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26-HYDROXYSQUALENE AND DERIVATIVES:

SUBSTRATES AND INHIBITORS FOR SQUALENE EPOXIDASE

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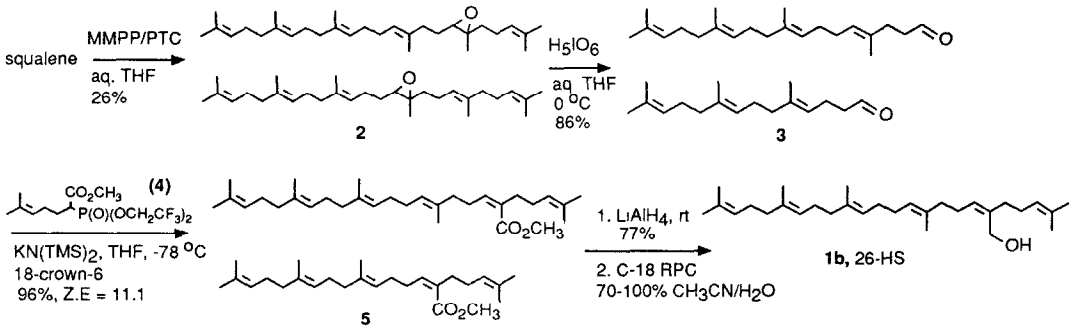
Summary: 26-Hydroxysqualene (26-HS) was synthesized from squalene and shown to be a potent competitive inhibitor (K_I = 4 μM) of partially-purified pig liver squalene epoxidase (SE). The corresponding aldehyde, amine and diazoacetate derivatives were also evaluated as SE inhibitors. The [^{3}H]26-HS was converted to a mixture of regioisomeric 2,3-epoxides bearing either a 26-hydroxyl or a 29-hydroxyl group.

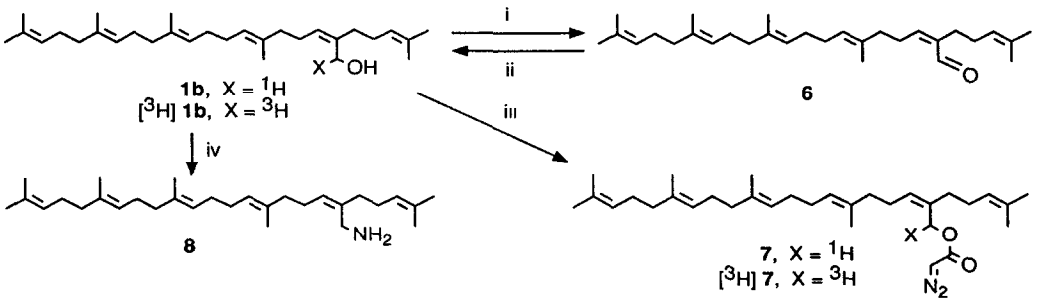
Squalene epoxidase (SE) (EC 1.14.99.7) catalyzes the conversion of squalene to (3*S*)-2,3-oxidosqualene.[1] In addition to squalene, the purified enzyme requires FAD, NADPH, oxygen, NADPH-cytochrome P-450 reductase, and either detergent or a soluble protein cofactor for full enzymatic activity[2]. Recently, we[3] and others[4] have reported several squalene-derived compounds as potent, selective, reversible inhibitors of vertebrate SE. The most potent compounds were trisnorsqualene alcohol (TNSA)[5] and the corresponding trisnorsqualene cyclopropylamine[6]. Examination of a variety of functional group derivatives[5] and extended and truncated chains[7] demonstrated very strict structural constraints for the squalene binding site of SE. As a continuation of our program to prepare affinity labels for the characterization of the squalene binding site of SE, we have investigated a number of hydroxyl-substituted acyclic isoprenoids and their derivatives as potential inhibitors of SE. We describe herein the first example of a potent inhibitor of SE which is also a substrate for the enzyme.

26-Hydroxysqualene (**1b**) was synthesized as illustrated in Scheme I. Oxidation of squalene in aqueous THF with magnesium monoperphthalic acid gave a 26% yield, after chromatography, of an equimolar mixture of the 6,7- and 10,11-epoxysqualenes (**2a,b**). This mixture was converted in 86% yield to the corresponding truncated aldehydes (**3a,b**) by hydration-cleavage process using hydroiodic acid in aqueous THF. An analogous internal-epoxide selective epoxidation followed by periodate cleavage sequence had been previously reported by Ceruti[8]. The aldehyde mixture **3a,b** was then condensed with the anion of phosphonate **4** under conditions developed by Still and Gennari[9] to give a mixture of α,β-unsaturated esters **5a,b** in 96% isolated yield (*Z:E* = 11:1). Reduction of this mixture of esters with lithium aluminum hydride afforded, after SiO_2 chromatography, a mixture of allylic alcohols **1a,b** in 77% yield. Separation of the isoprenologous alcohols was readily achieved on octadecylsilyl-modified silica gel (30 μm particle size) by elution with a gradient of 70% to 100% acetonitrile in water to give the desired 26-hydroxysqualene (26-HS) **1b**.



Scheme I. Synthesis of 26-hydroxysqualene.

In order to probe the functional group selectivity of the SE active site, we also prepared the aldehyde **6**, the diazoacetate **7** (a potential photoaffinity label), and the primary amine **8** as shown in Scheme II[10]. Moreover, [^{3}H]26-HS was prepared from the aldehyde **6** by sodium borotritide reduction in ethanol to give a product [^{3}H]**1b** with specific activity 16 Ci/mmol. This latter material was used to determine whether 26-HS was enzymatically processed by SE; in previous studies, we had shown that the trisnorsqualene derivatives were *not* epoxidized.



Scheme II. Synthesis of 26-functionalized squalenes and [^{3}H]26-HS. Reagents and conditions: (i) MnO_2, Na_2CO_3; (ii) NaB^3H_4, ethanol; (iii) TsNHN=CCOCl, $PhNMe_2$; then Et_3N; (iv) EtOOCN=NCOOEt, PPh_3, phthalimide; then NH_2NH_2, ethanol.

Squalene epoxidase assays were performed using partially-purified SE obtained by DEAE and Blue Sepharose chromatography of Triton X-100-solubilized pig liver microsomes[11]. Radio-TLC was employed to quantify the percentage of [^{14}C]squalene converted to [^{14}C]squalene epoxide during a 50-min incubation following a 10-min pre-incubation with varying concentrations of inhibitors.[12] TNSA was employed as a benchmark inhibitor. The results are summarized in Figure 1.

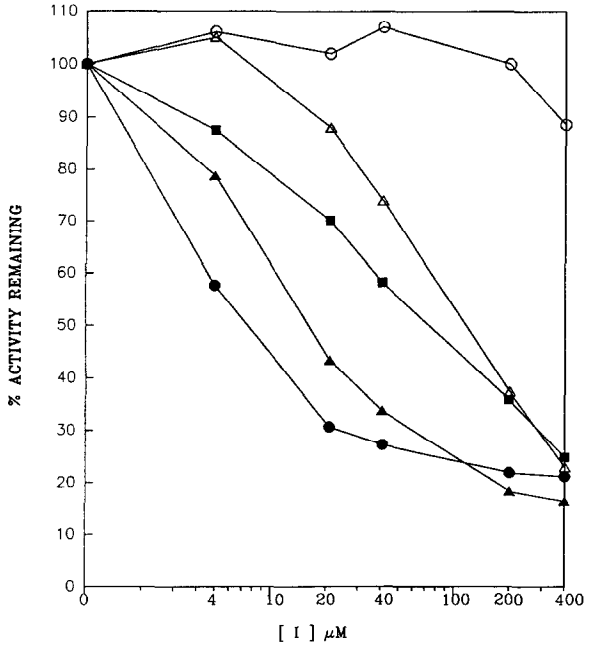
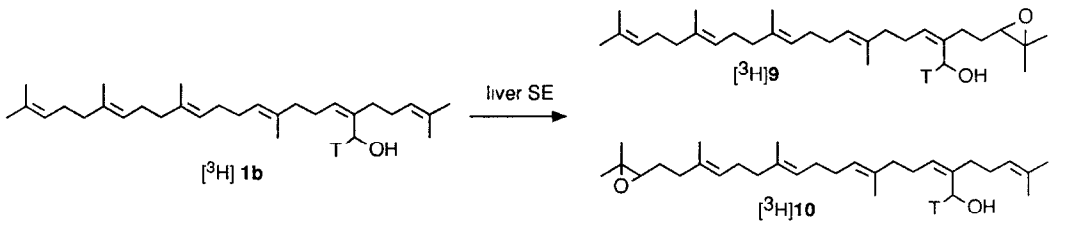


Figure 1. Inhibition of partially-purified SE at pH 7.4 by TNSA (solid circles), 26-HS **1b** (solid triangles), 26-aldehyde **6** (solid squares), 26-diazoacetate **7** (open circles), and 26-amine **8** (open triangles). Conversion of [^{14}C]squalene to the epoxide by partially-purified squalene epoxidase[11] was monitored by radio-TLC.[12]

Using [^{3}H]26-HS (**1b**) instead of [^{14}C]squalene as the substrate, two apparent epoxide products were formed in a 3:1 ratio, as determined from the radio-TLC assay. An independent synthesis of the separate regioisomeric 26-hydroxy-2,3-oxidosqualene **9** and the 29-hydroxy-2,3-oxidosqualene **10**[13] provided standards which co-chromatographed (TLC) with the metabolically-produced [^{3}H]-labeled epoxides and allowed identification of the more polar 26-hydroxy isomer **9** as the favored metabolic product (Scheme III). Each metabolically-produced epoxide was isolated by silica gel chromatography and analyzed by capillary GC as the free alcohol.[14] Again, co-injections confirmed the identities of the two regioisomeric epoxides with the authentic synthetic compounds.



Scheme III. Epoxidation of [^{3}H]26-HS by partially-purified SE.

The strict substrate requirements of squalene epoxidase have complicated preparation of suitable derivatives for affinity chromatography and photoaffinity labeling. Thus, we have undertaken extensive synthetic studies in order to identify positions which can be functionalized without completely eliminating access of the analog to the SE active site. It appears that modification at C-26 (= C-29) is acceptable to the active site, and that a variety of functional groups are tolerated in this position. The hydroxyl was first selected because of the known potency of TNSA; the IC_{50} of 26-HS is about 2.5-fold lower than that of TNSA, but the K_I values are essentially identical.[11] The aldehyde **6**, an intermediate in the synthesis of the vinyl[13] and cyclopropyl compounds (X.-y. Xiao, unpublished results), and the 26-aminosqualene **8** were also assayed for SE inhibition. The 26-aminosqualene analog was prepared for coupling to an affinity chromatography matrix.[15] Finally, the 26-diazoacetate **7** was prepared as a potential photoaffinity label for SE or squalene-hopane cyclases.[16] The differences in SE inhibitory potency presumably reflect steric constraints at the active site as well as a preference for a non-basic functional group.

26-Hydroxysqualene was readily accepted as a substrate by SE and was converted to both regioisomeric epoxides. Using the [^{3}H]-labeled aldehyde **6** and diazoacetate **7**, we also observed conversion to apparently epoxidized products, but complete structural analyses were not performed. It is noteworthy that 26-HS shows kinetics consistent with competitive inhibition[11], as expected for a substrate analog. In contrast, TNSA showed noncompetitive kinetics, and was not epoxidized by SE.

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References and Notes

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10. Spectroscopic data: (**1a**): ^{1}H NMR ($CDCl_3$) δ 1.59 (br s, 12 H), 1.67 (br s, 6 H), 1.93-2.16 (m, 16 H), 4.10 (s, 2 H), 5.05-5.17 (m, 4 H), 5.32 (t, J = 6.8 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 15.91, 16.07, 16.17, 17.55, 17.76, 17.80, 25.76, 26.58, 26.93, 27.00, 27.81, 28.01, 35.21, 39.67, 60.20, 123.94, 124.21, 124.48, 128.31, 128.59, 131.18, 131.61, 134.94, 135.84, 138.49; FT-IR (neat) 3314, 2923, 1670, 1446, 1376, 1008 cm^{-1}.

(**1b**): ^{1}H NMR ($CDCl_3$) δ 1.60 (br s, 15 H), 1.68 (br, 6 H), 1.93-2.22 (m, 20 H), 4.11 (s, 2 H), 5.06-5.20 (m, 5 H), 5.31 (t, J = 7.2 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 15.89, 15.94, 16.01, 16.06, 17.58, 17.64, 17.74, 25.58, 25.70, 26.16, 26.59, 26.72, 26.87, 27.04, 28.20, 35.16, 39.70, 60.24, 124.07, 124.27, 124.84, 124.97, 128.44, 128.57, 131.17, 131.63, 134.50, 134.83, 135.13, 138.26; FT-IR (neat) 3318, 2921, 1670, 1560, 1446, 1377, 1010 cm^{-1}.

(6): ^{1}H NMR ($CDCl_3$) δ 1.56 (s, 3 H), 1.59 (br s, 9 H), 1.61 (s, 3 H), 1.67 (br s, 6 H), 1.93-2.21 (m, 18 H), 2.65 (dt, *J* = 7.8, 7.6 Hz, 2 H), 5.03-5.25 (m, 5 H), 6.43 (t, *J* = 7.8 Hz, 1 H), 10.08 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 15.81, 15.85, 16.08, 17.52, 17.77, 25.03, 25.52, 25.77, 26.58, 26.71, 27.26, 28.07, 28.22, 30.33, 39.69, 123.43, 123.82, 124.17, 125.83, 126.13, 131.17, 132.06, 133.14, 134.86, 135.26, 139.68, 149.10; FT-IR (neat) 2921, 2855, 1677, 1446, 1378, 1146 cm^{-1}.

(7): ^{1}H NMR ($CDCl_3$) δ 1.60 (br s, 15 H), 1.68 (br s, 6 H), 1.93-2.23 (m, 20 H), 4.69 (s, 1 H), 4.74 (br s, 2 H), 5.04-5.20 (m, 5 H), 5.41 (t, *J* = 7.1 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 62.38, 124.24, 214.23; FT-IR (neat) 2920, 2109, 1699, 1442, 1375, 1234, 1177 cm^{-1}.

(8): ^{1}H NMR ($CDCl_3$) δ 1.60 (br s, 15 H), 1.68 (br, 6 H), 1.92-2.19 (m, 20 H), 3.24 (s, 2 H), 5.06-5.24 (m, 6 H). ^{13}C-NMR ($CDCl_3$) δ 16.02, 16.06, 17.66, 17.71, 25.66, 26.29. 26.74, 26.83, 27.06, 27.89, 28.31, 28.48, 35.37, 39.77, 40.10, 40.96, 123.95, 124.27, 124.46, 124.77, 126.26, 131.20, 131.50, 134.71, 134.91, 134.98, 135.19, 135.27, 135.57; FT-IR (neat) 3329, 2020, 2356, 1594, 1442, 1376, 1040 cm^{-1}.

11. The details of the purification of pig liver SE and aspects of its enzymology will be presented elsewhere: Bai, M.; Prestwich, G.D., manuscript in preparation (1991).

12. *Assay Method for Squalene Epoxidase.* The partially-purified SE activity[11] was reconstituted as follows: SE (50 μl, 0.55 mg/ml; enzyme specific activity 0.377 nmol/min/mg) in 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100, pH 7.4, 0.06 unit of NADPH-cytochrome P-450, 843 μM NADPH, 29 μM FAD in total volume of 240 μl. This reconstituted enzyme solution was pre-incubated at 37 °C for 10 min. Then, 15,000 cpm of [^{14}C]-squalene (9300 cpm/nmol) in 1 μl of 2-propanol was added into the mixture and incubation was continued at 37 °C for 20 min or 50 min. The enzymatic reaction was quenched by addition of 240 μl of 10% KOH in methanol and incubated at 37 °C for 30 min. The nonsaponifiable lipids were extracted with 1 ml of methylene chloride by vortexing thoroughly and centrifuging in a bench top centrifuge at 1000 x *g* for 5 min. The organic extracts were evaporated in a Savant Speed-Vac for 30 min. The residue was dissolved in 100 μl of methylene chloride and applied to the preadsorbent layer on a silica gel thin layer plate (Whatman silica gel 60A, 20 x 20 cm, 250 μm layer). The plate was developed with 5% ethyl acetate in hexane. Radioactive regions in each lane were visualized using Bioscan Imaging Scanner System 500-IBM with Autochanger 3000. R_f values were 0.84 for squalene and 0.45 for oxidosqualene.

13. Xiao, X.-y.; Prestwich, G.D., submitted to *J. Am. Chem. Soc.* (1991).

14. Capillary GC separation was achieved on a 25 m x 0.32 mm (1 μm film) HP-1 column; T_i 50 °C (1 min); T_p 20 °C/min; T_f 280 °C (30 min). Retention times: 26-hydroxy-2,3-epoxide **9**, 28.985 min; 29-hydroxy-2,3-epoxide **10**, 28.997 min. TLC (SiO_2, 20% ethyl acetate-hexane): R_f 0.19 (26-isomer **9**); R_f 0.36 (29-hydroxy isomer **10**).

15. An affinity column has been prepared and employed in the purification of liver SE: Bai, M.; Prestwich, G.D., in preparation.

16. Studies on the uses of this photoaffinity label will be presented in due course.