Stereospecific Removal of the *pro-R* Hydrogen at C-8 of (9*S*)-Hydroperoxyoctadecadienoic Acid in the Biosynthesis of Colneleic Acid

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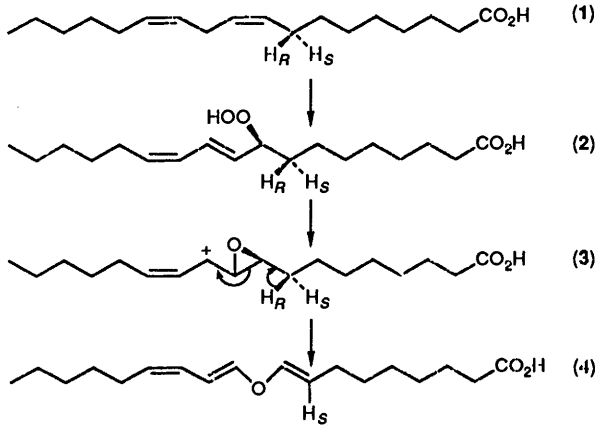
The stereochemistry of hydrogen removal in the conversion of (9*S*)-hydroperoxyoctadeca-(10*E*,12*Z*)-dienoic acid (**2**) into colneleic acid (**4**) was studied. [(8*R*)-^{2}H]-(**2**) and [(8*S*)-^{2}H]-(**2**) were incubated with the 105 000g particle fraction of potato homogenate and the colneleic acid formed was isolated. Mass spectrometric analysis demonstrated that colneleic acid which was biosynthesized from the [(8*R*)-^{2}H]-hydroperoxide was largely devoid of deuterium, whereas colneleic acid produced from the [(8*S*)-^{2}H]-hydroperoxide retained most of the deuterium. Accordingly, there is a selective removal of the *pro-R* hydrogen at C-8 in the biosynthesis of colneleic acid from (9*S*)-hydroperoxyoctadeca-(10*E*,12*Z*)-dienoic acid.

In 1971, conversion of linoleic acid (**1**) into (9*S*)-hydroperoxyoctadeca-(10*E*,12*Z*)-dienoic acid (**2**) by a lipoxygenase from potato tubers was described.[1] Shortly thereafter, potato homogenate was found to contain an enzyme activity that catalysed conversion of compound (**2**) into a divinyl ether derivative, colneleic acid {9-[nona-(1′*E*,3′*Z*)-dienyloxy]-non-(8*E*)-enoic acid} (**4**)[2] (Scheme 1). (9*S*)-Hydroperoxyoctadeca-(10*E*,12*Z*,15*Z*)-trienoic acid was transformed in an analogous way; however, no conversion took place with (13*S*)-hydroperoxyoctadeca-(9*Z*,11*E*)-dienoic acid (**8**). Colneleic acid was degraded, non-enzymatically and possibly enzymatically, into *inter alia* 9-oxononanoic acid[3] and may thus be a precursor of a number of the short-chain aldehyde and aldehyde-acid fragments which are formed upon disruption of plant tissues. The role of colneleic acid in the intact plant remains to be elucidated.



Scheme 1.

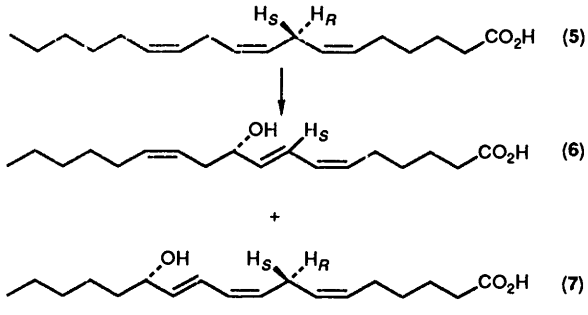
One of the two hydrogens at C-8 of linoleic acid (9*S*)-hydroperoxide (**2**) is eliminated in the formation of colneleic acid (**4**). The present study is concerned with the stereospecificity of this hydrogen removal.

Results and Discussion

Linoleic (**1**) and γ-linolenic (**5**) acids stereospecifically deuteriated at C-8 were prepared using methodology previously described.[4] Two sets of the deuteriated acids were prepared, starting either with methyl (8*R*)-8-hydroxystearate obtained from isano oil [containing 7% of methyl (9*S*)-9-hydroxystearate], or with homogenous, synthetically prepared methyl (8*R*)-8-hydroxystearate.

In previous studies dealing with the stereospecificity of lipoxygenase reactions, prostaglandin biosynthesis, and leukotriene A_4 biosynthesis, stereospecifically tritiated fatty acids were used. Because lithium aluminium tritide, needed for stereospecific introduction of tritium, is not commercially available at present, we prepared stereospecifically deuteriated acids by means of lithium aluminium deuteride and studied the fate of the introduced deuterium atom by mass spectrometry. The deuteriated linoleic and γ-linolenic acids were obtained from the corresponding stearates by biological desaturation using the flagellate, *Tetrahymena pyriformis*. Because of dilution with unlabelled material during this step, the recovered linoleic and γ-linolenic acids contained a large percentage of the unlabelled acids (isotope compositions 9–32% of monodeuteriated and 91–68% of undeuteriated molecules).

Previous work, in which tritium-labelled linoleic and γ-linolenic acids were prepared from stearates stereospecifically labelled with tritium at C-11, showed that biological desaturation using *Tetrahymena pyriformis* proceeded essentially without degradation of the added stearate and without any racemization of the tritium label.[4b,5] In order to verify that this was also true in the case of desaturation of the present stearates stereospecifically deuteriated at C-8, control experiments were performed with deuteriated γ-linolenic acids obtained by desaturation of [(8*R*)-^{2}H]- and [(8*S*)-^{2}H]-stearates. Advantage was taken by the previously described conversion of γ-linolenic acid into (10*S*)-hydroxyoctadeca-(6*Z*,8*E*,12*Z*)-trienoic acid (**6**) in human platelets[6] (Scheme 2). The initial step of this transformation was catalysed by arachidonic acid 12-lipoxy-



Scheme 2.

Table 1.

Compound	Monodeuteriated molecules (%) Exp. I[a]	Exp. II[b]
[(8*R*)-^{2}H]-(**5**)	19	28
(**6**) Expected for (8*R*)-^{2}H removal	0	0
(**6**) Expected for (8*S*)-^{2}H removal	19	28
(**6**) Found	2	2
(**7**) Expected for (8*R*)- or (8*S*)-^{2}H removal	19	28
(**7**) Found	19	28
[(8*S*)-^{2}H]-(**5**)	18	32
(**6**) Expected for (8*R*)-^{2}H removal	18	32
(**6**) Expected for (8*S*)-^{2}H removal	0	0
(**6**) Found	15	25
(**7**) Expected for (8*R*)- or (8*S*)-^{2}H removal	18	32
(**7**) Found	17	32

[a] Deuteriated γ-linolenic acids prepared starting with methyl (8*R*)-8-hydroxystearate obtained from isano oil. [b] Deuteriated γ-linolenic acids prepared starting with synthetic methyl (8*R*)-8-hydroxystearate.

Table 2.

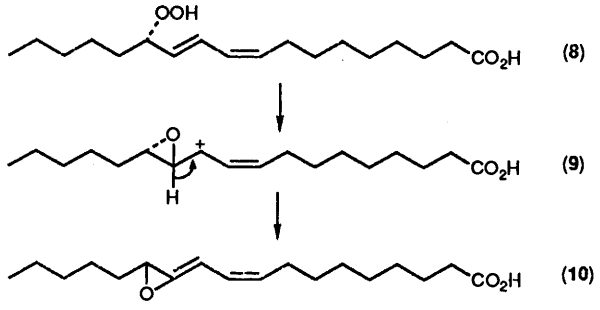
Compound	Monodeuteriated molecules (%) Exp. I[a]	Exp. II[b]
[(8*R*)-^{2}H]-(**2**)	10	12
(**4**) Expected for (8*R*)-^{2}H removal	0	0
(**4**) Expected for (8*S*)-^{2}H removal	10	12
(**4**) Found	2	1
[(8*S*)-^{2}H]-(**2**)	13	17
(**4**) Expected for (8*R*)-^{2}H removal	13	17
(**4**) Expected for (8*S*)-^{2}H removal	0	0
(**4**) Found	11	14

[a] Deuteriated hydroperoxides prepared starting with methyl (8*R*)-8-hydroxystearate obtained from isano oil. [b] Deuteriated hydroperoxides prepared starting with synthetic methyl (8*R*)-8-hydroxystearate.

genase. The resulting (10*S*)-hydroperoxyoctadecatrienoic acid was rapidly converted into compound (**6**) by the action of human platelet peroxidase. As seen in Table 1, compound (**6**) isolated following incubation of [(8*R*)-^{2}H]-γ-linolenic acid was largely undeuteriated, but retained most of the deuterium label when formed from [(8*S*)-^{2}H]-γ-linolenic acid. These findings, coupled with the known antarafacial relationship between hydrogen abstraction and oxygen insertion in the reaction catalysed by arachidonic acid 12-lipoxygenase,[7] therefore confirmed that the configuration of the deuterium label at C-8 remained unchanged during the desaturation step. Lipoxygenase activity of human platelets also catalyses the formation of (13*S*)-hydroxyoctadeca-(6*Z*,9*Z*,11*E*)-trienoic acid (**7**) from γ-linolenic acid.[6] As would be expected, acid (**7**) isolated following incubation of either [(8*R*)-^{2}H]- or [(8*S*)-^{2}H]-γ-linolenic acid completely retained the deuterium label (Table 1).

Incubation of the stereospecifically deuteriated linoleic acid (9*S*)-hydroperoxides with the 105 000g particle fraction of potato homogenate resulted in the formation of colneleic acid, which was isolated as its methyl ester by TLC. Parallel experiments with [1-^{14}C]-(**2**) demonstrated that, under the conditions used, the hydroperoxide was completely consumed and converted into a product consisting of >90% of colneleic acid. Analysis of methyl colneleates obtained from [(8*R*)-^{2}H]-(**2**) and [(8*S*)-^{2}H]-(**2**) by selected-ion monitoring demonstrated that the colneleate biosynthesized from the [(8*R*)-^{2}H]-precursor was largely devoid of deuterium, whereas colneleate formed from the [(8*S*)-^{2}H]-precursor retained most of the deuterium (Table 2). Thus, in the conversion of linoleic acid (9*S*)-hydroperoxide (**2**) into colneleic acid (**4**) catalysed by potato hydroperoxide dehydrase there is a selective removal of the *pro-R* hydrogen from C-8.

Recent studies with [$^{18}O_2$]-(**2**) have shown that the ether oxygen of colneleic acid originates in one of the hydroperoxide oxygens.[8] A mechanism was proposed for the conversion involving initial protonation of the distal hydroperoxide oxygen of the substrate followed by elimination of H_2O and formation of an epoxide carbocation intermediate (**3**).[8] Subsequent elimination of one of the hydrogens at C-8 (the *pro-R* hydrogen as shown in the present study) and cleavage of the C-9/C-10 bond resulted in the formation of colneleic acid (Scheme 1). In this context it is interesting to note that a similar epoxide carbocation has also been proposed as an intermediate in the recently described enzymatic conversion of (13*S*)-hydroperoxyoctadeca-(9*Z*,11*E*)-dienoic acid (**8**) to the allene oxide 12,(13*S*)-epoxyoctadeca-(9*Z*,11)-dienoic acid (**10**)[9] (Scheme 3). In that case, the hydrogen at C-12 of the epoxide cation (**9**) is eliminated as a proton with formation of the allene oxide structure.



Scheme 3.

Experimental

TLC was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck, FRG. Material was located by spraying with 2′,7′-dichlorofluorescein and viewing under UV light. GLC was performed with a F&M Biomedical gas chromatograph model 402 equipped with a packed column of 3% SP-2401 on Supelcoport (length 1.8 m; i.d. 3 mm) or with a Hewlett-Packard model 5890 gas chromatograph equipped with a methylsilicone capillary column (length 25 m; film thickness 0.33 μm). Mass spectra were obtained with a Hewlett-Packard model 5970B mass-selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. A Perkin-Elmer model 241 polarimeter was used for determination of optical rotations.

3-*Hydroxytridecanoic Acid*.—Undecanoyl chloride (200 mmol) was stirred at 20 °C for 2 h with sodium ethyl acetoacetate (230 mmol) in dry benzene (550 ml). The mixture was subsequently refluxed for 0.5 h, allowed to cool, and washed with water–ethanol (1:1 v/v) and water–ethanol (9:1 v/v). The combined aqueous phases were extracted with one portion of diethyl ether. The ether phase was combined with the benzene layer and dried over Na_2SO_4. Evaporation of the solvent gave an oil, which was treated with sodium methoxide (210 mmol) in methanol (240 ml) at 20 °C for 15 h. Acidification and extraction with two portions of diethyl ether yielded crude methyl 3-oxotridecanoate.

This material was dissolved in methanol (600 ml), and the solution was cooled to 0 °C and treated with $NaBH_4$ (12 g, added during 15 min). The mixture was stirred at 0 °C for 0.5 h and then at 20 °C for 4 h. A solution of NaOH (9 g) in water (100 ml) was added and the solution was stirred at 20 °C for 15 h. The

solution was carefully acidified to pH 3 and extracted with three portions of diethyl ether. The residue obtained after evaporation of the solvent was crystallized from ethyl acetate to yield the title compound (80 mmol, 40%), m.p. 79.5–80 °C (lit.,[10] 80.4–80.6 °C); mass spectrum of trimethylsilyl ether/methyl ester derivative: *m/z* 301 (100%, $M - 15$), 285 (3, $M - OCH_3$), 269 [4, $M - (CH_3 + CH_3OH)$], 243 (8, $M - CH_2CO_2CH_3$), 175 [62, $M - (CH_2)_9CH_3$], and 159 [21, $(CH_3)_2Si{=}O^+ - CH{=}CHCO_2CH_3$]; mass spectrum of acetyl/methyl ester derivative: *m/z* 243 (32%, $M - CH_3CO$), 226 (13, $M - CH_3CO_2H$), 195 [38, $M - (CH_3CO_2H + OCH_3)$], 194 [27, $M - (CH_3CO_2H + CH_3OH)$], 169 (29), 152 (41), and 55 (100).

(3R)-*Hydroxytridecanoic Acid.*—3-Hydroxytridecanoic acid (78.26 mmol) was dissolved in CCl_4 (1 l). Cinchonidine (78.26 mmol) was added and the mixture was kept on a steam-bath for 3 min. The solution was allowed to cool, and was then filtered, and kept at 20 °C for 15 h. The crystalline salt was collected, redissolved in CCl_4 (500 ml), and again allowed to crystallize at 20 °C for 15 h. After 6 recrystallizations the acid was regenerated from the salt by careful acidification and extraction with three portions of diethyl ether. Crystallization from ethyl acetate–hexane yielded the title compound (1.74 mmol, 4%), $[\alpha]_D^{20}$ −16.5° (*c* 0.47, $CHCl_3$) {lit.,[11] $[\alpha]_D$ −15° (*c* 2.0, $CHCl_3$); lit.,[12] $[\alpha]_D$ −16.6° (*c* 0.6, $CHCl_3$)}.

Methyl (8R)-8-*Hydroxystearate.*—Two batches of methyl (8*R*)-8-hydroxystearate were used, *i.e.* (8R)-hydroxystearate obtained from isano oil [contaminated with 7% of methyl (9*S*)-9-hydroxystearate],[13] as well as (8*R*)-hydroxystearate prepared by anodic coupling of (3*R*)-hydroxytridecanoic acid and methyl hydrogen pimelate as described below.

(3*R*)-Hydroxytridecanoic acid (1.74 mmol) was refluxed for 15 min with acetyl chloride (6 ml). Excess of acetyl chloride was evaporated off and the remaining acetoxytridecanoyl chloride was hydrolysed by being stirred with acetone–water (1.5:1 v/v) at 20 °C for 5 h. The resulting (3*R*)-acetoxytridecanoic acid was dissolved in methanol (100 ml) containing methyl hydrogen pimelate (25.5 mmol) and sodium methoxide (1.1 mmol). A current of 1.3 A was passed through the solution for 4.5 h. The material was saponified by treatment with 10% (w/v) NaOH at 20 °C for 15 h. Material isolated by extraction with diethyl ether was subjected to silicic acid chromatography. Crude (8*R*)-hydroxystearic acid was eluted with diethyl ether–hexane (2:8 v/v). The methyl ester obtained by treatment with diazomethane was subjected to further silicic acid chromatographic purification [elution with diethyl ether–hexane (15:85 v/v)]. Crystallization from hexane yielded pure methyl (8*R*)-8-hydroxystearate (0.96 mmol, 55%), m.p. 55.5–56.0 °C (lit.,[14] 55.3–55.6 °C); mass spectrum of trimethylsilyl ether derivative: *m/z* 371 (3%, $M - CH_3$), 355 (3, $M - OCH_3$), 339 [6, $M - (CH_3 + CH_3OH)$], 245 [100, $M - (CH_2)_9CH_3$], 243 [49, $M - (CH_2)_6CO_2CH_3$], and 216 [11, $M - OCH(CH_2)_9CH_3$]; mass spectrum of acetate derivative: 313 (4%, $M - CH_3CO$), 296 (8, $M - CH_3CO_2H$), 265 [22, $M - (OCH_3 + CH_3CO_2H)$], 264 [31, $M - (CH_3OH + CH_3CO_2H)$], 222 (13), 215 [8, $M - (CH_2)_9CH_3$], 173 (60), 141 (40), and 55 (100).

Methyl (8R)-8-p-*Tolylsulphonyloxystearate.*—A solution of methyl (8*R*)-8-hydroxystearate (0.53 mmol) in pyridine (5 ml) was treated with toluene-*p*-sulphonyl chloride (500 mg) at −25 °C for 2 h and at +4 °C for 40 h. Excess of reagent was destroyed by addition of water at 0 °C and the crude tosylester was isolated by extraction with diethyl ether. Purification by silicic acid column chromatography [elution with diethyl ether–hexane (1:9 v/v)] yielded the title compound (0.27 mmol, 51%), λ_{max} 225 nm; R_f 0.26 [solvent diethyl ether–hexane (1:9 v/v)].

Methyl (8S)-8-p-*Tolylsulphonyloxystearate.*—Methyl (8*R*)-8-*p*-tolylsulphonyloxystearate (0.18 mmol) was treated with a mixture of sodium acetate (41.2 mg) in glacial acetic acid (5.8 ml) at 60 °C for 15 h. The acetate of methyl (8*S*)-8-hydroxystearate was hydrolysed in refluxing NaOH (10% w/v) in methanol–water (8:2 v/v) and the hydroxy acid was first esterified by treatment with diazomethane and then converted into the toluene-*p*-sulphonyl derivative as described above. The title compound was obtained following silicic acid column chromatography [elution with diethyl ether–hexane (1:9 v/v)], λ_{max} 225 nm; R_f 0.26 [solvent diethyl ether–hexane (1:9 v/v)].

[(8S)-^{2}H]*Stearic Acid.*—Methyl (8*R*)-8-*p*-tolylsulphonyloxystearate (0.09 mmol) was dissolved in dry tetrahydrofuran (THF) (10 ml) and the solution was refluxed with lithium aluminium deuteride (100 mg; E. Merck, Darmstadt) for 22 h. Excess of reagent was decomposed by careful addition of moist THF and the octadecanol was extracted with diethyl ether.

Oxidation with chromium trioxide in 97.5% aqueous acetic acid at 50 °C for 1 h, followed by purification by silicic acid column chromatography [elution with diethyl ether–hexane (5:95 v/v)], yielded pure [(8*S*)-^{2}H]stearic acid (0.04 mmol, 44%), *m/z* (methyl ester), 299 (30%), 256 (14), 200 (9), 199 (7), 143 (18), 87 (59), and 74 (100). The specimen consisted of >98% monodeuteriated molecules as judged from selected monitoring of the ions at *m/z* 298 and 299.

[(8*R*)-^{2}H]*Stearic Acid.*—Reduction of methyl (8*S*)-8-*p*-tolylsulphonyloxystearate with lithium aluminium deuteride, followed by chromic acid oxidation and purification by silicic acid chromatography as described above, yielded [(8*R*)-^{2}H]stearic acid (0.07 mmol, 77%). Its identity was confirmed by mass spectrometry as described above. The specimen consisted of >98% monodeuteriated molecules.

Stereospecifically Deuteriated Linoleic and γ-Linolenic Acids.—To culture medium (160–375 ml) consisting of glucose (0.5% w/v), yeast extract (0.5% w/v), and peptone (0.5% w/v) in 0.004M-potassium phosphate buffer (pH 7.0) were added stereospecifically deuteriated stearates (sodium salts; 35 μg ml^{-1}). The medium was sterilized and inoculated with a suspension of *Tetrahymena pyriformis*, strain GL. After 5 days at 20 °C the organisms were recovered by centrifugation and refluxed with KOH (10% w/v) in methanol–water (1:1 v/v) for 1.5 h under argon. Material obtained by extraction with diethyl ether was subjected to silicic acid column chromatography. The fatty acid fraction [eluted with diethyl ether–hexane (5:95 v/v)] was subjected to HPLC on a column (300 × 8 mm) of Polygosil C_{18} 5μ and a solvent system consisting of acetonitrile–water–acetic acid (85:15:0.02 v/v/v). This afforded deuteriated linoleic acid (effluent volume 40.8–47.2 ml; yield 12–15 μg per ml of medium) and deuteriated γ-linolenic acid (effluent volume 31.2–35.6 ml; yield 15–17 μg per ml of medium). The isotopic composition of the [(8*R*)-^{2}H]- and [(8*S*)-^{2}H]-linoleates obtained in the different incubations was 9–19% of monodeuteriated molecules and 91–81% of undeuteriated molecules as determined by selected-ion monitoring of the ions at *m/z* 294 and 295 (methyl esters). The corresponding figures for the [(8*R*)-^{2}H]- and [(8*S*)-^{2}H]-γ-linolenates obtained by monitoring of the ions at *m/z* 292/293 were 18–32% of monodeuteriated molecules and 82–68% of undeuteriated molecules.

(9S)-*Hydroperoxyoctadeca*-(10E,12Z)-*dienoic Acid* (**2**).—Tomato lipoxygenase, like lipoxygenases of, *e.g.* potato tubers and corn seed, specifically catalyses oxygenation of C-9 of compound (**1**) to produce title compound (**2**).[15] In contrast to potato and corn seed homogenate, the hydroperoxide-degrading activity of tomato homogenate is low which makes it possible to

obtain compound (**2**) in acceptable yield.[15] Tomato fruit was homogenized at 0 °C in 0.1M-potassium phosphate buffer (pH 6.0) with a Polytron (tissue to buffer ratio 1:3 w/v). Compound (**1**) as well as [(8*R*)-^{2}H]-(**1**) and [(8*S*)-^{2}H]-(**1**) were dissolved in ethanol and converted into the sodium salt by treatment with NaOH (2 mol equiv.). Tomato homogenate was added (5–10 ml; final concentration of substrate 3mM) and the mixture was shaken at 20 °C for 20 min. Material obtained by extraction with diethyl ether was subjected to silicic acid column chromatography. Elution with diethyl ether–hexane (15:85 v/v) afforded compounds (**2**), [(8*R*)-^{2}H]-(**2**), and [(8*S*)-^{2}H]-(**2**) in 20–40% yield, λ_{max}(EtOH) 235 nm. An aliquot was reduced by treatment with $SnCl_2$ in ethanol,[16] esterified, converted into the trimethylsilyl ether derivative, and analysed by GLC–mass spectrometry. The protium derivative showed prominent ions at *m/z* 382 (12%, *M*), 351 (1, $M - OCH_3$), 311 [9, $M - (CH_2)_4CH_3$], 292 [44, $M - (CH_3)_3SiOH$], 225 [36, $M - (CH_2)_7CO_2CH_3$], and 75 (100). The derivatives of deuteriated acid (**2**) showed additional ions at, *e.g.*, *m/z* 383, 352, 312, and 293 (but not at, *e.g.*, *m/z* 226). The isotopic compositions of the derivatives of deuteriated acid (**2**) (Table 2) were determined by selected monitoring of the ions *m/z* 382 and 383.

Human Platelet Incubations.—Suspensions of washed human platelets (5–10 ml; 0.6–0.9 × 10^6 platelets μl^{-1}) in a medium consisting of 0.154M-NaCl, 25mM-tris(hydroxymethyl)-aminomethane, and 0.2mM-disodium EDTA (pH 7.4)[17] were stirred at 37 °C for 30 min with undeuteriated or deuteriated γ-linolenic acid (final concentration 0.3mM). The resulting (10*S*)-hydroxyoctadeca-(6*Z*,8*E*,12*Z*)-trienoic acid (**6**) and (13*S*)-hydroxyoctadeca-(6*Z*,9*Z*,11*E*)-trienoic acid (**7**) were isolated as their methyl esters by straight-phase HPLC as previously described.[6]

Potato Incubations.—Tubers of potato (*Solanum tuberosum*, Bintje; 15 g) were homogenized at 0 °C in 0.1M-sodium borate buffer (pH 9.0) (30 ml) with a Polytron. The homogenate was filtered through cheesecloth and centrifuged at 9 300g for 15 min. The supernatant was removed and centrifuged at 105 000g for 60 min. The particle fraction thus obtained was carefully rinsed with buffer and resuspended in buffer (20 ml) (protein 0.2 mg ml^{-1}). A portion (5 ml) was shaken at 20 °C for 15 min with compounds (**2**), [(8*R*)-^{2}H]-(**2**), and [(8*S*)-^{2}H]-(**2**) (final concentration 0.2mM). Material extracted with diethyl ether was esterified and subjected to TLC. Methyl colneleate [methyl ester of acid (**4**)] was obtained in 85–90% yield following TLC [R_f 0.61; solvent system ethyl acetate–hexane (1:9 v/v)], λ_{max}(EtOH, hexane) 251 nm [lit.,[18] 252 nm (EtOH, 2,2,4-trimethylpentane)]; mass spectrum of the methyl ester of compound (**4**) obtained from undeuteriated (**2**): *m/z* 308 (38%, *M*), 251 [8, $M - (CH_2)_3CH_3$], 237 [3, $M - (CH_2)_4CH_3$], 219 (3), 165 [12, $M - (CH_2)_6CO_2CH_3$], 151 (9), 137 (19), 123 (21), 109 (21), 95 (45), 81 (82), 67 (100), and 55 (72).

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