

# Deciphering the Cryptoregiochemistry of Oleate $\Delta^{12}$ Desaturase: A Kinetic Isotope Effect Study

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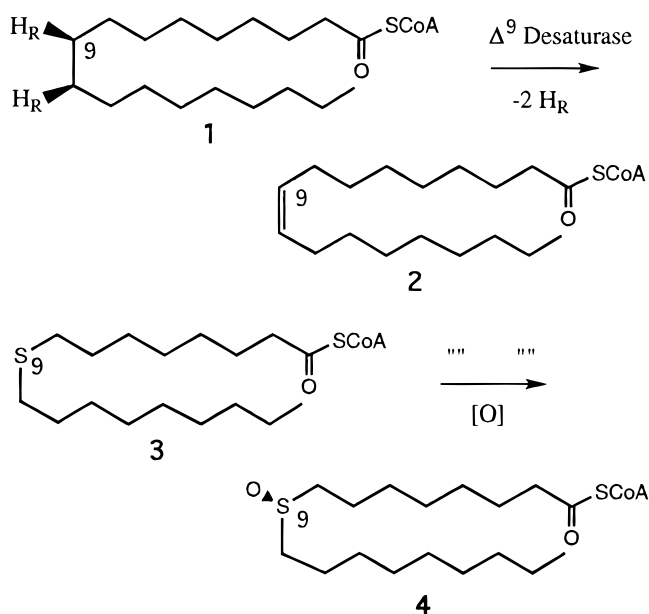
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**Abstract:** The intermolecular primary deuterium isotope effects on the individual C–H bond cleavage steps involved in linoleic acid biosynthesis were determined using a suitably transformed strain of *Saccharomyces cerevisiae* containing a functional oleate  $\Delta^{12}$  desaturase from *Arabidopsis thaliana*. Mass spectral analysis of the methyl 7-thialinoleate fraction obtained from competition experiments involving methyl 7-thiastearate, methyl [12,12- $^2\text{H}_2$ ]-7-thiastearate and methyl [13,13- $^2\text{H}_2$ ]-7-thiastearate showed that cleavage of the  $\text{C}_{12}$ –H bond is very sensitive to isotopic substitution ( $k_{\text{H}}/k_{\text{D}} = 7.3 \pm 0.4$ ) while a negligible isotope effect ( $k_{\text{H}}/k_{\text{D}} = 1.05 \pm 0.04$ ) was observed for the  $\text{C}_{13}$ –H bond breaking step. This result strongly suggests that the site of initial oxidation for  $\Delta^{12}$  desaturation is at C-12. The possible relationship between castor oleate 12-hydroxylase and microsomal  $\Delta^{12}$  oleate desaturases is discussed in the context of a common mechanistic paradigm. Our methodology may be also be useful in deciphering the cryptoregiochemistry of other desaturase systems.

## Introduction

Fatty acid desaturases constitute a ubiquitous family of  $\text{O}_2$ -dependent, nonheme iron-containing enzymes which catalyze the regio- and stereoselective syn dehydrogenation (desaturation) of unactivated fatty acyl hydrocarbon chains.<sup>1</sup> One of the most important biological roles of the desaturation reaction is to increase the fluidity of cell membranes by introducing a (Z)-olefinic bond into the phospholipid component.<sup>2</sup> The first example of this intriguing process was discovered some 40 years ago by Bloch et al. who showed that a microsomal fraction of *Saccharomyces cerevisiae* (Bakers' yeast) mediated the aerobic transformation of stearoyl CoA (1) to give oleoyl CoA (2).<sup>3</sup> Many more desaturases with a wide variety of unique regiochemical selectivities have subsequently been identified.<sup>4</sup> Shanklin and Fox have compared the amino acid sequences of a number of fatty acid desaturases and have concluded that there appear to be two discrete classes of enzymes. The first set are comprised of soluble plant desaturases<sup>5a</sup> which are characterized by the presence of a carboxylate-bridged, nonheme diiron cluster;<sup>6</sup> these enzymes also require the fatty acid substrate to be linked to acyl carrier protein (ACP). An X-ray structure of the  $\Delta^9$  stearoyl-ACP desaturase from castor seed has been obtained recently.<sup>7</sup> The second group of desaturases,<sup>5b</sup> exempli-



fied by the  $\Delta^9$  desaturase from *S. cerevisiae*<sup>8</sup> and rat liver<sup>9</sup> are less well-characterized since they are membrane-bound and relatively unstable. Sequence analysis<sup>5b</sup> has shown that these proteins possess eight highly conserved histidine residues which are thought to act as ligands for a diiron center.<sup>10</sup>

Very little work on the detailed mechanism of fatty acid desaturation has emerged over the past four decades. There is a steadily growing body of circumstantial evidence,<sup>11</sup> however, which suggests that biological dehydrogenation reactions of this

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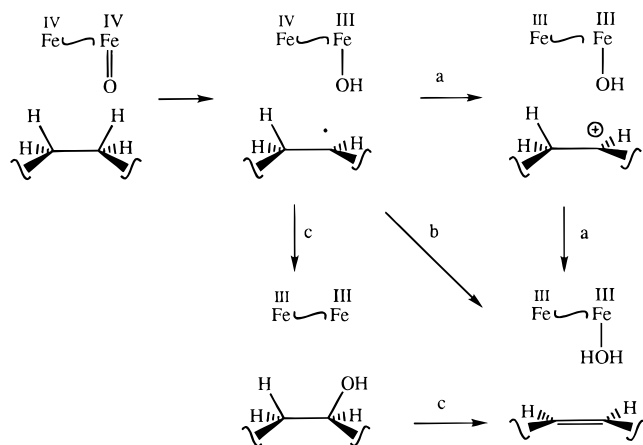
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## Scheme 1



type and the much better studied biohydroxylation of unactivated hydrocarbons follow similar reaction mechanisms. A possible mechanistic scheme<sup>16</sup> relating hydroxylation and desaturation is shown in Scheme 1: one might envisage an initial hydrogen atom abstraction step by a hypervalent iron-oxo species which generates a carbon-centered radical "intermediate"<sup>17</sup> or its iron-bound equivalent<sup>19</sup> (not shown). This species could collapse to olefin via a one electron oxidation/deprotonation sequence (pathway a),<sup>20</sup> simple disproportionation (pathway b),<sup>21</sup> or by a rapid Lewis acid ( $\text{Fe}^{3+}$ )-catalyzed dehydration of an alcohol intermediate (pathway c).<sup>22</sup> The role of the second iron atom

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(11) The evidence includes the following (a) the diiron active site of the soluble plant  $\Delta^9$  desaturase is similar to that found in methane monooxygenase (sMMO)<sup>12</sup>—an enzyme which hydroxylates alkanes; (b) the highly conserved multi-His motif of the membrane-bound desaturases is also found in the alkane  $\omega$ -hydroxylase from *P. oleovorans*;<sup>10</sup> (c) hepatic cytochrome *P*<sub>450</sub>—a known hydroxylator also dehydrogenates selected substrates;<sup>13</sup> (d) a mutated yeast  $\Delta^{22}$  sterol desaturase yields a 23-hydroxy sterol;<sup>14</sup> and (e) clavaminic acid synthase can either act as a hydroxylase or a desaturase depending on the nature of the substrate.<sup>15</sup>

(12) Rosenzweig, A. C.; Frederick, C. A.; Lippard, S. J.; Nordlund, P. *Nature* **1993**, *366*, 537.

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(16) We have depicted the oxidant as a diiron species since very recent Mössbauer studies<sup>10</sup> have shown that the closely related alkane  $\omega$ -hydroxylase possesses a catalytically active diiron site.

(17) (a) Groves, J. T.; McClusky, G. A.; White, R. E.; Coon, M. J. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 154. (b) Bowry, V. W.; Luszytk, J.; Ingold, K. U. *J. Am. Chem. Soc.* **1991**, *113*, 5699. (c) Newcomb has suggested that this species is not a true intermediate but behaves as "a component of a reacting ensemble" with a very short lifetime; accordingly it has been postulated that this type of hydrocarbon activation proceeds by "a nonsynchronous, concerted oxygen insertion" mechanism: cf., Newcomb, M.; Le Tadic-Biadetti, F. H.; Chestney, D. L.; Roberts, E. S.; Hollenberg, P. F. *J. Am. Chem. Soc.* **1995**, *117*, 12085. Additional support for such a mechanism as it applies to sMMO has recently been obtained using chiral methyl group experiments.<sup>18</sup>

(18) Valentine, A. M.; Wilkinson, B.; Liu, K. E.; Komar-Panicucci, S.; Priestley, N. D.; Williams, P. G.; Morimoto, H.; Floss, H. G.; Lippard, S. J. *J. Am. Chem. Soc.* **1997**, *119*, 1818.

(19) (a) Siegbahn, P. E. M.; Crabtree, R. H. *J. Am. Chem. Soc.* **1997**, *119*, 3103. (b) Blackburn, J. M.; Sutherland, J. D.; Baldwin, J. E. *Biochemistry* **1995**, *34*, 7548. (c) Baldwin, J. E.; Adlington, R. M.; Marquess, D. G.; Pitt, A. R.; Porter, M. J.; Russell, A. T. *Tetrahedron* **1996**, *52*, 2525.

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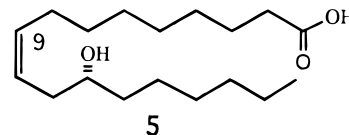
(21) Akhtar, M.; Wright, J. N. *Nat. Prod. Rep.* **1991**, *8*, 527.

(22) We consider pathway c to be the least likely route to olefin because the available evidence<sup>23</sup> suggests that alcohols are not discrete intermediates in fatty acid desaturation.

could be to act as an electron sink,<sup>24</sup> or it could be more intimately involved in the hydrocarbon activation step.<sup>18</sup>

On the basis of Scheme 1, one would predict that if a desaturase could be induced to act as an oxygenase, the regioselectivity of oxygenation should match that of the hydrogen abstraction step involved in the corresponding desaturation reaction. We have demonstrated that this is indeed the case using an *in vivo*, *S. cerevisiae*  $\Delta^9$  desaturase system: 9-thia fatty acid analogues such as **3** were consistently oxygenated more efficiently than the corresponding 10-thia analogues to give the corresponding sulfoxides with the correct absolute stereochemistry and with very high enantiomeric purity.<sup>25</sup> The site of initial oxidative attack for the parent  $\Delta^9$  desaturation reaction was subsequently found to be C-9, as anticipated, using a KIE approach.<sup>26</sup> In this study, we took advantage of the fact that according to our mechanistic scheme, initial hydrogen abstraction should be slow relative to the second C-H bond cleavage and, a priori, more sensitive to isotopic substitution. Thus a large primary deuterium isotope effect was observed for C-H bond cleavage at C-9 while a negligible isotope effect was obtained for the C<sub>10</sub>-H bond breaking step.

Another example of an apparent diverted desaturation is evident in the biosynthesis of ricinoleic acid (**5**, (*R*)-12-hydroxyoleic acid)—a rare but important natural product which accumulates in the seed oil of the castor plant (*Ricinus communis* L.). Ricinoleic acid has been termed "one of the world's most



versatile natural products"<sup>27a</sup> because it is used directly as a component of lubricants, soaps, adhesives, inks, cosmetics, etc., and also serves as an industrial feedstock for a variety of important materials such as plasticizers, Nylon-11, polyesters, and polyurethanes.<sup>27b</sup> Interest in the enzyme system responsible for the synthesis of this hydroxy acid has intensified recently in the context of efforts to broaden the scope of its agricultural production.<sup>27a</sup> It has been shown that ricinoleic acid is produced by a membrane-bound oleate 12-hydroxylase which bears a striking resemblance to the more common oleate  $\Delta^{12}$ -desaturase in terms of the stereochemistry of C-H cleavage,<sup>28a,b</sup> substrate requirements,<sup>28c</sup> and molecular weight.<sup>28d</sup> On the basis of a detailed comparison between the amino acid sequence of the microsomal *Arabidopsis*  $\Delta^{12}$  oleate desaturase and that of castor oleate hydroxylase, van de Loo et al. have suggested that ricinoleic acid is produced by a subtly modified oleate  $\Delta^{12}$  desaturase.<sup>28d</sup> We thought it would be interesting to determine whether the putative "parent" oleate  $\Delta^{12}$  desaturase also initiates the oxidation of substrate at C-12. In this article, we document

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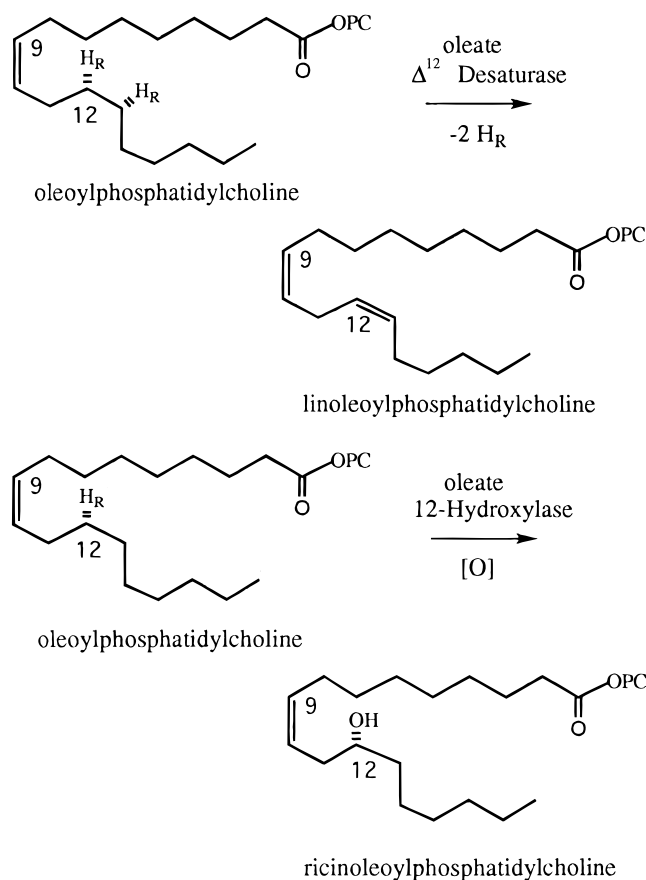
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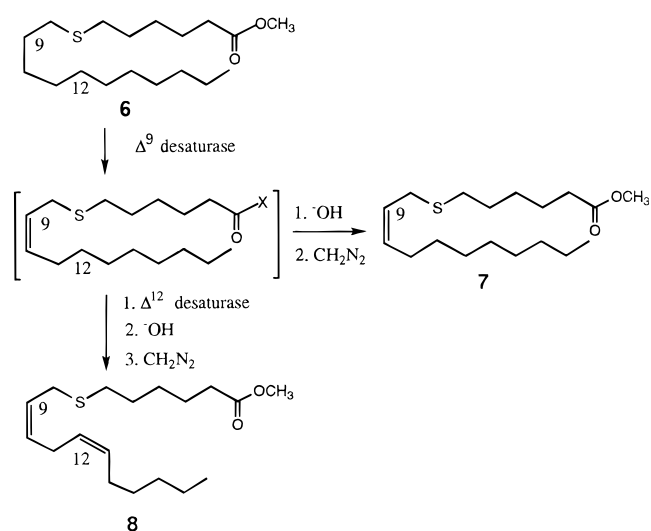
the results of a kinetic isotope effect study which clearly demonstrate that this is indeed the case.



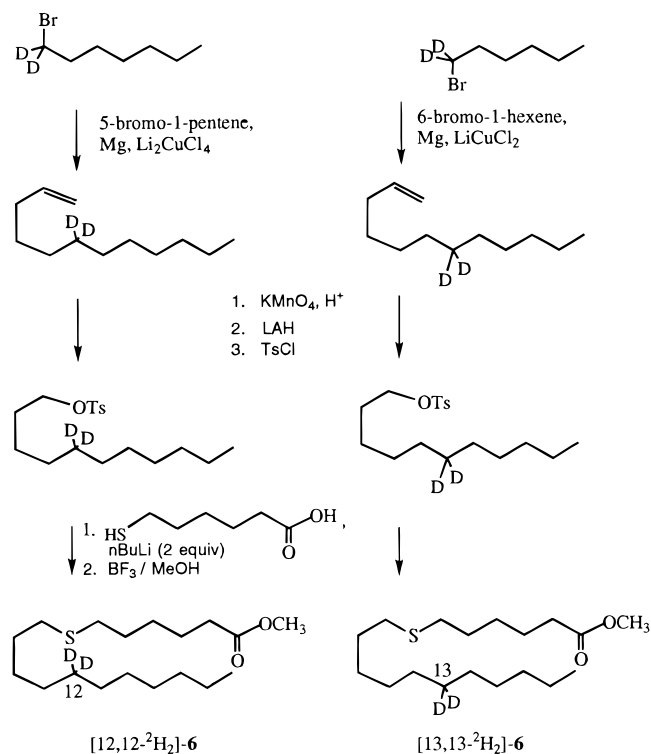
## Results and Discussion

The recent availability of a pDR1 strain of *S. cerevisiae*<sup>29</sup> which contains a functionally expressed plant  $\Delta^{12}$  oleate desaturase from *Arabidopsis thaliana* in addition to its own native  $\Delta^9$  desaturase offered a unique and extremely convenient opportunity to examine the primary KIEs associated with  $\Delta^{12}$  desaturation. Our *in vivo* methodology was similar to that used in the KIE study of the yeast  $\Delta^9$  desaturase.<sup>26</sup> The use of a whole-cell system to measure the intermolecular primary deuterium kinetic isotope effects of a uniquely defined enzyme-catalyzed reaction is legitimate provided the experiment is done in a direct competitive mode, the position of the isotopic label does not influence extraneous modifications of the substrate or product and preferential metabolic processing of one of the deuterated substrates or products does not lead to skewed results. All of these conditions are met in our experimental design which involved comparing the  $d_1/d_0$  ratio of 7-thialinoleate products derived from a ~1:1 mixture of methyl [12,12- $^2\text{H}_2$ ]-7-thiastearate ([12,12- $^2\text{H}_2$ ]-**6**) and methyl 7-thiastearate (**6**) with that obtained from a ~1:1 mixture of methyl [13,13- $^2\text{H}_2$ ]-7-thiastearate ([13,13- $^2\text{H}_2$ ]-**6**) and methyl 7-thiastearate (**6**) (Schemes 2 and 3). The pDR1 whole cell yeast system allows us to use labeled and nonlabeled thiastearate methyl esters<sup>30</sup> which are converted intracellularly to the corresponding thiaoleoyl CoA thioesters. The  $\Delta^9$ -desaturated products so obtained can then be processed *in situ* by the functionally expressed plant  $\Delta^{12}$  desaturase. (A corresponding yeast  $\Delta^{12}$  desaturase does not

## Scheme 2



## Scheme 3



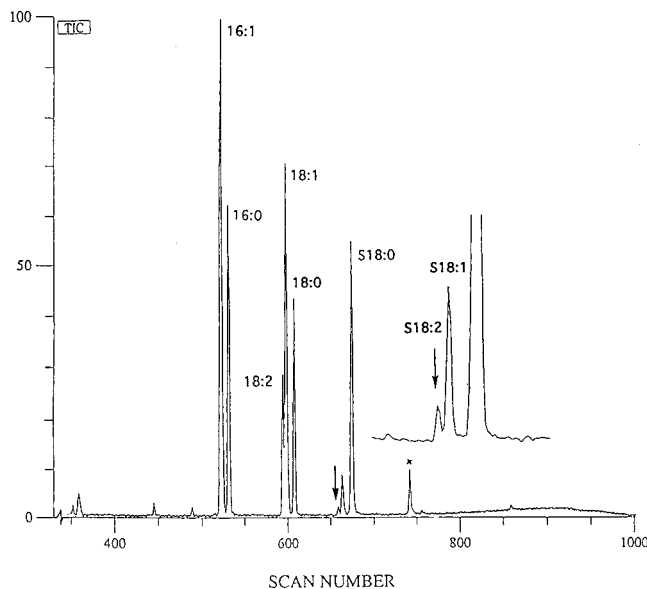
exist.) This approach avoids the more complicated synthesis of the deuterated thiaoleates and assumes, as is reasonable, that  $\Delta^9$  desaturation of [12,12- $^2\text{H}_2$ ]-**6**, [13,13- $^2\text{H}_2$ ]-**6**, and **6** occurs at equal rates (*vide infra*<sup>31</sup>). We should also point out that the use of thia-substituted analogues eliminates the problem of mass spectral interference by endogenous (nondeuterated) linoleate when the  $d_1/d_0$  ratio of the diolefinic products is evaluated. The sulfur was placed at the 7 position in the thiastearate substrates to facilitate the synthesis of the deuterated substrates.

Trial incubation experiments revealed that when methyl 7-thiastearate (**6**) was incubated with the pDR1 strain of *S. cerevisiae* at 15 °C for 1 week, methyl 7-thialinoleate (**7**) and methyl 7-thialinoleate (**8**) were produced (Figure 1). The

(31) This calculation is based on the assumption that the  $d_2/d_0$  ratio of the intermediate biosynthetic 7-thiaoleates was essentially the same as that of the starting materials. Mass spectrometric examination of the thiaoleate fraction confirmed that this was indeed the case within experimental error.

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**Figure 1.** Cellular fatty acid profile of *S. cerevisiae* (pDR1 strain), grown in CM-ura gal supplemented with methyl 7-thiastearate (0.5 mM), as determined by GC/MS: 16:0 = methyl palmitate (hexadecanoate); 16:1 = methyl palmitoleate (9(Z)-hexadecenoate); 18:0 = methyl stearate (octadecanoate); 18:1 = methyl oleate (9(Z)-octadecenoate); 18:2 = methyl linoleate (9(Z),12(Z)-octadecadienoate); S18:0 = methyl 7-thiastearate (**6**); S18:1 = methyl 7-thiaoleate (**7**); S18:2 = methyl 7-thialinoleate (**8**). The ratio of **6**:**7**:**8** was ~100:20:3. (The asterisk (\*) indicates an artifact.)

temperature of incubation is critical since similar incorporation experiments carried out at 25 °C resulted in very low levels of  $\Delta^{12}$  desaturation.<sup>29</sup>

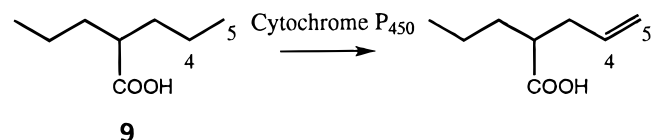
The synthesis of the required deuterated methyl 7-thiastearates was carried out in relatively straightforward fashion and is outlined in Scheme 3. [12,12-<sup>2</sup>H<sub>2</sub>]-**6** and [13,13-<sup>2</sup>H<sub>2</sub>]-**6** were obtained in 4% and 6% overall yield respectively and the deuterium content of each substrate was shown to be essentially 100% d<sub>2</sub> (within experimental error) by GC/MS. A ~1:1 mixture of each deuterated substrate and d<sub>0</sub> material (~25 mg) was administered to growing cultures (150 mL) of the *S. cerevisiae* transformant at 15 °C. Each incubation was carried out two times. After 1 week of growth, the cellular fatty acids (~30 mg) were isolated from the centrifuged cells by a standard hydrolysis/methylation sequence and the deuterium content of methyl 7-thialinoleate products was assessed by GC/MS (See Experimental Section for details). The analytical data is shown in Table 1.

Product kinetic isotope effects ( $k_H/k_D$ ) were calculated using the ratio: [% d<sub>0</sub> (7-thialinoleoyl product)/% d<sub>1</sub> (7-thialinoleoyl product)]/[% d<sub>0</sub> (7-thiastearoyl substrate)/% d<sub>2</sub> (7-thiastearoyl substrate)].<sup>31</sup> In this manner, a large primary deuterium isotope effect ( $k_H/k_D = 7.3 \pm 0.4$ , average of two experiments)<sup>32</sup> was determined for the carbon-hydrogen bond cleavage at C-12 while the C<sub>13</sub>-H bond breaking step was shown to be insensitive to deuterium substitution ( $k_H/k_D = 1.05 \pm 0.04$ , average of two experiments).

(32) Our observed isotope effect at C-12 is close to the theoretical maximum value for a primary deuterium isotope effect at 15 °C namely  $k_H/k_D = 7.4$ .<sup>33</sup> However, it should be noted that a small proportion (<10%) of the observed isotope effect may be due to an  $\alpha$ -secondary isotope effect. On the other hand, the magnitude of the "intrinsic" primary deuterium isotope effect at C-12 may well be higher since our estimated value is derived via an intermolecular competition experiment and is therefore subject to partial masking by other enzymic steps. Large "intrinsic" primary deuterium isotope effects are not uncommon for biological hydrocarbon activations thought to involve iron-oxo species.<sup>34</sup>

These results represent the first direct determination of the intermolecular primary deuterium isotope effects involved in oleate  $\Delta^{12}$  desaturation and are consistent with the notion that this process is initiated at C-12 by a slow, isotopically sensitive step while the second C-H cleavage at C-13 appears to be relatively fast as might be expected for the collapse of an unstable intermediate such as a carbon-centered radical or a carbocation (pathways a or b as shown in Scheme 1). (Pathway c is still an option but only if the dehydration reaction proceeds with a high degree of internal forward commitment. Other mechanistic variations<sup>17c,18,19</sup> are also possible.) That the site of initial oxidation for  $\Delta^{12}$  desaturation appears to be C-12 is in accord with the postulate that ricinoleic acid (**5**) is produced by an altered  $\Delta^{12}$  plant desaturase<sup>28d</sup> which initiates C-H bond cleavage at C-12 but for some, as yet undetermined, reason cannot prevent alcohol formation (Scheme 1). Identification of the factor(s) which steer the putative radical intermediate to a specific product remains a fascinating research objective.

The success of our KIE approach in yielding important cryptoregiochemical information on the  $\Delta^{12}$  desaturase-mediated reaction is in large measure due to the fact that the first C-H bond breaking step in fatty acid desaturation is a kinetically significant step in the catalytic cycle while the second C-H cleavage is not. It is interesting to note that the pattern of primary deuterium isotope effects (one large ( $k_H/k_D = 7.3$ , C-12) and one small ( $k_H/k_D = 1.05$ , C-13)) determined in the present work is remarkably similar to that obtained previously by us for the yeast  $\Delta^9$  desaturase system ( $k_H/k_D = 7.1$ , C-9;  $k_H/k_D = 1.03$ , C-10). Other laboratories have obtained qualitatively similar results for other desaturases. For example, Bloch inferred the existence of a kinetic isotope effect at C-9 but not at C-10 in an in vivo investigation of  $\Delta^9$  desaturation as it occurs in *Corynebacterium diphtheriae*.<sup>35</sup> In addition, a large isotope effect at C-6 but not at C-7 was deduced for a  $\Delta^6$  desaturase in *Tetrahymena*.<sup>36</sup> Finally, it is pertinent that Baillie et al. measured a substantial primary deuterium isotope effect at C-4 (5.58) and a significantly smaller value at C-5 (1.62) for the cytochrome P<sub>450</sub>-mediated 4,5-dehydrogenation of valproic acid (**9**)—a reaction thought to be initiated by hydrogen abstraction at C-4.<sup>37</sup> Taken together, these data suggest a common



mechanistic model for these systems of the type proposed in Scheme 1.

Our results and those of others, cited above, differ from those of Morris et al. who inferred<sup>38</sup> that a primary isotope effect was operating on the C-H cleavage at both C-9 and C-10 for the  $\Delta^9$  desaturase of *Chlorella vulgaris*. This work was apparently confirmed by Johnson and Gurr using cell-free preparations from *Chlorella vulgaris* and hen liver.<sup>39</sup> This raises the possibility that the  $\Delta^9$  desaturases from these sources follow a mechanism

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**Table 1.** Intermolecular Isotopic Discrimination in  $\Delta^{12}$  Desaturation of [12,12- $^2\text{H}_2$ ]-7-Thiaoleate and [13,13- $^2\text{H}_2$ ]-7-Thiaoleate

experiment	isotopic composition (mol %) <sup>a</sup>						KIE
	substrates <sup>b</sup>			products			
	d <sub>0</sub>	12-d <sub>2</sub>	13-d <sub>2</sub>	d <sub>0</sub>	12-d <sub>1</sub>	13-d <sub>1</sub>	
1	50.4 ± 1.0	49.6 ± 1.0		87.6 ± 1.0	12.4 ± 1.0		7.0 ± 0.6
2	51.8 ± 0.9	48.2 ± 0.9		89.1 ± 0.7	10.9 ± 0.7		7.6 ± 0.6 (7.3 ± 0.4) <sup>c</sup>
3	49.6 ± 0.8		50.4 ± 0.8	51.3 ± 1.5		48.7 ± 1.5	1.07 ± 0.05
4	51.8 ± 1.3		48.2 ± 1.3	52.3 ± 1.2		47.7 ± 1.2	1.02 ± 0.05 (1.05 ± 0.04) <sup>d</sup>

<sup>a</sup> The mol % of each isotopic species is given as an average value based on 2–3 GC/MS runs. <sup>b</sup> The four methyl 7-thiastearate mixtures were individually prepared. <sup>c</sup> The average KIE at C-12 ± standard deviation. <sup>d</sup> The average KIE at C-13 ± standard deviation.

in which both C–H bond-breaking steps are kinetically important. In this connection, it would be interesting to investigate the mechanism of the purified plant  $\Delta^9$  desaturase.<sup>5a</sup> The recent X-ray structure of this enzyme from castor (*Ricinus communis*) reveals a tight, hydrophobic, substrate-binding pocket, and computer modeling studies suggest that C-9 of the stearyl substrate is near the iron atom responsible for initial oxidation.<sup>7</sup> The appropriate KIE experiments are planned in order to test this prediction.

## Materials and Methods

**General Methods.** All NMR spectra were obtained using dilute  $\text{CDCl}_3$  solutions at the frequency indicated. Chemical shifts are expressed in parts per million (ppm) and are referenced to tetramethylsilane. Mass spectra were obtained by GC/MS using a Kratos Concept 1 H mass spectrometer interfaced with a HP 5980 Series 2 GC equipped with a J. & W. 30 m × 0.21 mm, DB-5 capillary column (temperature programmed from 120 °C (held, 2 min) to 320 °C at 10 °C/min). Deuterium content was estimated using a MS scan rate of 0.5 s/decade which resulted in 5–8 scans per GC peak; the integrated intensities of the individual ions in the pertinent ion cluster were recorded using a SUN SPARC I workstation equipped with Kratos Mach 3 software and have been corrected for natural isotopic abundances. Isotopic ratios were determined using the following ions:  $m/z$  316,  $\text{M}^+$  (methyl 7-thiastearate);  $m/z$  152,  $(\text{CH}_3(\text{CH}_2)_6\text{CH}=\text{CHCH}=\text{CH}_2)^+$  (methyl 7-thiaoleate);  $m/z$  150,  $(\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CH}-\text{CH}=\text{CHCH}=\text{CH}_2)^+$  (methyl 7-thialinoleate).

Flash chromatography using silica gel (230–400 mesh) was used to purify substrates. Analytical TLC was performed using Merck glass plates precoated with silica G/UV254. Visualization of UV-inactive materials was accomplished by using a combination of  $\text{I}_2$  vapor followed by a water spray.

Unless otherwise stated, all reagents and starting materials were purchased from Aldrich Chemical Co. and used without purification. All air- and moisture-sensitive reactions were performed under  $\text{N}_2$ . Organic extracts were typically shaken with saturated NaCl and dried over  $\text{Na}_2\text{SO}_4$ , and solvents were evaporated on a rotary evaporator. [1,1- $^2\text{H}_2$ ]-1-Bromohexane and [1,1- $^2\text{H}_2$ ]-1-bromoheptane were prepared by treatment of the corresponding deuterated primary alcohols with  $\text{HBr}/\text{H}_2\text{SO}_4$ .<sup>40</sup> The deuterated alcohols were in turn synthesized from the corresponding carboxylic acids by reduction using  $\text{LiAlD}_4$ .

Incubation experiments were carried out using a pDR1 strain of *S. cerevisiae* (PBI, Saskatoon) which contained the functionally expressed *Arabidopsis thaliana* FAD2 gene.<sup>29</sup>

**Synthesis of Substrates.** Methyl 7-thiastearate was prepared as previously described.<sup>30</sup> The deuterated 7-thia fatty acids were synthesized in similar fashion by S-alkylation of 6-mercaptohexanoic acid with the *p*-toluenesulfonic ester of the appropriately deuterated undecan-1-ol. After methylation of the carboxyl function, the crude products were purified by flash chromatography (silica gel, 4% EtOAc/hexane) to give colorless oils at room temperature. The relevant analytical data is given below.

(40) Vogel's *Textbook of Practical Organic Chemistry*, 4th ed.; Revised by Furniss, B. S., Hannaford, A. J., Rogers, V., Smith, P. W. G., Tatchell, A. R.; Longman: New York, 1978; pp 387–388.

**Methyl 7-thiastearate (6):** mp 23–25 °C;  $^1\text{H}$  NMR (200 MHz)  $\delta$  3.63 (3H, s, C(O)OCH<sub>3</sub>), 2.47 (4H, t,  $\text{CH}_2\text{SCH}_2$ ), 2.31 (2H, t,  $\text{CH}_2\text{C}(\text{O})\text{OCH}_3$ ), 1.60 (6H, m,  $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{OCH}_3$ ,  $\text{CH}_2\text{CH}_2\text{SCH}_2\text{CH}_2$ ), 1.29 (18H, br s,  $\text{CH}_2$ ) 0.89 (3H, t,  $\text{CH}_3\text{CH}_2$ );  $^{13}\text{C}$  NMR (100 MHz)<sup>41</sup>  $\delta$  174.09 (C-1), 51.49 (CH<sub>3</sub>O), 33.98 (C-2), 32.26 (C-8), 31.98 (C-6 or C-16), 31.94 (C-6 or C-16), 29.77 (C-9), 29.64 (C-13), 29.64 (C-14), 29.57 (C-12), 29.37 (C-4 or C-5), 29.37 (C-15), 29.30 (C-11), 28.99 (C-10), 28.43 (C-4 or C-5), 24.60 (C-3), 22.71 (C-17), 14.12 (C-18). MS (EI, 70 eV)  $m/z$  316 ( $\text{M}^+$ ), 285 ( $\text{M} - \text{OCH}_3$ ), 187 ( $(\text{CH}_3(\text{CH}_2)_{10}\text{S})^+$ ), 129 ( $(\text{M} - \text{CH}_3(\text{CH}_2)_{10}\text{S})^+$ ). Anal. Calcd for  $\text{C}_{18}\text{H}_{33}\text{O}_2\text{S}$ : C, 68.30; H, 11.46; S, 10.13. Found: C, 68.29; H, 11.45; S, 9.93.

**Methyl [12,12- $^2\text{H}_2$ ]-7-thiastearate ([12,12- $^2\text{H}_2$ ]-6)** was synthesized from [5,5- $^2\text{H}_2$ ]-1-*p*-toluenesulfonyloxyundecane. The spectral data of the title compound was similar to that of **6** except for  $^{13}\text{C}$  NMR (100 MHz)  $\delta$  29.43 (C-13, upfield  $\beta$ -isotope shift (0.21 ppm)), 29.58 (C-14, upfield  $\gamma$ -isotope shift (0.06 ppm)), 29.57 (C-12, absent), 29.09 (C-11, upfield  $\beta$ -isotope shift (0.21 ppm)), 28.93 (C-10, upfield  $\gamma$ -isotope shift (0.06 ppm)); MS (EI, 70 eV)  $m/z$  318 ( $\text{M}^+$ ), 287 ( $\text{M} - \text{OCH}_3$ ), 189 ( $(\text{CH}_3(\text{CH}_2)_5(\text{CD}_2)(\text{CH}_2)_4\text{S})^+$ ), 129 ( $(\text{M} - \text{CH}_3(\text{CH}_2)_5(\text{CD}_2)(\text{CH}_2)_4\text{S})^+$ ).

**Methyl [13,13- $^2\text{H}_2$ ]-7-thiastearate ([13,13- $^2\text{H}_2$ ]-6)** was synthesized from [6,6- $^2\text{H}_2$ ]-1-[(*p*-toluenesulfonyl)oxy]undecane. The spectral data of the title compound was identical with that of **6** except for  $^{13}\text{C}$  NMR (100 MHz)  $\delta$  29.64 (C-13, absent), 29.43 (C-14, upfield  $\beta$ -isotope shift (0.21 ppm)), 29.38 (C-12, upfield  $\beta$ -isotope shift (0.19 ppm)), 29.30 (C-15, upfield  $\gamma$ -isotope shift (0.07 ppm)), 29.24 (C-11, upfield  $\gamma$ -isotope shift (0.06 ppm)); MS (EI, 70 eV)  $m/z$  318 ( $\text{M}^+$ ), 287 ( $\text{M} - \text{OCH}_3$ ), 189 ( $(\text{CH}_3(\text{CH}_2)_4(\text{CD}_2)(\text{CH}_2)_5\text{S})^+$ ), 129 ( $(\text{M} - \text{CH}_3(\text{CH}_2)_4(\text{CD}_2)(\text{CH}_2)_5\text{S})^+$ ).

**Synthesis of Regiospecifically Deuterated Undecan-1-ol Tosylates.** [5,5- $^2\text{H}_2$ ]-[(*p*-Toluenesulfonyl)oxy]undecane. The title compound was prepared from [1,1- $^2\text{H}_2$ ]-1-bromoheptane via a standard sequence of Cu(I)-catalyzed Grignard coupling<sup>43</sup> with 5-bromo-1-pentene to give [6,6- $^2\text{H}_2$ ]-1-dodecene, oxidative cleavage<sup>44</sup> to give [5,5- $^2\text{H}_2$ ]undecanoic acid, followed by  $\text{LiAlH}_4$  reduction and tosylation. The overall yield for this sequence was 17%. For the known<sup>45</sup> unlabeled material (clear oil):  $^1\text{H}$  NMR (200 MHz)  $\delta$  7.80 (d, 2H, ArH), 7.35 (d, 2H, ArH), 4.00 (br s, 2H,  $\text{CH}_2\text{OTs}$ ), 2.44 (s, 3H, ArCH<sub>3</sub>), 1.65 (m, 2H,  $\text{CH}_2\text{CH}_2\text{OTs}$ ), 1.4 (br s, 18H,  $\text{CH}_3(\text{CH}_2)_8$ ), 0.87 (t, 3H,  $\text{CH}_3(\text{CH}_2)_8$ ). The title compound showed similar physical and spectral characteristics.

[6,6- $^2\text{H}_2$ ]-1-[(*p*-Toluenesulfonyl)oxy]undecane. The title compound was prepared from [1,1- $^2\text{H}_2$ ]-1-bromohexane via a sequence of Cu(I)-catalyzed Grignard coupling<sup>43</sup> with 6-bromo-1-hexene to give [7,7- $^2\text{H}_2$ ]-1-dodecene, oxidative cleavage<sup>44</sup> to give [6,6- $^2\text{H}_2$ ]undecanoic acid, followed by  $\text{LiAlH}_4$  reduction and tosylation. The overall yield for

(41) The  $^{13}\text{C}$  NMR assignments were carried out using literature values for methyl stearate<sup>42</sup> as well as  $^{13}\text{C}$  NMR data (unpublished) obtained for samples of methyl [9,9- $^2\text{H}_2$ ]-7-thiastearate and methyl [10,10- $^2\text{H}_2$ ]-7-thiastearate which we have prepared previously.<sup>26</sup>

(42) Tulloch, A. P.; Mazurek, M. *Lipids* **1976**, *11*, 228.

(43) (a) Tamura, M.; Kochi, J. *Synth. Commun.* **1971**, *303*. (b) Tolstikov, G. A.; Odinkov, V. N.; Galeeva, R. I.; Bakeeva, R. S. *Tetrahedron Lett.* **1978**, 1857.

(44) Krapcho, A. P.; Larson, J. R.; Eldridge, J. M. *J. Org. Chem.* **1977**, *42*, 3749.

(45) Krishnamurthy, S. *J. Org. Chem.* **1980**, *45*, 2551.

this sequence was 20%. The physical and spectral characteristics of the title compound were similar to that of [5,5-<sup>2</sup>H<sub>2</sub>]-1-[(*p*-toluenesulfonyl)oxy]undecane.

**Incubation Experiments Using *S. cerevisiae*.** A typical feeding experiment was carried out as follows: Cultures of the pDR1 strain of *Saccharomyces cerevisiae* were grown in minimal SGal<sub>ura</sub> medium<sup>46</sup> using a rotary incubator-shaker set at 150 rpm. A total of 8 mL of starter culture grown at 30 °C for 48 h was used to inoculate 150 mL of sterile medium contained in a 500 mL Erlenmeyer flask.

A ~25 mg portion of this fatty acid methyl ester was added as a solution (5% w/v) in absolute ethanol to each culture flask and the inoculated cultures were then incubated for 1 week at 15 °C. The addition of exogenous fatty acid did not affect the growth of the organism with respect to control cultures.

The desaturated fatty acid products were isolated from the cells by a standard hydrolysis/methylation procedure: Yeast cells from each incubation experiment (~5 g, wet weight) obtained by centrifugation were treated with refluxing 1 M 50% ethanolic KOH (50 mL) for three h under N<sub>2</sub>. Cell debris was filtered off and the filtrate was partially

evaporated in vacuo to remove ethanol, diluted with water (100 mL) acidified to pH 2 with 50% H<sub>2</sub>SO<sub>4</sub> and extracted with dichloromethane. This extract was washed with water, saturated NaCl, dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated to give a yellow solid. The residue was treated with a freshly prepared solution of ethereal diazomethane to yield ~30 mg of fatty acid methyl esters after evaporation.

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(46) *Current Protocols in Molecular Biology*; Ausubel, F. M., Brent R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., Albright, L. M., Coen, D. M., Varki, A., Eds.; John Wiley & Sons: New York, 1995; Chapter 13.