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Steric analysis of epoxyalcohol and trihydroxy derivatives of 9-hydroperoxy-linoleic acid from hematin and enzymatic synthesis

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ABSTRACT

We characterize the allylic epoxyalcohols and their trihydroxy hydrolysis products generated from 9*R*- and 9*S*-hydroperoxy-octadecenoic acid (HPODE) under non-enzymatic conditions, reaction with hematin and subsequent acid hydrolysis, and enzymatic conditions, incubation with *Beta vulgaris* containing a hydroperoxide isomerase and epoxide hydrolase. The products were resolved by HPLC and the regio and stereo-chemistry of the transformations were determined through a combination of ^{1}H NMR and GC–MS analysis of dimethoxypropane derivatives. Four trihydroxy isomers were identified upon mild acid hydrolysis of 9*S*,10*S*-*trans*-epoxy-11*E*-13*S*-hydroxyoctadecenoate: 9*S*,10*R*,13*S*, 9*S*,12*R*,13*S*, 9*S*,10*S*,13*S* and 9*S*,12*S*,13*S*-trihydroxy-octadecenoic acids, in the ratio 40:26:22:12. We also identified a prominent δ-ketol rearrangement product from the hydrolysis as mainly the 9-hydroxy-10*E*-13-oxo isomer. Short incubation (5 min) of 9*R*- and 9*S*-HPODE with *B. vulgaris* extract yielded the 9*R*- and 9*S*-hydroxy-10*E*-12*R*,13*S*-*cis*-epoxy products respectively. Longer incubation (60 min) gave one specific hydrolysis product via epoxide hydrolase, the 9*R/S*,12*S*,13*S*-trihydroxyoctadecenoate. These studies provide a practical approach for the isolation and characterization of allylic epoxy alcohol and trihydroxy products using a combination of HPLC, GC–MS and ^{1}H NMR.

1. Introduction

The oxygenation at C9 of linoleic acid is the gateway to a diverse collection of oxylipin products (Andreou et al., 2009). C9 oxygenation in plants is catalyzed by 9-lipoxygenases (9-LOX), well-represented among the LOX genes (Feussner and Wasternack, 2002). Linoleate 9-lipoxygenases are also known in prokaryotes (Andreou et al., 2008; Gao et al., 2010; Zheng et al., 2008), and are a catalytic activity of arachidonate 12*R*-LOX in higher animals (Meruvu et al., 2005; Siebert et al., 2001; Zheng et al., 2011). Among the many possible transformations of the resulting 9-hydroperoxy-octadecenoic acid (9-HPODE), in keeping with other fatty acid hydroperoxides are rearrangements to epoxy-hydroxy derivatives (fatty acid epoxyalcohols), with subsequent hydrolysis producing a collection of trihydroxy-octadecenoates.

The non-enzymatic transformation of fatty acid hydroperoxides to epoxyalcohols is catalyzed by heme or transition metals, resulting in a mixture of isomers, as analyzed earlier utilizing 13-HPODE (Dix and Marnett, 1985; Gardner et al., 1974; Hamberg, 1975). The present study aims to address the heme transformations with 9-HPODE as starting material. Enzymatic conversion results in regio- and stereo-specific formation of epoxyalcohols. Lipoxygenases can also catalyze epoxyalcohol synthesis (Garssen et al., 1976; Nigam et al., 2004; Pace-Asciak et al., 1995; Yu et al., 2003), the transformation being favored under anaerobic conditions (Zheng and Brash, 2010). Heme and lipoxygenase catalysis involves formation of alkoxyl and epoxyallylic radical intermediates, whereas oxygen transfer from the hydroperoxide with epoxidation of a remote double bond is effected by other enzymes. These include peroxygenase (Blée et al., 1993; Hamberg and Hamberg, 1996), catalase-related hemoproteins (Gao et al., 2009; Niisuke et al., 2009), as well as putative P450-like enzymes that remain to be characterized, for example in the fish fungus *Saprolegnia parasitica* (Hamberg et al., 1986), potato leaves (Hamberg, 1999), and beetroot (Hamberg and Olsson, 2011).

Trihydroxy hydrolysis products are readily formed from fatty acid epoxyalcohols, and in the case of potential 9-HPODE-derived

Abbreviations: CD, circular dichroism; COSY, correlation spectroscopy; DCM, dichloromethane; DMP, dimethoxypropane; GC–MS, gas chromatography–mass spectrometry; H(P)ODE, hydro(pero)xyoctadecadienoic acid; IPA, isopropyl alcohol; LOX, lipoxygenase; RP-HPLC, reversed-phase high pressure liquid chromatography; SP-HPLC, straight-phase high pressure liquid chromatography; TMS, trimethylsilyl; UV, ultraviolet.

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products, these triols are reported in such diverse settings as; an *in-vivo* pathogen response in potato leaves (Göbel et al., 2002; Hamberg, 1999), in brewing, where their formation contributes to the bitter flavor of beer (Garbe et al., 2005; Hamberg, 1991b), as products in mammalian blood vessels and leukocytes (Claeys et al., 1985; Funk and Powell, 1985), and in preserved meat as a marker of lipid peroxidation (Püssa et al., 2009). The adjuvant activity of 9*S*,12*S*,13*S*-trihydroxyoctadecenoic acid (commonly known as pinellic acid) and its isomers in the administration of flu vaccine has also been reported (Shirahata et al., 2003). In addition, our interests in the transformations of linoleate and its esters through LOX activity in mammalian epidermis (Zheng et al., 2011) have focused our attention on the preparation and characterization of 9-HPODE-derived epoxyalcohols and the resulting triols. There are effective methods for their analysis in the literature, although the separations cannot be related to typical HPLC methodology, which was one impetus for our conducting the present study. Hamberg (1991a) made assignments based on GLC and TLC mobilities coupled with mass spectrometry and chemical degradation (oxidative ozonolysis, followed by GLC analysis of the fragments). Shirahata et al. (2006) prepared all possible stereo isomers of pinellic acid by total synthesis and compared their spectral properties by NMR. The use of an acetonide derivative to capture the vicinal 12,13-diol in the 9,12,13-triol is part of this procedure and has been applied in other studies to identify single naturally occurring fatty acid triols by NMR (Bruno et al., 1992; Cardellina and Moore, 1980). We make use of this procedure in our NMR assignments herein.

Accordingly, the objectives of the current study were (i) to develop a practical method for production and purification of allylic epoxyalcohols via hematin treatment of 9-hydroperoxy-linoleic acid, (ii) to characterize the HPLC separation of the trihydroxy-octadecenoates resulting from hydrolysis of the allylic alcohols, (iii) to utilize a GC–MS and NMR approach for the assignment of the trihydroxy-octadecenoate regio- and stereo-chemistry, (iv) to compare the enzymatic synthesis, isomeric distribution and chirality of epoxyalcohols and triols from 9*R*- and 9*S*-hydroperoxy-linoleic acid in *Beta vulgaris*, a source of epoxyalcohol synthase (Hamberg and Olsson, 2011), and (v) to characterize specific delta-ketols as products from the mild acid treatment of allylic *trans*-epoxyalcohols.

2. Experimental

2.1. Generation of 9R-HPODE and 9S-HPODE substrates

2.1.1. 9R-HPODE

Linoleic acid (10 mg) in 0.5 ml ethanol was stirred into 200 ml 50 mM Tris pH 7.5, 150 mM NaCl, 20 mM CHAPS with a strong oxygen stream on the surface of the solution. *Anabaena* LOX enzyme (a linoleate 9*R*-LOX, (Zheng et al., 2008)) was added as 150 μl of a 20 mg/ml solution; reaction was complete in 2 min as evidenced by UV analysis of the conjugated diene chromophore of the product. The solution was then acidified to pH 4.0 and extracted with ethyl acetate, washed twice with half volume of water, taken to dryness and redissolved in a small volume of methanol for storage prior to HPLC. The 9*R*-HPODE was purified by semi-preparative SP-HPLC using a Beckman Ultrasphere silica 10 μm column, with isocratic hexane/IPA/acetic acid (100:2:0.1), a flow rate of 4 ml/min, with UV monitoring at 235 nm.

2.1.2. 9S-HPODE

9*S*-HPODE was prepared using the linoleate 9*S*-LOX in potato (Galliard and Phillips, 1971). Yukon Gold potatoes were diced and blended with 255 ml water. To eliminate starch, the solution was then stirred with 250 μl Tween 80, 320 μl Viscozyme L (Sigma) and 160 μl Amyloglucosidase (from *Aspergillus niger* 300 units/ml, Sigma) for 1 h at room temperature. After a low speed centrifugation (500 rpm, 10 min) the supernatant was poured through 2 layers of cheesecloth into a graduated cylinder. Sodium acetate (1 M) pH 5.5 was then added to 10% volume. The resulting enzyme solution was then suspended in a water bath at 15 °C. Linoleic acid in ethanol (1 g) was added drop wise with a strong stream of oxygen blowing on the surface of the solution and the reaction stirred for 90 min. The resulting solution was acidified to pH 4.0 and extracted with dichloromethane (DCM). The DCM was then washed twice with half volume of H_2O and taken to dryness. The 9*S*-HPODE was purified using SP-HPLC with a Beckman Ultrasphere silica 10 μm column with an isocratic mobile phase of hexane/IPA/acetic acid (100:2:0.1).

2.2. Reaction of 9S-HPODE with hematin

A hematin solution (5 mg/ml) was prepared by grinding the crystals to a fine powder and stirring with 0.1 M K_2HPO_4 adjusted to pH 11.0 with NaOH. 9*S*-HPODE (20 mg in 0.5 ml ethanol) was mixed with 0.1 M K_2HPO_4 (20 ml, pH 8.5), 1 ml of the hematin solution was added and the reaction left for 15 min at room temperature. The sample was cooled on ice, two volumes of ice-cold DCM were added, then a predetermined mix of 1 M KH_2PO_4 (5 ml) and 1 M HCl solution, sufficient to bring the pH to ~4.5, followed immediately by vigorous mixing of the phases. The aqueous layer was removed and the DCM washed twice with ice-cold water. The DCM was transferred to a fresh vial, dried under N_2, and the sample stored at −20 °C in DCM:MeOH (2:1, v/v) prior to initial purification on an open-bed silica Bond-Elut cartridge.

2.2.1. Silica cartridge purification of extract

The extract from the hematin reaction was pre-dissolved in ethyl acetate (2 ml) then hexane (8 ml) was added and the sample immediately applied to a 1 g Bond-Elut silica cartridge preconditioned with 25% ethyl acetate in hexane. A further 10 ml rinse of the sample vial with ethyl acetate (25%) in hexane was added and followed by a further 10 ml 25% ethyl acetate. The products were then eluted with 100% ethyl acetate (10 ml), and the sample was dried under N_2 and stored for analysis in DCM:MeOH, 2:1.

2.2.2. HPLC purification of hematin products

The range of products generated from the hematin reaction was analyzed on an Agilent 1100 series HPLC using a Thomson Advantage Silica 150 Å 5 μ column (250 mm × 4.6 mm) an isocratic solvent of hexane:IPA:acetic acid (100:2:0.02), a flow rate of 1 ml/min, with diode array detection at wavelengths of 205, 220, 235 and 270 nm.

2.3. HPLC separation of trihydroxy-octadecenoate products from allylic epoxyalcohol hydrolysis

2.3.1. Acid hydrolysis of 9S,10S-trans-epoxy-11E-13S-hydroxyoctadecenoate

To the allylic epoxide dissolved in acetonitrile (5 ml), 1% aqueous acetic acid (20 ml) was added and mixed thoroughly. After standing at room temperature for 30 min, the hydrolysis products were extracted in a C18 Bond Elute cartridge, washed with two volumes of H_2O and eluted with MeOH.

2.3.2. Purification of trihydroxy-octadecenoate products

Initial RP-HPLC analysis employed a Waters Symmetry C18 column (250 mm × 4.6 mm) eluted isocratically with methanol/water/acetic acid (80/20/0.01) at 1 ml/min. The 205 nm absorbing peaks in the triol region of the chromatogram at ~4 min retention time were pooled and subsequently resolved using a Chiralpak AD column (250 mm × 4.6 mm) with an isocratic mobile