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Formation of a new class of oxylipins from *N*-acyl(ethanol)amines by the lipoxygenase pathway

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N-Acylethanolamines (NAEs) constitute a new class of plant lipids and are thought to play a role in plant defense strategies against pathogens. In plant defense systems, oxylipins generated by the lipoxygenase pathway are important actors. To date, it is not known whether plants also use endogeneous oxylipins derived from NAEs in their defense reactions. We tested whether members of the NAE class can be converted by enzymes constituting this pathway, such as (soybean) lipoxygenase-1, (alfalfa) hydroperoxide lyase and (flax seed) allene oxide synthase. We found that both α-*N*-linolenoylethanolamine and γ-*N*-linolenoylethanolamine (18:3), as well as α-*N*-linolenoylamine and γ-*N*-linolenoylamine were converted into their (13*S*)-hydroperoxide derivatives by lipoxygenase. Interestingly, only the hydroperoxides of α-*N*-linolenoyl(ethanol)amines and their linoleic acid analogs (18:2) were suitable substrates for hydroperoxide lyase. Hexanal and (3*Z*)-hexenal were identified as volatile products of the 18:2 and 18:3 fatty acid (ethanol)amides, respectively. 12-Oxo-*N*-(9*Z*)-dodecenoyl(ethanol)amine was the nonvolatile hydrolysis product. Kinetic studies with lipoxygenase and hydroperoxide lyase revealed that the fatty acid ethanolamides were converted as readily or even better than the corresponding free fatty acids. Allene oxide synthase utilized all substrates, but was most active on (13*S*)-hydroperoxy-α-*N*-linolenoylethanolamine and the (13*S*)-hydroperoxide of linoleic acid and its ethanolamine derivative. α-Ketols and γ-ketols were characterized as products. In addition, cyclized products, i.e. 12-oxo-*N*-phytodienoylamines, derived from (13*S*)-hydroperoxy-α-*N*-linolenoylamines were found. The results presented here show that, in principle, hydroperoxide NAEs can be formed in plants and subsequently converted into novel phytooxylipins.

Keywords: allene oxide synthase; hydroperoxide lyase; lipoxygenase; *N*-acylethanolamine; plant defense reaction.

Fatty acid amides are emerging as important signal transduction molecules in mammals and plants. In mammals various compounds of this new lipid family, i.e. *N*-acylethanolamines (NAEs), exert different neurological and immunological functions. It is speculated that these NAEs may function as stress-recovery factors [1]. Little attention has been paid to the family of fatty acid amides in plants. The discovery of the important function of NAE in mammals, however, has renewed the interest in plant NAEs. *N*-Acylphosphatidylethanolamines (NAPEs) were first detected in plants more than 30 years ago and represent 1–5% of the lipid content [2]. The hydrolysis products of NAPEs, NAEs, may play a role in germination and in the defense system of plants [3,4]. During early germination of soybeans the amount of NAPE decreased from 3.5 to 1.5% of the total lipid content [3]. In tobacco-cell cultures the [^{14}C]NAPE content decreased fivefold after stimulation with a fungal elicitor, whereas the [^{14}C]NAE content in the culture medium increased ≈ sixfold [4]. *N*-Laureoylethanolamine (12:0) and *N*-myristoylethanolamine (14:0) were identified as endogeneously released compounds. Two hours after elicitor stimulation NAPE synthase activity was increased, thereby replenishing NAPE [5,6].

The plant defense system comprises a complex array of constitutive and inducible responses. Oxygenated fatty acids, termed oxylipins, were shown to be important metabolites in plant resistance [7]. These (phyto)oxylipins are generated in the lipoxygenase pathway. The first step in this cascade is catalyzed by lipoxygenases, which convert fatty acids containing one or more (1*Z*,4*Z*)-pentadiene systems into 1-hydroperoxy-(2*E*,4*Z*)-pentadiene derivatives in a regiospecific and stereospecific manner. The hydroperoxy fatty acids derived from linoleic (18:2) and linolenic (18:3) acids can be metabolized further by hydroperoxide lyase (HPO lyase), allene oxide synthase (AOS) or peroxygenase (POX). HPO lyases cleave the C–C bond next to the hydroperoxy group, resulting in the

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Abbreviations: AEA, *N*-arachidonoylethanolamine; AOS, allene oxide synthase; CB, cannabinoid receptor; CP, chiral phase; 13-H(P)OTNH2, 13-hydro(pero)xy-*N*-linolenoylamine; 13-H(P)OTNHEtOH, 13-hydro(pero)xy-*N*-linolenoylethanolamine; HPO lyase, hydroperoxide lyase; NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; NDGA, nordihydroguaiaretic acid; α(γ)-OT, α(γ)-linolenic acid; sLOX, soybean lipoxygenase-1; PEA, *N*-palmitoylethanolamine; POX, peroxygenase; SPE, solid-phase extraction; SPME, solid-phase micro extraction.

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formation of short chain aldehydes and ω-oxo acids. The short chain aldehydes are important constituents of the characteristic flavors of fruits, vegetables and green leaves and might be involved in wound healing and pest resistance [8,9]. The ω-oxo acid 12-oxo-(9*Z*)-dodecenoic acid can be converted to the wound hormones traumatin [12-oxo-(10*E*)-dodecenoic acid] and traumatic acid [10]. AOS dehydrates the hydroperoxy fatty acids to unstable allene oxides, which hydrolyze spontaneously to α-ketols and γ-ketols or are enzymatically cyclized to 12-oxo-phytodienoic acid, the precursor of jasmonic acid. Jasmonic acid is a wound hormone which is generally considered to be a key mediator in the signal transduction of defense reponses. POX converts the hydroperoxy fatty acids into phytoalexins, a class of natural pesticides [7].

Anandamide and *N*-linoleoylethanolamine (ODNHEtOH) (18:2) have been shown to be suitable substrates for plant lipoxygenases [11,12]. Because (13*S*)-hydroperoxylinolenic acid (18:3) is an important precursor for a broad range of phytooxylipins, it is interesting to know whether its corresponding *N*-linolenoyl(ethanol)amines (18:3, OTNHEtOH and $OTNH_2$) can also serve as substrates for lipoxygenases. Furthermore, it is not yet known whether hydroperoxy *N*-linoleoyl (ethanol)amines and *N*-linolenoyl(ethanol)amines can be further metabolized by HPO lyases and AOS to form possible defense compounds. Therefore, we tested four different linolenic acid amide derivatives as substrate for soybean lipoxygenase-1 (sLOX). Hydroperoxy *N*-linoleoyl(ethanol) amine and *N*-linolenoyl(ethanol)amine were tested as substrates for alfalfa HPO lyase and flax seed AOS. Here, we present the characterization of the products and the region and stereospecificities, as well as the kinetic parameters, of the dioxygenation and subsequent metabolism of these novel lipids.

MATERIALS AND METHODS

Materials and enzymes

Linoleic acid, α-linolenic acid and γ-linolenic acid (99% pure) were obtained from Sigma. *N*-Linole(*n*)oylethanolamine (OTNHEtOH) and *N*-linole(*n*)oylamine ($OTNH_2$) were synthesized using standard protocols (Fig. 1) [12]. All reagents used were of the purest grade available.

Lipoxygenase-1 was purified from soybean (Maple Glen) as described previously [13]. The specific activity of the lipoxygenase preparation was 40 U (μmol linoleic acid·min^{-1})·mg^{-1}.

AOS was extracted from an acetone powder of flax seed as described previously [14]. The clear extract was purified further using solid-phase extraction (SPE) on BakerBond (solid state C18, 500 mg, J. T. Baker) and OASIS (30 mg, Waters). The specific activity of the AOS preparation was 0.81 U (μmol 13-hydroperoxy linoleic acid·min^{-1})·mg^{-1}.

Alfalfa HPO lyase was expressed in *Escherichia coli* cells containing the pQE32 vector (Qiagen) with the *CYP74B4v1* gene without N-terminal sequence coding for the first 22 amino acids (EMBL database, accession number AJ249245). A 10-L culture of these *E. coli* cells was grown at 37 °C until an A_{600} of

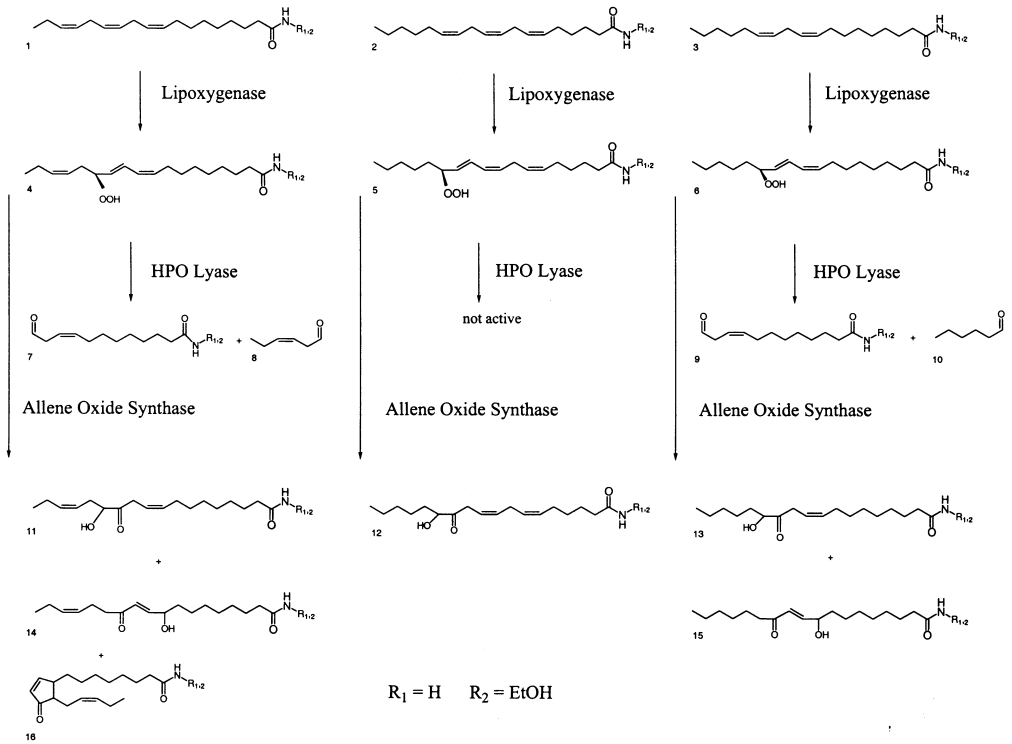

Fig. 1. Structures of products in the lipoxygenase-pathway of α-*N*-linolenoyl(ethanol) amine and γ-*N*-linolenoyl(ethanol) amine (2) and their linoleic analogs (3). Lipoxygenase products: (13*S*)-hydroxy-*N*-octadeca-(9*Z*,11*E*,15*Z*)-trienoyl(ethanol)amine (4); (13*S*)-hydroxy-*N*-octadeca-(6*Z*,9*Z*,11*E*)-trienoyl-(ethanol)amide (5) and (13*S*)-hydroxy-octadeca-(9*Z*,11*E*)-trienoyl(ethanol)amine (6). HPO lyase products: 12-oxo-*N*-(9*Z*)-dodecenoyl(ethanol)amine (7,9); (3*Z*)-hexenal (8) and hexanal (10). AOS products, α-ketols: 12-oxo-13-hydroxy-*N*-(9*Z*,15*Z*)-octadecadienoyl(ethanol)amine (11), 12-oxo-13-hydroxy-*N*-(6*Z*,9*Z*)-octadecadienoyl(ethanol)amine (12), 12-oxo-*N*-(9*Z*)-octadecenoyl(ethanol)amine (13) and γ-ketols: 12-oxo-9-hydroxy-*N*-(10*E*,15*Z*)-octadecadienoyl(ethanol)amine (14), 12-oxo-9-hydroxy-*N*-(10*E*)-octadecenoyl(ethanol)amine (15) and 12-oxo-*N*-phytodienoyl(ethanol)amine (16).

0.7, in 1.5 × Luria–Bertani medium supplemented with 0.5% glucose, 1 × SV buffer, 25 $\mu g \cdot mL^{-1}$ kanamycine and 100 $\mu g \cdot mL^{-1}$ ampicilline, with maximal stirring and O_2 flow. Expression of HPO lyase was induced by addition of 1 mM isopropyl thio-β-D-galactoside and the cells were grown overnight at 30 °C to prevent sequestering of the HPO lyase in inclusion bodies. Cells were harvested by centrifugation and the supernatant was discarded. The cell pellet was resuspended in 350 mL 50 mM potassium phosphate buffer pH 7.5 and sonicated on ice 15 × 1 min with 1-min intervals, in batches of 35 mL. The suspension was recentrifuged and the membrane pellet was resuspended in 400 mL 50 mM potassium phosphate buffer pH 7.5 containing 0.2% (w/v) Triton X-100 (membrane solubilization buffer). After centrifugation, the HPO lyase was present in the supernatant.

The enzyme was purified to homogenity by immobilized metal affinity chromatography using FPLC with a flow rate of 1 $mL \cdot min^{-1}$. One hundred milliliters of the supernatant was applied to a column containing 10 mL Ni-NTA superflow (Qiagen), equilibrated previously with membrane solubilization buffer. The column was washed with 20 mL membrane solubilization buffer and 50 mL buffer supplemented with 10 mM imidazole. Elution was performed in 100 min with a linear gradient of 10–250 mM imidazole in membrane solubilization buffer. The active fractions were pooled and the specific activity of the HPO lyase preparation was 249 U (μmol α-13-hydroperoxy linoleic $acid \cdot min^{-1}) \cdot mg^{-1}$. All steps were carried out at 4 °C.

Lipoxygenase assay: extraction and purification of reaction products

Typically 40 μM of substrate in 30 mL of rigorously stirred, air-saturated 0.1 M sodium borate buffer pH 9.0 was incubated with 1 U of lipoxygenase-1 for ≈ 60 min at 20 °C. The reaction was stopped by acidifying the reaction mixtures to pH 3 with 3 M HCl. The fatty acid amides were extracted with a BakerBond solid-state C_{18}-column (500 mg) according to Van Aarle *et al.* [15]. The eluate was concentrated under a N_2 flow and residual water was evaporated azeotropically with methanol. The products were dissolved in 1 mL methanol and stored at −25 °C until use.

For characterization, the products were reduced with $NaBH_4$ in methanol and purified by using HPLC; the main products were analyzed by ^{1}H-NMR, UV, CD spectroscopy, CP-HPLC and GC/MS as described previously [12].

Spectrophotometric assays of enzyme kinetics

The enzyme kinetics were determined on a Hewlett Packard 8452A diode array spectrophotometer by following the change in absorbance at 234 nm (linoleic acid derivatives) or 236 nm (linolenic acid derivatives; $\varepsilon = 27\,500\ L \cdot mol^{-1} \cdot cm^{-1}$) due to the formation or cleavage of conjugated hydroperoxydienes. The kinetic experiments with lipoxygenase were performed as described previously [13]. HPO lyase activity was determined in 50 mM potassium phosphate buffer pH 6.0 containing 100 μM substrate in a total of 0.5 mL. The AOS assay was performed with 30 μM substrate in 0.1 M potassium phosphate buffer pH 7.0 with 10–40 μL enzyme solution in a total of 1 mL. The changes in absorbance were corrected for the decrease in absorbance caused by the assay. Apparent K_m and k_{cat} were determined from the means of six determinations. The data were fitted to the standard Michaelis–Menten kinetic equation (GRAPHPAD PRISM). Protein concentrations were determined by using the bicinchoninic acid method [16].

Characterization of HPO lyase and AOS products

Characterization of HPO lyase products was carried out as described previously [17]. Volatile products were trapped by solid-phase micro extraction (SPME) and analyzed using GC. Non-volatile products were extracted from the acidified reaction mixture by SPE (BakerBond solid-state C18-column, 500 mg), reduced, methylated, trimethylsilylated and analyzed by using GC/MS. AOS reaction products were concentrated and purified with SPE (OASIS, 30 mg, Waters) without acidification, and analyzed by HPLC and GC/MS as described previously [11], except that the HPLC solvent was methanol/water/acetic acid (80 : 20 : 0.1). For GC/MS analyses the samples were reduced with sodium borodeuteride and analyzed without hydrogenation.

RESULTS

Lipoxygenase products

In each reaction with sLOX one major hydroperoxide regioisomer with a maximal absorbance (λ_{max}) at 236 nm was formed according to RP-HPLC analysis. Interestingly, for both α-$OTNH_2$ and γ-$OTNH_2$ the formation of some minor regioisomeric and double-dioxygenated ($\lambda_{max} = 269$ nm) products was observed. All reactions could be inhibited with nordihydroguaiaretic acid (NDGA), a widely used LOX-inhibitor.

^{1}H-NMR-spectra were recorded after reduction with sodium borohydride and additional purification by RP-HPLC, to establish the geometry of the 1-hydroperoxy-2,4-diene moiety in the main products (data not shown). The geometric configurations of the conjugated diene at the positions 9–12 were determined as 9*Z*,11*E* on the basis of the coupling constants ($J_{10,9} = 11$ Hz; $J_{10,11} = 11$ Hz; $J_{11,12} = 15$ Hz). The secondary hydroxyl group was located at position 13 in all main products, as determined on the basis of the mass spectra of the fully reduced fatty acid amides. The prominent fragment at *m/z* 173 in all spectra was produced by cleavage at C_{13}–C_{12}. Minor side products were identified by GC/MS analysis as 6,13-dihydroxy-γ-*N*-linolenoylamine (6,13-di$HOTNH_2$), 9-hydroxy-α-*N*-linolenoylamine (9-$HOTNH_2$) and 9,16-dihydroxy-α-*N*-linolenoylamine (9,16-di$HOTNH_2$) (Fig. 2A–C). In order to determine the absolute configuration of the chiral center, the pure main components were analyzed by CD spectroscopy. Because the CD spectra of the products showed a positive Cotton effect, like (13*S*)-HOD (data not shown) [18,19] and CP-HPLC analysis did not reveal the presence of two different enantiomers, it is concluded that the dioxygenation of the substrates yielded predominantly the *S*-enantiomer. This is in accordance with our previous observations, where C18:2 and C20 : 4 ethanolamides converted by sLOX also yielded mainly the *S*-enantiomer (> 92%) [11,14].

Thus, the main reaction products formed from α-OTNHEtOH and γ-OTNHEtOH and OTN-NH_2 with sLOX were identified as (13*S*)-hydroperoxy-*N*-octadeca-(9*Z*,11*E*,15*Z*)-trienoyl(ethanol)amine and as (13*S*)-hydroperoxy-*N*-octadeca-(6*Z*,9*Z*,11*E*)-trienoyl(ethanol)amine, respectively (Fig. 1).

Kinetics of the lipoxygenase reaction

Apparent K_m and k_{cat} of the four substrates with sLOX are given in Table 1, as well as the kinetic parameters of the

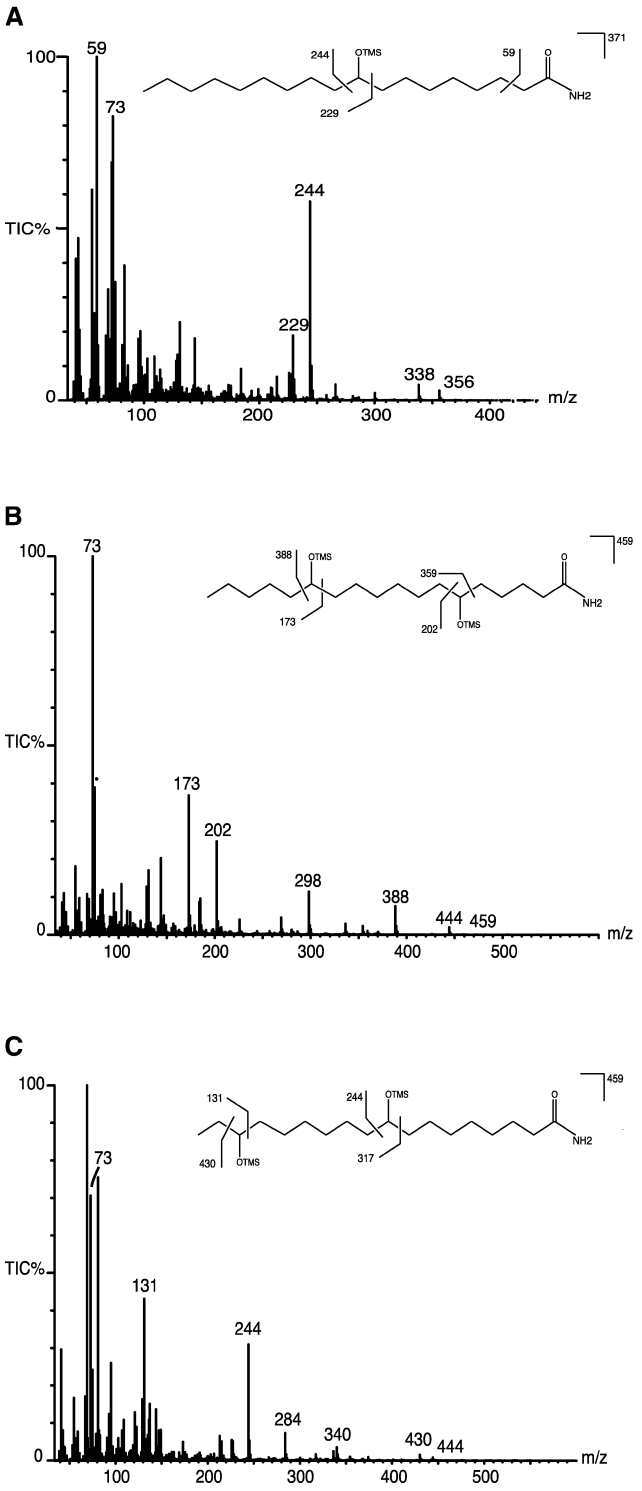

Fig. 2. Mass spectra of fully reduced TMS-ethers of (A) 9-hydroxy-*N*-linolenoylamine, (B) 6,13-dihydroxy-*N*-linolenoylamine, (C) 9,16-dihydroxy-*N*-linolenoylamine. GC/MS analyses were performed with a Carlo Erba GC 8060 with a Fisons MD800 mass detector equipped with a CP-Sil 5 CB-MS column (25m × 0.25 mm × 0.25 μm, Chrompack). The column temperature was held at 140 °C for 2 min, increased from 140 to 300 with 6.0 °C·min^{-1} and held at 300 °C for 2 min. Mass spectra were recorded under electron impact with an ionization energy of 70 eV.

Table 1. Kinetic parameters of sLOX with different substrates. Initial linear reaction rates were determined spectrophotometrically at 236 nm with various concentrations of substrate (4–80 μM) in 1 mL 0.1 M sodium borate buffer pH 9. K_m and k_{cat} were determined from the means of six measurements. Data were fitted to the standard Michaelis–Menten kinetic equation (GRAPHPAD PRISM).

Substrate	K_m (μM ± SD)	k_{cat} (s^{-1} ± SD)	k_{cat}/K_m
α-OT	39 ± 4	335 ± 70	8.6
α-$OTNH_2$	24 ± 4	113 ± 20	4.7
α-OTNHEtOH	16 ± 2	443 ± 66	28
γ-OT	23 ± 4	290 ± 62	13
γ-$OTNH_2$	14 ± 3	265 ± 69	19
γ-OTNHEtOH	17 ± 3	355 ± 81	21

corresponding fatty acids. K_m values are in the low micromolar range (14–39 μM) and the k_{cat} values are of the same order of magnitude, demonstrating a similar rate profile for both the α-fatty acid and γ-fatty acid series: OTNHEtOH > OT > $OTNH_2$.

HPO lyase and AOS activity with hydroperoxy *N*-acyl(ethanol)amines

To determine whether hydroperoxy fatty acid (ethanol)amides can be metabolized further into possible plant defense compounds, 13-$HPODNH_2$ and 13-HPODNHEtOH, α-13-$HPOTNH_2$ and γ-13-$HPOTNH_2$ and γ-13-HPODNHEtOH were tested as substrates for HPO lyase and AOS (Table 2). The HPO lyase activity with 13-HPODNHEtOH and α-13-HPOTNHEtOH as substrates was almost equal to the activity with the corresponding hydroperoxy fatty acids. The HPO lyase activity on the hydroperoxy fatty acid amides however, was ≈50% lower. HPO lyase was hardly active on hydroperoxy γ-linolenic acid and its derivatives. Apparent K_m and V_{max} of the 13-hydroperoxy-*N*-linoleoyl- and 13-hydroperoxy-α-*N*-linolenoyl (ethanol) amines with

Table 2. HPO lyase and AOS activity with different substrates. Initial linear rates were determined spectrophotometrically at 234 or 236 nm with a substrate concentration of 100 μM (30 μM for AOS) in 0.5 mL 50 mM (1 mL 100 mM for AOS) phosphate buffer, pH 6 (7 for AOS). Data reported are the means of six independent measurements. Because of the formation of a conjugated system in γ-ketols and 12-oxo PDA analogs (λ_{max} = 227 and 225 nm, respectively), the reaction rate of AOS with the substrates from which they are produced is actually higher than calculated from the decrease in absorbance at λ = 234 (or 236) nm.

Substrate	HPO lyase activity (U·mg^{-1} ± SD)	AOS activity (U·mg^{-1} ± SD)
13-HPOD	249 ± 10 (100%)	0.81 ± 0.04 (100%)
13-$HPODNH_2$	107 ± 20 (43%)	0.13 ± 0.01 (16%)
13-HPODNHEtOH	293 ± 18 (118%)	0.44 ± 0.01 (54%)
α-13-HPOT	428 ± 30 (100%)	0.12 ± 0.01 (100%)
α-13-$HPOTNH_2$	264 ± 20 (61%)	0.13 ± 0.001 (107%)
α-13-HPOTNHEtOH	394 ± 31 (92%)	0.32 ± 0.01 (268%)
γ-13-HPOT	24.6 ± 6.3 (100%)	0.09 ± 0.01 (100%)
γ-13-$HPOTNH_2$	34.9 ± 7.1 (141%)	0.04 ± 0.003 (48%)
γ-13-HPOTNHEtOH	42.5 ± 12 (173%)	0.08 ± 0.004 (86%)

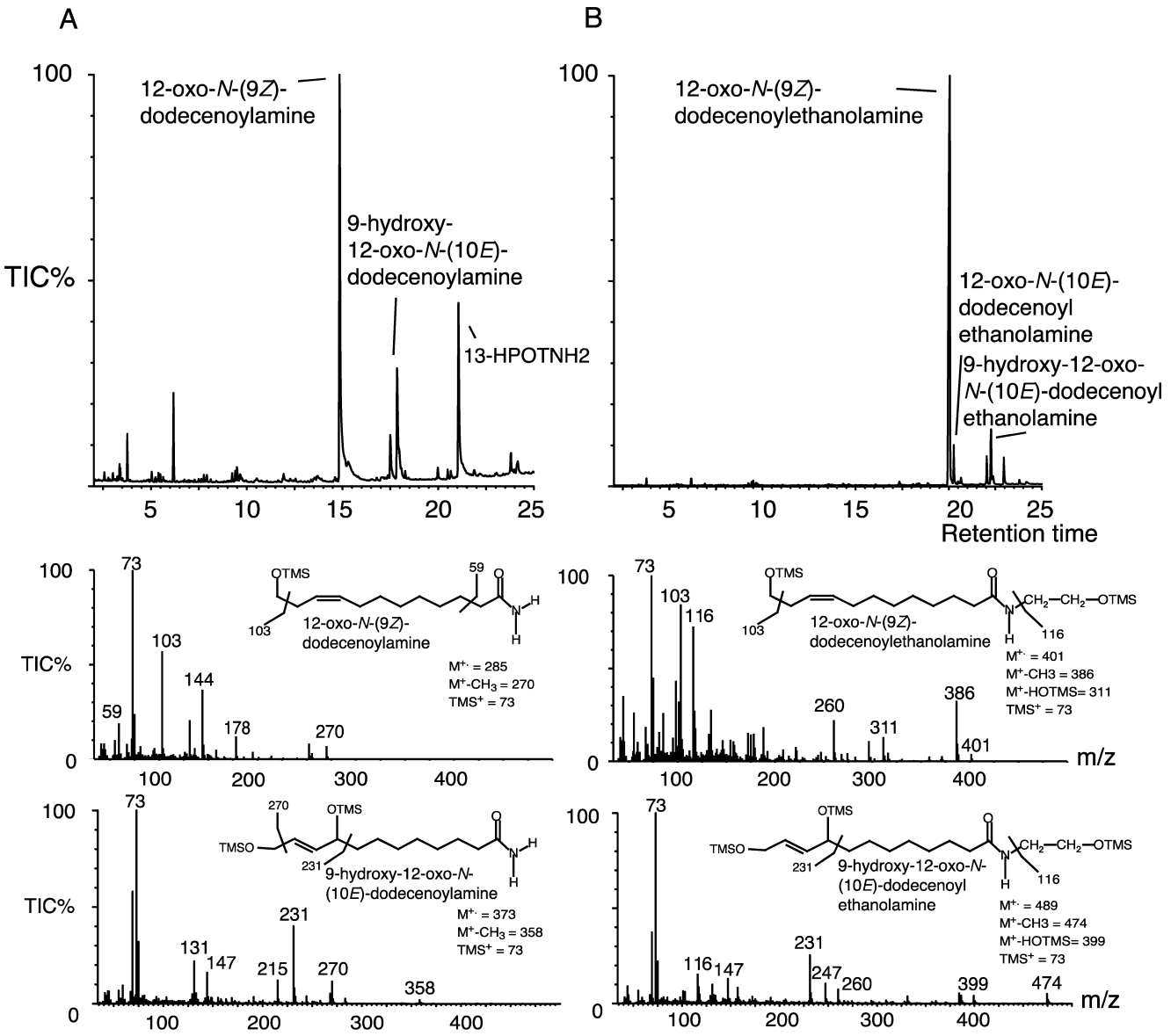

Fig. 3. Non-volatile reaction products formed by HPO lyase incubated with (A) 13-HPOTNH$_2$ or (B) 13-HPOTNHEtOH. GC/MS analysis of the fully reduced TMS-ethers of the extracted reaction products. The chromatograms of the incubations with 13-HPODNH$_2$ and 13-HPODNHEtOH were similar to (A) and (B), respectively. GC/MS-analyses were performed under similar conditions as described in the legend to Fig. 2.

HPO lyase, as well as the kinetic parameters of the corresponding hydroperoxy fatty acids, are shown in Table 3. The presence of the ethanolamide group in the substrate greatly increased K_m and V_{max}, whereas the amide group decreased K_m and V_{max}. AOS was able to use all hydroperoxy fatty acid amides as substrates. It was most active with 13-HPOD, 13-HPODNHEtOH and α-13-HPOTNHEtOH (Table 2).

Products formed by HPO lyase and AOS

The volatile products formed in the HPO lyase reactions with 13-hydroperoxy-*N*-linoleoyl(ethanol)amine or 13-hydroperoxy-*N*-linolenoyl(ethanol)amine were absorbed by SPME and identified by using GC as hexanal or (3*Z*)-hexenal, respectively. The nonvolatile products were reduced, methylated, trimethylsilylated and analyzed using GC/MS. The main product of the incubations with 13-HPODNH$_2$ and 13-HPOTNH$_2$ was identified as 12-oxo-*N*-(9*Z*)-dodecenoylamine (Fig. 3A). A small

Table 3. Kinetic parameters of HPO lyase with different substrates. Initial rates were determined as described in Table 2. Apparent K_m and V_{max} were determined from the means of six determinations with concentrations of substrate ranging from 5 to 150 μM. The data were fitted to the standard Michaelis–Menten kinetic equation (GRAPHPAD PRISM).

Substrate	K_m (μM ± SD)	V_{max} (U·mg^{-1} ± SD)	V_{max}/K_m (U·mg^{-1}·μM^{-1})
13-HPOD	152 ± 14	449 ± 25	3.0
13-HPODNH$_2$	85.3 ± 10	158 ± 9.0	1.9
13-HPODNHEtOH	199 ± 26	678 ± 60	3.4
α-13-HPOT	140 ± 15	676 ± 44	4.8
α-13-HPOTNH$_2$	213 ± 49	245 ± 40	1.2
α-13-HPOTNHEtOH	221 ± 21	987 ± 66	4.5

Table 4. Characteristic mass fragments of TMS-ethers of $NaBD_4$-reduced AOS-products of α-13-HPOTNHEtOH and α-13-$HPOTNH_2$: α-ketol, γ-ketol and 12-oxo-PDA derivatives (mass fragment; relative abundance). GC/MS-analyses were performed under similar conditions as described in the legend to Fig. 2.

Mass	α-ketol		γ-ketol	12-oxo-PDA	
(*m/z*)	NHEtOH	NH_2	NHEtOH	NHEtOH	NH_2
$M^{+\cdot}$	572 (< 1)	456 (< 1)	–	485 (1)	369 (< 1)
M^+-15[a]	557 (5)	441 (3)	557 (2)	470 (15)	354 (7)
M^+-90[b]	482 (< 1)	–	482 (1)	395 (7)	279 (8)
(M^+-15)-90	467 (1)	–	467 (4)	–	–
M-201/203[c]	371 (13)	255 (36)	–	–	–
a	401 (27)[d]	285 (71)[d]	360 (4)[e]	227 (11)[f]	227 (32)[f]
a-90	–	–	–	137 (5)[f]	137 (28)[f]
b	503 (4)[d]	387 (6)[d]	–	–	–
b-90	413 (14)[d]	297 (43)[d]	–	–	–
c	274 (4)[d]	274 (8)[d]	489 (8)[e]	–	–
c-90	184 (11)[d]	184 (21)[d]	399 (8)[e]	–	–
d	171 (12)[d]	171 (27)[d]	314 (3)[e]	–	–
d-90	–	–	224 (10)[e]	–	–
e	–	–	212 (2)[e]	–	–
TMS	73 (100)	73 (100)	73 (100)	73 (100)	73 (100)

[a] Loss of CH_3. [b] Loss of TMSOH. [c] Rearrangement with loss of OHC-CH(OTMS)-C_5H_{11} or OHC-CH(OTMS)-C_5H_9. [d] See Fig. 4A for fragmentation pattern. [e] See Fig. 4B for fragmentation pattern. [f] See Fig. 4C for fragmentation pattern.

amount of the isomer 12-oxo-*N*-(10*E*)-dodecenoylamine was also present. This product showed a mass spectrum similar to the 9*Z*-isomer, except that the *m/z* 103 peak was replaced by a *m/z* 129 peak [$C_3H_4OTMS^+$]. Furthermore, 9-hydroxy-12-oxo-*N*-(10*E*)-dodecenoylamine was present, as judged by its mass spectrum, which shows the same characteristic features as the mass spectrum of 9-hydroxy-traumatin (Fig. 3A) [20]. The main product of the incubations with 13-HPODNHEtOH and 13-HPOTNHEtOH was identified as 12-oxo-*N*-(9*Z*)-dodecenoylethanolamine (Fig. 3B). 12-Oxo-*N*-(10*E*)-dodecenoylethanolamine and 9-hydroxy-12-oxo-*N*-(10*E*)-dodecenoylethanolamine were also present. The mass spectrum of 12-oxo-*N*-(10*E*)-dodecenoylethanolamine was similar to the 9*Z*-isomer with the *m/z* 103 peak replaced by a *m/z* 129 peak [$C_3H_4OTMS^+$]. When a crude extract of alfalfa seedlings was incubated with α-OTNHEtOH, (2*E*)-hexenal, (2*E*,6*Z*)-nonadienal, 12-oxo-*N*-(10*E*)-dodecenoylethanolamine, 9-hydroxy-12-oxo-*N*-(10*E*)-dodecenoylethanolamine and 9-oxo-*N*-nonanoylethanolamine were formed, indicating that both lipoxygenase and HPO lyase in alfalfa were active on this new substrate.

RP-HPLC analysis revealed that three products were formed out of (13*S*)-hydroperoxy-α-*N*-linolenoyl(ethanol)amines by AOS, i.e. α-ketols and γ-ketols and the 12-oxo-*N*-phytodienoyl(ethanol)amines, as judged by GC/MS. The (13*S*)-hydroperoxy-γ-*N*-linolenoyl(ethanol)amines yielded mainly the α-ketol, whereas the linoleoyl analogs gave rise to both α-ketols and γ-ketols. Characteristic mass fragments of the AOS products of α-13-HPOTNHEtOH and α-13-$HPOTNH_2$ are listed in Table 4 and Fig. 4. The mass fragments of the AOS products of 13-HPODNHEtOH, 13-$HPODNH_2$, γ-13-HPOTNHEtOH and γ-13-$HPOTNH_2$ are not shown. The mass spectrum of the γ-ketol from 13-$HPOTNH_2$ is not given because of a low response and similar retention times of the α-ketols and γ-ketols. The acetone extract of flax seed does not contain an allene oxide cyclase [21] therefore, the 12-oxo-PDA analogs are generated by a spontaneous nonenzymatic reaction, yielding a racemic mixture.

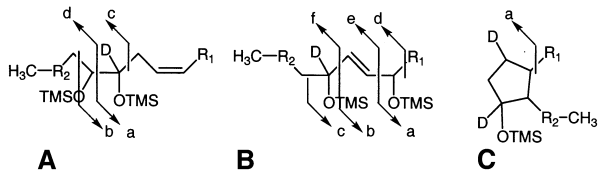

Fig. 4. Fragmentation patterns of TMS-ethers of $NaBD_4$ reduced AOS-products. (A) α-Ketols, (B) γ-ketols and (C) 12-oxo-PDA derivatives. Arrows indicate site of fragmentation and the letters correspond to the letters in Table 4.

DISCUSSION

N-acyl(ethanol)amines as substrates for lipoxygenase

The oxylipins traumatin and jasmonic acid are produced by the lipoxygenase pathway and used by plants as mediators in their defense reaction against pathogens [7]. Recently, the involvement of NAEs in plant resistance strategies was proposed by Chapman *et al*. [4]. To date, it had not been known whether the lipoxygenase pathway could utilize NAEs (18:3) as substrates. We demonstrated that sLOX converted α-*N*-linolenoyl(ethanol) amine and γ-*N*-linolenoyl(ethanol)amine in a regio-and stereoselective manner into their (13*S*)-hydroperoxide derivatives (Fig. 1). Remarkably, only the *N*-linolenoylamines were double dioxygenated. The extension of the headgroup of the hydroperoxy fatty acid with ethanolamine probably precludes it from being accepted as a substrate. This supports the concept of the inverse substrate orientation for double-dioxygenation of substrates by 15-lipoxygenases [22].

The results presented here extend our previous study in which we found similar regioselectivity and stereoselectivity

and a comparable rate profile of sLOX with 18:2 derivatives [14]. The enzyme kinetics with the fatty acid amides as substrates were quite similar to the reaction with the corresponding fatty acids. The comparable reactivities of the novel substrates and their corresponding fatty acids towards sLOX suggest that the fatty acid amides are possible lipoxygenase substrates *in vivo*. It is noteworthy that the catalytic efficiencies of sLOX with the ethanolamide substrates were even higher than those of the corresponding free fatty acids (Table 1).

Hydroperoxy *N*-acyl(ethanol)amines as substrates for HPO lyase and AOS

The formed hydroperoxy fatty acid (ethanol)amides were good substrates for HPO lyase and AOS (Fig. 1). Similar to sLOX, the hydroperoxy fatty acid ethanolamides were as good as or even better substrates for HPO lyase than the corresponding free fatty acids. This was also the case for hydroperoxy α-*N*-linolenoylethanolamine with AOS. In contrast, replacement of the carboxyl group by a methyl ester or alcohol greatly reduced the HPO lyase activity [23,24]. The polarity of the headgroup of the substrates seems to have an important influence on HPO lyase activity. Because the large ethanolamide group increases the activity, steric hindrance is likely to be of minor interest. Substrates that have a lower K_m appear to have a lower V_{max} indicating a slower release of the reaction products. Alfalfa HPO lyase was barely active on γ-linolenic acid and its derivatives. Low activity on γ-linolenic acid was also observed for HPO lyase from tea leaves and tea chloroplasts [23,25].

In addition to the known HPO lyase products (i.e. traumatin derivatives and volatile aldehydes), 9-hydroxy-traumatin derivatives were also formed, as judged by GC/MS analyses. We also observed the formation of 9-hydroxy-traumatin with 13-HPOD or 13-HPOT as substrates. Gardner [20] suggested that the oxygenation of traumatin is catalyzed by lipoxygenase and a peroxygenase. In our purified enzyme preparation, however, there was no lipoxygenase activity present. In these experiments 9-hydroxy-traumatin might be formed non-enzymatically or by a monooxygenase activity of HPO lyase. It is noteworthy that many cytochrome P450 enzymes are known to have monooxygenase activity [26]. However, in addition to these experiments further studies are required to obtain more insight into the mechanism of 9-hydroxy-traumatin formation.

Similarities between plant and animal defense systems and possible functions of NAEs in plants

We have shown that the *N*-acyl(ethanol)amines (18:2) and (18:3) can be converted by LOX, HPO lyase and AOS, yielding a new class of phytooxylipins (Fig. 1). Their physiological roles remain unknown, but they may have a function in plant defense. Several features of the plant signal transduction system for defense against herbivores are strikingly similar to the defense signaling systems of animals against pathogens and parasites, as outlined in Bergey *et al*. [27]. It is thought that the two systems developed through divergent evolution from an ancestral organism that had fundamental components [27]. Similarities between NAE metabolism in plants and the animal kingdom are also being discovered [4]. As in animals, plant NAEs are released from NAPEs, a minor lipid fraction, by the signal-induced action of phospholipase D [4,28]. They accumulate extracellularly and are degraded by a fatty acid amide hydrolase-like activity [4,29]. We have shown that the oxidative metabolism of NAEs by plant lipoxygenases is also similar to that in animals [14,30]. Furthermore, the subsequent metabolism of the lipoxygenase products by AOS is analogous to the action of cycloxygenase-2 on anandamide, which yields the prostaglandin analog, PGE_2-ethanolamide [31].

Based on analogy with the animal kingdom it is tempting to speculate that plant NAEs, in particular α-OTNHEtOH and/or their oxylipin derivatives, may function as stress recovery factors in the plant defense system. We demonstrated previously that lipoxygenase products derived from anandamide and *N*-linoleoylethanolamine, i.e. (13*S*)-hydroxy-*N*-linoleoylethanolamine and (15*S*)-hydroxyanandamide, may act as competitive inhibitors of mammalian fatty acid amine hydrolase [32,33]. We suggested that these oxylipins might prolong the pharmacological actions of anandamide [32,33]. This 'entourage' effect may also be active in plants. Oxylipins of plant NAEs may also have direct physiological effects, as demonstrated for other structurally related headgroup analogs of jasmonic acid. For example, leucine and isoleucine conjugates with jasmonic acid lead to the up-regulation of specific genes, the down-regulation of house-keeping genes and the production of volatiles [34,35]. Taken together, it would be interesting to screen plants for the occurrence of these novel phytooxylipins and test whether these oxylipins alter the physiological responses of plants to pathogens.

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SUPPLEMENTARY MATERIAL

The following material is available from
http://www.blackwell-science.com/ejb/

Table S1. NMR-data of sodium borohydride reduced sLOX-products.

Table S2. Characteristic mass fragments of TMS-ethers of NaBD4-reduced AOS-products of 13-HPODNHEtOH and 13-HPODNH$_2$.

Table S3. Characteristic mass fragments of TMS-ethers of NaBD4-reduced AOS-products of γ-13-HPOTNHEtOH and γ-13-HPOTNH$_2$.