Formation of cyclopentenones from all-(*E*) hydroperoxides of linoleic acid via allene oxides. New insight into the mechanism of cyclization

Alexander N. Grechkin[a,*], Mats Hamberg[b]

[a] *Institute of Biochemistry and Biophysics, Russian Academy of Sciences, P.O. Box 30, Kazan 420503, Russia*
[b] *Division of Physiological Chemistry II, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden*

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Abstract Conversions of (*Z,E*)- and (*E,E*)-isomers of linoleic acid 13- and 9-hydroperoxides with flax and maize allene oxide synthase were studied. All-(*E*) but not (*Z,E*) hydroperoxides readily undergo cyclization via allene oxides into *trans*-cyclopentenones. These results suggest that double bond geometry dramatically affects the formation of pericyclic pentadienyl cation intermediate and thus the capability of 18:2-allene oxides to undergo electrocyclization into cyclopentenones.

Key words: Linoleic acid hydroperoxide; Allene oxide synthase; Cyclization mechanism; Cyclopentenone formation; Oxylipin

1. Introduction

Except cyclooxygenase pathway, widespread in mammals, there is another route to cyclopentane fatty acid derivatives in nature. Allene oxide fatty acids, short-lived products of allene oxide synthase (AOS, EC 4.2.1.92), are precursors of cyclopentenones (15*Z*)-12-oxo-10,15-phytodienoic acid (12-oxo-PDA) in plants [1–5] and preclavulone A in corals [6,7]. Annulation of allene oxides into cyclopentenones occurs either spontaneously [3,7] or enzymatically [4,5]. AOSs utilize different fatty acid hydroperoxides [8–10]. However, not all of allene oxides undergo conversion into cyclopentenones [8–10]. Annulation is strongly dependent on the presence of a double bond in the β,γ-position besides with oxirane of allene oxide [8–11]. Allene oxide cyclase (EC 5.3.99.6), a plant enzyme, catalyzing annulation, specifically utilizes only (9*Z*,13*S*,15*Z*)-12,13-epoxy-9,11,15-octadecatrienoic acid [11,12].

12-Oxo-PDA is an important physiologically active compound and metabolic precursor of a newly acknowledged plant hormone 7-*iso*-jasmonate. Increasing interest to these important natural products led to some recent attempts to find a biosynthetic pathway from linoleic acid to 12-oxo-10-phytoenoic acid, i.e. 15,16-dihydro analogue of 12-oxo-PDA [13–16].

The present paper is concerned with AOS conversions of all-(*E*) 9- and 13-HPODs. We found that allene oxides generated from these hydroperoxides are effectively converted into the novel cyclopentenone 10-oxo-11-phytoenoic acid and 12-oxo-10-phytoenoic acid, respectively. The obtained results provide new insight into the mechanism of allene oxide annulation.

2. Materials and methods

2.1. Preparation of fatty acid hydroperoxides

Racemic hydroperoxides, (9*Z*,11*E*,13*R,S*)-13-hydroperoxy-9,11-octadecadienoic acid {(9*Z*,11*E*)-13(*R,S*)-HPOD}; (9*E*,11*E*,13*R,S*)-13-hydroperoxy-9,11-octadecadienoic acid {(9*E*,11*E*)-13(*R,S*)-HPOD}, (9*R,S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid {(10*E*,12*Z*)-9(*R,S*)-HPOD} and (9*R,S*,10*E*,12*E*)-9-hydroperoxy-10,12-octadecadienoic acid {(10*E*,12*E*)-9(*R,S*)-HPOD} were prepared as described before [17] by autoxidation of linoleic acid. The resulting mixture of racemic hydroperoxides, having *Z,E*- and *E,E*-double bond geometry, was separated by normal phase HPLC (SP-HPLC). Isomeric 9-HPODs were finally separated by the reversed phase HPLC. Chiral 13(*S*)-HPOD and 13(*S*)-HPOT were obtained by incubations of linoleic and α-linolenic acids, respectively, with soybean lipoxygenase (LOX). (6*Z*,9*S*,10*E*,12*E*)-9-hydroperoxy-6,10,12-octadecadienoic acid {9(*S*)-HPOT(γ)} was obtained by incubation of γ-linolenic acid with tomato fruit LOX.

2.2. AOS preparation and incubations

Flaxseed acetone powder was prepared as described before [18,19]. AOS was extracted from 10 g of acetone powder with 150 ml of 100 mM phosphate buffer, pH 7.4 at 0°C. Extract was centrifuged at 9300×*g* for 10 min. The supernatant was used as AOS solution for incubations.

Maize AOS (2 mg protein) prepared as described before [5] was suspended in 10 ml of 100 mM phosphate buffer, pH 6.7. Incubations were performed at 0°C for 20 min, starting with the addition of 30 μmol of (9*E*,11*E*)-13-HPOD, (9*Z*,11*E*)-13-HPOD, (10*E*,12*E*)-9-HPOD, or (10*E*,12*Z*)-9-HPOD in 100 μl of ethanol.

2.3. Extraction and preliminary purification of products

Incubations were terminated by the addition of equal volumes of methanol. Then the incubation mixture was acidified with 2 M HCl to pH 3.5 and extracted with diethyl ether. Flaxseed AOS incubation mixtures were diluted with four volumes of water before extraction. Acidic lipids were separated and purified for further HPLC and GC-MS analyses using the Supelclean LC-NH_2 (3 ml) cartridges (Supelco, Bellefonte, PA, USA) as described [20].

2.4. Analyses of products

Acidic fraction from NH_2 cartridge was methylated with diazomethane. The resulting methyl esters were analyzed by GC-MS in two different modes: either (1) full spectral scanning within *m/z* range from 50 to 650, or (2) selected ion monitoring (SIM) of ions at *m/z* 152, 238, 308 and 382. Alternatively, the same samples were tri-

*Corresponding author. Fax: (7)-8432-38 75 77.
E-mail: grechkin@sci.kcn.ru

Abbreviations: LOX, lipoxygenase; AOS, allene oxide synthase; 12-oxo-PDA, (15*Z*)-12-oxo-10,15-phytodienoic acid; 13-H(P)OD, 13-hydro(per)oxy-9,11-octadecadienoic acid; 9-H(P)OD, 9-hydro(per)oxy-10,12-octadecadienoic acid; 13(*S*)-HPOT, (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid; 9(*S*)-HPOT(γ), (6*Z*,9*S*,10*E*,12*Z*)-9-hydroperoxy-6,10,12-octadecatrienoic acid; GC-MS, gas chromatography-mass spectrometry; SIM, selected ion monitoring; TMS, trimethylsilyl

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methylsilylated after methylation. The TMS derivatives of methyl esters were analyzed using the same two above described approaches.

Products were separated as free acids either by isocratic RP-HPLC on a Macherey-Nagel Nucleosil 5 ODS column (250×4.6 mm), solvent mixture acetonitrile-water-acetic acid (60:40:0.01, by volume), flow rate 1.5 ml/min. Non-polar products eluting before 13- or 9-HODs were collected and subjected to diazomethane methylation. Methods of hydrogenation, trimethylsilylation and alkaline isomerization were described before [21].

Ultraviolet spectra were recorded with Hitachi U2000 spectrophotometer (Hitachi, Tokyo, Japan). Gas chromatography (GLC) analyses with Hewlett-Packard model 5980 instrument (flame ionization detection) and gas chromatography-mass spectrometry (GC-MS) analyses with Hewlett-Packard model 5970B GC-MS system were performed as described before [20].

3. Results

3.1. Metabolism of HPODs by flax and maize AOS

Summary products of incubations with flax or maize AOS were analyzed by RP-HPLC and GC-MS. Hydroxy acids (HODs) belonged to the main metabolites of all four racemic HPOD isomers. A large yield of HODs is, apparently, explained by non-enzymatic reduction of (*R*)-HPODs. As demonstrated previously, flax [22] and corn (Hamberg, M., unpublished observations) AOSs preferentially utilize (*S*)-stereoisomers during incubations with (*R*,*S*)-HPODs.

HPLC analyses of extracts of all incubations with flaxseed AOS revealed the presence of some amount of endogenous 12-oxo-10,15-PDA (RP-HPLC retention time 10.5 min), ultraviolet and mass spectral data of which were identical to previously described [21]. All HPOD isomers were partly converted into the corresponding α-ketols (RP-HPLC retention time ca. 11.5 min). Analyses of extracts of incubations with (9*E*,11*E*)-13-HPOD and (10*E*,12*E*)-9-HPOD allowed to detect peaks **I** and **IV** (respectively, RP-HPLC retention times ca. 14 and 15 min, for structures see Fig. 1), eluting before all-(*E*) 13- and 9-HODs (RP-HPLC retention times ca. 15.5 and 16.7 min). Products **I** and **IV** were collected for further analyses.

Product **I** (C-value 19.71) had the ultraviolet absorbance maximum (EtOH) at 225 nm. The electron impact (70 eV) mass spectral data for compound **I** (methyl ester) are presented in Table 1. The molecular mass (M^+ at *m*/*z* 308) as well as the fragmentation patterns (Table 1) fully correspond to previously published mass spectral data for 12-oxo-10-phytoenoic acid [15,21].

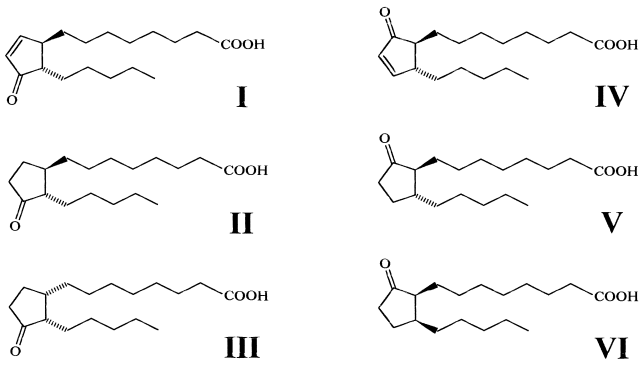

Fig. 1. The structural formulas of detected cyclopentenones **I** and **IV**, products of their catalytic hydrogenation **II** and **V** and the corresponding *cis* isomers **III** and **VI**, prepared by hydrogenation of (15*Z*)-12-oxo-10,15-phytodienoic and (6*Z*)-10-oxo-6,11-phytodienoic acids, respectively.

Catalytic hydrogenation of product **I** (as methyl ester) over PtO_2 afforded compound **II**. Its mass spectral data are presented in Table 1. Although the molecular ion was not detected, the observed spectral data suggest that compound **II** has the structure of 12-oxo-phytonoic acid. These results demonstrate that compound **I** has one double bond.

For the assignment of geometrical isomerism of compound **I** we prepared authentic sample of 12-oxo-PDA, having *cis* configuration of side chains. Part of this preparation was subjected to mild alkali treatment, as described above. Both original and isomerized samples of 12-oxo-PDA were subjected to catalytic hydrogenation over PtO_2. This enabled us to obtain samples of *cis*- and *trans*-12-oxophytonoic acid (**III** and **II**, respectively). Authentic sample of *trans*-12-oxophytonoic acid had coinciding C-value (19.60) with hydrogenation product (**II**) of metabolite **I** during GC-MS analyses. The second isomer having *cis* configuration of side chains (**III**) had distinct C-value (19.85). Thus, AOS conversion of (9*E*,11*E*)-13-HPOD affords *trans*-12-oxo-10-phytoenoate (**I**).

Table 1
Mass spectra of the identified metabolites **I** and **IV** (as methyl esters) and products of their hydrogenation **II** and **V**, *m*/*z* (relative intensity, %)

Ion attribution	Compounds			
	I	**II**	**IV**	**V**
$[M]^+$	308 (4)		308 (2)	310 (1)
$[M-MeO]^+$	277 (17)	279 (2)	277 (8)	279 (3)
$[M-C_5H_{11}+H]^+$	238 (26)	240 (6)		
$[M-C_5H_{11}]^+$				239 (3)
$[M-C_5H_{11}+H-MeOH]^+$	206 (23)			
$[M-C_5H_{11}-MeOH]^+$				207 (8)
$[M-(CH_2)_5COOMe]^+$	179 (13)			
$[M-(CH_2)_5COOMe-H]^+$	178 (17)			
$[M-(CH_2)_6COOMe]^+$	165 (12)		165 (4)	
$[M-C_{10}H_{17}O+H]^+$		158 (3)		
$[M-(CH_2)_7COOMe+H]^+$			152 (68)	154 (16)
$[M-(CH_2)_7COOMe]^+$	151 (39)	153 (14)		
$[M-(CH_2)_7COOMe-CO]^+$			123 (9)	
$[C_7H_9O]^+$	109 (52)		109 (15)	
$[C_6H_8O]^+$	96 (100)			
$[C_6H_7O]^+$	95 (86)		95 (100)	
$[C_5H_7O]^+$		83 (100)		83 (100)
$[C_5H_6O]^+$	82 (59)		82 (31)	

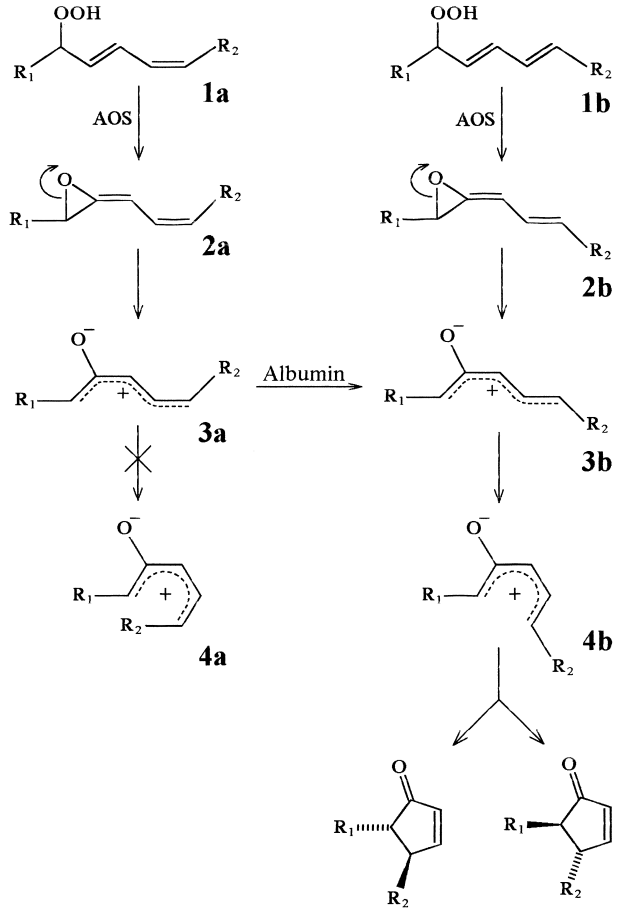

Fig. 2. The proposed distinct mechanisms of pentadienyl cations and cyclopentenones formation from (*Z*,*E*) and (*E*,*E*) HPODs via allene oxides. $R_1 = -CH_2(CH_2)_3CH_3$; $R_2 = -CH_2(CH_2)_6COOH$ (for 3-HPODs, their products and intermediates); $R_1 = -CH_2(CH_2)_6COOH$; $R_2 = -CH_2(CH_2)_3CH_3$ (for 9-HPODs, their products and intermediates).

Incubation of (10*E*,12*E*)-9-HPOD with flax or maize AOS afforded, inter alia, compound **IV** (C-value 19.68), having the ultraviolet absorbance maximum at 224 nm (in mobile phase acetonitrile-water-acetic acid 60:40:0.01 by volume). The mass spectral data for compound **IV** (methyl ester) are presented in Table 1. Along with M^+ at *m*/*z* 308 spectrum possesses a characteristic prominent ion at *m*/*z* 152, generating due loss of $(CH_2)_7COOCH_3$, accompanied by one hydrogen migration. The observed fragmentation patterns allow to ascribe the structure of 10-oxo-11-phytoenoic acid for product **IV**.

Hydrogenation of product **IV** over PtO_2 afforded compound **V**, having a featureless ultraviolet spectrum. The electron impact mass spectral data for compound **V** are presented in Table 1. The spectrum contains a molecular ion M^+ at *m*/*z* 310 and fragment ions, inter alia, $(M^+-(CH_2)_7COOCH_3+H)$ at *m*/*z* 154. The obtained results allow to identify compound **V** as 10-oxo-phytonoic acid. Thus, metabolite **IV** has one double bond.

For structural confirmation of compounds **IV** and **V** we prepared an authentic sample of (6*Z*)-10-oxo-6,11-phytodienoic acid by incubation of 9(*S*)-HPOT(γ) with maize AOS. Part of the resulting *cis*-cyclopentenone was subjected to alkaline isomerization as described above. Both isomerized and non-isomerized cyclopentenones were hydrogenated over PtO_2, affording the authentic samples of *trans*- and *cis*-10-oxophytonoic acid (**V** and **VI**, respectively). The authentic sample of *trans*-10-oxophytonoic acid had an identical C-value (19.52) and MS data with hydrogenation product **V** of metabolite **V** during GC-MS analyses. *cis* Isomer (**VI**) had a distinct C-value (19.79). Thus, AOS conversion of (10*E*,12*E*)-9-HPOD affords *trans*-10-oxo-11-phytoenoic acid (**IV**).

3.2. Quantification of cyclopentenones by selected ion monitoring (SIM) GC-MS

Only the SIM approach allowed to detect small amounts of *trans*-cyclopentenones formed from (9*Z*,11*E*)-13-HPOD and (10*E*,12*Z*)-9-HPOD. α-Ketols were by far predominant AOS utilization products of these hydroperoxides. Cyclopentenone to α-ketol ratios among the products of (9*Z*,11*E*)-13-HPOD and (10*E*,12*Z*)-9-HPOD did not exceed 0.007 and 0.015, respectively, as quantified by GLC with flame ionization detection. All-(*E*) HPODs hydroperoxides afford cyclopentenones along with α-ketols. Cyclopentenone to α-ketol ratios were 0.44 for (9*E*,11*E*)-13-HPOD products and 0.40 for (10*E*,12*E*)-9-HPOD products, as quantified by GLC with flame ionization detection.

For sensitive quantification of cyclopentenones formed during hydroperoxide incubations with AOS we used the selected ion monitoring (SIM) mode of GC-MS with tetracosanoic acid methyl ester as the internal standard. Masses 152, 238 and 382 were monitored in SIM mode. Monitoring of masses 152 and 238 are specific for 10-oxo-11-phytoenoic (**IV**) and 12-oxo-10-phytoenoic (**I**) acid methyl esters, respectively. Ion 382 corresponds to the molecular mass of methyl tetracosanoate.

SIM analysis of (9*E*,11*E*)-13-HPOD metabolites (as methyl esters) allowed to detect 12-oxo-10-phytoenoic acid (**I**) (Table 2). Incubation of AOS with (9*Z*,11*E*)-13-HPOD afforded ca. 125 times smaller yield of compound **I** (Table 2).

Cyclopentenone IV was detected among the products of (10*E*,12*E*)-9-HPOD conversion (Table 2). Hydroperoxide

Table 2
Quantification of cyclopentenones formed during the incubations of HPOD isomers with flax and maize AOS

Substrate	Ratio *trans*-cyclopentenone/internal standard	
	Incubations with flax AOS	Incubations with maize AOS
13-HPOD (*E*,*E*)	0.822	0.869
13-HPOD (*Z*,*E*)	0.00678	0.00664
9-HPOD (*E*,*E*)	1.526	6.10
9-HPOD (*Z*,*E*)	0.0570	0.296

Estimations of cyclopentenone/internal standard ratios were performed using GC-MS in selected ion monitoring (SIM) mode with tetracosanoic acid methyl ester as an internal standard. Cyclopentenone **I** was quantified by integration of its peak detected by SIM at *m*/*z* 238, while compound **IV** at *m*/*z* 152. Internal standard (methyl tetracosanoate) was quantified by integration of its peak at *m*/*z* 382.

with ordinary double bond geometry, (10*E*,12*Z*)-9-HPOD, was a much less efficient cyclopentenone precursor. It afforded only *trans*-cyclopentenone **IV** at a poor yield (Table 2).

4. Discussion

The obtained results demonstrate strong dependence of 18:2-allene oxides cyclization on the geometry of conjugated double bond system. The inability of (*Z*,*E*) hydroperoxides like (9*Z*,11*E*)-13-HPOD, lacking the 'β,γ-double bond', to undergo AOS induced conversion into cyclopentenones was noticed by many authors [3–5,8–12]. At the same time, there are few exceptions. Previously Hamberg and Hughes [21] have found that incubation of (9*Z*,11*E*)-13-HPOD with maize AOS in presence of BSA and other albumins affords *trans*-cyclopentenone along with α-ketol. Similar observations on 18:2-allene oxide cyclization in presence of BSA were recently reported by Gardner [15] and Gundlach and Zenk [16]. Brash et al. [23] observed *trans*-cyclopentenone formation after incubation of (5*Z*,9*E*,11*Z*,13*E*)-8-hydroxy-15-hydroperoxy-5,9,11,13-eicosatetraenoic acid, also lacking a β,γ-double bond, with flax AOS. The results of present work present direct evidence that allene oxides generated from hydroperoxides with (*E*,*E*) conjugated diene system are effectively converted into *trans*-cyclopentenones. Thus, the formation of *trans*-cyclopentenones during both 'albumin induced cyclization' [21] and cyclization of a complex 20:4 allene oxide [23] is, apparently, explained by *cis-trans* double bond isomerization (Fig. 2, conversion of **3a** into **3b**).

Allene oxide annulation into cyclopentenones could be only spontaneous under incubation conditions used in present work. The rules of orbital symmetry conservation predict the conrotatory electrocyclization for pericyclic pentadienyl cation. Thus, the allene oxides formed from (*Z,E*) HPODs should undergo annulation into *cis*-cyclopentenones. The obtained results demonstrate that in fact they produce only small quantities of *trans*-cyclopentenones. The inability of allene oxides formed from (*Z*,*E*) HPODs to form *cis*-cyclopentenones is, apparently, explained by sterical factor. Pericyclic pentadienyl cation 4a (Fig. 2) is essential for electrocyclization [24]. Its formation through the conformation transition of zwitterion **3a** is disabled due to sterical hindrance caused by *cis*-configuration of double bond (Fig. 2). Allene oxides formed from all-(*E*) HPODs, being free of this sterical hindrance, easily form pericyclic cation **4b**, which undergoes annulation into racemic *trans*-cyclopentenones (Fig. 2). This observed stereospecificity of cyclization is fully consistent with the rules of orbital symmetry conservation and with published experimental data for synthetic analogues of natural allene oxides [25]. Small yields of *trans*-cyclopentenones from (*Z*,*E*) HPODs are, apparently, explained by partial *cis-trans* double bond isomerization within the pentadienyl cation (conversion of **3a** into **3b**, Fig. 2).

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