Inhibition Kinetics and Affinity Labeling of Bacterial Squalene:Hopene Cyclase by Thia-Substituted Analogues of 2,3-Oxidosqualene†

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ABSTRACT: Five sulfur-containing analogues of 2,3-oxidosqualene (OS) were evaluated as inhibitors of squalene:hopene cyclase (SHC) from *Alicyclobacillus acidocaldarius*. In these analogues, sulfur replaces carbons at C-6, C-10, C-14, C-18, or C-19 of OS. Each analogue was a submicromolar inhibitor of SHC with IC_{50} values ranging from 60 to 570 nM. Enzyme inhibition kinetic analysis was performed using homogeneous recombinant *A. acidocaldarius* SHC. While analogues **9** (S-14, K_i = 109 nM, k_{inact} = 0.058 min^{-1}) and **11** (S-19, K_i = 83 nM, k_{inact} = 0.054 min^{-1}) were time-dependent inhibitors of SHC, analogues **7** (S-6, K_i = 127 nM) and **8** (S-10, K_i = 971 nM) showed no time dependency with SHC. Analogue **10** (S-18) was the most potent inhibitor and showed time-dependent irreversible inhibition (K_i = 31 nM, k_{inact} = 0.071 min^{-1}). Kinetic analysis for the five analogues with purified rat liver OSLC was conducted to compare the vertebrate and prokaryotic enzymes. Affinity labeling experiments, using either [17-^{3}H]**10** or [22-^{3}H]**10** with crude and with pure recombinant SHC, clearly showed specific labeling. A single major radioactive band at 72 kDa on SDS−PAGE indicated that irreversible covalent modification of SHC had occurred. These results suggest that the presence of sulfur at C-18 of OS can interrupt the cyclization and that an intermediate partially cyclized cation may be captured by a nucleophilic residue of the SHC active site.

The enzymatic cyclizations of squalene **1** and oxidosqualene **2** (OS)[1] convert acyclic polyalkenes to polycyclic products that are precursors of critical membrane constituents (*1*). Bacterial squalene-hopene cyclase (SHC) (EC 5.4.99.7) is believed to bind **1** in an all prechair conformation and then catalyze the sequential formation of five new C−C bonds through a series of carbocationic intermediates leading to a hopanyl C-22 carbocation **3**. Either H-29 proton elimination or addition of H_2O at **3** results in release of the final products hop-22(29)-ene (**4**) and hopan-22-ol (**5**), respectively (Scheme 1A) (*2*). In contrast, the eukaryotic oxidosqualene:lanosterol cyclase (OSLC) (EC 5.4.99.7) folds **2** in chair-boat-chair conformation and mediates the cyclization of **2** through enzyme-constrained carbocationic intermediates to the protosteryl (C-20) cation. Backbone rearrangement of the cation leads to lanosterol **6** (Scheme 1B). These two cyclizations both generate and stabilize carbocationic intermediates as key steps in the catalytic process. Bacterial squalene cyclases will also accept epoxides as substrates and can catalyze the cyclization of **2** into pentacyclic triterpenes by initiating oxirane ring opening rather than by protonating the terminal double bond (*3*−*6*). For instance, bacterial SHC can cyclize (3*R*) and (3*S*)-OS to 3α-hydroxyhopene and 3β-hydroxyhopene, respectively (*3*, *4*). The molecular details of how these cyclases fold their substrates and orchestrate specific cation-induced polyolefinic ring formation remains to be elucidated.

Recent advances in molecular and structural biology have spurred mechanistic studies of these enzymes. Several bacterial SHCs have been purified (*7*−*9*), cloned, and functionally expressed (*10*−*12*). SHCs are membrane-associated 70−75 kDa proteins. Sequence comparison of bacterial SHCs and eukaryotic OSLCs have showed 17−27% overall amino acid identity, with greater identity in the C-terminal region. In addition, eight repeats of a highly conserved α-helix turn motif rich in aromatic amino acids (the QW motif) (*13*, *14*) were recognized as key structural elements. Site-directed mutagenesis experiments on OSLC and SHC have demonstrated that D-456 in yeast OSLC was essential for catalytic function (*15*, *16*), while the homologous residues D-376 and D-377 in *A. acidocaldarius* SHC were crucial for enzyme activity (*17*). Most recently, a 2.9 Å resolution X-ray crystal structure of *Alicyclobacillus acidocaldarius* SHC was reported (*18*, *19*). SHC is a homodimeric enzyme, and each unit consists of 631 amino acids with molecular mass of 71 569 Da. Two α-helical domains create the hydrophobic active site of the enzyme, identified in a large central cavity by using the bound

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[1] Abbreviations: OS, 2,3-oxidosqualene; SHC, squalene: hopene cyclase; OSLC, oxidosqualene:lanosterol cyclase; 29-MOS, (3*S*)-29-methylidene-2,3-oxidosqualene; S-19, 19-thia-18-dehydro-(*R, S*)-2,3-oxidosqualene; S-18, 18-thia-19-dehydro-(*R,S*)-2,3-oxidosqualene; S-14, 14-thia-15-dehydro-(*R,S*)-2,3-oxidosqualene; S-10, 10-thia-11-dehydro-(*R,S*)-2,3-oxidosqualene; S-6, 6-thia-7-dehydro-(*R,S*)-2,3-oxidosqualene; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; AMO 1618; 5-hydroxylcarvacryl trimethylammonium chloride 1-piperidine carboxylate; $DodMe_2NO$, dodecyldimethylamine-*N*-oxide; $DodMe_3NBr$, dodecyltrimethylammonium bromide; Ro48-8071, [4′-(6-allyl-methyl-amino-hexyloxy)-2′-fluoro-phenyl]-(4-bromophenyl-methanone fumarate; BIBX79, *trans*-*N*-(4-chlorobenzoyl)-*N*-methyl-(4-dimethylaminomethylphenyl)-cyclohexylamine.

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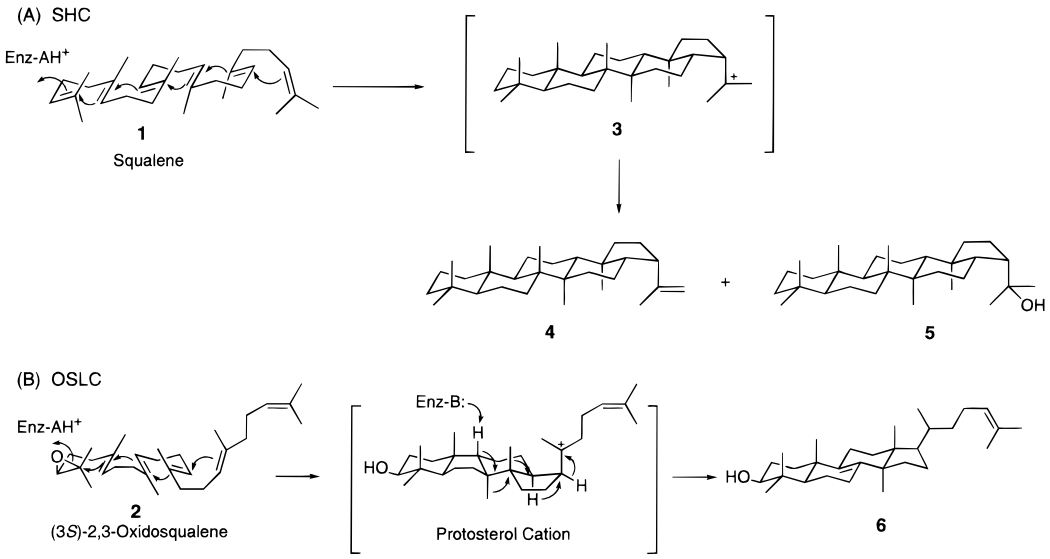
Scheme 1: (A) Proposed Cyclization Mechanism of Squalene (**1**) to Hopan-22-ol (**4**) and Hop-22(29)-ene (**5**); (B) Proposed Cyclization Mechanism of 2,3-oxidosqualene (**2**) to Lanosterol (**6**)



inhibitor dodecyldimethylamine-*N*-oxide ($DodMe_2NO$). In this model, the majority of QW motifs are restricted to the exterior surface of the protein, suggesting a structural rather than catalytic role. Instead, several other aromatic residues in the sequence were proposed to contribute to carbocationic stabilization (*20*).

We previously demonstrated that rat liver OSLC was specifically labeled with a mechanism-based irreversible inhibitor, [^{3}H]-(*3S*)-29-methylidene-2,3-oxidosqualene (29-MOS) (*21*−*24*), and that an Asp residue (D-456) in the DCTAEA motif was covalently linked to a partially cyclized intermediate derived from an inhibitor, implicating the Asp carboxylate in the stabilization at C-20 of the protosteryl cation (*25*−*27*). Studies of thia-substituted OS analogues with OSLC showed that pig liver OSLC, but not rat liver OSLC, was specifically labeled by S-18 analogue **10** (*28*). Very recently, we have demonstrated that *A. acidocaldarius* SHC was also specifically and covalently labeled by [^{3}H]-(*3S*)-29-MOS during catalysis of cyclization (*29*). The fact that 29-MOS is an irreversible mechanism-based inhibitor of SHC makes the OS analogues attractive targets for SHC inhibition studies. Moreover, comparison of the inhibition kinetics and affinity labeling among the bacterial SHCs and eukaryotic OSLCs should provide complementary information on the active-site structures of these enzymes. The thia-substituted-OSs were previously found to be potent inhibitors of OSLC (*30*−*33*). We now report that these five thia-OS analogues are also potent inhibitors of *A. acidocaldarius* SHC (Figure 1). The inhibition kinetics with SHC and rat liver OSLC were studied for thia analogues in which a sulfur replaces carbons at C-6, C-10, C-14, C-18, or C-19 of OS. The chemical affinity labeling of SHC with [17-^{3}H]**10** and [22-^{3}H]**10** required cyclase activity and could be displaced by potent nonterpenoid cyclase inhibitors, in analogy to the results obtained with pig liver OSLC.

MATERIALS AND METHODS

Syntheses of [*17-^{3}H*] *and* [*22-^{3}H*]*-18-Thia-19-dehydro-*(*R,S*)*-2,3-oxidosqualene and Other Thia-Substituted-*(*R,S*)*-2,3-oxidosqualenes*. Synthesis of [17-^{3}H]- and [22-^{3}H]-18-thia-19-dehydro-(*R,S*)-2,3-oxidosqualene **10** were carried out as previously described (*28*). Both [17-^{3}H]**10** (3.5 Ci/mmol) and [22-^{3}H]**10** (1.8 Ci/mmol) were purified by silica gel (Merck, 230−400 mesh) column using 5% ethyl acetate−hexane. Synthesis of unlabeled thia-substituted OSs **7**−**11** (S-6, S-10, S-14, S-18, and S-19) were accomplished as previously described (*32*).

Enzyme Purification and Assay. The recombinant *A. acidocaldarius* SHC was expressed in *Escherichia coli* and purified according to the published method (*10*). The enzyme converted squalene into a 17:1 mixture of hop-22-(29)-ene (**4**) and hopan-22-ol (**5**), and showed an apparent $K_m = 1.8\ \mu M$ and $k_{cat} = 2.4\ min^{-1}$ in the presence of 0.1% Triton X-100 in the assay mixture.

The reaction mixture contained 100 mM sodium citrate, pH 6.0, 0.1% (v/v) Triton X-100, 5 μM [^{14}C]squalene (7.0 mCi/mmol) and 0.5 μg of purified recombinant SHC in a final volume of 1 mL. Incubations were carried out at 60 °C for 30 min, stopped by extraction of 1 mL of CH_2Cl_2. The extract was concentrated using a Speed-Vac, and subjected to silica gel TLC (Whatmann LK6D). The TLC plates were developed twice: first for 5 cm in $CHCl_3$, then for 15 cm in hexane. The R_f values of squalene, hopene, and hopan-2-ol were 0.45, 0.77, and 0.15, respectively. The reaction mixtures were then analyzed using a radio-TLC scanner (Bio-Scan, System 500), which as an instrumental reproducibility of ±5−10% for repeated scans of the same sample lanes. The percentage of activity was plotted against inhibitor concentration to determine the IC_{50} value. All assays were carried out in duplicate (*29, 30*).

The rat liver OSLC was purified and kinetic studies of analogues **7**−**9** were performed as previously described (*22, 30*).

Inhibition Kinetics. The experiments were carried out in duplicate using three concentrations of thia-substituted 2, 3-oxidosqualenes (**7**, 0, 0.15, 0.3 μM; **8**, 0, 0.6, 1.2 μM; **9**, 0, 0.08, 0.16 μM; **10**, 0, 0.05, 0.10 μM; **11**, 0, 0.10, 0.20 μM). For each inhibitor concentration, [^{14}C]squalene was added to give four substrate concentrations: 1, 1.33, 2, and 4 μM. The mixtures were incubated at 60 °C for 30 min

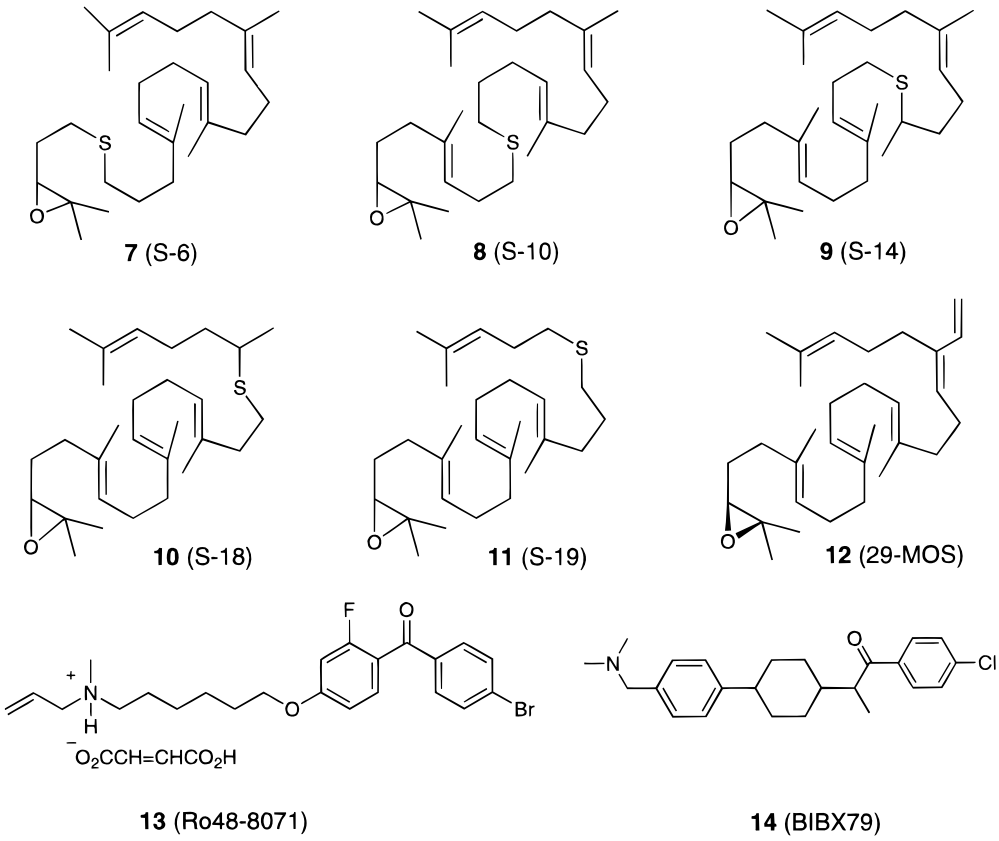


FIGURE 1: Thia analogues of OS analogues examined as inhibitors of *A. acidocaldarius* SHC and structures of three known OSLC and SHC inhibitors.

and analyzed using radio-TLC scanner (Bio-Scan, System 500) as described. Lineweaver−Burk plots of data (average of duplicates) were employed to derive the K_i values (*29*, *30*).

Time Dependency of Inhibition. The experiments were carried out in duplicate using four concentrations (**9**, 0, 44, 90, 180 nM; **10**, 0, 25, 50, 100 nM; **11**, 0, 40, 80, 160 nM). The enzyme SHC (3.2 μg) was incubated with these inhibitors in total volume of 500 μL, while aliquots (50 μL) of the inhibited SHC solution were removed at time intervals of 0, 2.5, 5, and 10 min and added to 950 μL of reaction mixture containing 100 mM sodium citrate, pH 6.0, 0.1% (v/v) Triton X-100, and 5 μM [^{14}C]squalene. The diluted enzyme solutions were then incubated at 60 °C for 30 min and analyzed as described above. The log(percentage of remaining activity) was plotted against time to determine the k_{inact} (*29*, *30*).

Chemical Affinity Labeling. The crude and purified recombinant *A. acidocaldarius* SHC (2 μg of protein in a total volume of 200 μL) were incubated with [17-^{3}H]**10** (1 μM, spec act 3.5 Ci/mmol) or [22-^{3}H]**10** (1 μM, spec act 1.8 Ci/mmol) in 100 mM sodium citrate buffer, pH 6.0 for 1 h at 60 °C. Samples of each incubation mixture were subjected to SDS−PAGE (7.5%), and proteins were visualized by Coomassie Brilliant blue staining. After impregnation with EN^{3}HANCE (NEN Life Science Products) for 1 h, the gels were dried and then exposed to X-ray film (Kodak X-Omat AR film) for 3 days at −80 °C. For competition experiment, the incubations (1 μM of [17-^{3}H]**10**) were carried out in the presence of 100 μM (100-fold excess) of an SHC inhibitor 29-MOS (**12**), [4′-(6-allyl-methyl-amino-hexyloxy)-2′-fluoro-phenyl]-(4-bromophenyl-methanone fumarate (Ro48-8071) (**13**), or *trans*-*N*-(4-chlorobenzoyl)-*N*-methyl-(4-dimethylaminomethylphenyl)cyclohexylamine (BIBX79) (**14**) or in the presence of 100 μM of squalene as competitors. Labeling was competitively displaced with Ro48-8071 (**13**) and BIBX79 (**14**) but not by squalene or 29-MOS (**12**) at 100 μM concentration (*29*).

RESULTS AND DISCUSSION

The OS analogues **7**−**11** each contain a sulfur atom in the OS skeleton, which may mimic the π-electron system of a double bond. We anticipated that this substitution of sulfur for methylene would disrupt the all prechair folding of the substrate, but could also lead to partial cyclization involving the sulfur atom. The kinetic constants K_i and k_{inact} for the racemic analogues S-6, S-10, S-14, S-18, and S-19 with homogeneous recombinant *A. acidocaldarius* SHC as well as purified rat liver OSLC are summarized in Table 1.

Each of the racemic thia analogues **7**−**11** was found to be a submicromolar inhibitor of *A. acidocaldarius* SHC (with IC_{50} values ranging from 60 to 570 nM), significantly more potent than the known irreversible SHC inhibitor, 29-MOS (**12**) (IC_{50} = 1200 nM) (*29*). All but S-10 (**8**) were more potent inhibitors than the previously reported effective SHC inhibitors such as 5-hydroxylcarvacryl trimethylammonium chloride 1-piperidine carboxylate (AMO 1618, K_i = 540 nM), $DodMe_3NBr$ (K_i = 320 nM), $DodMe_2NO$ (K_i = 140 nM) (*9*) and 2,3-hydro-2-azasqualene analogues (*34*). In addition, all but S-18 (**10**) were more potent inhibitors for SHC than for rat liver OSLC displaying 3.3-, 1.8-, 128-, and 3.3-fold higher potency toward SHC. The S-18 analogue **10** showed comparable and maximally potent inhibition with both cyclases.

Table 1: Inhibitory Studies of Sulfur-Substituted-OS as Inhibitors of Recombinant *A. acidocaldarius* SHC and Rat Liver OSLC

	A. acidocaldarius SHC			rat liver OSLC		
compds	IC_{50} (nM)	K_i (nM)	k_{inact} (min^{-1})	IC_{50} (nM)	K_i (nM)	k_{inact} (min^{-1})
7	150	127	NT[a]	500	520	0.085
8	570	971	NT[a]	1000	2100	0.037
9	86	109	0.058	11000	4200	0.042
10	60	31	0.071	50[c]	37[c]	0.0001[c]
11	78	83	0.054	260[c]	180[c]	0.0001[c]
12 (29-MOS)	1200	2100	0.06	300	2500	ND[b]

[a] NT, no time dependency. [b] ND, not determined. [c] Data from ref *30*.

The OS analogue **7** possesses sulfur at the position normally occupied by a C-6−C-7 double bond, and was designed to interfere with the all prechair folding of ring A; moderate inhibition of SHC (IC_{50} = 150 nM) was observed. The OS analogue **8** that features a sulfur at the position normally occupied by C-10−C-11 double bond was the least potent SHC inhibitor (IC_{50} = 570 nM) in this series of compounds. Analogues **9** (S-14), **10** (S-18), and **11** (S-19) were similarly designed to interfere with the formation of ring C or ring D; these three were the most potent compounds of the series, showing comparable IC_{50} values of 86, 60, and 78 nM for SHC, respectively. In fact, the S-18 analogue **10** showed the most potent inhibition (IC_{50} = 60 nM) toward this enzyme and is the most effective SHC inhibitor reported to date. These results suggest that substituents that interfere with the ring C formation (S-14) or ring D formation (e.g., S-18 and S-19) can result in effective inhibition of SHC. This result is consistent with the observation in rat liver or pig liver OSLC that modifications of the prering D region of OS skeleton by replacing sulfur at C-18 position gave the most potent inhibition. Apparently both OSLC and SHC are maximally sensitive to partially cyclized materials containing the S-18 substitution (*28*, *32*).

The more flexible and longer C−S bond in comparison with the naturally occurring alkene bond may serve to bring the sulfur closer to the active-site nucleophilic residues important in cationic charge stabilization. For S-18 (**10**), these partially cyclized cationic intermediates could produce a covalent enzyme−inhibitor complex such as **16** (Figure 2). Alternatively, the sulfur could act as a mimic of C-18 double bond and intramolecularly quench the cation **15** to form a sulfonium ion, which could be stabilized by nucleophilic residues at the active site of these enzymes to give a tightly bound ionic complex such as **17** (Figure 2). A similar process was invoked to rationalize the inhibition of pig liver OSLC (*28*).

Kinetic analyses revealed that **7**−**9** act as competitive inhibitors of SHC with K_i values of 127, 971, and 109 nM, respectively (Figure 3, Table 1), while **10** and **11** show the characteristics of noncompetitive inhibitors with K_i values of 31 and 83 nM (Table 1). The classification as competitive or noncompetitive is based on the intercepts for the Lineweaver−Burk plots; in reality, all five thia analogues are substrate analogues and the apparent mechanistic classification may arise from the catalytic formation of irreversible adducts or tightly bound complexes. For rat liver OSLC, analogues **7**−**11** are all competitive inhibitors with K_i values of 520, 2100, 4200, 37, and 180 nM, respectively (Table 1).The time dependency of inactivation was determined for both SHC and OSLC enzymes, and the rates of inactivation (k_{inact}) were calculated (Table 1).

The time dependency of inactivation of SHC by S-14, S-18, and S-19 analogues **9**−**11** is shown in Figure 4. Each of these three analogues showed significant ability to decrease the activity of SHC with k_{inact} values of 0.058, 0.071, and 0.054 min^{-1}, respectively. Their ability to inactivate the enzyme was comparable to that of a known irreversible mechanism-based inhibitor, 29-MOS (k_{inact} for SHC = 0.06 min^{-1}). In contrast, inhibition by the S-6 analogue **7** and the S-10 analogue **8** showed no time dependency. Thus, **9**−**11** are time-dependent, mechanism-based inhibitors for SHC, while **7** and **8** act only as substrate mimics; the time dependency further supports the hypothesis based on the K_i values that interfering with ring C or ring D formation results in the most potent inhibition of SHC.

In contrast, for rat liver OSLC, the S-18 analogue **10** and the S-19 analogue **11** did not show time-dependent inactivation. However, the S-6, S-10, and S-14 compounds **7**−**9** did exhibit time-dependent inactivation of OSLC activity. These results differ from those with SHC, suggesting that the enzyme active sites have different geometries to accommodate the C−S bond analogues and their cyclized intermediates (Scheme 1).

Since the S-18 analogue **10** was a potent, time-dependent, mechanism-based inhibitor of SHC, the covalent modification of SHC was examined. Mechanism-based affinity-labeling

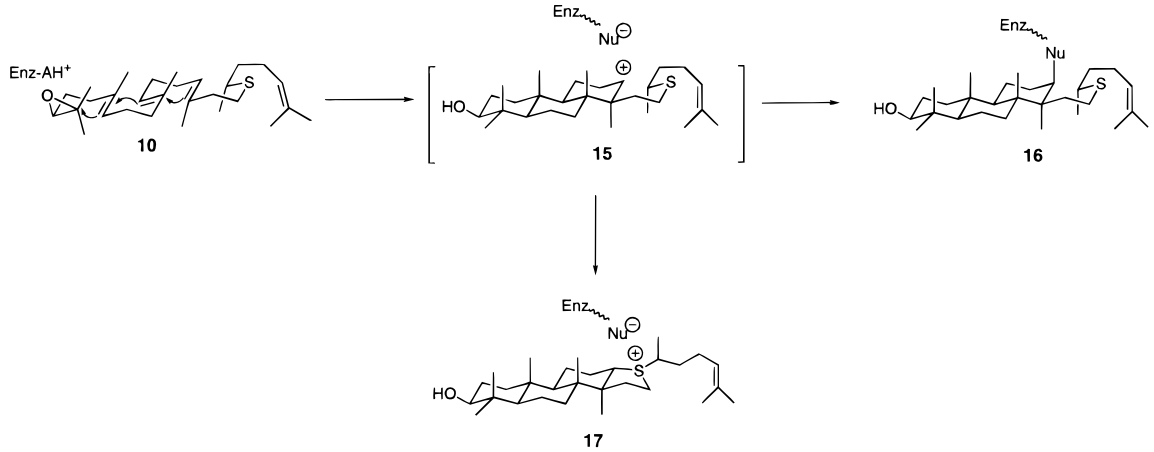


FIGURE 2: Putative inhibitory mechanism of *A. acidocaldarius* SHC by **10**.

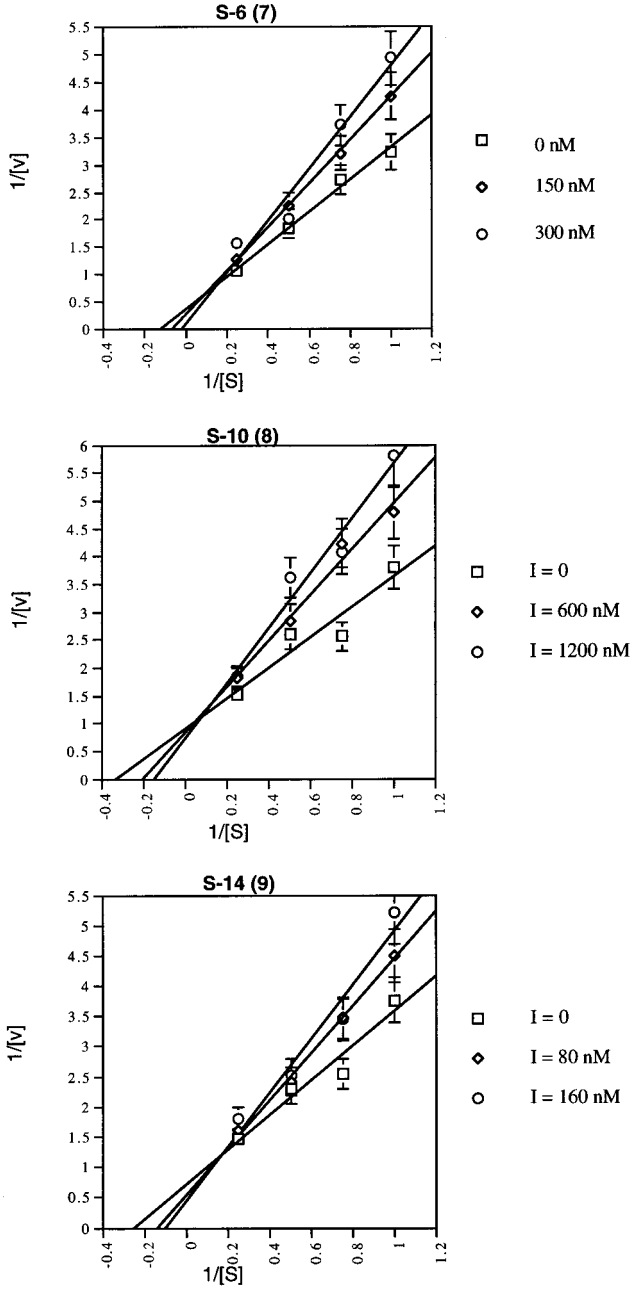


FIGURE 3: Lineweaver−Burk analysis for inhibition of *A. acidocaldarius* SHC by analogues **7**−**9**.

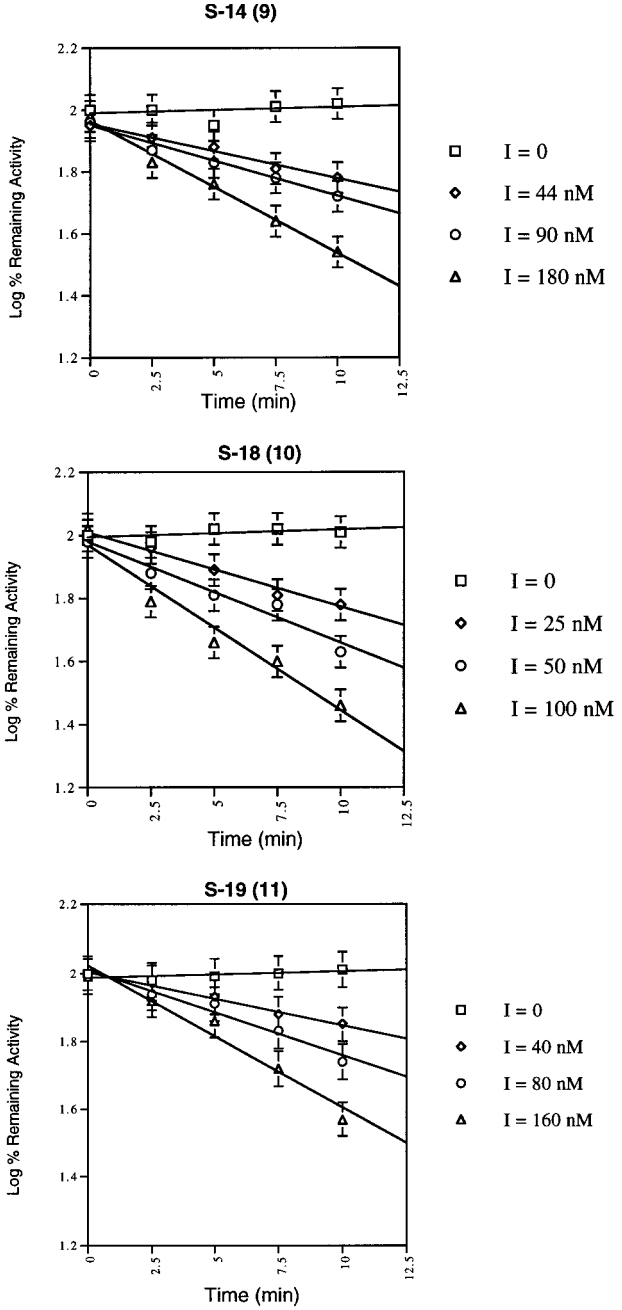


FIGURE 4: Time-dependent inactivation of *A. acidocaldarius* SHC by compounds **9**−**11**.

experiments using [17-^{3}H]**10** or [22-^{3}H]**10** with high specific activity (*28*) were performed with both pure and crude preparations of recombinant *A. acidocaldarius* SHC. After incubation of crude and purified SHC (2 μg of protein in a total volume of 200 μL) with [17-^{3}H]**10** (1 μM, spec act 3.5 Ci/mmol) or [22-^{3}H]**10** (1 μM, spec act 1.8 Ci/mmol) for 1 h at 60 °C, proteins were separated by SDS−PAGE and the covalently modified protein band was visualized by fluorography. In both pure and crude preparations of recombinant *A. acidocaldarius* SHC, a single specific radioactive band was observed on the fluorogram at the same molecular mass of *A. acidocaldarius* SHC (72 kDa) for both [17-^{3}H]-**10** and [22-^{3}H]**10** (Figure 5). The labeling of SHC by both [^{3}H]**10** regioisotopomers establishes three important points. First, the adducts are irreversible, covalent modifications of the active site of SHC, consistent with an enzyme−inhibitor complex that survives denaturing conditions. Second, the modification occurs with catalytically active enzyme and proceeds with mechanism-based inhibition, suggesting that a partially cyclized cationic intermediate is formed and then trapped by an active-site nucleophile. Third, the retention of the tritium label for both [17-^{3}H]**10** and [22-^{3}H]**10** excludes the possibility of transferring the side chain to the active site of SHC (*28*). These results provide further support for the postulated inhibition mechanism of SHC as shown in Figure 2.

Competition experiments were performed with [17-^{3}H]**10** (1 μM, spec act 3.5 Ci/mmol) and several cyclase inhibitors to determine the specificity of the mechanism-based affinity labeling. Thus, a 100-fold excess (100 μM) of the substrate squalene, (3*S*)-29-MOS (**12**) (*29*), Ro48-8071 (**13**) (*35*), and BIBX79 (**14**) (*36*) were coincubated with purified SHC and the labeled inhibitor. The results (Figure 5c) illustrate that

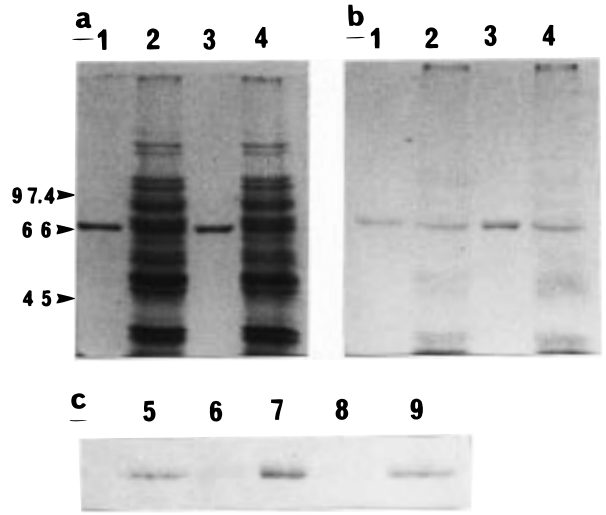


FIGURE 5: Mechanism-based affinity labeling of *A. acidocaldarius* SHC. (a) SDS−PAGE (7.5%) gel stained with Coomassie Blue; (b) corresponding fluorogram. Lanes 1 and 3, purified enzyme; lanes 2 and 4, *E. coli* cell-free extract; lanes 1 and 2, [22-^{3}H]**10**; lane 3 and 4, [17-^{3}H]**10**. Each labeling reaction was carried out at 1 μM concentration at 1 h at 60 °C in 100 mM sodium citrate buffer, pH 6.0. (c) Fluorogram illustrating competition of several known oxidosqualene cyclase inhibitors (100 μM, 100-fold molar excess) for labeling of SHC by [17-^{3}H]**10** (1 μM, 3.5 Ci/mmol). Lane 5, no competitor; lane 6, Ro48-8071 (**13**); lane 7, 29-MOS (**12**); lane 8, BIBX79 (**14**); lane 9, squalene.

mechanism-based labeling of SHC by [17-^{3}H]**10** was competitively displaced by a 100-fold molar excess of Ro48-8071 and BIBX79 but not by squalene or 29-MOS (100-fold excess). This corresponds to the tighter binding of the two nonsubstrate-like inhibitors relative to the cyclizable substrate and substrate analogues (Table 1).

In summary, we have demonstrated that four thia analogues of oxidosqualene (S-6, S-10, S-14, and S-19) are more potent as inhibitors of *A. acidocaldarius* SHC than of rat liver OSLC; the S-18 thia analogue **10** showed the most potent inhibition of both SHC and OSLC with comparable K_i values. The inhibition kinetic behavior of this series of analogues **7**−**11** with both enzymes are distinctive. The S-6 (**7**) and S-10 (**8**) analogues are time-dependent inhibitors of rat liver OSLC but display no time dependency toward *A. acidocaldarius* SHC. In contrast, S-18 (**10**) and S-19 (**11**) showed cyclase activity-dependent, time-dependent, and irreversible inhibition of *A. acidocaldarius* SHC but no time dependency in rat liver OSLC. Interestingly, S-18 but not S-19 displayed mechanism-based, time-dependent inhibition for pig liver OSLC (K_i = 1500 nM, k_{inact} = 0.06 min^{-1}) and *A. acidocaldarius* SHC. These results point to quite subtle differences in active site−thia analogue interactions for these cyclases. The irreversible inhibition of SHC [and previously with pig liver OSLC (*28*)] was achieved with tritium labeled S-18, and the time dependency and covalent modification of SHC suggested that inhibition arose from partially cyclized intermediates. Identification of the active-site residues of pig liver OSLC and bacterial SHC modified by [17-^{3}H] S-18 will be described in due course.

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