Chemical Research in Toxicology

APRIL 2000
VOLUME 13, NUMBER 4



# Articles

## Toxicity of Epoxy Fatty Acids and Related Compounds to Cells Expressing Human Soluble Epoxide Hydrolase

Jessica F. Greene, John W. Newman, Kristin C. Williamson, and Bruce D. Hammock*

*Department of Entomology, University of California at Davis, Davis, California 95616*

*Received September 16, 1999*

Soluble epoxide hydrolase (sEH) is suggested to alter the mode of action and increase the toxic potency of fatty acid epoxides. To characterize the structural features necessary for sEH-dependent epoxy fatty acid toxicity, 75 aliphatic compounds were assayed for cytotoxicity in the presence and absence of sEH. Three groups of aliphatic epoxide−diol pairs were described by their observed differential toxicity. Group I compounds were typified by terminal epoxides whose toxicity was reduced in the presence of sEH. Group II compounds were toxic in either their epoxide or diol form, but toxicity was unaffected by sEH. Group III compounds exhibited sEH-dependent toxicity and were therefore used to investigate the structural elements required for cytotoxicity in this study. The optimal structure for group III compounds appeared to be a fatty acid 18−20 atoms long (e.g., a carbon backbone plus a terminal heteroatom) with an epoxide positioned between C-7 and C-12. In the absence of sEH, replacement of epoxides with a *vicinal* diol was required for toxicity. While diol stereochemistry was unimportant, vicinal diol-induced toxicity exhibited fewer positional constraints to toxicity than sEH-dependent epoxide toxicity. Tested fatty acids and esters with neither an epoxide nor a vicinal diol were not toxic. These data support the hypothesis that long-chain epoxy fatty acid methyl esters are potential pro-toxins metabolized by sEH to more toxic diols. Furthermore, our results suggest that the endogenous compounds, leukotoxin methyl ester, 9,10(*Z*)-epoxyoctadec-12-(*Z*)-enoic acid methyl ester, and isoleukotoxin methyl ester, 12,13(*Z*)-epoxyoctadec-9(*Z*)-enoic acid methyl ester, are structurally optimized to elicit the observed effect.

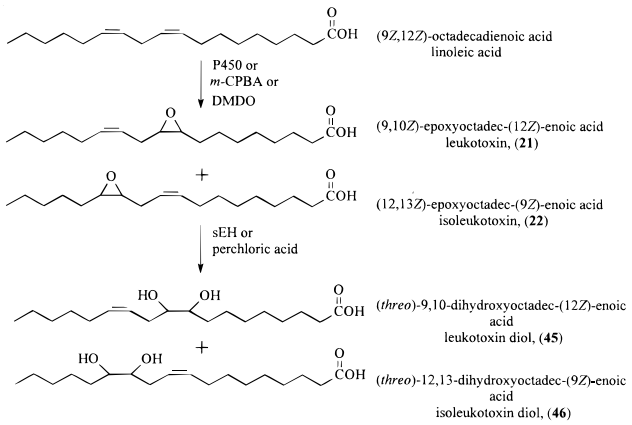
### Introduction

Leukotoxin, 9,10(*Z*)-epoxyoctadec-12(*Z*)-enoic acid (ltx,[1] **21**), and isoleukotoxin, 12,13(*Z*)-epoxyoctadec-9(*Z*)-enoic acid (iltx, **22**), are epoxy fatty acids associated with acute respiratory distress syndrome (ARDS) (*1*, *2*). It has been suggested that ltx either activates nitric oxide synthase, resulting in pulmonary edema, or causes lung injury and nitric oxide synthase is a marker for that lung injury (*3*). Jia-Ning et al. treated rats with ltx (iv at 200 and 500 $\mu$mol of ltx/kg of body weight) and after 10 min observed severe histological changes, including intravascular congestion and coagulation and alveolar exudation, edema,

* To whom correspondence should be addressed: Department of Entomology, University of California at Davis, Davis, CA 95616. Phone: (530) 752-7519. E-mail: bdhammock@ucdavis.edu.

10.1021/tx990162c CCC: $19.00 
Published on Web 03/10/2000

**Scheme 1. Proposed Pathway for Formation of Leukotoxin Diol and Isoleukotoxin Diol**




hemorrhage, and emphysema (*4*). These changes are similar to those seen in human patients suffering from ARDS (*4*, *5*). This is particularly interesting since high levels of circulating ltx (11.4−37 $\mu$M serum) were associated with toxicity in patients suffering from multiple-organ failure, following recovery from the primary shock of severe burns (>50% body surface). The patients also displayed pulmonary edema, cardiac failure, and coagulation abnormalities (*1*, *2*). Ltx and iltx have also been shown to inhibit mitochondrial respiration and relax smooth muscle in a dose-dependent manner in vitro (*6*, *7*), as well as to decrease cardiac function in dogs (*8*).

Soluble epoxide hydrolase (sEH) is a ubiquitous enzyme with a high $v_{max}$ and a low $K_m$ for fatty acid epoxides[2] (*9*). The dependence of ltx and iltx toxicity on sEH activation has been investigated in numerous model cell and mammalian systems (*10*). We have previously hypothesized that the observed toxicity is due to the metabolism of ltx and iltx to their corresponding diols, leukotoxin diol, *threo*-9,10-dihydroxyoctadec-12(*Z*)-enoic acid (ltxd, **45**), and isoleukotoxin diol, *threo*-12,13-dihydroxyoctadec-9(*Z*)-enoic acid (iltxd, **46**) (Scheme 1). These diols were synthesized as regioisomeric mixtures and found to be toxic whether or not functional sEH was present[3,4] (*10*, *11*).

The exacerbation of cytotoxicity by epoxide hydrolysis suggested a conspicuous diol dependence of the observed activity. To further probe a hypothetical "diol-specific" mechanism of toxicity, 75 aliphatic epoxides, including various fatty acids and some lipid analogues, were purchased or synthesized and the toxicities assayed. Herein, we describe our findings with respect to the structural features of aliphatic epoxides required for sEH-dependent toxicity to cells.

## Experimental Procedures

**Chemicals.** Fatty acids were purchased from Sigma Chemical Co. (St. Louis, MO), NuChek Prep (Elysian, MN), or Cayman Chemical (Ann Arbor, MI). All parent fatty acids and analogues were purchased, as was 9-hydroxyoctadec-12(*Z*)-enoic acid methyl ester. All other compounds were synthesized. Chemical reagents and solvents were purchased from Sigma Chemical Co. or Aldrich Chemical Co. (Milwaukee, WI), unless otherwise noted.

**Chemical Synthesis, Purity, and Structural Confirmation.** Racemic mixtures of epoxy and *vic*-diol fatty acids, esters, and analogues were synthesized as previously described (*10*, *12*−*15*). Briefly, olefins were epoxidized with *m*-chloroperoxybenzoic acid (*m*-CPBA) or dimethyldioxirane (DMDO), and purified epoxides were hydrolyzed to the corresponding *vic*-diols in a 1:1 (v/v) acetonitrile/5% aqueous perchloric acid mixture. Synthesized compounds were purified by nitrogen-pressurized flash chromatography on silica gel using equal volume step gradients of hexane/ethyl acetate mixtures (typically 5, 10, and 20% ethyl acetate). Fractionation was monitored by phosphomolybdic acid visualization of aliquots applied to silica gel TLC plates. Similar fractions were pooled, and the solvent was evaporated. All synthesized compounds were analyzed for purity, while structural confirmation was performed on a subset of synthesized compounds selected to represent unique chemical processes employed during the synthesis phase. For fatty acids, epoxidation of terminal olefins as well as the epoxidation and subsequent hydrolysis of $\alpha,\beta$-disubstituted olefins were confirmed. Similarly, the chemical stability of fatty acid analogues was confirmed after epoxidation and hydrolysis were performed. Purified materials were stored at −20 °C under nitrogen until use. Because the chemical epoxidation and subsequent hydrolysis reactions are well characterized (*9*, *12*, *14*−*17*), we performed complete structural analyses for only 12 representative compounds. The Supporting Information shows $^1$H NMR and GC/MS data for the selected surrogates (Figures S1−S37).

Initial purity assessments were performed by TLC using a hexane/ethyl acetate mixture (7:2) and visualized with UV light at 254 nm followed by treatment with phosphomolybdic acid and heat. A lack of UV absorption confirmed complete *m*-chlorobenzoic acid removal in the case of *m*-CPBA epoxidation. All compounds appeared to be pure as determined by TLC. To confirm purity and regioisomeric abundances, dilutions in *n*-hexane were analyzed on a Hewlett-Packard (HP) 5890A gas chromatograph equipped with a flame ionization detector (FID) and a 30 m × 0.25 mm i.d., 0.25 $\mu$m DB-5 column (J&W Scientific, Folsom, CA). The oven was initially held at 205 °C for 10 min, and ramped at 30 °C/min to 228 °C, then at 2 °C/min to 235 °C, and finally at 20 °C/min to 325 °C. All isolated compounds were greater than 91% pure as determined by GC/FID. By TLC and GC/FID, all synthesized compounds exhibited the number of spots or peaks corresponding to the theoretical number of positional isomers. Additionally, all synthesized compounds exhibited the same migration pattern, as determined by TLC and GC/FID, from parent compound to epoxide to diol, as compared to authentic standards when available.

Structural confirmations were achieved on the basis of $^1$H NMR and mass spectral results. $^1$H NMR spectra were collected on a GE-300 spectrometer (Bruker) in deuterated chloroform, and exchangeable protons were identified by addition of deuterated water. The appearance of a two-proton multiplet with a chemical shift between 2.8 and 2.9 ppm was considered confirmation of epoxide formation, while the shift of these protons to ∼3.3 ppm upon hydrolysis was indicative of diol formation. These $^1$H NMR results also provided chemical confirmation of the status of other chemical moieties, including esters, acetates, amides, nitriles, and methane sulfonates, which have well-characterized proton patterns or influences. The mass

[1] Abbreviations: ARDS, acute respiratory distress syndrome; BSA, bovine serum albumin; DMDO, dimethyldioxirane; FID, flame ionization detector; HP, Hewlett-Packard; hsEH, human soluble epoxide hydrolase; iltx, isoleukotoxin [12,13(*Z*)-epoxyoctadec-9(*Z*)-enoic acid]; iltxd, isoleukotoxin diol [*threo*-12,13-dihyroxyoctadec-9(*Z*)-enoic acid]; Lac Z, $\beta$-galactosidase; $LC_{50}$, median lethal concentration; ltx, leukotoxin [9,10(*Z*)-epoxyoctadec-12(*Z*)-enoic acid]; ltxd, leukotoxin diol [*threo*-9,10-dihydroxyoctadec-12(*Z*)-enoic acid]; *m*-CPBA, *m*-chloroperoxybenzoic acid; MPT, mitochondria permeability transition; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; sEH, soluble epoxide hydrolase; Sf-21, *Spodoptera frugiperda*; *vic*, vicinal.

[2] Greene, J., Williamson, K., Morisseau, C., Newman, J., and Hammock, B. (2000) Metabolism of Monoepoxides of Methyl Linoleate: Bioactivation and Detoxification. *Arch. Biochem. Biophys.* (in press).

[3] J. Zheng, C. Plopper, J. Lakritz, D. Storms, and B. Hammock, unpublished results.

[4] M. Sisemore, J. Zheng, J. Yang, C. Plopper, G. Cortopassi, and B. Hammock, unpublished results.

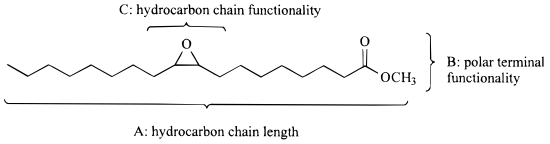
spectra of the selected compounds were acquired to support the acquired GC/FID and $^1$H NMR results. GC/MS analyses were executed on a HP 6890 system equipped with a 30 m × 0.25 mm i.d., 0.25 $\mu$m HP-5ms column and a 5973 mass spectral detector (Hewlett-Packard Co., San Jose, CA). Electron impact spectra (70 eV) were collected from 50 to 550 amu for all analytes. Hydroxyl-containing molecules were derivatized (48 h at 60 °C) with bis-silylacetamide (Supelco, Belvedere, PA) prior to analysis. In addition, MS and MS/MS spectra were acquired for the amide, nitriles, and methane sulfonate using electrospray ionization MS on a VG Quattro BQ instrument (Fisons, Altrancham, England) with a 50:50 dichloromethane/methanol mobile phase, and optimized ionization parameters, including a 3.75 kV capillary voltage and a 30−50 V cone voltage depending on the ionization mode and target compound.

For all compounds with available melting point, GC/MS, and/or NMR data, the data generated here were identical to those previously reported (*6*, *18*−*21*). For compounds with unique functional groups, the presence of these groups was confirmed by FT-IR. Specifically, nitrile stability during repeated oxidation steps was confirmed by FT-IR.

**Enzyme Assays.** To verify that the synthesized epoxy fatty acids, esters, and analogues were, in fact, sEH substrates, several representative compounds [including 12,13-epoxytridecanoic acid methyl ester (**3**), (*Z*)-9,10-epoxyhexadecanoic acid methyl ester (**5**), 9,10(*Z*)-epoxyoctadec-12(*Z*)-enol (**25**), 1-acetyl-9,10)*Z*)-epoxyoctadec-12(*Z*)-ene (**26**), 9,10(*Z*)-epoxy-1-sulfonyl-octadec-12(*Z*)-ene (**27**), 10,11(*Z*)-epoxynonadec-13(*Z*)-enitrile (**28**), 9,10(*Z*)-epoxyoctadec-12(*Z*)-enoic acid methyl ester (**23**), (*Z*)-9,-10-epoxyoctadecanoic acid methyl ester (**8**), (*E*)-9,10-epoxyoctadecanoic acid methyl ester, (*Z*)-6,7-epoxyoctadecanoic acid methyl ester (**7**), and (*E*)-6,7-epoxyoctadecanoic acid methyl ester] were chosen and assayed for hydrolysis after incubation with the enzyme for 1 h. These surrogates included compounds from each of the response groups discussed in section C of the Results and Discussion. In the assay, 13 $\mu$g/$\mu$L recombinant affinity-purified human soluble epoxide hydrolase (hsEH) incubated in a sodium phosphate buffer (100 $\mu$L, 100 mM, pH 7.4) containing 0.1 mg/mL bovine serum albumin (BSA) was placed in glass culture tubes and kept on ice until the assay was initiated. Stock solutions of compounds (25 mM) were prepared in ethanol, and the assay was initiated by addition of the stock solution (1 $\mu$L) with a Hamilton repeating syringe into the buffer containing hsEH. Controls for nonenzymatic hydrolysis were performed by addition of substrate to buffer containing 0.1 mg/mL BSA without hsEH. Tubes were then incubated in a shaking 37 °C water bath for 1 h. The enzymatic hydrolysis was then quenched by the addition of 200 $\mu$L of ether and approximately 200 mg of sodium chloride. Samples were centrifuged, and the ethereal layer was spotted on Baker preabsorbant TLC plates and developed in a 4:1 hexane/ethyl acetate mixture. Compounds were visualized by application of phosphomolybdic acid and heat. All lanes exhibited either a single spot cochromatographing with authentic diol standards or two spots cochromatographing with starting material and authentic diol standards.

**Cytotoxicity Studies.** Toxicity assays were performed in *Spodoptera frugiperda* (Sf-21) cells as previously described (*22*). Briefly, the cells were infected with baculovirus containing recombinant cDNA for either hsEH or $\beta$-galactosidase (Lac Z), as a control enzyme. Forty eight hours postinfection, compounds were administered to cells (final concentration of 0.06−1.0 mM) in dimethyl sulfoxide (DMSO) (final volume of 1%). All compounds were in solution when administered, with the exception of compound **19**, (*Z*)-9,10-epoxyoctadecane, which was in suspension at the two highest concentrations (0.5 and 1 mM). Cells were incubated for an additional 24 h at 27 °C, and then assayed for viability by incubation for 2 h with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were lysed (500 $\mu$L of a solution of 250 mg of sodium dodecyl sulfate in a 1:1 dimethyl formamide/water mixture at pH 4.5) over the course of 12 h in the dark to dissolve the insoluble formazan product. Aliquots were then transferred to 96-well plates, and the MTT hydrolysis was quantified at 560 nm on a Vmax Microplate Reader (Molecular Devices, Menlo Park, CA). Curve fitting was performed using SigmaPlot (SPSS, Inc., Chicago, IL) and Excel (Microsoft, Redmond, WA). $LC_{50}$ ranges and significance were calculated using Excel and the Student's *t* test. Toxicity data are representative of at least four independent experiments. Values that are different for hsEH- and Lac Z-expressing cells are significantly different ($p \leq 0.01$) except where otherwise noted.

**Scheme 2. Generalized Form of Fatty Acid Monoepoxide with Subunits Marked about Which Hypotheses Were Formed and Tested**




## Results and Discussion

Many tools for studying metabolism and toxicity exist. In this study, through the use of *S. frugiperda* (Sf-21) cells and the insect-specific baculovirus, we were able to assess toxicity in situ, without the difficulty and confounding factors of working with whole animals or mammalian cell cultures. The transgenic baculovirus assay system has the disadvantages inherent to any cell-based system. The cells must be transiently transfected, and there is a narrow window of time (24−48 h) in which in situ toxicity assays may be performed. This is also a very simple model of a very complex system, i.e., a whole animal. However, when we are attempting to discriminate between the effects of particular metabolizing enzymes, this can be an advantage. Sf-21 cells have very low background levels of many endogenous enzymes, including undetectable or very low levels of cytochrome P450 monooxygenases, epoxide hydrolases (*22*), glucuronosyl transferases (*23*), and esterases (*24*), among others. While esterase levels have been found to be low, sufficient esterase activity is present to cleave administered fatty esters to fatty acids.[2] The baculovirus expression system is widely used in the pharmaceutical industry to express human cytochrome P450 isozymes for investigation of xenobiotic metabolism (*25*). Grant et al. (*22*) extended the concept of P450 expression to the development of a simple system for examining geno- and cytotoxicity of reactive metabolites generated by expressed enzymes. Thus, within this system, effects can generally be attributed to reactions between the expressed enzyme and the compound of interest.

In this study, we have extended our investigation of the metabolic hydrolysis of epoxy fatty acids by sEH to consider structural constraints in the toxicity of these model compounds. Dissecting a generic fatty acid monoepoxide methyl ester into three conceptual subunits aided in the hypothesis generation and testing described herein (Scheme 2). Simply stated, our model consists of (A) a hydrocarbon chain of variable length with (B) a polar terminal functionality (e.g., an ester) and (C) a hydrocarbon chain functionality (e.g., an epoxide). Due to the limited availability of chemical starting materials, a complete spectrum of compounds could not be produced to generate a quantitative structure−activity relationship. However, by establishing acceptable variables, we could investigate structure−activity relationships.

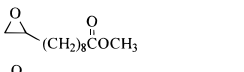
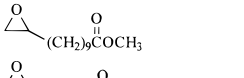
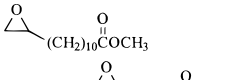
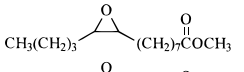
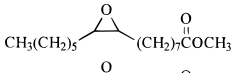
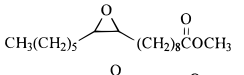
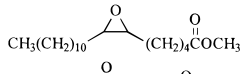
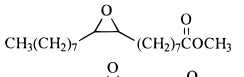
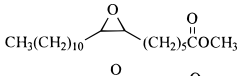
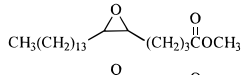
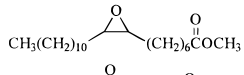
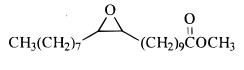
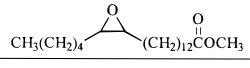
To establish a relationship between cytotoxicity and compound structure, cytotoxicity was first assessed. All fatty acids, esters, and analogues were originally tested for cytotoxic potential as the parent olefin in Sf-21 cells expressing either human sEH (hsEH) or $\beta$-galactosidase (Lac Z) as a control enzyme. These data are not shown because, with the exception of three compounds, which will be discussed in section C below, the 29 parent olefins were not toxic. Each epoxide was then synthesized and assayed for cytotoxicity in the Sf-21 system described above. As indicated above, the primary goal of this study was to understand the bioactivation of nontoxic epoxides to toxic diols by hsEH. Significant toxicity to Lac Z-expressing Sf-21 cells by epoxide-containing compounds indicates that bioactivation by hsEH was not required. Thus, diols were only synthesized (and tested) from a subset of parent epoxides which were not significantly toxic to Lac Z-expressing Sf-21 cells. Additionally, in the course of this research, a number of toxic compounds were identified that did not fit the pattern of bioactivation by sEH. In the interest of presenting a coherent report, we have included the data from these compounds but have not followed the implications of their toxicity except inasmuch as they relate to the central theses of this paper.

**(A) Effect of Hydrocarbon Chain Length on sEH-Dependent Toxicity.** By fixing the degree of unsaturation, the polar terminal group, and, where possible, distance to the polar terminal, we could investigate the effects of overall hydrocarbon length. Thus, we first tested the hypothesis that chemical potency increased with chain length. Mono-unsaturated fatty acid methyl esters 11−20 carbons in length were epoxidized and toxicities assayed. All epoxides were between position C-5 and C-15. While a fixed position epoxide at C-9 would have been ideal, the parent fatty esters were unavailable.

Table 1 shows the $LC_{50}$ values for hsEH- and Lac Z-expressing cells incubated with the 11−20-carbon mono-unsaturated epoxy fatty acid methyl esters (**1**−**13**). Toxicity of fatty acid methyl esters to hsEH-expressing Sf-21 cells, as measured by $LC_{50}$, increased with chain length up to 18 carbons (**8**) [with the exception of (*Z*)-6,7-epoxyoctadecanoic acid methyl ester (**7**), which will be discussed in section C below], but decreased with extension to 19 carbons (**9**), and was eliminated with further extension to 20 carbons (**10**−**13**). The tested 11-carbon (**1**) and 12-carbon (**2**) fatty acid methyl esters contained terminal epoxides and were nontoxic to hsEH-expressing cells. The 13-carbon terminal epoxide fatty acid methyl ester (**3**) and (*Z*)-9,10-epoxy 14- and 16-carbon compounds (**4** and **5**, respectively), while toxic to cells expressing either enzyme, were more toxic to cells expressing Lac Z than to those expressing hsEH. The longer-chain compounds, (*Z*)-10,11-epoxyheptadecanoic acid methyl ester (**6**), (*Z*)-6,7-epoxyoctadecanoic acid methyl ester (**7**), (*Z*)-9,10-epoxyoctadecanoic acid methyl ester (**8**), and (*Z*)-7,8-epoxynonadecanoic acid methyl ester (**9**), were all substantially more toxic to hsEH-expressing cells than to Lac Z-expressing cells.

The cytotoxicity of the midlength fatty acids, 10,11-epoxyundecanoic acid methyl ester and 11,12-epoxydodecanoic acid methyl ester (**1** and **2**, respectively), to Lac Z- but not to hsEH-expressing Sf-21 cells indicates that diol formation, in this case, is a detoxification step for these terminal epoxy fatty acid esters. Some epoxides are known to be carcinogens, teratogens, and mutagens; thus, the role of epoxide hydrolases as detoxifying enzymes has been studied with great interest (*22*, *26*−*28*). Although some epoxides are highly reactive alkylating agents, nonactivated aliphatic epoxides are usually far less reactive. However, among the aliphatic epoxides, one anticipates monosubstituted epoxides such as 10,11-epoxyundecanoic acid methyl ester, 11,12-epoxydodecanoic acid methyl ester, and 12,13-epoxytridecanoic acid methyl ester (**1**−**3**, respectively) to be far more reactive than $\alpha,\beta$-disubstituted epoxides. While the toxic behavior of compounds **1** and **2** suggests a reactive epoxide, the behavior of compounds **3**−**5** is more problematic to explain since the compounds show toxicity to both hsEH- and Lac Z-expressing cells. However, they are more toxic to Lac Z-expressing cells than to hsEH-expressing cells, so it is tempting to suggest that the diol is once again a detoxification product. If that were the case, however, one would expect, as with shorter-chain terminal epoxides **1** and **2**, that there would be no toxicity to hsEH-expressing cells. One possibility is that the 12−13-carbon chain may constitute a lipophilicity threshold such that nonspecific binding and sequestration become more relevant with the longer chains, leading to lower free concentrations of the toxic epoxide. Alternatively, these findings may suggest that more than one pathway for toxicity exists, perhaps one through the epoxide, and the other through the diol.

**Table 1. $LC_{50}$ Values for 11−20-Carbon Epoxy Fatty Esters**[a]

| # | Structure | Compound | $LC_{50}$, mM | |
|---|---|---|---|---|
| | | | hsEH | Lac Z |
| **1** |  | 10,11-epoxyundecanoic acid methyl ester | > 1 (100 %) | 0.746 ± 0.02 |
| **2** |  | 11,12-epoxydodecanoic acid methyl ester | > 1 (100 %) | 0.600 ± 0.005 |
| **3** |  | 12,13-epoxytridecanoic acid methyl ester | 0.875 ± 0.030 | 0.522 ± 0.005 |
| **4** |  | (*Z*)-9,10-epoxytetradecanoic acid methyl ester | 0.810 ± 0.031 | 0.580 ± 0.008 |
| **5** |  | (*Z*)-9,10-epoxyhexadecanoic acid methyl ester | 0.554 ± 0.015 | 0.331 ± 0.030 |
| **6** |  | (*Z*)-10,11-epoxyheptadecanoic acid methyl ester | 0.181 ± 0.015 | > 1 (100 %) |
| **7** |  | (*Z*)-6,7-epoxyoctadecanoic acid methyl ester | 0.428 ± 0.036 | > 1 (100 %) |
| **8** |  | (*Z*)-9,10-epoxyoctadecanoic acid methyl ester | 0.112 ± 0.007 | > 1 (100 %) |
| **9** |  | (*Z*)-7,8-epoxynonadecanoic acid methyl ester | 0.159 ± 0.016 | > 1 (100 %) |
| **10** |  | (*Z*)-5,6-epoxyeicosanoic acid methyl ester | > 1 (100 %) | > 1 (100 %) |
| **11** |  | (*Z*)-8,9-epoxyeicosanoic acid methyl ester | > 1 (100 %) | > 1 (100 %) |
| **12** |  | (*Z*)-11,12-epoxyeicosanoic acid methyl ester | > 1 (100 %) | > 1 (100 %) |
| **13** |  | (*Z*)-14,15-epoxyeicosanoic acid methyl ester | > 1 (100 %) | > 1 (100 %) |

[a] Compounds were incubated for 24 h with Sf-21 cells expressing either hsEH or Lac Z. Viability was measured by the ability of cells to reduce MTT. For compounds with $LC_{50}$ values of >1 mM, the percent cell viability at 1 mM is shown in parentheses. All values are representative of at least four independent experiments. With the exception of compounds **10**−**13**, hsEH and Lac Z values are significantly different ($p \leq 0.01$).

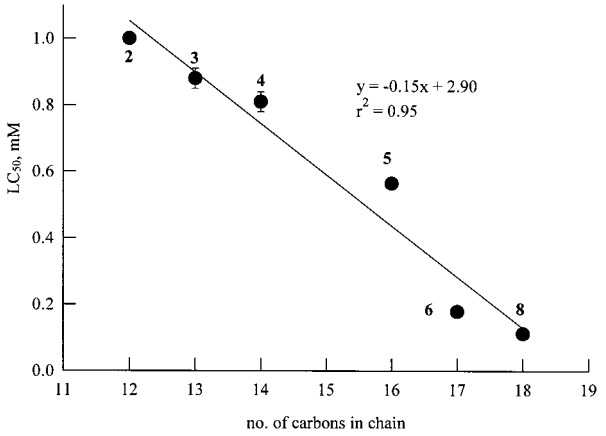



**Figure 1.** Correlation of $LC_{50}$ values with chain length of epoxy fatty esters. Sf-21 cells expressing hsEH were incubated with compounds for 24 h. All epoxides are between C-9 and C-12 from the ester. The compound number is in bold next to its point on the graph. All data are the result of at least four independent experiments; all $LC_{50}$ values are significantly different ($p \leq 0.01$). If not shown, the standard deviation of the replicate is smaller than the point on the graph.

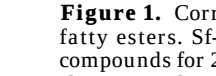
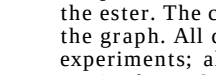
This raises the questions of how much diol is actually formed within the cell and how much is necessary for toxicity. The lower levels of compound necessary for toxicity in other cell types and organelles, e.g., alveolar epithelial cells (*10*) or isolated mitochondria,[4] suggest that only a low concentration of diol is necessary for toxicity.

The lack of toxicity of (*Z*)-8,9-epoxyeicosanoic acid methyl ester (**11**) and the relatively high $LC_{50}$ for *threo*-8,9-dihydroxyeicosanoic acid methyl ester (**43**) suggest that size is an important component in toxicity. The differential toxicity between these two compounds (**11** and **43**) could be due to very slow metabolism by hsEH or an inability to reach the active site of the enzyme. However, it has previously been shown that sEH metabolizes the 20-carbon poly-unsaturated epoxyeicosatrienoic acids regardless of epoxide position (*29*). While the effect of unsaturation on hydrocarbon chain flexibility prevents a direct translation, these data collectively suggest that (*Z*)-8,9-epoxyeicosanoic acid methyl ester (**11**) exceeds the substrate specificity requirements of the enzyme. Moreover, the relatively high $LC_{50}$ for *threo*-8,9-dihydroxyeicosanoic acid methyl ester (**43**) and the lack of toxicity of *threo*-11,12-dihydroxyeicosanoic acid methyl ester (**44**) indicate that size affects diol-induced toxicity as well as enzymatic hydrolysis.

It is important to point out that total molecular length, or the lack of it, is not the sole issue in toxicity but that the length of the hydrocarbon chain appears to be the critical component. This can be seen most clearly by comparing the relative cytotoxicities of two epoxide−diol pairs with 22 atom chains: the propyl ester octadecanoids (**16** and **38**) versus the methyl ester eicosanoids (**11** and **43**). This comparison shows that the 18-carbon diol is more potent than the 20-carbon diol. Taken together, these analyses of the effect of hydrocarbon chain length indicate that epoxy fatty esters with acyl chains between 17 and 19 carbons in length have the greatest toxicity in the presence of hsEH in our system.

The relationship between chain length and cytotoxicity to hsEH-expressing cells is best demonstrated when the epoxides are in similar positions with respect to the polar terminus (Figure 1). Again, it would have been ideal to use only compounds with a fixed position epoxide at C-9; unfortunately, the parent fatty esters were unavailable. Thus, we graphed the relationship using $LC_{50}$ values from compounds with a single site of unsaturation (the epoxide), a methyl ester at the polar terminus, and an epoxide position between C-9 and C-12.

**Table 2. $LC_{50}$ Values for 18-Carbon Epoxy Fatty Acid, Esters, Alkane, and Amide[a]**

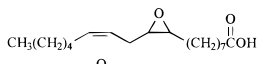
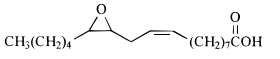
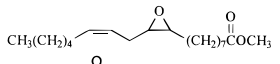
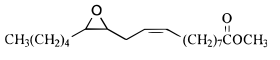
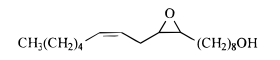
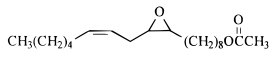
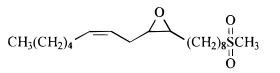
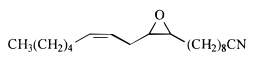
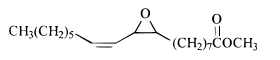
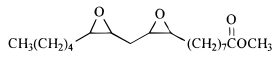
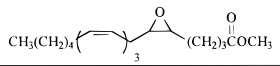
| # | Structure | Compound | $LC_{50}$, mM | |
|---|---|---|---|---|
| | | | hsEH | Lac Z |
| **14** |  | (*Z*)-9,10-epoxyoctadecanoic acid | 0.118 ± 0.017 | > 1 (100 %) |
| **8** |  | (*Z*)-9,10-epoxyoctadecanoic acid methyl ester | 0.112 ± 0.007 | > 1 (100 %) |
| **15** |  | (*Z*)-9,10-epoxyoctadecanoic acid ethyl ester | 0.108 ± 0.006 | > 1 (100 %) |
| **16** |  | (*Z*)-9,10-epoxyoctadecanoic acid propyl ester | 0.115 ± 0.009 | > 1 (100 %) |
| **17** |  | (*Z*)-9,10-epoxyoctadecanoic acid isopropyl ester | > 1 (51 %) | > 1 (100 %) |
| **18** |  | (*Z*)-9,10-epoxyoctadecanoic acid butyl ester | > 1 (54 %) | > 1 (100 %) |
| **19** |  | (*Z*)-9,10-epoxyoctadecane | > 1(100 %) | > 1 (100 %) |
| **20** |  | (*Z*)-9,10-epoxyoctadecamide | 0.258 ± 0.023 | > 1 (100 %) |

[a] Compounds were incubated for 24 h with Sf-21 cells expressing either hsEH or Lac Z. Viability was measured by the ability of cells to reduce MTT. Compound **8** is repeated from Table 1 to aid the reader in seeing the structural relationship. For compounds with $LC_{50}$ values of >1 mM, the percent cell viability at 1 mM is shown in parentheses. All values are representative of at least four independent experiments. Compounds **8**, **14**−**16**, and **20** have $LC_{50}$ values for hsEH- and Lac Z-expressing cells that are significantly different ($p \leq 0.01$).

**(B) Effect of Polar Terminal Functionality on sEH-Dependent Toxicity.** The dependence of sEH-mediated epoxide toxicity on the nature of the polar terminal group was then investigated. We tested the hypothesis that modification of the carboxylic acid moiety of a model compound would reduce toxicity. Since (*Z*)-9,10-epoxyoctadecanoic acid (**14**) and ltx (**21**) were both toxic in the Sf-21 cell system, all modifications were made to these basic structures. The effects on toxicity of esterification, ester chain length, and carboxyl moiety replacement with a methyl or amide were tested in the series based on compound **14** (Table 2). The effects of esterification or carboxyl replacement with an alcohol, acetate, methane sulfonate, or nitrile were further investigated in a series based on compound **21** (Table 3).

We first tested the effect of ester length on toxicity with the following series based on compound **14**: acid (**14**), methyl (**8**), ethyl (**15**), *n*-propyl (**16**), isopropyl (**17**), and *n*-butyl (**18**). The $LC_{50}$ values (Table 2) are somewhat misleading because, unlike those of most compounds that have been tested, toxicity appears at low concentrations but levels off, leaving a significant portion of the cells alive and suggesting that these compounds are less efficacious than most. However, at the highest concentrations (1 mM), a trend that is not otherwise apparent becomes visible. Increasing ester length gradually decreased the toxicity of the compounds (Figure 2). While there was no significant difference in toxicity between the free fatty acid (**14**) and the methyl ester (**8**) to hsEH-expressing Sf-21 cells, further ester extension decreased toxicity in size order: ethyl, *n*-propyl, isopropyl, and

**Table 3. $LC_{50}$ Values for Epoxy Alkene Fatty Acids, Esters, and Analogues[a]**

| # | Structure | Compound | $LC_{50}$, mM | |
|---|---|---|---|---|
| | | | hsEH | Lac Z |
| 21 |  | (9,10*Z*)-epoxyoctadec-(12*Z*)-enoic acid | 0.154 ± 0.018 | > 1 (100 %) |
| 22 |  | (12,13*Z*)-epoxyoctadec-(9*Z*)-enoic acid | 0.150 ± 0.015 | > 1 (100 %) |
| 23 |  | (9,10*Z*)-epoxyoctadec-(12*Z*)-enoic acid methyl ester | 0.153 ± 0.014 | > 1 (100 %) |
| 24 |  | (12,13*Z*)-epoxyoctadec-(9*Z*)-enoic acid methyl ester | 0.145 ± 0.012 | > 1 (100 %) |
| 25 |  | (9,10*Z*)-epoxyoctadec-(12*Z*)-enol | 0.060 ± 0.015 | 0.074 ± 0.009 |
| 26 |  | 1-acetyl-(9,10*Z*)-epoxyoctadec-(12*Z*)-ene | 0.057 ± 0.011 | 0.085 ± 0.019 |
| 27 |  | (9,10*Z*)-epoxy-1-sulfonyloctadec-(12*Z*)-ene | 0.049 ± 0.008 | 0.059 ± 0.013 |
| 28 |  | (10,11*Z*)-epoxynonadec-(13*Z*)-enitrile | 0.066 ± 0.012 | 0.118 ± 0.008 |
| 29 |  | (9,10*Z*)-epoxyoctadec-(11*Z*)-enoic acid methyl ester | 0.192 ± 0.023 | > 1 (100 %) |
| 30 |  | (9, 10*Z*, 12, 13*Z*)-diepoxyoctadecanoic acid methyl ester | > 1 (100 %) | > 1 (100 %) |
| 31 |  | (*Z*)-5,6-epoxyeicosatri-(8*Z*, 11*Z*, 14*Z*)-enoic acid methyl ester | 0.251 ± 0.007 | > 1 (100 %) |

[a] Compounds were incubated for 24 h with Sf-21 cells expressing either hsEH or Lac Z. Viability was measured by the ability of cells to reduce MTT. Where applicable, structures are representative of all regioisomers. $LC_{50}$ values are for mixtures of regioisomers. For compounds with $LC_{50}$ values of >1 mM, the percent cell viability at 1 mM is shown in parentheses. All values are representative of at least four independent experiments. With the exception of compounds **25**−**27** and **30**, hsEH and Lac Z values are significantly different ($p \leq 0.01$).

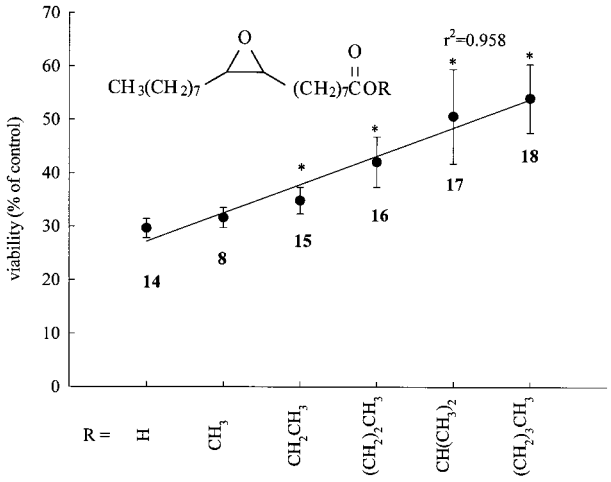



**Figure 2.** Correlation of the viability of treated cells with ester size for epoxide-containing compounds. Sf-21 cells expressing hsEH were incubated with (*Z*)-9,10-epoxyoctadecanoic acid (**14**), (*Z*)-9,10-epoxyoctadecanoic acid methyl ester (**8**), (*Z*)-9,10-epoxyoctadecanoic acid ethyl ester (**15**), (*Z*)-9,10-epoxyoctadecanoic acid *n*-propyl ester (**16**), (*Z*)-9,10-epoxyoctadecanoic acid isopropyl ester (**17**), and (*Z*)-9,10-epoxyoctadecanoic acid *n*-butyl ester (**18**) for 24 h. There appears to be a correlation between viability and ester size ($r^2 = 0.96$). The compound number is in bold next to its point on the graph. All data are the result of at least four independent experiments. Asterisks denote cases in which viability data for cells treated with compounds **15**−**18** are significantly different from the data for compound **8** ($p \leq 0.01$).

*n*-butyl (**15**−**18**, respectively). Figure 3 shows that, as for the epoxide-containing compounds, when ester length

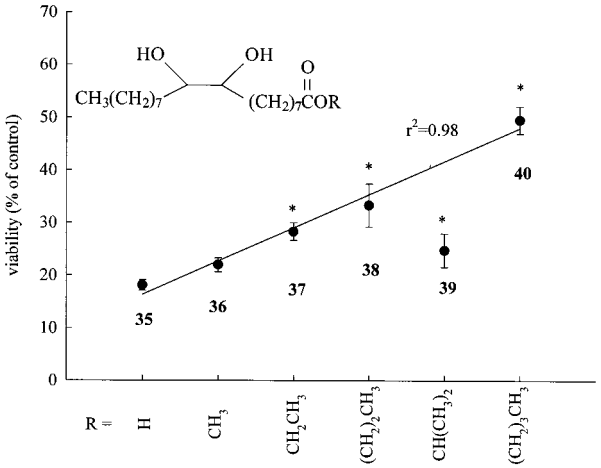
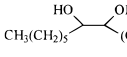
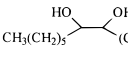
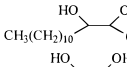
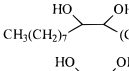
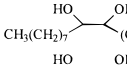
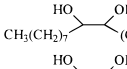
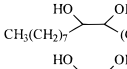
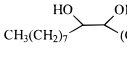
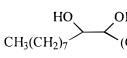
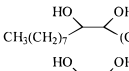
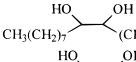
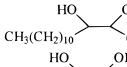
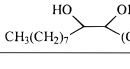



**Figure 3.** Correlation of the viability of treated cells with ester size for *vic*-diol-containing compounds. Sf-21 cells expressing hsEH were incubated with 1 mM *threo*-9,10-dihydroxyoctadecanoic acid (**35**), *threo*-9,10-dihydroxyoctadecanoic acid methyl ester (**36**), *threo*-9,10-dihydroxyoctadecanoic acid ethyl ester (**37**), *threo*-9,10-dihydroxyoctadecanoic acid *n*-propyl ester (**38**), *threo*-9,10-dihydroxyoctadecanoic acid isopropyl ester (**39**), and *threo*-9,10-dihydroxyoctadecanoic acid *n*-butyl ester (**40**) for 24 h. There appears to be a correlation between viability and ester size ($r^2 = 0.98$, if the isopropyl ester is not included). The compound number is in bold next to its point on the graph. All data are the result of at least four independent experiments. Asterisks denote cases in which viability data for compounds **37**−**40** are significantly different from the data for compound **35** ($p \leq 0.01$).

was increased for *vic*-diol-substituted compounds, toxicity decreased, such that viability at 1 mM was as follows: free acid (**35**) = methyl (**36**) < ethyl (**37**) < *n*-propyl (**38**) < *n*-butyl (**40**) esters. However, in this case, the isopropyl ester (**39**) did not fit neatly between the *n*-propyl and *n*-butyl esters, but exhibited a toxicity similar to that of the ethyl ester. It is unclear why this occurs. Together, these results suggest that the free carboxylic acid is the optimal structure for toxic activity in this system. The fact that we do not see increased cellular protection with increasing ester length at the $LC_{50}$ of epoxide-containing compounds is likely due to the effects of lipophilicity and/or esterase activity. Unfortunately, attempts to inhibit esterase activity were associated with hsEH-independent toxicity, further complicating these results.[2] Within the constraints of the system, increased lipophilicity, which increases with ester length, will increase the level of nonspecific interactions of the compound, thereby decreasing the amount available for metabolism or initiation of a toxic event. Furthermore, if the diol free acid is the active form and ester length decreases the rate of ester hydrolysis, it may be necessary for cellular esterases to be saturated before the trend in protection becomes apparent.

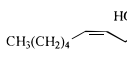
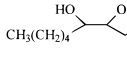
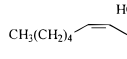
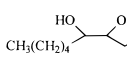
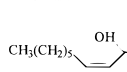
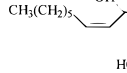
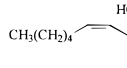
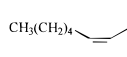
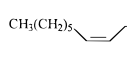
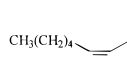
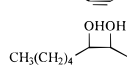
We then assessed the effect of replacing the carboxylic acid with different chemical functional groups in compound **14** and **21** analogues. Removal of the carboxyl moiety of **14** to form (*Z*)-9,10-epoxyoctadecane (**19**) eliminated toxicity (Table 2). However, replacement of the carboxyl function of **14** with an amide to form (*Z*)-9,10-epoxyoctadecamide (**20**) produced a compound with a 2-fold reduction in potency but a similar mode of toxicity (Table 2). More dramatic alterations in the chemical properties were investigated with compound **21** analogues (Table 3). Except for the case of the methyl esters, compound **21** analogues were tested as racemic mixtures

**Table 4. $LC_{50}$ Values of Dihydroxy Fatty Acids and Esters[a]**

| # | Structure | Compound | $LC_{50}$, mM | |
|---|---|---|---|---|
| | | | hsEH | Lac Z |
| **32** |  | (*threo*)-9,10-dihydroxyhexadecanoic acid methyl ester | 0.314 ± 0.014 | 0.348 ± 0.035 |
| **33** |  | (*threo*)-10,11-dihydroxyheptadecanoic acid methyl ester | 0.193 ± 0.024 | 0.191 ± 0.016 |
| **34** |  | (*threo*)-6,7-dihydroxyoctadecanoic acid methyl ester | 0.086 ± 0.014 | 0.088 ± 0.013 |
| **35** |  | (*threo*)-9,10-dihydroxyoctadecanoic acid | 0.130 ± 0.008 | 0.127 ± 0.009 |
| **36** |  | (*threo*)-9,10-dihydroxyoctadecanoic acid methyl ester | 0.122 ± 0.016 | 0.124 ± 0.011 |
| **37** |  | (*threo*)-9,10-dihydroxyoctadecanoic acid ethyl ester | 0.140 ± 0.008 | 0.136 ± 0.009 |
| **38** |  | (*threo*)-9,10-dihydroxyoctadecanoic acid propyl ester | 0.138 ± 0.012 | 0.137 ± 0.011 |
| **39** |  | (*threo*)-9,10-dihydroxyoctadecanoic acid isopropyl ester | 0.142 ± 0.005 | 0.142 ± 0.008 |
| **40** |  | (*threo*)-9,10-dihydroxyoctadecanonic acid butyl ester | > 1 (52 %) | > 1 (54 %) |
| **41** |  | (*threo*)-10,11-dihydroxynonadecanoic acid methyl ester | 0.156 ± 0.026 | 0.148 ± 0.016 |
| **42** |  | (*threo*)-9,10-dihydroxyoctadecane | > 1 (100 %) | > 1 (100 %) |
| **43** |  | (*threo*)-8,9-dihydroxyeicosanoic acid methyl ester | 0.457 ± 0.07 | 0.462 ± 0.010 |
| **44** |  | (*threo*)-11,12-dihydroxyeicosanoic acid methyl ester | > 1 (68 %) | > 1 (69 %) |

[a] Compounds were incubated for 24 h with Sf-21 cells expressing either hsEH or Lac Z. Viability was measured by the ability of cells to reduce MTT. For compounds with $LC_{50}$ values of >1 mM, the percent cell viability at 1 mM is shown in parentheses. All values are representative of at least four independent experiments. $LC_{50}$ values for hsEH- and Lac Z-expressing cells are not significantly different. Structures do not indicate stereochemistry.

**Table 5. $LC_{50}$ Values of Oxygenated Alkenes[a]**

| # | Structure | Compound | $LC_{50}$, mM | |
|---|---|---|---|---|
| | | | hsEH | Lac Z |
| **45** |  | (*threo*)-9,10-dihydroxyoctadec-(12*Z*)-enoic acid | 0.145 ± 0.01 | 0.144 ± 0.008 |
| **46** |  | (*threo*)-12,13-dihydroxyoctadec-(9*Z*)-enoic acid | 0.142 ± 0.009 | 0.146 ± 0.012 |
| **47** |  | (*threo*)-9,10-dihydroxyoctadec-(12*Z*)-enoic acid methyl ester | 0.133 ± 0.017 | 0.136 ± 0.015 |
| **48** |  | (*threo*)-12,13-dihydroxyoctadec-(9*Z*)-enoic acid methyl ester | 0.137 ± 0.005 | 0.133 ± 0.006 |
| **49** |  | (*threo*)-9,10-dihydroxyoctadec-(11*Z*)-enoic acid methyl ester | 0.166 ± 0.024 | 0.179 ± 0.030 |
| **50** |  | (*threo*)-10,11-dihydroxynonadec-(13*Z*)-enitrile | 0.048 ± 0.009 | 0.048 ± 0.010 |
| **51** |  | 9-hydroxyoctadec-(12*Z*)-enoic acid methyl ester | > 1 (100 %) | > 1 (100 %) |
| **52** |  | (9*Z*, 12*Z*)-9-hydroxyoctadecadienoic acid methyl ester | > 1 (100 %) | > 1 (100 %) |
| **53** |  | 9-methoxyoctadec-(12*Z*)-enoic acid methyl ester | > 1 (100 %) | > 1 (100 %) |
| **54** |  | (*threo*)-9,10,12,13-tetrahydroxyoctadecanoic acid methyl ester | > 1 (100 %) | > 1 (100 %) |
| **55** |  | 9,12-dihydroxy-10,13-furanyloctadecanoic acid methyl ester | > 1 (100 %) | > 1 (100 %) |

[a] Compounds were incubated for 24 h with Sf-21 cells expressing either hsEH or Lac Z. Viability was measured by the ability of cells to reduce MTT. Where applicable, structures are representative of both regioisomers. $LC_{50}$ values are for mixtures of regioisomers. For compounds with $LC_{50}$ values of >1 mM, the percent cell viability at 1 mM is shown in parentheses. All values are representative of at least four independent experiments. $LC_{50}$ values for hsEH- and Lac Z-expressing cells are not significantly different. Structures do not indicate stereochemistry.

of both epoxide positional isomers. Esterification of the carboxylic acid to form either racemic compound **23** or **24** did not significantly affect sEH-dependent toxicity. Carboxyl replacement with an alcohol, an acetate, a methane sulfonate, or a nitrile yielded compounds **25**−**28**, respectively, along with their respective regioiosmers (Table 3). These ltx and iltx analogues were toxic at very low concentrations ($LC_{50}$ = 0.040−0.126 mM) in the presence or absence of hsEH (Table 3); however, the olefinic precursors of compounds **25**−**28** [octadecadi-9,12-(*Z*)-enol, 1-acetyloctadecadi-9,12(*Z*)-ene, 1-sulfonyloctadecadi-9,12(*Z*)-ene, and nonadecadi-10,13(*Z*)-enitrile, respectively] were nontoxic (data not shown). Therefore, the toxicity of the oxidized ltx analogues does not appear to be solely an effect of ester replacement. It may simply be that toxicity occurs through a mechanism different from that seen with the compounds containing acids and esters. Because the $LC_{50}$ values of compounds **25**−**27** were similar for cells expressing either Lac Z or hsEH, the corresponding diols were not synthesized. While the toxicity of these compounds is very interesting, the lack of differential toxicity to cells expressing hsEH and Lac Z puts further investigation of compounds **25**−**27** outside the scope of this paper. The finding that the epoxy nitrile (**28**) exhibited enhanced toxicity in the presence of sEH (Table 3) led to the synthesis of the corresponding diol (**50**; Table 5).

**(C) Effect of Hydrocarbon Chain Functionality on sEH-Dependent Toxicity.** The effects of altering the nature, degree, and position of functionalities contained within the hydrocarbon chain were then assessed. The investigated alterations included the requirement of olefin oxidation, the effect of epoxide hydrolysis, the addition of olefins in combination with epoxide and/or diol, the effect of epoxide and/or diol position, and the replacement of the epoxide−diol pair with hydroxy, methoxy, tetraol, or tetrahydrofuran diol moieties.

To test the hypothesis that olefin oxidation was indeed necessary for sEH-dependent fatty acid ester toxicity, we tested the cytotoxicity of olefinic parent compounds. With the exception of the 11-, 12-, and 13-carbon precursors to compounds **1**−**3**, which exhibited equivalent toxicity to Lac Z- and sEH-expressing cells, the parent compounds were not toxic at or below 1 mM (data not shown). Therefore, sEH-dependent fatty acid ester toxicity requires olefin oxidation. The observed toxicity of the three terminal alkene fatty acid methyl esters is consistent with previous reports that metabolism of medium-chain unsaturated fatty acids in the mitochondrial matrix causes formation of excess AMP, uses excessive amounts of ATP, and thus contributes to energy dissipation (*30*).

A brief investigation of stereochemistry indicated that where tested [(*Z*)-9,10-epoxyoctadecanoic acid methyl ester (**8**)/(*E*)-9,10-epoxyoctadecanoic acid methyl ester, (*Z*)-6,7-epoxyoctadecanoic acid methyl ester (**7**)/(*E*)-6,7-epoxyoctadecanoic acid methyl ester, and 9,10(*Z*)-epoxyoctadec-12(*Z*)-enoic acid methyl ester (**23**)/9,10(*E*)-epoxyoctadec-12(*E*)-enoic acid methyl ester mixtures], the

toxicity of *cis* and *trans* epoxides could not be discriminated (data not shown). This was also true of the corresponding *erythro* and *threo* diols (data not shown). This was not particularly surprising because we have previously seen that there is no significant difference in the specific activity of sEH with respect to these epoxides[4] (*9*, *31*).

To assess the effects of epoxide position, while more directly differentiating effects induced by epoxide hydrolysis and diol-dependent toxicity, diols were synthesized from a number of the epoxides exhibiting high differential toxicity between cells expressing hsEH and Lac Z (Tables 4 and 5). Examination of data in Table 3 suggests that epoxide position affected toxicity within the 18-carbon series. While toxicity was unaffected by changing the position of the epoxide from the 9 to the 11 position, e.g., ltx and iltx (**23** and **24**) (Table 3), displacement of this functionality to the 6 position in (*Z*)-6,7-epoxyoctadecanoic acid methyl ester (**7**) significantly decreased toxicity relative to (*Z*)-9,10-epoxyoctadecanoic acid methyl ester (**8**) (Table 1).

With the exception of (*Z*)-6,7-epoxyoctadecanoic acid methyl ester (**7**) and (*Z*)-8,9-epoxyeicosanoic acid methyl ester (**11**), the epoxides and their hydrolysis products generally exhibited similar toxicity to hsEH- and Lac Z-expressing cells (Table 4). Notably, *threo*-8,9-dihydroxyeicosanoic acid methyl ester (**43**) was significantly more toxic than *threo*-11,12-dihydroxyeicosanoic acid methyl ester **(44)**, indicating that in the case of these 20-carbon compounds, diol position did affect toxicity. Similarly, *threo*-6,7-dihydroxyoctadecanoic acid methyl ester (**34**) appeared to be 5-fold more potent than its epoxide precursor, compound **7** (Tables 1 and 4). Together, these results suggest that diol toxicity can be initiated by a wider range of positional diols than can be efficiently produced by sEH in our cell system.

To evaluate the effect of olefin number and position on sEH-dependent toxicity, the 18-carbon 9,10-epoxy fatty acid methyl esters with either no (**8**) or one (**23**, **24**, and **29**) olefin were compared (Tables 1 and 3), as were the available corresponding diols (**36** and **47**−**49**, Tables 4 and 5). With these 18-carbon compounds, toxicity decreased with olefin addition in both the epoxide and diol series; however, relative diol toxicity was less affected. A secondary effect was also observed with epoxide position relative to the oxidation site. Simply stated, compounds formed from parent fatty acids that were conjugated (**29** or **49**) appeared to be slightly less toxic than compounds formed from nonconjugated parent fatty acids (**23**, **24**, **47**, or **48**). The similarity in the relative toxic potency within these groups suggests that the modest decrease in toxicity resulting from the olefin presence and position is not a result of differential sEH metabolism.

To assess the ability of other oxygen-containing functionalities in the hydrocarbon chain to stimulate a toxic response, six additional compounds were assessed for cytotoxicity (**30**, Table 3; **51**−**55**, Table 5). The presence of a single hydroxy (**51** and **52**) or methoxy group (**53**) at position 9 of an 18-carbon olefinic fatty acid did not impart toxicity at or below 1 mM. Similarly, epoxidation of both double bonds of 9(*Z*),12(*Z*)-octadecadienoic acid methyl ester produced compound **30**, which was not toxic at or below 1 mM. Hydrolysis of 9,10(*Z*),12,13(*Z*)-diepoxyoctadecanoic acid (**30**) can yield either a corresponding tetraol (**54**) or a mixture of dihydroxy furanyloctadecanoic acid methyl esters (e.g., **55**) resulting from sequential hydrolysis with cyclization (*32*). Again, these compounds were not toxic. Therefore, the data suggest that a *vic*-diol may be substituted for the epoxide and toxicity retained but that substitution with the other tested oxygenated groups results in a loss of toxic potential under the test conditions.

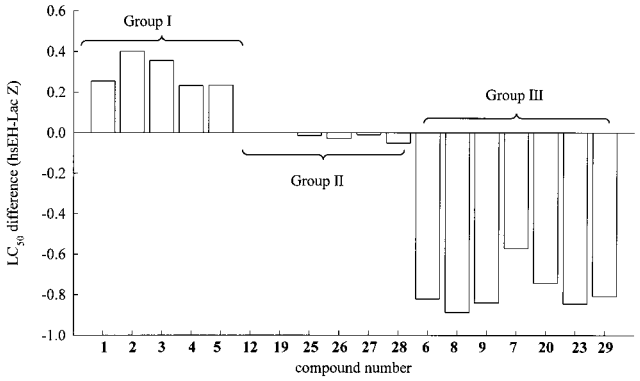



**Figure 4.** Differential toxicity of epoxide-containing fatty acids and analogues. $LC_{50}$ values of compounds for toxicity to Lac Z-expressing cells were subtracted from $LC_{50}$ values for hsEH-expressing cells. This reveals that there are three groups of compounds: that for which hsEH is a detoxification pathway (group I), that for which the expressed enzyme does not appear to be involved in toxicity (both epoxide and diol are equally toxic, or nontoxic) (group II), and that for which hsEH expression increases the toxicity of the compound (group III). Several compounds were chosen from each group and assayed in vitro to assess their activities as substrates. All were found to be substrates. The compounds chosen included 12,13-epoxytridecanoic acid methyl ester (**3**) and (*Z*)-9,10-epoxyhexadecanoic acid methyl ester (**5**) (group I), 9,10(*Z*)-epoxyoctadec-12(*Z*)-enol (**25**), 1-acetyl-9,10(*Z*)-epoxyoctadec-12(*Z*)-ene (**26**), 9,10(*Z*)-epoxy-1-sulfonyloctadec-12(*Z*)-ene (**27**), and 10,11(*Z*)-epoxynonadec-13-(*Z*)-enitrile (**28**) (group II), and 9,10(*Z*)-epoxyoctadec-12(*Z*)-enoic acid methyl ester (**23**), (*Z*)-9,10-epoxyoctadecanoic acid methyl ester (**8**), and (*Z*)-6,7-epoxyoctadecanoic acid methyl ester (**7**) (group III).

To summarize our findings, during the course of this investigation into the structural requirements of sEH-dependent epoxy fatty acid cytotoxicity, three groups of aliphatic epoxide−diol pairs were described by their observed differential toxicity (Figure 4). Group I compounds were typified by terminal epoxides whose toxicity was reduced in the presence of sEH. Group II compounds were toxic in either their epoxide or diol form, and toxicity was unaffected by sEH. Group III compounds exhibited sEH-dependent toxicity and were therefore used to investigate the structural elements required for cytotoxicity in this study. The optimal structure for group III compounds appeared to be a carboxylic acid that was 18−20 atoms long (i.e., carbon backbone plus a terminal heteroatom) with an epoxide positioned between C-7 and C-12. Replacement of the free carboxylic acid with an amide reduced the potency, but did not alter the mode of toxicity. In the absence of sEH, replacing these epoxides with a *vic*-diol was required for toxicity. While diol stereochemistry was unimportant, *vic*-diol-induced toxicity exhibited fewer positional constraints to toxicity than sEH-dependent epoxide toxicity. Additionally, our limited data set suggests that if additional olefins are present, toxicity is optimized if at least one carbon separates the epoxide and olefin, suggesting that the rigidity of the molecule may influence the toxic event.

The mode of toxic action elicited by epoxy fatty acids in the presence of sEH remains elusive. However, since

the epoxides are not toxic unless hsEH is present, this suggests an integral role for the *vic*-diol itself in toxicity. It has been shown that ltx and iltx slow mitochondrial respiration, and it has been suggested that this occurs through an uncoupling of oxidative phosphorylation (*6*, *7*, *33*). It has also been shown that ltxd and iltxd reduce the rate of basal oxygen consumption and completely inhibit ouabain-sensitive oxygen consumption (*11*). Ouabain is a specific inhibitor of the mitochondrial $Na^+/K^+$-ATPase activity and active $Na^+$ transport. A great deal of work has been performed investigating the uncoupling of oxidative phosphorylation by free fatty acids. One hypothesis put forth in these studies suggests that fatty acids act as protonophores by transbilayer movement of an undissociated fatty acid in one direction and its anion in the other (*30*, *34*, *35*). Within this body of work, 18-carbon poly-unsaturated compounds have been identified as the most effective uncouplers (*30*). It seems possible that a fatty acid diol could act similarly as a protonophore; however, one would expect the presence of the diols to impede transbilayer migration, leading to a decrease in potency, rather than the observed increase. In addition, the tested epoxy amide would be uncharged at physiological pH, suggesting it to be a poor proton transport agent. However, this compound exhibits the same hsEH-dependent toxicity as the free fatty acids, again suggesting that the activated compounds are not acting through an independent proton shuttling mechanism. The possibility exists, though we have no knowledge of such an enzyme, that an endogenous esterase or amidase in the Sf-21 cells may transform the amide to a free carboxylic acid during the course of the assay.

Free fatty acids have also been shown to be responsible for "loose coupling", which is characterized by a lack of respiratory control and a reduced ability to synthesize ATP (*30*, *36*). As with uncoupling, and the protonophoric action of free fatty acids, it is interesting to note that the most potent loose couplers were also 18-carbon poly-unsaturated free fatty acids (*36*−*38*). More recently, long-chain fatty acid-induced uncoupling of oxidative phosphorylation due to induction of the mitochondrial permeability transition (MPT) pore has been revisited (*39*, *40*). The MPT pore is an oligomeric assemblage that, when constructed, yields a high-mass permeable pore between the cytosol and the inner mitochondrial space stimulating a signal transduction cascade, which ultimately leads to cell death. These recent reports suggest that long-chain fatty acids directly interact with the pore assemblage (*39*) by binding to the ADP/ATP carrier and possibly stabilizing the cytosolic confirmation/component of the pore (*40*). This biological target would appear to be a promising focus for future investigations into the mechanism of sEH-mediated epoxy fatty acid cytotoxicity.[4]

Finally, it is important to note that the reported $LC_{50}$ values are relatively high, far higher than a receptor-mediated mechanism would suggest. It must be remembered, however, that we are reporting the concentration of compounds added to a test plate well containing a population of cells, not the final concentration within each cell. It is entirely possible that the final concentration reaching the cell is much lower, because of nonspecific binding to the 24-well plate, or albumin in the media or the exterior of the cell. Given that, it is not possible to completely rule out a receptor-based mechanism, i.e., a toxic mechanism involving a specific cellular target, and we feel very strongly that further research should be done in this area.

**Acknowledgment.** J.F.G. and J.W.N. were supported in part by NIEHS Training Grants ES07059. K.C.W. was supported in part by a Society of Toxicology/Procter & Gamble graduate student fellowship. This work was supported in part by NIEHS Grant ES02710, NIEHS Superfund Grant ES04699, and NIEHS Center for Environmental Health Research Grant ES05707.

**Supporting Information Available:** Complete $^1H$ NMR and GC/MS data for the selected compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Hayakwa, M., Kosaka, K., Sugiyama, S., Yokoo, K., Aoyama, H., Izawa, Y., and Ozawa, T. (1990) Proposal of leukotoxin, 9,10-epoxy-12-octadecenoate, as a burn toxin. *Biochem. Int.* **21**, 573−579.

(2) Ozawa, T., Hayakawa, M., Kosaka, K., Sugiyama, S., Ogawa, T., Yokoo, K., Aoyama, H., and Izawa, Y. (1990) Leukotoxin, 9,10-epoxy-12-octadecenoate, as a burn toxin causing adult respiratory distress syndrome. *Adv. Prostaglandin, Thromboxane, Leukotriene Res.* **21**, 569−572.

(3) Ishizaki, T., Shigemori, K., Nakai, T., Miyabo, S., Ozawa, T., Chang, S. W., and Voelkel, N. F. (1995) Leukotoxin, 9,10-epoxy-12-octadecenoate, causes edematous lung injury via activation of vascular nitric oxide synthase. *Am. J. Physiol.* **269**, L65−L70.

(4) Jia-Ning, H., Taki, F., Sugiyama, S., Asai, J., Izawa, Y., Satake, T., and Ozawa, T. (1988) Neutrophil-derived epoxide, 9,10-epoxy-12-octadecenoate, induces pulmonary edema. *Lung* **166**, 327−337.

(5) Demling, R. H. (1995) The modern version of adult respiratory distress syndrome. *Annu. Rev. Med.* **46**, 193−202.

(6) Ozawa, T., Hayakawa, M., Takamura, T., Sugiyama, S., Suzuki, K., Iwata, M., Taki, F., and Tomita, T. (1986) Biosynthesis of leukotoxin, 9,10-epoxy-12-octadecenoate, by leukocytes in lung lavages of rat after exposure to hyperoxia. *Biochem. Biophys. Res. Commun.* **134**, 1071−1078.

(7) Hayakawa, M., Sugiyama, S., Takamura, T., Yokoo, K., Iwata, M., Suquki, K., Taki, F., Takahashi, S., and Ozawa, T. (1986) Neutrophils biosynthesize leukotoxin, 9,10-epoxy-12-octadecenoate. *Biochem. Biophys. Res. Commun.* **137**, 424−430.

(8) Fukushima, A., Hayakawa, M., Sugiyama, S., Ajioka, M., Ito, T., Satake, T., and Ozawa, T. (1988) Cardiovascular effects of leukotoxin (9,10-epoxy-12-octadecenoate) and free fatty acids in dogs. *Cardiovasc. Res.* **22**, 213−218.

(9) Borhan, B., Mebrahtu, T., Nazarian, S., Kurth, M. J., and Hammock, B. D. (1995) Improved radiolabeled substrates for soluble epoxide hydrolase. *Anal. Biochem.* **231**, 188−200.

(10) Moghaddam, M. F., Grant, D. F., Cheek, J., Greene, J. F., Williamson, K. C., and Hammock, B. D. (1997) Bioactivation of leukotoxins to their toxic diols by epoxide hydrolase. *Nat. Med.* **3**, 562−566.

(11) Moran, J. H., Weise, R., Schnellmann, R. G., Freeman, J. P., and Grant, D. F. (1997) Cytotoxicity of linoleic acid diols to renal proximal tubular cells. *Toxicol. Appl. Pharmacol.* **146**, 53−59.

(12) Gunstone, F. D., and Schuler, H. R. (1975) Fatty acids, part 45. Epoxyoctadecenoates, dihydroxyoctadecenoates, and diepoxyoctadcanoates: preparation, chromatographic properties, and reaction with boron trifluoride etherate. *Chem. Phys. Lipids* **15**, 174−188.

(13) Blee, E., and Shuber, F. (1990) Efficient epoxidation of unsaturated fatty acids by a hydroperoxide-dependent oxygenase. *J. Biol. Chem.* **265**, 12887−12894.

(14) Murray, R. W. (1989) Chemistry of Dioxiranes. 12. Dioxiranes. *Chem. Rev.* **89**, 1187−1201.

(15) Murray, R. W., and Singh, M. (1997) Synthesis of epoxides using dimethyldioxirane: *trans*-stilbene oxide. In *Organic Synthesis* (Shinkai, I., Ed.) pp 91−100, John Wiley & Sons, New York.

(16) Rebek, J. (1981) Progress in the development of new epoxidation reagents. *Heterocycles* **15**, 517−545.

(17) Halarnkar, P. P., Wixtrom, R. N., Silva, M. H., and Hammock, B. D. (1989) Catabolism of epoxy fatty esters by the purified epoxide hydrolase from mouse and human liver. *Arch. Biochem. Biophys.* **272**, 226−236.

(18) Kuranova, I. L., and Balykina, L. V. (1978) Determination of the *threo* and *erythro* configurations of vicinal diastereomeric deriva-

tives of higher fatty acids with the aid of PMR spectra. *Chem. Nat. Compd.* **14**, 247−251.
(19) Itoi, Y., Inoue, M., and Enomoto, S. (1986) Epoxidation of fatty acid esters with aqueous hydrogen peroxide in the presence of molybdenum oxide-tributyltin chloride on charcoal catalyst. *Bull. Chem. Soc. Jpn.* **59**, 3941−3943.
(20) Fahlstadius, P. (1988) *cis*-9,10-Epoxystearic acid in human leukocytes: Isolation and quantitative determination. *Lipids* **23**, 1015−1018.
(21) Roe, E. T., Scanlan, J. T., and Swern, D. (1949) Chemistry of epoxy compounds. IX. Epoxidation of oleamide and n-substituted oleamides with peracetic acid. *J. Am. Chem. Soc.* **71**, 2219−2220.
(22) Grant, D. F., Greene, J. F., Pinot, F., Borhan, B., Moghaddam, M. F., Hammock, B. D., McCutchen, B., Ohkawa, H., Luo, G., and Guenthner, T. M. (1996) Development of an *in situ* toxicity assay system using recombinant baculoviruses. *Biochem. Pharmacol.* **51**, 503−515.
(23) Nguyen, N., and Tukey, R. (1997) Baculovirus-directed expression of rabbit UDP-glucuronosyl transferases in *Spodoptera frugiperda* cells. *Drug Metab. Dispos.* **25**, 745−749.
(24) Charles, J.-P., Wojtasek, H., Lentz, A. J., Thomas, B. A., Bonning, B. C., Palli, S. R., Parker, A. G., Dorman, G., Hammock, B. D., Prestwich, G. D., and Riddiford, L. M. (1996) Purification and reassessment of ligand binding by the recombinant, putative juvenile hormone receptor of the Tobacco Hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol.* **31**, 371−393.
(25) Gonzalez, F. J., Kimura, S., Tamura, S., and Gelboin, H. V. (1991) Expression of mammalian cytochromes P450 using baculovirus. *Methods Enzymol.* **206**, 93−99.
(26) Jerina, D. M., and Daly, J. W. (1974) Arene oxides: a new aspect of drug metabolism. *Science* **185**, 573−582.
(27) Gill, S. S., and Hammock, B. D. (1980) Distribution and properties of a mammalian soluble epoxide hydrase. *Biochem. Pharmacol.* **29**, 389−395.
(28) Ota, K., and Hammock, B. D. (1980) Cytosolic and microsomal epoxide hydrolases: differential properties in mammalian liver. *Science* **207**, 1479−1481.
(29) Zeldin, D. C., Kobayashi, J., Falck, J. R., Winder, B. S., Hammock, B. D., Snapper, J. R., and Capdevila, J. H. (1993) Regio- and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase. *J. Biol. Chem.* **268**, 6402−6207.
(30) Wojtczak, L., and Schonfeld, P. (1993) Effect of fatty acids on energy coupling processes in mitochondria. *Biochim. Biophys. Acta* **1183**, 41−57.
(31) Halarnkar, P. P., Nourooz-Zadeh, J., Kuwano, E., Jones, A. D., and Hammock, B. D. (1992) Formation of cyclic products from the diepoxide of long-chain fatty esters by cytosolic epoxide hydrolase. *Arch. Biochem. Biophys.* **294**, 586−593.
(32) Moghaddam, M., Motoba, K., Borhan, B., Pinot, F., and Hammock, B. D. (1996) Novel metabolic pathways for linoleic and arachidonic acid metabolism. *Biochim. Biophys. Acta* **1290**, 327−339.
(33) Sakai, T., Ishizaki, T., Ohnishi, T., Sasaki, F., Ameshima, S., Nakai, T., Miyabo, S., Matsukawa, S., Hayakawa, M., and Ozawa, T. (1995) Leukotoxin, 9,10-epoxy-12-octadecenoate, inhibits mitochondrial respiration of isolated perfused rat lung. *Am. J. Physiol.* **269**, L326−L331.
(34) Petrussa, E., Braidot, E., Nagy, G., Vianello, A., and Macri, F. (1992) Electrical potential dissipation induced by free fatty acids in pea stem mitochondria. *FEBS Lett.* **307**, 267−271.
(35) Kang, J. X., Xiao, Y. F., and Leaf, A. (1995) Free, long-chain, polyunsaturated fatty acids reduce membrane electrical excitability in neonatal rat cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3997−4001.
(36) Piper, H. M., Sezer, O., Schwartz, P., Hutter, J. F., and Spieckermann, P. G. (1983) Fatty acid-membrane interactions in isolated cardiac mitochondria and erythrocytes. *Biochem. Biophys. Acta* **732**, 192−203.
(37) Rottenberg, H., and Hashimoto, K. (1986) Fatty acid uncoupling of oxidative phosphorylation in rat liver mitochondria. *Biochemistry* **25**, 1747−1755.
(38) Schonfeld, P., Schild, L., and Kunz, W. (1989) Long-chain fatty acids act as protonophoric uncouplers of oxidative phosphorylation in rat liver mitochondria. *Biochim. Biophys. Acta* **977**, 266−272.
(39) Wieckowski, M. R., and Wojtczak, L. (1998) Fatty acid-induced uncoupling of oxidative phosphorylation is partly due to opening of the mitochondrial permeability transition pore. *FEBS Lett.* **423**, 339−342.
(40) Schonfeld, P., and Bohnensack, R. (1997) Fatty acid-promoted mitochondrial permeability transition by membrane depolarization and binding to the ADP/ATP carrier. *FEBS Lett.* **420**, 167−170.

TX990162C