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15(*R*)-HYDROXYLINOLEIC ACID, AN OXYLIPIN FROM OAT SEEDS

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Key Word Index—*Avena sativa*; Gramineae; oat; seeds; oxylipin; 15(*R*)-hydroxy-9(*Z*),12(*Z*)-octadecadienoic acid; quantitative determination.

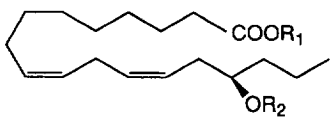
Abstract—A new oxylipin, 15(*R*)-hydroxy-9(*Z*),12(*Z*)-octadecadienoic acid (15(*R*)-hydroxylinoleic acid), was isolated from oat seeds. Its structure was determined by chemical and spectroscopic methods. Oat seeds contained 0.6–0.7 mg g^{-1} of the new compound in esterified form. The amounts found in seeds of barley, rye and wheat, however, were less than 1 $\mu g\ g^{-1}$.

INTRODUCTION

Several oxylipins, such as hydroxyoctadecadienoic acids, epoxyoctadecenoic acids, epoxyhydroxyoctadecenoic acids and trihydroxyoctadecenoic acids, are formed from linoleic acid upon incubation with preparations of cereal seeds [1–5]. In recent work [4], we have found that seeds of oat contain high levels of the enzyme peroxygenase [6–8] and that this enzyme, together with lipoxygenase, plays a key role in the conversion of linoleic acid into various oxylipins in oat seed homogenates. In order to obtain further information about the lipoxygenase-peroxygenase pathway in cereals, we have recently initiated a study aimed at profiling oxygenated fatty acids which occur as endogenous constituents of cereal seeds (Hamberg, M. and Hamberg, G., to be published). During these experiments, the presence in oat seeds of considerable quantities of a new hydroxylated derivative of linoleic acid was observed. The structure and stereochemistry of this compound is described in the present report.

RESULTS AND DISCUSSION

Lipids obtained by extracting ground oat seeds with chloroform–methanol were saponified and the methyl-esterified product was subjected to open column silicic acid chromatography. The hydroxyester fraction was subjected to TLC followed by reverse-phase HPLC to provide the methyl ester of a hydroxylinoleic acid (**2**) as a colourless oil. Saponification of **2** yielded free acid **1** as a colourless oil (room temperature) or a white solid (−30°). The UV spectrum of **2** was featureless, demonstrating the absence of conjugated double bonds. The IR spectrum, which showed the presence of a hydroxyl group (3435 cm^{-1}), a carbomethoxy group (1742 cm^{-1}) and the absence of *E* double bond(s) (no absorption band at *ca* 970 cm^{-1}), was similar, but not identical to the IR spectrum of methyl ricinoleate.



1 $R_1 = H$, $R_2 = H$

2 $R_1 = CH_3$, $R_2 = H$

3 $R_1 = CH_3$, $R_2 = COC_2H_5$

Table 1. ^1H NMR spectral data of compound **2**

Proton (carbon no.)	Chemical shift, multiplicity (*J*, Hz)
H-2	2.29 *t* (7.5)
H-3	1.60 *m*
H-4–H-7, H-17	1.29 *m*
H-8	2.03 *dt* (6.8)
H-9	5.38 *dt* (10.7, 7.2)
H-10	5.31 *dt* (10.7, 7.0)
H-11	2.79 *m*
H-12	5.53 *dt* (10.8, 7.2)
H-13	5.42 *dt* (10.8, 7.3)
H-14	2.23 *m*
H-15	3.63 *m*
H-16	1.44 *m*
H-18	0.92 *t* (6.9)
OCH_3	3.65 *s*

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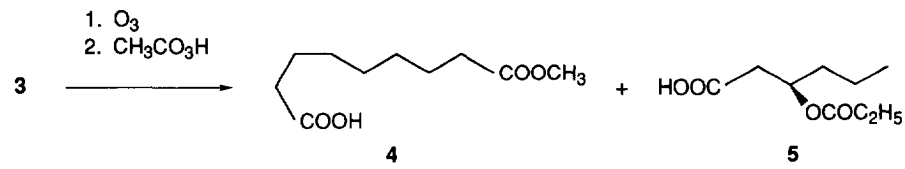

Fig. 1. Degradation products of propionyl derivative **3** by oxidative ozonolysis.

Analysis of the TMSi derivative of **2** by GC-mass spectrometry showed prominent ions at *m/z* 367 $[M-CH_3]^+$, 310 $[M-OHC-(CH_2)_2-CH_3]^+$, and 145 $[TMSiO{=}CH-(CH_2)_2-CH_3]^+$, suggesting a methyl octadecadienoate substituted with a hydroxyl group at C-15. The ^1H NMR spectrum (Table 1) was in agreement with the presence of two nonconjugated double bonds having the *Z* configuration ($J_{9,10}$ = 10.7 Hz, $J_{12,13}$ = 10.8 Hz), as well as a hydroxyl group located at C-15 (δ 3.63, partly hidden in the methoxyl signal at δ 3.65). Oxidative ozonolysis performed on the propionyl derivative **3** resulted in the formation of methyl hydrogen azelate (**4**) and 3-propionoxyhexanoic acid (**5**) (Fig. 1). This result demonstrated that the two double bonds were located in the Δ^9 and Δ^{12} positions. The absolute configuration of **5** was determined by GC analysis of the *N*-acyl-L-phenylalanine methyl ester derivative [9]. The *N*-(propionoxyacyl)-L-phenylalanine methyl ester derivatives of 3(*R*)- and 3(*R,S*)-hydroxyhexanoic acids (prepared by phytochemical [10] and chemical reductions, respectively, of 3-oxohexanoate) were used as references. The analysis demonstrated that the 3-propionoxyhexanoic acid derived from **3** had the *R* configuration. On the basis of these results, **1** was identified as 15(*R*)-hydroxylinoleic acid.

It was of interest to obtain quantitative data on the occurrence of **1** in oat seeds and in other cereal seeds. Accordingly, a method for quantitative determination of **1** based on GC analysis using methyl 17-hydroxystearate as internal standard was developed. The amounts of **1** in seeds of two cultivars of oat were 0.6–0.7 mg g^{-1} (Table 2). In contrast, **1** was undetectable in seeds of barley, rye, and wheat (less than 1 $\mu g\, g^{-1}$). In separate experiments, **1** was determined in lipid extracts of oat seed that were not subjected to alkaline hydrolysis. In this case, the content of **1** was 4 $\mu g\, g^{-1}$ or less. Thus, less than 1% of the total amount of **1** present in oat seeds existed in a nonesterified form.

Table 2. Content of 15*R*-hydroxylinoleic acid (**1**) in cereal seeds

Seed, cultivar	**1** ($\mu g\, g^{-1}$)
Barley, cv. Frost	N.D.
Barley, cv. Kinnan	N.D.
Oat, cv. Freja	725±31 (n = 3)
Oat, cv. Vital	593±14 (n = 3)
Rye, cv. Marder	N.D.
Rye, cv. Motto	N.D.
Wheat, cv. Dragon	N.D.
Wheat, cv. Kosack	N.D.

N.D., not detectable, *i.e.* $<1\ \mu g\, g^{-1}$.

To the best of our knowledge, 15(*R*)-hydroxylinoleic acid described in the present report is a new compound. It has not been previously isolated from plant or animal tissue [11] and it is conspicuously absent among the several hydroxylinoleates (18-, 17-, 16-, 14-, 13-, 11-, 9-, and 8-hydroxylinoleates) which are produced *in vitro* by cytochrome P-450-catalysed monooxygenation of linoleic acid [12]. Furthermore, 15-hydro(pero)-xylinoleic acid has neither been encountered as a dioxygenase product in experiments with, for example, linoleic acid 8(*R*)-dioxygenase [13], nor as an autoxidation product of linoleic acid using conditions under which 8- and 14-hydro(pero)xylinoleic acids are formed [14].

The significance of **1** in oat remains to be established. It may be noted that **1** and ricinoleic acid are both homoallylic alcohols and that the positions of the hydroxyl groups of the two compounds are at a carbon of the fatty acid chain that may undergo a desaturase reaction. In the case of ricinoleic acid biosynthesis, it has recently been reported that an oleoyl hydroxylase from *Ricinus communis*, which catalyses hydroxylation of oleate into ricinoleate, is homologous to a microsomal ω-6 oleoyl desaturase from *Arabidopsis*, that catalyzes conversion of oleate into linoleate [15]. It can be speculated that the putative hydroxylase involved in the formation of 15(*R*)-hydroxylinoleic acid in oat seeds may be related to ω-3 linoleoyl desaturase(s) responsible for the biosynthesis of α-linolenic acid from linoleic acid.

Other hydroxylated derivatives of linoleic acid, which have been previously isolated from natural sources, have been found to possess biological activity. Thus, 8-hydroxylinoleic acid from the fungus *Laetisaria arvalis* induces hyphal lysis in several phytopathogenic fungi [16], and the conjugated 9-hydroxy-10(*E*),12(*Z*)- and 13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acids isolated from rice have been implicated as defensive substances against attack by plant pathogens [17]. In addition, the acetate of 6-hydroxylinoleic acid, isolated from the marine alga *Spatoglossum pacificum*, is a pollen growth inhibitor [18]. The possibility of specific biological action(s) of 15(*R*)-hydroxylinoleic acid in oat should be explored.

EXPERIMENTAL

General. Open CC: 100 mesh silicic acid (Mallinckrodt). TLC: silica gel 60 (Merck) developed with EtOAc-hexane (1:3). Reverse-phase HPLC: Nucleosil C_{18} 100-7 (250 × 10 mm) using MeCN–H_2O (7:3) and detection at 210 nm. GC for quantitative determination:

FID instrument equipped with a methylsilicone capillary column (length, 25 m; film thickness, 0.33 μm; carrier gas, He; flow rate, 25 cm s^{-1}; oven temp., 230°). GC for steric analysis: FID instrument equipped with a DB-210 capillary column (length, 15 m; film thickness, 0.25 μm; carrier gas, He; flow rate, 36 cm s^{-1}; oven temp., 190°, raised at 2° min^{-1}. GC-MS: mass selective detector connected to a gas chromatograph fitted with a 5% phenyl methylsilicone capillary column (length, 12 m; film thickness, 0.33 μm). ^{1}H NMR: 400 MHz instrument, $CDCl_3$ solns, TMS as standard.

Plant material. Seeds of barley (*Hordeum vulgare* L., cv. Frost; *H. distichum* L., cv. Kinnan), oat (*Avena sativa* L., cv. Freja and Vital), rye (*Secale cereale* L., cv. Marder and Motto), and wheat (*Triticum aestivum* L., cv. Dragon and Kosack) were obtained from Svalöf Weibull, Svalöv, Sweden.

3(R,S)-*Hydroxyhexanoic acid.* Me 3-oxohexanoate (40 mg) in MeOH (2 ml) was reduced to Me 3(*R,S*)-hydroxyhexanoate by treatment with $NaBH_4$ (40 mg) at 0°. IR (film) ν_{max} cm^{-1}: 3460, 2959, 1739, 1439, 1171. GC-MS (TMSi ether derivative) *m/z*: (rel. int.) 203 $[M-CH_3]^+$ (71), 175 $[M-C_3H_7]^+$ (35), 145 $[M-CH_2COOCH_3]^+$ (18), 89 $[TMSiO]^+$ (100), 73 $[TMSi]^+$ (93). An aliquot of the ester was saponfied to give 3(*R,S*)-hydroxyhexanoic acid by treatment with 0.2 M NaOH in MeOH–H_2O (4:1) at 23° for 18 hr.

3(R)-*Hydroxyhexanoic acid.* Me 3-oxohexanoate (202 mg) was saponified and stirred for 41 hr with a suspension of baker's yeast (18 g) in H_2O (40 ml) containing glucose (8 g), KH_2PO_4 (20 mg) and $MgSO_4$ (10 mg) [*cf.* ref. 10]. Extraction (EtOAc), esterification (CH_2N_2) and open column CC afforded the title compound (33 mg). IR and GC-MS data were the same as those obtained for the racemic compound. GC analysis of the propionyl derivative of the L-phenylalanine methyl ester conjugate [9] showed an enantiomeric excess of 95% (derivative of 3(*R*)-hydroxyhexanoate: R_t, 8.8 min; area, 97.5%; derivative of 3(*S*)-hydroxyhexanoate: R_t, 9.3 min; area, 2.5%).

Extraction and isolation of compound **2**. Batches of oat seeds (cv. Freja) were ground in an electric rotating knife coffee mill and the powder (30 g) homogenized in $CHCl_3$–MeOH (2:1, 600 ml) containing 2,6-di-*tert*-butyl-4-methylphenol (6 mg) using an Ultra-turrax. The mixt. was filtered through cheesecloth and centrifuged at 1200 *g* for 10 min. The supernatant was washed with H_2O and the aq. phase re-extracted with one portion of $CHCl_3$. Material obtained following removal of solvent was treated with 0.2 M NaOH in EtOH–H_2O (4:1) under Ar at 23° for 18 hr. Extractive isolation followed by methyl-esterification and open column CC afforded a hydroxyester fr. (108 mg; elution with Et_2O–hexane (1:1)) which was subjected to prep. TLC (R_f value of **2**, 0.47). Pure hydroxyester **2** (10 mg) was obtained following semipreparative reverse-phase HPLC (elution volume, 91.0–96.1 ml).

Methyl 15(R)-*hydroxylinoleate* (**2**). Oil. UV (EtOH) end absorption. IR ν_{max} (film) cm^{-1}: 3435, 3010, 2929, 2855, 1742, 1019. GC-MS (TMSi ether derivative) *m/z* (rel. int.) 367 $[M-CH_3]^+$ (1), 310 $[M-OHC-(CH_2)_2-CH_3]^+$ (19), 145 $[TMSiO{=}CH-(CH_2)_2-CH_3]^+$ (100), 73 $[TMSi]^+$ (56). ^{1}H NMR, see Table 1. Catalytic hydrogenation of **2** yielded Me 15-hydroxystearate as determined by GC-MS analysis using authentic material [19]. Oxidative ozonolysis performed on propionyl derivative **3** afforded Me hydrogen azelate **4** and 3-propionoxyhexanoic acid **5**. The absolute configuration of **5** was '*R*' (>99.9% '*R*') as shown by GC analysis of the L-phenylalanine methyl ester conjugate [9] using the corresponding derivatives of authentic 3(*R*)- and 3(*R,S*)-hydroxyhexanoates as references.

15(*R*)-*Hydroxylinoleic acid* (**1**). **2** (10 mg) was saponified by treatment with 0.2 M NaOH in MeOH–H_2O (4:1) under Ar at 23° for 18 hr. Extractive isolation, followed by recrystallization from hexane at −80° afforded **1** as a white solid (solid at −80° and −30°, oil at room temp.). UV (EtOH) end absorption. IR ν_{max} (film) cm^{-1}: 3350, 3011, 2929, 2855, 1710, 1017. GC-MS (TMSi ester/TMSi ether derivative) *m/z*: (rel. int.) 425 $[M-CH_3]^+$ (6), 368 $[M-OHC-(CH_2)_2-CH_3]^+$ (36), 145 $[TMSiO{=}CH-(CH_2)_2-CH_3]^+$ (100), 75 $[(CH_3)_2Si{=}OH]^+$ (31), 73 $[TMSi]^+$ (74).

Quantitative determination of 15(R)-*hydroxylinoleic acid* **1** *in cereal seeds.* Seeds were ground and the powder (1 g) homogenized in $CHCl_3$–MeOH (2:1, 50 ml) containing Me 17-hydroxystearate (124.7 μg) and 2,6-di-*tert*-butyl-4-methylphenol (0.5 mg). The organic layer obtained after filtration and centrifugation was washed with H_2O and the H_2O phase re-extracted with one vol. of $CHCl_3$. Material obtained after evapn of solvent was saponified, treated with CH_2N_2 and subjected to open column CC. Material in the hydroxyester fr. (Et_2O–hexane, 1:1) was trimethylsilylated and analysed by GC. The amounts of **1** in the seeds analysed were calculated from the areas of the peaks corresponding to the TMSi ether derivatives of **2** (R_t, 8.3 min) and of Me 17-hydroxystearate (R_t, 10.5 min) and from the amount of the 17-hydroxystearate standard added to the cereal seed samples.

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