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Structural Characterization of a Pentadienyl Radical Intermediate Formed during Catalysis by Prostaglandin H Synthase-2

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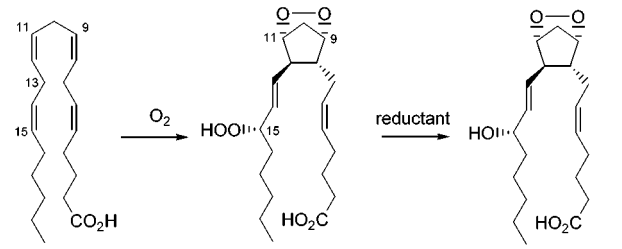
Prostaglandin H synthase (PGHS) or cyclooxygenase (COX) catalyzes the first committed step in the biosynthesis of all prostaglandins and thromboxanes, the conversion of arachidonic acid (**1**) into prostaglandin H_2 (Scheme 1).[1] These compounds are important mediators in inflammation, and as such, the enzyme has been of great interest for the development of antiinflammatory agents.[2] We report here the structural characterization of a radical intermediate by electron paramagnetic resonance (EPR) spectroscopy in combination with stereospecifically deuterated substrates.

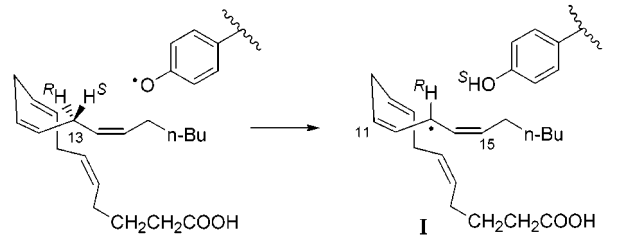
The discovery of two isozymes in mammalian cells, COX1 and COX2,[3] has allowed important improvements in nonsteroidal antiinflammatory drugs (NSAIDs).[4] COX1 is generally a constitutively expressed protein, whereas COX2 expression is induced in specific tissues in response to certain stimuli.[5] Recently developed COX2 selective inhibitors lack many of the toxic side effects observed with nonselective inhibitors.[6]

The chemical mechanism for the conversion of arachidonic acid to prostaglandin G_2 in the cyclooxygenase reaction still holds many unanswered questions. The overall mechanistic proposal by Hamberg and Samuelsson in 1967[7] still largely stands, although direct support for most of the steps and intermediates is lacking. Important advances since 1967 have been the identification of a tyrosyl radical as the initiator of catalysis,[8,9] the detection of a substrate-based radical by EPR spectroscopy (Scheme 2),[9] and very recently, the crystallographic characterization of substrate and PGH_2 bound to the enzyme.[10] However, the precise structure of the substrate radical needs further investigation. Tsai and coworkers have proposed that the radical is a delocalized pentadienyl radical spanning positions C11−C15 of arachidonic acid (i.e., **I,** Scheme 2).[9]

Scheme 1



Scheme 2



Pentadienyl radicals have an odd-alternate spin distribution, and therefore, the proposed radical in PGHS is expected to have significant spin density at C11, C13, and C15. Site-specifically deuterated arachidonic acids can contribute to corroboration of the proposed structure, as their reaction with the enzyme should lead to predictable changes in the hyperfine pattern observed in the EPR spectrum of **I**. We therefore prepared (*R*)-[13-^{2}H]-**1**, (*R*)-[13,15-$^2H_2$]-**1**, and [15-^{2}H]-**1** as structural probes.

A chemoenzymatic synthesis of (*R*)-[13-^{3}H]-**1** has been reported previously,[11] but this route was not feasible for our purposes as it would lead to partially deuterated products due to isotope dilution during in vivo conversion of labeled stearate to arachidonic acid. Our entirely synthetic route was designed to allow preparation of all three target compounds from one common advanced intermediate, aldehyde **2**[12] (Scheme 3). [15-^{2}H]-**1** was prepared by Wittig reaction of **2** with phosphonium salt **3**, obtained in four steps from [1-^{2}H]-hexanal. Arachidonic acid stereospecifically deuterium-labeled at C13 was produced from **2** by conversion into phosphonium salt **4**, followed by Wittig olefination of aldehyde **5**. This compound was prepared in three steps from phosphonium salt **6**[13] and hexanal. The doubly labeled arachidonic acid, (*R*)-[13,15-$^2H_2$]-**1**, was prepared by the same route using [1-^{2}H]-hexanal. The isotopic purity of the synthetic substrates was assessed by field ionization and electrospray mass spectrometry,[14] and the stereochemical purity of (*R*)-[13-^{2}H]-**1**

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(1) Smith, W. L.; DeWitt, D. L.; Garavito, R. M. *Annu. Rev. Biochem.* **2000**, *69*, 145−182.
(2) Vane, J. R.; Bakhle, Y. S.; Botting, R. M. *Annu. Rev. Pharmacol. Toxicol.* **1998**, *38*, 97−120. (b) Dubois, R. N.; Abramson, S. B.; Crofford, L.; Gupta, R. A.; Simon, L. S.; Van De Putte, L. B.; Lipsky, P. E. *FASEB J* **1998**, *12*, 1063−1073.
(3) (a) Xie, W.; Chipman, J. G.; Robertson, D. L.; Erickson, R. L.; Simmons, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 2692−2696. (b) Kujubu, D. A.; Fletcher, B. S.; Varnum, B. C.; Lim, R. W.; Herschman, H. R. *J. Biol. Chem.* **1991**, *266*, 12866−12872.
(4) (a) Vane, J. R.; Botting, R. M. *Clinical Significance and Potential of Selective Cox-2 Inhibitors*; William Harvey Press: London, 1998. (b) Marnett, L. J.; Kalgutkar, A. S. *Curr. Opin. Chem. Biol.* **1998**, *2*, 482−490.
(5) Herschman, H. R. *Biochim. Biophys. Acta* **1996**, *1299*, 125−140.
(6) (a) Penning, T. D.; Talley, J. J.; Bertenshaw, S. R.; Carter, J. S.; Collins, P. W.; Docter, S.; Graneto, M. J.; Lee, L. F.; Malecha, J. W.; Miyashiro, J. M.; Rogers, R. S.; Rogier, D. J.; Yu, S. S.; Anderson, G. D.; Burton, E. G.; Cogburn, J. N.; Gregory, S. A.; Koboldt, C. M.; Perkins, W. E.; Seibert, K.; Veenhuizen, A. W.; Zhang, Y. Y.; Isakson, P. C. *J. Med. Chem.* **1997**, *40*, 1347−1365. (b) Silverstein, F. E.; Faich, G.; Goldstein, J. L.; Simon, L. S.; Pincus, T.; Whelton, A.; Makuch, R.; Eisen, G.; Agrawal, N. M.; Stenson, W. F.; Burr, A. M.; Zhao, W. W.; Kent, J. D.; Lefkowith, J. B.; Verburg, K. M.; Geis, G. S. *J. Am. Med. Assoc.* **2000**, *284*, 1247−1255.
(7) Hamberg, M.; Samuelsson, B. *J. Biol. Chem.* **1967**, *242*, 5336−5343.
(8) (a) Karthein, R.; Dietz, R.; Nastainczyk, W.; Ruf, H. H. *Eur. J. Biochem.* **1988**, *171*, 313−320. (b) Tsai, A.-L.; Kulmacz, R. J. *Prostaglandins Lipid Med.* **2000**, *62*, 231−254.
(9) (a) Tsai, A.-L.; Kulmacz, R. J.; Palmer, G. *J. Biol. Chem.* **1995**, *270*, 10503−10508. (b) Tsai, A. L.; Palmer, G.; Xiao, G. S.; Swinney, D. C.; Kulmacz, R. J. *J. Biol. Chem.* **1998**, *273*, 3888−3894.
(10) (a) Malkowski, M. G.; Ginell, S. L.; Smith, W. L.; Garavito, R. M. *Science* **2000**, *289*, 1933−7. (b) Kiefer, J. R.; Pawlitz, J. L.; Moreland, K. T.; Stegeman, R. A.; Hood, W. F.; Gierse, J. K.; Stevens, A. M.; Goodwin, D. C.; Rowlinson, S. W.; Marnett, L. J.; Stallings, W. C.; Kurumbail, R. G. *Nature* **2000**, *405*, 97−101.
(11) (a) Schneider, C.; Boeglin, W. E.; Lai, S.; Cha, J. K.; Brash, A. R. *Anal. Biochem.* **2000**, *284*, 125−135. (b) Schneider, C.; Brash, A. R. *J. Biol. Chem.* **2000**, *275*, 4743−4736.
(12) Viala, J.; Sandri, J. *Tetrahedron Lett.* **1992**, *34*, 4897−4900.
(13) Corey, E. J.; Lansbury, P. T. *J. Am. Chem. Soc.* **1983**, *105*, 4093−4094.
(14) Isotopic purity was determined by comparison of mass spectra obtained for unlabeled and labeled synthetic material.

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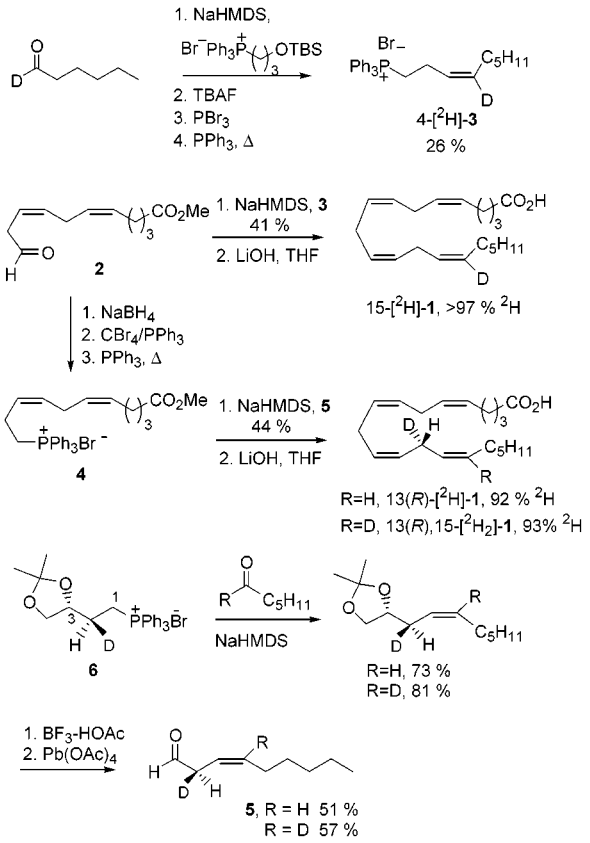
Scheme 3



and the doubly labeled compound was determined by incubation with soybean lipoxygenase. This enzyme converts **1** to 15-(*S*)-hydroperoxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid (15-H-PETE) by stereospecific abstraction of the *pro-S* hydrogen atom from position 13 of the substrate.[15] Comparison of the mass spectra of the enzymatic products from labeled and unlabeled **1** gave an estimated lower limit of 96:4 and 98:2 er., respectively, for the singly and doubly labeled arachidonic acids.

The three synthetically labeled substrates were used for characterization of the radical signal observed previously by EPR spectroscopy after anaerobic incubation of COX2 with arachidonic acid.[9] Similar to these earlier studies, unlabeled substrate resulted in a seven-line signal (Figure 1A). Consistent with the proposal that this signal derives from a pentadienyl radical spanning C11−C15 (Scheme 2), substitution of the proton at C15 with a deuterium resulted in a spectrum that can be simulated as a six-line multiplet (Figure 1B).[16] Similarly, incubation of COX2 with

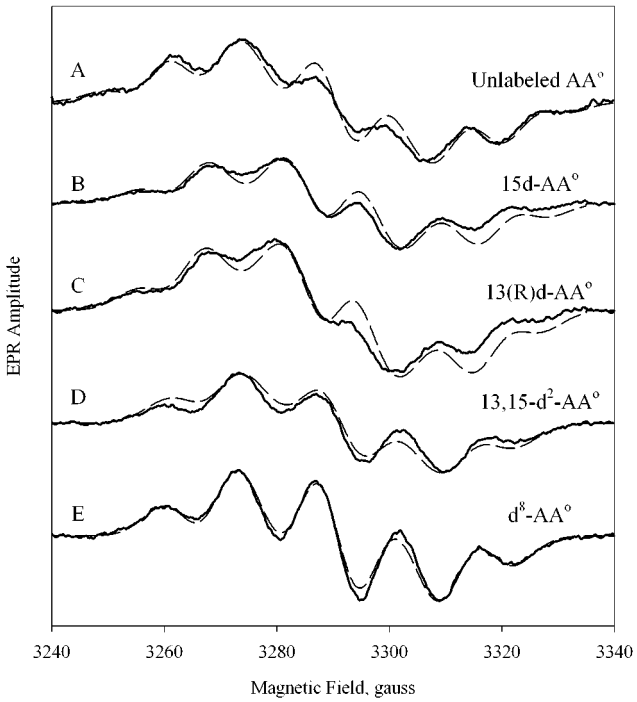


Figure 1. 9 GHz EPR spectra obtained by anaerobic incubation of COX2 (11−14 μM) with (A) unlabeled **1**, (B) 15-[^{2}H]-**1**, (C) 13(*R*)-[^{2}H]-**1**, (D) 13,15-[^{2}H$_2$]-**1**, and (E) 5,6,8,9,11,12,14,15-[^{2}H$_8$]-**1**. All samples were frozen approximately 60 s after addition of the substrate at 4 °C. The overlaid spectra (- - -) show simulations. For instrument settings and simulation parameters, see Supporting Information.

(*R*)-[13-^{2}H]-**1** provided a six-line signal (Figure 1C), whereas the doubly labeled compound gave rise to a five-line signal (Figure 1D). These observations provide support for spin density at C13 and C15. Finally, reaction of octadeuterated arachidonic acid gave rise to a five-line spectrum (Figure 1E), in good agreement with the predicted changes for the proposed pentadienyl radical in Scheme 2, as only two (at C11 and C15) of the eight deuteriums are in positions that would alter the hyperfine pattern. It is important to note that for all simulations in Figure 1 the parameters, which are consistent with known pentadienyl radicals,[17] were kept invariant with the exception of adjusting the size of the hyperfine value of protons substituted with deuterons.[9,16] Thus, the spectra in Figure 1 collectively provide strong support for the assignment of the radical signal to a C11−C15 pentadienyl radical.[18] This is direct[19] experimental verification of the structure of an intermediate in the original[7] mechanism of formation of prostaglandins by cyclooxygenase.

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Supporting Information Available: Spectral data and experimental procedures for all new compounds as well as a detailed description of the determination of isotopic and optical purity of the final products (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Hamberg, M.; Samuelsson, B. *J. Biol. Chem.* **1967**, *242*, 5329−35.
(16) This reduction in the multiplicity is the result of the substantially smaller gyromagnetic ratio (γ) for ^{2}H compared to ^{1}H ($\gamma_D/\gamma_H = 0.15$). The triplet hyperfine splitting of the deuteron 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.
(17) Davies, A. G.; Griller, D.; Ingold, K. U.; Lindsay, D. A.; Walton, J. C. *J. Chem. Soc., Perkin Trans. 2* **1981**, 633−641.
(18) Exposure of the observed radical to O_2 regenerates the tyrosyl radical, consistent with its chemical competence in catalysis.[9]
(19) The same radical may have been detected indirectly in radical trap studies: Schreiber, J.; Eling, T. E.; Mason, R. P. *Arch. Biochem. Biophys.* **1986**, *249*, 126−36.