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

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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.

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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.

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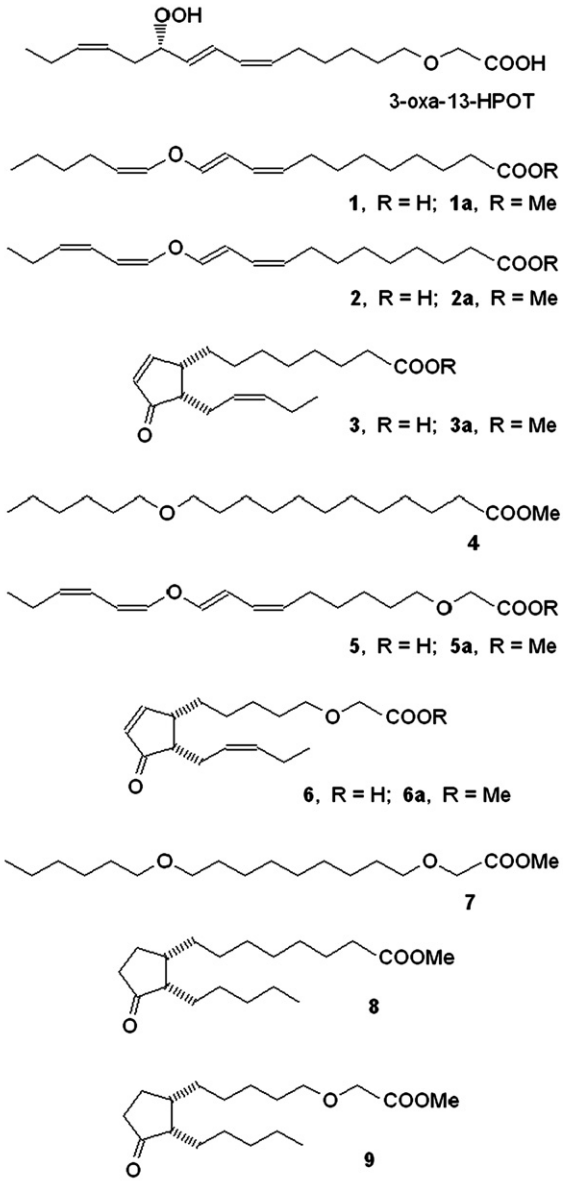

hydro(pero)xy fatty acids, compounds **1a**, **2a** and **3a** were detected during the GC–MS analyses of the extracted oxylipins. The same oxylipins were detected after incubations with 13-HPOD, as described below.

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-

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