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Thermal conversions of fatty acid peroxides to cyclopentenones: A biomimetic model for allene oxide synthase pathway

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ABSTRACT

The trimethylsilyl (TMS) peroxides of linoleic acid 9(*S*)-hydroperoxide (TMS or Me esters) were subjected to gas chromatography–mass spectrometry (GC–MS) analyses. The cyclopentenones, *trans*- and *cis*-10-oxo-11-phytoenoic acid (10-oxo-PEA, Me or TMS esters) were first time detected as the products of TMS-peroxide thermal conversions. The major products were ketodienes, epoxyalcohols, hemiacetals and decadienals. For further study of thermal cyclopentenone formation, 9(*S*)- or 13(*S*)-hydroperoxides of linoleic acid (Me esters) were sealed in ampoules and heated at 230 °C for 15 or 30 min. The products were separated by HPLC. The cyclopentenone fractions were collected and analyzed by GC–MS. *Trans*-10-oxo-PEA (Me) and 10-oxo-9(13)-PEA (Me) were formed during the thermal conversion of 9-hydroperoxide (Me ester). Similarly, the cyclopentenones *trans*-12-oxo-PEA (Me) and 12-oxo-9(13)-PEA (Me) were detected after the heating of 13-hydroperoxide (Me ester). Thermal formation of cyclopentenones can be considered as a biomimetic model of AOS pathway, providing new insights into the mechanisms of allene oxide formation and cyclization.

1. Introduction

Fatty acid hydroperoxides are the central intermediates of the lipoxygenase pathway in aerobic organisms (Gardner and Grechkin, 2002; Grechkin and Gardner, 2002). Hydroperoxides are also produced through the non-enzymatic fatty acid peroxidation (Yin et al., 2011). Hydroperoxides are relatively unstable products. They undergo the numerous enzymatic or spontaneous conversions (Gardner and Grechkin, 2002; Grechkin and Gardner, 2002). Both fatty acid hydroperoxides themselves, as well as many oxylipins derived from them, are biologically active compounds. These are, for instance, eicosanoids in mammals (Curtis-Prior, 2004) and some oxylipins, products of the plant lipoxygenase pathway (Gardner and Grechkin, 2002; Grechkin and Gardner, 2002).

Two major kinds of enzymes, lipoxygenases and several enzymes of CYP74 family (P450 superfamily) enzymes cooperate in oxylipin biosynthesis in plants (Gardner and Grechkin, 2002; Grechkin and Gardner, 2002). CYP74 enzymes are non-classical P450s, which do not need neither the redox partners nor the oxygen as a substrate (Brash, 2009). Their only substrates are fatty acid hydroperoxides. CYP74 enzymes have also been detected recently in some proteobacteria and Metazoa, including the stony coral *Acropora palmata* and lancelet *Branchiostoma floridae* (Lee et al., 2008). CYP74 family includes few different enzymes (Lee et al., 2008), inter alia the allene oxide synthase (AOS).

AOSs are widespread in higher plants (Grechkin and Gardner, 2002), as well as in soft corals (Corey and Lansbury, 1985; Corey et al., 1987a,b) and stony corals (Lee et al., 2008). AOSs of plants (Song and Brash, 1991; Song et al., 1993) and stony corals (Lee et al., 2008) belong to CYP74 family of P450 superfamily. AOSs of soft corals are distinct, they are mini-catalases (Boutaud and Brash, 1999; Brash, 2009). One more unusual AOS (a fusion protein containing one P450 part) have been detected recently in the fungus *Aspergillus tereus* (Hoffmann et al., 2013). This non-classical P450 is phylogenetically distant from CYP74s (Hoffmann et al., 2013). Nevertheless, all AOS possess the same catalytic function: the dehydration of fatty acid hydroperoxides to the short-lived allene oxides (Hamberg, 1987; Brash et al., 1988: Brash, 2009). Allene oxides are decomposed via two competing ways. First, the hydrolysis mainly to α-ketols (Hamberg, 1987; Brash et al., 1988; Grechkin et al., 1991). Second, the spontaneous (Baertschi et al., 1988) or enzymatic (Hamberg, 1988a,b; Hamberg and Fahlstadius, 1990) cyclization to cyclopentenones. Cyclization occurs either spontaneously (Baertschi et al., 1988) or enzymatically (Hamberg,

Abbreviations: TMS, trimethylsilyl; GC–MS, gas chromatography–mass spectrometry; TIC, total ion current; SIC, selected ion current; HPLC, high performance liquid chromatography; RP-HPLC, reversed phase HPLC; NP-HPLC, normal phase HPLC; 9(*S*)-HPOD, (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid; 13(*S*)-HPOD, (9*Z*,11*E*,13*S*)-13-hydroperoxy-9,11-octadecadienoic acid; 13-H(P)OT, (9*Z*,11*E*,13*S*,15*Z*)-13-hydro(pero)xy-9,11,15-octadecatrienoic acid; 12,13-EOT, (9*Z*,11*E*,13*S*,15*Z*)-12,13-epoxy-9,11,15-octadecatrienoic acid; AOS, allene oxide synthase; 10-oxo-PEA, 10-oxo-11-phytoenoic acids; 12-oxo-PEA, 12-oxo-10-phytoenoic acid; 12-oxo-PDA, 12-oxo-10,15-phytodienoic acid.

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1988a,b; Hamberg and Fahlstadius, 1990). Plants possess the allene oxide cyclase (Hamberg, 1988a,b; Hamberg and Fahlstadius, 1990; Ziegler et al., 1997, 2000; Hofmann et al., 2006), an enzyme which specifically converts (9*Z*,11*E*,13*S*,15*Z*)-12,13-epoxy-9,11,15-octadecatrienoic acid (12,13-EOT, an allene oxide synthesized from α-linolenic acid 13(*S*)-hydroperoxide) to cyclopentenone (9*S*,13*S*)-12-oxo-PDA (Ziegler et al., 1999). The cyclization product, cyclopentenone 12-oxo-10,15-phytodienoic acid (12-oxo-PDA) plays some regulatory roles in plants (Böttcher and Pollmann, 2009). Moreover, 12-oxo-PDA is a metabolic precursor of the phytohormone 7-*iso*-jasmonic acid and other bioactive jasmonates (Vick and Zimmerman, 1983; Wasternack and Kombrink, 2010).

As found before, treatment of hydroperoxides with trimethylsilylating reagents leads to TMS-peroxy derivatives (Turnipseed et al., 1993). These TMS derivatives can serve as a useful tool for biomimetic studies of peroxide decompositions. Previously we studied the thermal conversions of fatty acid TMS-peroxides accompanying their GC–MS analyses (Grechkin et al., 2005). A number of hydroxydienes (TMS), ketodienes, epoxyalcohols (TMS), hemiacetals (TMS) have been detected. These conversions mimic the metabolism of hydroperoxides occurring in different living organisms. For instance, the hemiacetals are the true primary products of CYP74B and CYP74C hydroperoxide lyases (Grechkin and Hamberg, 2004; Grechkin et al., 2006). Reinvestigation of these thermal reactions occurring during the GC–MS analyses enabled us to detect the cyclopentenones. Moreover, the thermal treatments of hydroperoxides (Me esters), sealed in ampoules, repeatedly afforded the cyclopentenones 10-oxo-11-phytoenoic, 10-oxo-9(13)-phytoenoic, 12-oxo-10-phytoenoic and 12-oxo-9(13)-phytoenoic acid methyl esters. These conversions mimicking the AOS pathway are described in the present paper.

2. Experimental procedures

2.1. Materials

Linoleic and α-linolenic acids, as well as soybean lipoxygenase type V were purchased from Sigma. Silylating reagents were purchased from Fluka (Buchs, Switzerland). 9-HPOD was prepared by incubation of linoleic acid with tomato fruit at 0 °C, pH 6.0, under continuous oxygen bubbling as described before (Grechkin et al., 2008). 13-HPOD was obtained by incubation of linoleic acid with soybean lipoxygenase type V as described before (Chechetkin et al., 2008). All hydroperoxides were purified by normal phase HPLC (NP-HPLC).

2.2. Derivatization of fatty acid hydroperoxides and other oxylipins

Samples of 13-HPOD or 9-HPOD (1 μmol each) were taken to dryness under a stream of nitrogen and treated with a mixture of pyridine/hexamethyldisilazane/trimethylchlorosilane 2:1:2 (by volume) for 10 min at ambient temperature. Silylation reagents were evaporated *in vacuo* and the remaining material was extracted with 1 mL of hexane. The solvent was evaporated under a stream of argon and the oily residue of TMS/TMS derivatives was dissolved in 0.1 mL of hexane for GC–MS analyses. Alternatively, 13-HPOD and 9-HPOD were trimethylsilylated as methyl esters after the preliminary methylation with ethereal diazomethane. The resulting Me/TMS derivatives were either analyzed by GC–MS, or heated in ampoules as described below.

2.3. Thermal conversions of fatty acid TMS-peroxides during their GC–MS analyses

Hydroperoxides (Me/TMS or TMS/TMS) were analyzed as methyl esters or methyl esters/TMS derivatives by GC–MS as described before (Grechkin et al., 2005). GC–MS analyses were performed using a Shimadzu QP5050A mass spectrometer connected to Shimadzu GC-17A gas chromatograph equipped with an MDN-5S (5% phenyl; 95% methylpolysiloxane) fused capillary column (length, 30 m; ID 0.25 mm; film thickness, 0.25 μm). Helium at a flow rate of 30 cm/s was used as the carrier gas. Injections were made in the split mode using an initial column temperature of 120 °C, injector temperature 230 °C. The column temperature was raised at 10 °C/min until 240 °C. The electron impact ionization (70 eV) has been used. The high resolution GC–MS analyses were performed using a DFS mass spectrometer (Thermo Scientific) connected to the Trace GC Ultra gas chromatograph (Thermo Scientific) equipped with an DB-5MS (5% phenyl; 95% methylpolysiloxane) fused capillary column (length, 30 m; ID 0.25 mm; film thickness, 0.1 mm). Helium at a flow rate of 30 cm/s was used as the carrier gas. Injections were made in the split mode. The temperature conditions were the same as described above for Shimadzu GC–MS system. The electron impact ionization (70 eV) has been used. The accurate *m*/*z* values were measured using the perfluorokerosene as a reference for mass calibration.

2.4. Thermal treatment of fatty acid hydroperoxides sealed in ampoules

The methyl esters of 13-HPOD or 9-HPOD (1 mg each) sealed in ampoules under argon were subjected to thermal treatment at 230 °C for 15 or 30 min. The products of thermal treatment were separated by reversed phase HPLC (RP-HPLC) on Macherey-Nagel Nucleosil 5 ODS column (250 mm × 4.6 mm) eluted with methanol–water (linear gradient from 76:24 to 96:4, by vol.) at a flow rate of 0.4 mL/min. The diode array detection (190–350 nm) with SPD-M20A instrument (Shimadzu) has been used. The products were collected, dissolved in hexane and analyzed by GC–MS.

2.5. Preparation of 10-oxo-11-phytoenoic acid (10-oxo-PEA) reference standards

10-Oxo-PEA was obtained as described before (Grechkin et al., 2008) by incubation of 9(*S*)-HPOD with the recombinant tomato AOS, LeAOS3 (CYP74C3). Solution of (9*S*)-HPOD (100 μg) in ethanol (10 μL) was added to LeAOS3 suspension (5 μg of purified protein) in 100 mM phosphate buffer (1 mL), pH 7.0, and the reaction was allowed to proceed for 15 min at 23 °C. Then the incubation mixture was acidified with acetic acid to pH 5–6 and extracted with hexane/ethyl acetate (1:1, v/v). The extract was concentrated *in vacuo* about 2-fold and treated with ethereal diazomethane at −20 °C for 1 min. The products (Me esters) were separated by RP-HPLC on Macherey-Nagel Nucleosil 5 ODS column (250 mm × 4.6 mm) eluted with methanol–water (linear gradient from 76:24 to 96:4, by vol.) at a flow rate of 0.4 mL/min. Fractions of *cis*- and *trans*-10-oxo-PEA (Me esters) were collected and re-chromatographed by NP-HPLC on two serially connected Separon SIX columns (150 mm × 3.2 mm; 5 μm) eluted with hexane–isopropanol 99.2:0.8 (by volume), flow rate 0.4 mL/min.

Alternatively, the LeAOS3 products were separated as the free fatty acids. The same HPLC conditions have been used with one exception: all solvents were acidified with 0.1% of acetic acid. Pure samples of *trans*- and *cis*-10-oxo-PEA were trimethylsilylated as described above to obtain their TMS esters.

2.6. Preparation of 12-oxo-10-phytoenoic acid (12-oxo-PEA) reference standards

12-Oxo-PEA was obtained by incubation of 13(*S*)-HPOD with the enzyme preparation from the roots of germinating sunflower seeds (1.5–6 days after germination) as described before (Grechkin et al., 2007). The products were extracted, methylated and the desired *cis*- and *trans*-10-oxo-PEA (Me esters) were separated and purified by HPLC as described in the preceding section.

2.7. Preparation of colneleic acid reference standard

Colneleic acid was prepared using the recombinant tobacco divinyl ether synthase (NtDES). NtDES (5 μg of recombinant protein) was incubated with 9(*S*)-HPOD (100 μg) in 2 mL of phosphate buffer (2 mL), pH 7.0, 23 °C, for 15 min. Then the reaction mixture was acidified and products were extracted, methylated, purified by HPLC and analyzed by GC–MS generally as described in Section 2.5 above with one alteration: NP-HPLC purifications were performed using the solvent system hexane–diethyl ether–acetic acid 98:2:0.1 (by volume), flow rate 0.6 mL/min. Purified sample was either methylated with ethereal diazomethane or trimethylsilylated to obtain the TMS ester of colneleic acid as reference standard for GC–MS.

3. Results

3.1. Rearrangements of TMS-peroxides during GC–MS analyses

When 9-HPOD (TMS/TMS) was subjected to GC–MS analyses, a number of thermal conversion products have been detected. These were for instance (1*E*,3*Z*)-decadienal and (1*Z*,3*Z*)-decadienal (products of β-cleavage), 9-HOD (TMS/TMS), ketodienes (10*E*,12*Z*)-9-oxo-10,12-octadecadienoic and (10*Z*,12*Z*)-9-oxo-10,12-octadecadienoic acids (TMS esters), epoxyalcohols 9,10-epoxy-11-hydroxy-12-octadecenoic acid (TMS/TMS) and 9,10-epoxy-13-hydroxy-11-octadecenoic acid (TMS/TMS), hemiacetal 9-hydroxy-9-[(1′*E*,3′*Z*)-nonadienyloxy]-nonanoic acid (TMS/TMS). The structural formulae of products described here and below are presented in Fig. 1. These compounds have been described before as the products of 9-HPOD (TMS/TMS) thermal conversions (Grechkin et al., 2005). At the same time, the current study enabled us to detect several products of great interest that were missing previously. These were three additional relatively unpolar products **1**–**3** (Fig. 2) appeared during the GC–MS analyses of 9-HPOD (TMS/TMS). Mass spectrum of product **3** possessed M^+ at *m*/*z* 366 and $[M-Me]^+$ at *m*/*z* 351. Its spectral patterns and retention time corresponded to those of colneleic acid TMS ester (data not illustrated).

Spectrum of product **1** possessed M^+ at *m*/*z* 366, $[M-Me]^+$ at *m*/*z* 351 (14%), $[M-(CH_2)_7COOSiMe_3+TMS]^+$ at *m*/*z* 224 (24%), $[M-(CH_2)_7COOSiMe_3+H]^+$ at *m*/*z* 152 (67%), $[152-(CH)_4Me]^+$ at *m*/*z* 95 (79%), *m*/*z* 75 (100%). Product **2** exhibited nearly identical spectral patterns with only minor difference in the relative peak intensities. Mass spectral patterns of compounds **1** and **2** and their retention times matched those of TMS esters of *trans*- and *cis*-10-oxo-PEA authentic standards, respectively. The even mass fragment at *m*/*z* 152 (Fig. 2B) is characteristic for 10-oxo-PEA derivatives. It should be noted that the appearance of peaks **1** and **2** during the GC–MS injections of 9-HPOD TMS peroxide was reliable. Attribution of peaks **1** and **2** to 10-oxo-PEA isomers was confirmed by correspondence of their spectral patterns and retention times to those of authentic samples of *trans*- and *cis*-10-oxo-PEA TMS esters, prepared by incubation of 9-HPOD with LeAOS3.

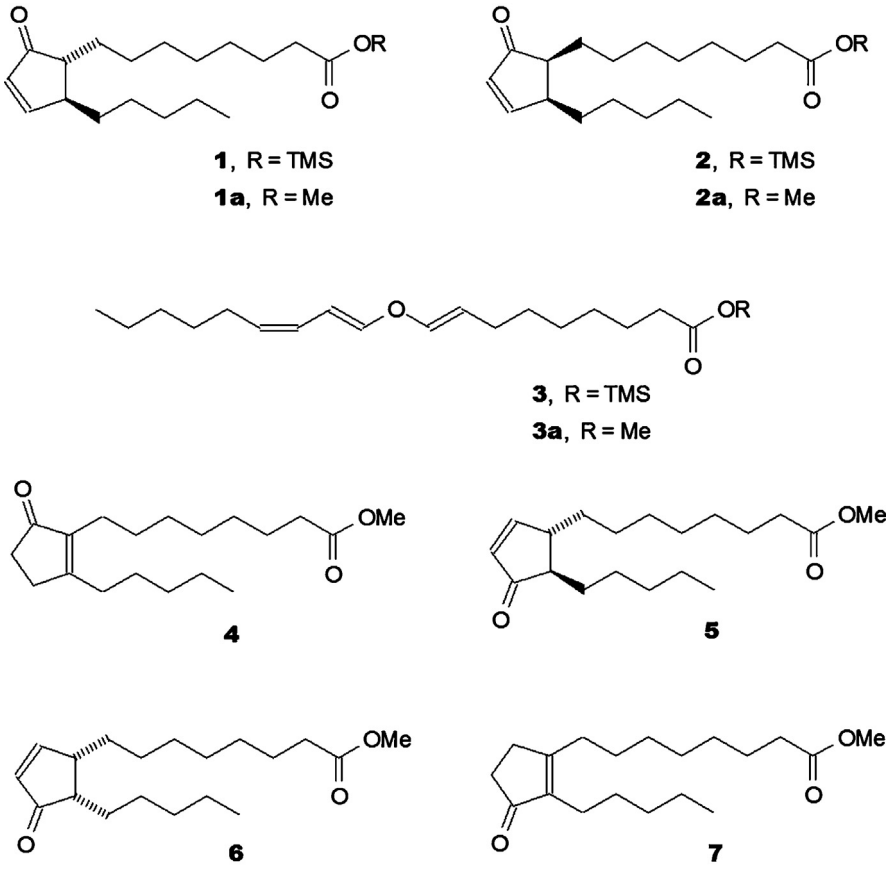

Fig. 1. The structural formulae of cyclopentenones and colneleic acid derivatives, detected as the products of thermal conversions of fatty acid TMS-peroxides.

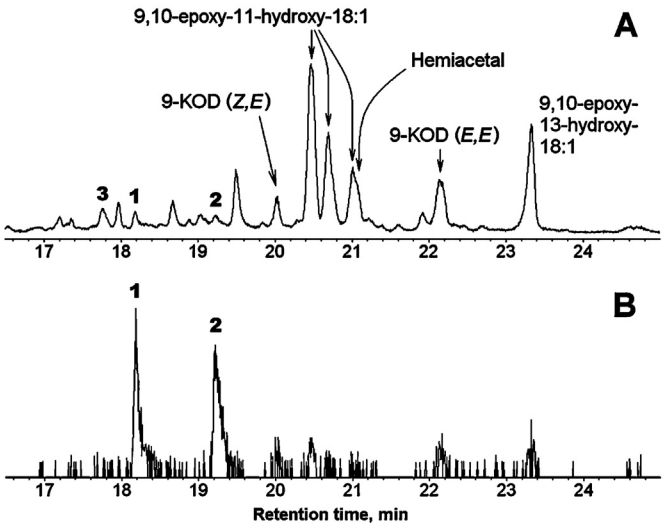

Fig. 2. Profiles of products detected during the GC–MS analysis of 9-HPOD (TMS/TMS). (A) Total ion current (TIC) chromatogram; (B) selected ion current (SIC) chromatogram, *m*/*z* 152.

When 9-HPOD (Me/TMS) was subjected to GC–MS analyses, two minor peaks **1a** and **2a** possessing ions at *m*/*z* 152 were detectable (Fig. 3). The spectrum of product **2a** possessed M^+ at *m*/*z* 308 (3%), $[M-MeO]^+$ at *m*/*z* 277 (4%), $[M-(CH_2)_7COOMe+H]^+$ at *m*/*z* 152 (75%), $[152-(CH_2)_3Me]^+$ at *m*/*z* 95 (100%). Spectrum of compound **1a** was nearly identical and possessed only slightly different relative peak intensities. The retention times of peaks **1a** and **2a** matched those of authentic *trans*- and *cis*-10-oxo-PEA, respectively. Sample of 9-HPOD (Me/TMS) was also subjected to high resolution GC–MS. This enabled to estimate the exact mass value at *m*/*z* 152.1209 for peak **2a**. This matches well the theoretical mass 152.1201, corresponding to fragment element composition $C_{10}H_{16}O$ $[M-(CH_2)_7COOMe]^+$. For further elucidation of 10-oxo-PEA formation we studied the thermal conversion of 9-HPOD (Me/TMS), preliminarily sealed in ampoule. The results are described below in Section 3.2.

Along with products **1a** and **2a**, one more peak **3a** eluting in front of 9-HOD (Me/TMS) has been detected (Fig. 2). Mass spectrum of product **3a** possessed M^+ at *m*/*z* 308 (18%), $[M-MeO]^+$ at *m*/*z* 277 (2%), $[M-(CH_2)_3Me]^+$ at *m*/*z* 251 (3%), $[M-(CH_2)_6COOMe]^+$ at *m*/*z* 165 (5%), *m*/*z* 151 (7%), $[M-Me(CH_2)_4CH{=}CH{-}CH{=}CHO{-}MeOH]^+$ at *m*/*z* 137 (10%). Retention time and spectral patterns of compound **3a** exactly corresponded to those of colneleic acid, (8*Z*)-9-[(1′*E*,3′*Z*)-nonadienyl]-8-nonenoic acid, Me ester (Itoh and Howe, 2001).

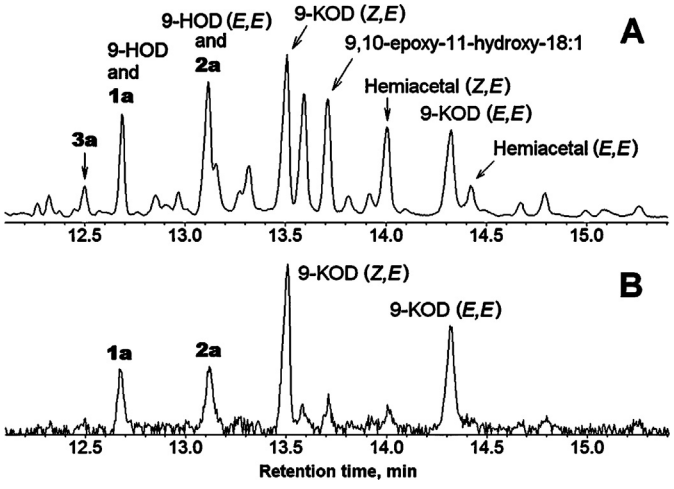

Fig. 3. Profiles of products detected during the GC–MS analysis of 9-HPOD (Me/TMS). (A) TIC chromatogram; (B) SIC chromatogram, *m*/*z* 152.

3.2. Thermal conversions of hydroperoxides sealed in ampoules

9-HPOD (Me ester) was sealed in ampoule and kept at 230 °C for 15 or 30 min. The resulting products were separated by RP-HPLC as described in Section 2. Fraction corresponding to the expected retention time of 10-oxo-PEA (Me), i.e. 45–50 min, was collected and subjected to GC–MS analyses. This fraction contained the products **1a**, **2a** and **4** at a ratio ca. 27:7:66, as estimated by integration of total ion current GC–MS data (Fig. 4A). The mass spectrum and fragmentation scheme for compound **1a**, *trans*-10-oxo-PEA, are presented in Fig. 5A. Product **2a** had nearly identical mass spectrum (not illustrated). As well as compounds **1a** and **2a**, the product **4** possessed M^+ at *m*/*z* 308. The mass spectral patterns of compound **4** (Fig. 5B) were identical to those described before (Grechkin et al., 2002) for 10-oxo-9(13)-phytoenoic acid (Me). Cyclopentenones, having a double bond at the tetrasubstituted position, possess the cleavages in α- and γ-positions (to the cycle) at that side chain, which is distant from carbonyl. So, the fragments at *m*/*z* 237 and *m*/*z* 265 were formed through the α-cleavage and γ-cleavage, respectively. Fragment at *m*/*z* 265 largely turned to *m*/*z* 233 due to the loss of methanol. The prominent fragments at *m*/*z* 110 and 123 are typical for this type of cyclopentenones. These are the cyclopentenone fragments with two and three (respectively) remaining methylenes. The obtained data enabled to identify compound **4** as 10-oxo-9(13)-phytoenoic acid (Me). Compound **4** is evidently the thermodynamically controlled product formed through the isomerization of cyclopentenones **1a** or **2a**, 10-oxo-11-PEA (Me).

13-HPOD (Me ester) was also heated in ampoule and the resulting products were separated by RP-HPLC as described in Section 2. RP-HPLC fraction corresponding to cyclopentenones (retention time 45–50 min) was collected and subjected to GC–MS analyses. Products **5**–**7** at a ratio ca. 54:10:36 (as estimated by integration of total ion current chromatogram, Fig. 4B) were detected in this fraction. The mass spectral data and the reconstructed fragmentation patterns (Fig. 5C) enabled to identify compound **5** as the cyclopentenone *trans*-12-oxo-10-phytoenoic acid Me ester (12-oxo-PEA). Product **6** had nearly identical mass spectral patterns (not illustrated). The retention times of compounds **5** and **6** corresponded to those of authentic standards of *trans*- and *cis*-12-oxo-PEA, obtained

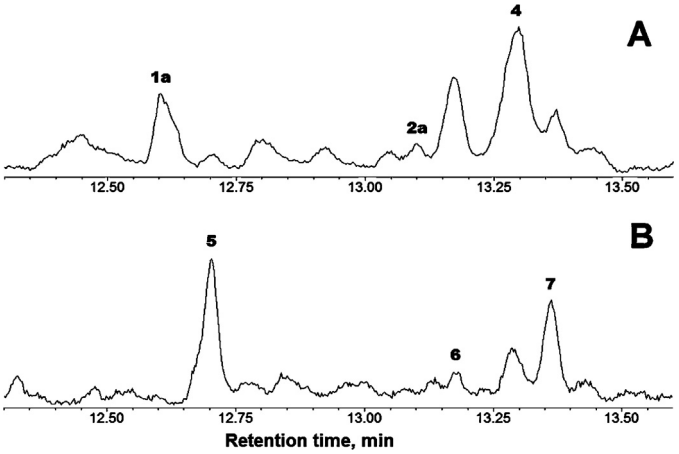

Fig. 4. Formation of cyclopentenones during the heating of hydroperoxides sealed in ampoules. The GC–MS profiles of cyclopentenone fractions collected during the RP-HPLC separation of thermal conversion products of 9-HPOD (A) and 13-HPOD (B) methyl esters. Conditions of heating and RP-HPLC separation of products are described in Section 2.

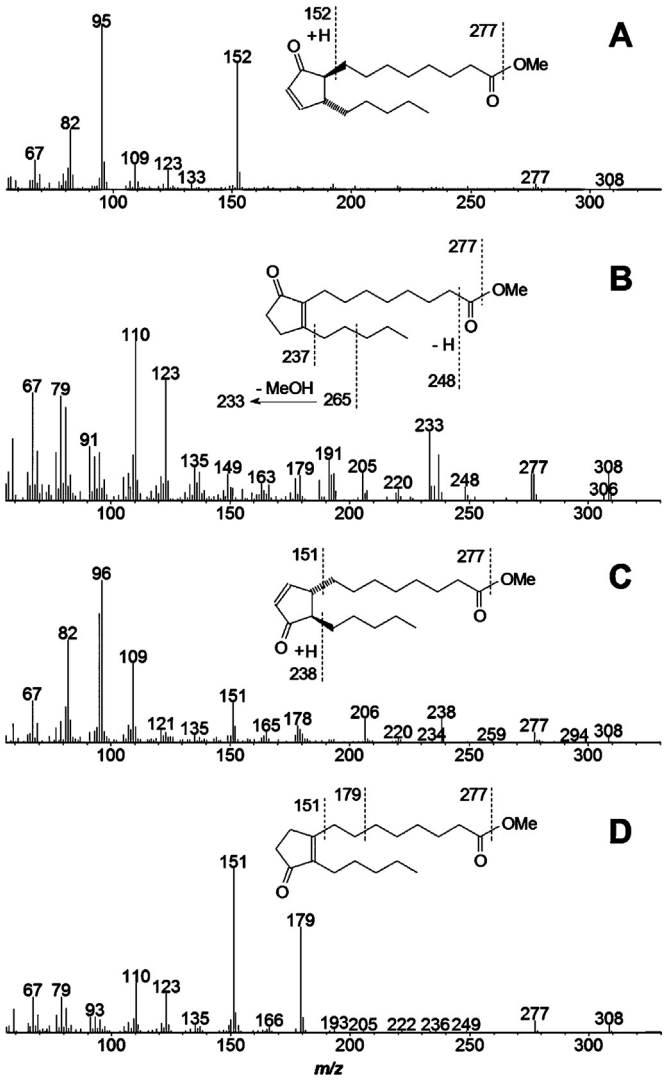

Fig. 5. The electron impact mass spectra of cyclopentenones (Me esters), detected as the products of thermal conversions of (A and B) 9-HPOD (Me/TMS) and (C and D) 13-HPOD (Me/TMS). (A) spectrum of product **1a**; (B) spectrum of product **4**; (C) spectrum of product **5**; (D) spectrum of product **6**.

as described in Section 2. Mass spectra of products **5** and **6** were identical to those described before (Grechkin et al., 2007). The last product **7** also possessed M^+ at *m*/*z* 308 and prominent fragments at *m*/*z* 179 and 151 (Fig. 5D). The last two ions arise from cleavages in γ- and α-positions toward the cyclopentenone ring. These cleavages are typical for cyclopentenones having a double bond between two side chains. Compound **4** possessed the corresponding fragments, as mentioned above. Both products **7** and **4** possessed fragments at *m*/*z* 110 and 123. Mass spectrum of compound **7** was identical to that described before for 12-oxo-9(13)-PEA (Grechkin et al., 2002). To summarize, the obtained data enabled us to identify products **5**, **6** and **7** as *trans*-12-oxo-PEA, *cis*-12-oxo-PEA and 12-oxo-9(13)-PEA, respectively.

4. Discussion

The thermal conversions of fatty acid hydroperoxides (Frankel and Gardner, 1989) and TMS-peroxides (Grechkin et al., 2005) occur through the homolysis of peroxide function resulted in formation of an oxyradical (Fig. 5). The latter intermediate undergoes three major conversions: (1) the β-cleavage, (2) conversion to ketodienes, or (3) rearrangement to the epoxyallylic radical (Fig. 5). The epoxyallylic radical undergoes a number of secondary conversions, which mimic the enzymatic reactions of fatty acid hydroperoxide controlled by CYP74 enzymes and mini-catalases. For instance, the TMS-peroxides undergo rearrangements to epoxyalcohols and hemiacetals (Fig. 5) via the epoxyallylic radical (Grechkin et al., 2005). The results of present work show that the epoxyallylic radical undergoes also the eliminations of hydrogen atoms (Fig. 5), resulting in the formation of cyclopentenones (via allene oxide or oxyallyl intermediates) or divinyl ethers.

Mechanism of allene oxide fatty acid cyclization is studied since the early works (Corey et al., 1987a,b; Baertschi et al., 1988). However, not all phenomena accompanying the cyclization are yet fully understood. First of all, the allene oxide itself exists as a trinity of valence tautomers: allene oxide itself, oxyallyl and cyclopropanone (Chan and Ong, 1980; Hess et al., 1998; Bettinger, 2010). In its turn, the oxyallyl exists in two resonance forms, zwitterion and diradical (Chan and Ong, 1980; Hess et al., 1998; Bettinger, 2010). Thus, the first unsolved question is: which tautomer is an immediate precursor of cyclopentenone? Majority of researchers (Corey et al., 1987a,b; Hamberg, 1988a,b; Baertschi et al., 1988; Kim and Cha, 1988; Brash et al., 1990) proposed the conrotatory electrocyclization mechanism through the oxyallyl cation (Nazarov-type cyclization). However, the cyclization of 12,13-EOT is diminished below pH 7.0 and fully suppressed at pH 4.5 and below (Grechkin et al., 2002). Thus, the protonation of oxyallyl disables the cyclization. In contrast, the negative charge plays no role in Nazarov-type electrocyclization (Davis and Tantillo, 2010). Thus, the pH dependence data favor a non-Nazarov mechanism. Recent theoretical study (López et al., 2004; Davis and Tantillo, 2010) revealed that the ring closure is accomplished through the oxyallyl diradical (pericylclic conrotatory 1,5-diradical closure). Thermal formation of cyclopentenones observed in the present work indicates a radical mechanism of cyclization in agreement with (López et al., 2004; Davis and Tantillo, 2010).

Conditions of allene oxide thermal formation and conversion are quite distinct to enzymatic ones occurring in aqueous media. Normally the hydrolysis of allene oxides prevails over their spontaneous cyclization. Moreover, both spontaneous (Vick et al., 1980; Grechkin, 1994) and enzymatic (Ziegler et al., 1999) cyclization strongly depends on the double bond in β,γ-position to the oxirane of allene oxide. For example, 12,13-EOT does cyclise, while allene oxide (12,13-EOD) formed from linoleate 13-hydroperoxide does not, even in aprotic solvent (Hamberg, 1988a,b; Medvedeva et al., 2007). This β,γ-double bond dependence is valid for all allene oxides generated in the presence of CYP74A AOSs. AOSs of CYP74C subfamily present an exception. Allene oxides formed from 9-HPOD (but not 13-HPOD) in the presence of CYP74C AOSs are converted into racemic cyclopentenones along with allene oxides (Hamberg, 2000; Itoh et al., 2002; Grechkin et al., 2008; Brash et al., 2013).

The proposed scheme of thermal cyclization is shown in Fig. 5 (bottom lines). As already mentioned, allene oxides readily undergo the conversion to oxyallyl (Fig. 5). The oxyallyl 1,3-diradical exists in a resonance equilibrium with 1,5-diradical, which undergoes the 1,5-ring closure (Fig. 5) to cyclopentenone (López et al., 2004; Davis and Tantillo, 2010). The 1,3-diradical is also convertible to cyclopropanone (Hess et al., 1998; Bettinger, 2010). Cyclopropanones are reactive compounds. For instance, they undergo the nucleophilic cleavage (Favorskii type rearrangement) to the branched chain carboxylic acids (Wasserman et al., 1974). Such dicarboxylic acid has been detected recently as a minor by-product accompanying the 12-oxo-PDA formation (Grechkin et al., 2011). This confirms the co-occurrence of cyclopropanone with 12,13-EOT.

Allene oxides generated enzymatically at ambient temperature are cyclised predominantly to cyclopentenones with a *cis*-assignment of side chains (Baertschi et al., 1988; Hamberg et al., 1988). Thermally formed cyclopentenones were represented by

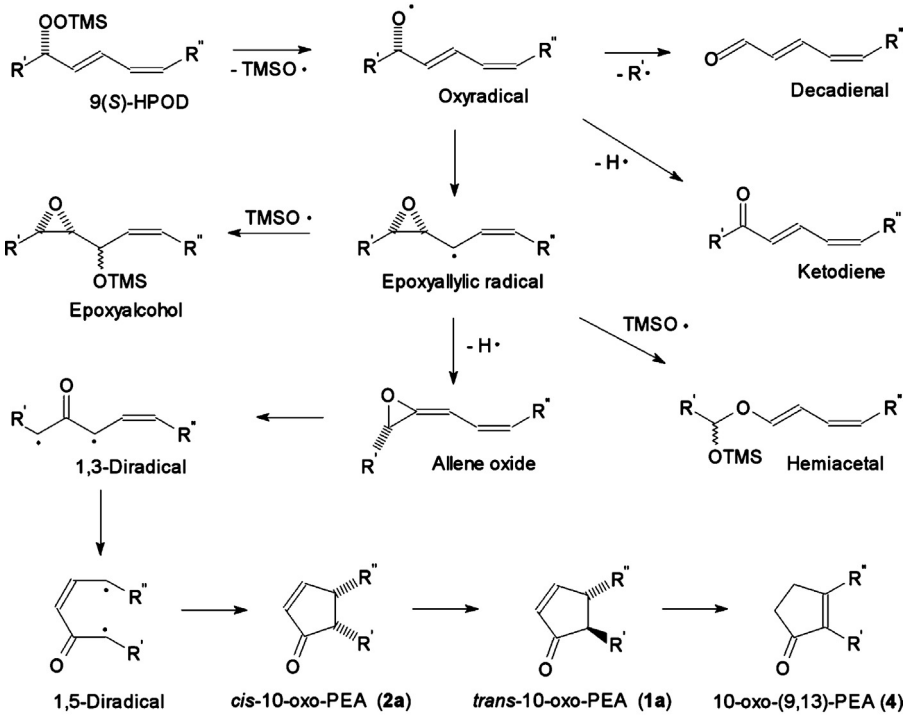

Fig. 6. The proposed scheme of thermal conversions of fatty acid TMS-peroxides, exemplified by 9(*S*)-HPOD (Me/TMS) transformations. R′ = —$(CH_2)_7$COOMe; R″ = —$(CH_2)_4$Me.

ca. 1:1 ratio of isomers with *cis*- and *trans*-configuration of side chains. Apparently this is explained by the thermal *cis–trans* double bond isomerization in the radical intermediates. This isomerization does occur, as seen from the appearance of geometrical isomers of other products, e.g. the (*Z*,*E*)- and (*E*,*E*)-9-HOD isomers (Fig. 3) and (*Z*,*E*)- and (*E*,*E*)-ketodienes (Figs. 2 and 3). As shown previously, the allene oxides biosynthesized from (*E*,*E*)-hydroperoxides, are cyclized to cyclopentenones with *trans*-assignment of side chains (Grechkin and Hamberg, 2000; Grechkin et al., 2002). After heating of TMS-peroxides in ampoules, only *trans*-disubstituted cyclopentenones, as well as the cyclopentenones with double bond migrated to 9(13)-position were detected. This evidently happened due to a longer thermal treatment resulting in *cis–trans* isomerization and a double bond migration to the thermodynamically preferential 9(13)-position. The 12-oxo-9(13)-PEA isomer has been described before (Grechkin et al., 2002) as a product of the alkaline 12-oxo-PEA isomerization. A similar thermal isomerization of *cis*-12-oxo-PDA (Me) to *trans*-12-oxo-PDA (Me) and 12-oxo-9(13),15-phytodienoic acid (Me) has been described before (Vick et al., 1979) (Fig. 6).

5. Concluding remarks

1. The results of present work demonstrate the production of cyclopentenones through the thermal conversions of fatty acid TMS-peroxides.
2. The results indicate that the heating of TMS-peroxides produce allene oxide fatty acids, which in turn are converted to cyclopentenones.
3. Thermal formation of cyclopentenones can be considered as a biomimetic model of AOS pathway, providing new insights into the mechanism of allene oxide cyclization.

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