SUPPLEMENTAL DATA

Cytochrome P450-type hydroxylation and epoxidation in a tyrosine-liganded hemoprotein, catalase-related allene oxide synthase

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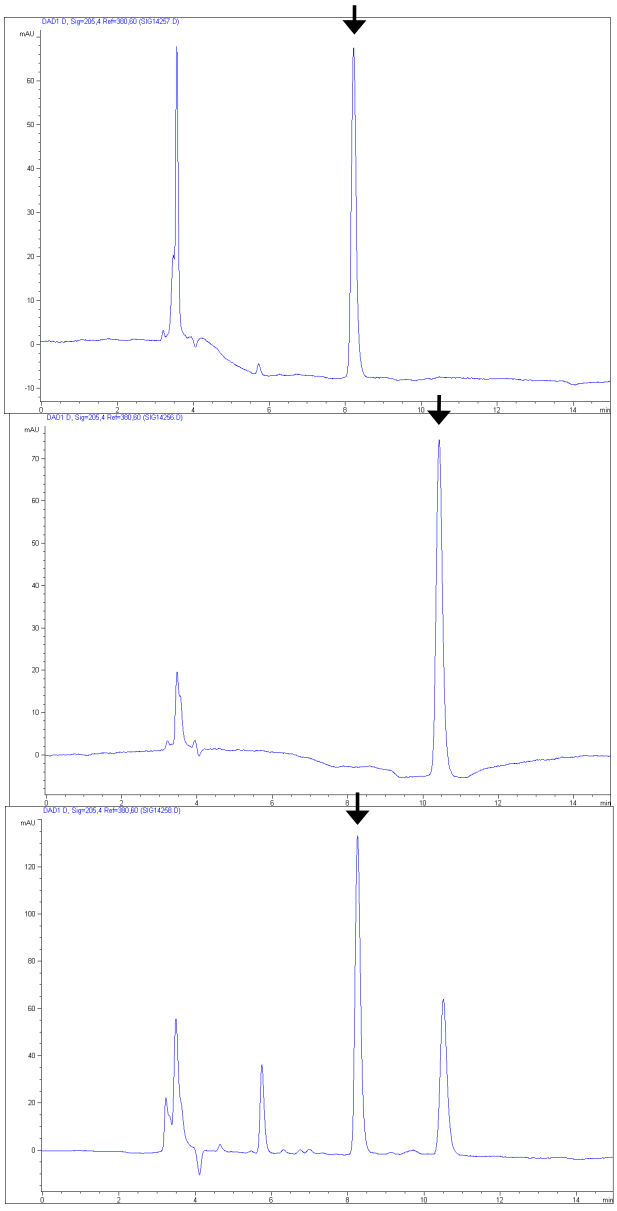
Supplement Fig. S1: **SP-HPLC separation of the methyl esters of *erythro* and *threo* 8*R*-hydroxy-9,10-epoxy-eicosatrienoates (Products 1 and 2)**
Column: Thomson Advantage 5μ silica (25 x 0.46 cm); Solvent: of hexane/isopropanol (100:1, v/v), using a flow rate of 1 ml/min, with UV detection at 205 nm.



Product 1 (*erythro*), from cAOS-PhIO-8*R*-HETE incubation

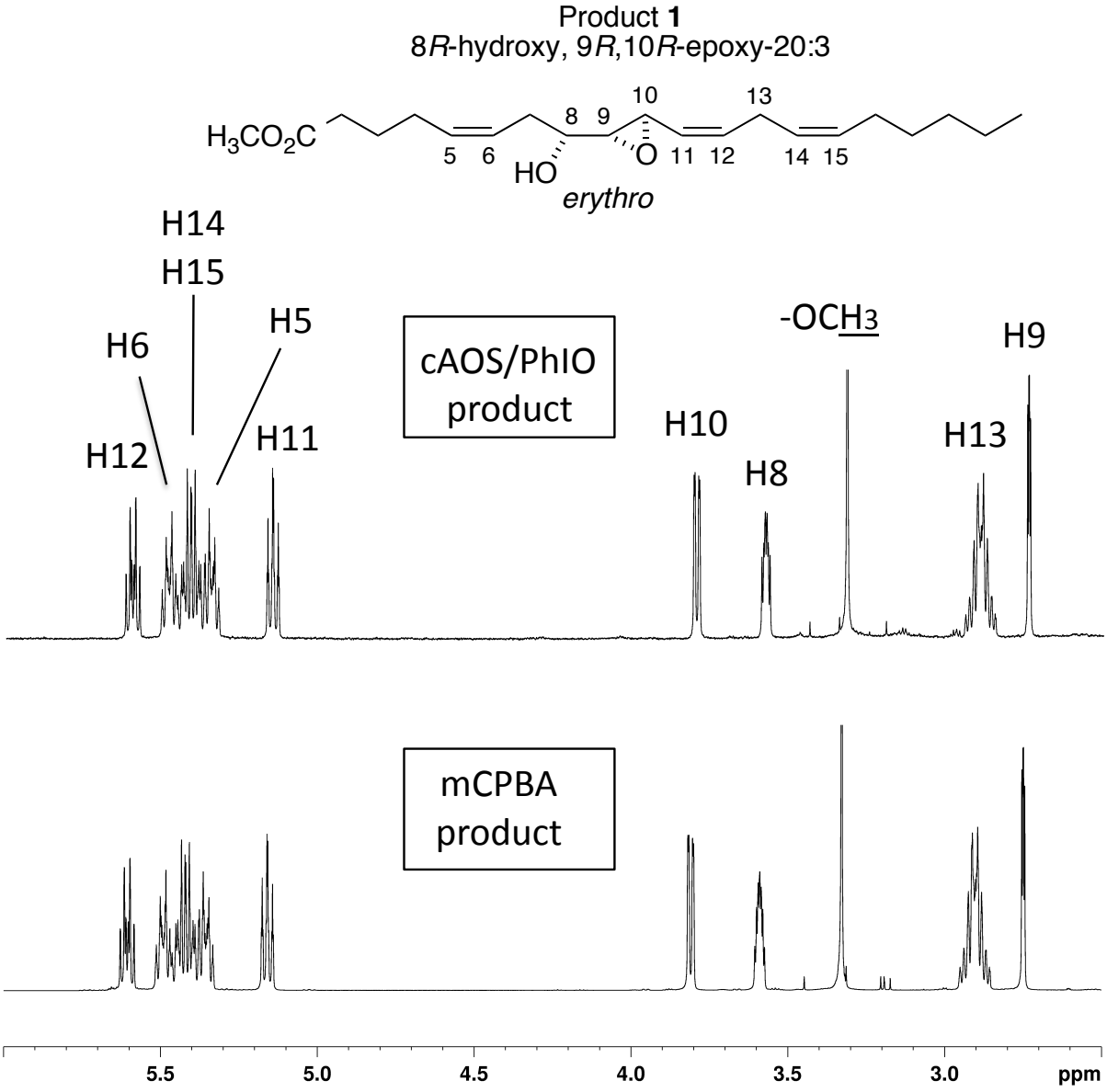
Product 2 (*threo*), from mCPBA reaction with 8*R*-HETE

Product 1 (arrow) from cAOS-PhIO, co-injected with a 50:50 mix of *erythro*/*threo* standards (mCPBA products)

Supplement Fig. S2

Fig. S2. Illustrating the identity of the ^{1}H-NMR spectra (2.5 – 6 ppm) of the methyl ester of cAOS/PhIO- and mCPBA-derived Product 1. The spectra were recorded in d6-benzene at 283 K.

Top: cAOS/PhIO product **1**
Below: mCPBA product **1**



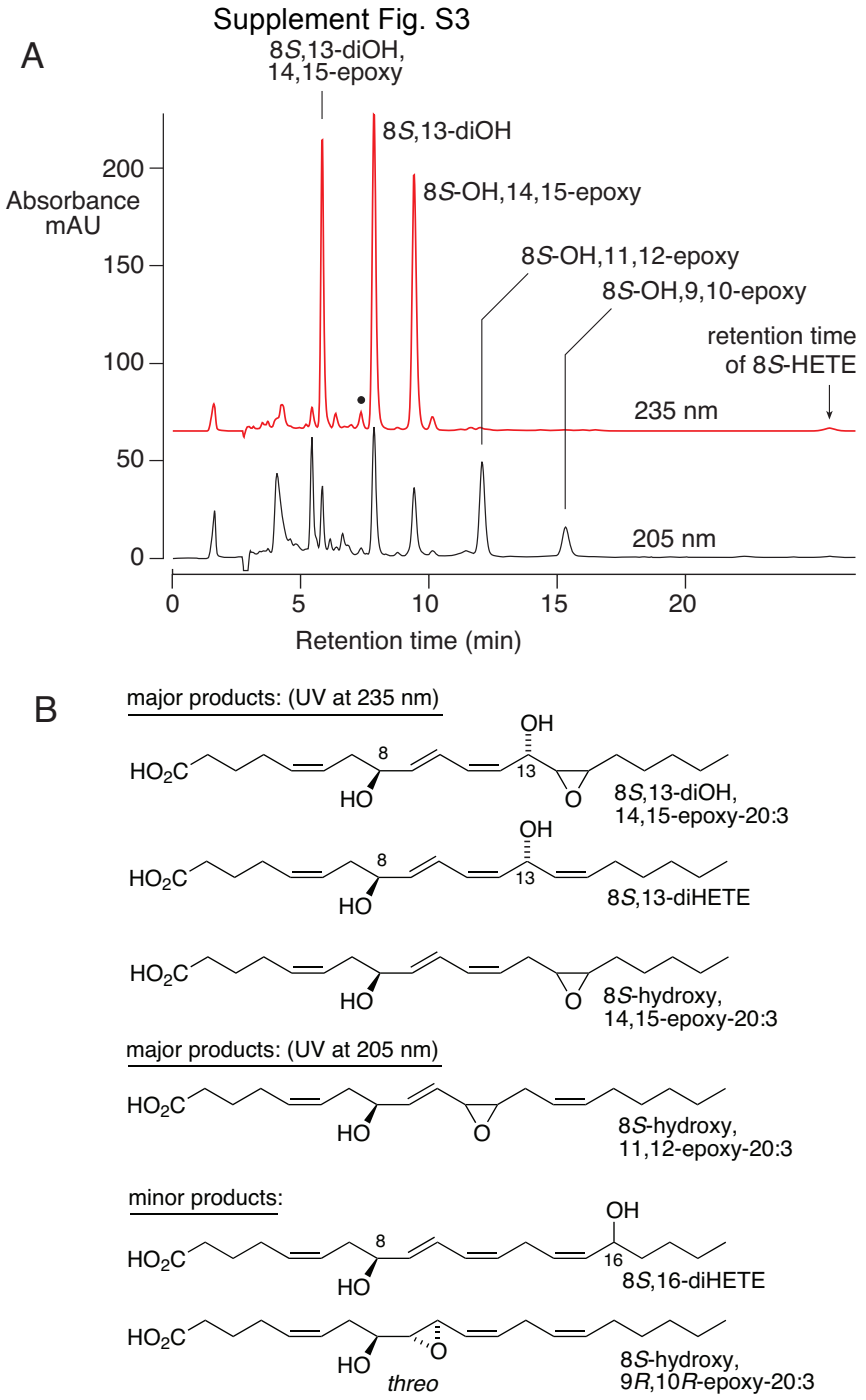


Fig. S3. RP-HPLC analysis of the products products formed from 8*S*-HETE by cAOS/PhIO (*A*) 8*S*-HETE (150 μM) was reacted with cAOS in the presence of 300 μM PhIO for 3 min at room temperature. Following extraction and formation of the methyl esters using diazomethane, an aliquot was analyzed on a Thomson Instrument Co. Advantage C18 column (25 x 0.46 cm) using a solvent system of MeOH/H_2O/HAc (80/20/0.01 by volume) and a flow rate of 1 ml/min. As in Fig. 2A and 5A (main text), the UV traces of 205 nm and 235 nm are at the same sensitivity and are offset for clarity. The black dot at ~7.5 min marks a small peak identified by GC-MS, after hydrogenation, as 8*R*,16-diHETE (Fig S6). (*B*) Structures of the products identified (NMR and GC-MS data included in SI).

Supplement Fig. S4

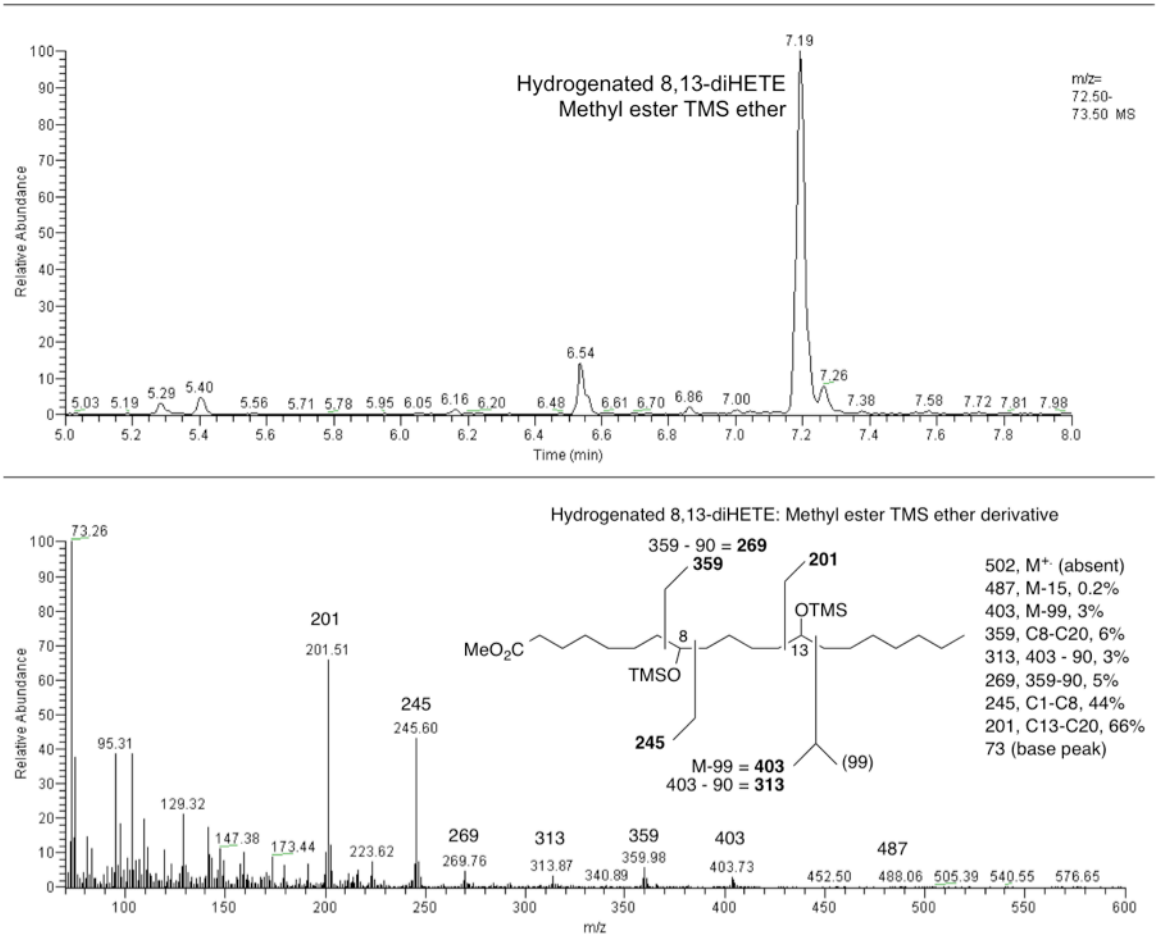


Fig. S4. Identification of 8,13-diHETE, product of 8*S*-HETE with cAOS/PhIO
8,13-diHETE methyl ester was hydrogenated, converted to the trimethylsilyl ether (TMS) derivative and analyzed by GC-MS. Top: GC profile at m/z 73 (equivalent to any compounds containing TMS). Lower panel: Electron impact mass spectrum. Diagnostic ions are listed in the lower panel and illustrated on the structure.

Assignment of the 13-hydroxyl configuration in 8*S*,13-diHETE, product of 8*S*-HETE/cAOS/PhIO

Chiral 13-HETE enantiomers were prepared by: (i) Vitamin E-controlled autoxidation of arachidonate PFB ester, using 500% excess of vitamin E, and purification of 13-HETE PFB ester. (ii) Resolution of 13-HETE enantiomers as the PFB esters using a Chiralpak AD column (solvent hexane/MeOH, 100:2 v/v). (iii) Assignment of the 13*R*/13*S* configurations by mild acid (0.1% acetic acid) treatment of the individual 13-HETE free acid enantiomers followed by purification and chiral analysis of the 11-HETE and 15-HETE products (cf. SI-ref 2). Then standards of 8,13-diHETE were prepared by: (iv) Reaction of 13*S*-HETE with 8*R*-LOX to produce 8*R*,13*S*-diHETE as standard. (v) Vitamin E-controlled autoxidation of 13-HETE PFB ester followed by isolation the two diastereomers of 8,13-diHETE as standards: structures confirmed by GC-MS. (vi) Comparison of the cAOS/PhIO/8*S*-HETE-derived 8*S*,13-diHETE with the 8*R*,13*S*-diHETE and the pair of diastereomers (Fig. S7).

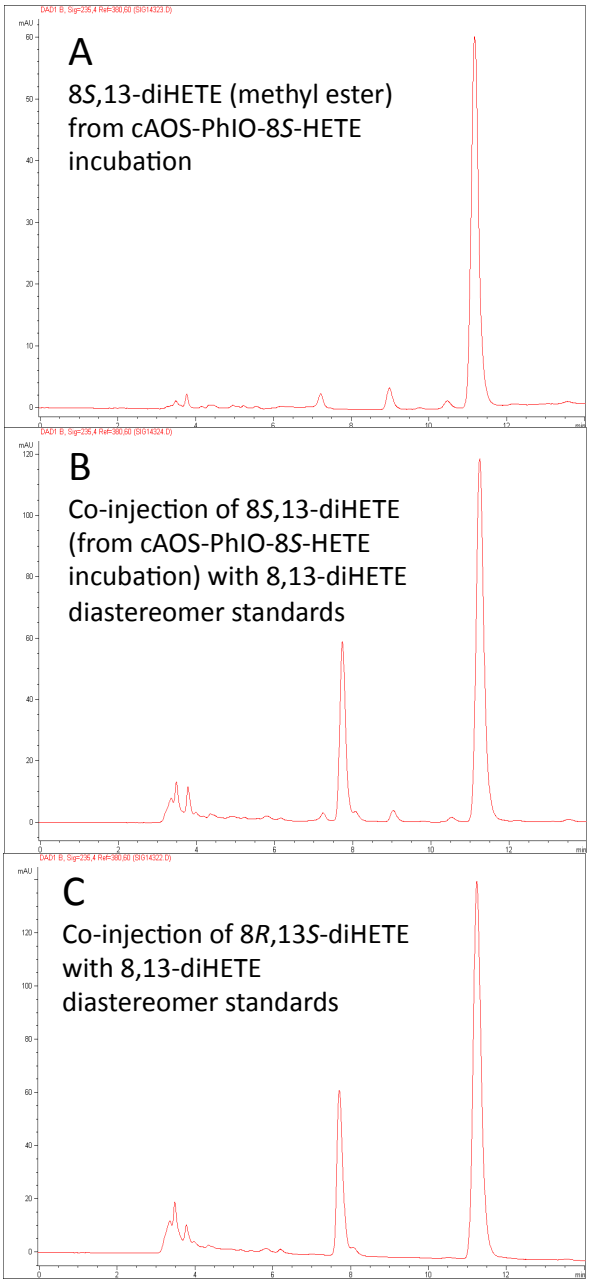


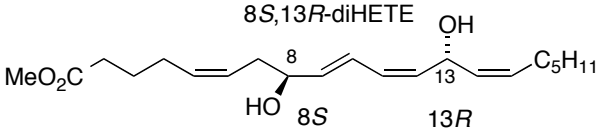
Fig. S5: Assignment of the 13-hydroxyl configuration in cAOS-derived 8*S*,13-diHETE as 13*R*.
Column: Thomson Advantage 5μ silica (25 x 0.46 cm), solvent, hexane/IPA (95:5 v/v), flow rate 1 ml/min, UV detection at 235 nm.

Panel A: SP-HPLC of the 8*S*,13-diHETE product from cAOS/PhIO/8*S*-HETE (methyl ester derivative), which elutes at a retention time of 11.2 min.

Panel B: as shown by co-injection, the 8*S*-HETE-derived 8,13-diHETE co-chromatographed with the second of the two diastereomeric standards.

Panel C: The standard of 8*R*,13*S*-diHETE also co-chromatographed with the second diastereomer.

Co-chromatography of the cAOS/PhIO/8*S*-HETE product and the 8*R*,13*S*-diHETE standard indicates that the two are enantiomers. Thus, the 8*S*-HETE-derived 8,13-diHETE is 8*S*,13*R*-diHETE.



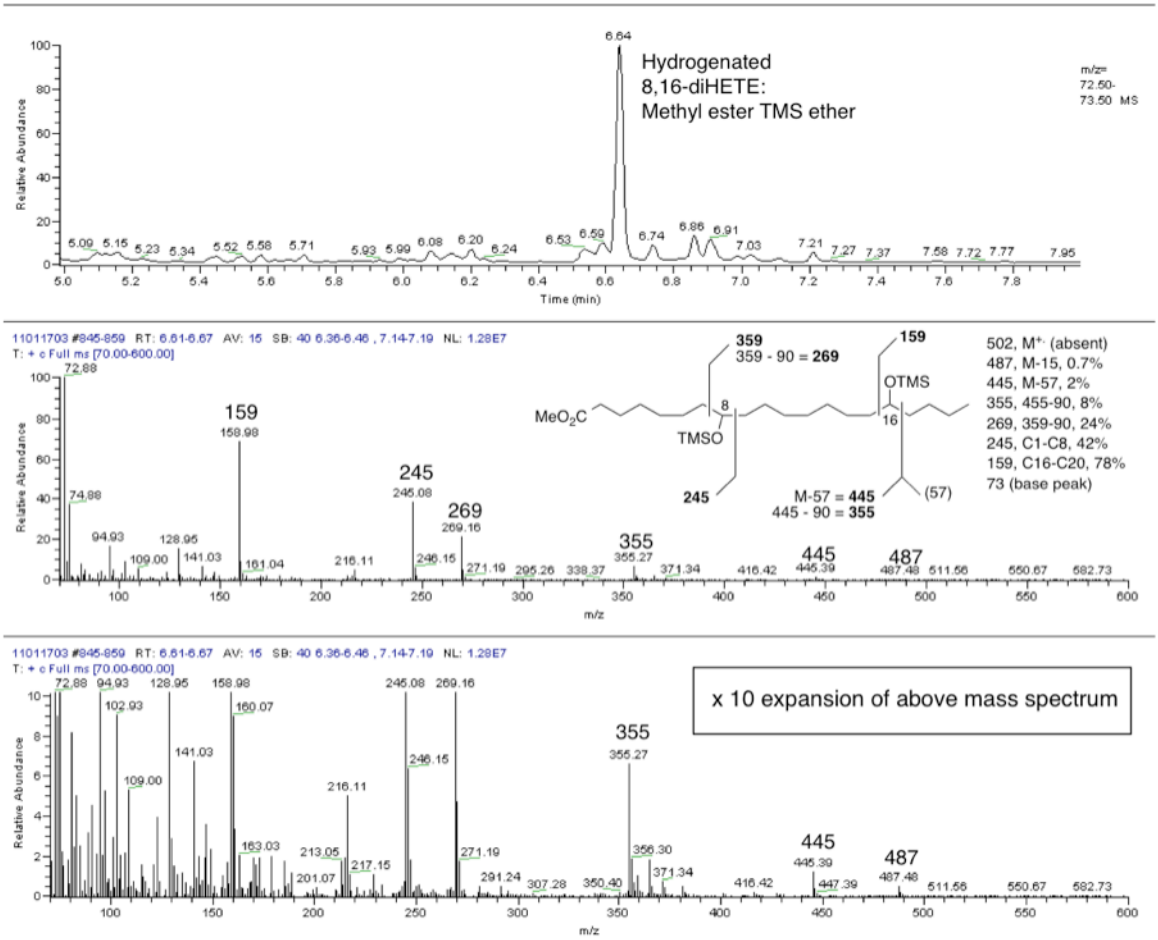


Fig. S6. Identification of 8,16-diHETE, product of 8*S*-HETE with cAOS/PhlO
The peak marked with a dot in Fig S1A, was hydrogenated, converted to the trimethylsilyl ether (TMS) derivative and analyzed by GC-MS. Top: GC profile at m/z 73 (equivalent to any compounds containing TMS). Middle and lower panels: Electron impact mass spectrum. Diagnostic ions are listed in the middle panel and illustrated on the structure.

Fig. S7. Chiral analysis of 5,6-EET (top), product of Arachidonic Acid with cAOS/PhIO
5,6-EET was chromatographed on a Chiralcel OJ column (25 x 0.46 cm) as described (SI ref 1) using a solvent of hexane/isopropanol/glacial acetic acid (100:0.5:0.1, by vol.) at a flow rate of 1 ml/min, with UV detection at 205 nm. Chiral assignment was established under these conditions using synthetic standards (SI ref 1).

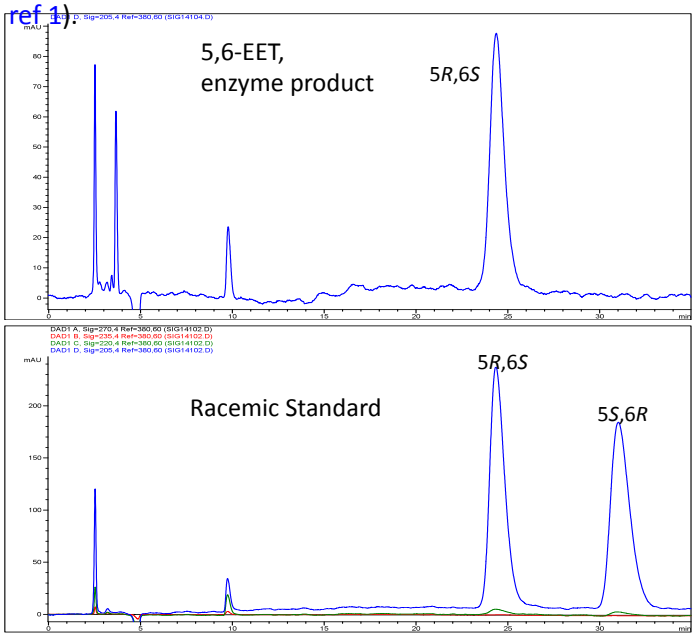


Fig. S8. Chiral analysis of 14,15-EET (top), product of Arachidonic Acid with cAOS/PhIO
14,15-EET was chromatographed on a Chiralcel OJ column (25 x 0.46 cm) as described (SI ref 1) using a solvent of hexane/isopropanol/glacial acetic acid (100:0.5:0.1, by vol.) at a flow rate of 1 ml/ min, with UV detection at 205 nm. Chiral assignment was established under these conditions using synthetic standards (SI ref 1).

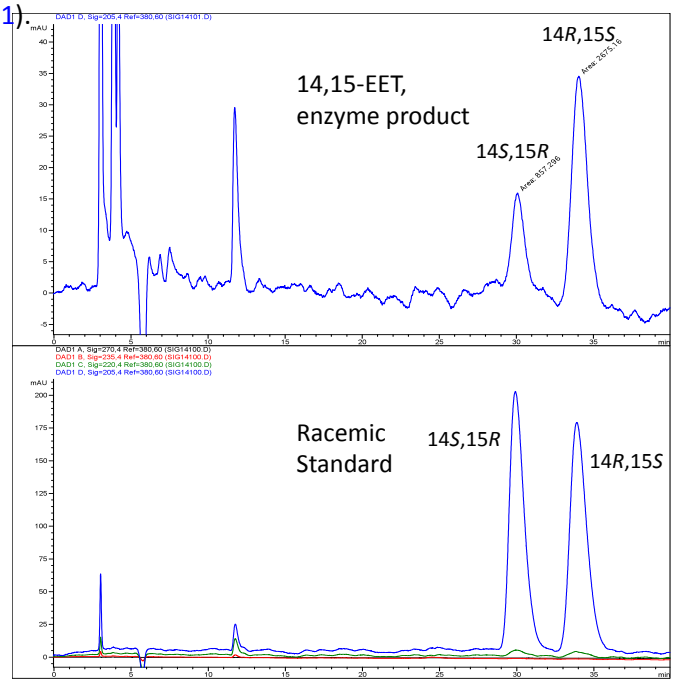
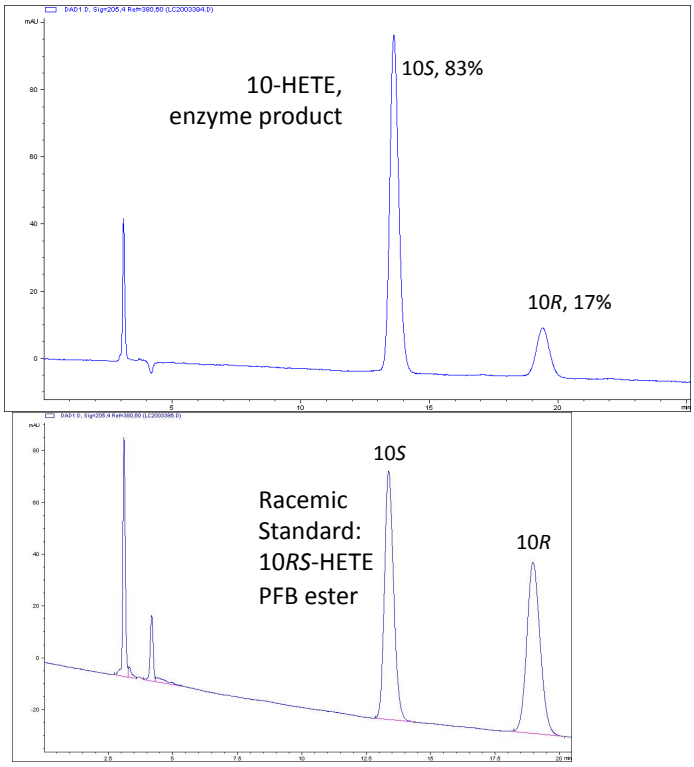


Fig. S9. Chiral analysis of 10-HETE, product of Arachidonic Acid with cAOS/PhIO
10-HETE was converted to the pentafluorobenzyl ester (PFB) and chromatographed on a Chiralpak AD column (25 x 0.46 cm) using a solvent of hexane/methanol (100:2, v/v) at a flow rate of 1 ml/ min, with UV detection at 205 nm. The panel sizes below are adjusted to match retention times on the x-axis.



The enantiomers of 10*RS*-HETE are well resolved by chiral HPLC as the PFB ester, (Supplemental Fig S9, above). Assignment of the enantiomers was established by rearrangement of the earlier-eluting chiral column peak to 8*S*-HETE and 12*R*-HETE, and the later peak to 8*R*-HETE and 12*S*-HETE, on exposure to 0.1% acetic acid. The rearrangement occurs with partial retention of the hydroxyl oxygen (SI ref 2) and is therefore assumed to be suprafacial, hence the assignment of the early eluting peak as 10*S*-HETE. The assignment using this method was confirmed by comparison to 10*S*-HETE prepared by total chemical synthesis (cf. SI ref 2).

NMR data

Product 1, 8*R*-hydroxy-9*R*,10*R*-epoxy-eicosa-5Z,11Z,14Z-trienoate (methyl ester) (The *erythro* 8,9 diastereomer). ^{1}H-NMR, 600 MHz, C_6D_6, 283 K, δ 5.61, dt, 1H, H12, $J_{11,12}$ = 10.9 Hz, $J_{12,13}$ = 7.7 Hz; 5.49, 1H, H6; 5.45 - 5.38, m, 2H, H14, H15; 5.36, m, 1H, H6; 5.155, dd, 1H, H11, $J_{11,12}$ = 10.8 Hz, $J_{10,11}$ = 9.9 Hz ; 3.81, dd, 1H, H10, $J_{9,10}$ = 2 Hz, $J_{10,11}$ = 8.9 Hz; 3.59, m, 1H, H8; 3.33, s, 3H, OCH3; 2.905, m, 2H, H13; 2.75, dd, 1H, H9, $J_{8,9}$ = 3.4 Hz, $J_{9,10}$ = 2 Hz; 2.24, m, 2H, H7; 2.08, t, 2H, H2; 2.00, q, 2H, H16; 1.91, m, 2H, H4; 1.57, p, 2H, H3; 1.34 – 1.21, m, 6H, H17, H18, H19; 0.89, t, 3H, H20.
(In spectra recorded at a temperature of 288 K compared to 283 K, the H10 epoxide signal shifts upfield by ~0.02-0.03 ppm at the higher temperature, from 3.81 to ~3.78 ppm).

Product 2, 8*R*-hydroxy-9*S*,10*S*-epoxy-eicosa-5Z,11Z,14Z-trienoate (methyl ester) (The *threo* 8,9 diastereomer). ^{1}H-NMR, 600 MHz, C_6D_6, 285 K, δ 5.95, dt, 1H, H12, $J_{11,12}$ = 10.9 Hz, $J_{12,13}$ = 7.7 Hz; ~5.46 – 5.35, 3H, H6, H14, H15; 5.34, m, 1H, H5; 5.13, t, 1H, H11, J = 10 Hz; 3.72, dd, 1H, H10, $J_{9,10}$ = 2 Hz, $J_{10,11}$ = 8.9 Hz; 3.405, m, 1H, H8; 3.34, s, 3H, OCH3; 2.88, m, 2H, H13; 2.745, dd, 1H, H9, $J_{8,9}$ = 4.0 Hz, $J_{9,10}$ = 2 Hz; 2.23, m, 2H, H7; 2.06, dt (!), 2H, H2; 1.99, q, 2H, H16; 1.91, m, 2H, H4; 1.70, d, 1H, OH (H8) J = 2 Hz; 1.55, p, 2H, H3; 1.33 – 1.20, m, 6H, H17, H18, H19; 0.89, t, 3H, H20.

8*S*-HETE product: 8*S*,13-dihydroxy-14,15-epoxy-eicosa-5Z,9E,11Z-trienoate (methyl ester) ^{1}H-NMR, 600 MHz, $CDCl_3$, 285 K, δ 6.55, dd, 1H, H10, $J_{10,11}$ = 11.3, $J_{9,10}$ = 15.1 Hz; 6.185, t, 1H, H11, J = 11.1 Hz; 5.84, dd, 1H, H9, $J_{8,9}$ = 5.8, $J_{9,10}$ = 15.1 Hz; 5.54, m, 1H, H5; 5.51, (dd?/m), 1H, H12; 5.42, m, 1H, H6; 4.44, t, 1H, H13, J = 8.1 Hz ; 4.23, m 1H, H8; 3.65, s, 3H, OCH3; 3.035, m, 2H, H14, H15; 2.39-2.27, m 2H, H7,a,b; 2.32, t, 2H, H2; 2.095, q, 2H, H4; 2.065, d, 1H, OH (H13) J = 2 Hz; 1.825, d, 1H, OH (H8) J = 2 Hz; 1.70, p, 2H, H3; ~1.65-1.45, m, H16;1.32-1.23, m, 6H, H17, H18, H19; 0.87, t, 3H, H20.

8*S*-HETE product: 8*S*,13-dihydroxy-eicosa-5Z,9E,11Z,14Z-tetraenoate (methyl ester) ^{1}H-NMR, 600 MHz, $CDCl_3$, 285 K, δ 6.62, dd, 1H, H10, $J_{10,11}$ = 11.3, $J_{9,10}$ = 15.1 Hz; 6.04, t, 1H, H11, J = 11.1 Hz; 5.79, dd, 1H, H9, $J_{8,9}$ = 6.1, $J_{9,10}$ = 15.1 Hz; ~5.56 – 5.40, m, 6H, H5, H6, H12, H13, H14, H15; 4.24, m, 1H, H8; 3.67, s, 3H, OCH3; 2.40-2.27, m 2H, H7,a,b; 2.32, t, 2H, H2; ~2.19 – 2.08, m, 4H, H4, H16; 1.80, d, 1H, OH (H8) J = 4.3 Hz; 1.70, p, 2H, H3; 1.51, d, 1H, OH (H13) J = 2.4 Hz; 1.37, m, 2H, H16; 1.32-1.23, m, 6H, H17, H18, H19; 0.87, t, 3H, H20.

8*S*-HETE product: 8*S*-hydroxy-14,15-epoxy-eicosa-5Z,9E,11Z-trienoate (methyl ester) ^{1}H-NMR, 600 MHz, C_6D_6, 285 K, δ 6.60, dd, 1H, H10, $J_{10,11}$ = 11.2, $J_{9,10}$ = 15.1 Hz; 6.11, t, 1H, H11, J = 11 Hz; 5.62, dd, 1H, H9, $J_{8,9}$ = 5.8, $J_{9,10}$ = 15.2 Hz; 5.46, dt, 1H, H12, $J_{11,12}$ = 10.9 Hz, $J_{12,13}$ = 7.6 Hz; 5.41, m, 1H, H6; 5.35, m, 1H, H5; 4.00, m, 1H, H8; 3.32, s, 3H, OCH3; 2.785, dt, 1H, H14, $J_{13,14}$ = 6.3, $J_{14,15}$ = 4. 1 Hz; 2.675, m, 1H, H15; 2.41, m, 1H, H13a; 2.28, m, 1H, H13b; 2.21, m, 1H, H7a; 2.16, m, 1H, H7b; 2.05, t, 2H, H2; 1.905, q, 2H, H4; 1.55, p, 2H, H3; 1.34, d, 1H, OH (H8), J = 4.4 Hz; ~1.44-1.22, m, 2H, H16; ~1.22-1.16, m, 6H, H17, H18, H19; 0.86, t, 3H, H20.

8*S*-HETE + cAOS + PhIO product (8*S*-hydroxy-11,12-epoxy-eicosa-5Z,9E,14Z-trienoate (methyl ester) ^{1}H-NMR, 600 MHz, C_6D_6, 285 K, δ 5.805, dd, 1H, H9, $J_{8,9}$ = 5.3 Hz, $J_{9,10}$ = 15.5 Hz; 5.67, 1H, H10, $J_{9,10}$ = 15.5 Hz, $J_{10,11}$ = 7.2 Hz; 5.49, m, 2H, H14, H15; 5.35, m, 2H, H5, H6; 3.93, m, 1H, H8; 3.32, s, 3H, OCH3; 3.24, dd, 1H, H11, $J_{10,11}$ = 7 Hz, $J_{11,12}$ = 4 Hz; 2.94, dt, 1H, H12, $J_{11,12}$ = 4 Hz, $J_{12,13}$ = 6.3 Hz; 2.42, m, 1H, H13a; 2.195, m, 1H, H13b; ~2.18 – 2.08, m, 2H, H7a,7b; 2.05, 7, 2H, H2; 1.97, q, 2H, H16; 1.89, q, 2H, H4; 1.55, p, 2H, H3; 1.32 – 1.20, m, 6H, H17, H18, H19; 0.89, t, 3H, H20.

8*S*-HETE + cAOS + PhIO product (8*S*-hydroxy-9*R*,10*R*-epoxy-eicosa-5*Z*,9E,14*Z*-trienoate (methyl ester)
This 8,9-*threo* diastereomer was the only 9,10-epoxide formed by reaction of 8*S*-HETE with cAOS+PhIO (Fig 5, main text). It is the enantiomer of the 8*R*-HETE *threo* product, with an identical NMR spectrum.

mCPBA products from 8*R*-HETE
As the methyl esters, these eluted on RP-HPLC (MeOH/H_2O,) as:
RP1 (products a_1 and a_2) - a mixture of 14,15-epoxy diastereomers
RP2/RP3 (products b_1 and b_2) - two 11,12-epoxy diastereomers, partly resolved by RP-HPLC
RP4/RP5 (products c_1 and c_2) - two 5,6-epoxy diastereomers, partly resolved by RP-HPLC
RP6 (Products 1 and 2) - the *erythro* and *threo* 9,10-epoxy diastereomers

The pairs of diastereomers were well resolved using a Chiralpak AD column (25 x 0.46 cm) with a solvent system of Hexane/EtOH/MeOH (100:5:5, v/v/v), flow rate 1 ml/min.
Retention volumes:
RP1 20.1 ml and 42.6 ml (8-hydroxy, 14,15-epoxy)
RP2 11.6 ml (8-hydroxy, 11,12-epoxy)
RP3 6.6 ml (8-hydroxy, 11,12-epoxy)
RP4 14.9 ml (8-hydroxy, 5,6-epoxy)
RP5 10.2 ml (8-hydroxy, 5,6-epoxy)
RP6 9.4 ml (*threo*), 12.8 ml (*erythro*) 8-hydroxy, 9,10-epoxy

Fig. 2B (main text) products $a_{1,2}$: two mCPBA products, diastereomers of 8*R*-hydroxy-14,15-epoxy-eicosa-5Z,9E,11Z-trienoate methyl ester (NMR data not recorded – but see 8*S*-HETE product, a 14,15-epoxide)

Fig. 2B (main text) products $b_{1,2}$: two mCPBA products, diastereomers of 8*R*-hydroxy-11,12-epoxy-eicosa-5Z,9E,14Z-trienoate methyl ester These had indistinguishable NMR spectra from each other and from the 11,12-epoxy product from 8*S*-HETE + cAOS + PhIO.

Fig. 2B (main text) product c_1: mCPBA product, 5,6-epoxy-8*R*-hydroxy-eicosa-9E,11Z,14Z-trienoate methyl ester
(the **first diastereomer** to elute, as the methyl ester, on RP-HPLC using MeOH/H_2O solvent)
^{1}H-NMR, 600 MHz, C_6D_6, δ 6.765, dd, 1H, H10, $J_{9,10}$ = 15.1 Hz, $J_{10,11}$ = 11.2 Hz; 6.085, t, 1H, H11, J = 11.1 Hz; 5.625, dd, 1H, H9, $J_{9,10}$ = 15.1 Hz, $J_{8,9}$ = 6.1; 5.50-5.42, m, 6H, H12, H14, H15; 4.285, q, 1H, H8, J = 6 Hz; 3.30, s, 3H, OCH3; 2.98, t, 2H, H13, J = 6 Hz; 2.80, dt, 1H, H6, $J_{5,6}$ = 4.2 Hz, $J_{6,7}$ = 6.2 Hz ; 2.52, dt, 1H, H5, $J_{4,5}$ = 6.2 Hz, $J_{5,6}$ = 4.2 Hz; ~2.07-1.97, m, 4H, H2, H16; 1.58 -1.52, m, 4H, H3, H7; 1.31, m, H4; ~1.28 – 1.20, m, 6H, H17, H18, H19; 0.89, t, 3H, H20.

Fig. 2B (main text) product c_2: mCPBA product, 5,6-epoxy-8*R*-hydroxy-eicosa-9E,11Z,14Z-trienoate methyl ester
(the **second diastereomer** to elute, as the methyl ester, on RP-HPLC using MeOH/H_2O solvent). There are significant differences in the chemical shifts compared to the first diastereomer, e.g. on the protons in the conjugated diene, and especially noticeable is the more downfield position of H6.
^{1}H-NMR, 600 MHz, C_6D_6, 285 K, δ 6.65, dd, 1H, H10, $J_{9,10}$ = 15 Hz, $J_{10,11}$ = 11 Hz; 6.04, t, 1H, H11, J = 10.9 Hz; 5.585, dd, 1H, H9, $J_{9,10}$ = 15.1 Hz, $J_{8,9}$ = 6.0; 5.46, m, 6H, H12, H14, H15; 4.21, m, 1H, H8; 3.30, s, 3H, OCH3; 3.02, m (ddd?), 1H, H6; 2.97, t, 2H, H13, J = 7.5 Hz; 2.61, dt, 1H, H5, $J_{4,5}$ = 6.2 Hz, $J_{5,6}$ = 4.2 Hz; ~2.09-1.99, m, 4H, H2, H16; 1.595, m, 3H, H3, H7a; 1.505, ddd, H7b; ~1.34 – 1.20, m, 8H, H4, H17, H18, H19; 0.89, t, 3H, H20.

Fig. 2B (main text) products 1 and 2: two mCPBA products, diastereomers of 8*R*-hydroxy-9,10-epoxy-eicosa-5Z,9E,14Z-trienoate methyl ester NMR data given above as Products **1** and **2**.

SI References

1. Wei S*, et al.* (2006) Chiral resolution of the epoxyeicosatrienoic acids, arachidonic acid epoxygenase metabolites. *Anal. Biochem.* 352:129-134.

2. Brash AR, Boeglin WE, Capdevila JH, Suresh Y, Blair IA (1995) 7-HETE, 10-HETE, and 13-HETE are major products of NADPH-dependent metabolism of arachidonic acid in rat liver microsomes. Analysis of their stereochemistry and the stereochemistry of their acid-catalyzed rearrangement. *Arch. Biochem. Biophys.* 321:485-492.