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Allene Oxide Synthase Pathway in Cereal Roots: Detection of Novel Oxylipin Graminoxins

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Young roots of wheat, barley, and sorghum, as well as methyl jasmonate pretreated rice seedlings, undergo an unprecedented allene oxide synthase pathway targeted to previously unknown oxylipins **1**–**3**. These Favorskii-type products, (4*Z*)-2-pentyl-4-tridecene-1,13-dioic acid (**1**), (2′*Z*)-2-(2′-octenyl)-decane-1,10-dioic acid (**2**), and (2′*Z*,5′*Z*)-2-(2′,5′-octadienyl)-decane-1,10-dioic acid (**3**), have a carboxy function at the side chain, as revealed by their MS and NMR spectral data. Compounds **1**–**3** were the major oxylipins detected, along with the related α-ketols. Products **1**–**3** were biosynthesized from (9*Z*,11*E*,13*S*)-13-hydroperoxy-9,11-octadecadienoic acid, (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD), and (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoic acid, respectively, via the corresponding allene oxides and cyclopropanones. The data indicate that conversion of the allene oxide into the cyclopropanone is controlled by soluble cyclase. The short-lived cyclopropanones are hydrolyzed to products **1**–**3**. The collective name "graminoxins" has been ascribed to oxylipins **1**–**3**.

1. Introduction

Oxylipins constitute a large and diverse family of natural products derived from the oxygenation of unsaturated fatty acids.[1] Plant oxylipins are biosynthesized primarily through the lipoxygenase oxidation of linoleic and α-linolenic acids and subsequent conversion of fatty acid hydroperoxides by several enzymes of the CYP74 family,[2,3] a kind of nonclassical P450.[4] Three enzymes of this family are known in flowering plants: allene oxide synthase (AOS), hydroperoxides lyase (HPL), and divinyl ether synthase (DES).[5,6]

AOSs (CYP74A and CYP74C subfamilies) are crucial enzymes that are widespread in land plants. AOSs catalyze the dehydration of fatty-acid hydroperoxides to short-lived allene oxides, which in turn undergo enzymatic or spontaneous cyclization to cyclopentenones.[6] Cyclopentenones themselves, as well as their jasmonate metabolites, play important physiological roles in plants.[3] The second competing way to convert allene oxides is hydrolysis, predominantly to α-ketols. A minor novel AOS product, accompanying the formation of cyclopentenone 12-oxo-10,15-phytodienoic acid, was detected recently.[7] This new oxylipin is a branched-chain dicarboxylic acid, formed from the 13-hydroperoxide of α-linolenic acid 13(*S*)-hydroperoxide via allene oxide and cyclopropanone intermediates. Nucleophilic opening of the cyclopropanone ring corresponds to the last stage of the Favorskii rearrangement.[7]

Studying the metabolism of linoleic and α-linoleic acids and their hydroperoxides in the roots of cereals, we made an unexpected observation. Prominent unknown oxylipins, similar but not identical to the previously described Favorskii-type products[7] but biosynthesized from linoleic acid hydroperoxides, belong to the main products of the AOS pathway in some cereal roots. The present paper reports the detection of these new products and their biosynthesis pathway via unstable cyclopropanone intermediates.

2. Results

2.1. Oxylipin in the Roots of some *Poaceae* Species: Detection of Unknown Oxylipins 1–3

The 15 000 g supernatant of the homogenate of wheat roots (5 days after germination) was incubated with linoleic acid. The GC–MS profiles of the products (Me/TMS) are presented in Figure 1. Among other oxylipins, several relatively volatile products (retention times: 5–10 min, Figure 1 a) were detected. These included 4-hydroxynonenoic acid (**11**); azelaic acid (**12**); 9-hydroxynonanoic acid (**13**); and (3*Z*)-traumatic, that is, (3*Z*)-dodecene-1,12-dioic acid (**14**). Oxylipins **11**–**14** derive by the redox conversion of aldoacids, the HPL chain-cleavage products of fatty-acid hydroperoxides.

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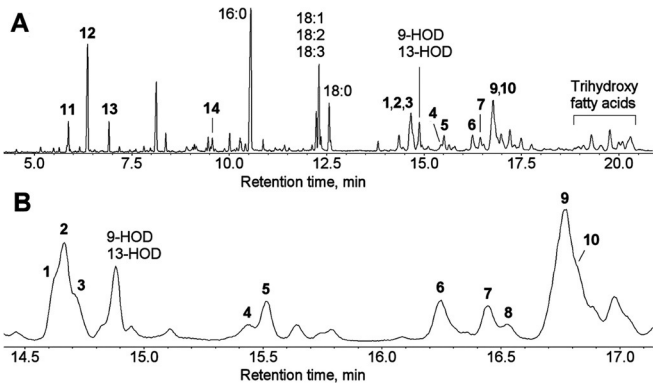

Figure 1. GC–MS (electron impact, 70 eV, total ion current) profile of oxylipins (Me/TMS) extracted after the incubation of a 15 000 g supernatant obtained from a homogenate of 5 day old wheat roots preincubated with linoleic acid. a) Full oxylipin profile. b) Expanded fragment of the same profile, corresponding to the major nonvolatile oxylipins.

Besides with the abovementioned relatively volatile HPL-related products, the following oxylipins listed below in the order of their appearance during GC–MS analysis were detected (Figure 1 b). First, unknown products **1**, **2**, and **3** (the structural formulae of these and related compounds are shown in Figure 2) appeared as three partly overlapping peaks (Figure 1 b). The mass spectra of products **1**–**3** did not correspond to any known compounds from mass-spectral libraries. The second group of peaks (Figure 1 b) included hydroxy acids, namely, (9*S*,10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid (9-HOD) and (9*Z*,11*E*,13*S*)-13-hydroxy-9,11-octadecadienoic acid (13-HOD) (Me/TMS). Then, cyclopentenones *cis*-10-oxo-11-phytoenoic acid (**4**) and *cis*-12-oxo-10-phytoenoic acid (**5**) eluted. The next two peaks corresponded to the epoxy alcohols (9*Z*)-11-hydroxy-12,13-epoxy-9-octadecenoic acid (**6**) and (9*Z*)-11-hydroxy-12,13-epoxy-9-octadecenoic acid (**7**). Then, the uncommon ketol (12*Z*)-10-oxo-11-hydroxy-12-octadecenoic acid (**8**) appeared. The α-ketols (12*Z*)-9-hydroxy-10-oxo-12-octadecenoic acid (**9**) and (9*Z*)-12-oxo-13-hydroxy-9-octadecenoic acid (**10**) eluted next. Finally, the most polar oxylipins, several trihydroxy fatty acids having retention times between about $t_R = 18.8$ and 20.5 min, eluted (Figure 1 b). It is noteworthy that products **1**–**3** were also detected during profiling of the endogenous oxylipins of wheat roots (Figure S1 a in the Supporting Information) and jasmonate-treated rice roots (Figure S1 a).

Alongside wheat roots, we studied the oxylipin profiles in the roots of barley, oat, and proso millet (*Panicum miliaceum* L.), in addition to three kinds of sorghum, Sudangrass, technical sorghum [*Sorghum vulgare* Pers. var. *technicum* (Koern.) Jáv.], and Chinese sorghum (*Sorghum chinense* Jakusch.). Oat and proso millet possessed α-ketols **9** and **10**, respectively, but lacked any of unknown products **1**, **2** and **3**. All three sorghum species possessed products **2** and **3**. The oxylipin patterns of barley roots were remarkably similar to those of wheat and also exhibited all unknown products **1**, **2**, and **3** besides α-ketols **9** and **10**. Furthermore, Favorskii product **2** was abundant in methyl jasmonate pretreated rice roots but was absent from untreated roots (results not shown).

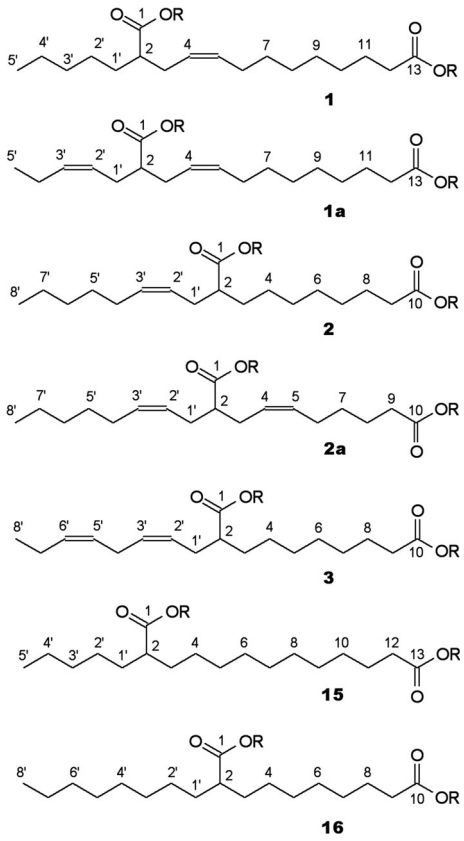

Figure 2. Structural formulae of novel products with their carbon-numbering scheme. The following trivial names are suggested: **1**, graminoxin A1; **1 a**, graminoxin A2; **2**, graminoxin B1; **2 a**, Favorskii product biosynthesized from γ-linolenic acid 9(*S*)-hydroperoxide[7]; **3**, graminoxin B2; **15** and **16**, graminoxins A and B, respectively, products of the catalytic hydrogenation of graminoxins **1**–**3** used as the prototypic parent structures for naming the naturally occurring graminoxins.

Identification of unknown products **1**, **2**, and **3**, belonging to the major group of oxylipins, appeared to be an intriguing goal of research. These products (Me esters) eluted as a partly overlapping group of peaks during GC–MS analysis. So, for detailed structural studies, these products (Me esters) were separated and purified by normal-phase (NP) HPLC with qualitative GC–MS control of the separate fractions. Their identification is described below.

2.1.1. Identification of Product 1

Product **1** (Me ester) and next (partly overlapping) products **2** and **3** eluted before 9-HOD and 13-HOD (Figure 1). The mass spectrum of NP-HPLC-purified product **1** and the mass fragmentation scheme are presented in Figure 3 a. The spectrum possesses a $[M]^+$ signal at $m/z = 340$. Most of the fragments result from fragmentation at the tertiary C2 atom (fragmentation scheme, Figure 3 a). No known spectra matched this spectrum. At the same time, the spectrum had some similarity to a previously described spectrum of Favorskii-type rearrangement product **1 a**,[7] accompanying the synthesis of 12-oxo-10,15-phytodienoic acid (12-oxo-PDA) from α-linolenic acid 13-hydroperoxide (13-HPOT) in the presence of flaxseed AOS. Catalytic

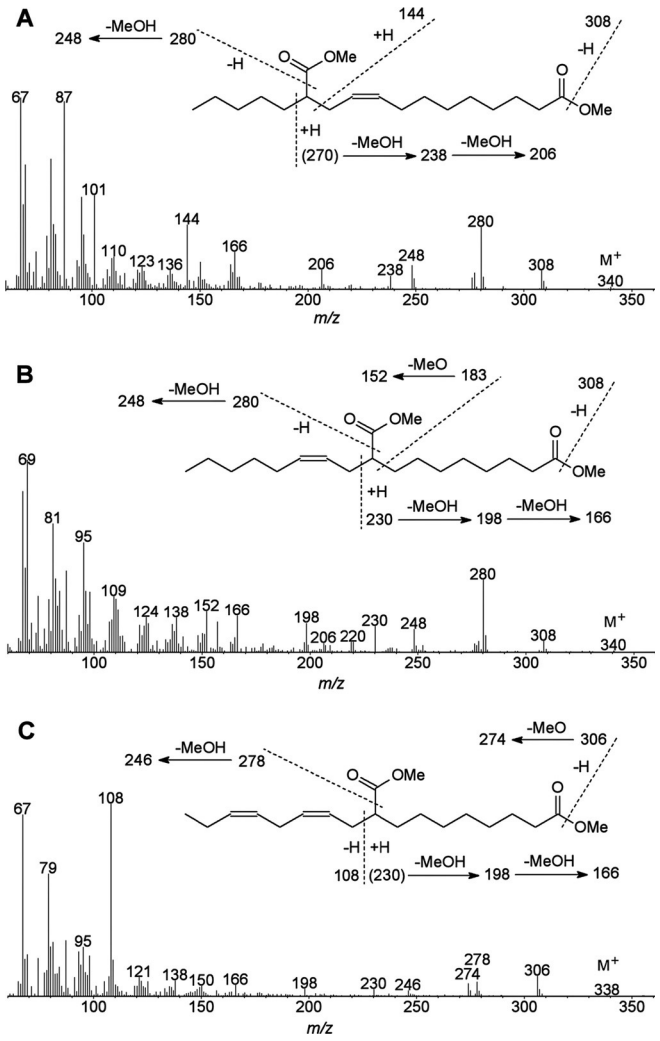

Figure 3. Electron impact mass spectral data for products **1**, **2**, and **3** (Me esters). Products **1** and **2** were isolated after incubation of cell-free preparations from wheat roots with linoleic acid. Product **3** was prepared analogously upon the incubation of roots of technical sorghum with α-linolenic acid. a–c) Mass spectra and fragmentation schemes for products **1–3** (Me esters), respectively. Conditions for incubation, extraction, derivatization, and analysis are described in the Experimental Section.

hydrogenation over Adams catalyst turned product **1** into compound **15**, which has a highly characteristic mass spectrum (Figure S2a). This spectrum matched that of hydrogenated Favorskii-type product **1a** formed from 13-HPOT in the presence of flaxseed AOS,[7] namely, (2-pentyl)tridecane-1,13-dioic acid (dimethyl ester). This coincidence, as well as the recorded MS data (Figure 3a), indicated that product **1** had a structure of 2-pentyl-4-tridecene-1,13-dioic acid. For further structural elucidation, its NMR spectrum was recorded.

The NMR data of product **1** are presented in Table 1. The assignments of the separate proton signals were validated by ^{1}H–^{1}H COSY and ^{1}H–^{1}H TOCSY data. The ^{1}H NMR spectrum exhibits intense singlets at $\delta = 3.41$ and 3.37 ppm (^{1}H signals of Me esters). The ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC data reveal heteronuclear correlations between these proton signals and the ^{13}C signals at $\delta = 50.49$ and 50.41 ppm (Me groups of Me esters) as well as those at $\delta = 175.28$ and 172.83 ppm (carboxylic C1 and C13 atoms, respectively). These data demonstrate the presence of two carboxylic groups (Me esters) in product **1**. The data reveal the presence of one *cis*-configured double bond ($J_{4,5} = 10.9$ Hz). The ^{1}H–^{13}C HMBC and ^{1}H–^{1}H NOESY (not illustrated) data confirm the tertiary structure of C2 and its connection to C1, C3, and C1′. Overall, the obtained data allowed a structure of (4*Z*)-2-pentyl-4-tridecene-1,13-dioic acid to be ascribed to compound **1**.

2.1.2. Identification of Product 2

Product **2** (Me ester) eluted close after product **1**. The mass spectrum of product **2** (Me) and its mass fragmentation scheme are presented in Figure 3b. The spectrum does not match that of any previously known compound but resembles the spectrum of the Favorskii product formed as a minor by-product in the conversion of γ-linolenic acid 9-hydroperoxide via allene oxide and cyclopropanone initiated by the AOS.[7] A characteristic and prominent ion at $m/z = 280$ is formed due to the loss of methyl formate (loss of 60 from $[M]^+$ at $m/z = 340$). Prominent ions at $m/z = 280$, 248, 230, 198, 183, 166, and 152 are formed owing to chain fragmentation at the tertiary carbon atom as well as subsequent loss of methanol (Figure 3b). Catalytic hydrogenation turned compound **2** into product **16**. Its spectrum (Figure S2b) matches that of the hydrogenated form of Favorskii product **2a** biosynthesized from γ-linolenic acid 9(*S*)-hydroperoxide via the corresponding allene oxide.[7] Thus, product **16** has a structure of 2-(octyl)-decane-1,10-dioic acid. Compound **2** has one double bond, presumably at the 2′-position of the octenyl substituent. For final verification of the structure of product **2** and to clarify the position and geometry of the double bond, the NMR spectrum of **2** was recorded. The ^{1}H NMR spectral data (Table 2) confirm the presence of one *cis* double bond ($J_{2',3'} = 10.9$ Hz) and two methyl ester functions (singlets at $\delta = 3.35$ and 3.40 ppm). The ^{1}H–^{13}C HMBC and ^{1}H–^{1}H NOESY data (not illustrated) confirm the chain-branching point at C2 and its connection to C1, C3, and C1′. Overall, the obtained data allowed a structure of (2′*Z*)-2-(2′-octenyl)-decane-1,10-dioic acid to be ascribed to compound **2**.

2.1.3. Identification of Product 3

The peaks of **2** and **3** (Me esters) partly overlap. Thus, the Me ester of product **3** (as well as **1** and **3**) was purified by NP-HPLC to record the qualitative spectral data. The mass spectrum of product **3** (Me) and its mass fragmentation scheme are presented in Figure 3c. The spectrum shows a weak $[M]^+$ signal at $m/z = 338$ (0.1), a $[M-\text{MeOH}]^+$ signal at $m/z = 306$ (9), a $[M-\text{HCOOMe}]^+$ signal at $m/z = 278$ (7), a $[306-\text{MeOH}]^+$ signal at $m/z = 274$ (7), and a $[278-\text{MeOH}]^+$ signal at $m/z = 246$ (2). The series of fragments at $m/z = 230$ (2), 198 (4), and 166 (6) is the same as that in the spectrum of product **2**, but with weaker intensity. The base fragment is $[M-\text{MeOOC}(\text{CH}_2)_8\text{COOMe}]^+$ at $m/z = 108$ (100). Catalytic hydrogenation turned product **3** into compound **16**, 2-(octyl)decane-1,10-dioic acid, described above (Figure S2b). Thus, the

Table 1. The NMR spectral data (^{1}H NMR, ^{1}H–^{1}H COSY, ^{1}H–^{1}H TOCSY, ^{1}H–^{13}C HSQC, and ^{1}H–^{13}C HMBC) for compound **1** (Me ester).[a]

Position no.	δ(^{13}C) [ppm]; functional group	δ(^{1}H) [ppm]; multiplicity; *J* [Hz]	Heteronuclear multiple bond correlation
1	175.28; COOMe		COOMe(1), H3a; H3b
2	45.68; CH	2.46; tt; 8.5 (H1′a,b); 5.3 (H3a,b)	H3a; H3b
3a	30.08; CH_2	2.27; ddd; 13.8 (H1′b); 8.5 (H2); 7.0 (H2′)	C1; C2; C4; C5
3b		2.51	C1;C2; C4; C5
4	126.41; CH	5.45; dt; 10.9 (H5); 6.5 (H3a,b)	H6
5	131.87; CH	5.49; dt; 7.0 (H6a,b)	H6
6	27.08; CH2	2.04	C4; C5; C7
7	29.64; CH_2	1.33; m	H6
8	28.97; CH_2	1.15; m	
9	27.10; CH_2	1.26; m	C10
10	28.97; CH_2	1.15; m	H9, H11; H12
11	24.72; CH_2	1.55; m	C10, H12
12	33.64; CH_2	2.12; t; 7.5 (H11)	C11; C10; C13
13	172.83; *C*OOMe		COO*Me*(13); H12
1′a	31.81; CH_2	1.44, m	C2; H2′
1′b		1.73; m	
2′	29.54; CH_2	1.30; m	C1′
3′	28.98; CH_2	1.21	H4′;
4′	22.50; CH_2	1.27; m	C3′; C5′; H5′
5′	13.71; CH_3	0.85; t; 7.1 (H4′)	C4′; H4′
(1)	50.49; COO*Me*	3.41; s	C1
(13)	50.41; COO*Me*	3.37; s	C13

[a] Data were obtained at 600 MHz in [$^2H_6$]benzene at 303 K.

Table 2. NMR spectral data (^{1}H NMR, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC, and ^{1}H–^{13}C HMBC) for compound **2** (Me ester).[a]

Position no.	δ(^{13}C) [ppm]; functional group	δ(^{1}H) [ppm]; multiplicity; *J* [Hz]	Heteronuclear multiple bond correlation
1	175.28; *C*OOMe		H1′a; H1′b; COO*Me*(1);
2	45.67; CH	2.45; tt; 8.5 (H1′a,b); 5.4 (H3a,b)	H3a; H3b; H1′a; H1′b
3a	31.88; CH_2	1.44, m	C1; H4
3b		1.72; m	C1
4	29.46; CH_2	1.28; m	C3
5	30.45; CH_2	1.17; m	H6
6	27.39; CH_2	1.25; m	C5
7	28.91; CH_2	1.13; m	H8, H9
8	24.70; CH_2	1.52; m	C7; C9; H9
9	33.40; CH_2	2.10; t; 7.5 (H8)	C7; C8; H8
10	172.90; *C*OOMe		H8; H9; COO*Me*(10)
1′a	29.86; CH_2	2.26; dddt; 13.9 (H1′b); 8.5 (H2); 7.0 (H2′); 1.3 (H3′)	C1; C2; C2′; C3′
1′b		2.50; dddt	C1; C2; C2′; C3′
2′	126.38; CH	5.45; dtt; 10.9 (H3′); 7.0 (H1′a,b); 1.3 (H4′)	H1′a; H1′b; H4′
3′	131.85; CH	5.50; dtt; 7.2 (H4′); 1.3 (H1′a,b)	H1′a; H1′b; H4′; H5′
4′	27.13; CH_2	2.06; dt; 7.1 (H5′)	C2′; C3′; C5′
5′	29.56; CH_2	1.33; m	C3′; H4′
6′	29.00; CH_2	1.19; m	C7′
7′	22.51; CH_2	1.27; m	H6′; H8′
8′	13.74; CH_3	0.88; t; 7.1 (H7′)	C7′
(1)	50.50; COO*Me*	3.40; s	C1
(10)	50.41; COO*Me*	3.35; s	C10

[a] Data were obtained at 600 MHz in [$^2H_6$]benzene at 303 K.

MS data indicate that products **2** and **3** have the same skeleton. The only difference is an extra (ω3) double bond in product **3**. For final verification of the structure of product **3** and to clarify the position and geometry of the double bond, its NMR spectrum (Table 3) was recorded. The ^{1}H NMR spectrum confirms the presence of two *cis* double bonds ($J_{2',3'}$ = 10.9 Hz and $J_{5',6'}$ = 10.9 Hz) and two methyl ester functions (singlets at δ = 3.36 and 3.40 ppm). The ^{1}H–^{13}C HMBC and ^{1}H–^{1}H NOESY data confirm the branching point at C2 and its connection to C1, C3, and C1′. Thus, the obtained data allow a structure of (2′*Z*,5′*Z*)-2-(2′,5′-octadienyl)-decane-1,10-dioic acid to be ascribed to compound **3**.

Table 3. NMR spectral data (^{1}H NMR, ^{1}H–^{1}H COSY, ^{1}H–^{1}H TOCSY, ^{1}H–^{13}C HSQC, and ^{1}H–^{13}C HMBC) for compound **3** (Me ester).[a]

Position no.	δ(^{13}C) [ppm]; functional group	δ(^{1}H) [ppm]; multiplicity; *J* [Hz]	Heteronuclear multiple bond correlation
1	175.34; *C*OOMe		H1′a; H1′b; H3a; H3b; COO*Me*(1)
2	45.50; CH	2.43; tt; 8.6 (H1′a,b); 5.4 (H3a,b)	H3a; H3b; H1′a; H1′b;
3a	31.85; CH_2	1.41, m	C1
3b		1.70; m	C1
4	29.10; CH_2	1.22; m	
5	28.73; CH_2	1.13; m	
6	27.30; CH_2	1.25; m	
7	28.73; CH_2	1.12; m	H8; H9
8	24.73; CH_2	1.53; m	C7; C10; H9
9	33.66; CH_2	2.10; t; 7.5 (H3)	C1; C7; C8; C10
10	173.04; *C*OOMe		H8; H9; COO*Me*(10);
1′a	30.03; CH_2	2.25; dddt; 14.0 (H1′b); 8.6 (H2); 7.2 (H2′); 1.3 (H3′)	C1; C2; C2′; C3′
1′b		2.50; dddt	C1; C2; C2′; C3′
2′	126.72; CH	5.43; dtt; 10.9 (H3′); 7.2 (H1′a,b); 1.3 (H4′)	H1′a; H1′b
3′	130.02; CH	5.49; dtt; 7.2 (H4′); 1.3 (H1′a,b)	H1′a; H1′b
4′	25.48; CH_2	2.83; dt; 7.1 (H5′)	H5′
5′	126.98; CH_2	5.40; AB; 10.9 (H6′)[b]	C4′; H7′
6′	131.83; CH_2	5.42; AB	C7′; H7′; H8′
7′	20.50; CH_2	2.02; m	C5′; C6′; H6′; H8′
8′	13.93; CH_3	0.91; t; 7.1 (H7′)	C6′; C7′; H7′
(1)	50.54; COO*Me*	3.40; s	C1
(10)	50.42; COO*Me*	3.36; s	C10

[a] Data were obtained at 600 MHz in [$^2H_6$]benzene at 303 K. [b] The value of the coupling constant ($J_{5',6'}$ = 10.9 Hz) was reconstructed by a computer simulation of the AB system signal.

2.2. Methanol-Trapping Experiments

The enzyme preparation from wheat roots obtained by $(NH_4)_2SO_4$ precipitation was incubated with (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD) in a biphasic buffer/hexane system for 5 or 30 min at 0 °C, followed by methanol trapping, as described in the Experimental Section. Then, the products (Me/TMS) were analyzed by GC–MS. A single product predominated in the 5 min trapping experiments. Its mass spectrum shows a $[M]^+$ signal at *m/z* = 340 (0.9), a $[M-MeOH]^+$ signal at *m/z* = 309 (2), a $[309-MeOH]^+$ signal at *m/z* = 277 (1), a $[M-C10/C18]^+$ signal at *m/z* = 201 (100), a $[201-MeOH]^+$ signal at *m/z* = 169 (31), and a $[169-MeOH]^+$ signal at *m/z* = 137 (59), in addition to signals at *m/z* = 109 (30), 95 (26), and 71 (90). The spectrum fully corresponds to that of 9-methoxy-10-oxo-12-octadecenoic acid (Me), the product of the methanolysis of the allene oxide [i.e. (10,12*Z*)-9,10-epoxy-10,12-octadecadienoic acid, 9,10-EOD].[8] After identical incubation in the biphasic system for 30 min, the relative abundance of this methanol-trapping product decreased about 20-fold. Instead, a transient increase in the signals of Favorskii-type product **2** and ketols **8** and **9** was observed.

3. Discussion

Oxylipins **1**–**3** detected in the present work are novel natural products. We suggest a collective name "graminoxins" for compounds **1**–**3** and congeners **1a** and **2a**. Assigning the trivial name seems to be reasonable given the complexity of IUPAC names for these products. The suggested name is biologically based,[9] taking into account the family name of cereals Gramineae (*syn.* Poaceae). The individual names of the individual products (Figure 2) are based on two fundamental parent structures,[9] **15** and **16**, named graminoxins A and B, respectively. The numerical indexes following the letters A and B denote the number of double bonds in the main carbon chain of the graminoxins.

Branched structures such as products **1**–**3** with a side carboxymethyl function are known in the chemical literature as products of Favorskii rearrangements, involving cyclopropanone intermediates.[10] Cyclopropanone, oxyallyl, and allene oxide are considered a triad of valence tautomers.[11–13] Thus, the relationship between the synthesis of compounds **1**–**3** and the AOS pathway in cereal roots seemed to be apparent. Moreover, the precursor–product relationship between allene oxide and Favorskii-type product **2** was confirmed by the data of the methanol-trapping experiments.

The obtained data uncover the previously unknown route of the AOS pathway, which is directed to novel oxylipins **1**–**3** in cereal roots. The proposed scheme of this route is presented in Figure 4a. The initial stage is allene oxide formation via the AOS dehydration of 9-HPOD. Then, the allene oxide undergoes two competing conversions: hydrolysis (mainly to α-ketol **9**) or rearrangement to cyclopropanone. The latter product is unstable and undergoes nucleophilic cleavage of the three-membered ring, which results in the formation of Favorskii-type product **2**. One more peculiarity of this route is a high yield of isomeric α-ketol **8**, which is presumably formed by hydrolysis of cyclopropanone. This ketol was described before as a minor product of the 9-AOS pathway.[14]

A mechanism for the conversion of the allene oxides into the cyclopropanones is proposed in Figure 4b. Opening of the strained oxirane ring leads to a oxyallyl zwitterion, which exists

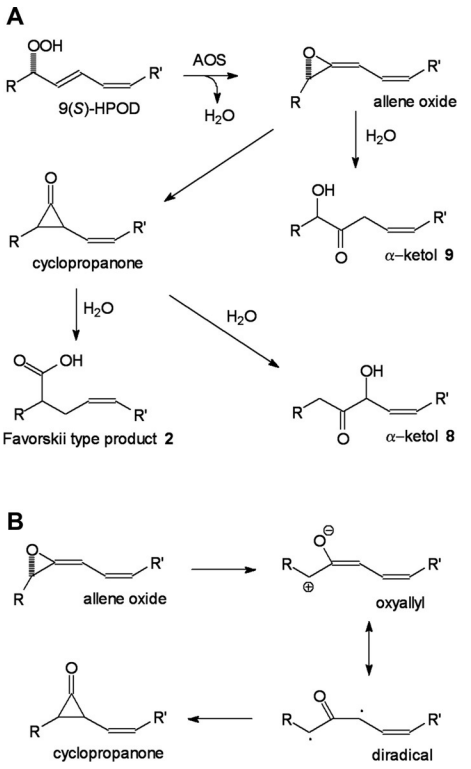

Figure 4. Proposed scheme for the biosynthesis of Favorskii product **2** (graminoxin B1) via allene oxide and cyclopropanone intermediates. a) Total scheme for 9-HPOD conversion. b) Scheme for the conversion of allene oxides into cyclopropanones.

in resonance equilibrium with an oxyallyl diradical. The existence of the latter was recently confirmed by direct spectroscopic observation.[15–17] Interestingly, not only 1,3-ring closure to the cyclopropanone but also 1,5-closure to the cyclopentenone is now considered to occur through a diradical mechanism, according to the latest DFT computations,[18,19] not electrocyclization, as was assumed previously.[20,21]

The AOS pathway is widespread in plants.[6,22] However, hitherto there was no evidence for the co-occurrence of cyclopropanones with allene oxide fatty acids. The only exceptions are compounds **1a** and **2a**, the minor byproducts of some 18:3-allene oxide conversions,[7] formed via cyclopropanone intermediates. However, Favorskii-type products **1a** and **2a** are roughly 50 times less abundant than the related cyclopentenones.[7] In contrast, novel Favorskii-type products **1**–**3** are major products, besides α-ketols, in cereal roots.

The formation of Favorskii product **2** directly correlated with the protein concentration, whereas a low concentration was beneficial for α-ketol formation. This dependency indicates that the conversion of allene oxide into cyclopropanone depends on a putative cyclase. Unless allene oxide meets this putative cyclase, it spontaneously hydrolyzes to the α-ketol. Moreover, upon incubating a boiled enzyme preparation from wheat roots with 9-HPOD in the presence of active recombinant ZmAOS, only the α-ketol, but not Favorskii product **2**, was formed. This result demonstrates that the conversion of allene oxide into cyclopropanone occurs enzymatically.

There is one well-known enzyme controlling allene oxide conversion, namely, allene oxide cyclase (EC 5.3.99.6).[23–27] This enzyme specifically converts an allene oxide formed from 13-HPOT [(9*Z*,11,15*Z*)-12,13-epoxy-9,11,15-octadecatrienoic acid, 12,13-EOT] into cyclopentenone (9*S*,13*S*)-12-oxo-PDA, a jasmonate precursor. One more recently described[28] cyclase of maize roots catalyzes the cyclization of allene oxide 9,10-EOD into the cyclopentenone (9*S*,13*S*)-10-oxo-11-phytoenoic acid (10-oxo-PEA), which was identified as a cytotoxic phytoalexin and transcriptional mediator.[29] Similarly, the results of the present work indicate that the conversion of allene oxides into cyclopropanones in cereal roots is controlled by a soluble protein. Currently, all mentioned proteins except the AOC (EC 5.3.99.6) are unknown. One cannot exclude that these different cyclases are phylogenetically related.

Further works on the biosynthesis of graminoxins as well as the identification of a hypothetical novel allene oxide cyclase (cyclopropanone synthase) are underway in our laboratory.

4. Conclusions

By studying the metabolism of linoleic and α-linoleic acids and their hydroperoxides in the roots of cereals, an unexpected observation has been made. Prominent unknown oxylipins, similar but not identical to the previously described Favorskii-type products,[7] were biosynthesized from linoleic acid hydroperoxides and belong to the main products of the AOS pathway in some cereal roots. The detection of these new products and their biosynthesis pathway via unstable cyclopropanone intermediates have been reported herein for the first time.

Experimental Section

Chemicals and Substrate Preparation

Linoleic acid and α-linolenic acid, as well as the soybean lipoxygenase type V, were purchased from Sigma–Aldrich. [$^2H_6$]Benzene (99.5 % ^2H) was acquired from the Applied Chemistry Centre (St. Petersburg, Russia). (9*S*,10*E*,12*Z*)-9-Hydroperoxy-10,12-octadecadienoic acid (9-HPOD) and (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoic acid (9-HPOT) were prepared by incubation of linoleic acid and α-linolenic acid, respectively, with the recombinant maize 9-lipoxygenase (GeneBank: AAG61118.1) in sodium phosphate buffer (100 mM, pH 6.0) at 0 °C, under continuous oxygen bubbling. (9*Z*,11*E*,13*S*)-13-Hydroperoxy-9,11-octadecadienoic acid (13-HPOD) and (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT) were obtained by incubation of linoleic acid and α-linolenic acid, respectively, with the soybean lipoxygenase type V in Tris·HCl buffer (50 mM, pH 9.0) at 23 °C, under continuous oxygen bubbling. The extracted non-esterified hydroperoxides were purified by normal-phase HPLC (NP-HPLC) on a Macherey–Nagel Nucleodur 100-3 silica column (250×4.6 mm, 3 μm) under isocratic elution with a solvent mixture comprising hexane/2-propanol/acetic acid (98.1:1.8:0.1, *v*/*v*/*v*) at a flow rate of 0.4 mL min^{-1}.

Plant Seeds and Conditions of Plant Growth

Wheat (*Triticum aestivum* L., var. Kazan Jubilee), barley (*Hordeum vulgare* L., var. Svyatich), oat (*Avena sativa* L., var. Allure), and proso millet (*Panicum miliaceum* L., var. Tatar red) seeds were purchased from SSI (State Scientific Institution) Tatar Scientific Research Institute of Agriculture of the Russian Academy of Sciences (Kazan, Russia). Seeds of three sorghum species, namely, Sudangrass [*Sorghum bicolor* (L.) Moench *ssp. drummondii* (Nees ex Steud.) de Wet & Harlan, var. Udacha/Luck], technical sorghum [*Sorghum vulgare* Pers. var. *technicum* (Koern.) Jáv., var. Master], and Chinese sorghum (*Sorghum chinense* Jakusch., var. Cream) were generously provided by the Agricultural Research Institute of South-East Region (Saratov, Russia). Rice (*Oryza sativa* L., var. Renar) seeds were purchased from All-Russia Rice Research Institute (Krasnodar, Russia). Seeds were germinated on tap water without supplements at 23 °C for 5–7 days.

Cell-Free Preparations from Cereal Roots

Roots of germinating cereal (wheat, sorghum, barley, oat, or proso millet) seeds were cut after 5–7 days of germination. Cut roots (10 g, fresh weight) were suspended in cold (0–4 °C) 0.05 M Tris·HCl buffer (20 mL, pH 7.5) and homogenized with Ultra-Turrax. The homogenate was filtered through cheesecloth and centrifuged at 15000 *g* for 15 min. The supernatant was decanted and used for incubations with α-linolenic acid, linoleic acid, or hydroperoxides of the latter. Alternatively, the 15000 *g* supernatant was adjusted to 60% $(NH_4)_2SO_4$ saturation, stirred for 30 min at 0–4 °C, and centrifuged at 15000 *g* for 15 min. The supernatant was decanted, and the pellet obtained after the addition of $(NH_4)_2SO_4$ was frozen with liquid nitrogen and stored at 85 °C prior to use for incubations.

Incubation of Enzyme Preparations with Linoleic Acid or Linolenic Acid and their Hydroperoxides

Standard incubations were initiated by the addition of either linoleic acid or linolenic acid (100 mg) or fatty-acid hydroperoxide (9-HPOD or 13-HPOD, 100 μg) to an aliquot of 15000 supernatant preparations, corresponding to 1 g of the roots (fresh weight). The mixture was stirred for 30 min with continuous oxygen bubbling for 15 min or without oxygen bubbling (for 9-HPOD or 13-HPOD incubation). Alternatively, the protein pellet obtained by $(NH_4)_2SO_4$ precipitation as described above from 12.8 g (fresh weight) of wheat roots was dissolved in 0.05 M Tris·HCl buffer (3.6 mL, pH 7.5). The resulting protein solution was incubated with 9-HPOD or 13-HPOD (100 μg) for 15 min at 23 °C. Then, the products were extracted and derivatized as described below.

Extraction, Preliminary Purification and Derivatization of Products

The incubation mixture was acidified with acetic acid (CH_3COOH) to pH 5–6 and was extracted with hexane/ethyl acetate (1:1, *v/v*). Acidic lipids were separated and purified for analysis by using Supelclean LC-NH_2 (3 mL) cartridges (Supelco, Bellefonte, PA, USA) as described before.[30] First, the total lipid extract dissolved in a mixture of chloroform/2-propanol (2:1, *v/v*) was passed through the cartridges. Then, free carboxylic acids were eluted with a mixture of ethyl acetate/acetic acid (98:2, *v/v*). Free fatty acids were esterified with diazomethane. If specified, the methyl esters of the products were trimethylsilylated by treatment with a silylating mixture consisting of pyridine/hexamethyldisilazane/trimethylchlorosilane (2:1:2, *v/v/v*).

Methanol-Trapping Experiments

The protein pellet obtained by $(NH_4)_2SO_4$ precipitation as described above from 5 g (fresh weight) of wheat roots was dissolved in 0.05 M Tris·HCl buffer (1 mL, pH 7.5). The resulting protein solution was combined with 9-HPOD (200 μg) dissolved in cold hexane (7 mL). This biphasic hexane/buffer system was vigorously vortexed for 5 min at 0 °C. The phases were separated by centrifugation at 5000 *g* for 1 min. The upper hexane layer was aspirated, and the main part of it was evacuated in vacuo. The products were methylated with ethereal diazomethane. Ether was evacuated in vacuo, the ice-cold MeOH (3 mL) was added, and the solution was left for 15 min at 0 °C. Then, methanol was evacuated, and the products (Me esters) were trimethylsilylated as described above.

Extraction of the Endogenous Oxylipins from Cereal Roots

Cut roots (10 g, by fresh weight) were homogenized and extracted at 0–4 °C with ice-cold hexane/ethyl acetate (1:1, *v/v*). The extract was concentrated, and acidic lipids were purified with Supelclean LC-NH_2 (3 mL) cartridges as described above. Then, the free fatty acids were esterified with diazomethane and trimethylsilylated.

General Schemes of Product Analyses

The methyl esters of the products (or their methyl ester TMS derivatives) were subjected to direct GC–MS analysis after amino cartridge purification and derivatization. Alternatively, the methyl esters of the products were preliminarily separated by NP-HPLC. Products were separated as methyl esters by NP-HPLC on a Macherey–Nagel Nucleodur 100-3 column (250×4.6 mm, 3 μm) by using a mixture of hexane/2-propanol (98:2, *v/v*) at a flow rate of 0.4 mL min^{-1}. Products were collected and rechromatographed by NP-HPLC, eluting with hexane/2-propanol (99.8:0.2, *v/v*) at a flow rate of 0.4 mL min^{-1}.

Spectral Studies

UV spectra were recorded online during the HPLC separations with a SPD-M20A diode array detector (Shimadzu). GC–MS analyses were performed by using a Shimadzu QP5050A mass spectrometer connected to a Shimadzu GC-17A gas chromatograph equipped with an MDN-5S (5% phenyl, 95% methylpolysiloxane) fused capillary column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm). Helium at a flow rate of 30 cm s^{-1} was used as the carrier gas. Injections were made in the split mode by using an initial column temperature of 120 °C. The temperature was raised at 10 °C min^{-1} until 240 °C. Full-scan GC–MS analyses were performed by using an ionization energy of 70 eV. The ^1H NMR, ^1H–^1H COSY, ^1H–^1H TOCSY, ^1H–^1H NOESY, ^1H–^{13}C HSQC, and ^1H–^{13}C HMBC spectra were recorded with a Bruker Avance III 600 instrument (600 MHz, [$^2H_6$]benzene, 296 K).

Abbreviations

AOS, allene oxide synthase; DES, divinyl ether synthase; HPL, hydroperoxides lyase; 9-H(P)OD, (9*S*,10*E*,12*Z*)-9-hydro(pero)xy-10,12-octadecadienoic acid; 9-HPOT, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoic acid; 13-H(P)OD, (9*Z*,11*E*,13*S*)-13-hydro-

(pero)xy-9,11-octadecadienoic acid; 13-HPOT, (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid; 9,10-EOD, (10,12*Z*)-9,10-epoxy-10,12-octadecadienoic acid (allene oxide); 10-oxo-PEA, 10-oxo-11-phytoenoic acid; 12,13-EOT, (9*Z*,11,15*Z*)-12,13-epoxy-9,11,15-octadecatrienoic acid (allene oxide); 12-oxo-PDA, 12-oxo-10,15-phytodienoic acid; NP-HPLC, normal-phase HPLC; TMS, trimethylsilyl; DFT, density functional theory; COSY, correlation spectroscopy; HSQC, heteronuclear single-quantum coherence spectroscopy; HMBC, heteronuclear multiple-bond correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy.

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Conflict of Interest

The authors declare no conflict of interest.

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