

Stereospecific synthesis and mass spectrometry of 5,6-*trans*-epoxy-8*Z*,11*Z*,14*Z*-eicosatrienoic acid

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Abstract—A novel, facile synthesis of 5,6-*trans*-epoxyeicosatrienoic acid (5,6-*trans*-EET) from 5,6-*trans*-arachidonic acid by iodolactonization and alkaline de-iodation is described along with characterization by mass spectrometry (LC–MS, negative ions) and NMR and comparison with 5,6-*cis*-EET.

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1. Introduction

A major product formed by the reaction of arachidonic acid (AA) with biologically relevant reactive nitrogen oxides, such as $\cdot\text{NO}_2$, is a mixture of four *trans*-AA isomers having three cis and one nonconjugated trans bond.^{1–4} We have found that *trans*-AA isomers occur in circulation of humans³ and rats.⁴ Detection of *trans*-AA and other recently identified lipids having a nitro group such as vicinal nitrohydroxy-eicosatrienoic acids⁵ and nitrolinoleic acids^{6,7} suggests that biological nitration of polyunsaturated fatty acids (FA) occurs in vivo.¹ We hypothesized that the *trans*-AA isomers function as specific mediators/markers of the $\cdot\text{NO}_2$ radical-dependent modifications of AA in cellular lipids.¹ This is supported by observations showing that the levels of circulating *trans*-AA are higher in inflammation,⁴ hyperoxia,⁸ and in plasma of cigarette smokers.⁹ In order to further characterize the mediator role of *trans*-AA isomers, we became interested in various aspects of their metabolism. Our previous work detected four vicinal dihydroxy lipids from the microsomal oxidation of the radiolabeled 14,15-*trans*-AA.¹⁰ This suggested that the biosynthesis of an epoxide having the trans configuration was possible by a microsomal cytochrome P450 epoxygenase. In the current study, we synthesized 5,6-*trans*-EET and compared the LC–MS and GC–MS properties with its cis isomer that was synthesized by

the same protocol. Synthetic 5,6-*trans*-EET will be used as a standard for metabolic studies with its precursor, 5,6-*trans*-AA.

2. Materials and methods

2.1. Chemicals

KI, I_2 , KHCO_3 , tetrahydrofuran (stabilizer free), LiOH, HCl, *meta*-chloroperoxybenzoic acid, and acetic acid were from Sigma–Aldrich. Hexane and acetonitrile of HPLC grade were from Fisher. Arachidonic acid (purity >99%) was from Sigma. 5,6,8,9,11,12,14,15-Octadeutero-eicostatetraenoic acid (d_8 -AA) (isotopic purity >98%) and 5,6-*cis*-EET standard were obtained from Cayman Chemicals. 5,6-*trans*-AA prepared as described previously,¹¹ free from its cis isomer, that is, arachidonic acid, was a generous gift from Dr. J. R. Falck (University of Texas). 2,3-*cis*-Epoxybutane and 2,3-*trans*-epoxybutane were from Aldrich.

2.2. Preparation of 5,6-*trans*-EET

A method developed by Corey et al.¹² was used by us with modifications¹³ to prepare 5,6-*trans*-EET, 5,6-*cis*-EET, and d_8 -5,6-*cis*-EET from 5,6-*trans*-AA, AA, and d_8 -AA, respectively. Briefly, either 5,6-*trans*-AA or AA/ d_8 -AA (1 mg) was mixed with 0.8 mL of a solution containing potassium iodide (8 mM), iodine (8 mM), and KHCO_3 (5 mM) in $\text{THF-H}_2\text{O}$ (2:1, v/v), and the mixture was stirred for 3–72 h at 4–70 °C (Table 1). A solution of $\text{Na}_2\text{S}_2\text{O}_3$ (0.1 M, 0.2 mL) was added in order

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Table 1. Comparison of conditions and yields for preparation of 5,6-*trans*-EET and 5,6-*cis*-EET^a

Reaction temperature (°C)	Reaction time (h)	Yield (%)	
		5,6- <i>trans</i> -EET	5,6- <i>cis</i> -EET
4	3	6.8	95.8
4	72	2.7	NA
37	48	13.8	49.9
37	72	12.6	NA
70	48	27.8	NA
70	72	22.7	NA

^a The epoxides were prepared via iodolactonization of 5,6-*trans*-AA and AA with KI₃ and KHCO₃ in THF/water for times and temperatures indicated. The iodolactone was converted to the epoxide by treatment with LiOH as described in Section 2. The yields were established by analysis of the reaction products by HPLC via integration of peak areas (detected as absorbance at 205 nm) corresponding to the epoxide and the fatty acid precursor (NA = not analyzed).

to terminate the reaction and bleach the excess iodine. The iodolactone was then extracted with hexane and the extract was dried under nitrogen. The residue was mixed with 1.5 mL of LiOH solution (0.2 M in H₂O–THF 1:2 v/v) and stirred for 3 h at 25 °C. The solution was neutralized with equimolar amount of HCl and then slowly acidified to pH 4 with acetic acid. The lipids were extracted with ethyl acetate, the extracts were washed with water and dried with anhydrous sodium carbonate, and finally the solvent was evaporated under nitrogen. The lipid residue was dissolved in acetonitrile and the epoxides were isolated in a pure form by HPLC. The synthetic epoxides (5,6-*trans*-AA and 5,6-*cis*-AA) were purified using a Beckman Ultrasphere column (250 × 10 mm, ODS 5 µm, flow 2 mL/min). The lipids were eluted with a gradient starting with 50% solution A (water–acetonitrile–acetic acid 75:25:0.05) and 50% solution B (acetonitrile–acetic acid 99.95:0.05) and changed to 25% solution A and 75% solution B within 15 min. After 40 min the solvent was changed to 100% solution B in 5 min and was maintained for an additional 5 min. The epoxides eluted in fractions at 27–30 min and were subjected to further analysis by LC–MS and GC–MS.

2.3. Mass spectrometry

LC–MS analysis was performed on a triple quadrupole mass spectrometer model API 2000 (AB Sciex Instruments) interfaced with a HP1100 HPLC system (Agilent). Negative ions were generated with a turbo-ion spray source having a temperature of 450 °C. Typical parameters of the mass spectrometer system were as follows: ion-spray potential, –4200 V; CAD potential, 6 V; deconvolution potential, –6 V; and capillary exit potential, –16.5 V. The MS/MS spectra were acquired by using the collision energy from –27 to –40 V with nitrogen in the quadrupole 2, which resulted in the decrease of the precursor ion intensity by about 50%. The lipid solutions were prepared in microvials and injected using an autoinjector. Lipids were analyzed on a Phenomenex Luna C18 column (150 × 2 mm, 5 µm) having a 10 mm precolumn. The samples were eluted with a solution composed of

water–acetonitrile–acetic acid (37.5:62.5:0.01 v/v/v) at a rate of 300 µL/min. UV spectra were acquired by an on-line diode-array detector (HP1100) set to scan the wavelength range of 200–400 nm/s. In some experiments, the effluent from the HPLC column was fractionated using a Gilson fraction collector to collect 1 min fractions for additional analysis by GC–MS.

GC–MS analysis was performed on a HP5973 system (Agilent) operating in negative ion chemical ionization mode (methane, 40 psi). The source and transfer line temperatures were 250 and 280 °C, respectively. The samples were analyzed on a HP-5MS column (30 m, 0.25 mm i.d., 0.25 µm film thickness). The initial temperature of the column (150 °C) was maintained for 1 min and then increased to 280 °C at a rate of 40 °C/min. The injector having a temperature of 280 °C was used in a splitless mode. Helium was used as a carrier gas at the pressure of 29.57 psi, delivering the total flow of 30.1 mL/min resulting in the column flow of 1.3 mL/min. Samples for GC–MS analysis were derivatized as pentafluorobenzyl (PFB) esters and trimethylsilyl (TMS) ethers as described¹³ and were injected in isooc-tane (1 µL).

2.4. NMR

The ¹H NMR (500 MHz) spectra were obtained on a DMX 500 instrument (Bruker) in CDCl₃ (1 mg/mL). 5,6-*trans*-EET: δ 0.91 (t, *J* = 7.0 Hz, 3H), 1.24–1.38 (m, 6H), 1.44–1.62 (m, 3H), 1.88–1.89 (m, 2H), 2.29 (m, 2H), 2.38 (m, 3H), 2.82–2.87 (m, 2H), 2.77 (q, *J* = 7.0 Hz, 4H), 5.35–5.41 (m, 5H), and 5.49–5.56 (m, 1H). 5,6-*cis*-EET: δ 0.91 (t, *J* = 7.0 Hz, 3H), 1.28–1.39 (m, 6H), 1.54–1.59 (m, 3H), 1.72–1.89 (m, 2H), 2.03–2.1 (m, 2H), 2.34–2.44 (m, 3H), 2.82–2.85 (t, 2H), 2.87–2.89 (m, 4H), 5.33–5.50 (m, 5H), and 5.58–5.63 (m, 1H).

3. Results

3.1. Synthesis and purification of 5,6-*trans*-EET

The iodolactonization of 5,6-*trans*-AA followed by alkaline de-iodation and opening of the lactone ring with simultaneous epoxidation yielded a single product, 5,6-*trans*-epoxyeicosatrienoic acid (5,6-*trans*-EET). As expected, the *trans* C5–C6 bond created unfavorable stereochemistry for the iodolactonization/de-iodation compared with the *cis* C5–C6 bond in AA. This stereochemical factor caused the differences in the reaction yields observed under similar conditions (Table 1). Increasing of reaction time and temperature favored formation of the *trans* isomer, whereas such trend decreased the yields of the *cis* isomer. The optimal yield of the 5,6-*trans*-EET achieved was ~ 30% following iodolactonization at 70 °C for 48 h (Table 1). The 5,6-*trans*-EET eluted as a single peak of absorbance at 205 nm from the HPLC column at 27.5 min and was well separated from the 5,6-*trans*-AA (retention time 46 min). We tested this procedure and obtained milligram amounts of 5,6-*trans*-EET, some of which was used as a standard for identification of microsomal metabolites.

3.2. Mass spectrometric and chromatographic properties of 5,6-*trans*-EET

5,6-*trans*-EET showed a similar fragmentation pattern as 5,6-*cis*-EET following collisional activation of the carboxylate molecular anion at m/z 319 in electrospray tandem mass spectrometry; however, minor fragments at m/z 71, 87, 97, 110, 125, 129, and 143 could be used to distinguish between these two isomers (Fig. 1). The spectra of 5,6-*cis*-EET and 5,6-*trans*-EET were similar to the mass spectra published,^{14,15} however, minor differences were noted. Fragment ions at m/z 301, 303, 275, and 257 corresponded to the loss of neutral molecules: H₂O, O, CO₂, and CO₂ + H₂O, respectively, and

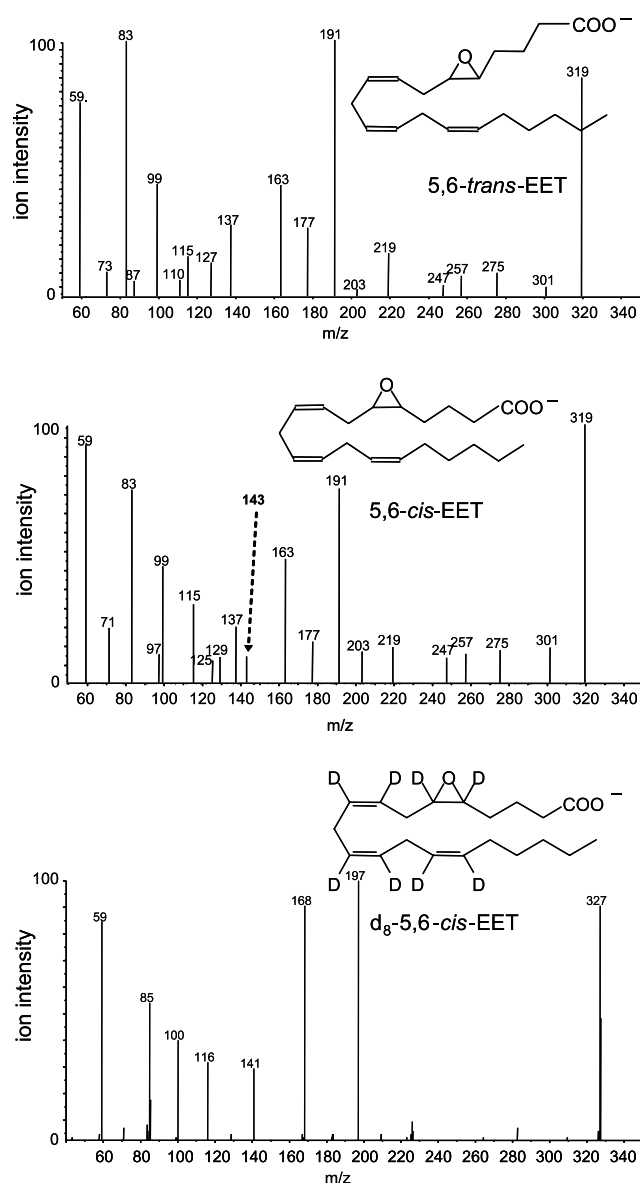


Figure 1. Comparison of tandem mass spectra obtained by collisional activation at -30 V of the carboxylic anion at m/z 319 generated from 5,6-*trans*-EET (top), 5,6-*cis*-EET (middle) and of ion m/z 327 from octadeuterium-labeled 5,6-*cis*-EET (bottom), which were prepared by iodolactonization of 5,6-*trans*-AA, AA, and *d*₈-AA, respectively. The spectra were averaged and background noise was subtracted.

were typical for the fatty acid epoxides.¹⁶ The spectra also showed fragment ions resulting from carbon bonds cleavages (Fig. 2). Ions at m/z 99 and 115 originated from the cleavages of the epoxide ring (Fig. 2). A prominent fragment ion at m/z 191 likely originated from the cleavage of the C6–C7 bond following intramolecular lactonization by the carboxyl anion and opening of the epoxide ring (Fig. 3). The nucleophilic attack of the carboxyl oxygen of ion m/z 319 on carbon C5 caused opening of the epoxide ring and formation of the hydroxy anion lactone (Fig. 3), which rearranged with a cleavage of the C6–C7 bond to ion m/z 191 and an aldehyde (tetrahydro-2-pyrone-5-carbaldehyde).

High abundance of the fragment anion at m/z 191 was likely to result from a partial stabilization of the alkyl negative charge by the conjugated double bond (Fig. 3). Formation of ion m/z 191 from 5,6-*trans*-EET anion appeared to be favored because its intensity was about 10–20% higher than in the spectrum of 5,6-*cis*-EET at the same collision energy.

The fragmentation pattern of *d*₈-5,6-*cis*-EET was similar to that of unlabeled EET isomers but several fragments were shifted to higher masses which revealed the number of deuterium in a given ion (Fig. 1). For example, a fragment at m/z 191 was found at m/z 197 suggesting that it had 6D atoms, consistent with a structure shown in Figure 4. Similar reasoning led to structure assignment for ions at m/z 163 and 141 (Fig. 4). Also, ions generated by the cleavage of the epoxy ring (m/z 99 and 115) were

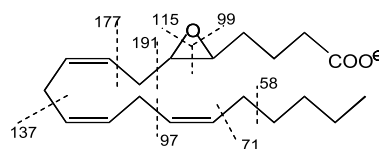


Figure 2. Fragmentation pattern of 5,6-*trans*-EET carboxylate anion resulting from collisional activation of ion m/z 319 generating a spectrum shown in Figure 1.

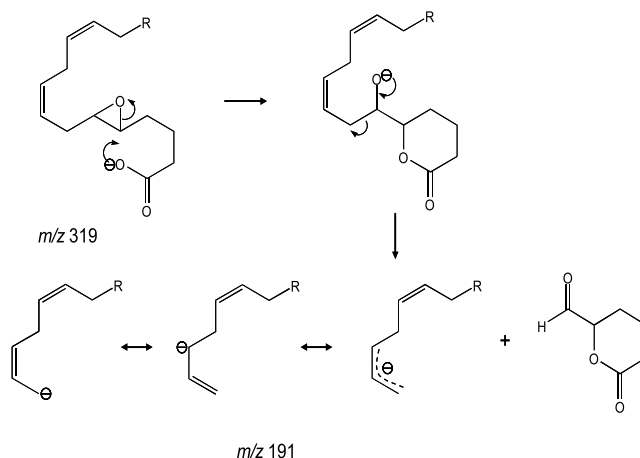


Figure 3. Fragmentation pattern explaining origins of ions m/z 191 from the collisionally activated dissociation of the molecular 5,6-EET carboxylate anion at m/z 319. R = C₁₀H₁₇.

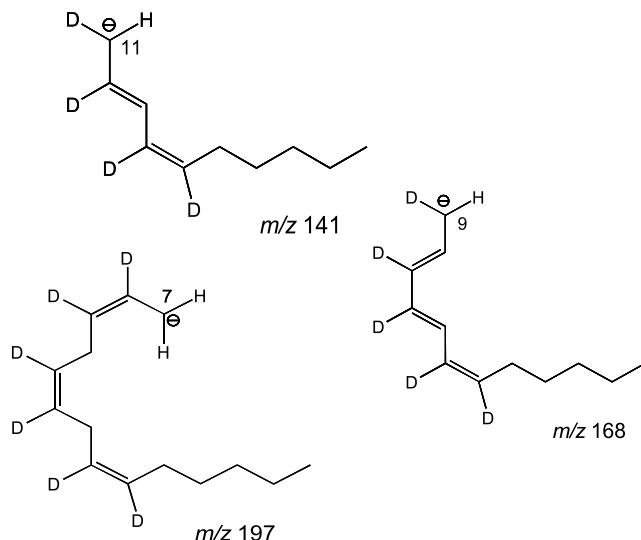


Figure 4. Structures of three major fragment ions observed in the spectrum of *d*₈-5,6-*cis*-EET (Fig. 1). The structures are hypothetical and are likely to involve other resonance structures. Corresponding ions in the spectrum of 5,6-*cis*-EET are found at *m/z* 137, 163, and 191 suggesting that the ions had four, five, and six deuterium, respectively.

found at *m/z* 100 and 116, respectively, consistent with presence of a single deuterium.

The fragmentation of both isomers of 5,6-EET appeared to be driven by the specific ability of the carboxyl anion to form an internal lactone resulting in formation of a

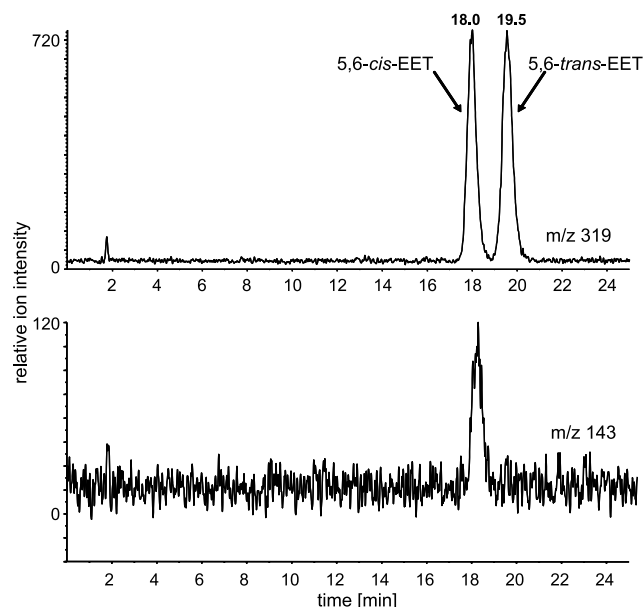


Figure 5. LC–MS analysis showing separation of *cis* and *trans* isomers of 5,6-EET with selected acquisition of ions at *m/z* 319 and 143. The latter appears only in the *cis* isomer. Lipids were analyzed on a Phenomenex Luna C18 column (150 × 2 mm) and eluted with a solution composed of water–acetonitrile–acetic acid (37.5:62.5:0.01 v/v/v) at a rate of 300 μL/min. Lipids were ionized and detected by the API 2000 mass spectrometer operating in a turbo-ion spray negative mode.

Table 2. Comparison of NMR shifts (δ , ppm) of *cis*- and *trans*-epoxides

Epoxide	$\text{RHC} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CHR} \end{array}$ (ppm)
5,6- <i>trans</i> -EET	2.84–2.88, m
5,6- <i>cis</i> -EET	2.82–2.87, m
2,3- <i>trans</i> -EB	3.80, q
2,3- <i>cis</i> -EB	3.02, o

EET—epoxyeicosatrienoic acid; EB—epoxybutane; m—multiplet; o—octet; q—quartet; R: CH₃; C₁₄H₂₃ and C₃H₆COOH.

hydroxyl anion (Fig. 3). Such anion can further abstract other methylene hydrogens via a remote charge abstraction mechanism.¹⁷ One such process is likely to form ion at *m/z* 143. This ion appears to be formed via a unique mechanism that is only possible for the *cis* isomer. We also found that this is a functional fragment that can be selectively monitored during LC–MS analysis to confirm the identity of 5,6-*cis*-EET, which was readily separated from 5,6-*trans*-EET by HPLC (Fig. 5). Such complete separation was not possible with GC–MS analysis of PFB esters in which *trans* isomer eluted before the *cis* (not shown).

3.3. NMR spectroscopy

The NMR (500 MHz) spectra were acquired for isomers of 5,6-EET and, for comparative purposes, for isomers of 2,3-epoxy-butane (Table 2). The spectra revealed that the protons at the epoxy group in the *trans*-epoxides were shifted upfield by δ 0.10–0.67 ppm relative to the *cis*-epoxides. The octet at 3.02 ppm for epoxy protons in 2,3-*cis*-epoxy-butane resulted from the fine splitting by the neighboring β *cis*-proton at the epoxy group. In 2,3-*trans*-epoxy-butane, a signal at 3.80 ppm was a quartet suggesting that a long range split from the other epoxy proton was not possible because of its *trans* configuration.

4. Discussion

trans-AA are products of *cis*–*trans* isomerization of AA induced by $\cdot\text{NO}_2$ and N_2O_3 ² and are found in vivo.^{3,4} Inflammation⁴ and other conditions^{8,9} increase the levels of *trans*-AA in circulation; therefore, these AA isomers are likely to function as specific lipid mediators of endogenous AA nitration.¹ *trans*-AA are unique *trans*-fatty acids that contain a nonconjugated *trans* bond among the three *cis* bonds, thus can be used as model compounds to uncover the function of a *trans* bond in oxidative metabolism.

Our previous work found that the *trans*-AA are good substrates for the CYP450/NADPH enzyme system in the hepatic microsomes and we proposed a mechanism by which this system epoxidized fatty acids having both *cis* and *trans* bonds.¹⁰ We established that the *trans* bond competes for microsomal oxidative metabolism and is oxidized to a unique epoxide having *trans* configuration.^{10,18} Such metabolic structural studies typically

generate limited amounts of metabolites, and usually rely on mass spectrometric analysis. The problem with such approach is that because mass spectra of *cis* and *trans* isomers of various oxidized lipids are very similar, it is often difficult to assign configuration based on interpretation of their mass spectra. NMR spectroscopy requires multimilligram amounts of lipids and thus is of limited application in such studies. One solution is to compare the properties of the biological material with a synthetic standard. In this study we synthesized a *trans* isomer of a known endogenous epoxide, 5,6-*cis*-EET,¹⁶ to obtain a standard for definitive identification of a unique epoxide generated by microsomal metabolism of 5,6-*trans*-AA.

An interesting aspect of our work is that the mass spectra of these two epoxides obtained by the electrospray (turbo-ion spray) ionization and collisionally-induced decomposition of the carboxylate anions showed subtle differences. We were able to distinguish these two isomers not only via HPLC separation but also by identifying specific fragment ions, such as ion at *m/z* 143, which is specific for the *cis* isomer. Additional comparison with the deuterium-labeled analog revealed that deuterium had other effects on fragmentation and some ions analogous to those of the unlabeled epoxide were absent, possibly reflecting isotopic effects in the collisionally-induced decomposition. This may explain why we were unable to detect an ion analogous to an ion at *m/z* 143 in the spectrum of *d*₈-5,6-*cis*-EET although it was reproducibly detected in many analyses of 5,6-*cis*-EET.

A peroxidation mechanism has also been known to generate fatty acid epoxides. For example, epoxides of linoleic acid and the EETs have been detected in the lungs¹⁹ of rats inhaling NO₂ and in erythrocytes treated with a peroxide.¹⁴ Peroxidative epoxidation of *trans*-AA could be an alternative mechanism by which *trans*-EET isomers are formed. Retention of the olefin stereochemistry in the epoxide requires *cis* addition of the oxygen to the two ends of the double bond.²⁰ Epoxidation of model olefins having a single *trans* double bond by chemical methods using various chemical approaches with peroxy acids or peroxides generates epoxides of *trans* configuration. Many olefinic products with a *trans* double bond are also substrates for enzymatic CYP450-mediated epoxidation, which generates epoxides having *trans* configuration. For example, hepatic microsomes metabolize *trans*-stilbene into diphenyloxirane and *trans*-linoleic acids into epoxylinoleic acid with the retention of configuration²¹ although scrambling of the stereochemistry is possible in rare situations.²⁰ Thus, available evidence suggests that fatty acid epoxides of *trans* configuration should originate from metabolism of corresponding *trans*-fatty acid precursors either by CYP450 epoxigenase or by peroxidative autoepoxidation. *trans*-Epoxides are unlikely to be formed from the all *cis*-fatty acid precursors. The biosynthesis of 5,6-*trans*-EET, which has been detected in vivo,¹⁵ is likely to occur via epoxidation of endogenous 5,6-*trans*-AA.³ We are pursuing characterization of this process in vivo.

Our recent results show that in contrast to its *cis* isomer, 5,6-*trans*-EET is a remarkably stable epoxide that resists hydrolysis and conjugation by liver microsomal epoxide hydrolase and glutathione transferase, respectively.¹⁸ It remains to be established whether *trans*-epoxides of other *trans*-FA would show similar stability. Human diet contains significant amount of *trans*-FA and we suggested that epoxidation could be one of the mechanisms of their activation.¹ While concerns regarding health effects of dietary *trans*-FA have been raised, considerably less is known of *trans*- than of *cis*-fatty acid epoxides. *trans*-Epoxides may convey toxicity of dietary as well as endogenously formed *trans*-FA.

Acknowledgments

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