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A lipoxygenase-divinyl ether synthase pathway in flax (*Linum usitatissimum* L.) leaves

Ivan R. Chechetkin [a], Alexander Blufard [a], Mats Hamberg [b], Alexander N. Grechkin [a,*]

[a] Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, Lobachevsky Street 2/31, P.O. Box 30, 420111 Kazan, Russia
[b] Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institutet, SE-171 77 Stockholm, Sweden

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ABSTRACT

Incubation of linoleic acid with an enzyme preparation from leaves of flax (*Linum usitatissimum* L.) led to the formation of a divinyl ether fatty acid, i.e. (9*Z*,11*E*,1′*Z*)-12-(1′-hexenyloxy)-9,11-dodecadienoic [(ω5*Z*)-etheroleic] acid, as well as smaller amounts of 13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid. The 13-hydroperoxide of linoleic acid afforded the same set of products, whereas incubations of α-linolenic acid and its 13-hydroperoxide afforded the divinyl ether (9*Z*,11*E*,1′*Z*,3′*Z*)-12-(1′,3′-hexadienyloxy)-9,11-dodecadienoic [(ω5*Z*)-etherolenic] as the main product. Identification of both divinyl ethers was substantiated by their UV, mass-, ^{1}H NMR and COSY spectral data. In addition to the 13-lipoxygenase and divinyl ether synthase activities demonstrated by these results, flax leaves also contained allene oxide synthase activity as judged by the presence of endogenously formed (15*Z*)-*cis*-12-oxo-10,15-phytodienoic acid in all incubations.

1. Introduction

The plant lipoxygenase cascade provides a large diversity of oxylipins. Many of these products are involved in plant cell signalling and defence (Blée, 1998; Grechkin, 1998). The existing diversity of oxylipins is largely established by enzymes belonging to the CYP74 family of cytochrome P450s (Blée, 1998; Grechkin, 1998). Divinyl ether synthases (DESs) along with allene oxide synthases (AOSs) and hydroperoxide lyases (HPLs) belong to this family (Blée, 1998; Grechkin, 1998, 2002; Stumpe and Feussner, 2006). Not many DESs are described compared to other CYP74 members, including AOSs and HPLs. At the same time, DES products were discovered in phylogenetically distant species, including brown (Proteau and Gerwick, 1993) and red (Jiang and Gerwick, 1997) algae, as well as higher plant species of families *Ranunculaceae* (Hamberg, 1998, 2002, 2004, 2005), *Solanaceae* (Galliard and Phillips, 1972; Galliard et al., 1973; Galliard and Mathew, 1975) and *Alliaceae* (Grechkin et al., 1995, 1997; Grechkin and Hamberg, 1996; Stumpe et al., 2008). DES genes of some *Solanaceae* species including tomato (Itoh and Howe, 2001), potato (Stumpe et al., 2001) and tobacco (Fammartino et al., 2007) have been cloned. Divinyl ethers are not as well studied as jasmonates with respect to their physiological importance. At the same time, there are increasing data demonstrating the involvement of divinyl ethers in plant resistance towards pathogens (Weber et al., 1999; Göbel et al., 2001; Granér et al., 2003; Cowley and Walters, 2005).

The present work reports the existence of a lipoxygenase-DES pathway in flax (Linaceae, Eurosids I). The DES was found to specifically utilize 13-hydroperoxides of α-linolenic and linoleic acids. Of the several linolenic acid-derived divinyl ether fatty acids previously described, the flax DES specifically generated (9*Z*,11*E*,1′*Z*,3′*Z*)-12-(1′,3′-hexadienyloxy)-9,11-dodecadienoic [(ω5*Z*)-etherolenic] acid.

2. Results

2.1. Biosynthesis of oxylipins in vitro in flax leaves

For the preliminary characterization of the lipoxygenase pathway, the 15,000*g* supernatant of flax leaf homogenate was incubated with linoleic acid. GC–MS analysis of products upon their methylation/hydrogenation/trimethylsilylation has revealed that the predominant hydroxy acid was 13-hydroxystearic acid (result not illustrated). This indicates that the major oxygenation product was 13-hydroperoxide of linoleic acid (13-HPOD). Thus, 13-lipoxygenase activity predominates in flax leaves. Along with

Abbreviations: DES, divinyl ether synthase; 13-HPOT, (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid; 13-HPOD, (9*Z*,11*E*,13*S*)-13-hydroperoxy-9,11-octadecadienoic acid; AOS, allene oxide synthase; HPL, hydroperoxide lyase; 12-oxo-PDA, (15*Z*)-12-oxo-10,15-phytodienoic acid.

* Corresponding author. Tel.: +7 843 292 75 35; fax: +7 843 292 73 47.
E-mail address: grechkin@mail.knc.ru (A.N. Grechkin).

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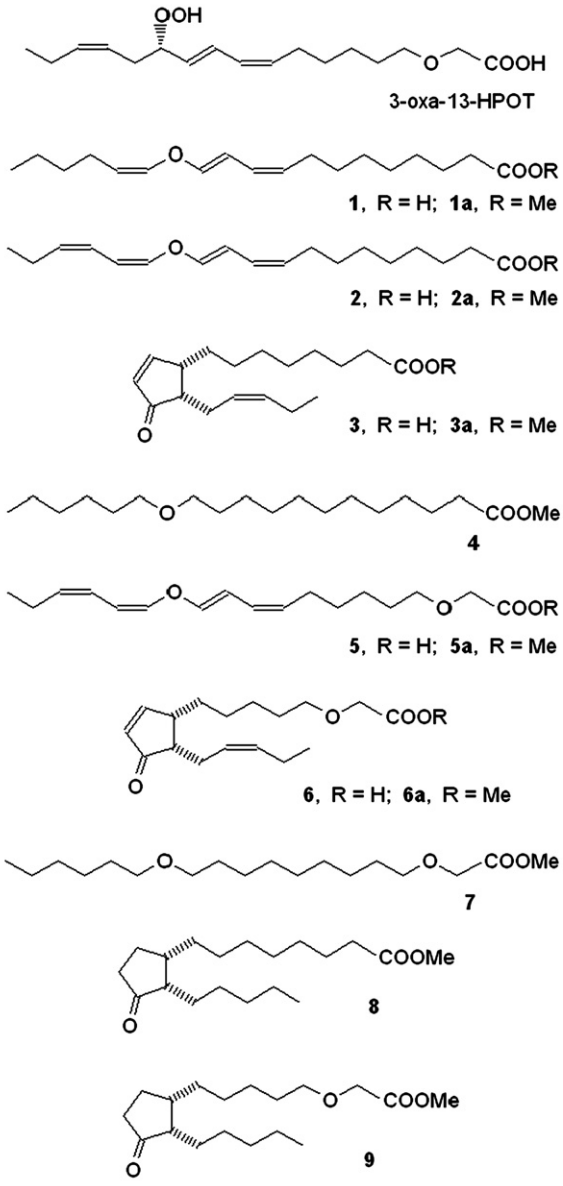

hydro(pero)xy fatty acids, compounds **1a**, **2a** and **3a** were detected during the GC–MS analyses of the extracted oxylipins. The same oxylipins were detected after incubations with 13-HPOD, as described below.

For further characterization of metabolic pathways, we incubated the 15,000*g* supernatant with 13-HPOD. Direct analyses of products as trimethylsilylated methyl esters by GC–MS enabled us to detect oxylipins **1a**, **2a** and **3a** (Fig. 1a). For identification of molecular structures of oxylipins **1a**, **2a** and **3a**, their mass spectra and spectra of their derivatives were recorded. Moreover, oxylipins **1a** and **2a** were separated and purified by micropreparative HPLC and their ^{1}H NMR spectra were recorded.

2.2. Identification of compound *1*

Compound **1a** (retention time 14.8 min, see Fig. 1a) was the major product of linoleic acid conversion *in vitro*. Product **1a** possessed a mass spectral fragmentation pattern (Fig. 1b) identical to that described previously for the divinyl ethers (9*Z*,11*E*,1′*E*)-12-(1′-hexenyloxy)-9,11-dodecadienoic (etheroleic) acid (Grechkin et al., 1995, 1997) and (ω5*Z*)-etheroleic acid (Hamberg, 1998). Catalytic hydrogenation of compound **1a** afforded product **4** (M^+ at *m*/*z* 314), which had a characteristic mass fragmentation pattern (Fig. 1c). This data enabled us to identify the hydrogenation product **4** as 13-oxa-nonadecanoic acid. Purified compound **1a** possessed a specific UV absorbance with λ_{max} at 250 nm which is typical for divinyl ethers like etheroleic acid (Grechkin et al., 1995, 1997; Hamberg; 1998). The ^{1}H NMR spectrum (Table 1) was recorded for final identification and verification of double bond geometry of compound **1a**. The ^{1}H NMR spectrum (Table 1) supported by 2D–COSY data (not illustrated) demonstrated that compound **1a** possessed a butadienyl vinyl ether partial structure having the (1*Z*,3*E*,1′*Z*) configuration of double bonds. Coupling constant value (6.2 Hz) unequivocally shows that 1′-double bond has *cis* configuration. On the basis of the data mentioned, compound **1** was identified as (9*Z*,11*E*,1′*Z*)-12-(1′-hexenyloxy)-9,11-dodecadienoic acid, i.e. (ω5*Z*)-etheroleic acid.

2.3. Identification of compound *2*

Compound **2** (retention time 45.9 min) exhibited UV absorbance with λ_{max} at 267 nm (the spectrum was recorded on line during the normal phase HPLC separation, solvent hexane – diethyl ether 99.4:0.6). The electron impact mass spectrum of the methyl ester of **2** (Fig. 1d) showed a molecular ion at *m*/*z* 306 and a fragmentation pattern similar to those of the methyl esters of etherolenic (Grechkin et al., 1995, 1997) and (ω5*Z*)-etherolenic acid (Hamberg, 1998). Catalytic hydrogenation of compound **2a** resulted in the formation of the above described product **4**, 13-oxa-nonadecanoic acid, which was identified by its electron impact mass spectrum (Fig. 1c). Thus, compound **2a** had four double bonds. Their position and geometry were elucidated by ^{1}H NMR (Table 1, Fig. 2b) and COSY (Fig. 2a) spectral data. Thus, the obtained data enabled us to identify compound **2** as (9*Z*,11*E*,1′*Z*,1′*Z*)-12-(1′,3′-hexadienyloxy)-9,11-dodecadienoic acid, i.e. (ω5*Z*)-etherolenic acid.

Recently a new α-linolenic acid analogue, 3-oxa-α-linolenic acid, was synthesized and described as a useful precursor in studies of oxylipin biosynthesis (Hamberg et al., 2006). Here we used the lipoxygenase-generated 13-hydroperoxide of 3-oxa-α-linolenic acid (3-oxa-13-HPOT) for further confirmation of the *in vitro* DES activity and (ω5*Z*)-etherolenic acid biosynthesis in flax leaves. The 15,000*g* supernatant was incubated with 3-oxa-13-HPOT. Analyses of incubation products (as methyl esters) by GC–MS enabled us to detect (along with the endogenous unlabelled compound **2**, detected in methylated form **2a**) the appearance of the new products **5** and **6** detected as their methyl esters **5a** and **6a** (Fig. 3a). The mass spectral data for compound **5a** (M^+ at *m*/*z* 308, Fig. 3b) enabled us to propose a fragmentation scheme depicted in Fig. 3b. Catalytic hydrogenation of compound **5a** afforded product **7**, which had a characteristic MS fragmentation patterns (Fig. 3c). This result demonstrates that compound **7** is 3,13-dioxa-nonadecanoic acid, thus substantiating the identification of compound **5** as 3-oxa-(ω5*Z*)-etherolenic acid.

The observations with 3-oxa-13-HPOT confirmed both the *in vitro* DES activity and the presence of endogenous (ω5*Z*)-etherolenic acid (**2**) in the leaves. Integration of total ion chromatograms revealed that the content of endogenous (ω5*Z*)-etherolenic acid in leaves reached up to 10% of the extracted total free fatty acids.

2.4. Identification of compound *3*

As mentioned above, compound **3** belonged to the most abundant oxylipins detected after the incubations with cell-free preparations from flax leaves. The mass spectrum of methyl ester **3a** (Fig. 4a) exhibited a characteristic fragmentation pattern identical to that earlier recorded on the methyl ester of *cis*-12-oxo-10,15-phytodienoic acid (12-oxo-PDA) (Hamberg, 1998). Catalytic hydro-

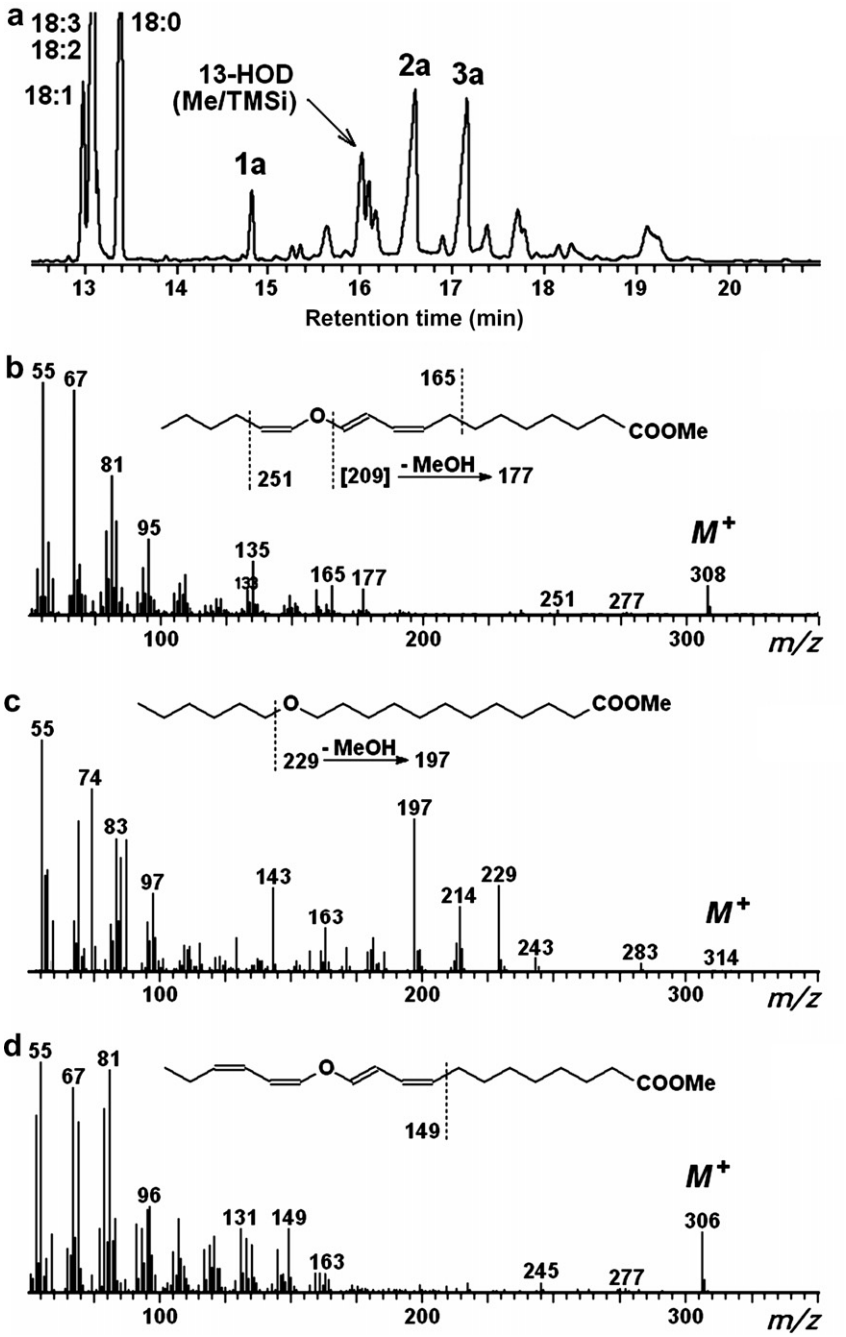

Fig. 1. GC–MS analyses of oxylipins extracted after incubations of 13-HPOD with 15,000*g* supernatant of flax leaf homogenate. Products were extracted, purified with aminopropyl cartridges, methylated, trimethylsilylated and analyzed by GC–MS as described in the Experimental section. (a), Total ion chromatogram of products; (b), (c), (d), mass spectra and fragmentation schemes of compounds **1a**; **4**, and **2a**, respectively.

genation of compound **3a** over Pt catalyst resulted in the formation of compound **8**. Its mass spectral data (Fig. 4c) enabled us to identify compound **8** as 12-oxo-phytonoic acid, the fully hydrogenated analogue of 12-oxo-PDA. Thus, the data obtained enabled one to identify compound **3a** as *cis*-12-oxo-PDA methyl ester.

When 15,000*g* supernatant of leaf homogenate was incubated with 3-oxa-13-HPOT, a new product **6** appeared along with compound **3** and divinyl ethers. The mass spectrum of methyl ester **6a** (M^+ at *m*/*z* 308) and the proposed fragmentation scheme (insert) are presented in Fig. 4b. In general the spectral patterns of compound **6a** were similar to those of compound **3a** with two exceptions: (1) difference of molecular mass (308 and 306, respectively); (2) difference of mass of fragment [M^+–CH_2CH = $CHCH_2CH_3$] (240 and 238, respectively). The obtained data enabled us to identify compound **6** as 3-oxa-12-oxo-PDA, i.e. 12-oxo-PDA derivative biosynthesized from 3-oxa-13-HPOT.

Catalytic hydrogenation of product **6a** afforded compound **9**. Its mass spectrum and fragmentation scheme (Fig. 4d) enabled one to identify compound **9** as 3-oxa-12-oxo-phytonoic acid. Thus, the obtained data demonstrated that the exogenously added 3-oxa-13-HPOT served as a precursor of cyclopentenone **6**, 3-oxa-12-oxo-PDA. Notably, along with compound **6a**, significant levels of endogenous 12-oxa-PDA **3** (as methyl ester **3a**) were detectable along with products (analyzed as methyl esters) of incubations with 3-oxa-13-HPOT (Fig. 3a).

Table 1
^{1}H NMR spectral data for compounds **1a** and **2a** (400 MHz, C^2H_3CN, 296 K)

Proton	Compound **1a**			Compound **2a**		
	Chemical shift, δ (ppm)	Multiplicity (number of protons)	Coupling constants (Hz)	Chemical shift, δ (ppm)	Multiplicity (number of protons)	Coupling constants (Hz)
H2	2.32	*t* (2)	7.5 (H3)	2.31	*t* (2)	7.5 (H3)
H3	1.59	*m* (2)		1.59	*m* (2)	
H4	1.31	*m* (2)		1.31	*m* (2)	
H5	1.31	*m* (2)		1.31	*m* (2)	
H6	1.31	*m* (2)		1.31	*m* (2)	
H7	1.31	*m* (2)		1.31	*m* (2)	
H8	2.13	*m* (2)		2.07	*m* (2)	
H9	5.30	*dt* (1)	7.5 (H8); 10.6 (H10)	5.34	*dt* (1)	7.6 (H8); 10.7 (H10)
H10	5.90	*dd* (1)	11.1 (H11)	5.90	*dd* (1)	11.1 (H11)
H11	6.02	*dd* (1)	11.9 (H12)	6.11	*ddd* (1)	11.9 (H12); 1.1 (H9)
H12	6.69	*d* (1)		6.75	*d* (1)	
H1′	6.30	*dt* (1)	6.2 (H2′); 1.2 (H3′)	6.35	*d* (1)	6.2 (H2′)
H2′	4.67	*dt* (1)	7.4 (H3′)	5.56	*ddd* (1)	11.6 (H3′); 1.2 (H4′)
H3′	2.13	*m* (2)		6.29	*dddt* (1)	11.1 (H4′); 1.2 (H1′); 1.2 (H5′)
H4′	1.31	*m* (2)		5.45	*m* (1)	7.5 (H5′);
H5′	1.31	*m* (2)		2.12	*m* (2)	
H6′	0.91	*t* (3)	6.9 (H5′)	1.01	*t* (3)	7.6 (H5′)
H(OMe)	3.63	*s* (3)		3.63	*s* (3)	

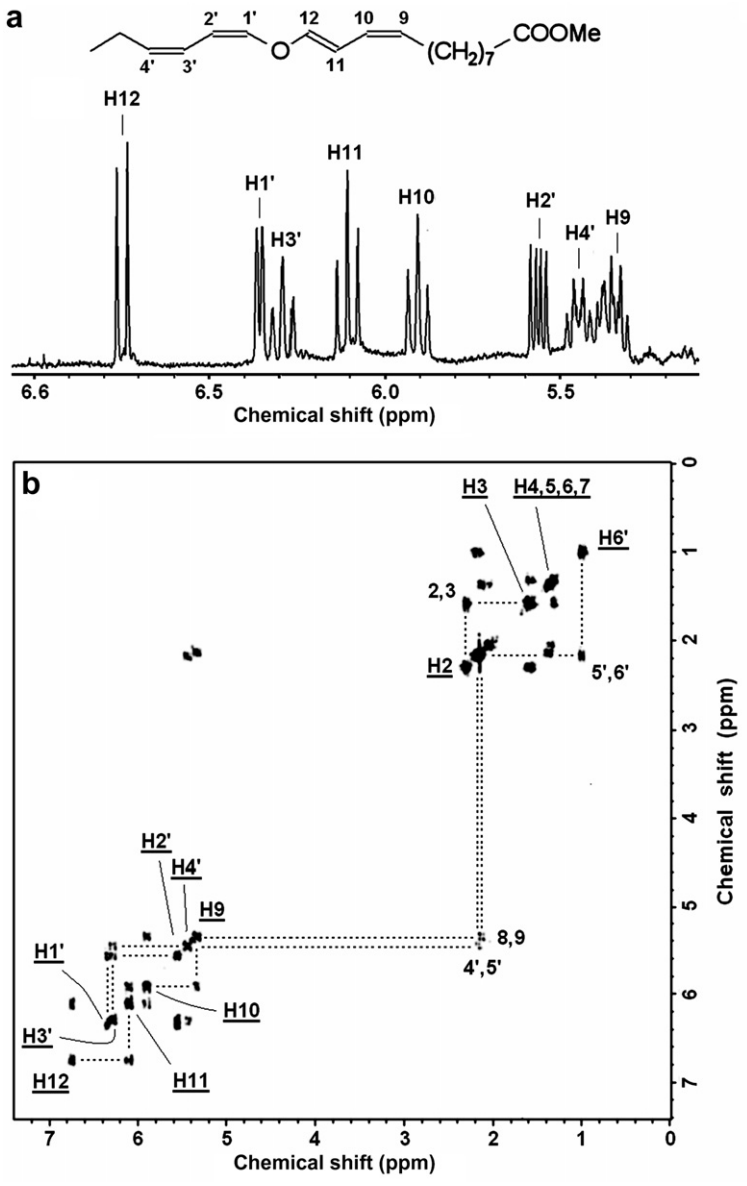

Fig. 2. ^{1}H NMR data for compound **2a**, including the 2D–COSY plot (a) and the olefinic region of ^{1}H NMR spectrum (400 MHz, C^2H_3CN, 298 K).

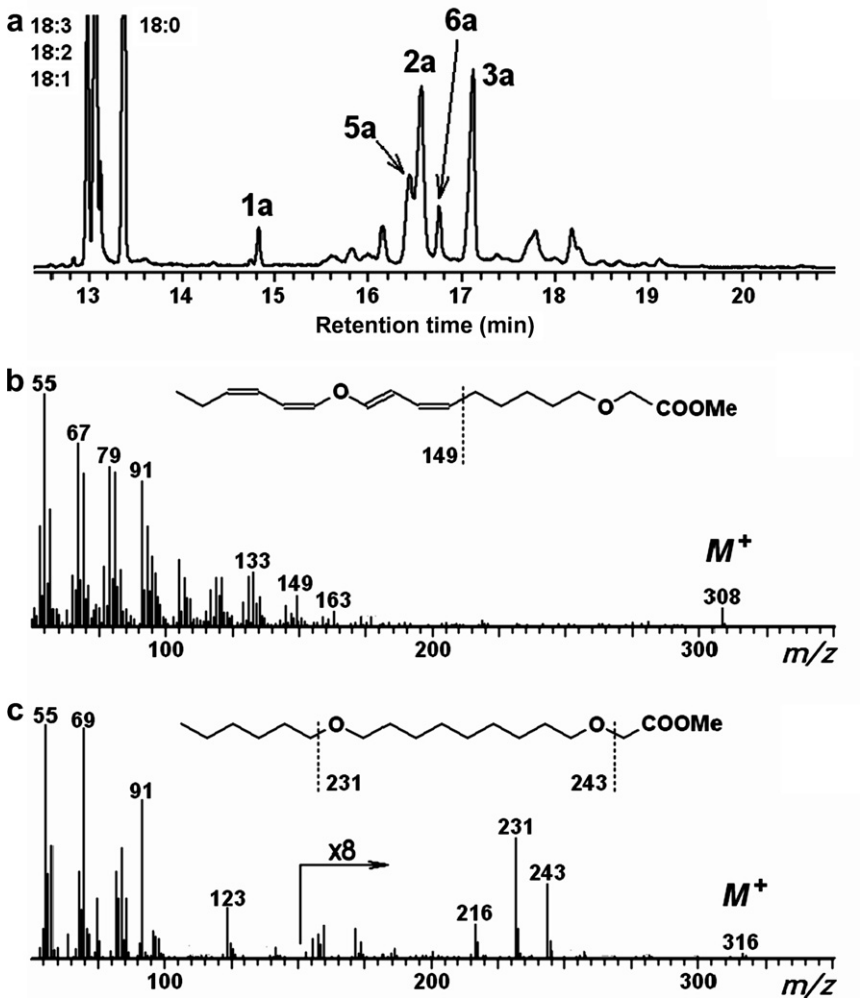

Fig. 3. GC–MS analyses of oxylipins extracted after incubations of 3-oxa-13-HPOT with 15,000*g* supernatant of flax leaf homogenate. Products were extracted, purified with aminopropyl cartridges, methylated, trimethylsilylated and analyzed by GC–MS as described in the Experimental section. (a), Total ion chromatogram of products; (b), (c), mass spectra and fragmentation schemes of compounds **5a** and **7**, respectively.

2.5. Biosynthesis of oxylipins in vitro in flax roots

The 15,000*g* supernatant of flax root homogenate was incubated with linoleic acid. GC–MS analysis of products (methyl esters TMSi derivatives) revealed the presence of α-ketol as a predominant product as well as a small amount of 12-oxo-PDA methyl ester (**3a**). The mass spectral data for α-ketol Me ester TMSi derivative possessed the following prominent ions, *m*/*z* [ion attribution] (relative intensity, %): 398 [M^+] (0.1); 383 [$M^+ - CH_3$] (2); 367 [$M^+ - CH_3O$] (0.5); 270 [M^+ – C12/C18 + TMSi] (18); 173 [M^+ – C1/C12] (100); 129 (3); 103 [$CH_2 = O^+ - SiMe_3$] (14); 73 [$Si(CH_3)_3$]+ (67), supported identification of the α-ketol as (9*Z*)-12-oxo-13-hydroxy-9-octadecenoic acid. No divinyl ethers were detectable after *in vitro* incubations with roots.

3. Discussion

The results obtained demonstrate that flax extends the number of plant species which possess DES activity and contain divinyl ethers. This is of particular interest since flax (Linaceae, Malpighiales, Eurosids I) is phylogenetically distant from other higher plants known to possess DESs, i.e. Ranunculaceae (basal Eudicots), Solanaceae (Euasterids I) and Alliaceae (monocots), see Fig. 5. The specificity of flax DES, converting 13-HPOT and 13-HPOD into (ω5*Z*)-etherolenic and (ω5*Z*)-etheroleic acids (respectively), and its localization in leaves, are properties similar to those of Ranunculaceae DESs (Hamberg, 1998, 2002, 2004). (ω5*Z*)-Etherolenic acid and related divinyl ethers also occur in the brown alga *Laminaria sinclairii* (Proteau and Gerwick, 1993). The presence of a *cis* double bond at one side of the ether bridge is a distinctive feature of divinyl ethers produced by enzymes of flax, Ranunculaceae as well as the brown and red algae. Mechanistic studies have suggested that even single mutations at the active site of DESs may be sufficient to change the double bond configuration of the divinyl ether products (Hamberg, 2005). However, the extent of homology in the family of DES enzymes can be revealed only after cloning and characterization of the genes involved.

Divinyl ethers are involved in plant defence against pathogens, including fungi attacking potato leaves (Weber et al., 1999; Stumpe et al., 2001; Göbel et al., 2001; Cowley and Walters, 2005; Fammartino et al., 2007). DES genes are constitutively expressed in the roots of tomato (Itoh and Howe, 2001) and tobacco (Fammartino et al., 2007), but DES activity is significantly increased in the infected roots (Fammartino et al., 2007). DES genes in the leaves and other organs (except roots) of Solanaceae plants are specifically expressed in response to pathogen attack (Weber et al., 1999; Fammartino et al., 2007). This is in contrast to the situation in leaves of flax (present study) and Ranunculaceae (Hamberg, 1998, 2002, 2004) and in garlic bulbs (Grechkin et al., 1995,

1997; Grechkin and Hamberg, 1996) where the DES gene is constitutively expressed.

4. Experimental

4.1. Materials

Unlabelled linoleic acid, as well as linoleic acid and soybean lipoxygenase type V was purchased from Sigma. Sodium borohydride as well as the silylating reagents were purchased from Fluka (Buchs, Switzerland). (9*Z*,11*E*,13*S*)-13-Hydroperoxy-9,11-octadecadienoic acid (13-HPOD) and (9*Z*,11*E*,15*Z*,13*S*)-13-hydroperoxy-3-oxa-9,11,15-octadecatrienoic acid (3-oxa-13-HPOT) were prepared by incubation of linoleic or 3-oxa-α-linolenic acids, respectively, with soybean lipoxygenase at 0 °C, pH 9.0, under continuous oxygen bubbling followed by extraction and purification by normal phase HPLC. Flax plants (*Linum usitatissimum* L., cv. Novotorzhski) were grown in the fields near Kazan in Summer 2007.

4.2. Incubations of cell-free preparation from flax leaves with linoleic acid, 13-HPOD and 3-oxa-13-HPOT

Leaves or roots of 35 days old flax plants (5 g) were dispersed in 20 ml cold (+4 °C) 50 mM Tris/HCL buffer, pH 7.5 with Ultra-Turrax T25. The obtained homogenate was centrifuged at 15,000*g* for 15 min. The supernatant was decanted and used as enzyme preparation for the following incubations immediately. The enzyme preparation (20 ml) was incubated with 1 mg of linoleic acid, 13-HPOD or 3-oxa-13-HPOT 40 min at the room temperature.

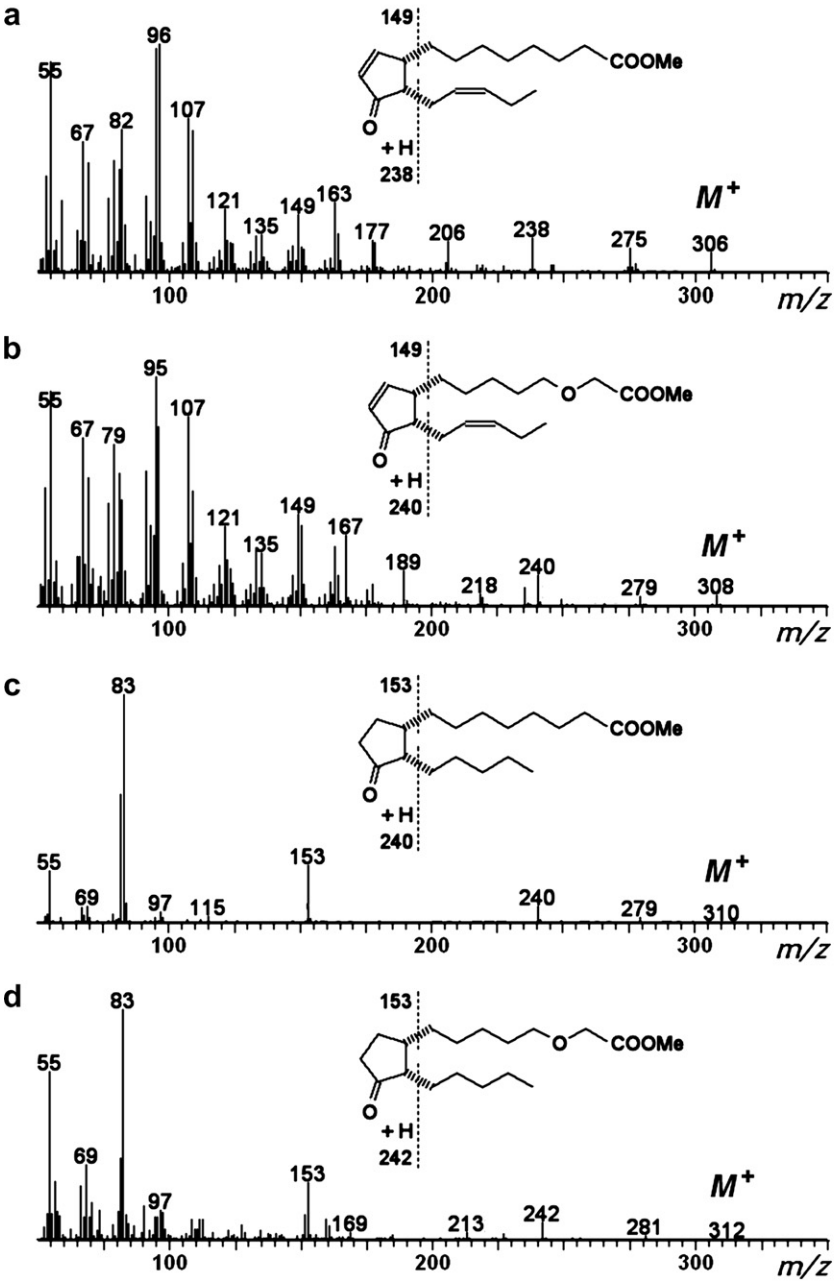

Fig. 4. Mass spectra and fragmentation schemes (inserts) for compounds **3a** (a); **6a** (b); **8** (c) and **9** (d). Compounds **8** and **9** are the products of catalytic hydrogenation of products **3a** and **6a**, respectively.

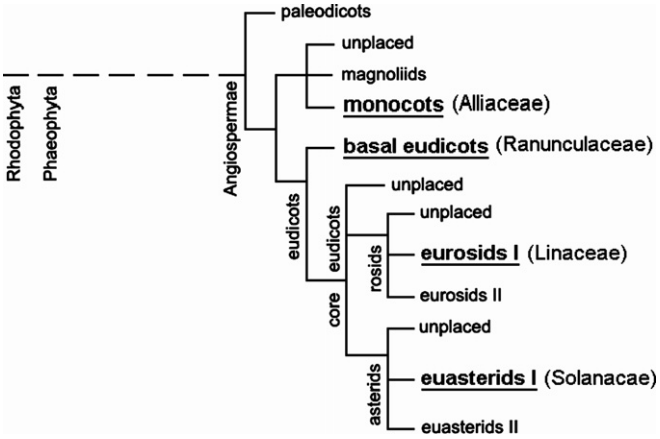

Fig. 5. Positions of taxons possessing DESs (marked with bold underlined font) on the plant phylogenetic tree. Names of families possessing DESs are specified on the right.

4.3. Extraction, preliminary purification and derivatization of products

The incubation mixture was acidified with acetic acid to pH 6 and triply extracted with hexane – EtOAc 1:1 (by volume). Acidic lipids were separated and purified for analyses using the Supelclean LC–NH_2 (3 ml) cartridges (Supelco, Bellefonte, PA, USA) as described before (Grechkin et al., 2007) and 1 ml silica cartridges (obtained from the same source). The lipids were loaded on silica cartridges and eluted from them with the solvent mixture hexane – Me_2CO – AcOH 90:10:0.1 (by volume). After the solvent evaporation the products were methylated with CH_2N_2. Alternatively, (when specified) the products were successively (1) reduced with sodium borohydride, (2) methylated with CH_2N_2, (3) the methyl esters of products were trimethylsilylated by treatment with silylating mixture consisting of pyridine – hexamethyldisilazane – trimethylchlorosilane 2:1:2 (by volume).

4.4. Analyses and separations of products

The methyl esters of products (or their hydrogenated methyl ester TMSi derivatives) were subjected to direct GC–MS analyses after the preliminary cartridge purification and derivatization.

Alternatively, the methyl esters of products were preliminarily separated by normal phase HPLC on two serially connected Separon SIX columns (150 × 3.2 mm; 5 μm) eluted with hexane – Et_2O 99.4:0.6 (by volume), flow rate 0.6 ml/min.

4.5. Spectral studies

UV spectra of isolated products were recorded with Perkin Elmer Lambda 25 spectrophotometer. Alternatively, the UV spectra of oxylipins were recorded on line during the HPLC separations using an SPD-M20A diode array detector (Shimadzu). GC–MS analyses were performed using a Shimadzu QP5050A mass spectrometer connected to Shimadzu GC-17A gas chromatograph equipped with an MDN-5 S (5% phenyl 95% methylpolysiloxane) fused capillary column (length, 30 m; ID 0.25 mm; film thickness, 0.25 μm). Helium at a flow rate of 30 cm/s was used as the carrier gas. Injections were made in the split mode using an initial column temperature of 120 °C. The temperature was raised at 10 °C/min until 240 °C. Full scan or selected ion monitoring (SIM) analyses were both performed using ionization energy of 70 eV. The ^1H NMR and 2D–COSY spectra of purified compounds were recorded with Bruker Avance 400 instrument, 400 MHz, C^2H_3CN, 296 K.

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