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# Deuterium NMR Used To Indicate a Common Mechanism for the Biosynthesis of Ricinoleic Acid by *Ricinus communis* and *Claviceps purpurea*

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***Abstract:*** Previous studies have shown that ricinoleic acid from castor bean oil of *Ricinus communis* is synthesized by the direct hydroxyl substitution of oleate, while it has been proposed that ricinoleate is formed by hydration of linoleate in the ergot fungus *Claviceps purpurea*. The mechanism of the enzymes specific to ricinoleate synthesis has not yet been established, but hydroxylation and desaturation of fatty acids in plants apparently involve closely related mechanisms. As mechanistic differences in the enzymes involved in the biosynthesis of natural products can lead to different isotopic distributions in the product, we could expect ricinoleate isolated from castor or ergot oil to show distinct $^{2}H$ distribution patterns. To obtain information concerning the substrate and isotope effects that occur during the biosynthesis of ricinoleate, the site-specific natural deuterium distributions in methyl ricinoleate isolated from castor oil and in methyl ricinoleate and methyl linoleate isolated from ergot oils have been measured by quantitative $^{2}H$ NMR. First, the deuterium profiles for methyl ricinoleate from the plant and fungus are equivalent. Second, the deuterium profile for methyl linoleate from ergot is incompatible with this chemical species being the precursor of methyl ricinoleate. Hence, it is apparent that 12-hydroxylation in *C. purpurea* is consistent with the biosynthetic mechanisms proposed for *R. communis* and is compatible with the general fundamental mechanistic similarities between hydroxylation and desaturation previously proposed for plant fatty acid biosynthesis.

## Introduction

Ricinoleic acid (12-OH, C18:1$\Delta^{9}$) is an attractive precursor for industrial synthesis. The presence of the 12-hydroxyl substituent and the $\Delta^{9}$ desaturation makes it amenable as a precursor to several potentially useful derivatives,[1−3] while the ease of cultivating the castor oil plant *Ricinus communis* makes it simple to obtain large quantities of this product. Ricinoleic acid constitutes about 85−90% of the triglyceride fatty acids found in castor bean oil.

The enzymatic mechanism capable of stereo- and regio-selectively introducing a hydroxyl at C12 of oleic acid ($\beta$ to the desaturation) has evoked considerable interest. Studies in *R. communis* have indicated that ricinoleic acid is synthesized by the direct hydroxylation of oleic acid[4−7] esterified specifically as the phosphatidylcholine ester.[5,8,9]

Ricinoleic acid is also produced in other organisms, notably the pathogenic ergot fungus, *Claviceps purpurea*.[10] This fungus, which can be responsible for the devastating destruction of cereal crops, produces and accumulates ricinoleic acid in the sclerotia. In contrast to the situation in castor oil plants, it has been proposed that biosynthesis of ricinoleic acid in the fungus involves the hydration of the $\Delta^{12}$ bond of linoleic acid.[11,12] Such a difference in synthetic mode is surprising in view of the relatively uncommon occurrence of this fatty acid. However, hydration of an existent olefinic bond in a fatty acid is a recognized mechanism of hydroxylation. Thus, in a pseudomonad, oleic acid is converted to 10-hydroxystearic acid,[13] while linoleic acid is also hydroxylated at the 10 position.[14] Thus, it is important to establish whether the enzymatic apparatus for this biosynthesis in the fungus is indeed more comparable to that in the bacterium than that in the plant.

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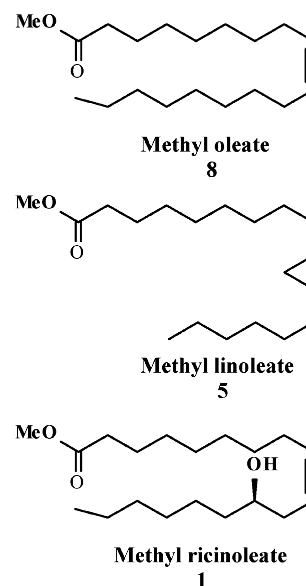
The mechanisms of the enzymes involved in ricinoleate synthesis have not yet been established. However, it is proposed that the 12-hydroxylase that acts on oleate is both genetically and mechanistically similar to the long-chain fatty acid desaturase enzymes.[15−19] This hypothesis is strongly supported by studies in which the alteration of key amino acids in the active center of oleate $\Delta^{12}$-desaturase leads to substantial 12-hydroxylase activity.[19] The current mechanistic model for desaturation, based on studies of the X-ray structure of a soluble $\Delta^9$-desaturase isolated from *R. communis*[20] and of the reaction kinetics of a wide range of desaturases,[16,21] invokes a high-valent diiron center and molecular oxygen. Essentially, a slow activation step involves the removal of *pro(R)* hydrogen and the creation of a short-lived radical. In desaturation, a rapid syn disproportionation results in the loss of the α-hydrogen and olefinic bond formation. Alternatively, attack by a putative iron-bound hydroxyl will lead to hydroxylation of the position from which the initial hydrogen was abstracted. The insertion of the hydroxyl with retention of configuration supports this proposal.[4]

In contrast, hydratases, which add water directly to an olefinic bond, are mechanistically dissimilar to the fatty acid desaturases. The desaturase mechanism involves molecular oxygen as substrate, whereas oxygen is introduced from water by hydratase activity.

Due to different sensitivities to heavy isotopes, these distinct mechanisms are likely to leave characteristic patterns in the residual $^2$H levels of ricinoleic acid derived from different origins. This pattern will reflect both that of the starting substrate and the kinetic isotope effects intrinsic to the enzymes involved. As each individual reaction acts on different positions in the substrate, this gives rise to a specific nonstatistical isotope distribution in the final product.[22]

It has been shown by quantitative $^2$H NMR spectroscopy that the distribution of $^2$H at natural abundance in the fatty acids proposed as precursors for ricinoleic acid is nonstatistical.[23,24] Thus, measurement of the site-specific natural isotope fractionation in $^2$H of methyl oleate **8** (C18:1$\Delta^9$) isolated from plants has shown a strong impoverishment in $^2$H at site 9 but not at site 10 of the $\Delta^9$-ethylenic bond. Similarly, methyl linoleate (C18:2$\Delta^{9,12}$) shows a strong impoverishment at positions 9 and 13 but not at positions 10 and 12. While the exact cause of these impoverishments is yet to be firmly established, they can reasonably be attributed to a combined influence of the initial $^2$H contents of the hydrogens of the substrates involved—stearate for oleate and oleate for linoleate—and isotope effects associated with the $\Delta^9$- and $\Delta^{12}$-desaturases. Hence, it can be predicted that ricinoleate formed from these two different substrates by the action of two different enzymes should possess different $^2$H distributions. Especially important in this context is the low ($^2$H/$^1$H) ratio at the C-13 position of linoleate compared with the C-13 of oleate.




With the objective of establishing whether a distinction exists between the biosynthetic mechanisms used by the fungal pathogen and the crop plant, the site-specific natural abundance isotope fractionations in $^2$H of ricinoleate isolated from both sources have been measured by quantitative $^2$H NMR. In particular, we have focused on the relative values of $(^2H/^1H)_{12}$ and $(^2H/^1H)_{13}$ in each species. This is the first report in which natural abundance ($^2$H/$^1$H) ratios have been effectively used directly to deduce similarity or dissimilarity in enzyme mechanisms.

## Materials and Methods

**Materials.** Castor seed oil from *R. communis* was obtained from independent origins: Sigma-Aldrich (castor 1, lot: 85H0439, 1995) and Rhône-Poulenc (castor 2, lot: A28457, 2000). The sample of ergot oil produced by the fungus *C. purpurea* was isolated from selected mycelium cultured axenically on a liquid nutrient medium in such a way as to differentiate and proliferate a compact purple-pigmented tissue form that closely resembled that of typical parasitic ergots.[25] The tissue contained approximately 30% (w/w) of oil. This was extracted in chloroform/methanol (2:1), stored anaerobically, and refrigerated.

**General Methods.** $^1$H NMR (500 MHz) and $^{13}$C NMR (125 MHz) spectra were recorded with a Bruker DRX 500 spectrometer. Chemical shifts are given relative to TMS in $CDCl_3$. Flash chromatography was performed with silica gel Normasil (40−63 $\mu$m, Prolabo). Petroleum ether was distilled before use. Reaction was followed by gas chromatography on a HP-5 capillary column (30 m × 0.32 mm, film thickness 0.52 $\mu$m); carrier gas, He, 1.2 mL·min$^{-1}$; split 1:40; FID temp, 280 °C; thermal gradient 100 °C initially for 1 min, ramped at 10 °C min$^{-1}$ to 240 °C for 1 min, ramped at 10 °C min$^{-1}$ to 280 °C for 1 min, then ramped at 10 °C min$^{-1}$ to 298 °C, and held at 298 °C for 2 min.

**$^2$H NMR Spectroscopic Measurements.** The samples were previously characterized by their $^1$H and $^{13}$C NMR spectra. The $^2$H NMR spectroscopy was carried out as previously described on a Bruker DRX 500 spectrometer with a dual probe ($^1$H/$^2$H) 10 mm in diameter operating at 76.7 MHz and fitted with a $^{19}$F field-frequency-locking

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device.[23] Acquisition temperature: 310 K. The internal reference used was pyridine or *N*,*N*,*N*′,*N*′-tetramethylurea (TMU). The isotopic ratio of pyridine $(^2H/^1H)_{pyr}$ was calibrated relative to TMU calibrated relative to the V.SMOW scale.[26] Sample preparation: **1**, 0.90 g; mass pyridine = 0.19 g; mass $CHCl_3$ = 0.87 g; mass $CCl_4$ = 2.4 g; volume $C_6F_6$ = 60 μL. **2** and **4**: 0.60−0.75 g (depending on product); mass pyridine = 0.21 g; mass $CHCl_3$ = 1.20 g; mass $CCl_4$ = 2.40 g; volume $C_6F_6$ = 60 μL. Sample preparation of **5**, **6**, and **7** was as described previously.[23]

The $(^2H/^1H)_i$ ratios of samples were calculated from eq 1, where $P_i$ and $P_{ref}$ are the stoichiometric numbers of hydrogen at site *i* and in the reference, $S_i$ and $S_{ref}$ are the areas of the signals, and $M_S$, $m_S$ and $M_{ref}$, $m_{ref}$ are the molecular weight and mass of the sample and the reference, respectively.

$$\left(\frac{^2H}{^1H}\right)_i = \frac{P_{ref} \times m_{ref} \times M_s \times S_i}{P_i \times m_s \times M_{ref} \times S_{ref}} \times \left(\frac{^2H}{^1H}\right)_{ref}$$

The calculation of $S_i$ and $S_{ref}$ for the monodeuterated isomers was performed using a curve-fitting algorithm (Perch NMR software, University of Kuopio, Finland).

**Chemical Synthesis.** (*R*)-12-Hydroxy-9-octadecenoic acid Methyl Ester (syn. Methyl Ricinoleate) **1** was synthesized from both castor and ergot oils. Oil (10.0 g) in a solution of sodium hydroxide in methanol (150 mL, 20 g·L$^{-1}$) was heated at reflux for 1 h. The mixture was diluted by 100 mL of MeOH, and then a solution of boron trifluoride in methanol (33 mL; 50% w/w) was added. The mixture was heated for an additional 45 min and then cooled. At ambient temperature, hexane (300 mL) was added, and the mixture was kept under agitation for 30 min. A saturated NaCl aqueous solution was added, both phases were separated, and the aqueous phase was extracted with $CHCl_3$. The combined organic phases were washed with water, dried ($MgSO_4$), filtered, and evaporated to give 9.2−10.3 g of mixed fatty acid methyl esters (FAMEs). Flash chromatography on silica gel (column: 40 × 5.0 cm$^2$; solvent gradient: petroleum ether (40−70°)/$Et_2O$: 95/5 to 50/50) gave **1** (castor 1, 8.6 g; castor 2, 8.8 g; ergot oil; 2.6 g) and a mixture of non-oxygenated FAMEs (castor 1, 1.0 g; castor 2, 1.0 g; ergot oil: 5.8 g). $^1H$ NMR ($CDCl_3$) of **1**: δ 5.50 and 5.35 (2H, *m*, H-9 and H-10), 3.61 (3H, *s*, COOMe), 3.55 (1H, *m*, H-12), 2.24 (2H, *t*, *J* = 7.5 Hz, H-2), 2.15 (2H, *t*, *J* = 7.0 Hz, H-11), 1.99 (2H, *q*, *J* = 6.0 Hz, H-8), 1.60−1.35 (4H, *m*, H-13 and H-3), 1.33−1.15 (16H, *m*, $CH_2$), 0.83 (3H, *t*, *J* = 7.0 Hz). $^{13}C$ NMR ($CDCl_3$) of **1**: δ 174.4 (*C*OOCH$_3$), 133.4 and 125.3 (C-9 and C-10), 71.6 (C-12), 51.5 (COO*C*H$_3$), 36.9−22.7 (13 $CH_2$), 14.1 ($CH_3$).

**9,9′-Bis(methoxy)nonanoic Acid Methyl Ester 2 and (*R*)-1,1′-Bis-(methoxy)-3-hydroxynonane 3.** To a solution of **1** (1.20 g, 3.80 mmol) in a mixture of $CHCl_3/H_2O$ (22 mL/0.22 mL) was added, at room temperature, *N*-methyl-morpholine oxide (0.64 g, 5.40 mmol), and then a solution of $OsO_4$ in 2-methyl-2-propanol (0.53 mL, 2.5%) was added slowly. After 24 h, solvent was evaporated, then coevaporated with toluene (3 × 30 mL) to give 1.5 g of a yellow solid. The residue was taken up in MeOH (80 mL), and then $NaIO_4$ was added. After 15 h, the reaction mixture was filtered to provide a mixture of aldehydes in MeOH. To this solution was added TsOH·$H_2O$ (0.87 g, 4.60 mmol), and the solution was heated at 40 °C. After 90 min, the solution was cooled to room temperature, and molecular sieves (3 Å, 10 g) were added. After 15 h, the mixture was filtered and evaporated, and the residue was taken up in $Et_2O$ and washed with an aqueous solution of $NaHCO_3$ (1%). The aqueous phases were extracted with $Et_2O$, and the combined organic phases washed with water, dried over ($MgSO_4$), filtered, and then evaporated to give a mixture of **2** and **3**. Flash chromatography on silica gel using petroleum ether (40−70°)/$Et_2O$ (80/20 to 50/50) as solvent gradient provided 0.64−0.80 g (72−90% for three steps) of **2** and 0.59−0.70 g (75−90% for three steps) of **3**. $^1H$- and $^{13}C$ NMR ($CDCl_3$) spectra of **2** were identical to those reported previously.[23]

**(*R*) 1,1′-Bis(methoxy)-3-acetylnonane 4.** To a solution of **3** (0.59−0.70 g, 2.89−3.43 mmol) in pyridine (8 mL) was added acetic anhydride (1.5 mL, 15.4 mmol). After 15 h at ambient temperature under agitation, the mixture was evaporated and coevaporated with toluene (3 × 30 mL) to give quantitatively **4**. $^1H$ NMR ($CDCl_3$): δ 4.93 (1H, *m*, H-3) 4.36 (1H, *dd*, *J* = 5.0 and 6.2 Hz, H-1), 3.25 and 3.27 (2 × 3H, 2*s*, 2 × OMe), 1.98 (3H, *s*, $COCH_3$), 1.80 (1H, *ddd*, *J* = 14.1, 7.5 and 5.0 Hz, H-2), 1.75 (1H, *ddd*, *J* = 14.1, 6.2 and 4.5 Hz, H-2′), 1.49 (2H, *m*, H-4), 1.30−1.15 (8H, *m*, 4 $CH_2$), 0.82 (3H, *t*, *J* = 7.0 Hz). $^{13}C$ NMR ($CDCl_3$): δ 170.6 (COO*C*H$_3$), 102.0 (C-1), 71.1 (C-3), 53.3 and 52.5 (2 × $OCH_3$), 37.4−21.2 (6 $CH_2$ and COO*C*H$_3$), 14.1 ($CH_3$).

**9,12-Octadecadienoic Acid Methyl Ester (Syn. Methyl Linoleate) 5.** The solution was synthesized from the same sample of ergot oil as used to isolate **1**. The mixture of non-oxygenated FAMEs recovered from silica chromatography (5.8 g) was subjected to silica−Ag chromatography. The isolated **5** was chemically modified as described previously to give 9,9′-bis(phenylthio)nonanoic acid methyl ester **6** and methyl 1,1′-bis(phenylthio)hexane **7**. The structure and purity of these products were checked by $^1H$ and $^{13}C$ NMR spectroscopy.[23,24]

## Results

**$^2H$ Distribution in Methyl Ricinoleate 1.** Access to $^2H$ isotopic data for methyl ricinoleate **1** isolated from castor oil or ergot oil was carried out in three steps: (1) transmethylation of oil, (2) separation of **1** from other FAMEs, and (3) chemical modification. Two unrelated samples of castor oil (castor 1, castor 2) and one sample of ergot oil (ergot) were treated in exactly the same way.

Transmethylation of oil samples (10 g) gave a mixture of FAMEs (ca. 10 g) from which methyl ricinoleate **1** was isolated by silica gel chromatography. The quantities obtained represented yields >95%, based on the quantity of **1** in the starting oil estimated by GC after transmethylation (data not shown).

The $^2H$ NMR spectrum of isolated **1** proved inadequate to obtain information on the $(^2H/^1H)_9$, $(^2H/^1H)_{10}$, $(^2H/^1H)_{12}$, and $(^2H/^1H)_{13}$ ratios due to the overlapping of resonance signals. To reveal masked isotopic data, a chemical modification was applied.[14] The cleavage of the olefinic bond of **1** gave two aldehydes, which were directly protected to give products **2** and **3**. These were separated by silica gel chromatography. To facilitate the $(^2H/^1H)_i$ measurement of **3** by $^2H$ NMR, the alcoholic function was protected to give **4** (Scheme 1). Samples of methyl ricinoleate **1** (castor 1, castor 2, and ergot) and their respective compounds **2** and **4** were submitted to quantitative $^2H$ NMR. Figure 1 shows the $^2H$ NMR spectra obtained from methyl ricinoleate **1** and derivatives **2** and **4** obtained from castor oil (castor 1). In these spectra, the area of the $^2H$ NMR signal is directly proportional to the number of monodeuterated isotopomers present. The site-specific isotopic ratio $(^2H/^1H)_i$ is defined in the following equation and expressed in parts per million (ppm):

$$\left(\frac{^2H}{^1H}\right)_i = \frac{D_i}{H_i} = \frac{N_{^2H,i}}{P_i N_{^1H}}$$

where $N_{^2H,i}$ is the number of monodeuterated isotopomers of type *i*, $N_{^1H}$ is the number of fully protonated molecules, and $P_i$ is the number of equivalent hydrogen atoms at site *i*.

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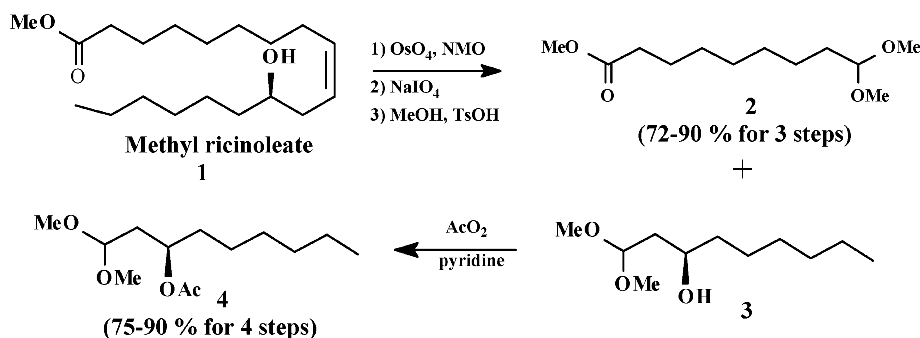
***Figure 1.*** $^2$H NMR spectra of methyl ricinoleate **1** and products **2** and **4** isolated from castor oil. (A) $^2$H NMR spectrum of methyl ricinoleate **1**. (B) $^2$H NMR spectrum of **2**. (C) $^2$H NMR spectrum of **4**. To maintain continuity, the sites are numbered following their position in methyl ricinoleate **1**.

***Scheme 1***




The measured $(^2H/^1H)_i$ values for methyl ricinoleate **1** (castor 1, castor 2, and ergot samples) and derivatives **2** and **4** are presented by carbon number in Tables 1−3. Data for methyl ricinoleate **1** from ergot are the means of two independent transesterifications and NMR acquisitions (Table 3). Data from derivatives **2** and **4** from castor 2 and ergot samples are the means of two independent chemical modifications and NMR acquisitions (Tables 2 and 3). The reproducibility of the experiments is very satisfactory. Standard errors for $(^2H/^1H)_i$ values presented in Tables 1−3 are acceptable, with most being less than 5 ppm. Most $(^2H/^1H)_i$ values were determined with less than 5% deviation, even when results were the mean of two independent manipulations.

During chemical modification, a risk exists that isotopic fractionation may be introduced due to kinetic and thermodynamic isotopic effects intrinsic to the manipulation. A number

***Table 1.*** $(^{2}H/^{1}H)_i$ Values (in ppm) of Methyl Ricinoleate **1** (Castor 1) and Corresponding Derivatives **2** and **4**$^{a}$

| carbon n° | 2 | 3 | 4–7 | 8 | 9 | 10 | 11 | 12 | COOMe | 13 | 14–17 | 13–17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| castor 1 | 139.9$^{b}$ | 97.5 | 128.2$^{c}$ | 139.9$^{b}$ | 105.0$^{d}$ | 105.0$^{d}$ | 139.9$^{b}$ | 124.8$^{e}$ | 124.8$^{e}$ | | | 128.2$^{c}$ | 127.8 |
| SD | 5.7 | 1.0 | 2.1 | 5.7 | 0.7 | 0.7 | 5.7 | 1.9 | 1.9 | | | 2.1 | 2.0 |
| **2**/**4**$^{f}$ | 143.9 | 130.2$^{g}$ | 139.0 | 130.2$^{g}$ | 79.3 | 125.0 | 108.9 | 122.1 | 125.3 | 117.6 | 119.0 | | 126.7 |
| SD | 2.3 | 2.0 | 2.1 | 2.0 | 2.5 | 6.1 | 3.1 | 1.0 | 1.6 | 1.1 | 0.8 | | 0.5 |
| measured or derived | 140$^{i}$ | 97$^{h}$ | 139$^{j}$ | 163$^{h}$ | 79$^{j}$ | 125$^{k}$ | 109$^{k}$ | 122$^{k}$ | 125$^{j}$ | 118$^{k}$ | 119$^{k}$ | | 128$^{i}$ |

$^{a}$ $(^{2}H/^{1}H)_i$ values were calculated as described in the Materials and Methods section. Three acquisitions were made for each sample. For each acquisition, $(^{2}H/^{1}H)_i$ values were calculated for resonance at 8.5 and 7.0 ppm of the internal reference, pyridine. SD is the standard deviation for each value. $^{b}$ Sites 2, 8, and 11 all resonate at the same frequency. $^{c}$ Sites 4−7 and 13−17 all resonate at the same frequency. $^{d}$ Sites 9 and 10 resonate at the same frequency. $^{e}$ Sites 12 and OMe resonate at the same frequency. $^{f}$ To maintain continuity, the sites in **4** are numbered following their position in methyl ricinoleate **1**. $^{g}$ Sites 3 and 8 resonate at the same frequency. $^{h}$ Calculated $(^{2}H/^{1}H)$ value. $^{i}$ Direct values from **1**. $^{j}$ Direct values from **2**. $^{k}$ Direct values from **4**. $^{l}$ SD is the standard deviation calculated from six values obtained for two independent chemical and NMR experiments.

***Table 2.*** $(^{2}H/^{1}H)_i$ Values (in ppm) of Methyl Ricinoleate **1** (Castor 2) and Corresponding Derivatives **2** and **4**$^{a}$

| carbon n° | 2 | 3 | 4–7 | 8 | 9 | 10 | 11 | 12 | COOMe | 13 | 14–17 | 13–17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| castor 2 | 137.4$^{b}$ | 98.6 | 129.6$^{c}$ | 137.4$^{b}$ | 103.4$^{d}$ | 103.4$^{d}$ | 137.4$^{b}$ | 123.5$^{e}$ | 123.5$^{e}$ | | | 129.6$^{c}$ | 131.9 |
| SD | 5.0 | 1.0 | 0.4 | 5.0 | 0.8 | 0.8 | 5.0 | 1.3 | 1.3 | | | 0.4 | 1.0 |
| expt 1: **2**/**4**$^{f}$ | 145.7 | 133.0$^{g}$ | 140.4 | 133.0$^{g}$ | 82.4 | 132.7 | 104.0 | 130.8 | 128.4 | 114.3 | 119.2 | | 127.2 |
| SD | 4.9 | 2.8 | 2.4 | 2.8 | 2.8 | 3.4 | 2.7 | 2.8 | 2.8 | 1.6 | 1.3 | | 0.7 |
| expt 2: **2**/**4**$^{f}$ | 142.7 | 126.9$^{g}$ | 137.0 | 126.9$^{g}$ | 79.1 | 118.7 | 102.5 | 123.1 | 125.5 | 123.8 | 122.1 | | 124.0 |
| SD | 0.2 | 0.9 | 0.7 | 0.9 | 0.9 | 2.2 | 2.3 | 4.5 | 1.2 | 1.7 | 1.2 | | 2.2 |
| means **2**/**4** | 144.2 | 130.0 | 138.7 | 130.0 | 80.7 | 125.7 | 103.2 | 126.9 | 127.0 | 119.0 | 120.7 | | 125.6 |
| SD$^{l}$ | 3.5 | 3.8 | 2.5 | 3.8 | 2.6 | 8.1 | 2.4 | 5.4 | 2.1 | 5.4 | 2.0 | | 2.3 |
| measured or derived | 144$^{i}$ | 99$^{h}$ | 139$^{j}$ | 161$^{h}$ | 81$^{j}$ | 126$^{k}$ | 103$^{k}$ | 127$^{k}$ | 127$^{j}$ | 119$^{k}$ | 121$^{k}$ | | 132$^{i}$ |

$^{a}$ For footnotes, see Table 1.

***Table 3.*** $(^{2}H/^{1}H)_i$ Values (in ppm) of Methyl Ricinoleate **1** (Ergot) and Corresponding Derivatives **2** and **4**$^{a}$

| carbon n° | 2 | 3 | 4–7 | 8 | 9 | 10 | 11 | 12 | COOMe | 13 | 14–17 | 13–17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| expt 1: ergot | 129.3$^{b}$ | 105.2 | 119.6$^{c}$ | 129.3$^{b}$ | 90.6$^{d}$ | 90.6$^{d}$ | 129.3$^{b}$ | 126.4$^{e}$ | 126.4$^{e}$ | | | 119.6$^{c}$ | 133.9 |
| SD | 3.4 | 5.3 | 0.5 | 3.4 | 3.0 | 3.0 | 3.4 | 1.0 | 1.0 | | | 0.5 | 0.4 |
| expt 2: ergot | 125.4$^{b}$ | 106.1 | 120.2$^{c}$ | 125.4$^{b}$ | 88.6$^{d}$ | 88.6$^{d}$ | 125.4$^{b}$ | 129.7$^{e}$ | 129.7$^{e}$ | | | 120.2$^{c}$ | 139.7 |
| SD | 2.2 | 1.9 | 0.6 | 2.2 | 2.7 | 2.7 | 2.2 | 2.3 | 2.3 | | | 0.6 | 1.1 |
| means ergot | 127.4$^{b}$ | 105.7 | 119.9$^{c}$ | 127.4$^{b}$ | 89.6$^{d}$ | 89.6$^{d}$ | 127.4$^{b}$ | 128.1$^{e}$ | 128.1$^{e}$ | | | 119.9$^{c}$ | 136.8 |
| SD | 3.1 | 3.6 | 0.6 | 3.1 | 2.8 | 2.8 | 3.1 | 2.2 | 2.2 | | | 0.6 | 3.3 |
| expt 1: **2**/**4**$^{f}$ | 137.9 | 122.8$^{g}$ | 128.0 | 122.8$^{g}$ | 50.2 | 131.4 | 92.6 | 127.4 | 127.3 | 123.7 | 115.6 | | 128.7 |
| SD | 1.4 | 0.4 | 0.7 | 0.4 | 5.2 | 0.7 | 1.8 | 5.3 | 0.7 | 1.7 | 1.5 | | 1.6 |
| expt 2: **2**/**4**$^{f}$ | 133.7 | 120.0$^{g}$ | 130.1 | 120.0$^{g}$ | 39.4 | 122.9 | 99.7 | 120.6 | 130.0 | 130.3 | 118.4 | | 126.3 |
| SD | 4.3 | 1.8 | 2.2 | 1.8 | 1.5 | 4.5 | 6.4 | 3.5 | 1.3 | 2.0 | 0.6 | | 2.7 |
| means **2**/**4** | 135.8 | 121.4 | 129.0 | 121.4 | 44.8 | 127.1 | 96.1 | 124.0 | 128.7 | 127.0 | 117.0 | | 127.5 |
| SD$^{l}$ | 3.7 | 1.9 | 1.9 | 1.9 | 6.8 | 5.5 | 5.7 | 5.5 | 1.8 | 4.0 | 1.8 | | 2.4 |
| measured or derived | 136$^{j}$ | 106$^{i}$ | 129$^{j}$ | 137$^{h}$ | 45$^{j}$ | 127$^{k}$ | 96$^{k}$ | 124$^{k}$ | 129$^{h}$ | 127$^{j}$ | 117$^{k}$ | | 137$^{i}$ |

$^{a}$ For footnotes, see Table 1.

***Table 4.*** Comparison between $(^{2}H/^{1}H)_i$ Values in ppm measured on Methyl Ricinoleate **1** (Castor 1, Castor 2, and Ergot) and their Corresponding Derivatives **2** and **4**

| comparison | 2, 8, 11 | 4–7/13–17 | 9, 10 | 18 |
|---|---|---|---|---|
| castor 1 (*M*) | 139.9 | 128.2 | 105.0 | 127.8 |
| products (*P*) | 138.6$^{a}$ | 127.7 | 102.2 | 126.7 |
| $\Delta$ ($M - P$) | 1.3 | 0.5 | 2.9 | 1.1 |
| castor 2 (*M*) | 137.4 | 129.6 | 103.4 | 131.9 |
| products (*P*) | 136.3$^{a}$ | 128.5 | 103.2 | 125.6 |
| $\Delta$ ($M - P$) | 1.2 | 1.1 | 0.2 | 6.3 |
| ergot (*M*) | 127.4 | 119.9 | 89.6 | 136.8 |
| products (*P*) | 123.0$^{a}$ | 123.4 | 86.0 | 127.5 |
| $\Delta$ ($M - P$) | 4.3 | −3.5 | 3.6 | 9.3 |

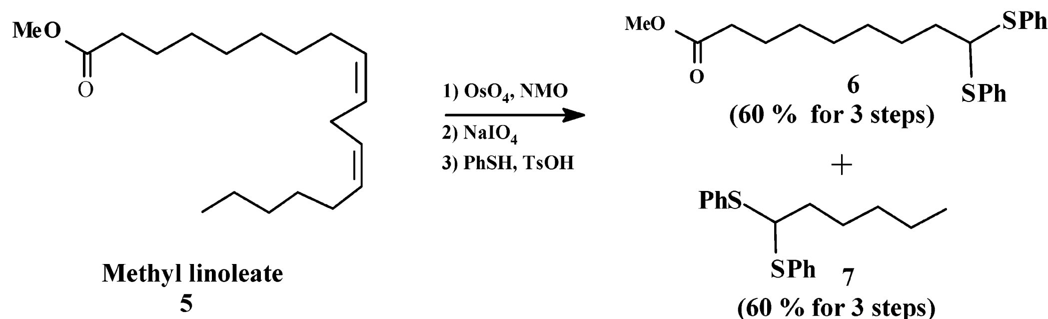
$^{a}$ $(^{2}H/^{1}H)_{2,8,11}$ for products was calculated from $(^{2}H/^{1}H)_2$ and $(^{2}H/^{1}H)_{11}$ directly measured in **2** and **4**, respectively, and $(^{2}H/^{1}H)_8$ calculated from $(^{2}H/^{1}H)_3$ in **1** and $(^{2}H/^{1}H)_{3,8}$ measured in **2**.

of internal checks must be performed to confirm that no significant artifactual fractionation has occurred. Thus, the $(^{2}H/^{1}H)_i$ values obtained from compounds **2** and **4** may be compared with the $(^{2}H/^{1}H)_i$ values measured for the intact methyl ricinoleate **1** samples (Table 4). Data from methyl ricinoleate **1** compare favorably with the data from the respective products **2** and **4**, with a difference ranging from 0.5 to 5.0%. The only exception was the $(^{2}H/^{1}H)_{18}$ of the ergot sample, for which **1** gives a value 7% higher than that measured for **4** (Table 4). This difference is probably due to the presence in the ergot sample of **1** of a small impurity that possesses the same chemical shift as that of site 18 in methyl ricinoleate **1** and which is removed during purification of **4**. However, as all other values are in good agreement, it can be concluded that the chemical modification of **1** to **2** and **4** has not introduced aberrant $(^{2}H/^{1}H)_i$ values.

By this procedure, the $(^{2}H/^{1}H)_i$ for a number of sites was directly determined. Other sites may be obtained by calculation, such as the $(^{2}H/^{1}H)_8$, which may be calculated by using the value $(^{2}H/^{1}H)_{3,8}$ measured in **2**, and the value for $(^{2}H/^{1}H)_3$ measured in **1**. The measured or calculated $(^{2}H/^{1}H)_i$ values for the three methyl ricinoleate **1** samples are compared in Figure 2A.

**$^{2}$H Distribution in Methyl Linoleate 5.** Methyl linoleate **5** was isolated from the same ergot oil sample as the *C. purpurea* **1** treated above. Following separation from **1** by silica chromatography, pure **5** was isolated by silica−Ag chromatography and cleavage fragments **6** and **7** were obtained by chemical modification (Scheme 2).[23] The $(^{2}H/^{1}H)_i$ data obtained from the

***Scheme 2***



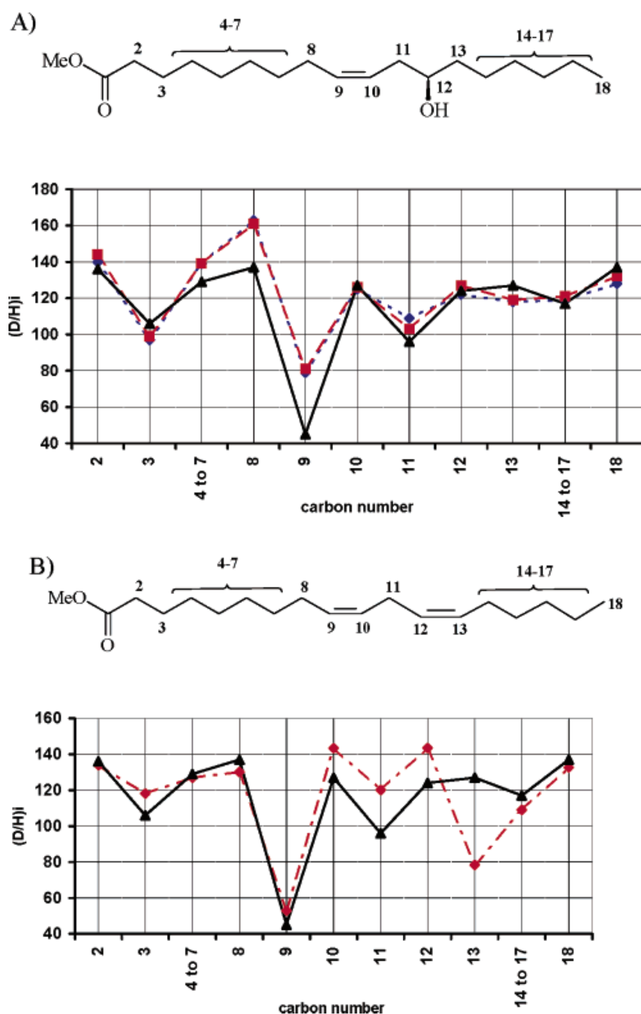




***Figure 2.*** Distribution of $(^2H/^1H)_i$ values at different sites in fatty acids isolated from *R. communis* and *C. purpurea* oils. (A) Methyl ricinoleate **1** isolated from castor 1 (•••), castor 2 (red - - -) or ergot (— — —). (B) Methyl ricinoleate **1** (— — —) and methyl linoleate **5** (red - - -) from ergot.

$^2$H NMR spectra of **5**−**7** are summarized in Table 5. The nonstatistical distribution of $^2$H is essentially as found previously for other samples of methyl linoleate.[23,24] Notably, the $(^2H/^1H)_i$ values at the C9 and C13 positions are markedly depleted in $^2$H relative to other uneven carbon positions. A similar validation that preparation had not led to fractionation was carried out as for methyl ricinoleate **1**.

## Discussion

The procedure described here has, for the first time, given access to a large number of $(^2H/^1H)_i$ values for ricinoleic acid from two different origins: plant and fungus. Furthermore, methyl linoleate **5** from ergot can be directly compared with methyl ricinoleate **1** from the same source. The low level of methyl oleate **8** in castor oil (3% total FAMEs) and the presence in ergot oil of a mixture of C16:1$\Delta$,$^7$ C18:1$\Delta^9$, and C18:1$\Delta^{11}$ makes it impractical to measure directly the $(^2H/^1H)_i$ values for methyl oleate from these sources.

Nevertheless, from these data, a number of conclusions can be drawn.

It is clear that the distributions of $^2$H along the fatty acid chain of ricinoleate **1** from castor and ergot samples are nonstatistical. Methyl ricinoleate **1** isolated from castor oil shows the same profile at the $\Delta^9$-desaturation as previously seen for two sources of methyl oleate **8** isolated from different plant origins, in that the $(^2H/^1H)_8 > (^2H/^1H)_{10} \gg (^2H/^1H)_9$. From this it can be concluded that the hydroxylation of methyl oleate **8** at the 12 position has not significantly influenced the $^2$H-distribution profile found in **1**. Methyl ricinoleate **1** isolated from ergot oil shows the same qualitative profile, with the notable difference that the $(^2H/^1H)_8 \approx (^2H/^1H)_{10} \gg (^2H/^1H)_9$. Whether this is a significant difference in terms of mechanism remains to be established. It could reflect the different sensitivity to $^2$H of a combination of factors: (a) the reductases of the FAS complex, which would result in a different *pro-S*-H/*pro-R*-H ratio, (b) the $\Delta^9$-desaturase, (c) the hydrogen removed by the $\Delta^9$-desaturase (removal of the *pro-S*-H rather than the *pro-R*-H), or (d) an influence of the hydroxylation mechanism.

A key observation in terms of mechanistic comparisons is that the $(^2H/^1H)_{13}$ of **1** from *C. purpurea* shows the same relationship to other sites in the molecule as it does in **1** from *R. communis* (Figure 2A). That is, its $(^2H/^1H)$ ratio does not correspond to that defined for the C13 position in any methyl linoleate so far examined,[23,24,27] including that reported here for ergot oil (Figure 2B). In all these cases, $(^2H/^1H)_{13} \ll (^2H/^1H)_{12}$. Rather, its $(^2H/^1H)$ ratio is similar to that of values determined for saturated positions.[23,24,27] Thus, it can be concluded as extremely improbable that linoleic acid is the precursor of ricinoleic acid in *C. purpurea*. It could be argued that the mechanism of hydration is associated with an overall kinetic isotope effect that restores the observed value to that of the original methylenic positions. As hydration involves an sp$^2$ to sp$^3$ conversion, known to be associated with an inverse secondary isotope effect,[28,29] this would act to increase the value of the $(^2H/^1H)_{13}$ in the product. However, the introduction of the H derived from water will show a normal primary isotope effect and select against introducing $^2$H at C-13 of ricinoleate.

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***Table 5.*** $(^2H/^1H)_i$ Values (in ppm) of Methyl Linoleate **5** (from Ergot Oil) and Corresponding Derivatives **6** and **7**

| carbon n° | 2 | 3 | 4–6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16, 17 | 18 | COOMe |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Me linoleate **5**[a] | 133.9 | 118.1 | 120.6[c] | 120.6[c] | 130.3[d] | 104.5[e] | 104.5[e] | 120.1 | 104.5[e] | 104.5[e] | 130.3[d] | 120.6[c] | 120.6[c] | 132.7 | 135.0 |
| SD | 4.2 | 3.8 | 4.9 | 4.9 | 4.2 | 3.5 | 3.5 | 3.8 | 3.5 | 3.5 | 4.2 | 4.9 | 4.9 | 4.8 | 4.3 |
| derivatives[b] **6/7**[f] | 142.3 | 117.2[g] | 130.8 | 117.2[g] | 130.1[h] | 52.8 | | | | 78.3 | 114.2 | 119.8 | 100.8 | 127.9 | 132.1 |
| SD | 1.6 | 3.7 | 2.1 | 3.7 | | 3.2 | | | | 7.0 | 2.3 | 1.5 | 1.0 | 2.1 | 5.5 |
| measured or derived | 134[i] | 118[i] | 131[j] | 116[l] | 130[j] | 53[j] | 143[l] | 120[i] | 143[l] | 78[k] | 114[k] | 120[k] | 101[k] | 133[i] | |

[a] $(^2H/^1H)_i$ values were calculated as described in the Materials and Methods section. Three acquisitions were made for each sample except for linoleate with two acquisitions. For **5**, $(^2H/^1H)_i$ values were calculated for resonance at 8.5 and 7.0 ppm of the internal reference, pyridine, on each acquisition. SD is the standard deviation calculated from four values. [b] For **6** and **7**, $(^2H/^1H)_i$ values were calculated for resonance at 2.8 ppm of the internal reference, TMU, on each acquisition. SD is the standard deviation calculated from three values. [c] Sites 4−7 and 15−17 all resonate at the same frequency. [d] Sites 8 and 14 resonate at the same frequency. [e] Sites 9, 10, 12, and 13 resonate at the same frequency. [f] To maintain continuity, the sites in **7** are numbered following their position in methyl linoleate **5**. [g] Sites 3 and 8 resonate at the same frequency. [h] Mean of two repetitions. [i] Direct values from **1**. [j] Direct values from **6**. [k] Direct values from **4**. [l] Calculated $(^2H/^1H)$ value.

The primary effect will override any weaker secondary effect, and thus hydration of the $\Delta^{12}$ bond will not lead to relative enrichment in $^2H$. Hence, it can be predicted that the $(^2H/^1H)_{13}$ of **1** derived by hydration of linoleate would retain impoverishment at the 13 position. However, as the C13 is not impoverished, this is not the case. In addition, as the linoleate from *C. purpurea* has $(^2H/^1H)_{13} = 78$ ppm, the hydrogen added to C-13 to form a methylenic site in ricinoleate would need to have a $(^2H/^1H)$ value of about 180 ppm to give the measured value of 127 ppm (Table 3 and Figure 2B), well in excess of that for water from southern England (ca. 150 ppm).

The last important deduction concerns the 12-(*R*)-hydroxylation step. During the formation of both oleate and 12-(*R*)-ricinoleate in castor seed, it is the *pro-R* hydrogen of the methylenic sites 10 and 12 that is abstracted.[4,15,30] As shown (Tables 1 and 2), $(^2H/^1H)_{10} \approx (^2H/^1H)_{12} < (^2H/^1H)_8$ in all samples of **1**. A strong isotope effect has been reported for the membrane-bound $\Delta^9$- and $\Delta^{12}$-desaturases at the 9 and 12 positions, respectively, with no detectable isotope effect at the 10 and 13 positions.[21,31,32] Because the mechanism of the enzyme involved in the 12-(*R*)-hydroxylation of oleate in *R. communis* is considered mechanistically close to these enzymes,[16,17,19] this implies either that no secondary effect is associated with the hydroxylation or that the mechanisms in the two sources do show some kinetic dissimilarities.

The soluble stearoyl-ACP $\Delta^9$-desaturase from higher plants shows negligible sequence homology with the stearoyl-ACP $\Delta^9$-desaturases from animals and fungi, which are membrane-bound proteins.[33,34] Yet, from the similarity of the $^2H$ profile established for **1** obtained from castor and ergot (Figure 2A), it would appear that the two activities are mechanistically close. Notably, both show depletion in the C9 and not in the C10 position. Further studies of other fungal oils will help establish whether this profile is general to this class of organism.

In conclusion, the results presented in this paper demonstrate three points. First, the data strongly support the conclusion that ricinoleic acid is biosynthesized by *R. communis* and *C. purpurea* by a common mechanism. It is evident that oleate and not linoleate is the substrate for hydroxylation, thus correcting a long-standing anomaly in the literature. Second, because $(^2H/^1H)_{10} \approx (^2H/^1H)_{12}$ in all samples studied, no measurable secondary kinetic isotope effect on the position C12 associated with hydroxylation can be detected that is distinct from any effect occurring in desaturation. This observation is in agreement with the current proposal that 12-(*R*)-hydroxylation and desaturation are mechanistically close.[19] Third, it is shown that a detailed analysis of the natural abundance $^2H$ distribution in similar chemical species can be used to predict biosynthetic mechanisms.

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