Journal of Visualized Experiments

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

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59770R2
Full Title:	Enzymatic Synthesis of Epoxidized Metabolites of Docosahexaenoic, Eicosapentaenoic, and Arachidonic Acids
Keywords:	epoxy polyunsaturated fatty acids; lipids, metabolites; enzymatic synthesis; enantioselective synthesis; DHA; eicosanoids; polyunsaturated fatty acid
Corresponding Author:	Kin Sing Stephen Stephen Lee Michigan State University East Lansing, MI UNITED STATES
Corresponding Author's Institution:	Michigan State University
Corresponding Author E-Mail:	sing@msu.edu
Order of Authors:	Kin Sing Stephen Stephen Lee
	Joseph W Woodman
	Maris A Cinelli
	Amy Stephen Scharmen-Burgdolf
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	East Lansing, MI 48824, USA

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript Click here to access/download;Manuscript;59770_R2.docx

TITLE:

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

AUTHORS & AFFILIATIONS:

Joseph W. Woodman[1], Maris A. Cinelli[1], Amy Scharmen-Burgdolf[1], and Kin Sing Stephen Lee[1]

[1]Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI, USA.

E-MAIL ADDRESSES:

Joseph W. Woodman (woodma21@msu.edu)

Maris A. Cinelli (cinellim@msu.edu)

Amy Scharmen-Burgdolf (scharmen@msu.edu)

CORRESPONDING AUTHOR:

Kin Sing Stephen Lee (sing@msu.edu)

KEYWORDS:

epoxy polyunsaturated fatty acids, lipids, enzymatic synthesis, enantioselective synthesis, omega-3 fatty acids, eicosanoids

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 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 into a 20 mL culture tube.

1.2. Incubate the cell culture in a 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 x *g*. Discard the supernatant and store the cell pellet at -78 ˚C until enzyme purification.

NOTE: The supernatant can be either chemically sterilized by treatment with bleach or sterilized using an autoclave and then poured down the drain.

2. Purification of BM3.

2.1. Cell lysis

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;), 0.01 mM EDTA; pH 7.8).

CAUTION: PMSF is toxic by contact.

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 in order to lyse the cells. Repeat this procedure 6 times. Centrifuge the cell lysate at 4 ˚C for 30 min at 11,000 x *g* to pellet cell debris.

2.2. Affinity chromatography

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 in 18.8 mL of 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 the consumption of NADPH, the reaction is complete.

NOTE: Typically, the reaction is complete 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 cartridge is sufficient). Start at 10% ethyl acetate (EtOAc) in hexanes and ramp up to 60% EtOAc in hexanes over 22 min.

NOTE: 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) and 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.

5.3. After 30 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 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 obtained as clear oils (total yield, 93%).

5.4. 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.

5.5. If any mixed fractions remain (containing both isomers; can be assessed by thin-layer chromatography (TLC) in 8:1 hexane/EtOAc and stained with potassium permanganate ($KMnO_4$)), re-chromatograph them with the same solvent system as before.

5.6. Concentrate the fractions containing the individual regioisomers.

NOTE: At this point, identity and purity may be assessed by NMR (using $CDCl_3$ as the solvent; see the legend for **Figure 2**).

5.7. 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.

NOTE: 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.

5.8. Quench the reaction slowly with formic acid, until the pH of the mixture reaches 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).

5.9. 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.

5.9. Concentrate the desired fractions and dry in vacuo to afford the purified acid.

NOTE: 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, **Figure 3A** and **Figure 4A**). As can be observed in **Figure 3B** and **Figure 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. **Figure 3A** shows a chiral HPLC chromatogram of "racemic" 16,17-EDP (an artificial mixture of authentic standards of both enantiomers[14]), whereas **Figure 3B** shows a chiral HPLC chromatogram of 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. **Figure 4A** shows a chiral HPLC chromatogram of "racemic" 19,20-EDP (an artificial mixture of authentic standards of both enantiomers[14]), whereas **Figure 4B** shows a chiral HPLC chromatogram of 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. Overall enzymatic epoxidation reaction scheme and relevant structures of AA, EPA, EETs, and EEQs produced by this method

Figure 6. Chiral HPLC indicating enantiopurity of 17,18-EEQ (acid form) produced by BM3. **Figure 6A** shows a chiral HPLC chromatogram of "racemic" 17,18-EEQ (an artificial mixture of authentic standards of both enantiomers[14]), whereas **Figure 6B** shows a chiral HPLC chromatogram of 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 it 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 (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 USD /0.5 mg. 1 g of NADPH can also be purchased for ~500-800 USD, 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).

DISCLOSURES:

The authors have no conflicts of interest to disclose.

ACKNOWLEDGEMENTS:

This work is funded by R00 ES024806 (National Institutes of Health), DMS-1761320 (National Science Foundation) and startup funds from Michigan State University. The authors wish to thank Dr. Jun Yang (University of California at Davis) and Lalitha Karchalla (Michigan State University) for assistance with optimization of the enzymatic reaction, and Dr. Tony Schilmiller (MSU Mass Spectrometry and Metabolomics Facility) for assistance with HRMS data acquisition.

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).

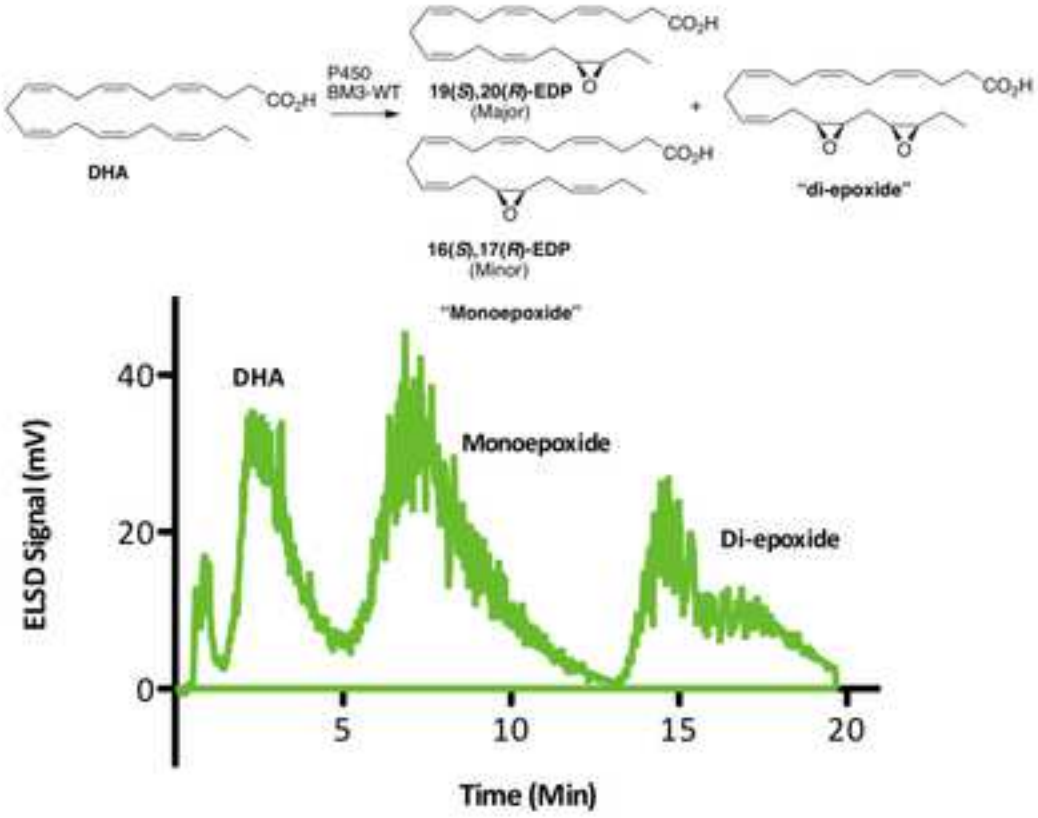
3. Ye, D. et al. Cytochrome p-450 epoxygenase metabolites of docosahexaenoate potently dilate coronary arterioles by activating large-conductance calcium-activated potassium channels. *Journal of Pharmacology and Experimental Therapeutics* **303**, 768-776 (2002).

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).

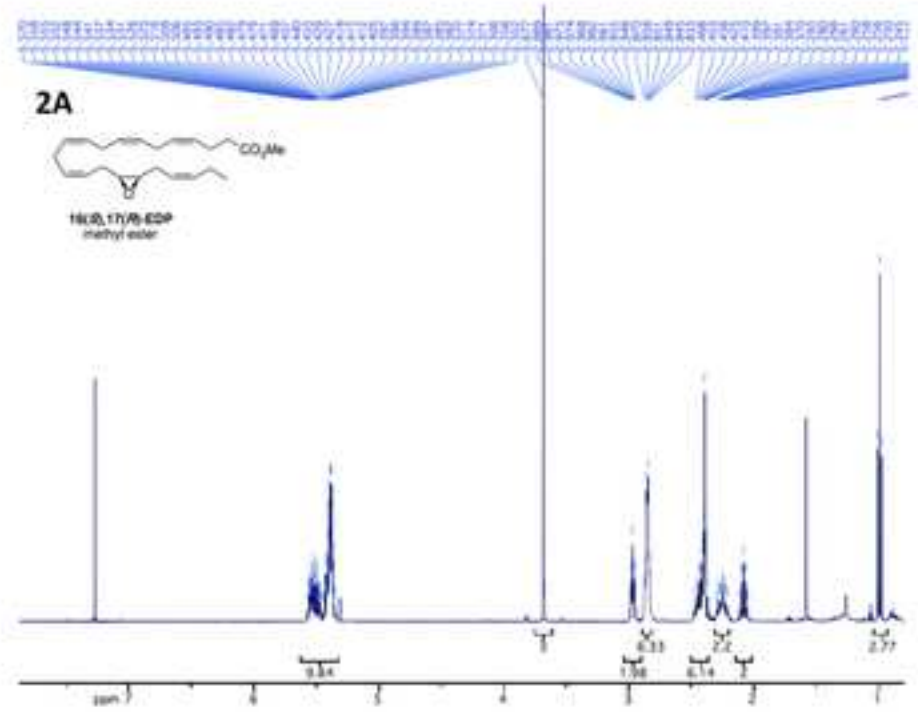
Figure

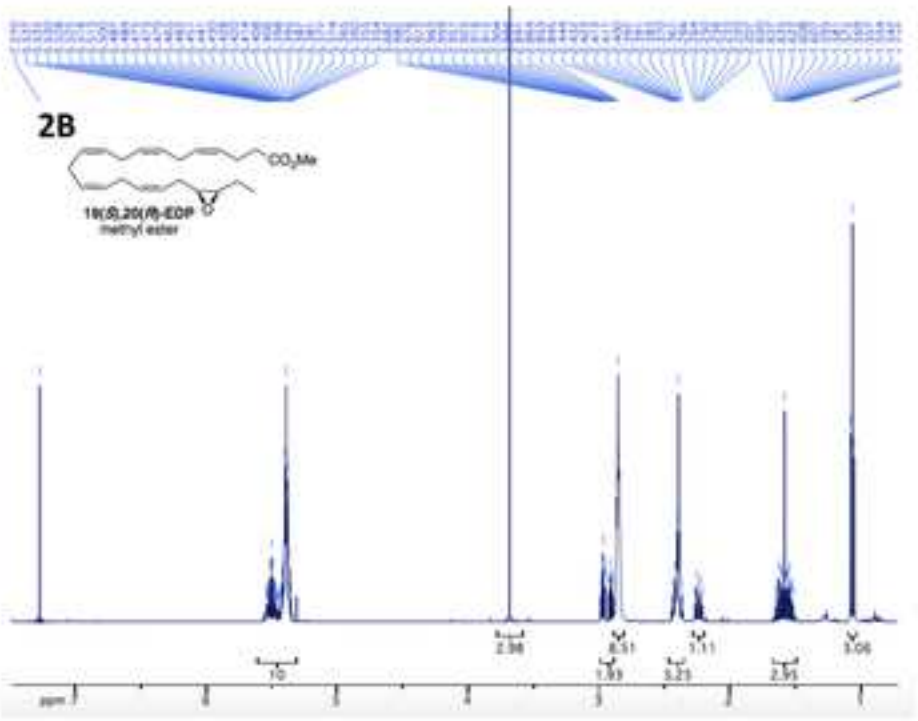
Click here to access/download;Figure;Figure 1JOVE.jpg



Figure

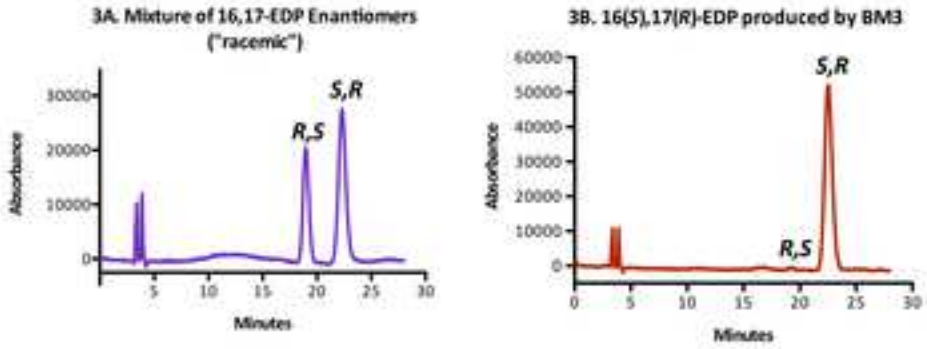
Click here to access/download;Figure;Figure 2JOVE.jpg





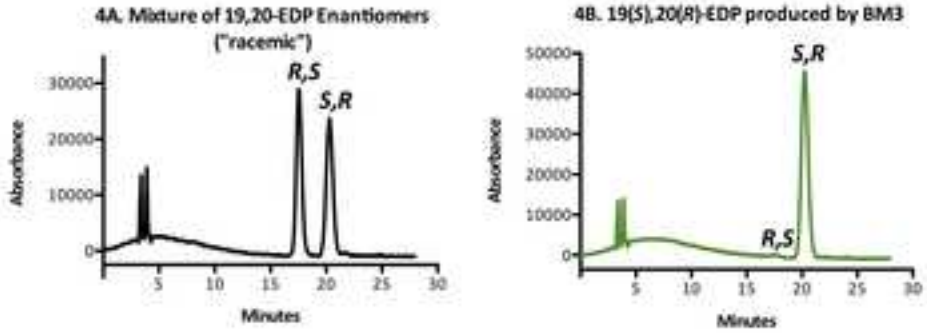
Figure

Click here to access/download;Figure;Figure 3 JOVE New.jpg



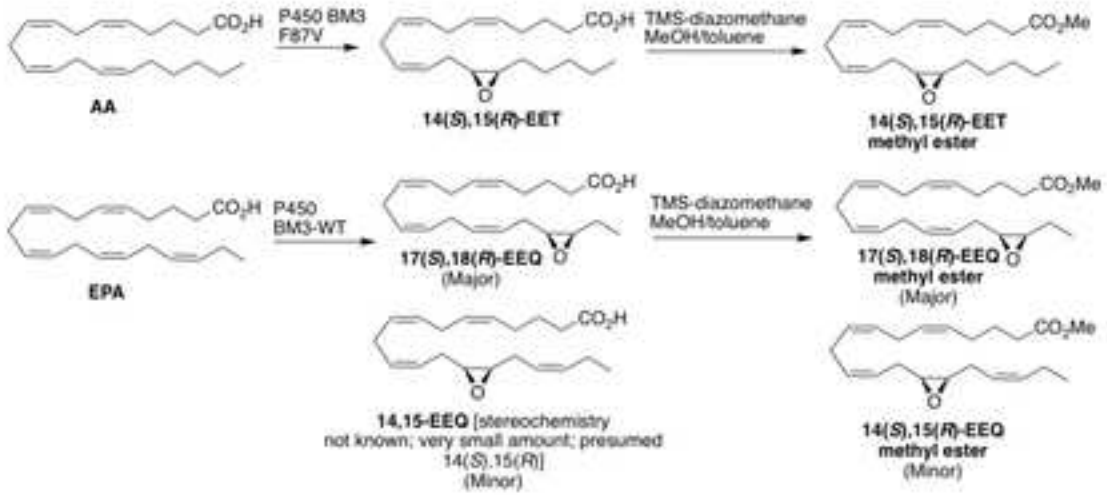
Figure

Click here to access/download;Figure;JOVE Fig 4 new.jpg



Figure

Click here to access/download;Figure;Figure 5JOVE.jpg



Figure

Click here to access/download;Figure;JOVE Figure 6.jpg

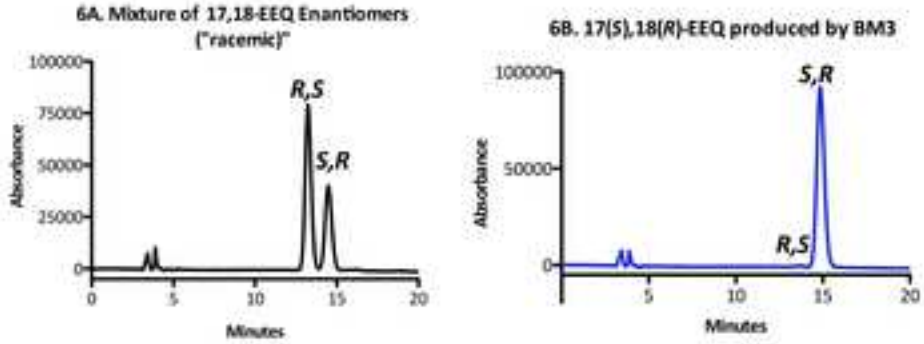


Table of Materials

Click here to access/download;Table of Materials;JOVE Epoxidation Materials Revised.xls

Name of Material/ Equipment	Company	Catalog Number
Ammonium Bicarbonate	Sigma	9830
Ampicillin	GoldBio	A30125
Anhydrous magnesium sulfate	Fisher Scientific	M65-3
Anhydrous methanol	Sigma-Aldrich	322515
Anhydrous sodium sulfate	Fisher Scientific	S421-500
Anhydrous toluene	Sigma-Aldrich	244511
Arachidonic Acid (AA)	Nu-Chek Prep	U-71A
Diethyl Ether	Sigma	296082
DMSO (molecular biology grade)	Sigma-Aldrich	D8418
Docosahexaenoic Acid (DHA)	Nu-Chek Prep	U-84A
EDTA (ethylenediaminetetraacetic acid)	Invitrogen	15576028
Eicosapentaenoic Acid (EPA)	Nu-Chek Prep	U-99A
Ethyl acetate	Sigma	34858
Flash column cartridges 25, 40, 4, 12 g sizes	Fisher Scientific	145170203, 145154064, 5170200
Formic acid (HPLC Grade)	J.T. Baker	0128-01
Glycerol	Sigma	G7757
Hexanes	VWR	BDH24575
LB Broth	Sigma	L3022
Lithium hydroxide	Sigma-Aldrich	442410
Magnesium chloride	Fisher Scientific	2444-01
Methanol (HPLC grade)	Sigma-Aldrich	34860-41-R
NADPH Tetrasodium Salt	Sigma-Aldrich	481973
Oxalic acid	Sigma-Aldrich	194131
pBS-BM3 transfected DH5α E. coli	NA	NA
PMSF (phenylmethanesulfonyl fluoride)	Sigma	P7626
Potassium Permanganate	Sigma-Aldrich	223468
Potassium phosphate dibasic	Sigma	795496
Potassium phosphate monobasic	Sigma	795488
Q Sepharose Fast Flow resin (GE Healthcare life sciences)	Fisher Scientific	17-0515-01
Sodium Chloride	Sigma	71376

Tetrahydrofuran, anhydrous	Sigma-Aldrich		186562
TMS-Diazomethane (2.0 M in hexanes)	Sigma-Aldrich		362832
Tris-HCl	GoldBio	T-400	

Also necessary:

Automatic flash purification system (we used a Buchi Reveleris X2)	Buchi
C18 HPLC column (Zorbax Eclipse XDB-C18)	Agilent
Centrifuge capable of 10,000 x g	
Chiral HPLC Column (Lux cellulose-3), 250 x 4.6 mm, 5 µM, 1000 Å)	Phenomenex
General chemistry supplies: a 2 L separatory funnel, beakers and Erlenmeyer flasks with 1000-2000 L capacity, 20 mL vials, HPLC vials, small round-bottomed flasks and stir-bars.	
HPLC (we use a Shimadzu Prominence LC-20AT analytical pump and SPD-20A UV-vis detector	Shimadzu
Nanodrop 2000 Spectrophotometer	Thermo-Fisher Scientific
NMR	NMR: Agilent DD2 spectrometer (500 MHz)
Rotary evaporator	Buchi
Sonic dismembrator or ultrasonic homogenizer	Cole-Parmer

Comments/Description
NA
NA
NA
NA
NA
NA
Air-sensitive.
NA
NA
Air-sensitive.
NA
Air-sensitive.
NA
Alternatively, conventional column chromatography can be used
NA
NA
NA
NA
NA
NA
NA
Air-sensitive.
NA
NA
Toxic!
For TLC staining.
NA
NA
For anion exchange purification of enzyme
NA

NA

Very toxic.

NA

Author License Agreement (ALA)
Click here to access/download;Author License Agreement (ALA);HP-Scan.pdf

jove VISUALIZED JOURNAL OF EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Enzymatic Synthesis of Epoxidized metabolites of Docosahexaenoic Acid Eicosatetraenoic Acid and Arachidonic Acid

Author(s): Joseph W. Woodman, Maris A Cinelli, Amy Scharmen-Burgdolf and Kin Sing Stephen Lee

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Kin Sing Stephen Lee
Department:	Pharmacology & Toxicology
Institution:	Michigan State University
Title:	Assistant Professor
Signature:	
Date:	03/05/2019.

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Rebuttal Letter

Click here to access/download;Rebuttal Letter;JOVE_coverletter-sing.pdf

MICHIGAN STATE UNIVERSITY

Mar 24th, 2019

Dr. Bing Wu
Review Editor
JoVE

Dear Dr. Wu,

This letter regards the submission of our revised manuscript to JoVE, entitled “Enzymatic Synthesis of Epoxidized Metabolites of Docosahexaenoic, Eicosapentaenoic, and Arachidonic Acids”. We have reviewed these comments carefully, and incorporated changes into our revised manuscript that we hope will provide additional clarity and completeness for the readers (and viewers) of JoVE.

Both the revised manuscript for submission (with Track Changes enabled) and our point-by-point responses to the reviewers are included at the end of this letter. Should you or any reviewers have questions or require any further information, please feel free to email me at sing@msu.edu or call me at 517-884-1813.’

Thank you very much.

Yours sincerely,

Kin Sing Stephen Lee, Ph.D.
Assistant Professor
Department of Pharmacology and Toxicology
Michigan State University
East Lansing, MI48824
Email: sing@msu.edu
Phone: 517-884-1813

Department of Pharmacology and Toxicology

Michigan State University
B330 Life Science Building
1355 Bogue Street
East Lansing, MI 48824

517-884-1813
sing@msu.edu

MSU is an affirmative-action, equal-opportunity employer.

Editorial comments:

The manuscript has been modified and the updated manuscript, **59770_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: This manuscript has been proofread substantially and all spelling and grammar issues have been corrected.

2. For in-text referencing, please place the reference numbers before a comma or period.

Response: We have corrected this.

3. Please add step 2.1 as a sub-heading for steps 2.1.1-2.1.2.
4. Please add step 2.2 as a sub-heading for steps 2.2.1-2.2.3.

Response: We have added subheading 2.1 “cell lysis” and 2.2 “affinity chromatography” as appropriate subheadings.

5. Please avoid long steps (more than 4 lines).

Response: There are now no steps longer than four lines. Excessively long steps have been broken into two steps.

6. Figure 4, Figure 5, Figure 6: Please add a short description of the figure in figure legend.

Response: We have made the legends for these figures more descriptive and representative.