Available online at www.sciencedirect.com
ScienceDirect
Analytical Biochemistry 367 (2007) 238–246
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Steric analysis of 8-hydroxy- and 10-hydroxyoctadecadienoic acids and dihydroxyoctadecadienoic acids formed from 8*R*-hydroperoxyoctadecadienoic acid by hydroperoxide isomerases

Ulrike Garscha, Ernst H. Oliw *

Department of Pharmaceutical Biosciences, Biomedical Center, Uppsala University, SE-751 24 Uppsala, Sweden

Received 21 February 2007
Available online 3 May 2007

Abstract

8-Hydroxyoctadeca-9*Z*,12*Z*-dienoic acid (8-HODE) and 10-hydroxyoctadeca-8*E*,12*Z*-octadecadienoic acid (10-HODE) are produced by fungi, e.g., 8*R*-HODE by *Gaeumannomyces graminis* (take-all of wheat) and *Aspergillus nidulans*, 10*S*-HODE by *Lentinula edodes*, and 10*R*-HODE by *Epichloe typhina*. Racemic [8-^{2}H]8-HODE and [10-^{2}H]10-HODE were prepared by oxidation of 8- and 10-HODE to keto fatty acids by Dess–Martin periodinane followed by reduction to hydroxy fatty acids with NaB^2H_4. The hydroxy fatty acids were analyzed by chiral phase high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) with 8*R*-HODE and 10*S*-HODE as standards. 8*R*-HODE eluted after 8*S*-HODE on silica with cellulose tribenzoate (Chiralcel OB-H), and 10*S*-HODE eluted before 10*R*-HODE on silica with an aromatic chiral selector (Reprosil Chiral-NR). 5*S*,8*R*-Dihydroxyoctadeca-9*Z*,12*Z*-dienoic acid (5*S*,8*R*-DiHODE) is formed from 18:2n-6 by *A. nidulans* and 8*R*,11*S*-dihydroxyoctadeca-9*Z*,12*Z*-dienoic acid (8*R*,11*S*-DiHODE) by *Agaricus bisporus*. 8*R*-Hydroperoxylinoleic acid (8*R*-HPODE) can be transformed to 5*S*,8*R*-DiHODE and 8*R*,11-DiHODE by *Aspergillus* spp., and 8*R*,13-dihydroxy-9*Z*,11*E*-dienoic acid (8*R*,13-DiHODE) can also be detected. We prepared racemic [5,8-$^2H_2$]5,8- and [8,11-$^2H_2$]8,11-DiHODE by oxidation and reduction as above and 8*R*,13*S*- and 8*R*,13*R*-DiHODE by oxidation of 8*R*-HODE by *S* and *R* lipoxygenases. The diastereoisomers were separated and identified by normal phase HPLC–MS/MS analysis. We used the methods for steric analysis of fungal oxylipins. *Aspergillus* spp. produced 8*R*-HODE (>95% *R*), 10*R*-HODE (>70% *R*), and 5*S*,8*R*- and 8*R*,11*S*-DiHODE with high stereoselectivity (>95%), whereas 8*R*,13-DiHODE was likely formed by nonenzymatic hydrolysis of 8*R*,11*S*-DiHODE.

Keywords: Aspergillus; Chiralcel OB; 5,8-DiHODE; 8,11-DiHODE; Normal phase HPLC; Reprosil chiral NR; Ion trap mass spectrometry

8-Hydroxyoctadecadienoic acid (8-HODE)[1] was originally discovered in *Laetisaria arvalis* [1] and consists mainly of the 8*R* stereoisomer [2]. Champe and co-workers [3,4] isolated 8*R*-HODE and 5*S*,8*R*-dihydroxyoctadecadienoic acid (5*S*,8*R*-DiHODE) from *Aspergillus nidulans* in pursuit of precocious sexual inducers of sporulation (psi factors). *Gaeumannomyces graminis*, the take-all fungus, and *Leptomitus lacteus*, the sewage fungus, also form 8*R*-HODE [5,6]. The mechanism of biosynthesis has been determined in *G. graminis* [7]. 18:2n-6 is oxidized to 8*R*-hydroperoxylinoleic acid (8*R*-HPODE) by a heme-containing dioxygenase, linoleate 7,8-diol synthase (7,8-LDS) [7]. 7,8-LDS abstracts the *pro-S* hydrogen at C-8 of 18:2n-6 and forms a carbon centered radical, which reacts with O_2 and forms 8*R*-HPODE [8]. The latter can be isomerized by the enzyme to 7*S*,8*S*-DiHODE [9]. 8-HODE and 7,8-DiHODE are also produced by 7,8-LDS of the rice-blast fungus, *Magnaporthe grisea* [10].

* Corresponding author. Fax: +46 18 55 29 36.
E-mail address: Ernst.Oliw@farmbio.uu.se (E.H. Oliw).

[1] *Abbreviations used:* CP, chiral phase; DiHODE, dihydroxyoctadecadienoic acid; HODE, hydroxyoctadecadienoic acid; HOME, hydroxyoctadecenoic acid; HPODE, hydroperoxyoctadecadienoic acid; Mn-LO, manganese lipoxygenase; NP, normal phase; Ppo, psi producing oxygenase; psi, precocious sexual inducer; RP, reversed phase; sLO-1, soybean lipoxygenase-1; TPP, triphenylphosphine.

0003-2697/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2007.04.045

Whether all fungal linoleate 8-dioxygenases form the *R* stereoisomer of 8-HPODE is not yet known. The majority of plant and mammalian lipoxygenases synthesize hydroperoxides with *S* configuration, but marine and a few mammalian lipoxygenases and manganese lipoxygenase (Mn-LO) form hydroperoxides with *R* configuration [11]. Indeed, *S* or *R* stereospecificity of a lipoxygenase can be changed by mutation of a single amino acid in the active site [12].

10*S*-HPODE is formed by shiitake mushroom, *Lentinula edodes*, and can be transformed to an aromatic compound, 1-octen-3-ol, by *L. edodes* and the field mushroom *Agaricus bisporus* or reduced to 10*S*-HODE [13,14]. 10*R*-HODE is formed by *Eichloe typhina* with >99% enantiomeric purity [15], and this stereoisomer also predominated in incubations of 18:2n-6 with *G. graminis* [16]. Little is yet known about the mechanisms of biosynthesis of 10*S*-HPODE/10*S*-HODE and 10*R*-HPODE/10*R*-HODE.

Genomes of important fungi have been sequenced. The genomes of *A. nidulans* and *A. fumigatus* contain three genes with homology to 7,8-LDS of *G. graminis*, designated psi producing oxygenase A (PpoA), B (PpoB), and C (PpoC) [17,18]. Homologous genes are also found in other *Aspergillus* spp., e.g., in *A. niger* and *A. oryzae* [19,20]. Ongoing work shows that mycelia of *A. nidulans* and *A. fumigatus* can oxidize 18:2n-6 to 8-HPODE, 8-HODE, 10-HODE and three dihydroxy fatty acids, 5,8-DiHODE, 8,11-DiHODE, and 8,13-DiHODE. 5,8-DiHODE and 8,11-DiHODE are formed from 8*R*-HPODE [21][2]. The absolute configurations of some of these metabolites have not yet been determined.

The stereo configurations of 8-HODE, 10-HODE, 5*S*,8*R*-DiHODE, 7*S*,8*S*-DiHODE, and 8*R*,11*S*-DiHODE have previously been resolved by NMR spectroscopy and by GC–MS analysis of suitable derivatives after ozonolysis [2,4,6,7,14,15,22]. These methods provide unambiguous results, but they can be time consuming and require large amounts of material and suitable standards. Rapid methods to determine the stereo configurations of 8-H(P)ODE, 10-H(P)ODE, and related fungal oxylipins in small amounts are therefore needed.

Our first goal was to find a method for chemical synthesis of racemic standards of hydroxy- and dihydroxyoctadecadienoic acids. Dess–Martin periodinane [23] proved to be an excellent reagent for oxidation of these compounds to keto fatty acids. Our second goal was to determine whether commonly used chiral HPLC columns could be used for separation of the antipodes of 8- and 10-HODE and to determine the absolute configuration of 8-H(P)ODE produced by *M. grisea* and *A. fumigatus* and the absolute configuration of 10-HODE produced by *A. nidulans* and *A. fumigatus*. Our third goal was to determine whether diastereoisomers of 5,8-, 8,11-, and 8,13-DiHODE could be separated by NP-HPLC. This would allow structural identification of dihydroxyoctadecadienoic acids with a hydroxyl group at C-8 (or at C-5) with known configuration, e.g., 5,8-DiHODE and 8,11-DiHODE formed from 8*R*-HPODE by enzymes of *A. fumigatus* and *A. nidulans* [21].[2]

Materials and methods

Materials

Linoleic acids (99 and 94–96%) were from Merck and from Carl Roth (Karlsruhe, Germany), respectively. Dess–Martin periodinane, triphenylphosphine, soybean lipoxygenase-1 (sLO-1) (lipoxidase IV), malt extract, and NaB^2H_4 were from Sigma–Aldrich. Dess–Martin periodinane was dissolved in dry CH_2Cl_2 and stored at −20 °C. Recombinant Mn-LO was expressed in *Pichia pastoris* and purified as described [24]. *G. graminis var. avenae* (CBS 870.73) was purchased from the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands). *G. graminis* was grown in liquid cultures as described [5], whereas *A. fumigatus* Fres. (courtesy Dr. Levenfors, MASE laboratory, Uppsala, Sweden) and *A. nidulans* (RDIT9.32; courtesy Dr. Keller, University of Wisconsin-Madison, Madison, WI) were grown in 1.5% malt extract. Mycelia were harvested and 10 g was incubated with 3–4 mM 18:2n-6 in 0.1 M $NaBO_3$ (pH 8.0 or 8.2). The HPODE, HODE and DiHODE products were purified by preparative TLC (usually ethyl acetate/hexane/acetic acid, 60/40/0.1–0.01) or by silic acid chromatography (Silicar CC-4, Mallinckrodt). The latter was eluted with increasing concentration of diethyl ether (7, 25, and 50%) in hexane and finally with ethanol. The products were identified by LC–MS/MS and by GC–MS [25]. Standards with known stereochemistry were 8*R*-HODE obtained from *G. graminis* and *A. nidulans* and 5*S*,8*R*-DiHODE from *A. nidulans*. *L. edodes* and *A. bisporus* were purchased locally and used for isolation of 10*S*-HODE and 8*R*,11*S*-DiHODE as described [14,22].

Synthesis of [8-^{2}H]8-HODE, [10-^{2}H]10-HODE, [5,8-$^2H_2$] 5,8-DiHODE, and [8,11-$^2H_2$]8,11-DiHODE

The method described by Dess and Martin for [26] oxidation of secondary alcohols was followed, but in a small scale (about 0.3 mg hydroxy fatty acids). The chemical reaction is outlined for oxidation of an allylic alcohol in Scheme 1.

It was essential to include 50 mM acetic acid as a catalyst [27]. For synthesis of racemic [8-^{2}H]8-HODE, 0.3 mg 8*R*-HODE was dissolved in 0.3 ml CH_2Cl_2/50 mM acetic acid, 0.1 ml 0.5 M periodinane in CH_2Cl_2 was added, and the reaction was left for 40 min at room temperature under normal atmosphere. The reaction was terminated with ethyl acetate (3-4 ml) and the organic phase was washed with 0.5 ml 7% $Na_2S_2O_3$ in saturated $NaHCO_3$ and then twice with 0.5 ml water. The organic phase was evaporated

[2] U. Garscha and E.H. Oliw, unpublished observations.