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Episulfide Inhibitors of Lipoxygenase

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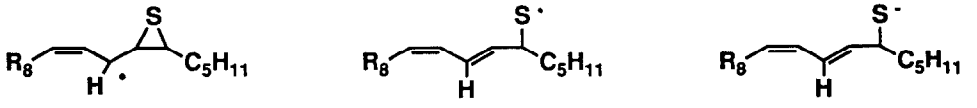
Abstract: A set of epoxide and episulfide substrate mimics were synthesized as inhibitors of soybean lipoxygenase. Enzyme inhibition was observed exclusively with the 12,13-episulfides (**1a** and **4a**), with a high degree of regio- and chemoselectivity. Inhibition by **1a** was shown to occur with reduction of the enzyme active site iron from the catalytically active Fe(III) to the inactive Fe(II).

The enzyme 5-lipoxygenase (5-LO) catalyzes the first step in the oxidation of arachidonic acid to leukotrienes, which are involved in a variety of biological responses such as smooth muscle contraction, increased vascular permeability, and chemotaxis.[1,2] The fatty acid hydroperoxides generated by lipoxygenases are considered to be potentially important in the development of atherosclerotic lesions.[3] Of the 5-lipoxygenase inhibitors known in the literature,[4] those which contain sulfur as the biologically critical structural feature are relatively few. They include 7-thiaarachidonic acid,[5a] disulfiram,[5b] and 1(E),3(Z),6(Z)-petadecenyl (2-carbomethoxy)phenyl sulfide.[5c] Using soybean Type 1 lipoxygenase (SBLO) as a model, we have investigated a new series of lipoxygenase inhibitors in which enzyme inhibition is brought about by interaction of the active site iron with an appropriately substituted episulfide.[5d]

We reasoned that substrate mimic episulfides such as **1a** should also be capable of bringing about lipoxygenase inhibition. Presuming that the substrate binds so as to place the site of oxygenation in proximity to the iron, the polarizable sulfur atom should be ideally situated to interact with the metal center of the soybean enzyme. Coordination of the sulfur atom by the iron atom could activate the episulfide ring towards cleavage (**A**).

1a **A**

On the other hand, hydrogen abstraction at C-11, as is normal for substrate turnover with SBLO, could occur with the allylic episulfide **1a** but not with the saturated analog **4a**. This could result in the formation of the allylic episulfide radical **B** (R_8 = $(CH_2)_7CO_2H$), the pentadienyl thiyl **C**, or the pentadienyl thiolate **D**. Both **B** and **D** would be expected to ultimately afford **C** by episulfide ring opening or oxidation by Fe (III), respectively.



B **C** **D**

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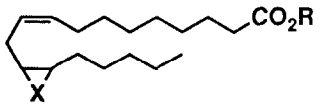
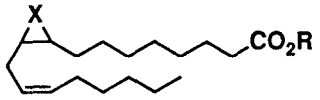
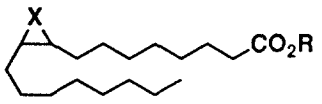
A set of four episulfide substrates was prepared (**1a** - **4a**), as were the corresponding epoxides (**1b** - **4b**). Treatment of methyl linoleate (for **1** and **2**), methyl oleate (for **3**), and methyl 12(Z)-octadecenoate[6] (for **4**) with *m*-CPBA afforded the corresponding epoxides, which upon treatment with KSCN in methanol under reflux gave the episulfides. Careful saponification of the epoxide and episulfide methyl esters with LiOH gave the desired epoxide acids and episulfide acids.[7] The reaction of methyl linoleate with *m*-CPBA gave a mixture of the mono-epoxides **5** and **6**. These were separated by chromatography and unambiguously identified by oxidative cleavage of each epoxide with HIO_4, followed by $NaBH_4$ reduction and GC analysis of the product mixture.[8]



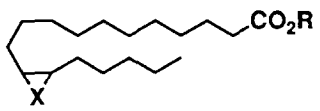
1a: X = S, R = H
1b: X = O, R = H
5: X = O, R = CH_3



2a: X = S, R = H
2b: X = O, R = H
6: X = O, R = CH_3



3a: X = S, R = H
3b: X = O, R = H
7: X = O, R = CH_3



4a: X = S, R = H
4b: X = O, R = H
8: X = O, R = CH_3

Evaluation of these compounds as SBLO inhibitors was carried out by incubation of three concentrations of each with SBLO in a standard competition assay against several concentrations of linoleic acid in 0.1 M pH 9.0 borate buffer (Table 1).[9] Only **1a** and **4a** demonstrated significant inhibition of SBLO. The SBLO shows a remarkable degree of selectivity between the 12,13-episulfides and the 9,10-episulfides. This is particularly striking as SBLO is effectively inhibited by a variety of structurally very diverse agents.[10] However, this selectivity is entirely consistent with the known regioselectivity of SBLO to produce the 13-hydroperoxide under these conditions.[11,12] As anticipated, none of the corresponding epoxides **1b** - **4b** significantly inhibited SBLO.

Table 1: Inhibition of SBLO by Episulfide and Epoxide Fatty Acids

Entry	SBLO K_i	Entry	SBLO K_i
1a	2 μM	1b	> 100 μM
2a	> 100 μM	2b	> 100 μM
3a	> 100 μM	3b	> 100 μM
4a	4 μM	4b	> 100 μM

The reaction of **1a** with SBLO was studied by EPR to elucidate further the mechanism by which **1a** inhibits SBLO. Treatment of ferric SBLO with **1a** resulted in the reduction of the catalytically active Fe(III) to the inactive Fe(II).[13] The saturated analog **4a** did not reduce the ferric enzyme under the same conditions. Treatment of the incubation mixtures with Ellman's reagent did not show significant concentrations of free thiol in the incubation mixture, suggesting that the thiol (**D**), if formed, does not escape the SBLO.

Experimental Section

Methyl 12,13-Epoxyoctadec-9,10(Z)-enoate (5) and Methyl 9,10-Epoxyoctadec-12,13(Z)-enoate (6): A solution of methyl linoleate (2.00 g, 6.8 mmol) in 35 mL of CH_2Cl_2 was cooled to 0° and treated with 1.64 g (7.51 mmol) of 79% *m*-CPBA. The clear solution began to deposit a precipitate almost at once. After 45 minutes the mixture was filtered and the precipitate was washed with hexane. The filtrate was concentrated and chromatographed on silica (25:1 hexane:isopropyl acetate) to afford first 0.582 g (28%) of **5** and then 0.562 g (27%) of **6**. A total of 0.271 g (13%) of the mixed epoxides was also isolated.

Methyl 12,13-epoxyoctadec-9,10(Z)-enoate (**5**): ^{1}H NMR ($CDCl_3$): 5.56-5.37 (m, 2 H); 3.67 (s, 3 H); 2.93 (m, 2 H); 2.37-2.15 (m, 2 H); 2.30 (t, 2 H); 2.03 (m, 2 H); 1.64-1.45 (m, 6 H); 1.36-1.30 (m, 12 H); 0.90 (t, 3 H). MS (CH_4 CI): *m/z* = 311 ($M + H^+$); 293 ($M + H^+ - H_2O$); 279 ($M + H^+ - CH_3OH$); 261 ($M + H^+ - H_2O - CH_3OH$). HRMS: Calc'd for $C_{19}H_{34}O_3$: 310.2508. Found: 310.2537.

Methyl 9,10-epoxyoctadec-12,13(Z)-enoate (**6**):[14] ^{1}H NMR ($CDCl_3$): 5.56-5.37 (m, 2 H); 3.67 (s, 3 H); 2.93 (m, 2 H); 2.37-2.15 (m, 2 H); 2.30 (t, 2 H); 2.03 (m, 2 H); 1.64-1.45 (m, 6 H); 1.36-1.30 (m, 12 H); 0.90 (t, 3 H). MS (CH_4 CI): *m/z* = 311 ($M + H^+$); 293 ($M + H^+ - H_2O$); 279 ($M + H^+ - CH_3OH$); 261 ($M + H^+ - H_2O - CH_3OH$). HRMS: Calc'd for $C_{19}H_{34}O_3$: 310.2508. Found: 310.2534.

Characterization of the Epoxides 5 and 6: Thirty mg of the epoxide was dissolved in 3 mL of dioxane and 2 mL of water and cooled to 0°. HIO_4 crystals were added one at a time with stirring until they dissolved. When no more epoxide was present by TLC analysis, the mixture was diluted with water and Et_2O. The Et_2O was separated, washed with water and brine, filtered through Na_2SO_4, and 2 mL of EtOH was added to the solution with cooling to 0°. $NaBH_4$ (75 mg) was added to the solution which was stirred for 30 minutes. The mixture was diluted with water and Et_2O and the Et_2O was separated, washed with water, brine, filtered through Na_2SO_4, and the solvents were removed in a stream of N_2. The residue was examined by GC (capillary column; initial temp. 50° for 1 min; then +20° min^{-1} to 100° final temp.). Under these conditions authentic 1-hexanol had R_t = 2.24 min; authentic 3(Z)-nonen-1-ol had R_t = 5.22 min. Epoxide **5** gave a signal at R_t = 5.23 min (no signal at 2.24 min); epoxide **6** gave a signal at R = 2.26 min (no signal at 5.22 min).

12,13-Epoxyoctadec-9,10(Z)-enoic Acid (1b): A solution of 0.100 g (0.32 mmol) of **5** in 3 mL of THF and 2 mL of MeOH was treated with 1 mL of 1 M LiOH. After 2 h, the mixture was concentrated and the residue was diluted with water, acidified with 1 M citric acid and extracted with Et_2O. The Et_2O extracts were washed with water, brine, dried and concentrated to give 0. 095 g (100%) of **1b**[15a] as a clear oil. ^{1}H NMR ($CDCl_3$): 5.53-5.37 (m, 2 H); 2.94 (m, 2 H); 2.42-2.27 (m, 1 H); 2.35 (t, 2 H); 2.28-2.13 (m, 1 H); 2.03 (m, 2 H); 1.66-1.58 (m, 2 H); 1.53-1.40 (m, 4 H); 1.34-1.23 (m, 12 H); 0.90 (t, 3 H). MS (CH_4 CI): *m/z* = 297 ($M + H^+$); 279 ($M + H^+ - H_2O$); 261 ($M + H^+ - 2H_2O$). HRMS: Calc'd for $C_{18}H_{32}O_3$: 278.2246. Found: 278.2236.

9,10-Epoxyoctadec-12,13(Z)-enoic Acid (2b): Prepared from 0.100 g (0.32 mmol) of **6** and 1 mL of 1 M LiOH similarly to **1b**, yield 0.097 g (100%) of **2b**[15b] as a clear oil. ^{1}H NMR ($CDCl_3$): 5.57-5.36 (m, 2 H); 2.93 (m, 2 H); 2.42-2.31 (m, 1 H); 2.35 (t, 2 H); 2.27-2.12 (m, 1 H); 2.03 (m, 2 H); 1.66-1.58 (m, 2 H); 1.53-1.40 (m, 4 H); 1.34-1.26 (m, 12 H); 0.89 (t, 3 H). MS (CH_4 CI): *m/z* = 297 (M + H$^+$); 279 (M + H$^+$ - H_2O); 261 (M + H$^+$ - 2H_2O). HRMS: Calc'd for $C_{18}H_{32}O_3$: 278.2246. Found: 278.2242.

12,13-Epithiooctadec-9,10(Z)-enoic Acid (1a): KSCN (0.31 g, 6.8 mmol) was added to a solution of 0.108 g (0.68 mmol) of **5** in 2 mL of MeOH and the mixture was heated under reflux for 24 h. The mixture was cooled, concentrated, and treated with water and Et_2O. The mixture was acidified with 1 M citric acid, the Et_2O was separated and the aqueous phase was extracted with Et_2O. The combined Et_2O extracts were washed with water, brine, dried, and concentrated. The residue was chromatographed on silica (15:1 hexane:ethyl acetate) to give 52.7 mg (50%) of the episulfide methyl ester as a colorless oil. ^{1}H NMR ($CDCl_3$): 5.47 (m, 2 H); 3.66 (s, 3 H); 2.94 (m, 2 H); 2.47 (d of t, 1 H); 2.41 (d of d, 1 H); 2.30 (t, 2 H); 2.04 (m, 2 H); 1.88 (m, 1 H); 1.64-1.47 (m, 5 H); 1.40-1.25 (m, 12 H); 0.91 (t, 3 H). MS (CH_4 CI): *m/z* = 327 (M + H$^+$); 293 (M + H$^+$ - H_2S). HRMS: Calc'd for $C_{19}H_{34}O_2S$: 326.2280. Found: 326.2277. A solution of 52.7 mg (0.16 mmol) of the ester in 3 mL of 2-propanol was treated with 0.48 mL of 1 M LiOH. The reaction was monitored by TLC until all starting material had been consumed, at which point the mixture was concentrated, diluted with water, acidified with 1 M citric acid and extracted with Et_2O. The ethereal solution was washed with water, brine, dried, and concentrated to give 45.4 mg (90%) of **1a** as a colorless oil. ^{1}H NMR ($CDCl_3$): 5.47 (m, 2 H); 2.95 (m, 2 H); 2.47 - 2.39 (m, 1 H); 2.35 (t, 2 H); 2.07 - 2.01 (m, 2 H); 1.89 - 1.85 (m, 1 H); 1.66-1.47 (m, 6 H); 1.35-1.23 (m, 12 H); 0.91 (t, 3 H). MS (EI): *m/z* = 312 (M$^+$); 280 (M$^+$ - S). HRMS: Calc'd for $C_{18}H_{32}O_2S$: 312.2123. Found: 312.2124.

9,10-Epithiooctadec-12,13(Z)-enoic Acid (2a): Prepared from 0.212 g (0.68 mmol) of **6** and KSCN (0.66 g, 6.8 mmol) as for **1a**, yield 124 mg (56%) of the ester as a clear oil. ^{1}H NMR ($CDCl_3$): 5.48 (m, 2 H); 3.67 (s, 3 H); 2.95 (m, 2 H); 2.47 (d of t, 1 H); 2.40 (d of d, 1 H); 2.31 (t, 2 H); 2.04 (m, 2 H); 1.88 (m, 1 H); 1.67-1.43 (m, 5 H); 1.40-1.25 (m, 12 H); 0.89 (t, 3 H). MS (CH_4 CI): *m/z* = 327 (M + H$^+$); 293 (M + H$^+$ - H_2S). HRMS: Calc'd for $C_{19}H_{34}O_2S$: 326.2280. Found: 326.2286. The acid was prepared from 0.124 g (0.38 mmol) of the ester and 1.14 mL of 1 M LiOH in the same manner as for **1a** to give 0.0983 g (83%) of **2a** as a clear oil. ^{1}H NMR ($CDCl_3$): 5.48 (m, 2 H); 2.95 (m, 2 H); 2.47 - 2.40 (m, 1 H); 2.36 (t, 2 H); 2.08 - 2.01 (m, 2 H); 1.91 - 1.86 (m, 1 H); 1.67-1.43 (m, 6 H); 1.38-1.22 (m, 12 H); 0.89 (t, 3 H). MS (EI): *m/z* = 312 (M$^+$); 280 (M$^+$ - S). HRMS: Calc'd for $C_{18}H_{32}O_2S$: 312.2123. Found: 312.2124.

12,13-Epoxyoctadecanoic acid (4b): Prepared from 0.800 g (2.70 mmol) of methyl octadec-12(Z)-enoate and 0.698 g (3.19 mmol) of *m*-CPBA similarly to **5** and **6**. Yield of epoxide ester **8**, 0.83 g (98%), mp 31° (lit. 31°).[16] ^{1}H NMR ($CDCl_3$): 3.67 (s, 3 H); 2.90 (m, 2 H); 2.30 (t, 2 H); 1.64 - 1.59 (m, 2 H); 1.57 - 1.39 (m, 6 H); 1.34 - 1.28 (m, 18 H); 0.90 (t, 3 H). MS (NH_3 CI): *m/z* = 313 (M + H$^+$); 330 (M + NH_4^+). HRMS: Calc'd for $C_{19}H_{36}O_3$ 312.2664. Found: 312.2652. The acid was prepared from 0.100 g (0.32 mmol) of the ester **8** and 1 mL of 1 M LiOH as for **1b**, yield 0.095 g (100%) of **4b** as a clear oil. ^{1}H NMR ($CDCl_3$): 5.53 - 5.37 (m, 2 H); 2.94 (m, 2 H); 2.42 - 2.27 (m, 1 H); 2.35 (t, 2 H); 2.28 - 2.13 (m, 1 H); 2.03 (m, 2 H); 1.66 - 1.58 (m, 2 H); 1.53 - 1.40 (m, 4 H); 1.34 - 1.23 (m, 12 H), 0.90 (t, 3 H). MS (CH_4 CI): 297 (M + H$^+$); 279 (M + H$^+$ - H_2O). HRMS: Calc'd for $C_{18}H_{30}O_2$ (M + H$^+$ - H_2O): 278.2236. Found: 278.2246.

12,13-Epithiooctadecanoic acid (4a): Prepared from 0.455 g (1.45 mmol) of **8** and KSCN (1.41 g, 14.5 mmol) as for **1a**. Yield of ester: 0.24 g (50%) of a colorless oil. ^{1}H NMR ($CDCl_3$): 3.67 (s, 3 H); 2.93 (m, 2 H); 2.30 (t, 2 H); 1.87 - 1.76 (m, 2 H); 1.64 - 1.57 (m, 2 H); 1.51 - 1.42 (m, 4 H); 1.35 - 1.28 (m, 18 H); 0.90 (t, 3 H). MS (NH_3 CI): *m/z* = 329 (M + H$^+$); 346 (M + NH_4^+). HRMS: Calc'd for $C_{19}H_{36}O_2S$: 328.2436. Found: 328.2437. The acid was prepared from 0.24 g (0.73 mmol) of the ester and 2.19 mL of 1 M LiOH as for **1a**, yield 0.210 g (92%) of **4a** as white crystals, mp 67° - 69°. ^{1}H NMR ($CDCl_3$): 2.95 (m, 2 H); 2.35 (t, 2 H); 1.87 - 1.79 (m, 2 H); 1.66 - 1.59 (m, 2 H); 1.51 - 1.43 (m, 4 H); 1.40 - 1.29 (m, 18 H); 0.90 (t, 3 H). MS (NH_3 CI): *m/z* = 315 (M + H$^+$); 332 (M + NH_4^+), 300 (M + NH_4^+ - S). Analysis: Calc'd for $C_{18}H_{34}O_2S$: C 68.74%; H 10.90%. Found: C 68.96%; H 10.94%.

9,10-Epoxyoctadecanoic acid (3b): Prepared from 1.29 g (5.9 mmol) of 79% *m*-CPBA and 1.50 g (5 mmol) of methyl oleate similarly to **5** and **6**. Yield of epoxide ester **7**, 1.57 g (100%) mp 23° (lit. 24°).[17] The acid was prepared from 0.100 g (0.32 mmol) of the ester **7** and 1 mL of 1 M LiOH as for **1b**, yield 0.091 g (99%), mp 57° (lit. 57°).[18]

9,10-Epithiooctadecanoic Acid (3a): Prepared from 0.625 g (2 mmol) of **7** and 1.94 g (20 mmol) of KSCN as for **1a**, yield of ester: 0.32 g (49%) of a colorless oil.[19] ^{1}H NMR ($CDCl_3$): 3.67 (s, 3 H); 2.97 - 2.95 (m, 2 H); 2.31 (t, 2 H); 1.87 - 1.81 (m, 2 H); 1.65 - 1.58 (m, 2 H); 1.54 - 1.42 (m, 4 H); 1.33 - 1.27 (m, 18 H); 0.88 (t, 3 H). MS (NH_3 CI): *m/z* = 329 (M + H$^+$); 346 (M + NH_4^+); 314 (M + NH_4^+ - S). HRMS: Calc'd for $C_{19}H_{36}O_2S$: 328.2436. Found: 328.2437. The acid was prepared from 0.200 g (0.61 mmol) of the ester and 1.83 mL of 1 M LiOH, yield 0.185 g (96%), mp 58° (lit. 57°).[20]

Soybean Lipoxygenase. Isozyme 1 was purified as previous described;[21] the specific activity was 220 U/mg. Assays were performed at 25 °C in 0.05 M borate buffer, pH 9.0, following the production of hydroperoxide by the change in absorbance at 234 nm (ε = 23600 M^{-1} cm^{-1}). Values for K_i were estimated from analysis of double reciprocal plots (1/velocity vs. 1/[substrate]) at three concentrations of inhibitor. The inhibitors were prepared as concentrated solutions in ethanol; the final concentration of ethanol in all assays was 0.2 M.

Preparation of EPR Samples and Spectroscopy. Samples of ferrous lipoxygenase were oxidized to the ferric state by addition of 13-hydroperoxy-9,11-octadecadienoic acid and dialyzed against one change of 1000 volumes of 0.05 M pH 9.0 borate buffer. Aliquots of the enzyme (250 µL, approximately 0.1 mM) were placed in EPR tubes, seven equivalents of the inhibitor were added, and the tube was gently agitated to mix the solution. The sample was frozen in liquid nitrogen after approximately 30 s. EPR spectra were obtained at X-band at 5K as previously described.[22]

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6. This compound was prepared from methyl 12-hydroxydodecanoate (Lycan, W. H., Adams, R. *J. Am. Chem. Soc.* **1929**, *51*, 628): (a) DMSO, ClCOCOCl, Et_3N, CH_2Cl_2, -60°, 98% (DNP mp 69°-70°); (b) $C_6H_{13}P(C_6H_5)_3Br$, KO-*t*-Bu, THF, 20°, 68% (bp 180°/0.8 Torr). ^1H NMR ($CDCl_3$): 5.38 (m, 2 H); 3.66 (s, 3 H); 2.32 (t, 2 H); 2.04 (m, 4 H); 1.63 (m, 2 H); 1.38-1.22 (m, 20 H); 0.89 (t, 3 H). MS (CH_4 CI): m/e = 297 ($M + H^+$); 265 ($M + H^+ - CH_3OH$). Analysis: Calc'd for $C_{19}H_{36}O_2$: C, 76.97%; H 12.24%. Found: C, 76.85%; H 12.52%. GC analysis of this product revealed it to contain less than 2% of the E-isomer.
7. No cleavage of the epoxides or episulfides by LiOH was detected by 300 MHz NMR or UV following the saponification.
8. The ^1H NMR and CI mass spectra of **5** and **6** were virtually indistinguishable.
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