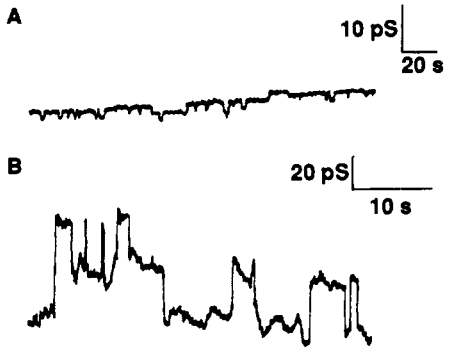

Figure 2. Amphotericin B ion channels in racemic glycerol monooleate with cholesterol. (A) Cholesterol, 5% in glycerol monooleate, 2×10^{-8} M amphotericin B, 2 M KCl, 0.1 M HEPES buffered to pH 7.0, 120 mV. (B) *ent*-Cholesterol, 5% in glycerol monooleate, 2×10^{-7} M amphotericin B, 2 M KCl, pH 7.0, 120 mV. Membranes were formed as in Figure 1. All records were filtered at 20 Hz.

nicely accounts for the sterol requirement[17] in ion-channel formation, and sterol binding provides a rational explanation for the greater sensitivity of ergosterol-containing fungal cells than of cholesterol-containing mammalian cells. A good deal of circumstantial evidence supports this model,[18] but there is no direct evidence that distinguishes between ion-channel formation mediated by sterol modification of membrane properties and sterol binding with amphortericin B. This distinction is significant because the two models lead to different strategies for increasing the therapeutic index of amphotericin B.

We have found that amphotericin B forms different ion channels in the presence of natural cholesterol and *ent*-cholesterol. We measured two-sided, single-channel conductances in black lipid membranes using soy azolecithin containing 5% cholesterol or *ent*-cholesterol.[19] The initial experiments were carried out using coded samples of cholesterol and its enantiomer to avoid operator bias. Single channels of 1-3 pS conductance were observed in natural cholesterol membranes at an amphotericin B concentration of 2×10^{-8} M, in accord with previous reports.[20] Membranes containing *ent*-cholesterol did not support any ion channels at these amphotericin B concentrations, but ion channels were observed at a 10-fold higher amphotericin B concentration. These new ion channels had a much higher conductance, 30-35 pS, than those formed in the presence of natural cholesterol (Figure 1). At this amphotericin B concentration, natural cholesterol membranes show higher bulk conductances than *ent*-cholesterol membranes. Membranes without sterols did not form ion channels, even at 10-fold higher amphotericin B concentration than required with *ent*-cholesterol membranes. Amphotericin B samples obtained commercially and those purified to homogeneity by C8 reverse-phase MPLC gave the same results, demonstrating that amphotericin B is required for both ion channels.

Soy azolecithin is composed of a mixture of enantiomerically pure chiral phospholipids that form diastereomeric membranes when combined with natural cholesterol and *ent*-cholesterol. Previous studies suggest that the lipid chiral center does not affect membrane properties; indeed membranes composed of natural cholesterol and the two enantiomers of dioleoyllecithin are indistinguishable.[21] We tested membranes prepared from soy azolecithin and the two enantiomers of cholesterol by comparing their ability to support gramicidin ion channels. Gramicidin A forms identical ion channels in membranes with 5% natural cholesterol or *ent*-cholesterol in soy azolecithin, demonstrating that gramacidin ion channels do not bind cholesterol,[22] and that the two diastereometric membranes are indistinguishable in this simple test.

Studies with racemic lipids confirm the stereochemical dependence of ion-channel formation. Synthetic, racemic glycerol monooleate was used to prepare membranes with 5% natural cholesterol or *ent*-cholesterol. In accord with the soy azolecithin membrane studies, purified amphotericin B produced low-conductance channels with natural cholesterol and high conductance channels with *ent*-cholesterol (Figure 2). The two glycerol monooleate membranes are exact mirror images of each other and have identical physical properties, so the differences observed cannot be attributed to macroscopic membrane properties.

Amphotericin B produces different ion channels in the presence of natural cholesterol or *ent*-cholesterol, and the distinction cannot be attributed to differences in membrane properties. Amphotericin B specifically binds the enantiomers of cholesterol, thus producing diastereomeric ion channels that have measurably different properties. This is the first direct proof that amphotericin B binds to cholesterol *in the ion-channel structure*. Cholesterol plays a vital role in biochemical systems throughout the body, and *ent*-cholesterol will be a valuable new probe to explore its function.

Acknowledgment. Support has been provided by the National Institutes of Health (R01 GM43854), the Searle Scholars Program (S.D.R.), and the Camille and Henry Dreyfus Teacher-Scholars Foundation (S.D.R.). Amphotericin B was a generous gift from Squibb Pharmaceuticals (Princeton, NJ).

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Terminal Difluoro Olefin Analogues of Squalene Are Time-Dependent Inhibitors of Squalene Epoxidase

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Squalene epoxidase (EC 1.14.99.7) catalyzes the conversion of squalene to (3*S*)-2,3-oxidosqualene,[1] an essential step in the biosynthesis of sterols in mammals, plants, and microorganisms. The chemical and kinetic mechanisms of squalene epoxidase are not known, but the enzyme requires O_2, NADPH, and FAD for full activity;[2] it is not a cytochrome P-450.[3,4] Many reversible squalene epoxidase inhibitors have been described;[5-8] some are useful antifungal[6,7] and hypolipidemic[8] agents. We report the time-dependent inhibition of squalene epoxidase from rat liver

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Scheme I

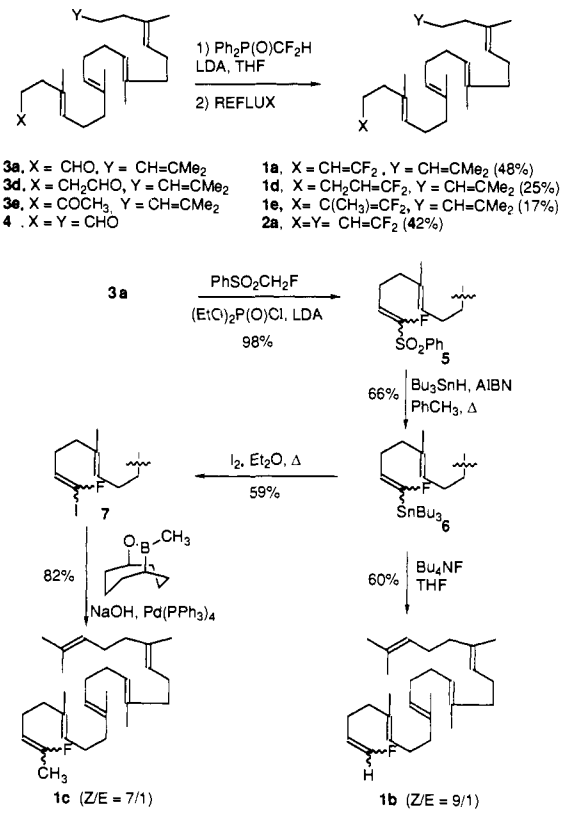
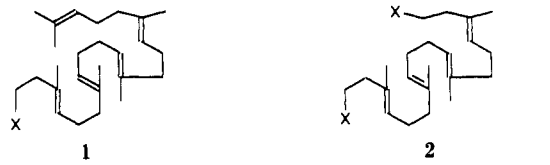


Table I. Inhibition of Squalene Epoxidase by Fluorinated Squalene Analogues



compd	X	IC_{50} (μM)
1a	$HC{=}CF_2$	5.4
1b	$HC{=}CHF^a$	100
1c	$HC{=}C(CH_3)F^b$	47
1d	$H_2CHC{=}CF_2$	>100
1e	$(CH_3)C{=}CF_2$	>100
2a	$HC{=}CF_2$	4.5
2b	$HC{=}CH_2$	>100

[a] Isomeric mixture consisted of 9:1 *Z*/*E* configurations. [b] Ratio of isomers was 7:1 *Z*/*E*.

by difluoro olefins **1a** and **2a** and structure–activity data that is consistent with mechanism-based inhibition.

Recent methods used to prepare difluoro[9] and monofluoro[10] olefins were used for the synthesis of **1a**, **1b**, **1d**, **1e**, and **2a** (Scheme I). The synthesis of **1c** utilized the Suzuki reaction,[11] and this reaction sequence provides a new route to 1-alkyl-1-fluoro olefins.

The IC_{50} values for the inhibition of rat liver squalene epoxidase by the mono- and difluoro olefin analogues of squalene are presented in Table I.[12] It is important to note that inhibition by

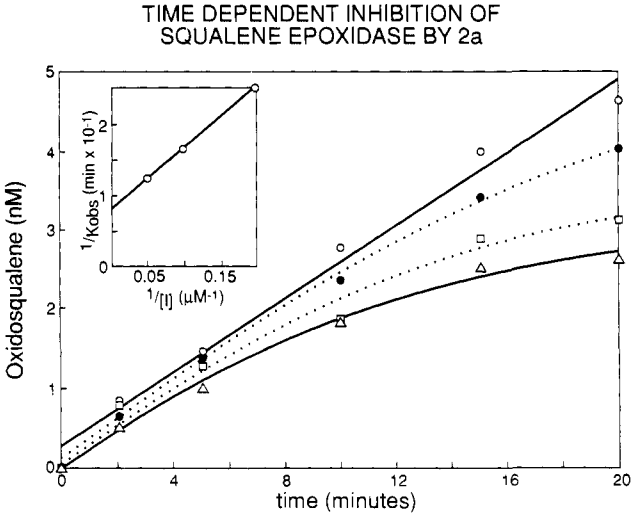


Figure 1. Rates of enzyme-catalyzed squalene epoxide formation (37 °C, pH 7.4) in the presence of (○) 0, (●) 5, (□) 10, and (△) 20 μM **2a**. $[S]_0$ was 10 μM and the reaction started by the addition of enzyme. The fitting of these curves to a reiterative least-squares analysis yields a solution of the pseudo-first-order rate constant of inactivation (K_{obsd}).[14] Inset: The slope (m) of the replot of $1/K_{obsd}$ versus $1/[I]$ yields a graphical solution of k_{inact} and K_i according to $m = K_i(1 + [S]/K_m)/k_{inact}$ and $b = 1/k_{inact}$ (and K_m = 39 μM).

the symmetrical tetrafluoro analogue **2a** establishes that the difluoro olefin terminus is accepted by the enzyme. Of the compounds listed in Table I, only **1a** and **2a** are time-dependent inhibitors of squalene epoxidase as determined by (1) preincubation of the enzyme (and its cofactors) with the inhibitor and measurement of residual enzyme activity as a function of preincubation time or (2) measurement of the first-order inactivation of the enzyme by inhibitor in the presence of substrate and cofactors.[13a-d] The inactivation constants, K_i and k_{inact}, were determined by method 2 (Figure 1); these parameters are 4 μM and 0.16 min^{-1} and 8 μM and 0.12 min^{-1} for compounds **1a** and **2a**, respectively.

Precise orientation of the difluoro olefin group within the active site is indicated by the fact that the homomethylene analogue **1d** and sterically-hindered analog **1e** did not inactivate the enzyme. The difluoro olefin functionality is necessary for the inactivation of squalene epoxidase,[15] and there is no chemical precedent to suggest affinity labeling of the enzyme by **1a** and **2a**. The time-dependent inhibition, protection by substrate against inactivation, and structure–activity data suggest that **1a** and **2a** are activated by the enzyme, generating a reactive functionality that modifies the epoxidase before diffusing away.[16]

We are currently using radiolabeled **1a** and a solubilized enzyme preparation to investigate the mechanism of enzyme inactivation; these experiments should help to elucidate cofactor requirements and the sequence of events occurring at the active site of squalene epoxidase during catalytic turnover.

Supplementary Material Available: Experimental procedures and spectral data for compounds **1a–e**, **2a**, and **2b** and experimental procedures for enzyme isolation and assays (12 pages). Ordering information is given on any current masthead page.

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(14) Performed on an HP-88 computer with DAES software purchased from R&L Software, Newton, MA.

(15) The *gem*-dichloro and *gem*-dibromo squalene derivatives analogous to **1a** and **2a** have been reported by others, but these compounds were not inhibitors of hog liver squalene epoxidase at concentrations up to 400 μM (see ref 5c).

(16) The presence of 500 μM dithiothreitol in the medium does not prevent inactivation, suggesting that a highly reactive species does not diffuse away from the active site and covalently modify the enzyme elsewhere.