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A RAPID ASSAY FOR THE COUPLED CELL FREE GENERATION OF OXYLIPINS

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Key Word Index—fatty acid; hydroperoxide; colneleic acid; tomato; lycopersicon esculentum; Solanaceae; roots; octadecanoid.

Abstract—We developed a rapid and simple assay for the coupled *in vitro* synthesis of oxylipins using free unsaturated fatty acids as substrates. Reactions were catalysed with extracts expressed from living plant tissues. Preliminary experiments involving the cell free transformation of fatty acid hydroperoxides revealed that storage or pretreatment of the plant extract rapidly altered its capacity to catalyse the generation of oxidised fatty acid derivatives. This could reflect changes in oxylipin generation that might take place *in situ* in damaged plant cells during herbivory. All subsequent experiments were performed without dilution, titration or any other modification of the plant extract prior to its addition to the assay system. The assays were used to study, for the first time, tissue-specific differences in fatty acid transformation to divinyl ethers. Root tissues from tomato efficiently catalysed the formation of colneleic and colnelenic acids from linoleic acid and linolenic acids, respectively, whereas leaf, hypocotyl and cotyledon extracts did not promote the formation of these compounds. We observed the efficient generation of 9-oxo-nonanoic acid from the substrate linolenic acid and speculate that this aldehyde could arise either from the action of hydroperoxide lyase on 9-hydroperoxylinolenic acid or by a novel route involving cleavage of colnelenic acid which was also present among the products of the reaction. A potential role of divinyl ethers as substrates for the generation of toxic aldehydes is discussed.

INTRODUCTION

Several pathways for the biogenesis of regulatory and antimicrobial fatty acids are known in plants. In general these biosynthetic pathways are complex multistep processes beginning with the addition of molecular oxygen to (*Z*,*Z*)-1,4-pentadiene-containing fatty acids to generate fatty acid hydroperoxides. These reactive molecules then undergo catalysed rearrangements to form a large variety of oxidised fatty acid derivatives collectively termed 'oxylipins'. For example, jasmonic acid and its precursor 12-oxo-phytodienoic acid are derived from 18 carbon precursors such as linolenic acid via the jasmonate pathway [1, 2, 3]. Hydroperoxide lyase and associated enzymes produce a variety of alkanals and alkenals from fatty acid hydroperoxides [4]. Cutin precursors are derived via the peroxygenase pathway [5, 6]. All these pathways require the participation of several enzymes, many of which are difficult to assay. Indeed, while our knowledge of the nature of oxylipins and toxic fatty acids increases our understanding of the regulation of the biogenesis of these compounds lags behind as does the attribution of biological roles to many of these molecules.

Despite the complexity of oxylipin production in plants it is clear from many studies that the coupled generation of some of these molecules can be observed in cell free systems [7, 8]. In these cases extracts of plant tissues have been first processed by dilution into buffers and/or partial purification. In other cases organelles or organellar membranes have been isolated and subfractionated and shown to catalyse coupled oxylipin generation *in vitro* [9]. The complex and delicate nature of the enzymes involved in oxylipin generation may lead to loss or modification of function during extraction and we thus decided to investigate whether plant juice rapidly expressed from intact tissues could catalyse oxylipin production.

We describe herein a simple assay system which might facilitate progress in the areas of fatty acid signal biogenesis and function. Using fresh plant extracts as catalysts the assay preserves the activity of

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several apparently delicate coupled enzyme reactions and allows the efficient cell free generation of complex oxylipins directly from unsaturated fatty acid precursors. We have investigated several 9-hydroperoxide-derived fatty acid derivatives in some detail as evidence for biological roles for these molecules lags behind what we know for oxylipins derived from fatty acid 13-hydroperoxides. Our results suggest that the assays we have developed may help in understanding both the biosynthesis and the biological roles of oxylipins.

RESULTS AND DISCUSSION

The idea to add plant juices (rather than diluted and/or partially purified extracts) to an assay system was influenced by a quantitative assay for proteinase inhibitor proteins developed by Ryan [10]. In this assay juice is expressed from the plant tissue and used directly, without dilution or any other manipulation. We reasoned that similar use of expressed juice might allow the assay of enzymes capable of catalysing the complex, coupled generation of fatty acid derivatives. Secondly, we reasoned that the coupled generation of oxylipins might by highly unstable and we wished to avoid having to measure separately each of the enzymes of a metabolic pathway. The liberation of cellular contents is exactly what happens on wounding a plant tissue and we were interested to know whether cellular debris (plant juice) is capable of catalysing the formation of oxylipins and whether this capacity changes qualitatively or quantitatively over time.

Our first experiments involved the use of linolenic acid 13-(*S*)-hydroperoxide (13-HPOTrE) to establish whether or not unprocessed plant extracts could catalyse oxylipin generation and to establish the (in)stability of these reactions. We chose to study this reaction rather than the conversion of a fatty acid since it is simpler, bypassing the lipoxygenase step and giving products derived from oxygen rearrangements at carbon 13. The 13-hydroperoxide of linolenic acid is readily converted to α-ketol and 12-oxo-phytodienoic acid by the enzymes allene oxide synthase and allene oxide cyclase [1]. Both these products are well characterised and easy to measure by GC-mass spectroscometry. Many other possible metabolites of 13-HPOTrE exist and we reasoned that some of these other compounds may be generated *in vitro*. We expressed tomato leaf juice and assayed its capacity to catalyse the transformation of 13-HPOTrE. We tested both freshly expressed juice and a batch of juice that had been allowed to 'age' in an open container at room temperature for 10 minutes prior to the assay. Before carrying out the experiment we noted that 13-HPOTrE poisons our assay, perhaps inhibiting allene oxide synthase and/or allene oxide cyclase (data not shown) and we thus kept its concentration to a minimum using only the carrier-free [1-^{14}C] linolenic acid 13-hydroperoxide at a concentration of 1.13 to 2.26 μM in the assay. Reactions were initiated by the addition of 1 μl of expressed leaf juice. Labelled compounds resulting from the transformation of HPOTrE were extracted and separated by TLC. The location of radioactive bands was identified and quantitated by exposure to a storage phosphor screen. In parallel experiments involving larger quantities of unlabelled fatty acids, oxylipin-containing bands were scraped off the TLCs and identified by GC-mass spectrometry. Often bands seen by phosphorimaging or autoradiography contain multiple components. We generally analysed the contents of bands scraped off TLC plates in parallel experiments using non-radioactive substrates. These analyses were conducted by HPLC (not shown) prior to analysis by GC-mass spectrometry.

Figure 1 shows that leaf extracts catalyse the formation of oxylipins from the radioactive substrate [1-^{14}C]-13(*S*) hydroperoxylinolenic acid (HPOTrE). Several of the oxylipins generated *in vitro* in this reaction were identified. Three other compounds that were not identified are indicated by question marks. Chief among the products generated by fresh leaf juice were 12-oxo-phytodienoic acid (OPDA) and the α-ketol 13-hydroxy-12-oxo-9(*Z*)-15(*Z*)-octadecadienoic acid

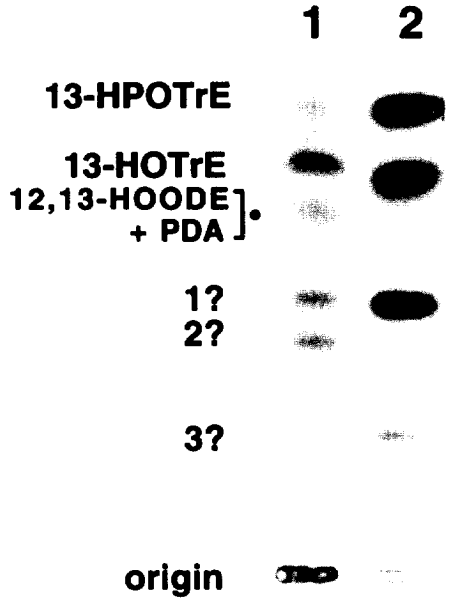

Fig. 1. Ageing if isolated leaf juice alters its ability to catalyse the formation of oxylipins from linolenic acid 13-hydroperoxide (13-HPOTrE). The substrate, [1-^{14}C]-13(*S*)-HPOTrE (0.25 μg, 1.11×10^{11} dpm mmol^{-1}) in 0.4 ml sodium acetate/acetic acid buffer, 40 mM, pH 5 containing 10% glycerol), was incubated for 10 min at 30° in the presence of freshly expressed leaf juice (Lane 1) or juice that was expressed 10 min prior to the assay (Lane 2). In each case 1 μl of juice (30 μg protein) was employed. Products were extracted and displayed as an autoradiogram after TLC using solvent system 1. 13-HPOTrE, 13-hydroxylinolenic acid; HOODE, 13-hydroxy-12-oxo-9(*Z*), 15(*Z*)-octadecadienoic acid; PDA, 12-oxo-phytodienoic acid. HOODE and PDA, which are not separated by the TLC method used were quantified as their methyl derivatives by GC-mass spectrometry, their ratio was 4:1.

(HOODE). The TLC solvent system used in Fig. 1 did not separate two of the products generated, OPDA and HOODE and their ratio was established by GC-mass spectrometry and HPLC using standards. The presence of these compounds in a ratio of 4:1 indicates that both the enzymes allene oxide synthase and allene oxide cyclase function well in our assay [11]. We chose to buffer the assay at pH 5 as it may represent the acidic pH generated on release of vacuolar contents when a leaf cell is wounded. Additional experiments at a series of different pHs from 5 to 9 (not shown) revealed increased activities of AOS at higher pH and also higher ratios of HOODE/OPDA at higher pH. It is clear from Fig. 1 that product formation was drastically altered when juice was allowed to age for 10 min prior to use in the assay. Several compounds of unknown identity were abundant in the reaction catalysed by aged juice. These results interested us for two reasons. Firstly, they showed the feasibility of our approach, using unmodified plant extracts to catalyse rapid oxylipin generating reactions. Secondly, they showed that the catalytic properties of leaf juice change over time. This is likely to occur in nature immediately after a tissue is wounded, for example by a herbivore. Oxylipin generation would probably continue in the cellular debris at the wound site. Over time, the spectrum of oxylipins generated in the cellular debris would change perhaps even generating dead cell specific oxylipins. This line of research will be well worth following but, for the present study, the principal conclusion drawn from experiments, such as that shown in Fig. 1, was that for certain experiments the immediate use of fresh extracts may be essential to avoid modification/activation or degradation of oxylipin synthesising enzymes. Thus all subsequent experiments reported herein employed tissue extracts that were instantly added to the assay system.

Based on what we learnt from preliminary experiments such as that shown in Fig. 1 we decided to try to catalyse more complex multistep reactions using unsaturated fatty acids as substrates. Juice was expressed from root, leaf, cotyledon and hypocotyl of tomato plants. The juice was immediately used to catalyse oxylipin formation from the ^{14}C-labelled parent fatty acids 18:3 and 18:2. Products were extracted, separated by TLC and displayed as autoradiograms as shown in Fig. 2. Among the compounds formed were two divinyl ethers, colneleic acid (CA, derived from 18:2) and colnelenic acid (CnA, derived from 18:3). The efficient root extract-catalysed production of CA and CnA allowed their purification by HPLC (not shown) and characterisation by GC-mass spectrometry. The methyl ester of CA yielded a spectrum in full agreement with the literature [7] with a parent ion at $[M]^+ = 308$ (30%) and fragments at $m/z = 251$ (10%), 165 (15%), 137 (20%), 123 (25%), 109 (30%), 95 (55%), 81 (90%) and 67 (100%). To further assure correct identification of CA the compound was reduced with H_2/PtO_2 to the fully saturated ether hexahydroCA which, after methylation, also gave a spectrum in agreement with that previously published [7]. Mass spectral analysis was also used to confirm the identity of the methyl ester of CnA; this compound gave a parent ion of $[M]^+ = 306$ (15%), as well as fragments of $m/z = 169$ (5%), 149 (5%), 137 (15%), 121 (35%), 105 (25%), 93 (65%) and 79 (100%). Only roots catalysed the efficient formation of divinyl ethers. Tissues from which extracts did not catalyse divinyl ether synthesis (leaves, cotyledons and hypocotyls) have in common chlorophyll. It has been suggested that chlorophyll inhibits lipoxygenase in *in vitro* assays, thus reducing the ability of leaf-derived enzymes to catalyse the first step of divinyl ether synthesis [12]. The low levels of chlorophyll in hypocotyl extracts argue against this and we favour the possibility that our assay reveals genuine differences between tissues in their ability to catalyse divinyl ether synthesis *in vivo* and *in vitro*. The results shown in Fig. 2 provide striking evidence for the tissue specific differences in the ability of tomato to catalyse oxylipin generation. CA, a 9-hydroperoxylinoleic acid-derived divinyl ether has been shown to be produced *in vitro* in reactions catalysed by potato tuber extracts [7, 13, 14]. Garlic extracts were shown to catalyse the formation of etheroleic and etherolenic acid from the 13-hydroperoxides of linoleic and linolenic acids, respectively [15].

The effect of pH on CnA synthesis *in vitro* was examined using linolenic acid (18:3) as a substrate. Such a study provides necessary preliminary information which will be useful in future experiments on potential biological roles of divinyl ethers and other fatty acid 9-hydroperoxide derivatives. Reactions at different pHs were catalysed by the addition of 10 μl fresh, undiluted tomato root extract (from which debris had been removed by centrifugation) into 0.39 ml assay buffer. The generation of CnA as well as a variety of other 9-hydroperoxide derived compounds was observed (Fig. 3). The production of CnA was optimal at pH 7.5 to 8 in agreement with conditions necessary for the *in vitro* formation of this compound catalysed by potato tuber derived enzymes [7]. However it is not known whether the appropriate conditions for efficient divinyl ether synthesis exist in living tomato roots. Another compound observed was 9-hydroxy, 10-oxo-octadecadienoic acid (9,10-HOODE), which was also identified by mass spectroscopy (see Experimental) and was produced optimally between pH 8 and 8.5. We noted the formation of the aldehyde 9-oxo-nonanoic acid (NON) at lower pHs. It is known that C9 carbonyl-containing molecules can arise from CA by both enzyme-catalysed and non-enzymatic breakdown [16]. These molecules, however, were not characterized. We suggest that CnA might be enzymatically cleaved to NON and that the analogous reaction from CA would also be possible. Alternatively, formation of NON by the action of hydroperoxide lyase on fatty acid 9-hydroperoxides is possible and further work will be needed to distinguish these two possibilities. The results

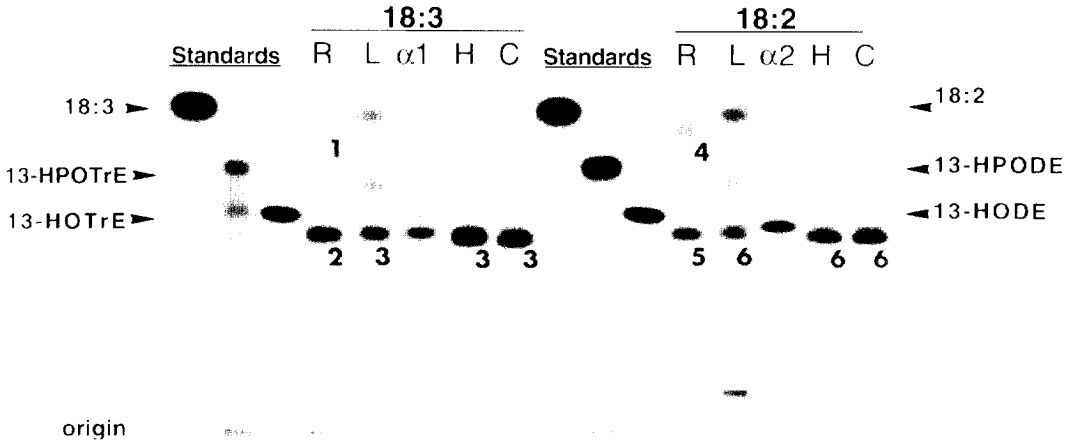

Fig. 2. Tissue specificity of colnelenic acid (CnA) and colneleic acid (CA) production from their free fatty acid precursors. Freshly expressed juice from tomato roots (R, 10 μl juice), leaves (L, 1 μl juice), hypocotyls (H, 12.5 μl juice) and cotyledons (C, 2.5 μl juice) were used to catalyse the formation of oxylipins. In each case the volume of juice used corresponded to 30 μg protein. Substrates were either [1-^{14}C]-linolenic acid (18:3) or [1-^{14}C]-linoleic acid (18:2) both at 0.126 μg, (1.11×10^{11} dpm mmol^{-1}) and were incubated at pH 7 (in 0.4 ml 40 mM MOPS–KOH buffer containing 10% glycerol) for 10 min at 30° prior to separation by TLC using solvent system 1. The resulting autoradiogram is shown. The position of the following compounds are indicated: 13-hydroperoxylinolenic acid (13-HPOTrE); 13-hydroxylinolenic acid (13-HOTrE); 13-hydroperoxylinoleic acid (13-HPODE); 13-hydroxylinoleic acid (13-HODE). The compounds indicated on the autoradiogram are 1, colnelenic acid. 2, 9-hydroxy-10-oxo-12(*Z*),15(*Z*)-octadecadienoic acid. 3, 13-hydroxy-12-oxo-9(*Z*),15(*Z*)-octadecadienoic acid (12,13-HOODE) and 12-oxo-phytodienoic acid. 4, colneleic acid. 5, 9-hydroxy-10-oxo-12(*Z*)-octadecaenoic acid (9,10-HOOE). 6, 13-hydroxy-12-oxo-9(*Z*)-octadecanoic acid (13, 12-HOOE) plus 9,10-HOOE. Two additional standards are indicated, α1, 13,12-HOODE, α2, 13,12-HOOE.

shown in Figs 2 and 3 provide a starting point for a more detailed analysis of the production of fatty acid divinyl ethers and other 9-hydroperoxide-derived compounds in tomato tissues. The biological functions of fatty acid divinyl ethers are unknown. Can we learn anything about the potential functions of these molecules from the experiments presented herein?

The biological role of the 13-hydroperoxidation of linolenic acid is now well established [17]. 13-hydroperoxylinolenic acid is converted to members of the jasmonate family of signals important in the regulation of many wound-inducible genes as well as in several developmental processes. Lipoxygenases from plants are able to add molecular oxygen to either (or in some cases both) the 9 and 13 positions of linoleic and linolenic acids. The biological roles of fatty acid 9-hydroperoxide derivatives are largely unknown [18] although fatty acid 9-hydroperoxides may be substrates for hydroperoxide lyases resulting in the formation of toxic alkanals and alkenals [4]. We feel it worthwhile to investigate the potential roles of 9-hydroperoxide-fatty acid derivatives and our rapid assay provides an ideal starting point. We chose to begin such a study with the cell free synthesis of 9-hydroperoxide derivatives including CA and CnA. The high efficiency of divinyl ether synthesis in our assays should allow us to study the question of whether CA (or CnA) can be degraded to potentially toxic and antimicrobial alkenals such as NON (Figure 3). If CA and CnA are degraded to NON and a second aldehyde (probably 3*Z*-nonenal in the case of CA) the divinyl ethers themselves might be precursors for the production of toxic defense-related aldehydes and their further metabolites. This might imply the existence of a previously unrecognised metabolic pathway from fatty acids via divinyl ethers to alkenals and alkanals. Further experiments will be necessary to test whether NON originates directly from CA and/or CnA.

In summary our assays depend on the use of fresh, unmodified plant extracts to catalyse the transformation of free fatty acids (or their hydroperoxides) to oxylipins. The assays are rapid, simple and sensitive and could be used to report the effect of numerous treatments of plants on oxylipin metabolism. The results offer a means of comparing different plant tissues or different plants for their ability to catalyse oxylipin generation but we caution the need to support the data obtained with data on the *in vivo* production of oxylipins. Our assay is intended as a complement and support for such measurements. We have used the assays to show, for the first time, a dramatic tissue-specific difference in divinyl ether synthesis catalysed by different tissues from the same plant. The assays should have a variety of other uses including as an aid to the rapid identification of mutants in oxylipin metabolism [9]. So far we have investigated cell free fatty acid transformation in the absence of cosubstrates, such as fatty acid hydroperoxides or cofactors, for example reduced dinucleotides which are essential for the generation of many oxygenated fatty acid derivatives [20]. Inclusion of such cofactors might extend the use of the assay. The assay should prove

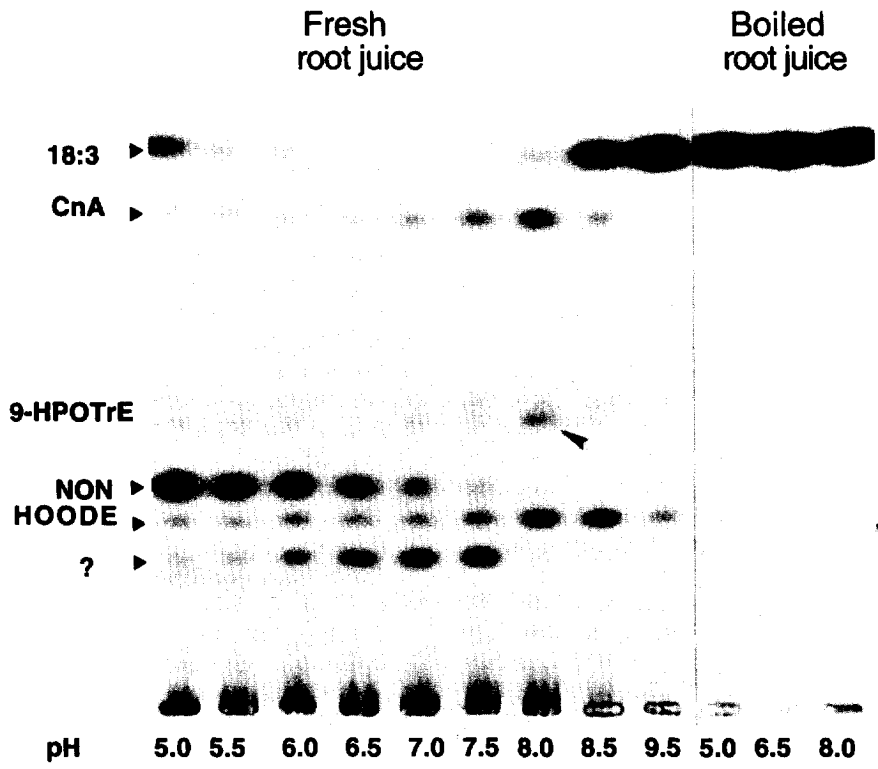

Fig. 3. Use of rapid assay to study the pH-dependent formation of colnelenic acid (CnA) and other oxylipins from linolenic acid (18:3). The reaction mixtures contained 0.126 μg [1-^{14}C]-linolenic acid (1.11×10^{11} dpm mmol^{-1}) and reaction was initiated by the addition of 10 μl root juice (30 μg protein). The reactions (total volume 0.4 ml) were allowed to proceed for 10 min at 30° at the pHs indicated. See Experimental for details of buffers used. Reaction products were separated by TLC in solvent system 2 (three developments) and are shown here in the form of an autoradiogram. The left hand side of the panel shows compounds generated in the presence of fresh root juice. On the right of the panel are control reactions initiated with boiled leaf juice. The compounds are 18:3, linolenic acid; CnA, colnelenic acid; 9-HPOTrE (indicated with arrowhead), 9-hydroperoxylinolenic acid; NON, 9-oxo-nonanoic acid; HOODE, 9-hydroxy, 10-oxo-octadecadienoic acid. Note that 9-HODE and 9,10-HOODE comigrate in this system. The compound marked ? may be a degradation product of 9,10-HOODE.

generally useful in the study of oxylipin production in all tissue types in healthy, injured and diseased plants.

EXPERIMENTAL

Plant materials. For leaf production tomato (*Lycopersicon esculentum* var. Bonny Best) were soil grown for two weeks under 16 hr days (300 μE m^{-2} s^{-1} light at 27°, 74% relative humidity; 8 hr dark, 17°, 68% relative humidity). Leaf juice was expressed directly. For experiments involving root tissue (Figs 2 and 3) tomato seeds were planted in sand and otherwise grown under identical conditions. Two week old seedlings were removed from the sand and roots were ground in a pestle and mortar. Extracts from soil grown tomato behave identically in terms of their activity in the assay. Protein concns in plant extracts were established with the BCA assay (Pierce Chemical Co., Rockford, IL, USA). Tomato tissues contained the following quantities of protein per μl juice: leaf, 30 μg; root, 3 μg; cotyledon, 12 μg; hypocotyl, 2.4 μg.

Assays. [1-^{14}C]-13(*S*)-hydroperoxylinolenic acid (13-HPOTrE, specific activity 1.11 dpm mmol^{-1}) was synthesised from [1-^{14}C]-linolenic acid (DuPont NEN) using soybean lipoxygenase [21]. For assays using free fatty acids as starting substrates [1-^{14}C]-linoleic acid or [1-^{14}C]-linolenic acid (DuPont NEN) was adjusted to various specific activities by addition of the respective nonlabelled fatty acid. The standard assay was conducted in a total vol. of 0.4 ml containing the fatty acid or fatty acid hydroperoxide. The reaction was initiated by addition of freshly expressed juice which was briefly centrifuged (2 min $\times$ 10 500 *g*) before use in the assay. Reactions were typically for 10 min at 30°. Nonpolar products were extracted from the assay with $CHCl_3$–MeOH (2:1).

The following solvent systems were used for TLC on silica plates: System 1; ET_2O–petrol–HCO_2H (70:30:1); System 2; Petrol–ET_2O–HCO_2H (70:30:1). Thin layer plates were analysed by quantitative phosphor imaging (GS 250 Molecular Imager, BioRad, Switzerland). For experiments at different pHs the following buffer systems were used: pH 5.0 and 5.5, NaOAc–HOAc, 40 mM; pH 6.0 and 6.5, MES–KOH 40 mM; pH 7.0 and 7.5, MOPS–KOH 40 mM; pH 8.0 and 8.5, Tris–HCl 40 mM; pH 9.0 and 9.5, H_3BO_3–KOH, 40 mM. In addition all buffers contained 10% glycerol. Note that the buffers were not adjusted for

constant ionic strength and this fact should be taken into account in interpreting the data. HPLC was routinely used to investigate the detailed composition of reaction products. Divinyl ethers were sepd on an analytical silica column with hexane–*iso*-PrOH–HCO_2H (99.5:0.5:0.1, solvent A) and hexane–*iso*-PrOH–HCO_2H (97:3:0.1, solvent B). Conditions were solvent A 100% for 15 min, addition of solvent B to 2% in solvent A over one min then held constant for 30 min. CA and CnA were eluted at approximately 14 min. Elution was monitored at 252 nm (λ_{max}) or 234 nm.

Analytical methods. GC-MS was carried out in the positive ion mode on a Hewlett Packard 5890 GC fitted with a 5% cross linked phenyl methyl siloxane column (30 m × 0.25 mm i.d.). All molecules were methylated with CH_2N_2. Keto groups were derivatised with methoxylamine (MOX). Secondary alcohols were derivatised with *N,O*-bis(trimethylsilyl)trifluoroacetamide.

Compounds had the following mass spectra: 13-HOTrE, agrees with [21]. OPDA; agrees with [22]. 12,13-HOODE; agrees with [22]. 9-HOTrE, agrees with [23]. 9-KODE, agrees with [23]. 9,10-HOODE; parent $[M]^+ = 425$ (2%), $m/z = 410$ (10%), 335 (10%), 304 (20%), 269 (25%), 259 (25%), 199 (20%) 155 (40%) and 73 (100%). 9,10-HOOE; parent $[M]^+ = 427$ (1%), $[M\text{-}15]^+ = 412$ (13%), $m/z = 271$ (52%), 270 (29%), 259 (24%), 240 (30%), 155 (23%), 109 (21%) and 73 (100%). CA; see text, agrees with [7]. HexahydroCA; $[M]^+ = 314$ (2%) $m/z = 283$ (5%), 201 (5%), 187 (45%), 172 (30%), 155 (100%), 138 (70%) and 129 (30%), agrees with [15]. CnA; see text, agrees with [13]. NON, methylated, free aldehyde, $[M\text{-}31]^+ = 155$ (25%), $m/z = 143$ (37%), 111 (42%), 87 (82%) and 74 (100%). NON, methylated, MOX-derivative $[M]^+ = 215$ (2%), $[M\text{-}31]^+ = 184$ (23%), $m/z = 152$ (12%), 124 (14%), 86 (49%) and 73 (100%).

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