Title: Enzymatic Synthesis of Epoxidized Metabolites of Docosahexaenoic, Eicosapentaenoic, and Arachidonic Acids

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Short Abstract:

We present a method useful for large-scale enzymatic synthesis and purification of specific enantiomers and regioisomers of epoxides of arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) with the use of a bacterial cytochrome P450 enzyme (BM3).

Long Abstract:

The epoxidized metabolites of various polyunsaturated fatty acids (PUFAs), termed epoxy fatty acids, have a wide range of roles in human physiology. These metabolites are produced endogenously by the cytochrome P450 class of enzymes. Because of their diverse and potent biological effects, there is considerable interest in studying these metabolites. Determining the unique roles of these metabolites in the body is a difficult task, as the epoxy fatty acids must first be obtained in significant amounts and with high purity. Obtaining compounds from natural sources is often labor intensive, and soluble epoxide hydrolases (sEH)

rapidly hydrolyze the metabolites. On the other hand, obtaining these metabolites via chemical reactions is very inefficient, due to the difficulty of obtaining pure regioisomers and enantiomers, low yields, and extensive (and expensive) purification. Here, we present an efficient enzymatic synthesis of 19(*S*),20(*R*)- and 16(*S*),17(*R*)-epoxydocosapentaenoic acids (EDPs) from DHA via epoxidation with BM3, a bacterial CYP450 enzyme isolated originally from *Bacillus megaterium* (that is readily expressed in *Escherichia coli*). Characterization and determination of purity is performed with nuclear magnetic resonance spectroscopy (NMR),high-performance liquid chromatography (HPLC), and mass spectrometry (MS). This procedure illustrates the benefits of enzymatic synthesis of PUFA epoxy metabolites, and is applicable to the epoxidation of other fatty acids, including arachidonic acid (AA) and eicosapentaenoic acid (EPA) to produce the analogous epoxyeicosatrienoic acids (EETs) and epoxyeicosatetraenoic acids (EEQs) (respectively).

Introduction:

As interest in the role that polyunsaturated fatty acids (particularly omega-3 and omega-6 polyunsaturated fatty acids) play in human biology has grown in recent years, researchers have taken notice of the wide range of appealing benefits that their metabolites exhibit. In particular, epoxy fatty acid metabolites produced by the cytochrome P450 class of enzymes have been a large point of focus. For example, many PUFA epoxides [including epoxyeicosatrienoic acids (EETs), epoxydocosapentaenoic acids (EDPs) and epoxyeicosatetraenoic acids (EEQs)] play a critical role in regulation of blood pressure and inflammation.[1-5] Interestingly, the specific enantiomers and regioisomers of AA and EPA epoxides are known to have varying effects on vasoconstriction.[6,7] While the physiological effects of the enantiomers and regioisomers of EETs and EEQs have been documented, little is known about the effect of the analogous epoxydocosapentaenoic acids (EDPs) formed from DHA. Widespread use of fish oil,[8] which is rich in both EPA and DHA, has also has stirred interest in EDPs.[9] The benefits of these supplements are believed to be partly due to the downstream DHA metabolites (16,17-EDP and 19,20-EDP being the most abundant) because in vivo levels of EDPs coordinate very well with the amount of DHA in the diet.[10, 11]

Studying the mechanisms and targets of these epoxy fatty acids by metabolomics, chemical biology, and other methods has proven challenging, in part because they exist as mixtures of regio- and stereo-isomers, and a method of obtaining pure amounts of the enantiomers and regioisomers is required. Conventional means for chemically synthesizing these compounds have proved ineffective. Use of peroxyacids like *meta*-chloroperoxybenzoic acid for epoxidation has many drawbacks, notably the lack of epoxidation selectivity, which necessitates expensive and painstaking purification of individual regioisomers and enantiomers. Total synthesis of DHA and EPA metabolites is possible, but also suffers from drawbacks that make it impractical for large-scale synthesis such as high costs and low yields.[12-13] Efficient overall production can be achieved with enzymatic synthesis, as enzymatic reactions are regio- and stereoselective.[14] Studies show that enzymatic epoxidation of AA and EPA (with BM3) is both regioselective and enantioselective,[15-18] but this procedure has not been tested with DHA, or on a large scale. The overall goal of our method was to scale up and optimize this

chemoenzymatic epoxidation to rapidly produce significant amounts of pure epoxy fatty acids as their individual enantiomers. Using the method presented here, researchers have access to a simple and cost-effective strategy for synthesis of EDPs and other PUFA epoxy metabolites.

Protocol:

Caution: Please consult all relevant material safety data sheets (MSDS) before using the listed chemicals.

1. Expression of Wild-Type BM3.

1.1. Inoculate pBS-BM3 transfected DH5α *E. coli* (a generous donation from Dr. F. Ann Walker) in 5 mL of sterile LB broth with 0.5 mg of ampicillin added (in a 20 mL culture tube).

1.2. Incubate the cell culture in the shaker at 37 ˚C for 24 h at 200 rpm. Add the overnight starter culture (5 mL) and 100 mg of ampicillin to 1 L of sterile LB broth (in a Fernbach or Erlenmeyer flask). Shake at 37 ˚C for 6 h at 200 rpm, then at 30 ˚C for 18 h at 200 rpm.

1.3. Collect and centrifuge the cell culture at 4 ˚C for 10 min at 1000 *g*. Discard the supernatant (it can be either chemically sterilized by treatment with bleach or sterilized using an autoclave and then poured down the drain) and store the cell pellet at -78˚C until enzyme purification.

2. Purification of BM3.

2.1.1. Thaw the cell pellet on ice and resuspend in 40 mL of ice-cold (4 ˚C) solubilization buffer [10 mM Tris, 0.01 mM phenylmethylsulfonyl fluoride (PMSF; caution: PMSF is toxic by contact!), 0.01 mM EDTA; pH 7.8].

2.1.2. While on ice, sonicate the cells for 1 min with an ultrasonic homogenizer (output power setting 10, duty 100%), followed by a 1 min break on ice (repeat this procedure 6 times) in order to lyse the cells. Centrifuge the cell lysate at 4 ˚C for 30 min at 11,000 *g* to pellet cell debris.

2.2.1. Prepare a strong anion exchange chromatography column (see Table of Materials; diameter: 2.8 cm x 6 cm, column volume: 37 mL) by washing with 5 column volumes (CV) of buffer A (10 mM Tris, pH 7.8) at 4 ˚C.

2.2.2. Add the cell lysates to equilibrated column and wash the column with 3 CV of cold buffer A. Elute the BM3 by washing the column with cold buffer B (10 mM Tris, 600 mM NaCl, 6 CV).

2.2.3. Collect the reddish-brown eluent fraction. If the protein is not being used immediately, mix it with an equal volume of glycerol and flash freeze with liquid nitrogen. Store the frozen solution at -78 ˚C.

3. Epoxidation of DHA by BM3.

3.1. Prepare the reaction by adding 0.308 g [(0.940 mmol) of DHA (dissolved in 18.8 mL dimethylsulfoxide (DMSO)] to 2 L of stirring reaction buffer (0.12 M potassium phosphate, 5 mM $MgCl_2$, pH 7.4) along with 20 nM of the thawed BM3 enzyme. The enzyme concentration can be determined by the carbon monoxide/dithionite spectral assay method.[19]

3.2. While the solution is stirring, begin the reaction by adding 1 equivalent of NADPH (nicotinamide adenine dinucleotide phosphate reduced, tetrasodium salt, 0.808 g, 0.940 mmol) dissolved in reaction buffer. Stir the reaction for 30 min while bubbling air through the reaction mixture with an air-filled balloon attached to a syringe and needle.

3.3. Using a spectrophotometer (see Table of Materials), check the absorbance of the reaction mixture at 340 nm to determine if NADPH is depleted. If there is no remaining absorbance (indicating consumption of NADPH), the reaction is complete (typically it is after 30 min).

3.4. Quench the reaction mixture by slowly adding 1 M oxalic acid, dropwise, until the pH of the solution reaches 4.

4. Extraction of EDPs.

4.1. Extract the quenched buffer solution with 2 L of diethyl ether (anhydrous, peroxide-free) 3 times. Collect the ether layer (6 L) and dry with anhydrous magnesium sulfate ($MgSO_4$).

4.2. Filter the $MgSO_4$ from the solution and concentrate the dried ether layer on a rotary evaporator to yield the crude EDP residue.

4.3. Purify the residue by flash column chromatography (a 40 g silica column cartridge is sufficient). Start at 10% ethyl acetate (EtOAc) in hexanes and ramp up to 60% EtOAc in hexanes over 22 min. Three major peaks are obtained and collected, eluting in the order of 1. unreacted DHA; 2. mixture of EDP isomers; and 3. di-epoxide [normal over-oxidation products (See Figure 1)].

4.4. Combine the fractions and concentrate them on the rotary evaporator. From this example, 0.074 g, (24%) of unreacted DHA, 0.151 g (47%) of EDP isomers, and 0.076 g, (22%) of di-epoxide were obtained.

5. Esterification of EDPs, Separation of 16(*S*),17(*R*)- and 19(*S*),20(*R*)-EDP, and Saponification of Esters.

Caution: Trimethylsilyldiazomethane (TMS-diazomethane) is very toxic by both contact and inhalation. Use only in a fume hood with the proper personal protective equipment.

5.1. Dilute the epoxides (0.151 g, 0.435 mmol) in a round-bottomed flask or small vial with 2 ml of anhydrous methanol (MeOH), 3 mL of anhydrous toluene, add a stir-bar, and add TMS-diazomethane (1.2 molar equivalents, or 0.26 mL of a 2 M solution in hexanes) under argon.

5.2. Wait 10 min and add additional TMS-diazomethane (0.050 mL) until a pale yellow color remains. After 30 more min, carefully concentrate the mixture using the rotary evaporator and purify the residue by flash column chromatography. Elute with 4% EtOAc in hexanes (using a 40 g silica gel column or column cartridge) for 22 min. In this example, 19,20-EDP methyl ester (0.116 g, 74%) and 16,17-EDP methyl ester (0.029 g, 19%), were both obtained as clear oils (total yield, 93%).

5.3. Collect the fractions containing the purified EDP methyl ester regioisomers. 19(*S*),20(*R*)-EDP methyl ester elutes first, followed by 16(*S*),17(*R*)-EDP methyl ester. If any mixed fractions

remain [(containing both isomers; can be assessed by thin-layer chromatography (TLC) in 8:1 hexane/EtOAc and staining with potassium permanganate ($KMnO_4$)], re-chromatograph them with the same solvent system as before. Concentrate the fractions containing the individual regioisomers. At this point, identity and purity may be assessed by NMR (using $CDCl_3$ as the solvent; see the legend for Figure 2).

5.4. To convert individual EDP methyl ester regioisomers to their acid forms, dilute the EDP ester in THF: water (approximately 0.7 mL/0.1 mmol of ester). Add 2 M aqueous LiOH (3 molar equivalents) and stir overnight (completeness of the reaction can be assessed by TLC, using 3:1 hexanes/EtOAc, staining with $KMnO_4$; the product has a retention factor of ~0.3).

5.4. Quench the reaction slowly with formic acid, until the pH of the mixture is 3-4. Add water and ethyl acetate (1-2 mL/0.100 mmol of ester), and separate the layers. Extract the water layer with EtOAc (3 x 5 mL), wash with saturated brine (NaCl) solution, and dry the EtOAc layer over anhydrous sodium sulfate (Na_2SO_4).

5.5. Concentrate the ethyl acetate solution using a rotary evaporator, add hexanes (10 mL) and concentrate again. Repeat twice to azeotropically remove residual formic acid. Purify the residue by flash column chromatography, eluting with 10-30% EtOAc over 15 min. Concentrate the desired fractions and dry in vacuo to afford the purified acid. At this stage, enantiomeric purity may be assessed (by chiral HPLC, see the legends for Figures 3-4 for column and conditions). Chemical purity can be assessed by C18 (achiral) HPLC (see Table of Materials and reference 14).

Representative Results:

The flash column chromatogram (performed using an automated flash purification system as described below) obtained upon purification of the crude mixture from enzymatic epoxidation is shown in Figure 1. Following esterification and separation of the regioisomers, pure 16(*S*),17(*R*)-EDP and 19(*S*),20(*R*)-EDP methyl esters were obtained (typically they are present in an approximate 1:4 to 1:5 ratio, with the major product being 19(*S*),20(*R*)-EDP. No other EDP regioisomers (e.g., 13,14- or 10,11-EDP) are obtained. The ^{1}H-NMR spectra of 16(*S*),17(*R*)-EDP (Figure 2A) and 19(*S*),20(*R*)-EDP (Figure 2B) methyl esters, (along with their structures) are shown below, indicating the high purity of these compounds; C18 (achiral) HPLC of the acid forms also indicated purities >98%). Their identity was further confirmed by high-resolution mass spectroscopy (both of the acid and ester forms), which yielded mass/charge ratios and fragmentation patterns consistent with the identified EDPs. Enantiomeric purity was determined using chiral HPLC, by comparison to authentic enantiopure and mixed standards of the EDPs (in their acid form, Figures 3A and 4A). As can be observed in Figures 3B and 4B), both EDPs obtained by enzymatic epoxidation are highly enantiopure following saponification (>99% one enantiomer). The identities of these enantiomers were previously reported to be 16(*S*),17(*R*)- and 19(*S*), 20(*R*)-EDP.[18]

Figure Legends:

Figure 1. Chromatogram from purification of crude mixture obtained from enzymatic epoxidation of DHA (along with relevant structures). The middle peak (monoepoxide) contains the desired EDPs. Purification was performed using an automated flash purification system (see Table of Materials).

Figure 2. Example ^{1}H-NMR spectra of pure 16(*S*),17(*R*)-EDP methyl ester (2A) and 19(*S*),20(*R*)-EDP methyl ester (2B). Spectra were recorded at 500 MHz in $CDCl_3$ (solvent is visible at 7.26 ppm and residual water at 1.6 ppm). The chemical shifts are as follows: **16(*S*),17(*R*)-EDP methyl ester:** ^{1}H-NMR (500 MHz; $CDCl_3$): δ 5.57-5.35 (m, 10 H), 3.68 (s, 3 H), 2.99-2.95 (m, 2 H), 2.87-2.83 (m, 6 H), 2.47-2.37 (m, 6 H), 2.29-2.21 (m, 2 H), 2.11-2.05 (m, 2 H), 0.99 (t, *J* = 7.5 Hz, 3 H); **19(*S*),20(*R*)-EDP methyl ester:** ^{1}H-NMR (500 MHz; $CDCl_3$): δ 5.54-5.35 (m, 10 H), 3.68 (s, 3 H), 2.97 (td, *J* = 6.4, 4.2 Hz, 1 H), 2.91 (td, *J* = 6.3, 4.2 Hz, 1 H), 2.85-2.82 (m, 8 H), 2.44-2.36 (m, 5 H), 2.27-2.21 (m, 1 H), 1.65-1.51 (m, 3 H), 1.06 (t, *J* = 7.5 Hz, 3 H)

Figure 3. Chiral HPLC indicating enantiopurity of 16,17-EDP (acid form) produced by BM3 (A) shows "racemic" 16,17-EDP (an artificial mixture of authentic standards of both enantiomers[14]), whereas (B) shows enantiopure 16(*S*),17(*R*)-EDP produced by epoxidation of DHA with BM3, assessed as >99% *S,R* isomer. The column is cellulose-based (see Table of Materials, 250 x 4.6 mm, 5 µm, 1000 Å) eluting with isocratic 45% 50 mM ammonium bicarbonate (NH_4HCO_3) in methanol (30 min), with a sample concentration of 0.5 mM and flow rate of 1 mL/min.

Figure 4. Chiral HPLC indicating enantiopurity of 19,20-EDP (acid form) produced by BM3 (A) shows "racemic" 19,20-EDP (an artificial mixture of authentic standards of both enantiomers[14]), whereas (B) shows enantiopure 19(*S*),20(*R*)-EDP produced by epoxidation of DHA with BM3, assessed as >99% *S*,*R* isomer (method as described for Figure 3).

Figure 5. Enzymatic reaction scheme and structures of AA, EPA, EETs, and EEQs.

Figure 6. Chiral HPLC indicating enantiopurity of 17,18-EEQ (acid form) produced by BM3 (A) shows "racemic" 17,18-EEQ (an artificial mixture of authentic standards of both enantiomers[14]), whereas (B) shows enantiopure 17(*S*),18(*R*)-EEQ produced by epoxidation of EPA with BM3, assessed as >99% *S*,*R* isomer (method as described for Figure 3).

Discussion:

We present here an operationally simple and cost-effective method for preparing the two most abundant epoxy metabolites of DHA – 19,20 and 16,17-EDP. These epoxy fatty acids can be prepared in highly enantiopure (as their *S,R*-isomers) form using wild-type BM3 enzyme. Several critical points (which may be used for troubleshooting), and the extension of our method to preparing enantiopure epoxy metabolites of AA and EPA, are described below.

BM3 storage guidelines.

Storing the purified BM3 enzyme is possible by mixing the protein solution with equal volume of glycerol and flash freezing with liquid nitrogen before storage in a -78 ˚C freezer.

Once the enzyme is frozen, it can be stored for up to a year. The enzyme can only be thawed once, must be thawed on ice, and can only be left on ice for 4 h. Freezing again, and allowing the enzyme to thaw without an ice bath will deactivate the enzyme.

Chemical storage guidelines.

Many of the compounds required for the procedure are air-sensitive. These include DHA and other PUFAs, EDPs and other epoxy metabolites, and NADPH. To prevent peroxidation (and other oxidative processes) of these compounds, always flush the containers in which they are stored with argon or nitrogen and store at -78 ˚C.

Another important note is the solution in which the DHA is stored. Although DHA is not very stable in DMSO, BM3 is incompatible with ethanol and methanol, so DMSO must be used. To counteract its low stability, the DHA mixture must be prepared freshly the same day as the epoxidation. The total DMSO percentage in the reaction mixture must be kept under 1% to avoid deactivating the enzyme. Additionally, because NADPH has a short shelf life, the concentration should be checked with the spectrophotometer prior to addition to reaction. This ensures that 1 equivalent of the NADPH is always added to the reaction mixture.

Epoxidation reaction guidelines.

Airflow from the balloon into reaction mixture must be maintained in order to keep the reaction oxygenated (as oxygen is necessary for epoxidation). The mixture must also be stirred rapidly, since PUFAs are not very soluble in water (the solubility of DHA is (≤125 μM in the reaction buffer). The reaction is quenched with oxalic acid to denature protein (as its chelates metal and removes cofactors) and acid keeps DHA its neutral form, which is necessary for diethyl ether extraction. The quenching of the reaction mixture must be done slowly to avoid acid-catalyzed hydrolysis of the EDPs.

EDP extraction and purification guidelines.

Ether was chosen specifically as the extraction solvent for multiple reasons. Dichloromethane can precipitate the protein out of solution, which complicates the extraction. EtOAc extracts glycerol from (added with the enzyme stock solution), which is difficult to remove and interferes with the flash chromatography.

EDP regioisomers in their free acid state are not easily separable, which is why the regioisomers are mixed into a single peak in the first flash column chromatography. Once they are converted to the methyl esters, the regioisomers are readily separable. Additionally, the esters are generally more stable than the corresponding acids for long-term storage if the acid form is not needed immediately.

Significance of the method with respect to existing/alternative methods.

Our method provides a simple and effective method for obtaining enantiopure EDPs, which has many advantages over existing and alternative methods. First, chemical epoxidation of DHA and other PUFAs and their derivatives is neither regioselective or enantioselective, and complicated mixtures are often obtained. Multiple flash chromatography columns and preparative HPLC, including chiral preparative HPLC, are therefore necessary to purify enantiopure epoxy fatty acids from these mixtures, which are labor intensive and can produce only very small amounts of the desired metabolites. Total synthesis can also be employed to produce epoxy fatty acids, but it is rigorous, time-consuming, requires multiple steps, and gives a low overall yield, whereas the BM3 enzyme is easy to express and purify, and the epoxidation is complete within a short period of time. Our method is also cost-effective: a commercial source[20] currently offers 16,17- and 19,20-EDP (as their racemates) for $528/0.5 mg. One gram of NADPH can also be purchased for ~$500-$800, and can be used to produce over two hundred times the amount of 19,20-EDP (and approximately fifty times the amount of 16,17-EDP) offered commercially for a similar price – and in enantiopure forms, which are currently not commercially available.

Limitations of the method.

As this enzyme preferentially epoxidizes the last double bond of DHA, the major product is 19,20-EDP (although 16,17-EDP is also produced). Therefore, other DHA regioisomers that might be desired (e.g., 13,14-EDP, 10,11-EDP) cannot be produced by enzymatic epoxidation with wild-type BM3. Also, as only the *SR*-enantiomers are produced by BM3, the *RS*-enantiomers are inaccessible, although our previously published chemical inversion method[14] may be used to synthesize them. Additionally, because of the low solubility of lipophilic PUFAs in the reaction buffer, very large amounts of buffer would be necessary for large-scale production (ca. 500-1000 mg) of EDPs, which could potentially make extraction time-consuming or prohibitive.

Other applications of the method.

Pleasingly, this enzymatic epoxidation protocol is also applicable to EPA and AA (relevant structures are shown in Figure 5). The concentrations, buffer, and reaction time required for epoxidation of all three fatty acids is the same. For the EPA, the wild-type BM3 enzyme is also used, and the EEQ fraction obtained from EPA (56% yield of monoepoxide), after esterification is ~14:1 17,18-EEQ:14,15-EEQ. Similar to the observations made for EDPs, the 17,18-EEQ obtained is highly enantiopure (>99% 17(*S*),18(*R*)-EEQ, see Figure 6) as assessed by chiral HPLC (see Figure 3 legend and Table of Materials; identity of enantiomers was previously reported.[17] For AA, however, the F87V BM3 mutant must be used instead (as wild-type BM3 is a hydroxylase for AA).[21] Expression and purification of this mutant also uses the above protocol, and epoxidation is performed in an analogous fashion. By this method, 14,15-EET is obtained as the sole regioisomer. As 14,15-EET free acid is inseparable from unreacted AA following the

epoxidation, esterification is necessary; 14,15-EET methyl ester is obtained in 52% yield from AA. Chiral HPLC (see Figure 3 legend and Table of Materials) of the acid indicates highly enantiopure (>99%) 14(*S*),15(*R*)-EET (as previously reported).[15] The chemical shifts for these EPA and AA metabolites are as follows: **17(*S*),18(*R*)-EEQ methyl ester:** ^{1}H-NMR (500 MHz; $CDCl_3$): δ 5.53-5.34 (m, 8 H), 3.67 (s, 3 H), 2.96 (td, *J* = 6.4, 4.2 Hz, 1 H), 2.90 (td, *J* = 6.3, 4.2 Hz, 1 H), 2.85-2.79 (m, 6 H), 2.44-2.38 (m, 1 H), 2.32 (t, *J* = 7.5 Hz, 2 H), 2.26-2.20 (m, 1 H), 2.11 (q, *J* = 6.7 Hz, 2 H), 1.71 (quintet, *J* = 7.4 Hz, 2 H), 1.66-1.50 (m, 2 H), 1.05 (t, *J* = 7.5 Hz, 3 H); **14(*S*),15(*R*)-EET methyl ester:** ^{1}H-NMR (500 MHz; $CDCl_3$): δ 5.53-5.33 (m, 6 H), 3.67 (s, 3 H), 2.95-2.92 (m, 2 H), 2.81 (dt, *J* = 17.8, 5.8 Hz, 4 H), 2.40 (dt, *J* = 14.1, 6.8 Hz, 1 H), 2.32 (t, *J* = 7.5 Hz, 2 H), 2.23 (dt, *J* = 14.1, 6.8 Hz, 1 H), 2.11 (q, *J* = 6.8 Hz, 2 H), 1.71 (quintet, *J* = 7.4 Hz, 2 H), 1.56-1.41 (m, 4 H), 1.38-1.30 (m, 4 H), 0.90 (t, *J* = 7.1 Hz, 3 H).

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References:

1. Campbell, W. B., Gebremedhin, D., Pratt, P. F., and Harder, D. R. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circulation Research* **78**, 415-423 (1996).

2. Ulu, A. et al. An omega-3 epoxide of docosahexaenoic acid lowers blood pressure in angiotensin-II-dependent hypertension. *Journal of Cardiovascular Pharmacology* **64**, 87-99 (2014).

3. Ye, D. et al. Cytochrome p-450 epoxygenase metabolites of docosahexaenoate potently dilate coronary arterioles by activating large-conductance calcium-activated potassium channels. *Journal of Pharmacology and Experimental Therapeutics* **303**, 768-776 (2002).

4. Imig, J. D. Epoxyeicosatrienoic acids, hypertension, and kidney injury. *Hypertension* **65**, 476-682 (2015)

5. Capozzi, M. E., Hammer, S. S., McCollum, G. W., and Penn, J. S. Epoxygenated fatty acids inhibit retinal vascular inflammation. *Scientific Reports* **6**, 39211 (2016).

6. Zou, A-P. et al. Stereospecific effects of epoxyeicosatrienoic acids on renal vascular tone and K(+)-channel activity. *American Journal of Physiology* **270**, F822-F832 (1996).

7. Lauterbach, B.et al. Cytochrome P450-dependent eicosapentaenoic acid metabolites are novel BK channel activators. *Hypertension* **39**, 609-13 (2002).

8. Clarke T. C., Black T. I., Stussman B. J., Barnes P. M., Nahin R.L. Trends in the use of complementary health approaches among adults: United States, 2002–2012. Hyattsville, MD: National Center for Health Statistics (2015).

9. Mozaffarian, D., and Wu, J. H. Y. Omega-3 fatty acids and cardiovascular disease. *Journal of the American College of Cardiology* **58**, 2047-2067 (2011).

10. Shearer, G., W. Harris, T. Pederson, and J. Newman. Detection of omega-3 oxylipins in human plasma in response to treatment with omega-3 acid ethyl esters. *Journal of Lipid Research* **51**, 2074-2081 (2010.)

11. Ostermann, A. I. and Schebb, N. H. Effects of omega-3 fatty acid supplementation on the pattern of oxylipins: a short review about the modulation of hydroxy-, dihydroxy-, and epoxy-fatty acids. *Food & Function* **8**, 2355-2367 (2017).

12. Khan, M. A. and Wood, P. L. Method for the synthesis of DHA. WO2012126088A1 (2012).

13. Nanba, Y., Shinohara, R., Morita, M., and Kobayashi, Y. Stereoselective synthesis of 17,18-epoxy derivative of EPA and stereoisomers of isoleukotoxin diol by ring-opening of TMS-substituted epoxide with dimsyl sodium. *Organic and Biomolecular Chemistry* **15**, 8614-8626 (2017).

14. Cinelli, M. A. et al. Enzymatic synthesis and chemical inversion provide both enantiomers of bioactive epoxydocosapentaenoic acids. *Journal of Lipid Research* **59**, 2237-2252 (2018).

15. Falck, J. R. et al. Practical, enantiospecific syntheses of 14,15-EET and leukotoxin B (vernolic acid). *Tetrahedron Letters* **41**, 4131-4133 (2001).

16. Celik, A., Sperandio, D., Speight, R. E., and Turner, N. Enantioselective epoxidation of linolenic acid catalyzed by cytochrome P450BM3 from Bacillus megaterium. *Organic and Biomolecular Chemistry* **3**, 1688-2690 (2005).

16. Capdevila, J. H. et al. The highly stereoselective oxidation of polyunsaturated fatty acids by cytochrome P450BM-3. *Journal of Biological Chemistry* **271**, 22663-22671 (1996).

18. Lucas, D. et al. Stereoselective epoxidation of the last double bond of polyunsaturated fatty acids by human cytochromes P450. *Journal of Lipid Research* **51**, 1125-1133 (2010).

19. Guengerich, F. P., Martin, M. V., Sohl, C. D., and Cheng, Q. Measurement of cytochrome P450 and NADPH-cytochrome P450 reductase. *Nature Protocols* **4**, 1245-1251 (2009).

20. https://www.caymanchem.com/product/10175 Cayman Chemical, 19,20-EpDPA.

21. Graham-Lorence, S. et al. An active site substitution, F87V, converts cytochrome P450 BM-3 into a regio- and stereoselective (14*S*, 15*R*)-arachidonic acid epoxygenase. *Journal of Biological Chemistry* **272**, 1127-1135 (1996).