Oxidation of Octadecatrienoic Acids in the Red Alga *Lithothamnion corallioides*: Structural and Stereochemical Studies of Conjugated Tetraene Fatty Acids and Bis Allylic Hydroxy Acids

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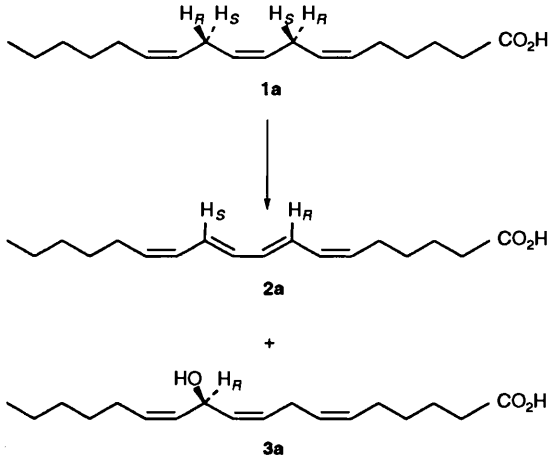
Enzymatic oxidation of (6*Z*,9*Z*,12*Z*)-octadeca-6,9,12-trienoic acid (γ-linolenic acid; **1a**) in a preparation of the red alga *Lithothamnion corallioides* Crouan led to the formation of (6*Z*,8*E*,10*E*,12*Z*)-octadeca-6,8,10,12-tetraenoic acid **2a** and (11*R*,6*Z*,9*Z*,12*Z*)-hydroxyoctadeca-6,9,12-trienoic acid **3a** as the main products. (9*Z*,12*Z*,15*Z*)-Octadeca-(9,12,15)-trienoic acid (α-linolenic acid; **4a**) was oxidized in an analogous way to yield (9*Z*,11*E*,13*E*,15*Z*)-octadeca-9,11,13,15-tetraenoic acid (α-parinaric acid; **5a**), (11*S*,9*Z*,12*Z*,15*Z*)-hydroxyoctadeca-9,12,15-trienoic acid **6a**, and (14*R*,9*Z*,-12*Z*,15*Z*)-hydroxyoctadeca-9,12,15-trienoic acid **7a**. Isotope studies demonstrated that enzymatic conversion of the acid **1a** into the tetraene **2a** was accompanied by stereospecific eliminations of the *pro-S* and *pro-R* hydrogens from C-8 and C-11, respectively. The bis-allylic hydroxy acid **3a** was formed from acid **1a** by a reaction involving stereospecific elimination of the *pro-S* hydrogen from C-11 and incorporation of 1 atom of oxygen from water in the C-11 hydroxy group. Although the bis-allylic hydroxy esters **3b**, **6b**, and **7b** were chemically convertible into conjugated tetraenes by rapid acid-catalysed dehydration, enzymatic formation of conjugated tetraenes and hydroxy acids in *Lithothamnion* occurred by independent pathways.

Fatty acids possessing a conjugated polyene structure occur in many plants, especially in seed oils. Although several conjugated trienoic acids differing with respect to double-bond position and configuration have been isolated[1,2], the only well-established conjugated tetraene fatty acids are (9*Z*,11*E*,-13*E*,15*Z*)-octadeca-9,11,13,15-tetraenoic acid (α-parinaric acid; **5a**) from certain seed oils[1], and (5*Z*,8*Z*,10*E*,12*E*,14*Z*)-eicosa-5,8,10,12,14-pentaenoic acid (bosseopentaenoic acid) from the red alga *Bossiella orbigniana*.[3] Several hypotheses have been offered to explain the mode of formation of conjugated polyenoic acids in plants, including dehydration of unsaturated fatty acids,[3,4] lipoxygenase-like radical reactions not involving oxygenated intermediates,[5,6] and NADPH-/NADH- and O_2-dependent desaturation of fatty acyl CoA.[7,8]

An extract of the red alga *Lithothamnion corallioides* Crouan was recently found to catalyse oxidation of linoleic acid into an unstable bis-allylic hydroxy acid, (11*R*,9*Z*,12*Z*)-hydroxyoctadeca-9,12-dienoic acid.[9] Furthermore, this alga produced conjugated tetraene fatty acids by enzymatic oxidation of γ-linolenic acid[10] and arachidonic acid[11]. The present paper is concerned with the chemistry of conjugated tetraenes and bis-allylic hydroxy acids formed from γ-linolenic and α-linolenic acids in *Lithothamnion corallioides*. The stereochemistry of the enzymatic bis-allylic oxidations leading to the formation of the different products has been determined utilizing stereospecifically deuteriated precursors. In the course of the investigation, chemical methods of wide applicability for determination of the absolute configuration of in-chain hydroxylated fatty acids, and of the geometrical configuration of conjugated polyenoic fatty acids, were developed. A preliminary account of part of this work has appeared.[10]

Results and Discussion *

Enzymatic oxidation of [1-^{14}C]-γ-linolenic acid **1a** and [1-^{14}C]-α-linolenic acid **4a** in the red alga *Lithothamnion corallioides* led to the formation of conjugated tetraene fatty acids and bis-allylic hydroxy acids as the major products (Schemes 1 and 2). Thus, (6*Z*,8*E*,10*E*,12*Z*)-octadeca-6,8,10,12-tetraenoic acid **2a** (29% of the recovered radioactivity) and (11*R*,6*Z*,9*Z*,12*Z*)-hydroxyoctadeca-(6,9,12)-trienoic acid **3a** (29%) were obtained from **1a**, and (9*Z*,11*E*,13*E*,15*Z*)-octadeca-9,11,13,15-tetraenoic acid **5a** (13%), (11*S*,9*Z*,12*Z*,15*Z*)-hydroxyoctadeca-9,12,15-trienoic acid **6a** (46%), and (14*R*,9*Z*,12*Z*,15*Z*)-hydroxyoctadeca-9,12,15-trienoic acid **7a** (6%) were obtained from **4a**. In addition, (6*Z*,10*E*,12*Z*)-9-hydroxyoctadeca-6,10,12-trienoic acid (2%) and (6*Z*,9*Z*,11*E*)-13-hydroxyoctadeca-6,9,11-trienoic acid (2%) were obtained as minor products from **1a**, and (10*E*,12*Z*,15*Z*)-9-hydroxyoctadeca-10,12,15-trienoic acid (2%) and (9*Z*,11*E*,-15*Z*)-13-hydroxyoctadeca-9,11,15-trienoic acid (2%) were obtained from **4a**.



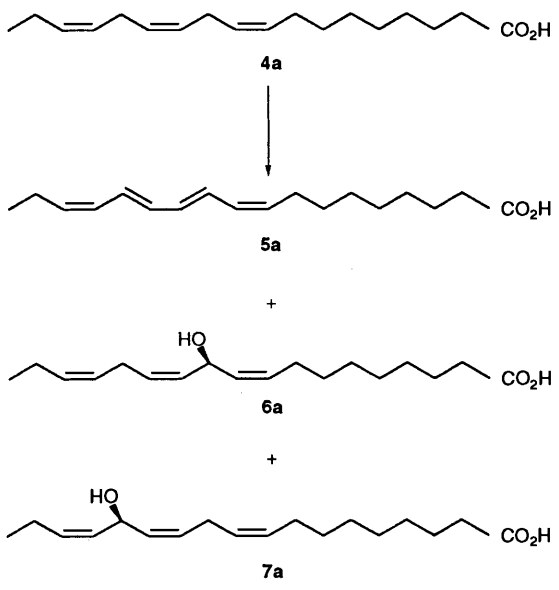
Scheme 1 Enzymatic oxidation of γ-linolenic acid in *Lithothamnion corallioides*

(6*Z*,8*E*,10*E*,12*Z*)-Octadeca-6,8,10,12-tetraenoic acid **2a** produced from γ-linolenic acid was isolated as its methyl ester **2b** by preparative HPLC. NMR spectroscopy demonstrated that the conjugated tetraene structure had the *Z*,*E*,*E*,*Z*, configur-

* Throughout, structural key numbers with the suffixes **a** and **b** refer to the acid and methyl ester, respectively, of the displayed formula.

Table 1 Steric analysis of methyl α-parinarate **5b** and its geometrical isomers **5c**, **5d** and **5e**

Isomer of methyl octadeca-9,11,13,15-tetraenoate	Configurations of methyl dihydroxystearates				
	9,10-	11,12-	13,14-	15,16-	Configuration of tetraene
5b	*erythro*	*threo*	*threo*	*erythro*	9*Z*,11*E*,13*E*,15*Z*
5c	*erythro*	*threo*	*threo*	*threo*	9*Z*,11*E*,13*E*,15*E*
5d	*threo*	*threo*	*threo*	*erythro*	9*E*,11*E*,13*E*,15*Z*
5e	*threo*	*threo*	*threo*	*threo*	9*E*,11*E*,13*E*,15*E*



Scheme 2 Enzymatic oxidation of α-linolenic acid in *Lithothamnion corallioides*

ation, and oxidative ozonolysis established that the tetraene was sited at $\Delta^{6,8,10,12}$. The conjugated octadecatetraenoic acid formed from α-linolenic acid was identified as α-parinaric acid, *i.e.* (9*Z*,11*E*,13*E*,15*Z*)-octadeca-9,11,13,15-tetraenoic acid **5a**, using the authentic compound as reference.

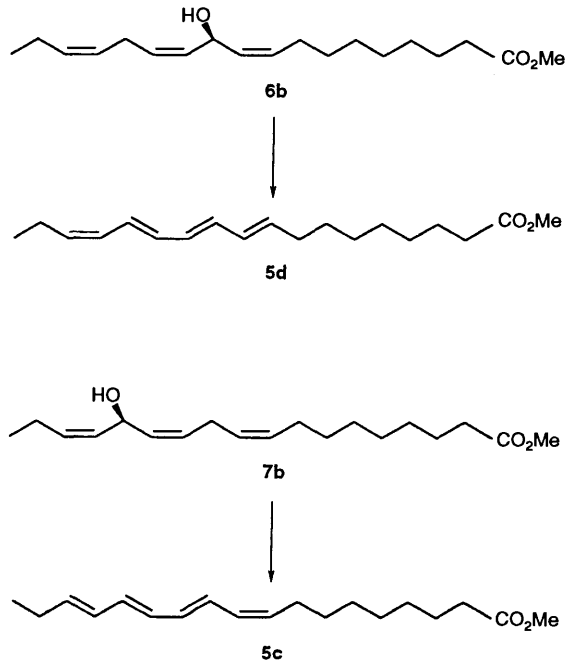
A sensitive, chemical method was developed for determination of the geometrical configuration of conjugated polyenoic acids such as α-parinaric acid and its geometrical isomers. The procedure was based on mono-dihydroxylation of the tetraene (0.2–1 mg) by treatment with 0.4 equiv. of osmium tetroxide. The osmate esters were decomposed by treatment with mannitol and the product hydrogenated. GC-MS analysis showed that geometrical isomers of methyl octadeca-9,11,13,15-tetraenoate treated in this way yielded mainly methyl stearate, corresponding to tetraene not dihydroxylated, and a mixture of regio- and stereo-isomeric methyl dihydroxystearates. The latter compounds were resolved and identified by GC-MS using the *erythro* and *threo* isomers of methyl 9,10-, 11,12-, 13,14-, and 15,16-dihydroxystearates as references. From the pattern of different methyl dihydroxystearates produced, coupled with the well-established mechanism of OsO_4 dihydroxylation by *cis*-addition to the olefin,[12] it could be confirmed that methyl α-parinarate **5b** isolated from *Impatiens balsamina* seed oil had the *Z*,*E*,*E*,*Z* configuration [13–15] (Table 1). Furthermore, three minor isomers of α-parinarate, *i.e.* **5c**, **5d** and **5e**, were assigned the *Z*,*E*,*E*,*E*-, *E*,*E*,*E*,*Z*- and *E*,*E*,*E*,*E*-configurations, respectively.

The conjugated *Z*,*E*,*E*,*Z* tetraenes suffered thermal *Z*→*E* double-bond isomerization during GLC analysis. Thus, with a methyl silicone capillary column operated at 210 °C, injection of methyl α-parinarate **5b** resulted in a major peak (92%) due to the injected **5b** as well as peaks due to the isomeric tetraenes **5c** (4%), **5d** (3%) and **5e** (1%).* Also, the tetraene **2b** underwent partial isomerization during GLC to produce a total of 5% of compounds tentatively assigned the *Z*,*E*,*E*,*E*-, *E*,*E*,*E*,*Z*- and *E*,*E*,*E*,*E*-configurations. Another problem was encountered during isolation of conjugated tetraenes by TLC. Although the compounds could be chromatographed normally, they rapidly degraded when adsorbed to the dry silica gel following development. It was found that this degradation was effectively inhibited by the antioxidant 2,6-di-*tert*-butyl-4-methylphenol. Accordingly, by spraying TLC plates with a solution of 2,6-di-*tert*-butyl-4-methylphenol in diethyl ether immediately following development it was possible to realize an almost quantitative recovery of tetraenes during TLC.

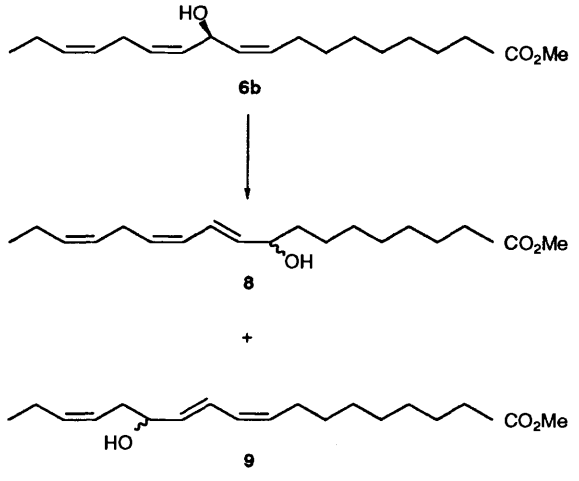
The structures of the bis-allylic hydroxy acids were determined by a combination of physical and chemical methods. Partial hydrogenation into mixtures having 0-3 double bonds followed by derivatization with (−)-menthyloxycarbonyl chloride, oxidative ozonolysis and steric analysis of the resulting 2-hydroxy-alkanoates/alkanedioates as the (−)-menthyloxycarbonyl derivatives [16] allowed determination of the stereochemistry of the asymmetric bis-allylic carbon. The stereochemistry of the (11*R*,6*Z*,9*Z*,12*Z*)-hydroxyoctadeca-6,9,12-trienoic acid **3a** formed from γ-linolenic acid, as well as the (11*S*,9*Z*,12*Z*,15*Z*)-hydroxyoctadeca-9,12,15-trienoic acid **6a** and the (14*R*,9*Z*,12*Z*,15*Z*)-hydroxyoctadeca-9,12,15-trienoic acid **7a** formed from α-linolenic acid, were identical and corresponded to that of (11*R*,9*Z*,12*Z*)-hydroxyoctadeca-9,12-dienoic acid [9] and of 13*R*,5*Z*,8*Z*,11*Z*,14*Z*)-hydroxyeicosatetraenoic acid [11,17] earlier isolated from *Lithothamnion*.

It was recently found that the bis-allylic hydroxy ester methyl (11*R*,9*Z*,12*Z*)-hydroxyoctadeca-9,12-dienoate underwent facile acid-catalyzed dehydration into two geometrical isomers of each of methyl octadeca-8,10,12-trienoate and methyl octadeca-9,11,13-trienoate.[9] Methyl (11*S*,9*Z*,12*Z*,15*Z*)-hydroxyoctadeca-9,12,15-trienoate **6b** of the present study underwent a similar rapid dehydration into stereoisomeric methyl octadeca-9,11,13,15-tetraenoates upon treatment with acid in acetonitrile solution. Interestingly, GLC analysis revealed that the major (71%) compound produced was methyl (9*E*,11*E*,13*E*,15*Z*)-octadeca-9,11,13,15-tetraenoate **5d** and not the natural (9*Z*,11*E*,13*E*,15*Z*)-octadeca-9,11,13,15-tetraenoate isomer **5b** (Scheme 3). In an analogous way, acid treatment of methyl (14*R*,9*Z*,12*Z*,15*Z*)-hydroxyoctadeca-9,12,15-trienoate **7b** produced methyl (9*Z*,11*E*,13*E*,15*E*)-octadeca-9,11,13,15-tetraenoate **5c** as the main tetraene isomer (Scheme 3). Acid treatment of (11*R*,6*Z*,9*Z*,12*Z*)-hydroxyoctadeca-6,9,12-trienoate **3b** yielded an isomer of methyl (6*Z*,8*E*,10*E*,12*Z*)-octadeca-6,8,10,12-tetraenoate as the main product, but, in this case, the stereoconfiguration was not determined because of lack of reference compounds. When methyl (11*S*,9*Z*,12*Z*,15*Z*)-hydroxyoctadeca-9,12,15-trienoate **6b** was treated with aqueous acid, *e.g.* perchloric acid in dimethoxyethane–water (0.003 : 1 : 2, v/v/v), solvolysis took place with the formation of comparable amounts of methyl (9*RS*,10*E*,12*Z*,15*Z*)-hydroxyoctadeca-10,12,15-tri-

* Suffixes **c**, **d** and **e** refer to structural isomers; for **a** and **b** see footnote * on p. 1.



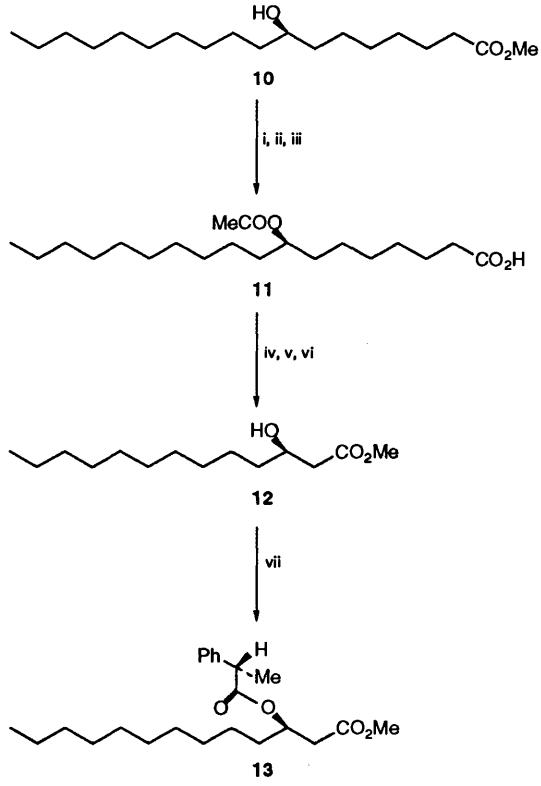
Scheme 3 Acid-catalysed dehydration of the bis-allylic hydroxy esters **6b** and **7b**



Scheme 4 Acid-catalysed solvolysis of the bis-allylic hydroxy ester **6b**

enoate **8** and methyl (13*RS*,9*Z*,11*E*,15*Z*)-hydroxyoctadeca-9,11,15-trienoate **9** (Scheme 4). In the same way, acid treatment of methyl (11*R*,6*Z*,9*Z*,12*Z*)-hydroxyoctadeca-6,9,12-trienoate **3b** in aqueous dimethoxyethane resulted in the formation of comparable amounts of methyl (9*RS*,6*Z*,10*E*,12*Z*)-hydroxyoctadeca-6,10,12-trienoate and methyl (13*RS*,6*Z*,9*Z*,11*E*)-hydroxyoctadeca-6,9,11-trienoate.

γ-Linolenic acids stereospecifically deuteriated in the C-8 and C-11 positions were prepared in order to determine the stereochemistry of the hydrogen removals occurring in the bis-allylic oxidations of γ-linolenic acid. Methyl (8*R*)- and (11*R*)-hydroxystearates served as the starting materials for the (8*R*)- and (11*S*)-deuterio-γ-linolenic acids, respectively, while the (8*S*)- and (11*R*)-deuterio-γ-linolenic acids were prepared starting with methyl (8*S*)-and (11*S*)-hydroxystearates, respectively. The two last mentioned compounds were obtained as their acetates by treatment of the corresponding (*R*)-*p*-tolylsulphonyloxystearate with acetate ion. Although this reaction proceeds with inversion of absolute configuration, it has not previously been possible to determine precisely the extent of stereoselectivity of the reaction. For example, in an early study[18] measurement of optical rotations indicated a significant extent of racemization and an optical purity of the inverted product of *ca.* 89%. In the present work, a method was developed which allowed determination of the optical purity of the hydroxy esters by a GLC method. The procedure, illustrated in Scheme 5 for steric analysis of methyl (8*R*)-hydroxystearate **10**, involved preparation of the corresponding acetoxy acid **11** followed by stepwise degradation of the carboxyl side-chain by refluxing with potassium permanganate in acetone according to



Scheme 5 Reactions used for steric analysis of hydroxystearates. *Reactants:* i, HO^-; ii, AcCl; iii, H_2O/acetone; iv, $KMnO_4$ in acetone; v, CH_2N_2; vi, MeO^-; vii, (2*S*)-phenylpropionyl chloride

Murray.[19] The 3-hydroxy ester **12** was isolated by TLC and derivatized with (2*S*)-phenylpropionyl chloride. The (2*S*)-phenylpropionyl derivative **13** was subjected to GLC analysis under conditions which allowed separation of the diastereoisomeric derivatives of methyl (3*R*)-and (3*S*)-hydroxy esters.[20] Table 2 shows results of steric analyses of methyl (7*R*)-, (8*R*)-, and (11*R*)-hydroxystearates, and of the corresponding (*S*)-hydroxy esters prepared by inversion. As seen, the optical purities of the inverted hydroxy esters were high, *i.e.*, the inversion step was accompanied by only little racemization.

Results of incubation of stereospecifically deuteriated γ-linolenic and linoleic acids are given in Table 3. As seen, biosynthesis of (6*Z*,8*E*,10*E*,12*Z*)-octadeca-6,8,10,12-tetraenoic acid **2a** from γ-linolenic acid **1a** was accompanied by loss of the *pro-S* hydrogen from C-8 and the *pro-R* hydrogen from C-11. Although the conformation of the fatty acid during the desaturation reaction is unknown, it may be pointed out that the two hydrogens abstracted lie on the same side of the plane of the carbon chain when the acid is in its chemically preferred, extended conformation (Scheme 1). In an attempt to determine which one of the hydrogens at C-8 and C-11 was abstracted in the initial step of the conversion, incubations of [(8*S*)-^{2}H]- and [(11*R*)-^{2}H]-**1a** were carried out and the deuterium contents of non-converted **1a** determined by GC-MS. As seen in Table 4, formation of the tetraene **2a** from the deuteriated precursors was accompanied by an isotope effect as judged by the finding that substrate remaining unconverted was enriched with respect to deuterium. Although the isotope effect accompanying

Table 2 Enantiomeric compositions of the hydroxystearates used for preparation of stereospecifically deuteriated **1a**

Compound	Enantiomeric composition (%)	
	R	*S*
Methyl (7*R*)-hydroxystearate	97.7	2.3
Methyl (7*S*)-hydroxystearate	2.4	97.6
Methyl (8*R*)-hydroxystearate	97.7	2.3
Methyl (8*S*)-hydroxystearate	4.3	95.7
Methyl (11*R*)-hydroxystearate	98.3	1.7
Methyl (11*S*)-hydroxystearate	4.6	95.4

Table 3 Isotopic compositions of stereospecifically deuteriated precursors and their products

Compound	Monodeuteriated molecules (%)	Retention of deuterium (%)
[(8*R*)-^{2}H]-**1a**	34	100
2a	32	94
3a	33	97
[(8*S*)-^{2}H]-**1a**	28	100
2a	4	14
3a	33	118
[(11*R*)-^{2}H]-**1a**	35	100
2a	6	17
3a	41	117
[(11*S*)-^{2}H]-**1a**	31	100
2a	45	145
3a	2	6
[(11*R*)-^{2}H]Linoleic acid	18	100
(11*R*)-Hydroxylinoleic acid	18	100
11-Oxolinoleic acid	1	6
[(11*S*)-^{2}H]Linoleic acid	16	100
(11*R*)-Hydroxylinoleic acid	1	6
11-Oxolinoleic acid	0	0

removal of the (8*S*)-deuterium was stronger than that in the removal of the (11*R*)-deuterium (Table 4), it was not possible to draw any conclusion regarding the sequence of hydrogen removals from these data. Biosynthesis of (11*R*)-hydroxyoctadecatrienoic acid **3a** from γ-linolenic acid **1a**, and of (11*R*)-hydroxyoctadecadienoic acid from linoleic acid, was found to involve elimination of the *pro-S* hydrogen from C-11 (Table 3). Conversion of γ-linolenic and linoleic acids into bis-allylic hydroxy acids thus occurred with inversion of the absolute configuration of the carbon oxidized (Scheme 1). Experiments with ^{18}O-water demonstrated that the oxygen introduced originated in water and not in dioxygen.

The finding that the isolated bis-allylic 11-hydroxyoctadecatrienoates were rapidly converted into octadecatetraenoates upon treatment with acid suggested the possibility that bis-allylic hydroxy acids served as the precursors of conjugated tetraenes in *Lithothamnion, i.e.*, the sequences **1a**→**3a**→**2a**, and **4a**→**6a**→**5a**. However, separate incubation of the hydroxy acids **3a** and **6a**, as well as of the tetraenes **2a** and **5a**, showed that the hydroxy acid and tetraene products were not interconvertible with the enzyme preparation. Evidence for the involvement of separate enzyme activities in the formation of the hydroxy acid and the tetraene was provided by the isotope experiments, which showed that the hydrogens eliminated from C-11 of γ-linolenic acid **1a** in the formation of the hydroxy acid **3a** and the tetraene **2a** had opposite configurations, *i.e., pro-S* and *pro-R*, respectively (Table 3). Further support for the operation of disparate enzyme activities was furnished by the recent finding that 5–10 mmol dm^{-3} sodium azide selectively blocked formation of the bis-allylic hydroxy acid **3a** but did not inhibit formation of the conjugated tetraene **2a**.[10] Allylic hydroxy acids, such as (9*S*,10*E*,12*Z*)-hydroxyoctadeca-10,12-dienoic acid (α-dimorphecolic acid) and (13*R*,9*Z*,11*E*)-hydroxyoctadeca-9,11-dienoic acid (coriolic acid), can be chemically dehydrated by acid into conjugated polyenoic acids.[21–23] In spite of this fact, and in spite of the co-occurrence of hydroxy acids and conjugated polyenoic acids in many plants, it has never been possible to demonstrate a precursor role of hydroxy acids in the biosynthesis of conjugated polyenoic acids.

The biosynthesis of the tetraene **2a** from γ-linolenic acid in *Lithothamnion* was recently found to be oxygen-dependent, to be accompanied by production of 0.9–1.0 equiv. of hydrogen peroxide and to be supported, under anaerobic conditions, by a number of artificial electron acceptors such as *p*-benzoquinone.[10] On the basis of these findings, a fatty acid oxidase was implicated in tetraene formation. Further studies are in progress to establish whether the biosynthesis of conjugated polyenoic acids in higher plants also involves catalysis by oxidase enzyme(s).

Experimental

TLC was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck, Darmstadt, Germany) and solvent systems consisting of mixtures of ethyl acetate and hexane in the proportions indicated. Radioactivity on TLC plates was determined with a Berthold Dünnschichtscanner II interfaced with a Macintosh SE/30 computer. Non-labelled material on TLC plates was located by spraying with 2′,7′-dichlorofluorescein and viewing under UV light. Reversed-phase HPLC was performed with columns packed with Nucleosil C_{18} or Polygosil C_{18} and solvents composed of mixtures of acetonitrile and water as indicated. GLC was carried out with a Hewlett-Packard model 5890 gas chromatograph equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm). Helium at a flow rate of 25 cm s^{-1} was used as carrier gas. Retention times were converted into C values using standards of saturated fatty acid methyl esters.[24] GC-MS was performed with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. UV spectroscopy was carried out with a Hewlett-Packard model 8450A diode array spectrophotometer and NMR spectra were recorded with a Bruker model WM-300 instrument. Radioactivity was determined with a Packard Tri-Carb model 4450 liquid scintillation counter.

Reagents.—(−)-Menthoxycarbonyl chloride,[16] (2*S*)-phenylpropionyl chloride,[20] dimethyl (2*S*)- and (2*R*,*S*)-hydroxydodecanedioates[25] and other 2-hydroxyalkanoates and 2-hydroxyalkanedioates[16,26] were prepared as indicated. Lithium aluminium deuteride (>99% ^{2}H) was purchased from E. Merck, Darmstadt, Germany. $H_2{}^{18}O$ (95.0 atom%) was obtained from Isotec, Miamisburg, OH, USA.

Octadecatrienoic Acids.—Unlabelled α- and γ-linolenic acids were purchased from Nu-Chek-Prep (Elysian, MN., USA). [1-^{14}C]-α-Linolenic acid was obtained from Amersham, UK and [1-^{14}C]-γ-linolenic acid was prepared by biological desaturation of [1-^{14}C]stearic acid.[27] [(8*R*)-^{2}H]- and [(8*S*)-^{2}H] γ-linolenic acids were prepared by desaturation of [(8*S*)-^{2}H]- and [(8*R*)-^{2}H]-stearic acids, respectively, as described elsewhere.[28] In the same way, desaturation of [(11*R*)-^{2}H]- and [(11*S*)-^{2}H]stearic acids provided [(11*R*)-^{2}H]-and [(11*S*)-^{2}H]-γ-linolenic acids. The stereochemical purity of the methyl 8- and 11-hydroxystearates used to prepare the stereospecifically dueteriated stearic acids was determined as described below.

Hydroxyoctadecatrienoic Acids.—(9*S*,10*E*,12*Z*,15*Z*)-Hydroxyoctadeca-10,12,15-trienoic acid [λ_{max}(EtOH)/nm 236; C value (methyl ester–TMS derivative), 19.87; *m*/*z* (methyl

Table 4 Isotope effects in the conversion of intermolecularly labelled [(8*S*)-^{2}H; 1-^{14}C]-**1a** and [(11*R*)-^{2}H; 1-^{14}C]-**1a** into the tetraene **2a**

Time of incubation (min)	Conversion of [1-^{14}C]-**1a** into [1-^{14}C]-**2a** (%)	Monodeuteriated molecules in recovered **1a** (%)	
		Incubation of [(8*S*)-^{2}H]-**1a**	Incubation of [(11*R*)-^{2}H]-**1a**
0	0	28	25
20	33	—	30
20	40	36	—
40	66	—	38
40	67	48	—
60	81	63	—
60	82	—	47
80	89	—	56
80	91	77	—

ester–TMS derivative) 380 (8%, M^+), 365 (3, $M^+ - Me$), 311 (100, $M^+ - CH_2CH{=}CHEt$) and 223 (50, $Me_3SiO^+{=}CH(CH{=}CH)_2CH_2CH{=}CHEt$)] was obtained by incubation of α-linolenic acid with tomato lipoxygenase[29] followed by sodium borohydride reduction and purification by silicic acid chromatography.(9*S*,6*Z*,10*E*,12*Z*))-Hydroxyoctadeca-6,10,12-trienoic acid was obtained in an analogous way by incubation of γ-linolenic acid [λ_{max}(EtOH)/nm 234; C value 19.60; *m/z* 380 (0.5%, M^+), 365 (1, $M^+ - Me$), 225 (100, $Me_3SiO^+{=}CH(CH{=}CH)_2C_5H_{11}$)]. (13*S*,9*Z*,11*E*,15*Z*)-Hydroxyoctadeca-9,11,15-trienoic acid was obtained by incubation of α-linolenic acid with soybean lipoxygenase[30] followed by reduction and purification [λ_{max}(EtOH)/nm 234; C value 19.85; *m/z* 380 (0.3%, M^+), 365 (1, $M^+ - Me$), 311 (100, $M^+ - CH_2CH{=}CHEt$)]. (13*S*,6*Z*,9*Z*,11*E*)-Hydroxyoctadeca-6,9,-11-trienoic acid was obtained from γ-linolenic acid by incubation with soybean lipoxygenase {λ_{max}(EtOH)/nm 236; C value 19.64; *m/z* 380 (8%, M^+), 365 (2, $M^+ - Me$), 309 (47, $M^+ - C_5H_{11}$), 225 (92, $[(CH{=}CH)_2CH(OSiMe_3)C_5H_{11}]^+$)}. The purity of the specimens was >95%.

Methyl Dihydroxystearates.—Methyl *erythro*-9,10-, 11,12-, 13,14- and 15,16-dihydroxystearates were prepared by *cis*-hydroxylation of the corresponding methyl (*Z*)-octadecenoates by treatment with OsO_4[31]. Methyl *threo*-9,10-, 11,12-, 13,14- and 15,16-dihydroxystearates were obtained in an analogous way by *trans*-hydroxylation of the (*Z*)-octadecenoates by treatment with performic acid and subsequent hydrolysis.[30] The fragmentation patterns observed upon GC-MS analysis of the TMS ether derivatives were in full accord with published results.[32] The C-values of the TMS ether derivatives of methyl 9,10-, 11,12-, 13,14- and 15,16-dihydroxystearates were, respectively: 21.49, 21.60, 21.90, 22.64 (*erythro* series), and 21.33, 21.43, 21.72, 22.45 (*threo* series).

Methyl α-Parinarate **5b** *and its Geometrical Isomers* **5c**, **5d**, **5e**.—Seed of *Impatiens balsamina* were ground in an electric coffee mill and the fine powder (20 g) was stirred with diethyl ether (250 cm^3) containing 2,6-di-*tert*-butyl-4-methylphenol (0.0007% w/v) at room temperature for 2 h. Material obtained after evaporation of the ether (4.5 g) was saponified and subjected to silicic acid column chromatography to afford a fraction of mixed fatty acids (3.8 g) which contained α-parinaric acid (1.4 g) and its geometrical isomers according to GLC and UV analysis. Six crystallizations from hexane containing 2,6-di-*tert*-butyl-4-methylphenol (0.0008% w/v) at −20 °C yielded a white solid (0.7 g) consisting of >98% of α-parinaric acid and its isomers. The material was esterified and an aliquot of the colourless oil was shown by GLC analysis to contain methyl α-parinarate **5b** (74%, C-19.73) as well as isomers **5c** (18%, C-19.83), **5d** (4%, C-20.11) and **5e** (4%, C-20.17). Preparative reversed-phase HPLC (column, 300 × 8 mm of Polygosil C_{18} 5 μm; solvent, acetonitrile–water [82:18, v/v] at a flow rate of 3 cm^3 min^{-1}) afforded the pure compounds **5b** (eluent volume, 68.4–72.1 cm^3), **5c** (72.1–74.8 cm^3), **5d** (74.8–76.8 cm^3) and **5e** (78.3–80.4 cm^3). Methyl α-parinarate **5b**: λ_{max}(MeOH)/nm 291, 304 (ε 70,000) and 319; *m/z* 290 (68%, M^+), 261 (5, $M^+ - Et$), 259 (5, $M^+ - OMe$), 247 (1), 229 (1), 211 (2), 147 (32), 133 (45), 119 (43), 105 (66), 91 (100), 79 (60) and 55 (43). Catalytic hydrogenation of **5b** afforded methyl stearate, and oxidative ozonolysis[16] yielded methyl hydrogen azelate as the major non-volatile fragment. The presence of a 9*Z*,11*E*,13*E*,15*Z* conjugated tetraene structure in **5b** was verified by steric analysis of dihydroxystearates formed by mono-dihydroxylation with OsO_4 as described below. GLC analysis of the tetraene **5b** (column temp, 210 °C) showed an apparent purity of only 92%: in addition to **5b**, three minor peaks due to isomers **5c** (4%), **5d** (3%) and **5e** (1%) appeared. These three minor peaks gradually increased at the expense of the main peak at higher column temperatures and were ascribed to thermal *Z*→*E* double-bond isomerization during the GLC analysis.

The geometrical isomers **5c**, **5d** and **5e** gave UV spectra typical for a conjugated tetraene structure and mass spectra which were similar to that of **5b**. The positions and configurations of the double bonds of the tetraenes **5c** (9*Z*,-11*E*,13*E*,15*E*), **5d** (9*E*,11*E*,13*E*,15*Z*) and **5e** (9*E*,11*E*,13*E*,15*E*) were determined by mono-dihydroxylation as described below.

Catalytic Hydrogenation.—Complete hydrogenation was carried out by stirring the sample (0.1–3 mg) with either PtO_2 (15 mg) in methanol (3 cm^3) for 20 min, or 5% palladium-on-calcium carbonate (25 mg) in methanol (3 cm^3) for 1 h under hydrogen gas. Partial hydrogenation of the hydroxyoctadecatrienoates (0.1–1 mg) into hydroxy esters containing 0–3 double bonds was achieved by stirring with palladium-on-calcium carbonate (5 mg) in ethyl acetate (3 cm^3) under hydrogen gas for 5 min followed by immediate removal of the catalyst by filtration through glass wool.

Method for Steric Analysis of Methyl α-Parinarate and its Isomers.—Methyl α-parinarate **5b** (0.8 mg) was treated with OsO_4 (0.28 mg, 0.4 equiv.) in diethyl ether (0.1 cm^3) containing pyridine (2 mm^3) at 23 °C for 20 min. Methanol (1 cm^3) and D-mannitol (15 mg) were added to the mixture which was then stirred at 23 °C for 1 h. The product consisted of unchanged methyl α-parinarate as well as four dihydroxyoctadecatrienoates as shown by GC-MS (TMS ethers), *i.e.*, methyl 11,-12-dihydroxyoctadecatrienoate {2%, C-21.12; *m/z* 453 (2, $M^+ - Me$), 285 [100, $Me_3SiO^+{=}CHCH{=}CH(CH_2)_7CO_2Me$], 183 [51, $Me_3SiO^+{=}CH(CH{=}CH)_2Et$]}, methyl 13,14-dihydroxyoctadecatrienoate {2%; C-21.37; *m/z* 453 (3, $M^+ - Me$), 311 [100, $Me_3SiO^+{=}CH(CH{=}CH)_2(CH_2)_7CO_2Me$], 157 [65, $Me_3SiO^+{=}CHCH{=}CHEt$]}, methyl 9,10-dihydroxyoctadecatrienoate {42%; C-22.06; *m/z* 453 (1, $M^+ - Me$), 259 [100, $Me_3SiO^+{=}CH(CH_2)_7CO_2Me$], 209 [8, $Me_3SiO^+{=}CH(CH{=}CH)_3Et$], 155 [43, $OHC(CH_2)_7C{\equiv}O^+$]}, and methyl 15,16-

dihydroxyoctadecatrienoate {54%; C-22.88; *m/z* 453 (1, M^+ − Me), 410 (32, M^+ − 58 by rearrangement and loss of OHCEt), 337 [32, Me_3SiO^+=CH(CH=CH)$_3$(CH$_2$)$_7$CO$_2$Me], 131 (100, Me_3SiO^+=CHEt)}. The corresponding dihydroxystearates obtained following catalytic hydrogenation (PtO_2 in methanol) were identified by GLC (TMS ether derivatives) as methyl *erythro*-9,10-dihydroxystearate (C-21.49; < 2% of the *threo* isomer), methyl *erythro*-15,16-dihydroxystearate (C-22.64; < 2% of the *threo* isomer), methyl *threo*-11,12-dihydroxystearate (C-21.43; *erythro* isomer undetectable), and methyl *threo*-13,14-dihydroxystearate (C-21.72; *erythro* isomer undetectable). The identity of the compounds were ascertained by GC-MS analysis using the authentic compounds as references. The geometrical isomers **5c**, **5d** and **5e** isolated from *Impatiens balsamina* seed oil were analysed in the same way (Table 1).

Method of Steric Analysis of Hydroxystearates.—Methyl (8*R*)-hydroxystearate **10**[28] (25 mg) was converted into (8*R*)-acetoxystearic acid **11** by sequential treatment with 6% sodium hydroxide in aqueous methanol, acetyl chloride (0.3 cm^3, 60 °C for 30 min) and 60% aqueous acetone (60 °C for 1 h) (Scheme 5). The acetate was dissolved in acetone (5 cm^3) and refluxed with potassium permanganate (150 mg) for 12 h. The esterified product was deacetylated by treatment with 0.5 mol dm^{-3} sodium methoxide at 23 °C for 6 h. TLC analysis using ethyl acetate–hexane (3:7, v/v) as solvent showed 4 major bands of hydroxy esters: band A (R_f 0.44; mainly methyl 5-hydroxypentadecanoate), band B [R_f 0.48; methyl 4-hydroxytetradecanoate and methyl 6-hydroxyhexadecanoate (1:18, w/w)], band C [R_f 0.53; methyl 3-hydroxytridecanoate **12** and methyl 7-hydroxyheptadecanoate (1:12, w/w)] and band D (R_f 0.59; mainly methyl 8-hydroxyoctadecanoate). Material present in band C was treated with (2*S*)-phenylpropionyl chloride (7 mg) in toluene (80 mm^3) and pyridine (20 mm^3) at 37 °C for 1 h. The (2*S*)-phenylpropionyl derivative of methyl 3-hydroxytridecanoate **13** was isolated by TLC [solvent system, ethyl acetate–hexane (7:93, v/v)] and analysed by GLC (column temp raised from 180 °C to 260 °C at 4 °C min^{-1}) using the (2*S*)-phenylpropionyl derivative of methyl (3*R*,*S*)-hydroxytridecanoate as reference. This analysis showed the presence of the (2*S*)-phenylpropionyl derivatives of methyl (3*R*)-hydroxytridecanoate (retention time, 15.52 min; 97.7%) and of methyl (3*S*)-hydroxytridecanoate (15.81 min; 2.3%). Accordingly, the methyl (8*R*)-hydroxystearate degraded was due to 97.7% of the (*R*) and 2.3% (*S*) enantiomers. In the same way, methyl (8*S*)-hydroxystearate, prepared by inversion of the (8*R*) enantiomer,[28] was found to be due to 95.7% of the (*S*) and 4.3% of the (*R*) enantiomers. Further results of the steric analyses of methyl (7*R*)- and (11*R*)-hydroxystearates, and of the corresponding (7*S*)- and (11*S*)-hydroxy esters obtained by inversion, are given in Table 2.

Enzyme Preparation and Incubations.—Batches of the red alga *Lithothamnion corallioides* Crouan (90 g) were ground in a mortar with liquid nitrogen.[9,10] The powder was added to 0.09 mol dm^{-3} potassium phosphate buffer pH 7.4 (1:1, w/v) and homogenized with an Ultra-Turrax. The supernatant obtained after centrifugation at 105 000 *g* for 60 min was subjected to ammonium sulfate fractionation. The precipitate obtained at 30–55% saturation was dissolved in potassium phosphate buffer (protein, 2 mg cm^{-3}) and used as the enzyme source.

Incubation of γ-Linolenic Acid.—Batches of [1-^{14}C]-**1a** (500 μmol dm^{-3}; 2.8 mg) were stirred with enzyme preparation (20 cm^3; 40 mg of protein) at 23 °C for 20 min and the product was isolated by extraction with diethyl ether containing 0.0007% (w/v) of 2,6-di-*tert*-butyl-4-methylphenol. Materials from several such incubations were combined and esterified by brief treatment with ethereal diazomethane. Analysis of an aliquot by radio-TLC [solvent, ethyl acetate–hexane (1:9 v/v)] showed the following product composition: methyl hydroxyoctadecatrienoates (33%; R_f 0.12), methyl octadeca-6,8,10,12-tetraenoate (29%; R_f 0.61), and methyl ester of unchanged γ-linolenic acid (25%; R_f 0.71). Silicic acid column chromatography (5 g of SiO_2) afforded a fatty acid ester fraction [eluted with 150 cm^3 of diethyl ether–hexane (5:95, v/v)] and a hydroxy ester fraction [eluted with 250 cm^3 of diethyl ether–hexane (25:75, v/v)]. Material present in the fatty acid ester fraction was subjected to reversed-phase HPLC [column, 300 × 8 mm of Polygosil C_{18} 5 μm; solvent, acetonitrile–water (82:18, v/v) at a flow rate of 4 cm^3 min^{-1}] to afford the conjugated tetraene **2b** (5.2 mg; eluent volume, 67.8–73.8 cm^3). Material present in the hydroxy ester fraction was further separated by reversed-phase HPLC (column, 250 × 4 mm of Nucleosil 100 C_{18} 5 μm; solvent, acetonitrile–water [60:40, v/v] at a flow rate of 1.5 cm^3 min^{-1}). A major peak due to the bis-allylic hydroxy ester **3b** (88%; eluent volume, 26.9–29.7 cm^3) and two minor peaks due to methyl (6*Z*,10*E*,12*Z*)-9-hydroxyoctadeca-6,10,12-trienoate (6%; eluent volume, 35.4–37.4 cm^3) and methyl (6*Z*,9*Z*,11*E*)-13-hydroxyoctadeca-6,9,11-trienoate (6%; eluent volume, 33.5–35.4 cm^3) appeared.

Methyl (6Z,8E,10E,12Z)-*Octadeca*-6,8,10,12-*tetraenoate* **2b**. The conjugated tetraene **2b** showed λ_{max}(MeOH)/nm 292, 305 (ε 70 000) and 320; δ_H(300 MHz; $CDCl_3$) 0.88 (t, 18-H$_3$), 2.20 (m, 5-H$_2$ and 14-H$_2$, $J_{5,6} = J_{13,14}$ 7.7), 3.65 (s, OMe), 5.43 (m, 6-H and 13-H, $J_{6,7} = J_{12,13}$ 11), 6.04 (m, 7-H and 12-H, $J_{7,8} = J_{11,12}$ 11), 6.27 (m, 9-H and 10-H, $J_{9,10}$ 11) and 6.48 (m, 8-H and 11-H, $J_{8,9} = J_{10,11}$ 15); *m/z* 290 (79%, M^+), 259 (2), 247 (2), 233 (7), 219 (8), 175 (18), 161 (13), 131 (20), 105 (48), 91 (100), 79 (38), 67 (33) and 55 (27). The NMR spectrum, which was analogous to that of methyl α-parinarate,[13] demonstrated that the configuration of the conjugated tetraene structure was *Z,E,E,Z*. Catalytic hydrogenation of the tetraene afforded methyl stearate [*m/z* 298 (30%, M^+), 87 (63), 74 (100)], while oxidative ozonolysis resulted in the formation of methyl hydrogen adipate, thus siting the conjugated *Z,E,E,Z* tetraene structure at Δ^{6,8,10,12}.

Like the methyl ester of α-parinaric acid, the conjugated tetraene **2b** underwent *Z*→*E* double-bond isomerization to a small extent during GLC. Thus, at a column temp of 210 °C the peak due to **2b** (C value, 19.75; 95%) was accompanied by three minor peaks of stereomutated tetraenes, *i.e.*, C 20.04 (2%), C 20.11 (2%) and C 20.32 (1%). These three minor peaks increased at the expense of the main peak at higher column temperatures.

Methyl (11R,6Z,9Z,12Z)-*Hydroxyoctadeca*-6,9,12-*trienoate* **3b**. The bis-allylic hydroxy ester **3b** was transparent in the region 210–320 nm; ν_{max}($CHCl_3$)/cm^{-1} 3350–3620 (OH), 1735 (ester CO); C value (TMS ether derivative) 19.20; *m/z* (TMS ether derivative) 380 (5%, M^+), 365 (1, M^+ − Me), 309 (9, M^+ − C_5H_{11}), 290 (7, M^+ − Me_3SiOH), 225 (76, [CH=CHCH-(OSiMe$_3$)CH=CHC$_5$H$_{11}$]$^+$), 167 (13), 105 (13), 91 (24), 73 (100, Me_3SiO^+). Catalytic hydrogenation afforded methyl 11-hydroxystearate[9] and oxidative ozonolysis yielded methyl hydrogen adipate by cleavage of the Δ^6 double bond. Partial hydrogenation afforded a mixture of hydroxy esters having 0-3 double bonds. Treatment of this material with (−)-menthoxycarbonyl chloride, purification by TLC, and oxidative ozonolysis yielded the (−)-menthoxycarbonyl derivatives of (2*R*)-hydroxynonanoic acid and of methyl hydrogen (2*S*)-hydroxydodecanedioate. This experiment thus established that C-11 of **3b** had the *R* configuration, and showed the presence in **3b** of two double bonds in the Δ^9 and Δ^{12} positions.

Methyl (6Z,10E,12Z)-9-*hydroxyoctadeca*-6,10,12-*trienoate and methyl* (6Z,9Z,11E)-13-*hydroxyoctadeca*-6,9,11-*trienoate*. The identities of the title compounds were established by UV spectrometry and GC-MS analysis of the TMS derivatives using

authentic compounds as references. Additional structural proof was provided by catalytic hydrogenation, which afforded methyl 9-hydroxystearate from methyl (6*Z*,10*E*,12*Z*)-9-hydroxyoctadeca-6,10,12-trienoate and methyl 13-hydroxystearate from methyl (6*Z*,9*Z*,11*E*)-13-hydroxyoctadeca-6,9,11-trienoate.

Incubation of α-Linolenic Acid.—[1-^{14}C]-**4a** (500 μmol dm^{-3}) was stirred with the enzyme preparation at 23 °C for 20 min and material extracted with diethyl ether was treated with diazomethane. Radio-TLC analysis [solvent, ethyl acetate–hexane (1:9 v/v)] showed the following product composition: methyl hydroxyoctadecatrienoates (55%; R_f 0.12), methyl octadeca-9,11,13,15-tetraenoate (13%; R_f 0.64), and methyl ester of unchanged α-linolenic acid (27%; R_f 0.71). The material was separated by silicic acid column chromatography (5 g of SiO_2) into a fatty acid ester fraction [eluted with 150 cm^3 of diethyl ether–hexane (5:95, v/v)] and a hydroxy ester fraction (eluted with 250 cm^3 of diethyl ether–hexane (25:75, v/v)]. The first mentioned material was subjected to reversed-phase HPLC [column, 300 × 8 mm of Polygosil C_{18} 5 μm; solvent, acetonitrile–water (82:18, v/v) at a flow rate of 3 cm^3 min^{-1}] to yield the conjugated tetraene **5b** (1.5 mg; eluent volume, 68.4–72.1 cm^3). Separation of the hydroxy esters was also effected by reversed-phase HPLC (column, 250 × 4 mm of Nucleosil 100 C_{18} 5 μm; solvent, acetonitrile–water [60:40, v/v] at a flow rate of 1.5 cm^3 min^{-1}). This yielded the bis-allylic hydroxy ester **6b** as the major product (84% of the hydroxy esters; eluent volume, 26.0–28.2 cm^3) as well as three minor hydroxy esters, **7b** (10%; eluent volume, 28.2–30.0 cm^3), methyl (10*E*,12*Z*,15*Z*)-9-hydroxyoctadeca-10,12,15-trienoate (3%; eluent volume, 32.0–33.9 cm^3), and methyl (9*Z*,11*E*,15*Z*)-13-hydroxyoctadeca-9,11,15-trienoate (3%; eluent volume, 34.5–36.2 cm^3).

Methyl (9Z,11E,13E,15Z)-*octadeca*-9,11,13,15-*tetraenoate* **5b**. The conjugated tetraene **5b** had λ_{max}(MeOH)/nm 291, 304 and 319; *m/z* 290 (68%, M$^+$), 261 (5, M$^+$ − Et), 259 (5, M$^+$ − OMe), 247 (1), 229 (1), 211 (2), 147 (32), 133 (45), 119 (43), 105 (66), 91 (100), 79 (60) and 55 (43). Catalytic hydrogenation afforded methyl stearate, while oxidative ozonolysis resulted in the formation of methyl hydrogen azelate and thus sited the conjugated *Z,E,E,Z* tetraene structure at Δ.^{9,11,13,15} The title compound was indistinguishable from the methyl ester of α-parinaric acid by UV spectroscopy, mass spectrometry and TLC and reversed-phase HPLC. Furthermore, GLC analysis of **5b** and of the methyl ester of α-parinaric acid gave identical results, *i.e.*, a major peak due to the *Z,E,E,Z* tetraene (C 19.73; 92%) as well as minor peaks (C 19.83, 4%; C 20.11; 3%; C 20.17, 1%) due to materials formed by *Z*→*E* double-bond isomerization.

Methyl (11S,9Z,12Z,15Z)-*hydroxyoctadeca*-9,12,15-*trienoate* **6b**. The major bis-allylic hydroxy ester **6b** obtained from α-linolenic acid showed a featureless UV spectrum in the region 210–320 nm; ν_{max}($CHCl_3$)/cm^{-1} 3350–3620 (OH) and 1735 (ester CO); C value (TMS derivative), 19.45; *m/z* (TMS derivative) 380 (6%, M$^+$), 365 (2, M$^+$ − Me), 324 (3), 311 (59, M$^+$ − CH_2CH=CHEt), 290 (6, M$^+$ − Me_3SiOH), 223 {12, [CH=CHCH(OSiMe_3)CH=CHCH_2CH=CHEt]$^+$}, 195 (7), 167 (14), 133 (14), 91 (26) and 73 (100, Me_3SiO)$^+$. Catalytic hydrogenation afforded methyl 11-hydroxystearate while oxidative ozonolysis produced methyl hydrogen azelate by cleavage of the Δ^9 double bond. The product obtained following partial hydrogenation was derivatized with (−)-menthoxycarbonyl chloride and subjected to oxidative ozonolysis. GLC analysis of the esterified ozonolysis product revealed the presence of the (−)-menthoxycarbonyl derivatives of methyl (2*R*)-hydroxynonanoate and of dimethyl (2*S*)-hydroxydodecanedioate. These results showed that C-11 of **6b** had the *S* configuration with the two double bonds sited at the Δ^9 and Δ^{12} positions.

Methyl (14R,9Z,12Z,15Z)-*hydroxyoctadeca*-9,12,15-*trienoate* **7b**. The minor bis-allylic hydroxy ester **7b** obtained from α-linolenic acid was transparent in the region 210–320 nm; ν_{max}($CHCl_3$)/cm^{-1} 3350–3620 (OH), 1735 (ester CO); C value (TMS derivative), 19.69; *m/z* (TMS derivative) 380 (2%, M$^+$), 365 (1, M$^+$ − Me), 351 (6, M$^+$ − Et), 290 (10, M$^+$ − Me_3SiOH), 237 (3), 183 (86, [CH=CHCH(OSiMe_3)CH=CHEt]$^+$), 167 (14), 133 (10), 105 (13), 93 (24), 91 (19) and 73 (100, Me_3SiO^+). Catalytic hydrogenation yielded methyl 14-hydroxystearate, *m/z* (TMS derivative) 371 (3%, M$^+$ − Me), 355 (6, M$^+$ − OMe), 339 (20, M$^+$ − Me − MeOH), 329 (89, M$^+$ − C_4H_9), 300 (18, M$^+$ − $OHCC_4H_9$), 159 (100, Me_3SiO^+=CHC_4H_9), 73 (59, Me_3SiO^+), while oxidative ozonolysis afforded methyl hydrogen azelate by cleavage of the Δ^9 double bond. Partial hydrogenation followed by oxidative ozonolysis of the (−)-menthyloxycarbonyl derivative afforded *inter alia* the (−)-menthyloxycarbonyl derivative of (2*R*)-hydroxyhexanoic acid, thus establishing the *R* configuration for C-14 and siting one double bond at the Δ^{12} position.

Methyl (10E,12Z,15Z)-9-*hydroxyoctadeca*-10,12,15-*trienoate and methyl* (9Z,11E,15Z)-13-*hydroxyoctadeca*-9,11,15-*trienoate*. The identities of the minor hydroxy esters obtained from α-linolenic acid were established by UV spectrometry and GC-MS analysis of the TMS ether derivatives using authentic compounds as references. Catalytic hydrogenation of the methyl (10*E*,12*Z*,15*Z*)-9-hydroxyoctadeca-10,12,15-trienoate and the methyl (9*Z*,11*E*,15*Z*)-13-hydroxyoctadeca-9,11,15-trienoate yielded methyl 9- and 13-hydroxystearates respectively.

Incubations of (11R,6Z,9Z,12Z)-*Hydroxyoctadeca*-6,9,12-*trienoic Acid* **3a** *and* (11S,9Z,12Z,15Z)-*Hydroxyoctadeca*-9,12,15-*trienoic Acid* **6a**.—[1-^{14}C]-**3a** and [1-^{14}C]-**6a** were prepared by incubation of compounds [1-^{14}C]-**1a** and [1-^{14}C]-**4a**, respectively, with the enzyme preparation from *Lithothamnion* followed by silicic acid chromatography.[10] The labelled hydroxy acids (100 μmol dm^{-3}) were re-incubated with the enzyme preparation (23 °C, 20 min) and the product analysed by radio-TLC. The starting materials **3a** and **6a** were essentially recovered unchanged although a small (*ca* 5%), probably non-enzymatic, conversion into 9- and 13-hydroxyoctadecatrienoic acids was noted. Formation of conjugated tetraenes was not detectable. In another experiment [1-^{14}C]-**2a** and [1-^{14}C]-**5a** were prepared by incubations of [1-^{14}C]-**1a** and [1-^{14}C]-**4a**, respectively, and the labelled tetraenes were re-incubated with the enzyme preparation. In both cases only non-converted starting material plus a peak of unidentified polar material (*ca* 30%) could be detected by radio-TLC.

Acid-catalyzed Dehydration of Bis-Allylic Hydroxy Esters.—The bis-allylic hydroxy ester **6b** (0.2 mg) was dissolved in acetonitrile (5 cm^3) and treated at 23 °C for 1 min with 5 mm^3 of 70% perchloric acid. This led to the appearance of UV absorption typical for a conjugated tetraene, *i.e.*, λ_{max}(MeCN)/nm 289, 302 and 316. GLC analysis revealed the following mixture of tetraenes: **5d** (71%), **5e** (16%), **5b** (9%) and **5c** (4%); *m/z* 290 (M$^+$). Treatment of the hydroxy ester **7b** in the same way led to the formation of a mixture of tetraenes having λ_{max}(MeCN)/nm 289, 302 and 316, and consisting of **5c** (62%), **5e** (26%), **5b** (7%) and **5d** (5%); *m/z* 290 (M$^+$). Acid treatment of the hydroxy ester **3b** (0.1 mg) led to the appearance of UV absorption at λ_{max}(MeCN)/nm 289, 302 and 317. Analysis of the reaction product by GC-MS revealed that **3b** was converted into a mixture of stereoisomeric conjugated methyl octadecatetraenoates having the following C values: 19.75 (**2b**; 4%), 20.04 (76%), 20.11 (3%) and 20.32 (17%); *m/z* 290 (M$^+$). Although the geometrical configurations of the three last cited tetraenes were not determined, oxidative ozonolysis performed

on the mixture afforded methyl hydrogen adipate, thus siting the conjugate tetraene structure at $\Delta^{6,8,10,12}$.

Acid-catalyzed Solvolysis of Bis-allylic Hydroxy Esters.—The hydroxy ester **3b** (0.1 mg) was dissolved in 1,2-dimethoxyethane–water (1:2, v/v; 3 cm^3) and treated with 70% perchloric acid (3 mm^3) at 23 °C for 30 min. Analysis of the reaction product (λ_{max} 235 nm) by reversed-phase HPLC and GC-MS (TMS ether) demonstrated complete conversion of **3b** into a mixture of methyl 9- and 13-hydroxyoctadecatrienoates, *i.e.*, methyl (9*RS*,6*Z*,10*E*,12*Z*)-hydroxyoctadeca-6,10,12-trienoate (43%), methyl (13*RS*,6*Z*,9*Z*,11*E*)-hydroxyoctadeca-6,9,11-trienoate (43%), as well as compounds tentatively identified as the corresponding *E*,*E* isomers, methyl (6*Z*,10*E*,12*E*)-9-hydroxyoctadeca-6,10,12-trienoate (8%) and methyl (6*Z*,9*E*,11*E*)-13-hydroxyoctadeca-6,9,11-trienoate (6%). That the two major hydroxyoctadecatrienoates were racemic was determined by oxidative ozonolysis of the (−)-menthyloxycarbonyl derivatives which produced the (−)-menthyloxycarbonyl derivatives of dimethyl (RS)-malate and methyl (2*RS*)-hydroxyheptanoate. Treatment of the bis-allylic hydroxy ester **6b** with perchloric acid in aqueous dimethoxyethane afforded a product (λ_{max} 235 nm) consisting of methyl (9*RS*,10*E*,12*Z*,15*Z*)-hydroxyoctadeca-10,12,15-trienoate **8** (45%) and methyl (13*RS*,9*Z*,-11*E*,15*Z*)-hydroxyyoctadeca-9,11,15-trienoate **9** (45%) as well as of compounds tentatively identified as methyl (10*E*,12*E*,-15*Z*)-9-hydroxyoctadeca-10,12,15-trienoate (4%) and methyl (9*E*,11*E*,15*Z*)-13-hydroxyoctadeca-9,11,15-trienoate (6%).

Incubation of Stereospecifically Deuteriated Acids.—Compounds [(8*R*)-^{2}H]-, [(8*S*)-^{2}H)-, [(11*R*)-^{2}H]- and [(11*S*)-^{2}H]-**1a** (300 μmol dm^{-3}) were stirred with the enzyme preparation at 23 °C for 20 min. The esterified products were subjected to TLC [solvent, ethyl acetate–hexane (1:9, v/v)]. The plates were sprayed with a solution of 1 mg cm^{-3} of 2,6-di-*tert*-butyl-4-methylphenol in diethyl ether immediately after development and the zones containing the tetraene **2b** (R_f 0.61) and 11-hydroxyoctadecatrienoate **3b** (R_f 0.12) were scraped off and eluted with diethyl ether. Determination of the percentages of monodueteriated molecules (Table 3) was accomplished by GC-MS and selected ion monitoring of *m*/*z* 309 and 310 (TMS ether of **3b** biosynthesized from 8-deuteriated **1a**), *m*/*z* 225 and 226 (TMS ether of **3b** biosynthesized from 11-deuteriated **1a**), *m*/*z* 290 and 291 (**2b**), and *m*/*z* 292 and 293 (methyl ester of the incubated deuteriated **1a**). In addition, [(11*R*)-^{2}H]- and [(11*S*)-^{2}H]-linoleic acids were incubated with the enzyme preparation and the percentages of deuteriated molecules of the products[9], (11*R*,9*Z*,12*Z*)-hydroxyoctadeca-9,12-dienoic acid (methyl ester–TMS ether; *m*/*z* 225 and 226) and (9*Z*,12*Z*)-11-oxooctadeca-9,12-dienoic acid (methyl ester; *m*/*z* 308 and 309), as well as of the incubated deuteriated linoleic acids (methyl esters; *m*/*z* 294 and 295) were determined by GC-MS (Table 3).

In another set of experiments, 500 μmol dm^{-3} [(8*S*)-^{2}H; 1-^{14}C]-and [(11*R*)-^{2}H; 1-^{14}C]-**1a**, prepared by mixing the deuteriated acids with [1-^{14}C]-**1a**, were stirred at 23 °C with the enzyme preparation containing 10 mmol dm^{-3} sodium azide. Aliquots were removed after 20, 40, 60 and 80 min, extracted and esterified. The product was subjected to radio-TLC and the percentage conversion of [1-^{14}C]-**1a** into the tetraene **2b** was determined. The zone of **1b**, corresponding to **1a** remaining not converted, was scraped off and the eluted material analyzed by GC-MS (*m*/*z* 292 and 293; Table 4).

$H_2{}^{18}O$ *Incubation.*—Compound **1a** (400 μmol dm^{-3}) was stirred at 23 °C for 20 min with a mixture of the enzyme preparation (3.9 cm^3) and $H_2{}^{18}O$ (0.9 cm^3; final atom% of ^{18}O, 17.8). Methyl 11-hydroxyoctadecatrienoate **3b** was isolated by HPLC, converted into the TMS ether derivative, and the $^{16}O/^{18}O$ composition was determined by selected monitoring of the ions *m*/*z* 225 and 227 (Found: $^{18}O_1$, 17.9%, $^{18}O_0$, 82.1%, *i.e.*, the incorporation of oxygen from water was 100–101%).

Acknowledgements

The author is greatly indebted to Dr. David Weisleder, National Center for Agricultural Utilization Research, USDA, Peoria, IL, for the NMR analyses. Ms. Kirsten Andersen, Aarhus Universitet, Denmark, is thanked for generously providing cultures of *Tetrahymena pyriformis*. The expert technical assistance of Ms. Gunvor Hamberg and Ms. Lian-Ying Zhang is gratefully acknowledged. This work was supported by the Swedish Medical Research Council (proj. no. 03X-05170).

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Paper 3/04889F
Received 12*th August* 1993
Accepted 1*st September* 1993