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

Alexander N. Grechkin*, Mats Hamberg

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

Received 7 October 1999; received in revised form 6 December 1999

Edited by Vladimir Skulachev

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 show 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 (15Z)-12,13-octadecadienoic 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 (9Z,13S,15Z)-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-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-phytocenic acid and 12-oxo-10-phytocenic 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, (9Z,11E,13R,S)-13-hydroperoxy-9,11-octadecadienoic acid [(9Z,11E)-13(R,S)-HPOD]; (9R,S,10E,12Z)-9-hydroperoxy-10,12-octadecadienoic acid [(10E,12Z)-9(R,S)-HPOD]; and (9R,S,10E,12E)-9-hydroperoxy-10,12-octadecadienoic acid [(10E,12E)-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 γ-linolenic acids, respectively, with soybean lipoxigenase (LOX). (6Z,9S,10E,12E)-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 (9E,11E)-13-HPOD, (9Z,11E)-13-HPOD, (10E,12E)-9-HPOD, or (10E,12Z)-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. Extract was concentrated at 9300×g for 10 min. The supernatant was used as AOS solution for incubations.

Acidic lipids were separated and purified for further HPLC and GC-MS analyses using the Supelclean LC-NH2 (3 ml) cartridges (Supelco, Bellefonte, PA, USA) as described [20].

2.4. Analyses of products

Acidic fraction from NH2 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-
methylsilylated after methylation. The TMS derivatives of methyl esters were analyzed using the same two 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 acetone-tritile-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 published data [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 (9E,11E)-13-HPOD and (10E,12E)-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-phytotonoic acid [15,21].

Catalytic hydrogenation of product I (as methyl ester) over PtO2 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-phytotononionic 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 PtO2. This enabled us to obtain samples of cis isomers III and VI, prepared by hydrogenation of (15Z)-12-oxo-10,15-phytophodiene and (6Z)-10-oxo-6,11-phytophodiene acids, respectively.

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 (15Z)-12-oxo-10,15-phytophodiene and (6Z)-10-oxo-6,11-phytophodiene acids, respectively.

### Table 1

<table>
<thead>
<tr>
<th>Ion attribution</th>
<th>Compounds</th>
<th>I</th>
<th>II</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>M+</td>
<td>308 (2)</td>
<td></td>
<td></td>
<td>308 (2)</td>
<td>310 (1)</td>
</tr>
<tr>
<td>[M–MeO]+</td>
<td>277 (17)</td>
<td>279 (2)</td>
<td>277 (8)</td>
<td>279 (3)</td>
<td></td>
</tr>
<tr>
<td>[M–C₅H₁₀H]+</td>
<td>239 (26)</td>
<td>240 (6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[M–C₆H₁₁]+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[M–C₅H₁₀H]+</td>
<td>206 (23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[M–C₅H₁₀H–MeOH]+</td>
<td>207 (8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[M–(CH₃)₂COOMe]+</td>
<td>178 (17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[M–C₆H₁₁O]+</td>
<td>165 (12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[M–C₅H₁₀H+CO]+</td>
<td>158 (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[M–C₅H₁₁O]+</td>
<td>151 (39)</td>
<td>153 (14)</td>
<td>152 (68)</td>
<td>154 (16)</td>
<td></td>
</tr>
<tr>
<td>[M–C₅H₁₁O–CO]+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[CH₂O₃O]+</td>
<td>109 (52)</td>
<td></td>
<td></td>
<td>109 (15)</td>
<td></td>
</tr>
<tr>
<td>[CH₂O₃O]+</td>
<td>96 (100)</td>
<td></td>
<td></td>
<td>95 (100)</td>
<td></td>
</tr>
<tr>
<td>[C₆H₈O]+</td>
<td>83 (100)</td>
<td>83 (100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[C₆H₈O]+</td>
<td>82 (59)</td>
<td>82 (31)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Incubation of (10E,12E)-9-HPOD with flax or maize AOS afforded, inter alia, compound IV 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 had a distinct C-value (19.79). Thus, AOS conversion of (10E,12E)-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 (9Z,11E)-13-HPOD and (10E,12Z)-9-HPOD. α-Ketols were by far predominant AOS utilization products of these hydroperoxides. Cyclopentenone to α-ketol ratios among the products of (9Z,11E)-13-HPOD and (10E,12Z)-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 (9E,11E)-13-HPOD products and 0.40 for (10E,12E)-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 (9E,11E)-13-HPOD metabolites (as methyl esters) allowed to detect 12-oxo-10-phytoenoic acid (I) and 12-oxo-10-phytoenoic acid methyl esters, respectively. Ion 382 corresponds to the molecular mass of methyl tetracosanoate.

### Table 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ratio trans-cyclopentenone/internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubations with flax AOS</td>
<td></td>
</tr>
<tr>
<td>13-HPOD (E,E)</td>
<td>0.822</td>
</tr>
<tr>
<td>13-HPOD (Z,E)</td>
<td>0.00678</td>
</tr>
<tr>
<td>9-HPOD (E,E)</td>
<td>1.526</td>
</tr>
<tr>
<td>9-HPOD (Z,E)</td>
<td>0.0570</td>
</tr>
<tr>
<td>Incubations with maize AOS</td>
<td></td>
</tr>
<tr>
<td>13-HPOD (E,E)</td>
<td>0.869</td>
</tr>
<tr>
<td>13-HPOD (Z,E)</td>
<td>0.00664</td>
</tr>
<tr>
<td>9-HPOD (E,E)</td>
<td>6.10</td>
</tr>
<tr>
<td>9-HPOD (Z,E)</td>
<td>0.296</td>
</tr>
</tbody>
</table>

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 382, 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, (10E,12Z)-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 (9Z,11E)-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 (9Z,11E)-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 (5Z,9E,11Z,13E)-8-hydroxy-15-hydroperoxy-5,9,11,13-eicosaetraenoic 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 steric 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 steric hindrance caused by cis-configuration of double bond (Fig. 2). Allene oxides formed from all-(E) HPODs, being free of this steric 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).

Acknowledgements: The authors thank Mrs. Gunvor Hamberg for her expert technical assistance. The work was partly supported by Russian Foundation of Basic Research, Grant 97-04-40509. The travel grant, provided to A.N.G. by the Scientific Exchange Program of the Royal Swedish Academy of Sciences, is gratefully acknowledged.

References