, when 13(*S*)-[^{2}H]-**1** was analyzed in an identical manner, the electrospray mass spectrum of the product of the enzymatic reaction showed no appreciable amount of deuterium-labeled HPETE (Figure 2). Therefore, the stereochemical purity of 13(*S*)-[^{2}H]-**1** prepared using the route in Scheme 6 but starting with D-dimethyl tartrate can be estimated as >98:2. This result provides clarity on a number of issues: (1) both enantiomers prepared using the route in Scheme 6 give highly enantioenriched products, whereas the reaction sequence in Scheme 8 did result in ∼20% racemization, and (2) the stereospecificity of hydrogen atom abstraction by SLO-1 does not change significantly upon deuteration of the *pro-S* proton at carbon 13 of arachidonic acid.

Preparation of 11-[^{2}H]-Arachidonic Acid. The initial route to the final target, 1-[^{2}H]-**1**, involved reaction of 11-[^{2}H$_2$]-**5** with unlabeled aldehyde **6a** (Scheme 9). Although the desired product was obtained, the isotopic purity was a disappointing 67%. This route differs from the synthetic strategies described so far in that the label is located on the Wittig reagent rather than on the

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(38) Kohen, A.; Klinman, J. P. *Acc. Chem. Res.* **1998**, *31*, 397−404.

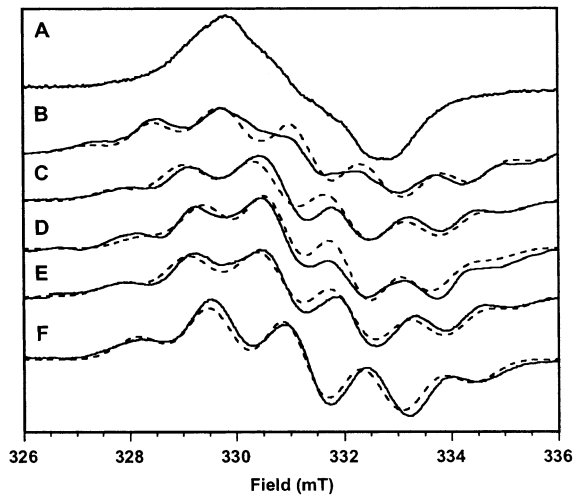

Figure 3. EPR spectra of PGHS-2 intermediate II with the wide singlet tyrosyl radical (A) and of the radical intermediate obtained after anaerobic incubation of PGHS-2 intermediate II with (B) unlabeled **1**, (C) 11-[^{2}H]-**1**, (D) 13(*R*)-[^{2}H]-**1**, (E) 15-[^{2}H]-**1**, and (F) 13(*R*),15-[^{2}H$_2$]-**1**. The dashed lines represent simulations using the parameters in Table 1. Instrument settings: temperature 98 K, frequency 9.22 GHz, modulation amplitude 2 G, power 1 mW, modulation frequency 100 kHz, time constant 1 s.

aldehyde reaction partner. Given the success with this latter approach, we prepared aldehyde **19** in four steps from β-propiolactone (Scheme 9). Wittig coupling of **19** using unlabeled phosphonium salt **3** and subsequent deprotection of the alcohol provided **20**, which was converted to the corresponding Wittig reagent **21**. Olefination of the aldehyde derived from deprotection of acetal **10**, followed by saponification of the methyl ester, produced the target molecule. Analysis of the product by electrospray mass spectrometry indicated >97% deuterium incorporation.

Structure of the Radical Intermediate. The site specifically deuterium labeled arachidonic acids were used for investigation of the structures of radical intermediates formed during cyclooxygenase catalysis. Purified ovine PGHS-2 was reacted with ethyl hydroperoxide under anaerobic conditions to generate the so-called wide singlet[11c] tyrosyl radical signal (Figure 3A). Subsequent anaerobic addition of unlabeled arachidonic acid and freezing of the sample in an EPR tube produced the seven-line signal shown in spectrum B. Accurate quantitation of the conversion from tyrosyl radical to substrate radical is difficult in these experiments since the generation of the tyrosyl radical is not complete at the time point that the substrate is added. In this work, the conditions of the experiments were adjusted to maximize the signal associated with the substrate radical while the intensity of the tyrosyl radical signal was minimized to avoid the need for subtractions as much as possible. This resulted in stoichiometries of tyrosyl radical consumed to radical produced varying from 0.4 to 2.2. The ratio of substrate radical relative to the heme varied from 0.11 to 0.42. For a more precise quantitation and extraction of kinetic data, rapid freeze quench experiments will be required. Repeating the procedure with 11-[^{2}H]-**1** resulted in spectrum C, which shows a reduction in multiplicity from seven to six lines. This reduction in the multiplicity is the result of the substantially smaller gyromagnetic ratio (γ) for deuterium compared to protium (γ_D/γ_H = 0.154). The triplet hyperfine splitting of the deuterium is too small to be resolved in these spectra of frozen solutions, and hence substitution of a proton with deuterium results in the apparent loss of one hyperfine interaction. Furthermore, the turning points in spectrum C occur at different fields than those in signal B. Similarly, incubation of the enzyme with 13(*R*)-[^{2}H]-**1** or 15-[^{2}H]-**1** after generation of the tyrosyl radical produced six-line spectra (D and E). Collectively, these findings indicate that the radical contains spin density at C11, C13, and C15, consistent with the assignment of a pentadienyl radical structure to the radical intermediate. Further support is presented in the spectrum obtained with 13(*R*),15-[^{2}H$_2$]-**1**, which displays a five-line pattern, as would be expected for a pentadienyl radical spanning C11−C15 (Figure 3, spectrum F). The line shapes of the EPR signals produced with all synthetic substrates could be reasonably well simulated with one set of parameters shown in Table 1.[15] Except for adjusting the size of the hyperfine value of the protons substituted with deuterium, the sizes of the hyperfine couplings in these simulations were kept invariant. Importantly, the hyperfine tensors used for these simulations are consistent with values reported for other pentadienyl radicals.[39]

Table 1. Parameters Used To Simulate the Substrate Radicals in Figures 3 and 4[a]

	1	11-[^{2}H]-**1**	13(*R*)-[^{2}H]-**1**	15-[^{2}H]-**1**	13(*R*),15[^{2}H$_2$]-**1**	**16-**[^{2}H$_2$]-**1**
line width (G)	3.8	4	4	4	5	4.5
$A_{H11,15}$ (G)	10.4	1.6/10.4	10.4/10.4	10.4/1.6	10.4/1.6	10.4/10.4
A_{H13} (G)	11.5	11.5	1.8	11.5	1.8	11.5
$A_{H12,14}$ (G)	3.3	3.3	3.3	3.3	3.3	3.3
A_{H10a} (G)	16	16	16	16	16	13
A_{H10b} (G)	16	16	16	16	16	13
A_{H16a} (G)	9.8	9.8	9.8	9.8	9.8	1.5
A_{H16b} (G)	3.9	3.9	3.9	3.9	3.9	0.6

[a] For all simulations, $g_1 = 2.002$, $g_2 = 2.002$, and $g_3 = 2.002$.

If the intermediate has a pentadienyl radical structure as suggested by the results in Figure 3, the four protons at C10 and C16 may give rise to hyperfine splittings, with their individual contributions depending on the dihedral angle of the C−H bonds at C10 and C16 with respect to the extended π-system harboring the unpaired electron (Scheme 2).[40] Since the overall appearance of spectrum B in Figure 3 is a seven-line spectrum, and not an eight-line spectrum, one of the four protons at C10/16 must produce only a small hyperfine coupling that is not resolved. In a previous report, using a spin density on C15 of 0.42, Tsai and co-workers proposed that one of the protons at C16 is positioned with a dihedral angle θ of 71°, such that its hyperfine value is small (3.9 G).[15] This hypothesis derived from the expectation that the conformation of the pentadienyl radical would bring C9 and C11 into the correct orientation for *endo*-peroxide formation. The EPR spectrum produced upon incubation of PGHS-2 with 16-[^{2}H$_2$]-**1** is fully consistent with this view, because the seven-line signal observed with unlabeled arachidonic acid is replaced by a six-line pattern with 16-[^{2}H$_2$]-**1** (Figure 4). Hence, only one of the protons at C16 gives rise to a significant hyperfine interaction. Simulation of the signal obtained with 16-[^{2}H$_2$]-**1** indicated a hyperfine value of 9.8 G for this proton. The best fit of the line shape in these simulations required a smaller coupling constant for the

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(40) Weil, J. A.; Bolton, J. R.; Wertz, J. E. *Electron Paramagnetic Resonance: Elementary Theory and Practical Applications*; John Wiley & Sons: New York, 1994.

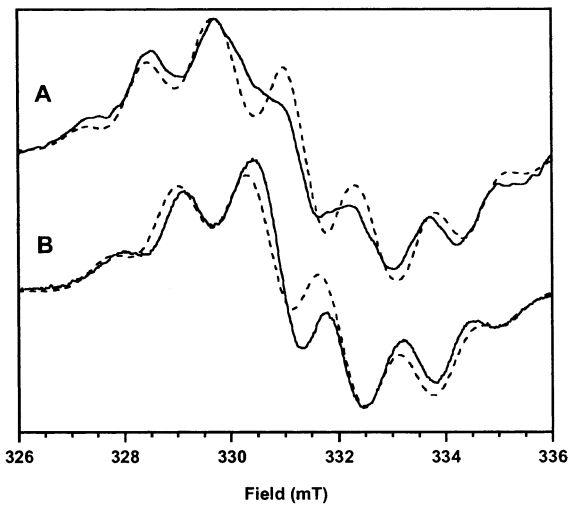

Figure 4. EPR spectra of the radical intermediate obtained after anaerobic incubation of PGHS-2 intermediate II with (A) unlabeled **1** and (B) 16-[$^2H_2$]-**1**. The samples were frozen 30 s to 1 min after addition of the substrate. Instrument settings: temperature 98 K, microwave frequency 9.22 GHz, modulation amplitude 2 G, power 1 mW, modulation frequency 100 kHz, time constant 0.33 s. A smaller coupling constant for the C10 protons (13 G instead of 16 G) was used to simulate the radical derived from 16-[$^2H_2$]-**1**.

C10 protons than deduced from previous studies (13 G instead of 16 G, Table 1).

Discussion

Our synthetic design to prepare the desired series of site specifically deuterated arachidonic acids from one advanced intermediate, **2**, proved successful except in the case of 11-[^{2}H]-**1**. The initial synthetic route to this compound was the only example in which the deuterium label was located on the α-carbon of a phosphonium salt rather than on the aldehyde reaction partner. The desired product was therefore synthesized using an alternative strategy that assembled the target in a tail-to-head fashion. All other labeled arachidonic acids were prepared in a head-to-tail manner, including both enantiomers of 13-[^{2}H]-**1**. The synthetic schemes proved quite efficient, as compounds **2**, **3**, **6**, and **8** were building blocks for the synthesis of several targets. The three-carbon homologating agent **8**[21] was particularly useful in these studies as it was used to install three of the four *Z*-olefins in 15-[^{2}H]-**1** and 16-[$^2H_2$]-**1**, and two of the double bonds in the other targets. Negative ion mode electrospray mass spectrometry was an effective technique to determine the isotopic purity of these synthetic materials, and soybean lipoxygenase was invaluable for the determination of the stereochemical purity of the products labeled at C13. One of these highly enantiomerically enriched compounds, 13(*S*)-[^{2}H]-**1**, will provide a useful tool to compare the kinetic isotope effects displayed by COX-1 and -2 and lipoxygenase. These three metalloenzymes share the unusual property of activating a fatty acid substrate for subsequent reaction with molecular oxygen[14,41,42] instead of the more common theme of activation of oxygen for reaction with substrate.

A difference in the response of SLO-1 to deuteration of linoleic acid versus arachidonic acid was observed in the current studies. Whereas deuteration in the *pro-S* position at C11 in linoleic acid induces a decreased stereoselectivity in the hydrogen atom abstraction step by SLO-1,[35] deuteration of the *pro-S* position at C13 of arachidonic acid did not change the stereoselectivity. The decreased stereoselectivity in the hydrogen atom abstraction from the 18-carbon linoleic acid has been postulated to be due to binding of the substrate in a reverse orientation.[35] Our results with the 20-carbon arachidonic acid suggest that either such a reverse binding is less favorable or the discrimination against deuterium is significantly diminished. Kinetic isotope effect studies on the reaction of SLO-1 with 13(*S*)-[^{2}H]-arachidonic acid are in progress to investigate the origin of the difference between the two substrates.

The synthetic substrates were used to investigate the structure of the fatty acid radical intermediate formed upon anaerobic incubation with PGHS-2 containing the essential tyrosyl radical. Tyrosyl radicals in both PGHS-1 and PGHS-2 have been shown previously to be kinetically competent for cyclooxygenase catalysis.[43,44] The EPR spectra presented here and in our preliminary report[16] clearly show that under anaerobic conditions a substrate radical is generated with spin density at positions 11, 13, and 15. Furthermore, the spectrum obtained with 16-[$^2H_2$]-**1** corroborates the earlier hypothesis that one of the protons at position 16 interacts only weakly with the unpaired electron due to a dihedral angle of about 70°. These results therefore provide strong support for the generation of a pentadienyl radical in the first step of cyclooxygenase catalysis. Furthermore, exposure of samples containing the substrate radical to O_2 leads to regeneration of the tyrosyl radical,[15] consistent with the mechanism in Scheme 1 and demonstrating the chemical competence of the radical intermediate. Mason and co-workers previously trapped an arachidonyl radical generated by PGHS-1 using 2-methyl-2-nitrosopropane under aerobic conditions.[9] These authors showed in experiments with 5,6,8,9,11,12,14,-15-octadeuterated arachidonic acid that the unpaired spin was located at one of these positions. The trapped radical, however, appeared only well after cyclooxygenase catalysis,[10] and crystallographic studies indicate that the PGHS-1 active site is too small to simultaneously accommodate both the substrate and the spin trap reagent.[12] Hence, these studies presumably trapped arachidonyl radicals released from the protein, as has also been suggested for soybean lipoxygenase under certain conditions.[45−48] On the other hand, the observed regeneration of the tyrosyl radical upon exposure of the PGHS fatty acid radical to oxygen indicates that the chemical conversions of the substrate radical in the present studies occurred while it was bound in the cyclooxygenase active site and not free in solution. These results argue strongly that the observed radical is on the reaction pathway, or at least connected to that pathway. It remains possible, however, that under anaerobic conditions, which preclude the reaction with oxygen that would take place during normal catalysis, we are observing the thermodynamically most stable radical species but not the actual reactive intermediate.

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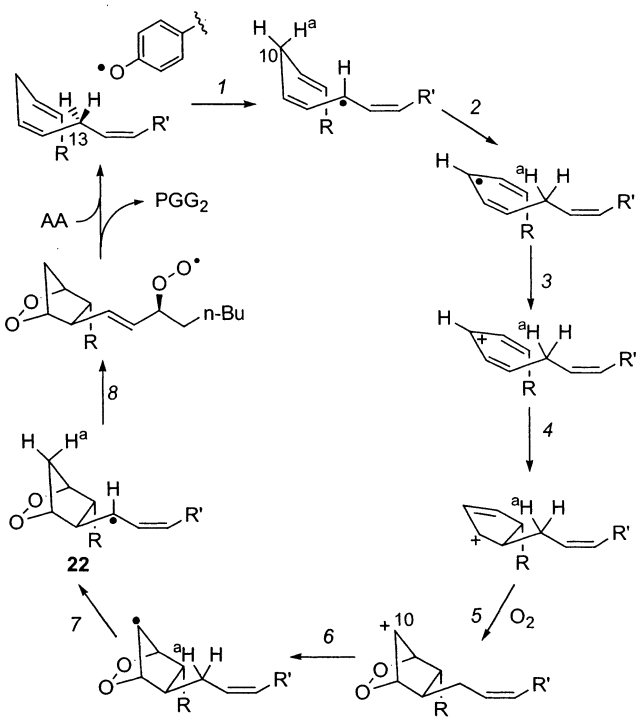
For example, the pentadienyl radical could conceivably be in rapid equilibrium with a nondetectable amount of a Δ^{14}-[11,-12,13]-ene allyl radical, with the latter actually reacting at C11 with molecular oxygen. Such an ene allyl radical would readily explain the observed regioselectivity for the first oxygenation. An ene allyl radical has in fact been detected in the reaction of purple lipoxygenase with various deuterated 13(*S*)-hydroperoxy-9,11-(*E*,*Z*)-octadecadienoic acids, the hydroperoxy products of the reaction of lipoxygenase with linoleic acid.[49]

A related possibility is that the pentadienyl radical does not form to a significant extent during generation of PGG_2 under aerobic conditions. The K_m for oxygen is 5 μM, and k_{cat} is typically around 60 s^{-1}, giving a large second-order rate constant ($k_{cat}/K_{m,O_2} = 1.2 \times 10^7$ M^{-1} s^{-1}), which reflects the steps from O_2 binding to the first irreversible step, probably product release. This bimolecular rate constant is close to the diffusion-controlled limit, and thus, hydrogen atom abstraction from C13 could result in a Δ^{14}-[11,12,13]-ene allyl radical that reacts faster with oxygen than it relaxes to the thermodynamically favored pentadienyl radical. Another potential issue in this connection is that although rotation around the C12−C13 or C13−C14 bonds would be very rapid in solution, this need not be the case when the fatty acid is bound in the active site of the enzyme. Direct structural information is not available for productive binding of arachidonic acid to PGHS-2, but the X-ray structure of PGHS-1 with the substrate bound in the productive conformation has been reported.[12] The π-systems of the C11−C12 and C14−C15 double bonds of arachidonic acid in this structure are not coplanar but twisted at an angle of approximately 30°, and hence some nuclear motion would have to take place upon hydrogen atom abstraction to provide a planar pentadienyl radical.[50] However, the PGHS-2 cyclooxygenase active site in particular should readily accommodate such a rotation, since it has a volume that is about 20% larger than that of PGHS-1,[51,52] and the fatty acid binding channel is not particularly tight. Thus, there appears to be no serious impediment to formation of a planar pentadienyl radical during aerobic catalysis.

The assignment of a pentadienyl structure spanning C11−C15 for the observed radical intermediate provides an important calibration point to evaluate an alternative cyclooxygenase mechanism that was recently proposed by Dean and Dean.[53] These authors noted that the original characterization of a pentadienyl radical[14,15] did not exclude the possibility that this radical was delocalized over positions C8−C12. In their hypothesis, such a pentadienyl radical is formed via a stereospecific sigmatropic 1,4-shift of the *pro-R* hydrogen at C10 to the *pro-R* position at C13 in the initially formed radical (Scheme 10, step 2). The C8−C12 pentadienyl radical is subsequently oxidized to a pentadienyl cation, which undergoes a symmetry-allowed conrotatory ring closure to provide a *trans*-cyclopentenyl cation. The latter species then reacts with O_2 to generate

Scheme 10



the *endo*-peroxide, positioning the cation at C10. Reduction of this cation to the radical and a 1,4-shift of the *pro-R* hydrogen at C13 would provide allyl radical **22**, which completes the catalytic cycle in the same fashion as in the Samuelsson mechanism. In their proposal, Dean and Dean suggest that since the pentadienyl radicals spanning C11−C15 and C8−C12 are probably similar in energy, the EPR spectra reported under anaerobic conditions would likely be a mixture of both species. However, the results presented in this paper argue strongly against this, since the effect of site-specific deuteration would be very different on each radical, making it quite unlikely that the various signals could be simulated successfully with one set of parameters. Therefore, we conclude that the formation of a C8−C12 radical is not supported. Other features of the Dean mechanism deserve some comment. The heme would be the only available redox partner to carry out the two redox processes at the substrate binding channel that are key to the Dean model (steps 3 and 6, Scheme 10). All reported X-ray structures of either isozyme show that the arachidonic acid binding channel and the heme are not in close proximity. The distance between the metal in the heme and C10 of arachidonic acid is >16 Å in PGHS-1.[12] Therefore, if oxidizing equivalents at the heme are to oxidize and reduce intermediates in the arachidonic acid binding site, this would have to occur by long-range redox chemistry, whereas the Samuelsson mechanism does not require this. At present no experimental support is available for the additional steps in the Dean mechanism, and the EPR data presented in this paper do not agree with formation of a C8−C12 radical. On the other hand, this and previous reports[11,14,15,43,44,54] do provide strong evidence of radical chemistry.

In summary, we have designed and executed synthetic routes to a series of site specifically deuterium labeled arachidonic acids and have used these compounds to characterize the structure of a pentadienyl radical intermediate in the PGHS-2 cyclooxygenase reaction. This structural assignment supports

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(50) Preliminary computational studies indicate that the pentadienyl radical does not need to be perfectly planar. Rotations of ~15° along the C12−C13 or C13−C14 bond do not significantly change the spin distribution. Palmer, G, Blomberg, M., Siegbahn, P. E. M. Personal communications.
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the radical mechanism originally proposed by Hamberg and Samuelsson. The synthetic 13(*S*)-deuterium-labeled arachidonic acid will be a useful mechanistic probe to investigate kinetic isotope effects in the two PGHS isozymes as well as in various lipoxygenases. Use of the 13(*S*)-deuterium-labeled arachidonate in this study has shown that the stereochemistry of hydrogen atom abstraction by soybean lipoxygenase is not affected by deuterium substitution.

Experimental Section

General Procedures. All NMR spectra were recorded on Varian U500 spectrometers. ^{1}H spectra were referenced to $CHCl_3$ at 7.26 ppm, and ^{13}C spectra were referenced to $CDCl_3$ at 77.23 ppm. All spectra were taken in $CDCl_3$. Mass spectrometry (MS) experiments were carried out by the Mass Spectrometry Laboratory at the University of Illinois at Urbana-Champaign. Fractions collected during silica gel column chromatography were analyzed by thin-layer chromatography on Merck silica gel 60 F_{254} plates. Compounds and solvents were obtained from Fisher or Aldrich. THF and toluene were distilled from sodium/benzophenone, and CH_2Cl_2 was distilled from CaH_2. SLO-1 was obtained from Sigma (type V, 1×10^6 units/mg).

The following compounds were prepared following literature procedures: (3,3-diisopropoxypropyl)triphenylphosphonium bromide (**8**),[21] 4-oxobutyric acid methyl ester (**9**),[55] 8,8-diisopropoxyoct-5-enoic acid methyl ester (**10**),[56] 1-[^{2}H]-hexanal (**11b**),[26,57] (3-hydroxymethyloxiranyl)methanol (**14**),[18] 2(*R*)-[^{2}H]-(2,2-dimethyl[1,3]dioxolan-4(*R*)-yl)ethanol (**15**),[18] and [2(*R*)-[^{2}H]-(2,2-dimethyl[1,3]dioxolan-4(*R*)-yl)-ethyl]triphenylphosphonium bromide (**16**).[18] Detailed descriptions of the synthesis and full characterization of compounds **2**−**5**, **6c**, **13**, **13a**, **17b**, and **19**−**21** can be found in the Supporting Information.

15-[^{2}H]-Arachidonic Acid. 4-[^{2}H]-Non-3-*cis*-enyltriphenylphosphonium bromide (**3**) (160 mg, 0.35 mmol) was dried by three cycles of azeotropic evaporation with anhydrous benzene. THF (2.1 mL) was added, followed by a 1 M solution of NaHMDS (0.23 mL, 0.23 mmol) in THF at −40 °C. The orange solution of the ylide was stirred at −40 °C for 10 min and warmed to room temperature over 1 h. The mixture was cooled to −100 °C, and a solution of 11-oxo-undeca-*cis*,*cis*-5,8-dieneoic acid methyl ester (**2**) (48 mg, 0.23 mmol) in THF (1 mL) was added dropwise. After 0.5 h at −100 °C, the reaction was allowed to warm to room temperature over 5 h. Saturated aqueous sodium bicarbonate was added, and the aqueous phase was extracted with Et_2O (3×50 mL). The combined organic phases were washed with brine, dried over anhydrous sodium sulfate, concentrated, and purified by silica gel chromatography (R_f 0.5, hexane:EtOAc = 100:5) to provide methyl 15-[^{2}H]-arachidonate (31 mg, 41%) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.45−5.34 (7H, m), 3.69 (3H, s), 2.87−2.81 (6H, m), 2.34 (2H, t, J = 7.5 Hz), 2.15−2.11 (2H, m), 2.11 (2H, t, J = 7.0 Hz), 1.73 (2H, quin, J = 7.7 Hz), 1.39−1.27 (6H, m), 0.91 (3H, t, J = 7.0 Hz); ^{13}C NMR (125 MHz) δ 174.32, 129.17, 129.11, 128.83, 128.44, 128.40, 128.10, 127.649, 51.70, 33.65, 31.74, 29.53, 27.33, 26.76, 26.75, 25.84, 25.82, 24.99, 22.79, 14.28; FIMS (*m*/*z*) 319 (M^+, 100). Methyl 15-[^{2}H]-arachidonate (9 mg, 0.028 mmol) was dissolved in THF (0.47 mL), and the solution was cooled to 0 °C. A 1 M solution of aqueous LiOH (0.47 mL, 0.47 mmol) was added. The reaction mixture was stirred for 19 h at room temperature, acidified to pH 1 with 1 M HCl, saturated with solid NaCl, and extracted with Et_2O (3×50 mL). After the combined organic phases were washed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure, purification by silica gel chromatography (R_f 0.2, hexane:EtOAc = 100:10) gave 15-[^{2}H]-**1** (7.3 mg, 85%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.46−5.34 (7H, m), 2.87−2.82 (6H, m), 2.40 (2H, t, J = 6.7 Hz), 2.18−2.14 (2H, m), 2.07 (2H, t, J = 7.2 Hz), 1.74 (2H, quin, J = 7.5 Hz), 1.39−1.27 (6H, m), 0.90 (3H, t, J = 7.3 Hz); EIMS (*m*/*z*) 305 (M^+, 8.82); HRMS (EI) (*m*/*z*) calcd for $C_{20}H_{31}DO_2$ 305.246507, found 305.246481; deuterium content 98% as determined by ESI-MS comparison with unlabeled arachidonic acid.

16-[$^2H_2$]-Arachidonic Acid. 2-[$^2H_2$]-Hexyltriphenylphosphonium bromide (80 mg, 0.19 mmol) was dried thoroughly by azeotropic evaporation with anhydrous benzene. THF (9 mL) was added, followed by a 1 M solution of sodium bis(trimethylsilyl)amide (0.15 mL, 0.15 mmol) in THF at −15 °C. The solution was stirred at 0 °C for 40 min and cooled to −100 °C, and a solution of 14-oxo-tetradeca-5,8,11-*cis*,*cis*,*cis*-trienoic acid methyl ester (**13**) (22 mg, 0.09 mmol) in THF (2 mL) was added. After being stirred overnight, the reaction was warmed to room temperature. Saturated aqueous sodium bicarbonate was added, and the aqueous phase was extracted with Et_2O (3×30 mL). The combined organic phases were washed with brine, dried over anhydrous sodium sulfate, concentrated, and purified by silica gel chromatography (R_f 0.5, hexane:EtOAc = 100:5) to provide methyl 16,16-[$^2H]_2$-arachidonate (9 mg, 32%) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.38−5.41 (8H, m), 3.69 (3H, s), 2.82−2.86 (6H, m), 2.34 (2H, t, J = 7.5 Hz), 2.13 (2H, t−d, J = 6.5, 6.5 Hz), 1.73 (2H, quin, J = 7.7 Hz), 1.27−1.32 (6H, m), 0.91 (3H, t, J = 7.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 129.16, 129.10, 128.82, 128.52. 128.49, 128.44, 128.40, 128.10, 51.71, 34.44, 33.65, 31.68, 29.35, 26.76, 25.83, 24.98, 22.80, 21.40, 14.28; MS (EI) (*m*/*z*) 322 ($M^+ + 1$). Methyl 16-[$^2H_2$]-arachidonate (8.5 mg, 0.027 mmol) was dissolved in THF (0.47 mL), and the solution was cooled to 0 °C. A 1 M solution of aqueous LiOH (0.47 mL, 0.47 mmol) was added. The reaction mixture was stirred for 20 h at room temperature, acidified to pH 1 with 1 M HCl, saturated with solid NaCl, and extracted with Et_2O (3×50 mL). After the combined organic phases were washed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure, purification by silica gel chromatography (R_f 0.1, hexane:EtOAc = 100:10) gave 16,16-[$^2H]_2$-**1** (5 mg, 59%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.34−5.46 (8H, m), 2.82−2.87 (6H, m), 2.39 (2H, t, J = 7.5 Hz), 2.16 (2H, t−d, J = 7.0, 7.0 Hz), 1.74 (2H, quin, J = 7.2 Hz), 1.28−1.38 (6H, m), 0.91 (3H, t, J = 7.0 Hz); MS (ESI) (*m*/*z*) 305 ($M^+ - 1$); MS (EI) (*m*/*z*) 305 ($M^+ - 1$); HRMS (EI) (*m*/*z*) calcd for $C_{20}H_{30}D_2O_2$ 306.252784, found 306.25353; deuterium content 98% as determined by ESI-MS comparison with unlabeled arachidonic acid.

13(*R*)-[^{2}H]-Arachidonic Acid. (10-Methoxycarbonyldeca-3,6-*cis*,-*cis*-dienyl)triphenylphosphonium bromide (**5**) (193 mg, 0.36 mmol) was thoroughly dried by repeated evaporation from anhydrous benzene (4×10 mL). The compound was dissolved in THF (1.56 mL) and the solution cooled to −70 °C. A 1 M solution of sodium bis(trimethylsilyl)-amide (0.34 mL, 0.34 mmol) in THF was added, and the reaction mixture was stirred for 1 h at −70 °C, and then further cooled to −90 °C. 2(*R*)-[^{2}H]-3-*cis*-nonenal **6b** (20 mg, 0.14 mmol), dissolved in THF (1 mL), was added to the solution of the ylide. After 0.5 h at −90 °C, the reaction was allowed to warm to 0 °C over 5 h. Saturated aqueous sodium bicarbonate was added, and the mixture was extracted with Et_2O (3×50 mL). The combined organic phases were washed with brine, dried over anhydrous sodium sulfate, concentrated, and then purified by silica gel chromatography (R_f 0.5, hexane:EtOAc = 100:5) to provide methyl 13(*R*)-[^{2}H]-arachidonate as a colorless oil (20 mg,

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44%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.41−5.37 (8H, m), 3.68 (3H, s), 2.87−2.81 (5H, m), 2.34 (2H, t, J = 7.5 Hz), 2.15−2.05 (4H, m), 1.72 (2H, quin, J = 7.7 Hz), 1.41−1.25 (6H, m), 0.91 (3H, t, J = 7.0 Hz); ^{13}C NMR (125 MHz) δ 174.31, 130.74, 129.16, 129.11, 128.76, 128.44, 128.40, 128.11, 127.72, 51.70, 33.65, 31.74, 29.54, 27.43, 26.76, 25.85, 25.83, 25.53 (t, J = 19.3 Hz), 25.00, 22.79, 14.28; FIMS (m/z) 319 (M^+, 100). Methyl 13(*R*)-[^{2}H]-arachidonate (7 mg, 0.022 mmol) was dissolved in THF (0.37 mL). To this solution was added a 1 M aqueous solution of LiOH (0.37 mL, 0.47 mmol) at 0 °C, and the reaction was stirred for 15 h at room temperature. The solution was acidified to pH 1 with 1 M aqueous HCl, saturated with solid sodium chloride, and extracted with Et_2O (3 × 50 mL). After the combined organic phases were washed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure, purification by silica gel chromatography (R_f 0.2, hexane:EtOAc = 10:1) gave 13(*R*)-[^{2}H]-**1** (5.4 mg, 81%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.46−5.34 (8H, m), 2.87−2.82 (5H, m), 2.39 (2H, t, J = 7.4 Hz), 2.16 (2H, dt, J = 6.8, 6.8 Hz), 2.07 (2H, dt, J = 7.1, 7.1 Hz), 1.74 (2H, quin, J = 7.5 Hz); MS (ESI) (m/z) 304 ($M^+ - 1$, 100); HRMS (ESI) (m/z) calcd for $C_{20}H_{30}DO_2$ 304.2387, found 304.2402; deuterium content 93% as determined by ESI-MS comparison with unlabeled arachidonic acid.

13(*R*),15-[$^2H_2$]-Arachidonic Acid. 5 (116 mg, 0.22 mmol) was thoroughly dried by four cycles of azeotropic evaporation of anhydrous benzene and dissolved in THF (2 mL). After the solution was cooled at −70 °C, a 1 M solution of NaHMDS (0.204 mL, 0.204 mmol) in THF was added, and the reaction was stirred for 1 h at −70 °C and then cooled to −90 °C. 2(*R*),4-[^{2}H]$_2$-Non-3-*cis*-enal **6c** (11 mg, 0.14 mmol) dissolved in THF (1 mL) was added to the solution of the ylide. After 0.5 h at −90 °C, the reaction was allowed to warm to 0 °C over 5 h. Saturated aqueous sodium bicarbonate was added, and the aqueous layer was extracted with Et_2O (3 × 30 mL). The combined organic phases were washed with brine, dried over anhydrous sodium sulfate, concentrated, and purified by silica gel chromatography (R_f 0.5, hexane:EtOAc = 100:5) to provide methyl 13(*R*),15-[^{2}H]$_2$-arachidonate as a light yellow oil (10 mg, 40%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.41−5.37 (7H, m), 3.69(3H, s), 2.86−2.81 (5H, m), 2.34 (2H, t, J = 6.7 Hz), 2.15−2.11 (2H, m), 2.07 (2H, t, J = 7.0 Hz), 1.73 (2H, quin, J = 7.7 Hz), 1.41−1.24 (6H, m), 0.90 (3H, t, J = 7.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.33, 129.17, 129.11, 128.77, 128.45, 128.39, 128.11, 127.59, 51.72, 33.65, 31.74, 29.52, 27.33, 26.76, 25.84, 25.82, 24.99, 22.79, 14.29. To a solution of methyl 13(*R*),15-[^{2}H]$_2$-arachidonate (10 mg, 0.031 mmol) in THF (0.57 mL) was added a 1 M aqueous solution of LiOH (0.57 mL, 0.57 mmol) at 0 °C. After being stirred for 18 h at room temperature, the solution was acidified to pH 1 with 1 M HCl, saturated with solid NaCl, and extracted with Et_2O (3 × 50 mL). After the combined organic phases were washed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure, silica gel chromatography (R_f 0.2, hexane:EtOAc = 100:10) gave 13-(*R*),15-[^{2}H]$_2$-**1** (7 mg, 73%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.46−5.35 (7H, m), 2.88−2.79 (5H, m), 2.40 (2H, t, J = 7.2 Hz), 2.17−2.13 (2H, m), 2.70 (2H, t, J = 7.2 Hz), 1.74 (2H, quin, J = 7.3 Hz), 1.39−1.27 (6H, m), 0.90 (3H, t, J = 7.0 Hz); ESIMS (m/z) 305 ($M^+ - 1$, 100); deuterium content (for two ^{2}H) 93% as determined by ESI-MS comparison with unlabeled arachidonic acid.

13(S)-[^{2}H]-Arachidonic Acid. Hexyltriphenylphosphonium bromide (103 mg, 0.24 mmol) prepared by reflux of 1-bromohexane with triphenylphosphine was thoroughly dried by four cycles of azeotropic distillation of anhydrous benzene and diluted in THF (3 mL). A 1 M solution of NaHMDS in THF (0.23 mL, 0.23 mmol) was added at −10 °C, and the bright orange ylide was stirred at room temperature for 1 h. Then the mixture was cooled to −90 °C, and a solution of aldehyde **13a** (30 mg, 0.071 mmol) in THF (1 mL) was added dropwise. After 0.5 h at −90 °C, the reaction was allowed to warm to room temperature over 5 h. Saturated aqueous sodium bicarbonate was added, and the aqueous layer was extracted with Et_2O (3 × 50 mL). The combined organic phase was washed with brine, dried over anhydrous sodium sulfate, concentrated, and passed through a silica gel column (R_f = 0.5, hexane:EtOAc = 100:5) to provide methyl 13(*S*)-[^{2}H]-arachidonate (15 mg, 40%) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.41−5.37 (8H, m), 3.68 (3H, S), 2.87−2.81 (5H, m), 2.34 (2H, t, J = 7.5 Hz), 2.15−2.05 (4H, m), 1.72 (2H, quin, J = 7.7 Hz), 1.41−1.25 (6H, m), 0.91 (3H, t, J = 7.0 Hz). To a solution of methyl 13(*S*)-[^{2}H]-arachidonate (9 mg, 0.47 mmol) in THF (0.47 mL) was added a 1 M aqueous solution of lithium hydroxide (0.47 mL, 0.47 mmol) at 0 °C. After being stirred at room temperature for 22 h, the mixture was acidified to pH 1 with 1 M HCl and saturated with solid sodium chloride. Extraction with Et_2O (4 × 20 mL), drying over anhydrous sodium sulfate, concentration under reduced pressure, and chromatography on silica gel (R_f = 0.2, hexane:EtOAc = 10:1) gave 13(*S*)-[^{2}H]-**1** (8 mg, 93%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.460−5.335 (8H, m), 2.869−2.819 (5H, m), 2.390 (2H, t, J = 7.4 Hz), 2.158 (2H, dt, J = 6.8, 6.8 Hz), 2.067 (2H, dt, J = 7.1, 7.1 Hz), 1.741 (2H, quin, J = 7.5 Hz); MS (ESI) (m/z) 304 ($M^+ - 1$); deuterium content 93% as determined by ESI-MS comparison with unlabeled arachidonic acid.

11-[^{2}H]-Arachidonic Acid. (3-[^{2}H]-Dodeca-3,6-*cis*,*cis*-dienyl)triphenylphosphonium bromide (**21**) (200 mg, 0.4 mmol) was thoroughly dried by azeotropic distillation with anhydrous benzene (4 × 15 mL) and diluted with THF (4 mL). A 1 M solution of NaHMDS in THF (0.37 mL, 0.37 mmol) was added at −70 °C, and the bright orange ylide was stirred for 1 h. After the mixture was cooled to −100 °C, a solution of 8-oxo-oct-5-*cis*-enoic acid methyl ester (70 mg, 0.4 mmol) in THF (1 mL) was added. The reaction mixture was warmed to 0 °C over 5 h, and saturated aqueous ammonium chloride was added to quench the reaction. After workup and silica gel chromatography (R_f 0.5, hexane:EtOAc = 100:5), methyl 11-[^{2}H]-arachidonate (85 mg, 67%) was obtained as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.32−5.45 (7H, m), 3.68 (3H, s), 2.82−2.86 (6H, m), 2.34 (2H, t, J = 7.5 Hz), 2.13 (2H, dd, J = 7.0 Hz, 7.0 Hz), 2.07 (2H, dd, J = 7.1 Hz, 7.1 Hz), 1.73 (2H, quin, J = 7.3 Hz), 1.26−1.41 (6H, m), 0.91 (3H, t, J = 7.0 Hz); ^{13}C NMR (125 MHz) δ 174.32, 130.715, 129.16, 129.11, 128.67, 128.42, 128.40, 127.78, 51.70, 33.64, 31.74, 30.52, 29.55, 27.44, 26.77, 25.81, 25.73, 24.99, 22.79, 14.29. To a solution of methyl 11-[^{2}H]-arachidonate (20 mg, 0.06 mmol) in THF (1 mL) was added 1 M lithium hydroxide in H_2O (1 mL, 1 mmol) at 0 °C. After the reaction was stirred at room temperature for 15 h, HCl (1 N) was added to adjust the pH to 1. Extraction with Et_2O (4 × 30 mL), drying over Na_2SO_4, concentration, and chromatography on silica gel (R_f 0.15, hexane:EtOAc = 100:10) provided the compound as a colorless oil (19 mg, 99%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32−5.47 (7H, m), 2.81−2.86 (6H, m), 2.39 (2H, t, J = 7.5 Hz), 2.15 (2H, dd, J = 7.0 Hz, 7.0 Hz), 2.07 (2H, dd, J = 7.0 Hz, 7.0 Hz), 1.73 (2H, quin, J = 7.5 Hz), 1.27−1.42 (6H, m), 0.91 (3H, t, J = 7.0 Hz); MS (ESI) (m/z) 304 ($M^+ - 1$); HRMS (ESI) (m/z) calcd for $C_{20}H_{30}DO_2$ 304.2387, found 304.2361; deuterium content >97% as determined by ESI-MS comparison with unlabeled arachidonic acid.

Determination of the Stereochemical Purity of Labeled Arachidonic Acids. The assays were carried out at 4 °C in 2 mL of 0.1 M Tris−HCl buffer (pH 8.5) containing 1 mM phenol.[58] SLO-1 (60 × 10^3 units) was added to the buffer, followed by dropwise addition of arachidonic acid (1 mg, 0.0033 mmol) dissolved in 80 μL of 0.01 M aqueous NH_4OH. A relatively large amount of SLO-1 was used to avoid any artificial enrichment of 13-[^{2}H]-HPETE because of a possible large selection against 13(*S*)-[^{2}H]-**1** as reported for the reaction of linoleic acid and SLO-1.[33] After completion of the enzymatic reaction, the assay mixture was acidified with 1 M HCl to pH 3. The lipid product was extracted into Et_2O (2 × 20 mL), and the pooled extracts were washed three times with ice-cold water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to give crude HPETE, a sample of which was submitted to MS directly without further purification. Subsequently, 2 mg of triphenylphosphine was added to the remaining

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HPETE dissolved in Et_2O to give HETE, which was also analyzed by ESI-MS. Starting with 13(*R*)-[^{2}H]-arachidonic acid containing 93% deuterium, the deuterium contents of 13-[^{2}H]-HPETE and 13-[^{2}H]-HETE were 88%. Thus, the enantiomeric ratio of the arachidonic acid is 95:5. Starting with 13(*R*),15-[^{2}H]$_2$-arachidonic acid containing 95% bisdeuterated material, the isotope content of 13,15-[^{2}H]$_2$-HPETE was 91% (bisdeuterated). Therefore, the enantiomeric ratio for 13(*R*),15-[^{2}H]$_2$-**1** is 96:4.

Enzyme Purification and EPR Experiments. Recombinant human PGHS-2 apoenzyme was purified from material expressed in High Five insect cells infected with a Baculoviral vector.[59] Cyclooxygenase activity was measured at 30 °C in 0.1 M potassium phosphate, pH 7.2, containing 1.0 mM phenol, 100 μM arachidonate, and 1 μM heme using an oxygen electrode.[60] The PGHS-2 batches used in the present studies had specific activities of 20−35 μmol of O_2/min/mg of protein. PGHS-2 holoenzyme was prepared by incubation with stoichiometric amounts of heme in the presence of 50 μM phenol; excess heme was removed by treatment with DEAE cellulose and gel filtration[61] in buffer containing 50 μM phenol and 0.1% Tween 20. Single turnover experiments using an anaerobic titrator were performed as previously described.[14,15] Briefly, 10 μM PGHS-2 was incubated under anaerobic conditions with 10 equiv of EtOOH. After 10−15 s the substrate was added, and two samples were frozen in an EPR tube at about 15 and 60 s after substrate addition. EPR spectra were recorded at liquid nitrogen temperatures on a Varian E-6 spectrometer. The instrument settings are given in the captions of Figures 3 and 4. Radical concentrations were determined by double integration of the EPR signals, with reference to a copper standard. Computer simulations of the EPR spectra were performed on a PC using a modified version of the POWFUN program[62] kindly provided by Drs. Gerald T. Babcock and Curt Hoganson, Michigan State University.

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Supporting Information Available: Details of the synthesis and characterization for **2**−**5**, **6b**,**c**, **13**, **13a**, **17a**,**b**, and **19**−**21**; negative ion mode ESI-MS of 15-HPETE (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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