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Hexadecanoid Pathway in Plants: Lipoxygenase Dioxygenation of (7*Z*,10*Z*,13*Z*)-Hexadecatrienoic Acid

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Abstract—7,10,13-Hexadecatrienoic acid (16:3) is abundant in many plant species. However, its metabolism through the lipoxygenase pathway is not sufficiently understood. The goal of present work was to investigate the oxygenation of 16:3 by different plant lipoxygenases and to study the occurrence of oxygenated derivatives of 16:3 in plant seedlings. The recombinant maize 9-lipoxygenase specifically converted 16:3 into (7*S*)-hydroperoxide. Identification of this novel oxylipin was substantiated by data of GC-MS, LC-MS/MS, ^{1}H-NMR, and 2D-COSY as well as by deuterium labeling from [$^2H_6$]16:3. Soybean lipoxygenase 1 produced 91% (11*S*)-hydroperoxide and 6% racemic 14-hydroperoxide. Recombinant soybean lipoxygenase 2 (specifically oxidizing linoleate into 13-hydroperoxide) lacked any specificity towards 16:3. Lipoxygenase 2 produced 7-, 8-, 10-, 11-, 13-, and 14-hydroperoxides of 16:3, as well as a significant amount of bis-allylic 9-hydroperoxide. Seedlings of several examined plant species possessed free hydroxy derivatives of 16:3 (HHTs), as well as their ethyl esters. Interestingly, HHTs occur not only in "16:3 plants", but also in typical "18:3 plants" like pea and soybean seedlings.

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Metabolism of polyenoic fatty acids through the plant lipoxygenase cascade is the source of numerous oxylipins [1, 2]. Many of them are involved in plant cell signaling and plant defense. The most abundant polyenoic fatty acids of higher plants, α-linolenic and linoleic acids, are the major lipoxygenase substrates and oxylipin precursors. Many plant species which are called "16:3-plants" possess another trienoic acid, namely (7*Z*,10*Z*,13*Z*)-hexadecatrienoic acid (16:3) [3, 4]. As reported previously, the hexadecatrienoic acid is converted in some plants through the lipoxygenase pathway into the jasmonate congener dinor-12-oxo-10,15-phytodienoic acid [5], as well as into the divinyl ether dinor-(ω5*Z*)-etherolenic acid [6]. At the same time, the regio- and stereospecificity of 16:3 dioxygenation by lipoxygenases has not yet been investigated.

Studying the properties of a recombinant maize 9-lipoxygenase (ZmLOX), we found that this enzyme does oxidize 16:3 efficiently and specifically into the corresponding (7*S*)-hydroperoxide, which has not been described previously. Here we report these results on the detection and identification of a new oxylipin, (7*S*)-hydroperoxide of 16:3, on the specificity of 16:3 oxygenation by some plant lipoxygenases, as well as on the detection of hydroxy derivatives of 16:3 and their ethyl esters in some plant species.

Abbreviations: CP-HPLC, chiral phase HPLC; GC-MS, gas chromatography–mass spectrometry; GmLOX-1, soybean lipoxygenase-1; GmLOX-2, soybean lipoxygenase-2; 7-H(P)HT, (8*E*,10*Z*,13*Z*)-7-hydro(pero)xy-8,10,13-hexadecatrienoic acid; H(P)HT, hydro(pero)xyhexadecatrienoic acid; H(P)OD, hydro(pero)xide of linoleic acid; H(P)OT, hydro(pero)xide of α-linolenic acid; NP-HPLC, normal phase HPLC; ZmLOX, maize 9-lipoxygenase; 16:3, (7*Z*,10*Z*,13*Z*)-7,10,13-hexadecatrienoic acid; 18:3, α-linolenic acid.

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MATERIALS AND METHODS

Materials. Unlabeled (7*Z*,10*Z*,13*Z*)-hexadecatrienoic acid and deuterium labeled [7,8,10,11,13,14-

$^2H_6$](7*Z*,10*Z*,13*Z*)-hexadecatrienoic acid were generously provided by Larodan (Sweden). Linoleic, α-linolenic, and arachidonic acids, soybean lipoxygenase-1 (type V; GmLOX-1), and isopropyl β-D-thiogalactoside were from Sigma (USA). Oligonucleotides were obtained from Lytech (Russia). Glycerol stocks of *E. coli* strains were obtained from Novagen (USA). *Pfu* polymerase and restriction enzyme *Xho*I were purchased from Fermentas (Lithuania).

Expression of recombinant ZmLOX and GmLOX-2. The expression vector carrying the full length ZmLOX cDNA [7] (GenBank GeneID: 542495) was generously provided by Dr. N. Keller and Dr. M. Kolomiets. Vector pUC3C76 carrying the full-length soybean lipoxygenase-2 (GmLOX-2) cDNA (GenBank GeneID: 547774) [8] was generously provided by Prof. D. Shibata and Dr. T. Nagaya. The *GmLOX-2* gene was subcloned in the improved expression vector. Two gene-specific primers were synthesized corresponding to the open reading frame of the cDNA. *Xho*I restriction sites were incorporated at the 5′-ends to facilitate subcloning. The sequence of the upstream primer was 5′-CTCGAGTTAGATAGAGATGCTATTAGG-3′, and that of the downstream primer 5′-CTCGAGATGTTTTCAGTTCCAGGGG-3′ (restriction site is underlined).

Lipoxygenase-2 cDNA was amplifed by PCR from pUC3C76 using *Pfu* polymerase. The PCR product was digested with *Xho*I and subcloned into the same site of the expression vector pET15-b (Novagen). The resulting construct pETL-2 containing an additional 23 amino acid sequence (MGSSHHHHHHSSGLVPRGSHMLG) at the N-terminus of GmLOX-2 was transformed into *E. coli* host strain BL21(DE3)pLysS followed by ampicillin selection (as described below). Selected clones were controlled by DNA sequence analysis. Clone pETL-2, carrying the *GmLOX-2* gene, having the sequence published previously by Shibata et al. [8], was used for protein expression.

Recombinant genes ZmLOX and soybean lipoxygenase 2 (GmLOX-2) were expressed in *E. coli* strain Rosetta(DE3)pLysS. The culture was induced at A_{590} of 0.6 by isopropyl β-D-thiogalactoside addition to final concentration of 0.1 mM. Induced cultures were incubated for 24 h at 18°C in a shaker at 160 rpm. Cells were collected by centrifugation. The pellets were suspended in 10 ml 50 mM phosphate buffer, pH 7.0, containing 100 mM NaCl and 0.1% Triton X-100. The cells were disrupted with a French press (20,000 psi), and then the suspension was clarified by centrifugation (12,500*g*). The recombinant protein was purified by three steps: precipitation with $(NH_4)_2SO_4$ (20-50% saturation) and by anion-exchange and hydrophobic chromatography as described in [9]. The expression and purification of recombinant protein was monitored by SDS-polyacrylamide 12% gel electrophoresis.

Kinetics of lipoxygenase oxidation. The standard assay mixture (0.7 ml) containing 0.3 mM 16:3, 18:3, linoleate, or arachidonate in 0.1 M sodium phosphate buffer (pH 7.0) was preliminarily saturated with oxygen for 3 min. The reaction was started by addition of lipoxygenase preparation (1 nkat) and proceeded for 40 sec at 23°C. Enzymatic activity was determined by monitoring the increase of absorbance at 234 nm on a Cary 50 Bio UV/VIS spectrophotometer (Varian, USA). Rates of enzymatic reaction were calculated from the initial linear part of the curve. Five independent experiments were performed for each specified variant. The mean values and standard deviations are given.

Incubation and extraction of products. ZmLOX (20 nkat) was incubated with 0.5 mg of either linoleic acid, 18:3, 16:3, or [7,8,10,11,13,14-$^2H_6$]16:3 in 10 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 23°C for 20 min under continuous oxygen bubbling and stirring. GmLOX-1 and GmLOX-2 were incubated with fatty acids under the same conditions and in the same proportions in 0.1 M sodium phosphate buffer (pH 9.0 and 6.5, respectively). Incubations were terminated by the addition of glacial acetic acid to pH 5.0 followed by triple extraction with 10 ml of hexane–ethyl acetate (1 : 1 v/v). The lipoxygenase products were methylated with diazomethane, and the methyl esters were analyzed by NP-HPLC as described below. Alternatively, the product methyl ester TMS derivatives or fully reduced hydrogenated methyl ester TMS derivatives were analyzed by GC-MS.

Extraction of oxylipins from plant tissues. Leaves of pea, soybean, or sunflower seedlings or roots of cabbage, potato, or sunflower seedlings (5 g) were dispersed in 10 ml cold (4°C) 50 mM Tris-HCl buffer (pH 7.5) with an Ultra-Turrax T25. The homogenate was centrifuged at 15,000*g* for 15 min. The supernatant was decanted, acidified with acetic acid to pH 6.0, and extracted thrice with 15 ml of hexane–ethyl acetate (1 : 1 v/v).

Derivatization procedure. After solvent evaporation, the dry residues were dissolved in methanol and reduced with sodium borohydride. The reduced free fatty acids were methylated with diazomethane. The methyl esters were analyzed by NP-HPLC and CP-HPLC. Alternatively, the methyl esters were catalytically hydrogenated over PtO_2 as described before [10, 11]. Hydrogenated samples were silylated with pyridine–hexamethyldisilazane–trimethylchlorosilane (2 : 1 : 2 v/v) as described before [10, 11]. The resulting TMS ether/methyl ester derivatives were analyzed by GC-MS.

HPLC analysis of products. The lipoxygenase products (free acids) were separated by NP-HPLC on two successively connected Separon SIX columns (5 μm; 3.2 × 150 mm; Tessek, Czech Republic) under isocratic conditions using the solvent mixture hexane–propan-2-ol–acetic acid (98.4 : 1.5 : 0.1 v/v) at flow rate of 0.4 ml/min. Peaks of separate products collected after NP-HPLC were methylated with diazomethane. The methyl esters were subjected to CP-HPLC analysis on a

Chiralcel OD-H column (5 μm; 4.6 × 250 mm; Daicel Chemical Industries, France) under isocratic conditions using the solvent mixture hexane–isopropanol (97 : 3 v/v) at flow rate of 0.4 ml/min. UV spectra of compounds purified by HPLC were recorded online with an SPD-M20A diode array detector (Shimadzu, Japan).

Spectral studies. GC-MS analyses were performed using a Shimadzu QP5050A mass spectrometer connected to Shimadzu GC-17A gas chromatograph equipped with an MDN-5S (5% phenyl, 95% methylpolysiloxane) fused silica capillary column (length 30 m, ID 0.25 mm, film thickness 0.25 μm). Helium at flow rate of 30 cm/sec 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 to 240°C. Full scan or selected ion monitoring (SIM) analyses were both performed using ionization energy of 70 eV. The ^{1}H-NMR and 2D-COSY spectra of purified compounds were recorded with a Bruker Avance 400 instrument, 400 MHz, C^2H_3CN, 296 K.

RESULTS AND DISCUSSION

The recombinant ZmLOX possessed high activity towards linoleic acid. The oxidation was regiospecific. Analyses of products as free acids by normal phase HPLC and as hydrogenated methyl esters/TMS derivatives by GC-MS (data not illustrated) revealed that the predominant hydroperoxide formed was 9-HPOD, in full agreement with previously published data [7]. Its analysis by chiral phase HPLC (not illustrated) revealed that it was present predominantly as the (9*S*)-enantiomer (93%).

ZmLOX possessed lesser regiospecificity during oxidation of α-linolenic acid. The predominant product was 9-HPOT (75%) having mostly the (9*S*)-configuration (97%).

In contrast to linoleic and α-linolenic acids, arachidonic acid was a completely inefficient substrate for ZmLOX.

Testing of 16:3 revealed that this was also a good substrate for ZmLOX. The enzyme dioxygenated 16:3 11.5- and 12-fold slower (5.3 ± 0.2 nmol/min) than 18:2 (61 ± 1 nmol/min) and 18:3 (66 ± 3 nmol/min). The NP-HPLC (Fig. 1a) and GC-MS (Fig. 1c) analyses of products revealed the formation of a single predominant (93% of total hydroperoxides) product, compound **1**. Its identification is described below.

The GC-MS analyses of TMS-derivatives of methyl esters of $NaBH_4$ reduced products revealed a single predominant peak **1a**. Its mass spectrum (Fig. 2a) possessed M^+ at *m/z* 352 and two predominant fragment ions at *m/z* 283 and 223. This indicated the structure of 7- or 11-hydroxy derivative of 16:3. To confirm the structure and reveal the exact position of the hydroxyl group, we analyzed the TMS-derivatives of methyl esters of $NaBH_4$ reduced and fully hydrogenated products using GC-MS. These analyses revealed the presence of a single predominant product (**1b**). The electron impact mass spectral data and mass fragmentation patterns (Fig. 2b) enabled us to identify compound **1b** as the TMS-derivative of 7-hydroxyhexadecanoic acid methyl ester (Me/TMS). Minorities of 11-hydroxy and other regioisomers were either small or undetectable. Thus, the oxidation displayed high regiospecificity. These results also suggested that 7-hydroperoxide of hexadecatrienoic acid was the primary oxidation product.

The GC-MS analysis of derivatives of products formed after the incubation of [7,8,10,11,13,14-$^2H_6$]16:3 with ZmLOX revealed the incorporation of deuterium atoms into compound **1b** (Fig. 2c). The fragmentation patterns of deuterium labeled compound **1b** (Fig. 2b) provide additional structural confirmation.

The native (non-reduced) products of 16:3 oxygenation by ZmLOX were separated by normal phase HPLC (Fig. 1a). The major product, compound **1**, was collected for further structural studies, including steric analyses and ^{1}H-NMR. The NMR data for compound **1** are presented

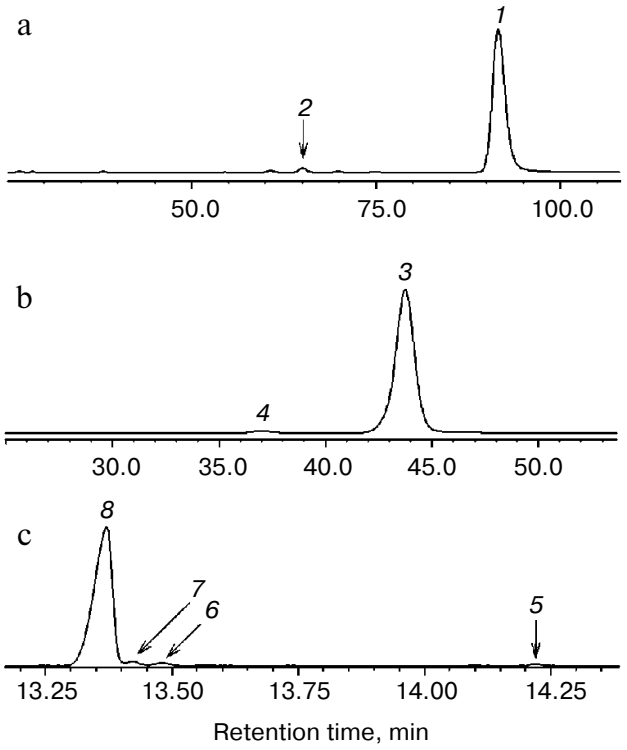

Fig. 1. Chromatographic analyses of products of 16:3 oxidation by ZmLOX. a) Analysis of 16:3 oxidation products (free acids) by normal phase HPLC. b) Enantiomeric analysis of compound 1 methyl ester (peak 1 collected during the normal phase separation) by chiral phase HPLC. c) GS-MS analyses of products of 16:3 oxidation by ZmLOX (as methyl esters/TMS derivatives of saturated hydroxy fatty acids). *1*) 7-HPHT; *2*) 11-HPHT; *3*) 7(S)-HPHT; *4*) 7(R)-HPHT; *5*) 14-HPHT; *6*) 11-HPHT; *7*) 10-HPHT; *8*) 7-HPHT.

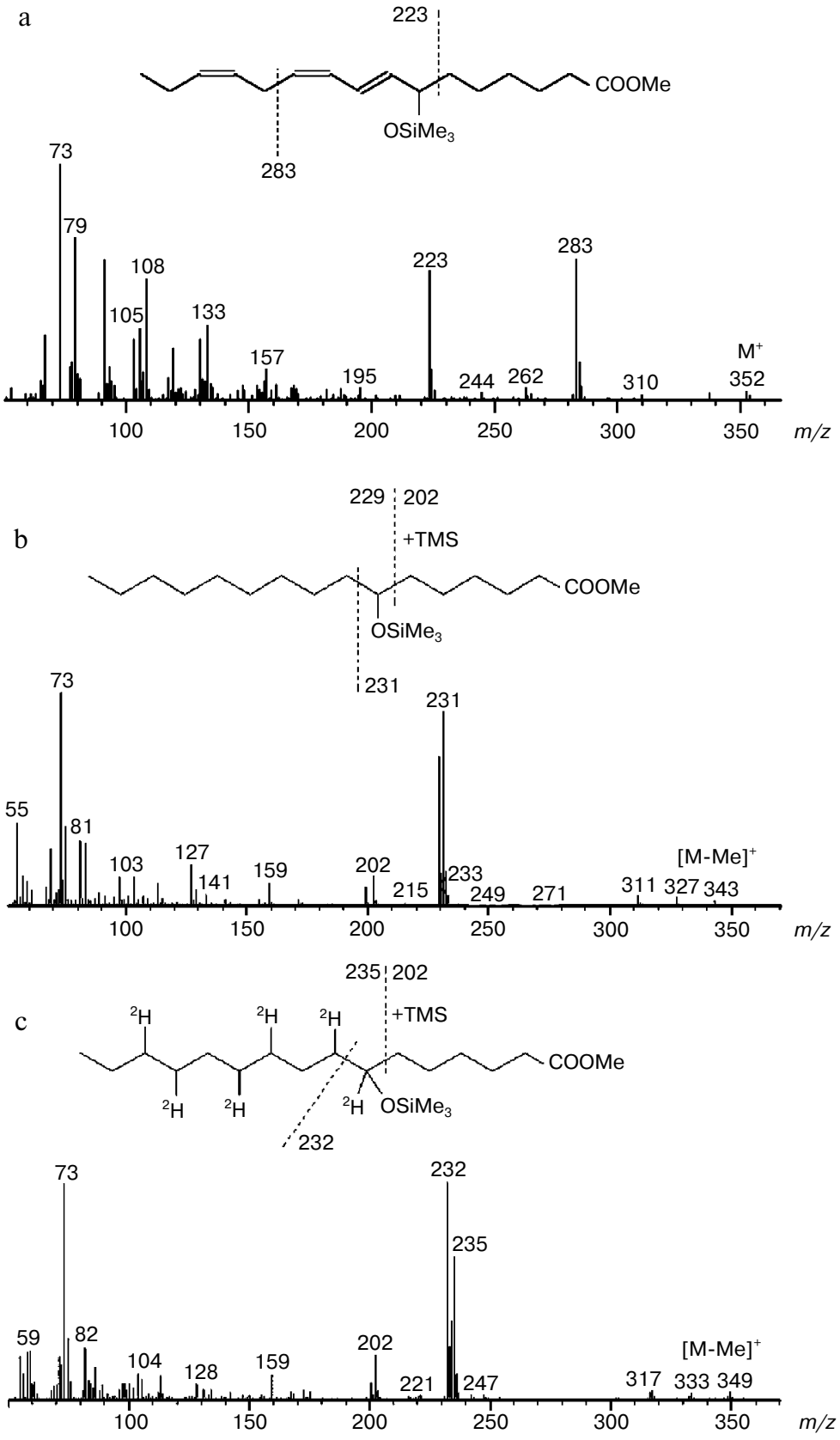

Fig. 2. Electron impact mass spectra of unlabeled compound **1** (a) and unlabeled (b) and deuterium-labeled (c) compound **1** derivatives.

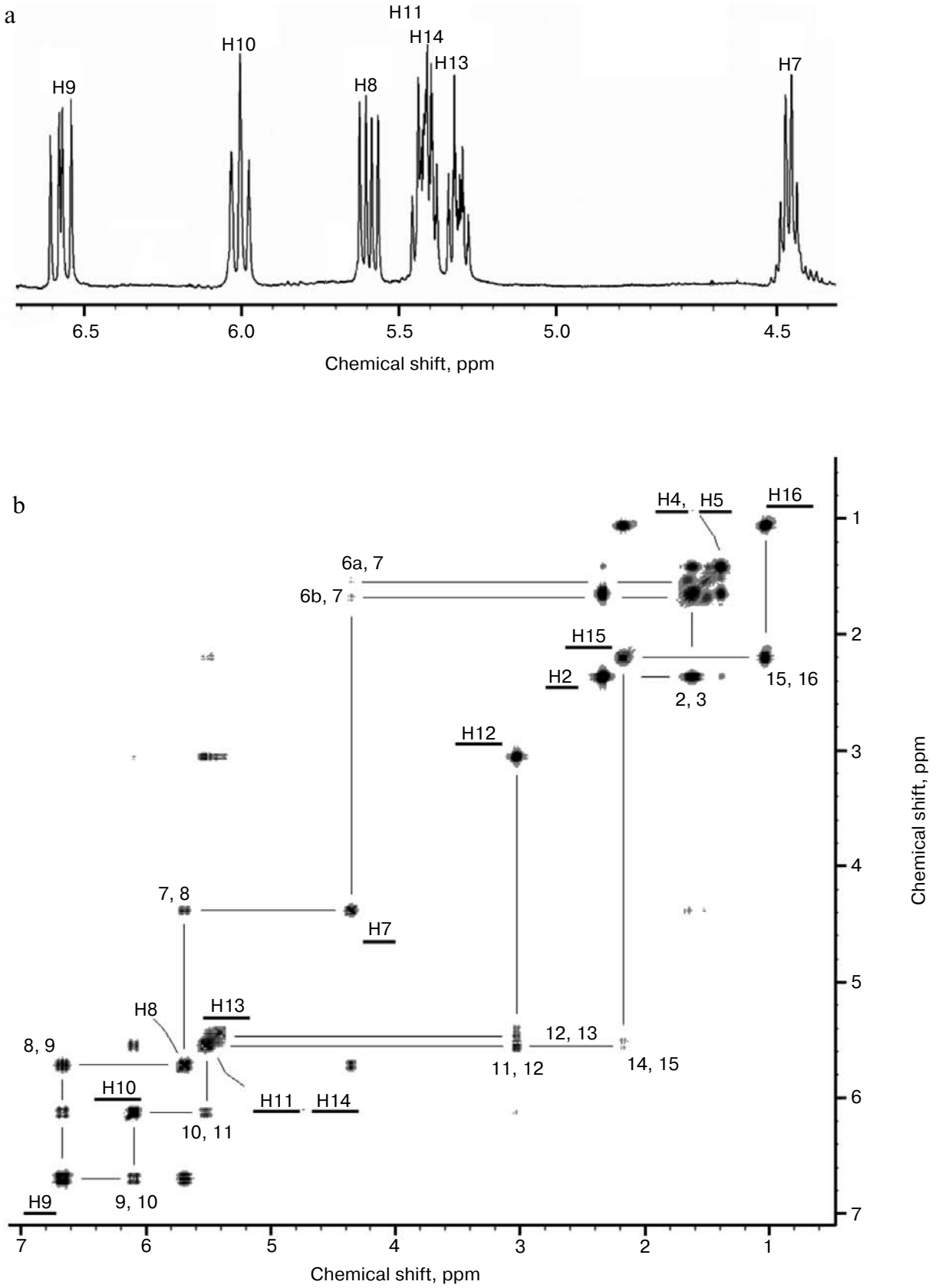

Fig. 3. ^{1}H-NMR data (400 MHz, $C^{2}H_{3}CN$, 296 K) for 7-HPHT. a) Downfield region of compound **1** ^{1}H-NMR spectrum; b) 2D-COSY plot for compound **1**.

in Fig. 3 and Table 1. Attribution of all signals is confirmed by 2D-COSY data (Fig. 3b). The chemical shift of H7 (4.25 ppm) and the signal multiplicity (*dt*) suggests that this methine is bound to a hydroperoxy group (Fig. 3a and Table 1). This methine signal is relatively upfield shifted as compared to typical chemical shifts (about 4.1 ppm) of hydroxy fatty acids like 9-HOT. Furthermore, these signals of hydroxy acids have a more complex multiplicity due to the additional spin coupling with the OH proton, which is absent in the case of hydroperoxides. The olefinic part of the spectrum shows the presence of three double bonds. Taken together, the MS and NMR data reveal the structure of (8*E*,10*Z*,13*Z*)-7-hydroperoxy-8,10,13-hexadecatrienoic acid (7-HPHT). The steric analysis of 7-HHT methyl ester (prepared through the sodium borohydride reduction of 7-HPHT followed by diazomethane methylation of the reduction product) by chiral phase HPLC (Fig. 1b) revealed the predominance (95%) of a single enantiomer, (7*S*)-HPHT. This indicates that ZmLOX oxygenates 16:3 stereospecifically.

Soybean lipoxygenase-1 (GmLOX-1) is known as a specific 13-lipoxygenase. This enzyme specifically forms (13*S*)-hydroperoxides when it is incubated with linoleic or α-linolenic acid. Our results (Table 2) showed that soy-

Table 1. ^{1}H-NMR spectral data for compound **1** (400 MHz, $C^{2}H_{3}CN$, 296 K)

Proton	Chemical shift (δ), ppm	Multiplicity (number of protons)	Coupling constants, Hz
H2	2.25	*t* (2)	7.5 (H3)
H3	1.45-1.60	*m* (2)	
H4	1.24-1.36	*m* (2)	
H5	1.24-1.36	*m* (2)	
H6a	1.34-1.46	*m* (1)	
H6b	1.50-1.63	*m* (1)	
H7	4.26	*dt* (1)	8.0 (H8); 6.4 (H6)
H8	5.50	*dd* (1)	15.2 (H9); 8.0
H9	6.58	*dddd* (1)	15.2; 11.2 (H10); 0.9 (H7); 0.9 (H11)
H10	6.00	*dddt* (1)	11.2; 10.7 (H11); 0.7 (H8); 1.6 (H12)
H11	5.43	*dt* (1)	10.7; 7.6 (H12)
H12	2.93	*t* (2)	7.6
H13	5.31	*dtt* (1)	10.7 (H14); 7.3 (H12); 1.4 (H15)
H14	5.41	*dtt* (1)	10.7; 7.2 (H15); 1.3 (H12)
H15	2.08	*dq* (2)	7.5 (H16)
H16	0.94	*t* (3)	7.5

Table 2. Specificity of 16:3 oxidation by selected plant lipoxygenases

Lipoxygenase	Positional isomers of fully reduced/hydrogenated 16:3 hydro(pero)xides (Me/TMS), % of total hydro(pero)xide products; masses (*m/z*) of (SIC) used for quantification						
	7-H(P)HT; *m/z* 229, 231	8-H(P)HT; *m/z* 215, 245	9-H(P)HT; *m/z* 201, 259	10-H(P)HT; *m/z* 187, 273	11-H(P)HT; *m/z* 173, 287	13-H(P)HT; *m/z* 145, 315	14-H(P)HT; *m/z* 131, 329
GmLOX-1	1.7	n.d.	0.3	0.8	90.6	0.4	6.2
GmLOX-2	21.5	12.3	5.2	12.9	14.5	14.0	19.6
ZmLOX	93.3	0.2	n.d.	2.5	3.5	0.1	0.5

Note: Methyl esters/TMS derivatives of fully reduced and hydrogenated products were analyzed by GC-MS. Full details of incubations, extraction, derivatization and analyses are described in "Materials and Methods". The products were quantified by integration of the selected ion chromatograms as described in "Materials and Methods"; n.d., not detected (below 0.1%). Selected ion chromatograms (two characteristic ions per regioisomer; *m/z* specified above in the table) were used for quantification. The sum of areas of two peaks of selected ions (for example, ions at *m/z* 229 and 231 for 7-H(P)HT derivative) was assumed to be proportional to the quantity of derivative of corresponding H(P)HT regioisomer.

bean GmLOX-1 specifically oxygenated 16:3 to 11-HPHT. Steric analysis of purified 11-HPHT demonstrated that it has predominantly (90%) (11*S*)-configuration.

GmLOX-2, as well as soybean GmLOX-1, exhibits the activity of specific 13-lipoxygenase when it is incubated with linoleic or α-linolenic acids [9]. On the other hand, GmLOX-2 lacked any regiospecificity towards the 16:3 (Table 2). It produced equal amounts of 7-, 8-, 10-, 11-, 13-, and 14-hydroperoxides of 16:3. The bis-allylic 9-HPHT was also a major product (Table 2), about 5% of total 16:3 hydroperoxides.

To summarize, the data show that ZmLOX, GmLOX-1, and GmLOX-2 are active toward 16:3. ZmLOX and GmLOX-1 unlike GmLOX-2 oxidized 16:3 regio- and stereospecifically. These results are interesting in connection with the hypothetical pathways of hexadecanoid biosynthesis. To examine how specific hexadecanoid formation could be *in vivo*, we tested the presence of HHT isomers in some plant species. The results are described in the next section.

Previously, esterified (11*S*)-HHT and 7-HHT (the enantiomeric composition of the latter was not studied) were detected in complex lipids of *Arabidopsis* [12, 13]. Studying the GC-MS oxylipin profiles, we detected isomeric HHTs in a number of different plant species. Free 7-HHT, 9-HHT, and 11-HHT are present in the roots of cabbage (*Brassica oleraceae*) (Fig. 4, a and b) and potato (not illustrated), which are "16:3 plants". Potato roots possess also 8, 10-, 12-, 13-, and 14-HHTs. The complexity of potato HHT isomer patterns indicates that these are largely the 16:3 autoxidation products. Cabbage roots possessed relatively simple HHT patterns with predominance of 7-HHT and bis-allylic 9-HHT. Prominent 9-HHT is an unusual detail of hexadecanoid profile in cabbage and potato. One cannot exclude that 9-HHT is formed through the autoxidation of 16:3. Previously, the formation of bis-allylic 11-HPOD was observed during the autooxidation of 18:2 [14]. The ordinary iron lipoxygenases produce no or extremely little amount of 11-HPOD [15]. The only known exception is the fungal manganese lipoxygenase producing bis-allylic 11-HPOD from linolenic acid [16]. One also cannot exclude that 9-HHT can be formed enzymatically by some plant lipoxygenases. However, we observed only traces of 9-HHT after the *in vitro* oxidation of 16:3 by plant lipoxygenases.

The presence of hexadecanoids in "16:3 plants" is not surprising. On the other hand, their detection in "18:3 plants" seemed to be fully unexpected. Nevertheless, we detected HHTs in a number of tested "18:3 plants". The detection of HHTs in soybean and pea (*Pisum sativum*) leaves is illustrated in Fig. 4, c and d. The leaves of both species contained 11-HHT, as well as lesser amount of other HHT isomers including 8-, 9-, 10-, and 14-HHTs. Along with soybean and pea, HHTs were detected in seedlings of sunflower. HHTs can be formed in 18:3 plants in two alternative ways: either through the β-oxidation of HPOD or though the β-oxidation of α-linolenic acid to 16:3 and its subsequent lipoxygenase dioxygenation.

We detected HHT ethyl esters in some plant species. To our knowledge, the ethyl esters of oxylipins have not been described before as naturally occurring plant constituents. For example, cabbage roots contain 7-HHT ethyl ester (Fig. 4, a and b). The ethyl esters are often more abundant than free hexadecanoids. So, the estimated ratio of 7-HHT ethyl ester to free 7-HHT in cabbage roots was about 20 : 1. In the other examined "16:3 plant", potato tubers lacked any detectable HHT ethyl esters. The ethyl esters of 7- and 11-HHTs were also detected in pea and soybean leaves and roots (Fig. 4, c and d).

The results of the present work demonstrate that 16:3 can serve as an efficient lipoxygenase substrate. Both soybean GmLOX-1 and maize ZmLOX possessed high regio- and stereospecificity, oxidizing 16:3 predominantly to (11*S*)-HPHT and (7*S*)-HPHT, respectively. Soybean GmLOX-2 oxidizing linoleate specifically to (13*S*)-HPOD [9] showed no specificity towards 16:3 and produced a number of stereoisomers.

Previously, Montillet et al. [12] detected esterified 11-HHT and 7-HHT in *Arabidopsis* leaves. The 11-HHT had mostly (11*S*) configuration, while the 7-HHT configuration was not revealed [12]. The authors noted that the markers of 16:3 autoxidation, 10-HHT and 14-HHT, could not be quantified by the negative ion mode electrospray LC-MS due to insufficient sensitivity. The results of the present work show that highly sensitive quantification of all regioisomers is routinely feasible when the sodium borohydride reduced fully hydrogenated oxylipins are analyzed by electron impact GC-MS. Our results demonstrate that the relative abundance of lipoxygenase products (7-HHT and 11-HHT) and autoxidation markers (8-HHT, 10-HHT, and 14-HHT) vary significantly depending on plant species and age. Notably, some of these autoxidation markers can be produced by lipoxygenases. For instance, pure soybean lipoxygenase converts 16:3 into 14-HPHT (6%) along with the predominant 11-HPHT.

To our knowledge, free HHTs have not been detected in plants before. Detection of free hexadecanoids in 18:3 plants is especially surprising. Free HHTs have little abundance as compared to HOTs (hydroxy derivatives of octadecatrienoic acid). It is not clear whether HHTs of 18:3 plants are only occasional β-oxidation products or that they have some physiological significance. We paid attention to the hypothetical shorter chain C_{14} and C_{12} β-oxidation products, but they were not detectable (in contrast to HHTs). This suggests that HHTs are not just short-lived intermediates of β-oxidation. Moreover, different plant species contain the ethyl esters of HHTs, which are more abundant than free HHTs. In contrast, octadecanoid ethyl esters are not detectable.

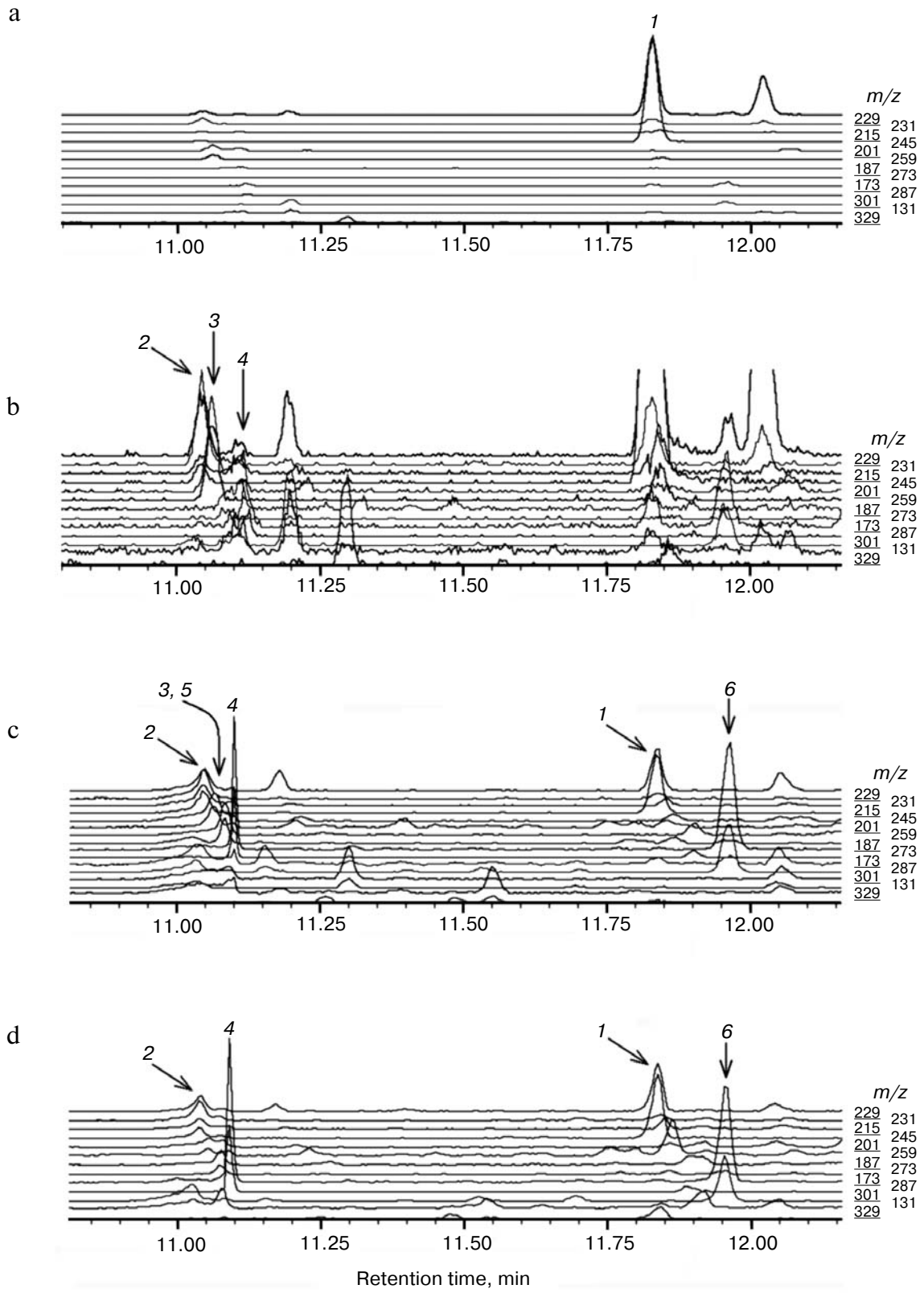

Fig. 4. Detection of hexadecanoids (HHTs and their ethyl esters) in some plants. The data are presented as GC-MS selected ion chromatograms. Masses (*m/z*) of selected ions are specified at the right side. a) Cabbage (*Brassica oleraceae*) roots; b) the same chromatograms with expanded Y-scale; c) soybean leaves; d) pea (*Pisum sativum*) leaves. *1*) TMS-derivative of ethyl ester of endogenous 7-HHT; *2*) 7-HHT; *3*) 9-HHT; *4*) 11-HHT; *5*) 10-HHT; *6*) TMS-derivatives of ethyl esters of endogenous 11-HHT.

Efficient and specific oxidation of 16:3 by lipoxygenases indicates that this polyenoic acid can be a precursor of an alternative lipoxygenase route, the hexadecanoid cascade. Two hexadacanoids formed though the 11-HPHT, dinor-12-oxo-10,15-phytodienoic acid [5] and dinor-(ω5*Z*)-etherolenic acid [6], were described before. Possible pathways of 7-HPHT metabolism by CYP74 family enzymes to the variety of hexadecanoid oxylipins remain to be explored. For instance, the metabolism of 7-HPHT by CYP74 enzymes is of interest. Further research in this direction is under way in our laboratory.

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