Accepted version

https://doi.org/10.1016/j.biorg.2021.105014

Biocatalytic regio- and stereoselective access to ω-3 endocannabinoid epoxides with peroxygenase from oat flour

Claudia Sanfilippo* and Angela Patti

CNR - Istituto di Chimica Biomolecolare, Via Paolo Gaifami 18, I-95126 Catania, Italy

Abstract

The biocatalytic epoxidation of ethanolamides of ω-3 fatty acids EPA and DHA, regarded as biologically active ω-3 endocannabinoids, in the presence of a peroxygenase-containing preparation from oat flour was investigated. Good regio- and steroselectivity toward the formation of the epoxide on the terminal double bond in the chain was observed with both these fatty acid derivatives and chiral monoepoxides **1** or **2** in 74% optical purity and 51-53% yields were isolated and spectroscopically characterized. The use of acetone as cosolvent in the reaction medium allowed to increase the concentration of starting substrates up to 40 mM and to further improve the selectivity in the epoxidation of DHA-EA. Due to the easy availability of the enzymatic preparation, the method offers a valuable strategy for the access to oxyfunctionalized derivatives of fatty acids.

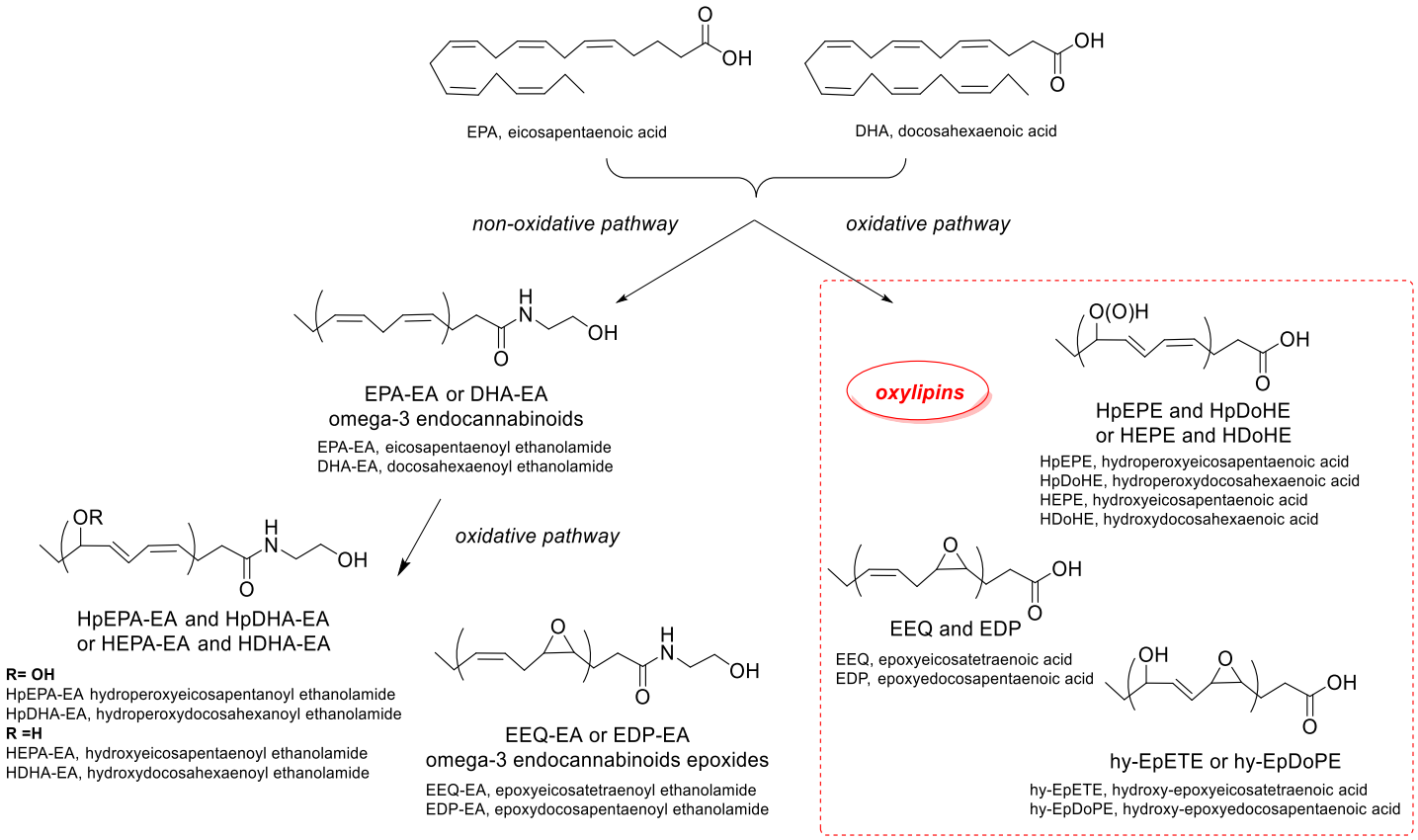
Keywords: Peroxygenase; epoxidation; polyunsaturated fatty acids; ω-3 endocannabinoids; oat flour

1. Introduction

Long chain polyunsaturated fatty acids (LC-PUFA) belonging to the omega-3 family, mainly eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexanoic acid (DHA, 22:6 ω-3), display beneficial effects on the health of the cardiovascular and nervous systems by modulating cell membrane properties, altering lipid metabolism and inhibiting inflammatory pathways, in some case with structure-related differential action [1-4]. Furthermore, they are precursors to a large collection of highly bioactive lipid mediators, metabolically generated through two different biosynthetic pathways involving oxidative and/or non-oxidative reactions.

The oxidative path proceeds through one or more oxygen-dependent reactions catalyzed by cyclooxygenases (COXs), lipoxygenases (LOXs) or by Cytochrome P450 enzymes (CYP P450), leading to several bioactive oxidized lipids as resolvins, protectins and maresins, all grouped into the family of oxylipins [5,6].

Alternatively, in the non-oxidative route both EPA and DHA are converted into the corresponding *N*-acylethanolamides (NAEs), the eicosapentaenoyl ethanolamide (EPA-EA) and docosahexaenoyl ethanolamide (DHA-EA), which have been regarded as omega-3 endocannabinoids (eCB) because they are endogenously produced and show affinity for the cannabinoid receptors CB1 and CB2 [7]. A biological activity similar to that of Δ-9-tetrahydrocannabinol (THC), the active metabolite of *Cannabis sativa*, has been evidenced for EPA-EA and DHA-EA endocannabinoids, that exhibit important anticancer and neuroprotective activities, reducing chronic inflammation and regulating the pro-inflammatory processes [7,8]. In analogy with the metabolism of the first ethanolamide identified



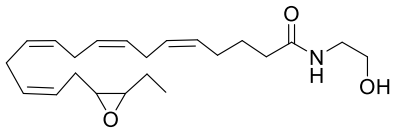
Scheme 1. EPA and DHA metabolites deriving from oxidative and non-oxidative enzymatic pathways.

as an endocannabinoid, the arachidonoyl ethanolamide (AEA, anandamide), EPA-EA and DHA-EA are physiologically transformed in a multitude of related oxygenated endocannabinoids, as hydroperoxy- (HpEPA-EA and HpDHA-EA), hydroxy- (HEPA-EA and HDHA-EA) and epoxides-derivatives (EEQ-EA and EDP-EA), through oxidative processes involving different oxygenases [9] (Scheme 1).

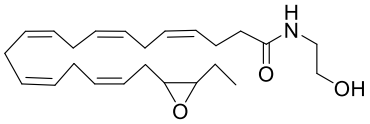
Among these metabolites, terminal epoxides 17,18-EEQ-EA **1** and 19,20-EDP-EA **2** (Scheme 2) have been detected in blood and in several human tissues and their increased vasodilator, anti-tumorigenic and anti-angiogenic activities with respect to the parent acid or amide have been related with the simultaneous presence of amide and epoxide functional groups [10]. The epoxides **1** and **2**, for their

selective agonist activity for endocannabinoid receptor CB2, play a crucial role in modulating the immune response to inflammation by inhibiting the production of pro-inflammatory NO (nitric oxide) and IL-6 (interleukin-6) and increasing simultaneously the levels of anti-inflammatory cytokine IL-10 (interleukin 10) [11].

In spite of the biological relevance and potential therapeutic applications of **1** and **2**, these compounds have been prepared only in analytical scale by epoxidation of EPA or DHA in the presence of *meta*-chloroperbenzoic acid (*m*-CPBA) and following conversion of the products into the corresponding amides [11]. Due to the lack of selectivity in this epoxidation reaction, pure epoxide regioisomers were obtained in low chemical yield after multiple chromatographic purifications. Furthermore, epoxides **1** and **2** have been not yet reported in enantio-enriched form, although some asymmetric syntheses of 17,18-EEQ and 19,20-EDP (the epoxides of free acids EPA and DHA) have been developed [12-14].



17,18-EEQ-EA, **1**



19,20-EDP-EA, **2**

Scheme 2. Chemical structures of the target epoxides derived from EPA and DHA.

Enzymatic catalysis applied to the production of fine chemicals is a useful alternative to traditional chemical synthesis due to the high specificity, selectivity and activity with which enzymes are able to transform a suitable substrate in mild conditions of temperature, pH and pressure.

Oxygenases (E.C. 1.13, monooxygenases and E.C. 1.14, dioxygenases) are a class of enzymes involved in different metabolic pathways able to catalyze the oxyfunctionalization of organic substrates and among these enzymes cytochromes P450, which contain heme and use molecular oxygen for fat metabolism in humans and other living organisms, are the most investigated ones even for synthetic purposes [15-17].

Although cytochromes tolerate a large diversity of substrates as fatty acids [18,19], terpenes [20] and steroids [21], they can be unstable under many process conditions and their catalytic cycle requires expensive cofactor NAD(P)H in equimolar amount with the substrate or an ancillary recycle system for NAD(P)H. Instead, peroxygenases (EC 1.11.2) are considered “self-sufficient” oxygenating enzymes for their cofactor-independence and display a promising potential for applied biocatalysis

[22]. Thanks to the presence of an oxoferryl heme unit in their prosthetic group, peroxygenases are able to promote the epoxidation of unsaturated fatty acids as well as organic non-natural substrates using peroxides (H_2O_2 or R-OOH) as source of oxygen.

Biocatalytic methods for the oxidation of oleic, linoleic and linolenic acid using unspecific peroxygenase from fungi (UPO, EC 1.11.2.1) [23,24] or plant peroxygenase (EC 1.11.2.3) [25] have been described, whereas epoxidation of EPA has been reported with a freeze-dried extract of oat seed flour as enzymatic source [26]. Compared to the purified oxygenases currently reported, this enzymatic preparation provides advantage in cheap and easy availability and herein we investigated its catalytic performances in the selective epoxidation of ethanolamides of both EPA and DHA as an efficient route to epoxides **1** and **2**.

2. Material and methods

2.1. General

(5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-Eicosapentenoic acid ethyl ester (EPA-EE), (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-docosahexaenoic acid ethyl ester (DHA-EE) and methyl ester (DHA-ME) were obtained from Sigma-Adrich. Immobilized lipase from *Candida antarctica* (Novozyme® 435) was obtained from Strem Chemicals Inc.. Lyophilized enzymatic preparation flour from oat seeds was obtained as previously reported [26] and its activity in the epoxidation of oleic acid was 0.37 μmol/mg/h. Enzymatic activity was measured in the epoxidation of methyl oleate (13 μl, 38 μmol) with enzymatic preparation (50 mg) and *t*-BuOOH (13 μl, 95 μmol) in 50 mM phosphate buffer (7 mL) pH 7.4 [26].

All chemicals were used as received. 3Å Molecular sieves (Sigma-Aldrich) were activated by heating at 100 °C for 3h before use. TLC analyses were performed on aluminium plates coated with silica gel and fluorescent indicator F254, revealing the compounds by UV and cerium sulphate solution. Column chromatography was performed on LiChroprep Si 60 (Merck, 25-40 μm) using the specified eluents.

^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AvanceTM 400 spectrometer at 400.13 and 100.62 MHz, respectively. Chemical shifts (δ) are given as ppm relative to the residual solvent peak and coupling constants (*J*) are in Hz. 2D-experiments were carried out using an inverse multinuclear probe with pulse-field Z-gradient and standard Bruker pulse sequence programs.

Optical rotations were measured on Jasco DIP-135 polarimeter using a 10 cm length cell.

High resolution mass spectra (HR-MS) were acquired by a Thermo Scientific Exactive Plu Orbitrap MS (Thermo Fisher Scientific, Inc., Milan, Italy) instrument with ESI ionization mode using a

Thermofisher Orbitrap QExactive instrument, set with 3.0 kV source voltage and 300 °C capillary temperature.

2.2. HPLC analyses

HPLC analyses were carried out on a Dionex instrument equipped with a Ultimate 3000 high-pressure binary pump, an ASI-100 autosampler, a TCC-100 thermostated column compartment and a UVD-100 multiple wavelength detector set at 210, 215, 220 and 250 nm. Chromeleon software (version 6.7) was used for instrument control, data acquisition and data handling. Normal phase HPLC was carried out on a Phenomenex SphereClone TM 5μm Silica 80A LC column (250 x 4.6 mm) eluting with *n*-hexane/2-PrOH 85:15 v/v at flow 0.4 mL/min. Chiral HPLC was performed on a Phenomenex Lux 5μm Cellulose-2 (250 x 4.6 mm) column eluting with *n*-hexane/2-PrOH 90:10 at flow 0.5 mL/min for analysis of **1** and *n*-hexane/2-PrOH 85:15 at flow 0.5 mL/min for analysis of **2**.
Enantiomers of epoxide **1** gave peaks at t_R 57.3 min [(17*R*,18*S*)-**1**] and 63.3 min [(17*S*,18*R*)-**1**] while enantiomers of **2** were eluted at t_R 29.8 min [(19*R*,20*S*)-**2**] and 32.2 min [(19*S*,20*R*)]-**2**.

2.3. Lipase catalyzed synthesis of EPA-EA and DHA-EA

Substrate (200 mg EPA-EE, 0.60 mmol or 200 mg DHA-EE 0.56, mmol) was dissolved in 6 mL of *tert*-butyl methyl ether (TBME) and Novozyme® 435 (100 mg), ethanolamine (70 μl, 1.15 mmol) and molecular sieves (100 mg) were added to the solution. The mixture was stirred at 45 °C and 280 rpm until TLC analysis (*n*-hexane/EtOAc 9:1) revealed the complete disappearance of the substrate, whereas the products were revealed after elution with *n*-hexane/EtOAc 1:1 containing 5% EtOH v/v. The mixture was then extracted with aqueous 2M HCl to remove the excess of amine and the organic phase was washed with water and dried on sodium sulphate. The organic solvent was removed under reduced pressure and the products EPA-EA or DHA-EA were recovered as yellow pale oils in 96% (190 mg, 0.55 mmol) and 94% (186 mg, 0.50 mmol) yield, respectively, and their NMR spectra (Figures SI1 and SI2) were found in accordance with those reported [27,28].

2.4. Chemical epoxidation of EPA-EA and DHA-EA

Substrate (25 mg EPA-EA, 0.072 mmol or 25 mg DHA-EA, 0.067 mmol) was dissolved in 1 mL of CH_2Cl_2 and *m*-CPBA (≤77% purity, 18 mg) was added. The reaction was kept at room temperature under magnetic stirring until TLC analysis (*n*-hexane/EtOAc 1:1 containing 5% EtOH v/v) indicated a steady state (2h). The reaction mixture was extracted with an aqueous solution of sodium bicarbonate and the organic layer was dried on anhydrous sodium sulphate, filtered and subjected to normal phase HPLC analysis.

2.5. Biocatalyzed epoxidation of EPA-EA and DHA-EA in water

Substrate (100 mg EPA-EA, 0.29 mmol or 100 mg DHA-EA, 0.27 mmol) and 550 mg of freeze-dried oat extract were suspended in 50 mM phosphate buffer at pH 7.4 (29 mL or 27 mL, 10mM substrate concentration). To the suspension, vigorously stirred at 25 °C, *tert*-butyl hydroperoxide (*t*-BuOOH, 70 wt% in H_2O) was added in three aliquots over 1 hour (total volume 40 μL, 0.29 mmol for EPA-EA or 37 μL, 0.27 mmol for DHA-EA) and the reaction progress was monitored by TLC analysis (*n*-hexane/EtOAc 1:1 containing 5% EtOH v/v). After 3h the reaction was stopped by adding MeOH (1 mL) and the mixture was extracted with EtOAc (3 × 10 mL). The organic layer was dried on anhydrous sodium sulphate, filtered and analyzed by normal phase HPLC. The organic solvent was evaporated under reduced pressure and the residue was purified by preparative liquid chromatography on LiChroprep Si 60 (*n*-hexane/EtOAc 1:1 containing 5% EtOH v/v). The monoepoxide (17*R*,18*S*)-**1** was isolated in 49% yield (51 mg, 0.14 mmol) and 74% *ee*, $[\alpha]_D^{25}$ = + 3.6 (*c* = 1.25, CH_3OH). ^{1}H-NMR ($CDCl_3$): δ 1.05 (t, 3H, *J* = 7.6 Hz, H-20), 1.48-1.65 (m, 2H, H-19), 1.67-1.76 (m, 2H, H-3), 2.06-2.13 (m, 2H, H-4), 2.17-2.26 (m, 3H, H-2 and H-16a), 2.38-2.46 (m, 1H, H-16b), 2.77-2.87 (m, 6H, H-7, H-10 and H-13), 2.89-2.93 (m, 1H, H-18), 2.94-2.99 (m, 1H, H-17), 3.40 (m, 2H, -C*H*$_2$NH), 3.69 (m, 2H, -C*H*$_2$OH), 5.30-5.43 (m, 6H, H-5, H-6, H-8, H-9, H-11, H-12), 5.43-5.56 (m, 2H, H-14 and H-15), 6.24 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$): δ 10.6 (C-20), 21.0 (C-19), 25.4 (C-3), 25.6 (2 × CH_2-C=), 25.8 (CH_2-C=), 26.1 (C-16), 26.6 (C-4), 35.9 (C-2), 42.4 (*C*H$_2$NH), 56.6 (C-17), 58.5 (C-18), 62.3 (*C*H$_2$OH), 124.3 (C-15), 127.7 (CH=), 127.9 (CH=), 128.2 (CH=), 128.3 (CH=), 128.7 (CH=), 129.0 (CH=), 130.5 (C-14), 174.2 (C-1). HR-ESI-MS: 384.2492 $[M + Na]^+$; theor. for $C_{22}H_{35}NO_3 + Na^+$ 384.2509.

Following the same workup procedure monoepoxide (19*R*,20*S*)-**2** was isolated with 42% yield (44 mg, 0.11 mmol) and 74% *ee*, $[\alpha]_D^{25}$ = + 3.7 (*c* = 0.35, EtOH). ^{1}H-NMR ($CDCl_3$): δ 1.06 (t, 3H, *J* = 7.6 Hz, H-22), 1.51-1.66 (m, 2H, H-21), 2.20-2.29 (m, 3H, H-2 and H-18a), 2.38-2.47 (m, 3H, H-3 and H-18b), 2.80-2.88 (m, 8H, H-6, H-9, H-12 and H-15), 2.90-2.95 (m, 1H, H-20), 2.95-3.00 (m, 1H, H-19), 3.41 (m, 2H, -C*H*$_2$NH), 3.70 (m, 2H, -C*H*$_2$OH), 5.34-5.44 (m, 8H, H-4, H-5, H-7, H-8, H-10, H-11, H-13, H-14), 5.44-5.56 (m, 2H, H-16 and H-17), 6.06 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$): δ 10.6 (C-22), 21.0 (C-21), 23.4 (C-3), 25.6, 25.8, 26.1 (C-18), 36.3 (C-2), 42.5 (*C*H$_2$NH), 56.6 (C-19), 58.4 (C-20), 62.5 (*C*H$_2$OH), 124.4 (C-17), 127.8 (CH=), 128.0 (CH=), 128.1 (2 × CH=), 128.2 (CH=), 128.3 (CH=), 128.4 (CH=), 129.4 (CH=), 130.5 (C-16), 173.6 (C-1). HR-ESI-MS: 410.2647 $[M + Na]^+$, theor. for $C_{24}H_{37}NO_3 + Na^+$ 410.2666.

*2.6. Determination of the absolute configuration of (+)-**1***

A sample (10 mg, 0.03 mmol, 65% *ee*) of the known epoxide (+)-(17*R*,18*S*)-**3** was obtained from EPA by a reported procedure [26]. Then epoxide (+)-(17*R*,18*S*)-**3** was treated with ethanolamine (4 μl, 0.06 mmol) in TBME (1 mL) in the presence of Novozyme® 435 (20 mg) and molecular sieves (20 mg). The mixture was stirred at 45 °C and 280 rpm for 6 h until complete disappearance of the substrate (TLC analysis, *n*-hexane/ EtOAc 1:1 containing 5% EtOH v/v) was detected. Work up as above gave the ethanolamide derivative of (+)-**3** in nearly quantitative yield and 65% *ee*, whose chiral HPLC analysis gave the same elution order for enantiomers as observed for (+)-**1** obtained from direct biocatalyzed epoxidation of EPA-EA.

*2.7. Determination of the absolute configuration of (+)-**2***

Commercial DHA-ME (50 mg, 0.15 mmol) was added to a 50 mM phosphate buffer at pH 7.4 suspension (13 mL) containing 250 mg of freeze-dried oat extract. To the vigorously stirred reaction mixture at 25 °C *t*-BuOOH (70 wt% in H_2O) was added in three aliquots over 1 hour (total volume 20 μL, 0.15 mmol) and the reaction progress was monitored by TLC analysis (*n*-hexane/EtOAc 9:1). After 4 h the reaction was stopped by addition of MeOH (1 mL) and extracted with EtOAc (5 mL × 3). Monoepoxide (+)-**4** (23 mg, 0.064 mmol, 42% yield) was isolated by column chromatography on LiChroprep Si 60 (*n*-hexane/EtOAc 9:1 v/v) and its (19*R*, 20*S*)-configuration was assigned on the basis of specific rotation $[\alpha]_D^{25}$ = + 3.7 (*c* = 0.23, CH_3OH), lit.[12] $[\alpha]_D^{20}$ = + 4.4 (*c* = 1, CH_3OH). Epoxide (+)-**4** (15 mg, 0.042 mmol) was then treated with ethanolamine (6 μl, 0.09 mmol) in the presence of Novozyme® 435 (30 mg) and molecular sieves (30 mg) as above to give the corresponding ethanolamide (+)-**2** (95% yield, 70 % *ee*), whose chiral HPLC analysis gave the same elution order for enantiomers as observed for the epoxide (+)-**2** obtained by biocatalyzed epoxidation of DHA-EA.

2.8. Optimization of the reaction conditions

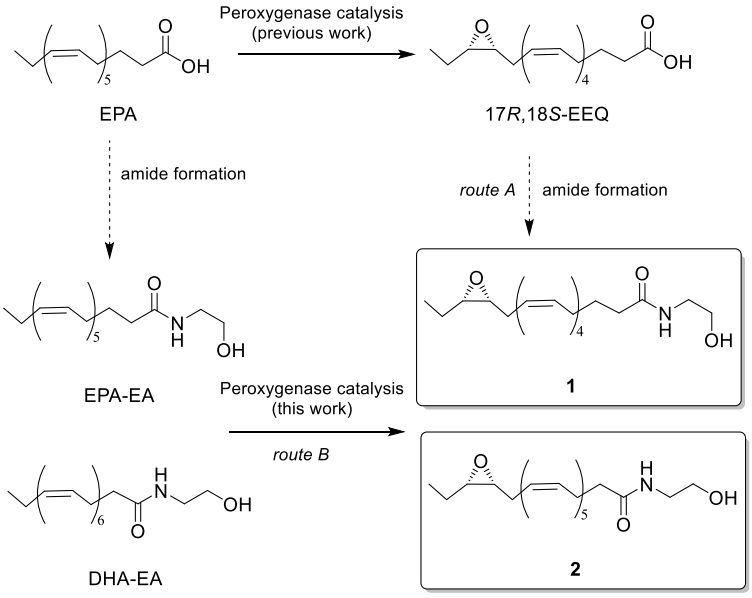
DHA-EA (50 mg, 0.13 mmol) and freeze-dried oat extract (275 mg) were suspended in different volumes (7 mL for 20 mM and 3.5 mL for 40 mM substrate concentration) of the suitable phosphate buffer pH 7.4:acetone mixture (90:10 or 75:25 v/v). To the suspension, vigorously stirred at 25 °C, *t*-BuOOH (70 wt% in H_2O) in 1:1 molar ratio with the substrate was added in 3-6 aliquots over 1-2 hours and the progress of the reaction was monitored by HPLC. To aliquots (0.4 mL) of the reaction mixture MeOH (0.1 mL) and EtOAc (0.5 mL) were added and the organic layer was analyzed by HPLC.

2.9. Preparative epoxidation reactions

DHA-EA (300 mg, 0.81 mmol) and 1.65 g of freeze-dried oat extract were suspended in phosphate buffer pH 7.4:acetone 90:10 v/v mixture (21 mL). To the suspension, vigorously stirred at 25 °C, *t*-BuOOH (70 wt% in H_2O) was added in six aliquots over 2 hours (total volume 110 μL, 0.81 mmol) and the reaction progress was monitored by TLC analysis (*n*-hexane/EtOAc 1:1 containing 5% EtOH v/v). The reaction was stopped by addition of MeOH (1 mL) and the mixture was extracted with EtOAc (3 × 8 mL). The organic layers were collected and dried on anhydrous sodium sulphate. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on LiChroprep Si 60 (*n*-hexane/EtOAc 1:1 containing 5% EtOH v/v) to give monoepoxide (+)-**2** in 53% yield, 74% *ee* and 97% chemical purity (165 mg, 0.42 mmol).

The same procedure was then applied to EPA-EA (300 mg, 0.87 mmol) in phosphate buffer pH 7.4:acetone 90:10 v/v mixture (22 mL) and in the presence of 1.65 g of enzymatic preparation and *t*-BuOOH (120 μL, 0.87 mmol). Work-up as above gave monoepoxide (+)-**1** in 51% yield, 74% *ee* and >98% chemical purity (160 mg, 0.44 mmol).

3. Results and discussion



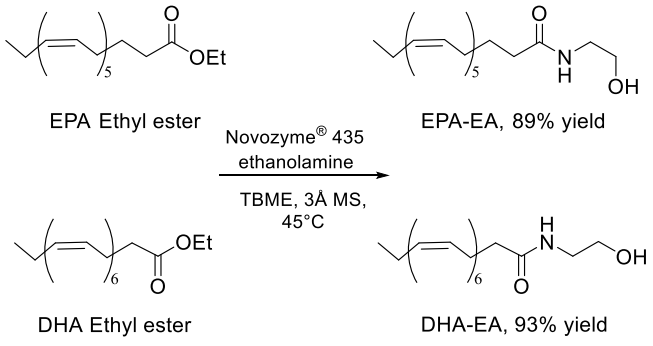
Scheme 3. Possible complementary routes for the biocatalytic access to target epoxides **1** and **2**.

In our previous work [26] it was shown that the microsomal peroxygenase, a membrane-bound heme-thiolate protein, contained in the freeze-dried aqueous extract of defatted flour from oat seed (*Avena sativa*) was able to catalyze the preferential formation of (17*R*,18*S*)-EEQ, but no data were available for the biocatalyzed epoxidation of DHA. Although (17*R*,18*S*)-EEQ could be chemically converted into the target epoxide **1** (Scheme 3, route A), we were more interested in the direct epoxidation of

EPA-EA (Scheme 3, route B) in order to evaluate the substrate tolerance of oat peroxygenase toward derivatives of EPA and DHA, also prompted in this direction by the recent report of Das et al. [11] that found that EPA-EA and DHA-EA are good substrates for CYP P450, especially for the human isozyme CYP2J2. Their work showed that this CYP epoxygenase reacts with EPA-EA and DHA-EA giving respectively five and six monoepoxide regioisomers, one for each C-C double bond present in the substrate molecule, but very high selectivity (50 and 76% respectively) for the terminal epoxides was observed by LC-MS. A greater selectivity of enzyme for DHA-EA compared to EPA-EA was evidenced and it was also found that both these substrates are transformed by CYP2J2 with higher efficiency with respect to the related EPA and DHA free acids. We therefore decided to investigate the peroxygenase-catalyzed epoxidation of EPA-EA (Scheme 3, route B), extending the study to DHA-EA, for the preparation of the target epoxides **1** and **2**, with the aim to explore the substrate tolerance of enzymatic preparation from oat seed and better understand the features of the enzyme, also in comparison with CYP2J2.

3.1. Biocatalyzed synthesis of EPA and DHA ethanolamides

In order to avoid the harsh conditions often required for the chemical synthesis of amides, we resorted into a biocatalyzed reaction for the synthesis of ethanolamide derivatives EPA-EA and DHA-EA. Commercial preparations (Novozyme®435 or Lipozyme 435) of lipase from *Candida antarctica* have been reported as the most effective catalysts for the direct reaction of fatty acids and PUFAs with ethanolamine in different organic solvents as well as in solvent free conditions [29-32]. Since the formation of acid/amine ion-pair could limit the substrate conversion and the availability of "free" amine, the aminolysis reaction of the esters of fatty acids with ethanolamine has been also considered [33-36]. In a modification of a reported procedure [35] we carried out the aminolysis of ethyl esters of EPA and DHA with ethanolamine in TBME using 1:2 substrate:amine molar ratio in the presence of Novozyme® 435 and 3 Å molecular sieves. In these conditions quantitative substrate conversion was observed within 6 h to give the target amides as exclusive products (Scheme 4).



Scheme 4. Synthesis of EPA-EA and DHA-EA *via* lipase-catalyzed aminolysis.

3.2. Peroxygenase-catalyzed synthesis of epoxides 1 and 2

At the onset of our work, aiming to prepare some analytical reference standards of the target epoxides **1** and **2**, a conventional chemical epoxidation of EPA-EA and DHA-EA was carried out using *m*-CPBA as oxidant in about 1:1 molar ratio and at about 60% of substrate conversion at least 3 isomeric monoepoxides were detected in the HPLC chromatograms of the reaction mixtures (Figure 1A and Figure 2A) together with other products in the elution zone of diepoxides.

The peroxygenase-containing preparation was obtained as previously reported [26] by simple aqueous extraction of flour of oat seed followed by low gravity centrifugation to maintain the microsomal fraction as a suspension. Freeze-drying of this suspension was crucial to preserve the activity of the enzyme up to 6 months at –20 ° C storage and ensure reproducibility within the same batch of enzyme preparation.

Biocatalytic epoxidation of EPA-EA was then carried out by suspending EPA-EA (10 mM) and freeze-dried extract of oat seed flour in 1:5.5 w/w ratio in phosphate buffer (pH 7.4) at room temperature and *t*-BuOOH (1:1 molar ratio) was added portionwise during the first hour of reaction. The reaction was monitored by TLC analysis and after quenching (2.5 h) and extraction with EtOAc the whole mixture was analyzed by HPLC. In the chromatogram, the substrate (25%) and a main product in the region of monoepoxides were observed in about 1:2.2 ratio (Figure 1B). Purification of the reaction mixture by silica gel chromatography allowed to isolate compound **1** in 49% yield and its structure was deduced from 1D- and 2D-NMR spectra.

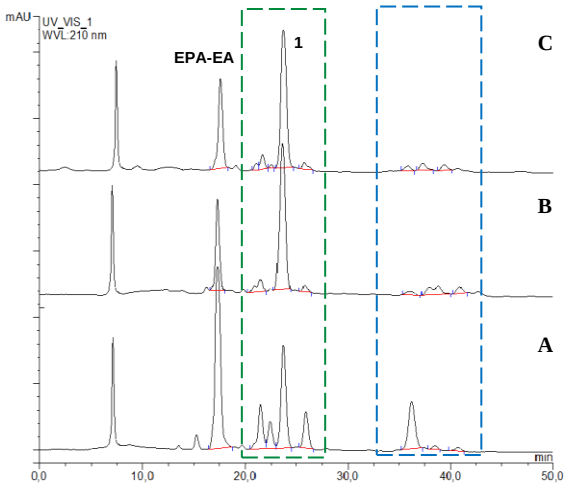


Figure 1. HPLC chromatograms of the reaction mixture of epoxidation of EPA-EA with (A) *m*-CPBA, (B) in presence of peroxygenase preparation in phosphate buffer or (C) in phosphate buffer/acetone 90:10. Elution zones for monoepoxides and diepoxides are indicated in green and blue dashed squares, respectively.

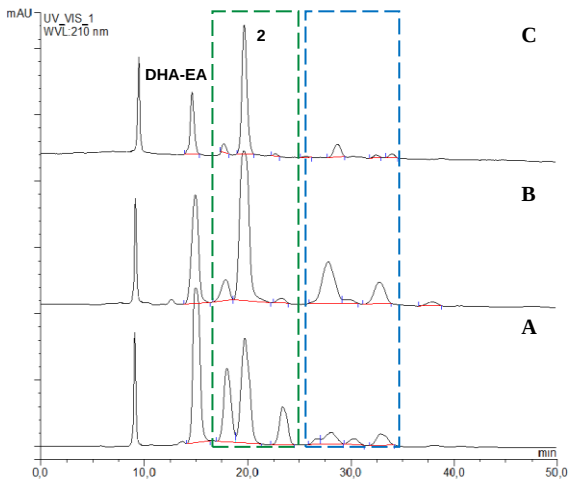


Figure 2. HPLC chromatograms of the reaction mixture of epoxidation of DHA-EA with (A) *m*-CPBA, (B) in presence of peroxygenase preparation in phosphate buffer or (C) in phosphate buffer/acetone 90:10. Elution zones for monoepoxides and diepoxides are indicated in green and blue dashed squares, respectively.

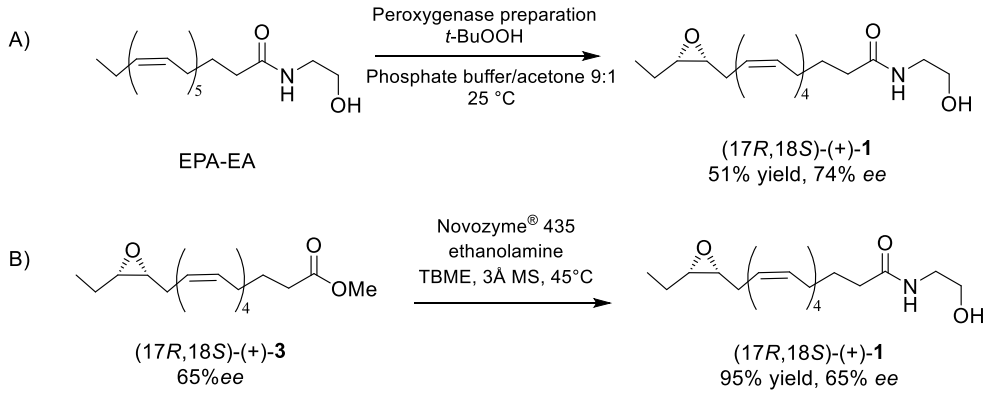
In the ^{1}H-NMR spectrum of **1** (Figure SI3), which was found in agreement with the reported data [11], the two methine protons on the oxirane ring were visible as distinct multiplets in the 2.8-3.0

ppm zone, whereas the corresponding carbons resonated at 56.6 and 58.5 ppm. The structure of the ethanolamide of 17,18-epoxy-(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid was unequivocally assigned to compound **1** on the basis of the long-range C-H correlation (HBMC spectrum, Figure SI6) between the C18 carbon at 58.5 and methyl protons on terminal C-20.

The analysis of COSY spectrum of **1** (Figure SI4) allowed to assign protons on the C2-C4 and C16-C20 fragments of the molecule, revealing that methylenic protons on C-16 resonates as an AB system at 2.4 and 2.2 ppm, the latter signal being partially obscured by the multiplet of protons on C2. Protons on methylene groups intercalating two double bonds as well as olefinic protons gave overlapped resonances collapsing in two unresolved multiplets at about 2.8 and 5.4 ppm, respectively. However, protons on the C14-C15 double bond, which is adjacent to the epoxide group, were slightly differentiated from the broad olefinic resonance and their coupling with H-16 was visible in COSY spectrum.

In the previously unreported ^{13}C-NMR spectrum of **1** (Figure SI3), well resolved signals were observed for all the carbons in the molecule except two and assignment of C2-C4 and C16-C20 fragments was deduced from HSQC spectrum (Figure SI5). Assignment of the olefinic carbons was possible only for C-14 and C-15, identified on the basis of their cross peaks with methinic protons on the epoxide ring in the HBMC spectrum, and C-5 for its long-range correlation with both H-4 and H-3 protons.

As the could be expected, the assignment of NMR resonances of **1** is quite consistent with those reported for (17*R*,18*S*)-EEQ and its methyl ester [26], confirming that magnetic features of the fatty acid chain are litt le influenced by the –COOR group.



Scheme 5. (A) Optimized preparation of monoepoxide (+)-**1** and (B) synthesis of (+)-**1** from reference compound (+)-**3**

Epoxide **1** was obtained as (+)-enantiomer and its optical purity (74% *ee*) was measured by chiral HPLC analysis (Figure 3A). The absolute configuration of (+)-**1** was then assigned by comparing the HPLC retention times of enantiomers of (+)-**1** with those of the epoxide obtained by reaction of the known methyl ester of (17*R*,18*S*)-epoxy-(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid (+)-**3** [26] with

ethanolamine (Scheme 5, B). Since the epoxides obtained by these different ways displayed the same first eluting enantiomer as major peak, the (17*R*,18*S*)-configuration was assigned to (+)-**1** (Figure SI13).

In comparison with the same reaction on EPA free acid [26], the peroxygenase-catalyzed conversion of EPA-EA proceeded with the same stereopreference and was slightly more selective toward the epoxidation of the terminal double bond, as afforded lower amounts of the other isomeric monoepoxides (7% vs. 15%).

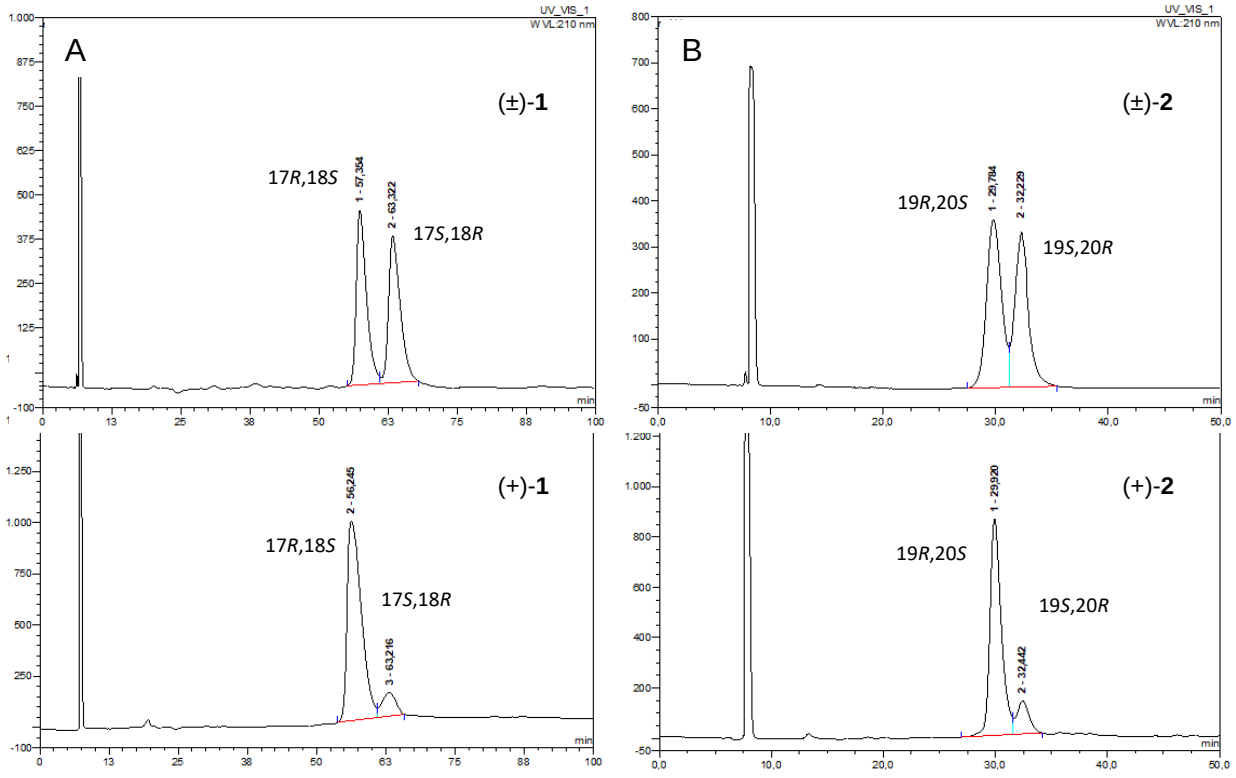
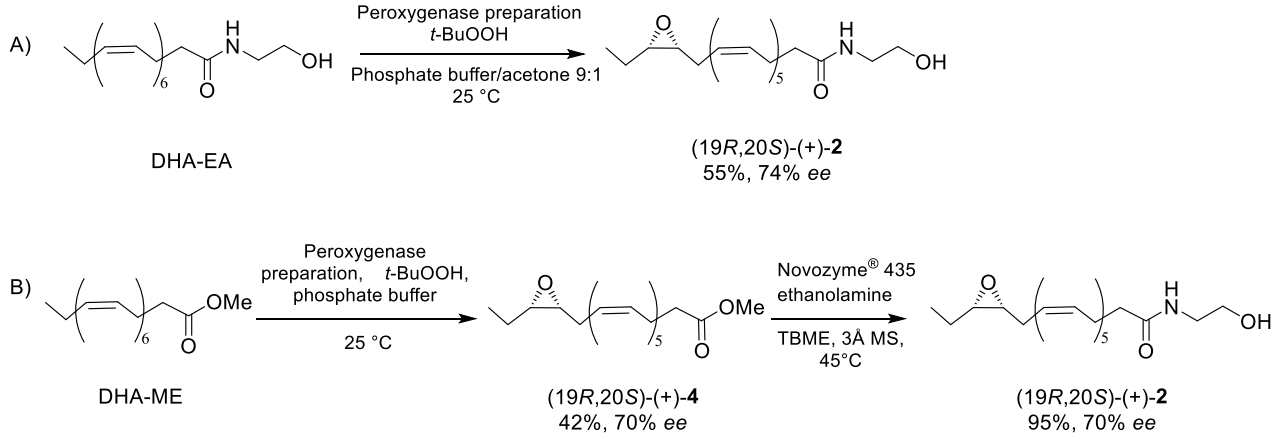


Figure 3. (A) Chiral HPLC chromatogram of (±)-**1** (top) and (+)-**1** (bottom) from biocatalyzed epoxidation of EPA-EA; (B) Chiral HPLC chromatogram of (±)-**2** (top) and (+)-**2** (bottom) from biocatalyzed epoxidation of DHA-EA. For chromatographic conditions see section 2.2

The same reaction conditions were then applied to the biocatalytic epoxidation of DHA-EA and also in this case the enzyme promoted the formation of a main monoepoxide (Figure 2B), whose structure as **2** was spectroscopically confirmed after chromatographic purification (42% isolated yield). Apart from the different intensity of the olefinic proton resonances and the partial overlap of the signals for olefinic carbons, the ^{1}H- and ^{13}C-NMR spectra of **2** (Figure SI7) clearly resemble those of **1** and the same diagnostic C-H correlation between a carbon of the epoxide ring and protons on the methyl group at the end of the chain was observed for both compounds. Assignment of most resonances of **2** was deduced by its 2D-COSY, HSQC and HMBC spectra (Figures SI8-10).

Epoxide **2** was obtained as (+)-enantiomer and its optical purity (74% *ee*) was measured by chiral HPLC analysis (Figure 3B). In order to assign the absolute configuration of (+)-**2**, a peroxygenase-catalyzed epoxidation of DHA methyl ester was carried out and the main monoepoxide product was assigned as (19*R*,20*S*)-epoxy-(4*Z*,7*Z*,10*Z*,13*Z*,16*Z*)-docosapentaenoic acid methyl ester (+)-**4** by comparison of its optical rotation with the value reported in the literature [12]. Conversion of (+)-**4** into the corresponding ethanolamide gave (+)-**2** with 70% *ee* (Scheme 6, B) and (19*R*,20*S*)-absolute configuration for its firstly eluted major enantiomer. Since the epoxide (+)-**2** obtained by biocatalyzed epoxidation of DHA-EA displayed the same chiral HPLC features (retention times and elution order of enantiomers), (19*R*,20*S*)-absolute configuration was therefore assigned also to this compound (Figure SI14), so confirming that the enzyme stereoselectivity observed in the epoxidation of EPA-EA was maintained also with DHA-EA substrate.



Scheme 6. (A) Optimized preparation of monoepoxide (+)-**2** and (B) synthesis of (+)-**2** from reference compound (+)-**4**

3.3. Optimization of the biocatalyzed epoxidation reaction

The very poor solubility in water of EPA and DHA derivatives could limit the practical relevance of biocatalytic processes for their oxyfunctionalization, since the use of dilute mixtures poses difficulties in handling and waste disposing of great volumes of aqueous solution.

Although our results are relevant in terms of substrate concentration compared to other oxygenase-catalyzed reactions on PUFAs and related derivatives (0.1-0.5 mM) [18-19,23-25], we were interested in further improving the productivity of the peroxygenase-catalyzed epoxidation. In the first attempt, the initial 10 mM concentration of EPA-EA or DHA-EA was doubled maintaining the same enzyme/substrate ratio but, disappointingly, the reaction rate significantly decreased to stop completely after 4 h and the conversion reached just 25-30%.

We then resorted into the use of a cosolvent and acetone appeared a good choice since it is miscible with water, not oxidizable by the enzyme and has been positively applied to fungal peroxygenases [23,37].

To our delight, in phosphate buffer:acetone 90:10 mixture the enzyme maintained the same selectivity and reaction rate as in water even with 20 mM concentration of EPA-EA (Figure 1C). Under the same conditions, the epoxidation of DHA-EA proceeded with greater selectivity with respect to water (Figure 2C), giving **2** in higher ratio to the substrate (**2**:DHA-EA 2.7 vs. 1.9) and diepoxides in halved amount (11% vs. 23%) at comparable substrate conversion (75-77%).

Encouraged by these results, the activity of our peroxygenase preparation in the standard epoxidation of oleic acid methyl ester [26] was monitored at different phosphate buffer:acetone ratios in order to evaluate the enzyme tolerance to increasing amount of cosolvent. Comparable enzymatic activity was found in pure buffered water and in the presence of 10% of acetone, while a marked increase in the initial reaction rate was observed in 75:25 and 60:40 buffer:acetone solutions. However, with 40% acetone some deactivation probably occurs since the epoxidation of oleic acid slowed down after 40

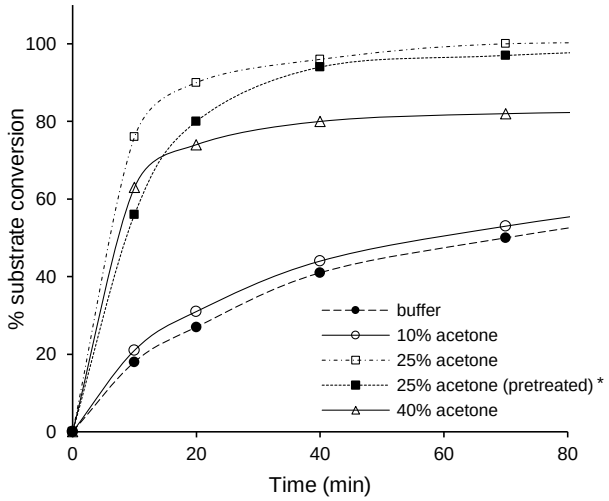


Figure 4. Effect of acetone cosolvent on the activity of peroxygenase in the epoxidation of oleic acid methyl ester. (* = substrate was added after suspending the enzyme in the solvent for 2h)

min and the complete conversion of substrate was not reached. When the enzymatic preparation was suspended in 75:25 buffer:acetone solution for 2 h prior the addition of substrate, unchanged overall enzymatic activity was observed in spite of the lower initial reaction rate (Figure 4).

The best conditions observed with oleic acid methyl ester were then applied to DHA-EA and parallel reactions were then carried out in 90:10 and 75:25 buffer:acetone mixtures with further increased concentration of substrate (40 mM), but in both solvents the same yield of the target monoepoxide **2** (about 50%) was obtained in 2h and lower substrate conversions (66% and 59%, respectively) were reached compared to the reaction with 20 mM DHA-EA (compare entries 6 and 7 with 3, Table 1).

Although the substrate:enzyme ratio was kept constant in all these experiment, the enzyme activity was affected by the increased concentration of the substrate or *t*-BuOOH more than the reaction medium composition.

Decreasing the addition rate of *t*-BuOOH could be exploited for reducing temporary concentration of the oxidant and its possible effects and, indeed, when this parameter was suitably adjusted the same reaction outcome was observed in the biotransformation of 20 or 40 mM DHA-EA in 90:10 buffer:acetone (compare entries 8 and 3, Table 1).

Attempts to decrease the enzyme:substrate ratio were unsuccessful since the epoxidation reactions slowed down without reaching satisfactory substrate conversion (entry 4, Table 1). The use of H_2O_2 as alternative and sustainable oxidant also revealed not feasible since the enzyme was fully inhibited and the reaction did not proceed at all (entry 5, Table 1).

Preparative reactions were then carried out in phosphate buffer:acetone 90:10 at 40 mM concentration of EPA-EA or DHA-EA and target epoxides (+)-**1** and (+)-**2** were isolated in 51% and 53% yield, respectively, and the same optical purity (74% *ee*). It is worth noting that the observed regioselectivity of peroxygenase from oat seeds in the epoxidation of fatty acid ethanolamides is comparable to that reported for CYP2J2 on the same substrates [11]. Regarding stereoselectivity, this is the first time that the absolute configuration of the epoxides of EPA-EA and DHA-EA is assigned and the stereopreference of peroxygenase here observed is in agreement with that shown by CYP2J2 in the epoxidation of EPA and DHA free acids, while opposite stereopreference is reported for other cytochrome enzymes with these fatty acids [18, 38].

Table 1. Optimization of the reaction conditions in the peroxygenase-catalyzed epoxidation of DHA-EA[a]

Entry	DHA-EA (mM)	Solvent	Conversion (%)[b]	Monoepoxide 2 (%)[b]
1	10	Buffer[c]	75	49
2	20	Buffer	31	29
3	20	Buffer/acetone 90:10	77	61
4	20	Buffer/acetone 90:10[d]	45	40
5	20	Buffer/acetone 90:10[e]	0	0
6	40	Buffer/acetone 90:10	66	51
7	40	Buffer/acetone 75:25	59	49
8	40	Buffer/acetone 90:10[f]	74	61

[a]The reactions were carried out on using 50 mg DHA-EA and 275 mg of enzyme preparation at 25 °C for 2-4 h and adding *t*-BuOOH (1:1 molar ratio) in three aliquots over 1 hour. [b]Determined by HPLC of the whole reaction mixture. [c]50 mM Phosphate buffer at pH 7.4. [d]Half amount of enzyme preparation was used. [e]H_2O_2 was used as oxidant. [f]*t*-BuOOH was added in six aliquots over 2 hours

4. Conclusions

A raw preparation from oat flour containing peroxygenase has been shown to be a good catalyst for the epoxidation of the ethanolamides of both polyunsaturated fatty acids EPA and DHA, giving the corresponding monoepoxides on the terminal double bond of the chain, **1** and **2**, with good regio- and enantioselectivity. The use of acetone as cosolvent allowed to increase the concentration of the starting substrates and improved regioselectivity in the epoxidation. The obtained monoepoxides, regarded as ω-3 endocannabinoids with interesting biological activity as mediators of inflammation, have been reported up to date only in racemic form and in analytical scale. The developed method offers a valid alternative to chemical synthesis or cytochrome-mediated epoxidation of PUFA-derivatives for the low cost and easy availability of the employed enzyme.

Acknowledgements

Thanks are due to Dr. Sandro Dattilo, Institute for Polymers, Composites and Biomaterials of CNR, for HR-ESI-MS spectra and to Mr. Antonio Greco for technical assistance in graphics.

References

[1] P. Saravanan, N.C. Davidson, E.B. Schmidt, P.C. Calder, Cardiovascular effects of marine omega-3 fatty acids, Lancet 375 (2010) 540–50. DOI: 10.1016/S0140-6736(10)60445-X

[2] D. Mozaffarian, J. H. Y. Wu, (n-3) Fatty acids and cardiovascular health: are effects of EPA and DHA shared or complementary? J. Nutr. 142 (2012) 614S–625S. DOI: 10.3945/jn.111.149633

[3] S.C. Dyall, Front. Aging Neurosci. Long-chain omega-3 fatty acids and the brain: a review of the independent and shared effects of EPA, DPA and DHA7 (2015) Article 52. DOI: 10.3389/fnagi.2015.00052

[4] S. Layé, A. Nadjar, C. Joffre, R.P. Bazinet, Anti-inflammatory effects of omega-3 fatty acids in the brain: physiological mechanisms and relevance to pharmacology, Pharmacol. Rev. 70 (2018) 12–38. DOI: 10.1124/pr.117.014092

[5] R.-R. Ji, Z.-Z.Xu, G. Strichartz, C.N. Serhan, Emerging roles of resolvins in the resolution of inflammation and pain, Trends Neurosci. 34 (2011) 599-609. DOI: 10.1016/j.tins.2011.08.005

[6] C.N. Serhan, J. Dalli, R.A. Colas, J.W. Winkler, N. Chiang, Protectins and maresins: new pro-resolving families of mediators in acute inflammation and resolution bioactive metabolome, Biochim. Biophys. Acta 1851 (2015) 397–413. DOI: 10.1016/j.bbalip.2014.08.006

[7] J.E. Watson, J.S. Kim, A. Das, Emerging class of omega-3 fatty acid endocannabinoids & their derivatives, Prostaglandins Other Lipid Mediat. 143 (2019) 106337. DOI: 10.1016/j.prostaglandins.2019.106337

[8] I. Brown, M.G. Cascio, D. Rotondo, R.G. Pertwee, S. D. Heys, K.W.J. Wahle, Cannabinoids and omega-3/6 endocannabinoids as cell death and anticancer modulators, Prog. Lipid Res. 52 (2013) 80–109. DOI: 10.1016/j.plipres.2012.10.001

[9] C.A. Rouzer, L.J. Marnett, Endocannabinoid oxygenation by cyclooxygenases, lipoxygenases, and cytochromes P450: cross-talk between the eicosanoid and endocannabinoid signaling pathways, Chem. Rev. 111 (2011) 5899–5921. DOI: 10.1021/cr2002799

[10] J. Roy, J.E. Watson, I.S. Hong, T.M. Fan, A. Das, Antitumorigenic properties of omega-3 endocannabinoid epoxides, J. Med. Chem. 61 (2018) 5569-5579. DOI: 10.1021/acs.jmedchem.8b00243

[11] D. R. McDougle, J.E. Watson, A.A. Abdeen, R. Adili, M.P. Caputo, J.E. Krapf, R.W. Johnson, K.A. Kilian, M. Holinstat, A. Das, Anti-inflammatory ω-3 endocannabinoid epoxides, PNAS 114 (2017) E6034-E6043. DOI: 10.1073/pnas.1610325114

[12] T. Kato, T. Ishimatu, A. Aikawa, K. Taniguchi, T. Kurahashi, T. Nakai, Preparation of the enantiomers of 19-epoxy docosahexaenoic acids and their 4-hydroxy derivatives, Tetrahedron: Asymmetry 11 (2000) 851–860. DOI: 10.1016/S0957-4166(00)00015-X

[13] Y. Nanba, R. Shinohara, M. Morita, Y. Kobayashi, Stereoselective synthesis of 17,18-epoxy derivative of EPA and stereoisomers of isoleukotoxin diol by ring opening of TMS-substituted epoxide with dimsyl sodium, Org. Biomol. Chem. 15 (2017) 8614–8626. DOI: 10.1039/C7OB02291C

[14] M. A. Cinelli, K. S. S. Lee, Asymmetric total synthesis of 19, 20-epoxydocosapentaenoic acid, a bioactive metabolite of docosahexaenoic acid, J. Org. Chem. 84 (2019) 15362−15372. DOI: doi.org/10.1021/acs.joc.9b02378

[15] Y. Liang, J. Wei, X. Qiu, N. Jiao, Homogeneous oxygenase catalysis, Chem. Rev. 118 (2018) 4912-4945. DOI: 10.1021/acs.chemrev.7b00193

[16] J. Dong, E. Fernandez-Fueyo, F. Hollmann, C. E. Paul, M. Pesic, S. Schmidt, Y. Wang, S. Younes, Biocatalytic oxidation reactions: A chemist's perspective, Angew. Chem. Int. Ed. 57 (2018) 9238 – 9261. DOI: 10.1002/anie.201800343

[17] V. B. Urlacher, M. Girhard, Cytochrome P450 monooxygenases in biotechnology and synthetic biology, Trends in Biotechnol. 37 (2019) 882-897. DOI: 10.1016/j.tibtech.2019.01.001

[18] J. H. Capdevila, S. Wei, C. Helvig, J. R. Falck, Y. Belosludtsev, G. Truani, S. E. Graham-Lorencei, J. A. Peterson, The highly stereoselective oxidation of polyunsaturated fatty acids by cytochrome P450BM-3, J. Biol. Chem. 271 (1996) 22663–22671. DOI: 10.1074/jbc.271.37.22663

[19] M. A. Cinelli, J. Yang, A. Scharmen, J. Woodman, L. M. Karchalla, K. S. S. Lee, Enzymatic synthesis and chemical inversion provide both enantiomers of bioactive epoxydocosapentaenoic acids, J. Lipid Res. 59 (2018) 2237-2252. DOI: 10.1194/jlr.D089136

[20] I. Pateraki, A.M. Heskes, B. Hamberger, Cytochromes P450 for Terpene Functionalisation and Metabolic Engineering. In: Biotechnology of Isoprenoids. Advances in Biochemical Engineering/Biotechnology, ed. J. Schrader and J. Bohlmann, Springer, Cham, 148 (2015) 107-139. DOI: 10.1007/10_2014_301

[21] R. W. Estabrook, Steroid hydroxylations: A paradigm for cytochrome P450 catalyzed mammalian monooxygenation reactions, Biochem. Biophys. Res. Comm. 338 (2005) 290-298. DOI: 10.1016/j.bbrc.2005.08.168

[22] S. Bormann, A. G. Baraibar, Y. Ni, D. Holtmann, F. Hollmann, Specific oxyfunctionalisations catalysed by peroxygenases: opportunities, challenges and solutions, Catal. Sci. Technol. 5 (2015) 2038-2052. DOI: 10.1039/C4CY01477D

[23] C. Aranda, A. Olmedo, J. Kiebist, K. Scheibner, J.C. del Rìo, A.T. Martìnez, A. Gutìerrez, Selective epoxidation of fatty acids and fatty acid methyl esters by fungal peroxygenases, ChemCatChem 10 (2018) 3964 – 3968. DOI: 10.1002/cctc.201800849

[24] A. González-Benjumea, J. Carro, C. Renau-Mínguez, D. Linde, E. Fernández-Fueyo, A. Gutiérrez, A.T. Martínez, Fatty acid epoxidation by *Collariella virescens* peroxygenase and heme-channel variants, Catal. Sci. Technol. 10 (2020) 717–725. DOI: 10.1039/C9CY02332A

[25] G. J. Piazza, A. Nuñez, T.A. Foglia, Epoxidation of fatty acids, fatty methyl esters, and alkenes by immobilized oat seed peroxygenase, J. Mol. Catal. B: Enzym. 21 (2003) 143-151. DOI: 10.1016/S1381-1177(02)00122-4

[26] C. Sanfilippo, A. Paterna, D.M. Biondi, A. Patti, Lyophilized extracts from vegetable flours as valuable alternatives to purified oxygenases for the synthesis of oxylipins, Bioorg. Chem. 93 (2019) 103325. DOI: 10.1016/j.bioorg.2019.103325

[27] R. Ottria, S. Casati, P. Ciuffreda, ^{1}H, ^{13}C and ^{15}N NMR assignments for *N*- and *O*-acylethanolamines, important family of naturally occurring bioactive lipid mediators, Magn. Reson. Chem. 50 (2012), 823-828. DOI: 10.1002/mrc.3891

[28] I.H. Kim, Y. Kanayama, H. Nishiwaki, T. Sugahara, K. Nishi, Structure–activity relationships of fish oil derivatives with antiallergic activity in vitro and in vivo, J. Med. Chem. 62 (2019) 9576-9592. DOI: 10.1021/acs.jmedchem.9b00994

[29] Liu, K. J.; Nag, A.; Shaw, J.-F. Lipase-catalyzed synthesis of fatty acid diethanolamides, J. Agric. Food Chem. 49 (2001) 5761−5764. DOI: 10.1021/jf0107858

[30] P. Tufvesson, A. Annerling, R. Hatti-Kaul, D. Adlercreutz, Solvent-free enzymatic synthesis of fatty alkanolamides, Biotechnol. Bioeng. 97 (2007) 447–453. DOI: 10.1002/bit.21258

[31] P. Plastina, J. Meijerink, J.P. Vincken, H. Gruppen, R. Witkamp, B. Gabriele, Selective synthesis of unsaturated N-acylethanolamines by lipase-catalyzed N-Acylation of ethanolamine with unsaturated fatty acids, Lett. Org. Chem. 4 (2009) 444-447. DOI: 10.2174/157017809789124885

[32] Wang, X.; Wang, X.; Wang, T. Synthesis of oleoylethanolamide using lipase, J. Agric.Food Chem. 60 (2012) 451−457. DOI: 10.1021/jf203629w

[33] M. Fernández-Pérez, C. Otero, Enzymatic synthesis of amide surfactants from ethanolamine, Enz. Microb. Technol. 28 (2001) 527-536. DOI: 10.1016/S0141-0229(01)00293-9

[34] L. Couturier, D. Taupin, F. Yvergnaux, Lipase-catalyzed chemoselective aminolysis of various aminoalcohols with fatty acids, J. Mol. Catal. B: Enzym. 56 (2009) 29–33. DOI: 10.1016/j.molcatb.2008.04.010

[35] K.M. Whitten, A. Makriyannis, S.K. Vadivel, Enzymatic synthesis of N-acylethanolamines: direct method for the aminolysis of esters, Tetrahedron Lett. 53 (2012) 5753–5755. DOI: 10.1016/j.tetlet.2012.08.042

[36] X. Wang, Y. Chen, Y. Ma, Q. Jin, X. Wang, Lipozyme 435-catalyzed synthesis of eicosapentaenoyl ethanolamide in a solvent-free system, J. Mol. Catal. B: Enzym. 122 (2015) 233-239. DOI: 10.1016/j.molcatb.2015.09.016

[37] S. Peter, M. Kinne, X. Wang, R. Ullrich, G. Kayser, J. T. Groves, M. Hofrichter, Selective hydroxylation of alkanes by an extracellular fungal peroxygenase, FEBS Journal 278 (2011) 3667–3675. DOI: 10.1111/j.1742-4658.2011.08285.x

[38] D. Lucas. S. Goulitquer, J. Marienhagen, M. Fer, Y. Dreano, U. Schwaneberg, Y. Amet, L. Corcos, Stereoselective epoxidation of the last double bond of polyunsaturated fatty acids by human cytochromes P450, J. Lip. Res. 51 (2010) 1125-1133. DOI: 10.1194/jlr.M003061