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Solvent-induced 7*R*-dioxygenase activity of soybean 15-lipoxygenase-1 in the formation of omega-3 DPA-derived resolvin analogues

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Abstract

The resolvin family contains important anti-inflammatory and pro-resolution compounds enzymatically derived *in vivo* from the polyunsaturated omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). More recently, docosapentaenoic acid (DPA) has emerged as another potentially important precursor in the biological production of resolvin compounds. In this work we have used medium engineering to develop a simple method for the controlled synthesis of two di-hydroxylated diastereomers of DPAn-3 catalyzed by soybean 15-lipoxygenase-1 (15-sLOX-1) in the presence of short chain *n*-alcohols, including methanol, ethanol and isopropanol. The complete structures of the two major products, 7*S*,17*S*-dihydroxydocosapenta-8*Z*,10*E*,13*Z*,15*E*,19*Z*-enoic acid (7*S*,17*S*-diHDPAn-3) and 7*R*,17*S*-dihydroxydocosapenta-8*Z*,10*E*,13*Z*,15*E*,19*Z*-enoic acid (7*R*,17*S*-diHDPAn-3), have been elucidated using spectroscopic analysis. The alcohol-dependent *R*-dioxygenase activity of soybean 15-lipoxygenase with mono-hydroperoxide intermediate substrates has also been demonstrated with other biologically relevant PUFAs, including DHA, EPA and ARA. The developed method has applications in the production of closely related isomers of naturally-occurring resolvins and protectins, demonstrating the versatility of 15-sLOX-1 as a biocatalyst.

Keywords

Soybean 15-lipoxygenase-1
Docosapentaenoic acid
n-Alcohol
Medium engineering
Resolvins

Abbreviations

ARA, arachidonic acid, all-*cis*-5,8,11,14-eicosatetraenoic acid; EPA, all-*cis*-5,8,11,14,17-eicosapentaenoic acid; DHA, all-*cis*-4,7,10,13,16,19-docosahexaenoic acid; DPAn-3, all-*cis*-7,10,13,16,19-docosapentaenoic acid; DPAn-6, all-*cis*-4,7,10,13,16-docosapentaenoic acid; DTAn-6, all-*cis*-7,10,13,16-docosatetraenoic acid; 15-sLOX-1, soybean 15-lipoxygenase P1; 17-H(P)DPAn-3, 17-hydro(pero)xy-7*Z*,10*Z*,13*Z*,15*E*,19*Z*-docosapentaenoic acid; 7,17-dihydroxy-8*E*,10*Z*,13*Z*,15*E*,19*Z*-docosapentaenoic acid; LC-PUFA, long chain-polyunsaturated fatty acid.

1. Introduction

The discovery of specialized pro-resolving mediators (SPM) derived from dietary long chain polyunsaturated fatty acids (LC-PUFAs) marks a significant event in understanding the mechanisms of acute inflammation, and more importantly the resolution process [1]. Although previously believed to be a passive process, inflammation is now known to be actively resolved by locally acting SPMs, including resolvins (Rv), protectins (PD) and maresins (MaR) [1-3] derived from omega-3 fatty acids.

Many naturally occurring resolvins and protectins from inflammatory exudates have been identified within the previous ten years, which has contributed to the elucidation of important anti-inflammatory and pro-resolution pathways [4, 5]. This gain in understanding has also directed novel therapeutic approaches for the treatment of chronic inflammation through the production of SPM analogues which possess similar bioactivity. The family of resolvins and protectins, terms coined by Serhan and co-workers [2, 6], have become pharmaceutical targets for the treatment of several chronic conditions [7, 8], including lipoxin analogues for the treatment of asthma [9] and resolvin analogues in the treatment of dry eye [7]. A current limitation for the pharmaceutical development of SPM analogues is the requirement to introduce specific regio- and stereochemistry of double bonds (*cis*/*trans*) and hydroxy groups (*R*/*S*) to produce stereochemically pure biologically active structures. This is commonly achieved through organic synthesis protocols that are often lengthy, complex and employ the use of harsh/toxic solvents [10-12].

In biological systems, peroxidase enzymes including cyclooxygenase (EC 1.14.99.1) and lipoxygenase (EC 1.13.11.-) isoforms have been shown to be important biocatalysts of both pro- and anti-inflammatory mediators including leukotrienes, lipoxins, resolvins, protectins and maresins. LC-PUFAs containing multiple 1*Z*,4*Z*-pentadiene structures are suitable substrates for metabolism by lipoxygenase enzymes, which catalyze the insertion of molecular oxygen in a highly stereo- and regio-specific manner to generate mono-, di- and tri-hydro(pero)xylated products, making the use of enzymes an attractive alternative to organic synthesis methods in the synthesis of SPM analogues. The stereospecificity of soybean 15-lipoxygenase (15-sLOX-1) has been well established since its initial application to LC-PUFAs in 1967 [13], with the enzyme known to exclusively synthesise *S*-chirality hydro(pero)xy groups in standard buffer medium due to the stereo- and regio-specific nature of the initial hydrogen abstraction and subsequent antarafacial incorporation of molecular oxygen [13-18].

Whilst the high stereospecificity of many enzymatic reactions is what makes them so useful as biocatalysts, it can also limit their applicability, as the required stereochemistry may not be achievable [Wescott, 1994]. This is why methods to improve and/or change the stereospecificity of enzymes are desired. The addition of water-miscible organic cosolvents to enzymatic reactions was originally used to increase solubility of hydrophobic substrates in the aqueous reaction medium, however, it was soon found that these cosolvents could also affect various aspects of the reaction, including the stereospecificity [Faber, 1993]. The manipulation of enzyme properties through changing the nature

of the solvent has been termed 'medium engineering' [19-21]. There are many reports of changing, and even inverting, enzyme enantioselectivity by changing the reaction medium [19-22]. For example, Kalkote and co-workers studied the desymmetrization of *meso*-cyclopent-2-en-1,4-diacetate to 4-(*R*)-hydroxycyclopent-2-en-1-(*S*)-acetate, catalyzed by a hydrolase enzyme from *Trichosporin beigelii*, and found that the addition of 10% v/v ethanol enhanced the enantioselectivity of the enzyme from 29.1% optical purity (88% yield) to 82.6% optical purity (83% yield) [23]. In examining the biocatalytic properties of three Baeyer-Villiger monooxygenases in aqueous-organic media with organic sulfide substrates, de Gonzalo et al. found that organic cosolvents generally decreased the enzyme activity, but in some cases significant improvements in enantioselectivity were induced by short chain alcohols, and in a small number of cases, methanol was able to cause a reversal of enantiopreference [24].

The dioxygenation reactions of biologically relevant PUFAs by commercially available 15-sLOX-1 are well characterized in the literature, in particular with arachidonic acid (ARA; C20:4n-6), docosahexaenoic acid (DHA; C22:6n-3) and eicosapentaenoic acid (EPA; C20:5n-3) substrates [14, 17, 25-33]. However, considerably less is known concerning other LC-PUFAs which exist in lower concentrations in biological systems, yet remain significant as potential substrates for 15-sLOX-1. Among these, omega-3 and omega-6 docosapentaenoic acid (DPA; C22:5) are emerging as interesting potential precursors to resolvin and protectin analogues [29, 34], due to their structural similarity to DHA and the presence of the multiple 1*Z*,4*Z*-pentadiene moieties for lipoxygenase catalysis. DPAn-3 is an important dietary omega-3 and the major LC-PUFA present in red meat, with Australians consuming similar amounts of EPA and DPAn-3 in their diets [35]. Both DPAn-3 and n-6 have themselves been found to possess anti-inflammatory activity, including inhibition of angiogenesis [36] and reduction of pro-inflammatory cytokine expression [37]. To date there have been few studies focusing on the biosynthesis of DPAn-3-derived resolvin analogues from either purified enzymes [29] or isolated cell/inflammatory exudates [38].

Recently we characterized products from the double dioxygenation of LC-PUFAs, including DPAn-3 and n-6, using 15-sLOX-1 [31]. In this work we further expand on the synthesis of DPAn-3-derived resolvin analogues using 15-sLOX-1 as a biocatalyst, demonstrating the ability to affect product stereochemistry through relatively subtle changes in environmental conditions, and offering an attractive alternative to total organic synthesis in the production of SPM analogues.

2. Materials and methods

2.1 Materials

Soybean 15-lipoxygenase-1 (EC 1.13.11.33, P_1, 15.3 Munits/ml, 27.73 mg of protein/ml, 0.552 Munits/mg of protein) was purchased from Cayman Chemical (Ann Arbor, MI). All fatty acids (DHA, EPA, ARA, DPAn-3, DPAn-6 and DTAn-6) were purchased from Nu-Chek Prep (Elysian, MN). Supelclean LC-18 SPE cartridges (500 mg, Supelco Analytical) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia).

2.2 Lipoxygenase catalyzed reactions

Lipoxygenase catalyzed reactions were performed as described previously [25, 31]. Briefly, PUFA substrates (0.08 mM) were prepared in sodium tetraborate buffer (50 mM, pH 9.0) containing the desired concentration of *n*-alcohol. The reaction was initiated by the addition of 15-sLOX-1 (15-150 kunits per ml of substrate solution) and allowed to proceed for 15 minutes with stirring at room temperature. The resulting hydroperoxides were reduced with 200 µl of 1 M sodium borohydride (15 minutes) and acidified with concentrated acetic acid (50 µl) until foaming ceased. Products were extracted using a pre-conditioned C18 SPE cartridge (500 mg) and eluted with ethanol, or by chloroform solvent extraction. Products were stored in ethanol at -20°C.

2.3 NP-HPLC

HPLC analysis was performed using an Agilent 1200 Series HPLC system (Agilent Technologies, Mulgrave, VIC, Australia) in normal phase mode, equipped with a thermostated column compartment, diode array detector and automated fraction collector. Analytical separations were carried out using a Supelcosil LC-Diol column (Supelco, 25 cm × 3 mm, 5 µm; Sigma Aldrich) with an isocratic mobile phase of 95% *n*-heptane/acetic acid/2,2-dimethoxypropane (100:0.1:0.1 v/v) and 5% isopropanol. Extraction solvent was evaporated under a stream of N_2 and samples were prepared in mobile phase. Injection volume was 15 µl. The flow rate was 0.5 ml/min and the column temperature was 10°C. Elution of products was monitored at 210 nm, 234 nm, 250 nm and 270 nm [31]. For preparative separations, products were methylated prior to analysis using 0.5 ml methanol and 0.5 ml (trimethylsilyl)diazomethane (2 M in diethyl ether) for 1 hour at room temperature. The methylation reagents were then evaporated under nitrogen and the products re-dissolved in mobile phase. Preparative separations were performed using a LiChrospher silica column (Merck, 12.5 cm × 4 mm, 5 µm) with an isocratic mobile phase of 98% *n*-heptane and 2% isopropanol. The injection volume was 50 µl, flow rate was 1 ml/min and the column temperature was 30°C.

2.4 Chiral-phase HPLC

Chiral phase analysis was performed on an Agilent 1260 Infinity HPLC system (Agilent Technologies) equipped with a diode array detector. Products were methylated prior to analysis as described above. Separations were performed on a Chiralpak AD-H column (25 cm × 0.46 cm, 5 µm; Daicel) with an isocratic mobile phase of *n*-hexane and ethanol (1-10% v/v) at ambient temperature [39]. The flow rate was 1 ml/min and injection volume 20 µl.

2.5 GC-MS

Products (~ 1 mg) were subjected to full catalytic hydrogenation over PtO_2 catalyst (10 mg) in 2 ml ethanol for 25 minutes with a constant stream of hydrogen and stirring. The solution was filtered (0.45 µm nylon filter) and the solvent evaporated under nitrogen. Products were then trimethylsilylated using BSTFA (200 µl) and pyridine (200 µl) at 100°C for 1 hour. GC-MS analysis was performed on an Agilent 6890N gas chromatograph with a 5975 mass selective detector (electron

impact ionization). Separations were performed on a BPX5 column (SGE, 10 m × 0.1 mm, 0.1 µm film thickness) with helium carrier gas (0.2 ml/min). The inlet was maintained at 250°C with a split ratio of 20:1, and injection volume was 1.5 µl. The oven temperature program was as follows: 150°C for 2 minutes, increased at 8°C/min to 300°C, and held at 300°C for 6 minutes [31]. The conditions of the mass selective detector were as follows: electron voltage, -70 eV; MS quad, 150°C; MS source, 230°C; solvent delay, 3.00 minutes; scan range, 35.0 – 600.0 amu.

2.6 UV-visible spectrophotometry

Absorption spectra of products were collected in ethanol using a Cary Series 300 UV-visible spectrophotometer (Agilent Technologies) with a 1.0 mm path length quartz cuvette. Spectra were collected from 200 nm to 350 nm in double beam mode with a bandwidth of 1 nm and a scan rate of 0.5 nm/second.

2.7 NMR spectroscopy

1D ^{1}H-NMR and 2D ^{1}H, ^{1}H-COSY NMR experiments were performed on an Avance 500 MHz FT-NMR spectrometer (Bruker). Purified products were dissolved in 40 µl of deuterated methanol and transferred to an internal capillary within a standard bore NMR tube. 64 scans were accumulated and all chemical shifts were measured using residual CH_3OH peaks as reference (3.31 ppm).

2.8 Enzyme kinetic parameters

DPAn-3 and DTAn-6 substrates (10-300 µM) were prepared in oxygenated borate buffer (75 mM, pH 9.0) in a 1 cm path length quartz cuvette, and reactions initiated with the addition of 20 kunits 15-sLOX-1 prepared in buffer (400 kunits/mL). Reactions were performed at room temperature. Product formation was monitored at 237 nm for conjugated dienes (ε_M = 23,000 $M^{-1} \times cm^{-1}$) [25], and data was collected at 0.1 second intervals for 0.5 minutes using a Cary Series 300 UV-visible spectrophotometer (Agilent Technologies). Measurements were performed in triplicate, and the data analysed using the Michaelis-Menten kinetic model (GraphPad Prism v6.0d, La Jolla, CA).

2.9 TOF-MS (ESI)

Mass spectra were collected using an Agilent Technologies 6210 MSD time-of flight mass spectrometer operating in positive ionization mode (ESI). Analysis conditions were as follows: drying gas, nitrogen (7 L/min, 350°C); nebulizer gas, nitrogen (16 psi); capillary voltage, 4.0 kV; vaporizer temperature 350°C; and cone voltage, 60 V. Data analysis was performed using Agilent MassHunter Qualitative Analysis software (version B.03.1).

2.10 Compound characterisation

7*S*,17*S*-dihydroxydocosa-8*E*,10*Z*,13*Z*,15*E*,19*Z*-pentaenoic acid methyl ester (II); UV-vis (heptane solvent): 224, 243 nm. FT-IR (hexane solvent): $\tilde{\nu}$ = 3012, 2956, 2925, 2855, 1734, 1645, 1460 cm^{-1}. ^{1}H NMR (500 MHz, CD_3OD): δ (ppm) 6.55 (m, 2H, $C_{9,15}$), 6.00 (t, 1H, C_{10}/C_{14}, *J* = 10.9 Hz), 5.99 (t, 1H, C_{10}/C_{14}, *J* = 10.9 Hz) 5.68 (dd, 1H, C_8/C_{16}, *J* = 15.1 Hz), 5.66 (dd, 1H, C_8/C_{16}, *J* = 15.2 Hz), 5.49-5.44 (m,

1H, C_{20}), 5.39-5.35 (m, 3H, $C_{11,13,19}$), 4.12 (dt, 1H, C_7/C_{17}, $J_{7,8/16,17}$ = 6.8 Hz), 4.09 (dt, 1H, C_7/C_{17}, $J_{7,8/16,17}$ = 6.6 Hz), 3.09 (t, 2H, C_{12}, J = 7.5 Hz), 2.34-2.30 (m, 4H, $C_{2,18}$), 2.09-2.04 (m, 2H, C_{21}), 1.61 (quin, 2H, C_3, J = 7.3 Hz), 1.53-1.49 (m, 2H, C_6), 1.34-1.33 (m, 4H, $C_{4,5}$), 0.95 (t, 3H, C_{22}, $J_{21,22}$ = 7.6 Hz). ESI-TOF m/z: $[M+Na]^+$ Calcd for $C_{22}H_{34}O_4Na$ 385.23493; Found 385.22452. EI-MS (fully hydrogenated, trimethylsilyl derivative): *m/z* 573 $[M - CH_3]^+$, 517 $[M - C_5H_{11}]^+$, 402 $[M - C_9H_{19}O_2Si]^+$, 289 $[M - C_{18}H_{39}OSi]^+$, 173 $[M - C_{22}H_{47}O_3Si_2]^+$.

7*R*,17*S*-dihydroxydocosa-8*E*,10*Z*,13*Z*,15*E*,19*Z*-pentaenoic acid methyl ester (III); UV-vis (heptane solvent): 244, 227 nm. FT-IR (hexane solvent): $\tilde{\nu}$ = 3012, 2956, 2924, 2854, 1742, 1641, 1461 cm^{-1}. ^1H NMR (500 MHz, CD_3OD): δ (ppm) 6.58 (dd, 1H, C_9, $J_{8,9}$ = 14.9 Hz), 6.56 (dd, 1H, C_{15}, $J_{15,16}$ = 14.3 Hz), 6.02 (t, 1H, C_{10}, J = 10.9 Hz), 5.99 (t, 1H, C_{14}, J = 10.8 Hz), 5.68 (dd, 1H, C_{16}, J = 15.4 Hz), 5.62 (dd, 1H, C_8, J = 15.3 Hz), 5.49-5.33 (m, 4H, $C_{11,13,19,20}$), 4.31 (dt, 1H, C_7, $J_{7,8}$ = 7.3 Hz, broad), 4.14 (dt, 1H, C_{17}, $J_{16,17}$ = 6.4 Hz, broad), 3.10 (t, 2H, C_{12}, J = 7.5 Hz), 2.34-2.29 (m, 4H, $C_{2,18}$), 2.08-2.03 (m, 2H, C_{21}), 1.61 (quin, 2H, C_3, J = 7.2 Hz), 1.49-1.44 (m, 2H, C_6), 1.39-1.36 (m, 4H, $C_{4,5}$), 0.96 (t, 3H, C_{22}, $J_{21,22}$ = 7.5 Hz). ESI-TOF m/z: $[M+Na]^+$ Calcd for $C_{22}H_{34}O_4Na$ 385.23493; Found 385.23326. EI-MS (fully hydrogenated, trimethylsilyl derivative): *m/z* 573 $[M - CH_3]^+$, 517 $[M - C_5H_{11}]^+$, 402 $[M - C_9H_{19}O_2Si]^+$, 289 $[M - C_{18}H_{39}OSi]^+$, 173 $[M - C_{22}H_{47}O_3Si_2]^+$.

3. Results

3.1 Soybean 15-lipoxygenase-1 catalyzed reaction of DPAn-3

In previous work by our group we have demonstrated an altered metabolism of DPAn-3 and DPAn-6 by 15-sLOX-1 in comparison to other LC-PUFAs including DHA, ARA and EPA [31], an observation which to our knowledge had previously gone unreported. Under optimized conditions the reaction of 15-sLOX-1 with a PUFA substrate containing four or more methylene-interrupted double bonds typically yields two dihydroxy products, both containing a hydro(pero)xy group at the n-6 position, and a second hydro(pero)xy group at either n-13 or n-16 in approximately equal quantities (**Fig. 1**). However with DPAn-3 and DPAn-6 as substrates, which vary from DHA only in the absence of one double bond at either the methyl end (n-6) or carboxylic acid end (n-3), only one major product is detected for each substrate despite the availability of both n-11 and n-14 sites for hydrogen abstraction to generate the two respective n-13-O(O)H and n-16-O(O)H isomers [31].

In investigating this unusual feature of DPA catalysis by 15-sLOX-1 we have observed a further aspect of the reaction which may be utilized in the synthesis of potentially bioactive SPM analogues. The addition of low concentrations of short chain *n*-alcohols, such as methanol, ethanol and propan-1-ol, to the reaction medium results in altered stereochemistry of products from DPAn-3. This effect can be influenced by both alcohol and lipoxygenase concentration, to generate dihydro(pero)xy compounds with *R*-chirality at carbon 7, rather than the well-known *S*-chirality usually catalyzed by 15-sLOX-1.

3.2 Ethanol and 15-sLOX-1 concentration

Reactions were optimized for final ethanol concentration in the reaction medium (0 - 40 % v/v) with constant 15-sLOX-1 concentration (75 kunits/ml). At low ethanol concentrations (<5 % v/v) the reaction proceeds as normal with full conversion of DPAn-3 to the previously described 7,17-diHDPAn-3 (compound II) through the 17-H(P)DPAn-3 (compound I) intermediate [29, 31]. As the ethanol concentration is increased a second dihydro(pero)xy compound is observed (III) (**Fig. 2**). Compound III has a very similar UV-visible absorbance spectrum (λ_{max} = 244 nm, suppressed maxima at 227 nm), however exhibits a reversed maximum compared to that of compound II (λ_{max} = 224 nm, suppressed maxima at 244 nm) (**Fig. 2 inset**). Under these conditions, the optimum concentration of ethanol for the production of compound III is 10 % (v/v) with compound III representing 69.7 % of total dihydro(pero)xylated products (**Fig. 3a**). At higher ethanol concentrations the formation of all products decreases, and the reaction is completely inhibited at 40 % (v/v) ethanol.

The 15-sLOX-1 concentration was also optimized over the range 15 – 150 kunit/ml in the presence of 10 % (v/v) ethanol, to maximize production of compound III. Formation of compound III reaches a maximum at 45 kunits/ml 15-sLOX-1, accounting for 77.1 % of the total dihydro(pero)xy products produced as shown in **Fig. 3b**. Re-optimisation of ethanol concentration at this enzyme concentration enabled increased production of compound III, reaching a maximum purity of 95 % at 15 % (v/v) ethanol concentration, with complete consumption of the DPAn-3 substrate.

3.4 n-Alcohol chain length

The effect of *n*-alcohols on the activity of 15-sLOX-1 has been explored in a small number of studies [40-42], where inhibition of the enzyme with increasing alcohol chain length has been observed as a result of competition with the alkyl region of the substrate for binding within the enzyme active site. Based on our observations of the effect of low concentrations of ethanol on 15-sLOX-1 catalysis and the reported inhibitory activity of longer chain *n*-alcohols, we tested the effect of a series of *n*-alcohols (chain length = 1-5) on the 15-sLOX-1 catalyzed reaction of DPAn-3. The inhibitory effect of the longer chain *n*-alcohols (butan-1-ol and pentan-1-ol) was observed in the reaction of DPAn-3 with a significant reduction in both di- and mono-hydroxy products, resulting in a substantial amount of DPAn-3 substrate remaining. However, it is unclear whether this is a result of direct binding to the active site, or the limited solubility of these longer chain alcohols in aqueous buffer, which results in a biphasic environment in which the PUFA substrate is extracted into the organic phase and is therefore less accessible to the enzyme. In contrast, for the reaction with shorter *n*-alcohols there was an increase in the production of compound III with increasing alcohol chain length, with propan-1-ol exerting the greatest effect (**Table 1**).

3.5 Product characterisation

Methyl esters of compounds II and III were purified by normal phase HPLC and characterized by GC-MS, NMR and UV-visible spectroscopy. Analysis of fully hydrogenated, trimethylsilyl derivatives of all

products by GC-MS confirmed the structure of both compounds II and III as 7,17-diHDPAn-3 (*refer to section 2.9*). Briefly, ions with *m/z* of 289 and 402 were detected which are indicative of a hydroxy group at C7, as well as ions with *m/z* of 173 and 518 characteristic of a hydroxy group at C17. No other structural isomers of dihydroxy products were detected, indicating the dioxygenation reactions occur at the same positions in both products.

Purified compounds II and III were analysed by NMR spectroscopy and protons assigned based on chemical shift (^{1}H-NMR) and correlation (2D ^{1}H, ^{1}H-COSY) to elucidate the complete structure (*refer to section 2.9*). In support of the GC-MS data, correlation spectroscopy confirmed the presence of hydroxy groups at positions C7 and C17 in both compounds. A significant downfield shift of ~ 0.2 ppm in the resonance of the C7 proton (~ 4.3 ppm) of III in comparison to II (~ 4.1 ppm) was attributed to a change in chirality at C7. This feature was also accompanied by a slight upfield shift of the neighboring C8 proton (~ 5.6 ppm) of compound III only. Meanwhile the chemical shift of the C17 proton (~ 4.1 ppm) remained constant indicating the well-known *S*-chirality of lipoxygenase catalysis was conserved in both products. The conjugated double bond geometry of compound III was confirmed as *E,Z,Z,E* (consistent with compound II) based on correlation of chemical shifts and *J*-coupling constants [29, 43]. Importantly, correlation was not observed between H7 and H17 and protons at ~ 6.0 ppm, a chemical shift characteristic of *cis* protons. Instead strong correlation was observed with *trans* protons at 5.6 – 5.7 ppm, identified as C8 and C16. Coupling constants of $J_{8,9}$ and $J_{15,16}$ were calculated as 14.9 Hz and 14.3 Hz respectively, again characteristic of *trans* protons, while $J_{10,11}$ and $J_{13,14}$ were calculated as 10.9 and 10.8 Hz, consistent with *cis* protons. Additionally there was no evidence of an *E,E* conjugated diene at ~ 6.2 ppm [25]. Coupling constants of C9 and C15 were not able to be calculated due to the multiplicity and overlapping of the signals. Finally, *J*-coupling constants of $J_{16,17}$ were found to be between 6.4-6.6 Hz for both compounds, whereas $J_{7,8}$ was found to be 7.3 Hz for III and 6.8 Hz for II. This variance in coupling constants between opposite chiral centres may provide an effective method for identifying changes in chirality, along with changes in chemical shift as previously discussed.

Chiral-phase chromatography of the 17-HDPAn-3 intermediate (I) produced in both 100% aqueous and 10% ethanol conditions indicated that the compound was stereochemically pure (> 98%, **Fig. 4**). However as there are currently no synthetic standards of DPAn-3 products commercially available for comparison, assignment of *S*-stereochemistry at C17 is based on previous knowledge of 15-sLOX-1 as a catalyst for *S*-configuration hydroxy groups [14, 16-18]. Therefore compound II has been tentatively identified as 7*S*,17*S*-dihydroxydocosapenta-8*E*,10*Z*,13*Z*,15*E*,19*Z*-enoic acid. We propose that in the presence of *n*-alcohols the first dioxygenation at C17 proceeds as normal to produce 17*S*-H(P)DPAn-3, however further dioxygenation occurs in the *R*-configuration producing 7*R*,17*S*-dihydroxydocosapenta-8*E*,10*Z*,13*Z*,15*E*,19*Z*-enoic acid (III) as the major product.

3.6 Application of optimized conditions to other LC-PUFA substrates

The optimized conditions for the production of 7*R*,17*S*-diHDPAn-3 (15 % v/v ethanol and 45 kunits 15-sLOX-1/ml) were applied to docosatetraenoic acid (DTA; C22:4n-6), a substrate analogous to DPAn-3 in the 5-carbon alkyl region (C_2-C_6), which is likely to experience the same flexibility when it is bound in the active site during the second dioxygenation. Under standard conditions (0% ethanol and 150 kunits/ml 15-sLOX-1), the major product of DTAn-6 is 7,17-dihydroxydocosatetraneoic acid (7,17-diHDTAn-6), with proposed *S*,*S*- stereochemistry consistent with previously characterized products [31]. Under optimized conditions (for the production of 7*R*,17*S*-diHDPAn-3 from DPAn-3) the second dioxygenation was significantly inhibited, with only a small amount of conversion of 17*S*-hydroperoxy-DTAn-6 to dihydroxylated products.

This reaction was therefore optimized for DTAn-6 (17 % v/v ethanol and 135 kunits/ml 15-sLOX-1) to achieve 7*R*,17*S*-diHDTAn-6 in 82 % purity. Analysis of enzyme kinetic parameters (**Table 2**) indicates 15-sLOX-1 has a greater affinity for DPAn-3 than DTAn-6, as demonstrated by a higher maximum velocity (V_{max}), lower K_M and higher substrate capture constant (V_{max}/K_M). Both optimized methods were also applied to DPAn-6, DHA, EPA and ARA substrates with some success. However, the total conversion rate remained lower than previously observed for DPAn-3 and DTAn-6 reactions. Major products of these substrates have been tentatively assigned based on retention time and UV-visible absorbance spectra, and are summarized in **Table 3**. Of interest is the production of diastereomers of the conjugated triene products, especially as 10*R*,17*S*-dihydroxydocosahexa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid is a closely related isomer of (neuro)protectin D1 (10*R*,17*S*-dihydroxydocosahexa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-enoic acid) varying only in double bond geometry at C13 and C15.

Under certain conditions, lipoxygenase catalyzed reactions are known to produce other non-specific, non-enzymatic isomers through free radical initiated chain reactions [32, 44]. The free radicals are produced enzymatically when a deprotonated substrate radical is released from the enzyme prior to the addition of oxygen. These radicals can react with oxygen in a non-specific manner forming a range of positional, stereo, and geometrical isomers; typically the oxygen will be added at both the [+2] and [-2] position from the original carbon centred radical, with all possible stereo and geometrical (*cis,trans* and *trans,trans*) isomers formed in approximately equal amounts [32, 44]. This mix of isomers is not observed here (but may account for some very minor products), suggesting the products listed are enzymatically formed. This could be further investigated by intercepting the free radicals with scavengers, as has been done previously [44].

4. Discussion

Previous work in our laboratory has demonstrated the synthesis of resolvin analogues from both DPAn-3 and n-6 using 15-sLOX-1 [31], however in an altered ratio and significantly higher yield than previously described [29]. Dangi et al. reported the production of two dihydroxylated products, 7,17*S*-diHDPA and 10,17*S*-diHDPA, formed through the 17*S*-HDPA intermediate from both n-3 and n-6

starting materials using 15-sLOX, with low rates of conversion from 17*S*- to dihydroxy products (2-4%) [29]. However, we have consistently observed 7*S*,17*S*-diHDPAn-3 as the single major product of DPAn-3, with 10*S*,17*S*-diHDPAn-3 representing ~ 5 % of the total products despite the site required for formation of 10*S*,17*S*-diHDPAn-3 appearing to be readily accessible for hydrogen abstraction.

In the current research we have further optimized the metabolism of DPAn-3 by 15-sLOX-1 to enable the formation of two diastereomers of 7,17*S*-diHDPAn-3 in high purity and high yield. This major product, tentatively named $RvD5_{DPAn-3}$ based on structural similarity to RvD5 (7*S*,17*S*-dihydroxydocosahexa-4*Z*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-enoic acid) from DHA [38], is a double dioxygenation product of soybean 15-lipoxygenase. The formation of $RvD5_{DPAn-3}$ has been confirmed in zymosan-activated isolated human neutrophils after treatment with DPAn-3, with the complete stereochemistry assumed to be *S*,*S*- following previously characterized pathways of resolvin biosynthesis [38]. In this work we have optimized the synthesis of two $RvD5_{DPAn-3}$ diastereomers using 15-sLOX-1, in greater than 95 % stereochemical purity, and fully characterized the products to establish double bond and hydroxy group stereochemistry using NMR spectroscopy, GC-MS and HPLC analysis. The synthesis of the corresponding diastereomer pairs from other LC-PUFAs including EPA, DHA, ARA and DTAn-6 has also been demonstrated (albeit with lower yields and purity). As the potency and bioactivity of SPMs are known to be significantly dependent on the stereochemical features as a result of selective receptor affinities [45-49], the ability to introduce subtle stereospecific features into products via enzyme-catalyzed methods offers a major advantage over synthetic methods in generating bioactive compounds with high chiral purity.

The effect of *n*-alcohols on lipoxygenase has been briefly explored previously, in terms of inhibition of LOX activity, indicating C_1 to C_{12} *n*-alcohols competitively inhibit metabolism of linoleic acid (9*Z*,12*Z*-octadecadienoic acid) through binding of the alkyl chain of the alcohol to a hydrophobic region close to the catalytic centre of the active site [41, 42, 50, 51]. It has also been proposed that this binding restricts access of the pentadiene system of the substrate to the catalytic centre, thus preventing hydrogen abstraction [42, 50]. Previous investigations of enhanced enantioselectivity through medium engineering have also suggested one mechanism of action could be through solvent molecules binding within the active site, and affecting the association or transformation of one enantiomer more than the other [20]. Consistent with previous results, we have observed as alcohol chain length increases the inhibitory effect also increases. However, we have also found that the presence of shorter chain *n*-alcohols (methanol, ethanol and 1-propanol) exerts an effect on the stereospecificity of the LOX enzyme at low concentrations. This may indicate that the binding of the alcohol to the active site as previously demonstrated, not only restricts access of the substrate to the catalytic centre, but under appropriate concentrations may alter the position of the substrate, enabling access of molecular oxygen to both sides of the pentadienyl radical.

15-sLOX-1 is well known to catalyze stereospecific *S*-chirality dioxygenations of PUFAs containing one or more 1*Z*,4Z-pentadiene moieties, and to date there have been no reports of *R*-chirality major products of wild-type soybean 15-LOX-1. The structure of 15-sLOX-1 in the inactive ferrous (+2) form has been characterized by X-ray crystallography to a resolution of 1.4 Å, identifying the likely substrate-binding cavity [52]. Due to the structural flexibility of PUFAs, modeling of actual substrate-binding within the active site cavity has been subject to speculation based on observed product formation and mutagenesis studies [18, 52]. The general consensus of binding is with the substrate arranged in a bent conformation extending between two subcavities intersected by a constricted region proximal to the catalytic iron, as well as the presence of oxygen pockets or channels [52-54]. The regiospecificity of the reaction is believed to be greatly controlled by how deep the substrate can penetrate the distal subcavity (effectively displacing the reacting pentadiene and the proximity of the CH_2 group for hydrogen abstraction), and also the orientation of the substrate within the active site (methyl versus carboxylic end first) [55, 56]. The stereospecificity of the reaction is somewhat more complex and is likely to be affected by several factors including [57-59]:

(i) the selective channeling of oxygen through the protein to a specific region in the active site,
(ii) steric shielding of the radical substrate by the enzyme structure to guide oxygen accessibility to certain reactive locations of the pentadienyl radical, and
(iii) the localization or delocalization of the pentadienyl radical which can favour selective reaction.

This has been demonstrated by the presence or absence of bulky amino acid residues within the active site which appear to guide oxygen access to regions of the pentadienyl radical [60]. When the presence of short chain *n*-alcohols and an increased flexibility of the substrate are also considered it is possible that the positioning of the substrate restricts antarafacial access of oxygen as a result of alcohol binding and rotation of the alkyl region of the substrate. As a result oxygen insertion may be favoured on the same side as hydrogen abstraction to produce *R*-chirality products.

5. Conclusions

The lipoxygenase family of enzymes have been established as valuable and versatile biocatalysts in the production of hydroxy fatty acids from a range of PUFA substrates, with demonstrated regio- and stereo-specificity. In this research we have demonstrated the versatility of commercially available 15-sLOX-1 through subtle changes in experimental conditions, enabling the production of diastereomeric compounds analogous to biologically-active resolvin and protectin species. The major compounds from DPAn-3 substrate, (8*E*,10*Z*,13*Z*,15*E*,19*Z*)-7*R*,17*S*-diHDPA and (8*E*,10*Z*,13*Z*,15*E*,19*Z*)-7*S*,17*S*-diHDPA, have also been characterized to elucidate their complete chemical structures. This stereo-selective feature makes the use of 15-sLOX-1 an attractive and efficient option in the production of SPM analogues, providing a simper method of producing chirally pure products than the current synthetic strategies.

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Tables:

Table 1. Effect of *n*-alcohol chain length on 15-sLOX-1 activity with DPAn-3 substrate (10 % v/v *n*-alcohol). (n.d.: Not detected)

***n*-Alcohol**	**% of Total peak area**			
	DPAn-3 [a]	**(I) 17*S*-** [b]	**(II) 7*S*,17*S*-** [b]	**(III) 7*R*,17*S*-** [b]
Methanol	n.d.	0.1	47.3	52.6
Ethanol	n.d.	5.8	21.6	72.6
Propan-1-ol	n.d.	5.2	6.5	88.3
Butan-1-ol	75.1	24.9	n.d.	n.d.
Pentan-1-ol	55.1	44.9	n.d.	n.d.

[a] Absorbance measured at 210 nm.

[b] Absorbance measured at 234 nm.

Table 2. Kinetic parameters of DPAn-3 and DTAn-6 substrates with 15-sLOX-1. Data was fitted to a Michaelis-Menten kinetic model using GraphPad Prism 6.0d software.

Parameter	**DPAn-3**	**DTAn-6**
V_{max} (μM/min)	628.6 ± 39.4	492.1 ± 37.9
K_M (μM)	24.2 ± 5.7	50.1 ± 11.4
V_{max}/K_M (min^{-1})	26.0	9.8
R^2	0.87	0.85

Table 3. Major products from LC-PUFA substrates under optimized conditions for 7*R*,17*S*-diHDTAn-6 synthesis by 15-sLOX-1.

Substrate	**Conjugated diene products** [a]	***S,S*-:*R,S*-ratio**	**Conjugated triene products** [b]	***S,S*-:*R,S*-ratio**
ARA	5,15-diHETE	1 : 1.49	8,15-diHETE	1 : 0.15
EPA	5,15-diHEPA	1 : 0.40	8,15-diHEPA	1 : 0.00
DPAn-6	7,17-diHDPAn-6	1 : 1.30	10,17-diHDPAn-6	1 : 0.22
DHA	7,17-diHDHA	1 : 0.88	10,17-diHDHA	1 : 0.11

[a] Absorbance measured at 234 nm.

[b] Absorbance measured at 270 nm.

Figure Captions:

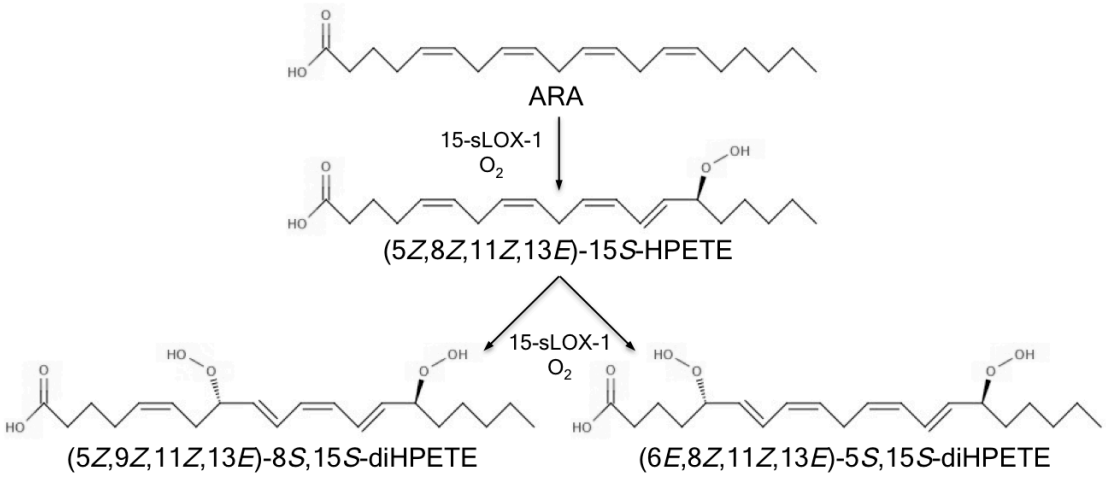
Figure 1. Major products of 15-sLOX-1 metabolism of arachidonic acid (ARA) - 15*S*-H(P)ETE; 8*S*,15*S*-diH(P)ETE and 5*S*,15*S*-diH(P)ETE.

Figure 2. NP-HPLC chromatogram of DPAn-3 dioxygenation products catalyzed by 75 kunits/ml 15-sLOX-1 in the presence (dashed line) or absence (solid line) of 10 % ethanol (pH 9.0). **Inset:** UV spectra of analytes measured in ethanol.

Figure 3. a: Effect of ethanol concentration on formation of compounds I, II and III at 75 kunits/ml 15-sLOX-1. **b:** Effect of 15-sLOX-1 concentration on formation of I, II and III at 10 % (v/v) ethanol. Measured by peak area ratio (from HPLC-DAD).

Figure 4. Chiral phase-HPLC chromatogram of (I) 17-HDPAn-3 (as methyl ester derivative). Analysis conditions: mobile phase – *n*-hexane/ethanol (99/1 v/v); flow rate - 1 ml/min.

Figures:



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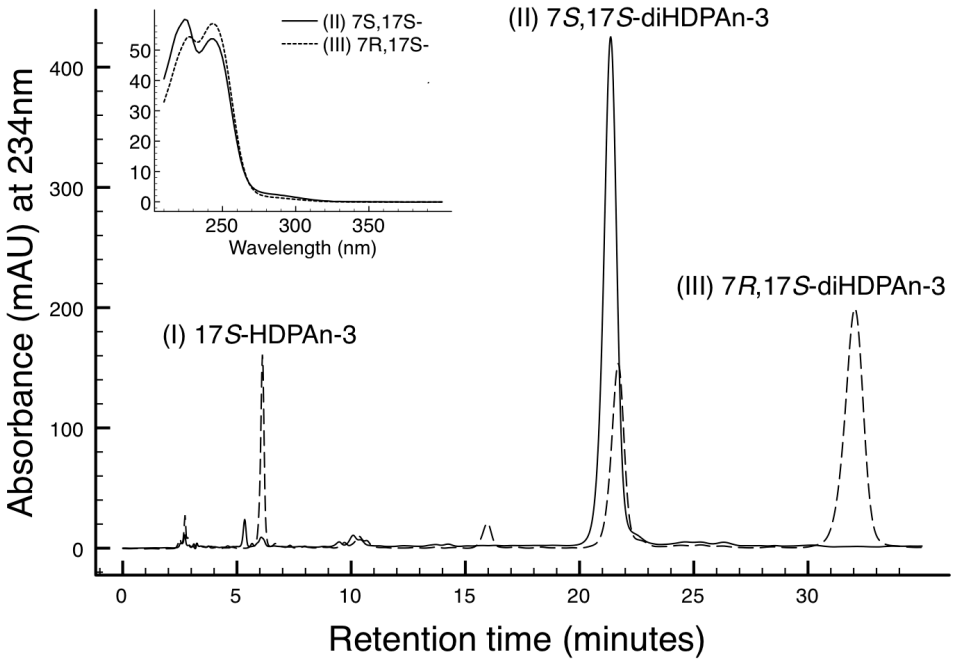

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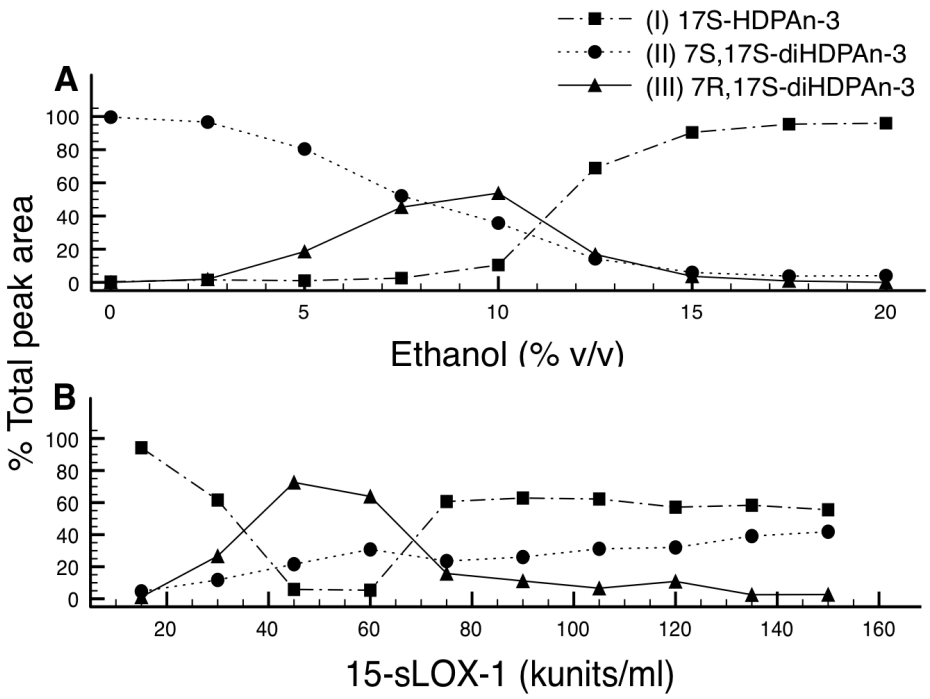

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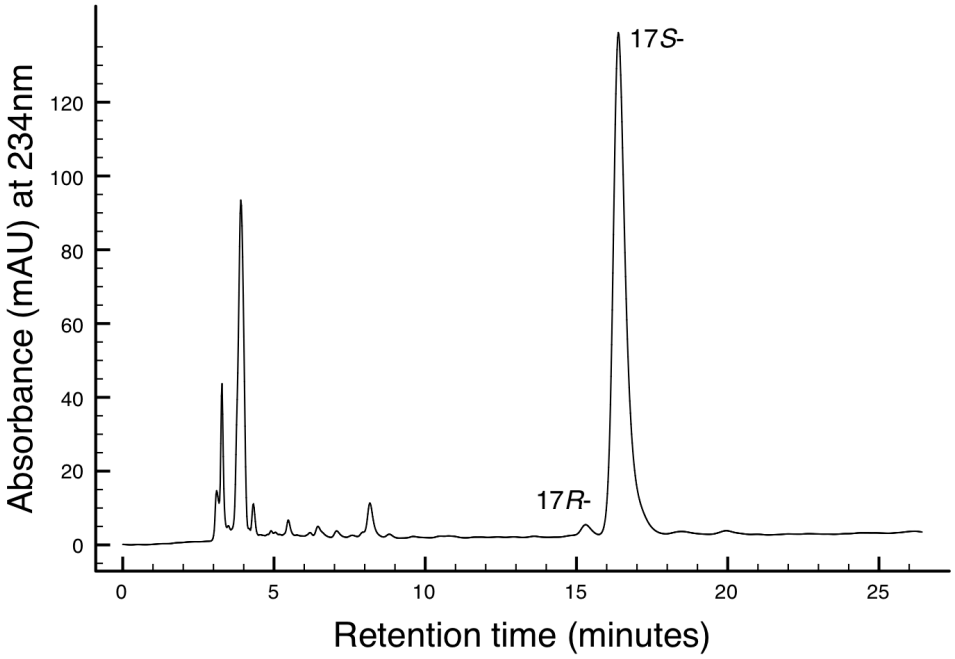

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