Mechanism-Based Active Site Modification of Oxidosqualene Cyclase by Tritium-Labeled 18-Thia-2,3-Oxidosqualene

Ikuro Abe,*,† Wei Liu,‡ Allan C. Oehlschlager,‡ and Glenn D. Prestwich*,†,§

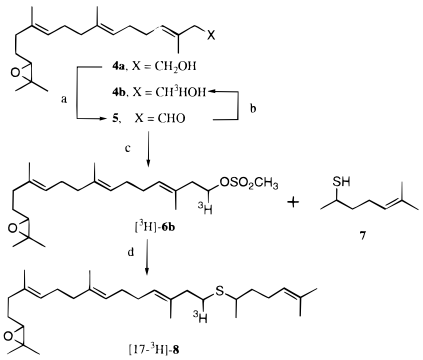
Departments of Chemistry and Biochemistry & Cell Biology, University at Stony Brook Stony Brook, New York 11794-3400 Department of Chemistry, Simon Fraser University Burnaby, British Columbia, Canada V5A 1S6

Received May 16, 1996

The enzymatic cyclization reaction[1] of (3*S*)-2,3-oxidosqualene (**1**) to lanosterol (**3**) proceeds through a progression of rigidly-held carbocationic intermediates.[2] Oxidosqualene:lanosterol cyclases (OSCs) (E.C. 5.4.99.7) have been purified,[3] cloned,[4] and expressed[4] from five species. The OSCs are membrane-associated 80−85 kDa proteins containing six repeats of a highly-conserved β-strand turn motif rich in aromatic amino acids (the QW motif) that may participate in cation-π stabilization during cyclization.[4a,5] Active-site mapping with a mechanism-based irreversible inhibitor of OSC, [^{3}H]29-methylidene-2,3-oxidosqualene (29-MOS)[6] (**14**), revealed that an Asp residue (D-456 in rat OSC) in the conserved DCTAEA motif was linked to the cyclized inhibitor.[4a,7] This implicated the aspartate carboxylate in stabilization of the C-20 cationic center of the protosterol intermediate cation (**2**).[4a,7]

Sulfur-containing analogues of **1** have been prepared in which sulfur has replaced carbons C-5, C-6, C-8, C-9, C-10, C-11, C-13, C-14, C-15, C-16, C-18, C-19, and C-20,[8] and many are potent inhibitors of OSC. In particular, the S-18 analogue (**8**) showed the most potent inhibition toward vertebrate OSCs (IC_{50} = 0.05 μM for rat OSC)[8e] when compared to the best OSC inhibitors,[9] e.g., 29-MOS (IC_{50} = 0.5 μM for pig OSC)[6] and 2,3:18,19-dioxidosqualene (IC_{50} = 0.11 μM for rat OSC).[10] Inhibition kinetics with purified vertebrate OSCs demonstrated that **8** was a time-dependent, noncompetitive inhibitor of *pig* OSC (K_I = 1.5 μM, k_{inact} = 0.06 min^{-1}, partition ratio[11] = 16.2). Surprisingly, the more potent inhibition of *rat* OSC (K_I = 0.037 μM) was not time-dependent.[8e] We report herein the synthesis of two tritium-labeled radioisotopomers of **8**, and we demonstrate the irreversible, mechanism-based inactivation of pig OSC but not rat OSC.

Scheme 1[a]



[a] Reagents: (a) PCC, $CaCO_3$, CH_2Cl_2, rt, 1 h, 97%; (b) NaB^3H_4, EtOH, 0 °C, 2 h 24%; (c) CH_3SO_2Cl, Et_3N, CH_2Cl_2, −50 °C, 1 h, 91%; (d) 25% NaOH, *n*-Oct_4NBr, toluene, H_2O, 40 °C, 10 h, 37%.

Synthesis of [17-^{3}H](*R*,*S*)18-thia-2,3-oxidosqualene **8** was performed[8] as shown in Scheme 1. Oxidation of homoallylic alcohol **4a** (PCC, $CaCO_3$, 4 Å sieves) gave aldehyde **5** in 97% yield,[12] and reduction with [^{3}H]$NaBH_4$ gave [^{3}H]-**4b**. Mesylation of **4b** followed by coupling of [^{3}H]-**6b** with 6-methyl-5-hepten-2-thiol (**7**) (50% NaOH, toluene, tetraoctylammonium bromide) afforded [17-^{3}H]-**8** (3.5 Ci/mmol). We also synthesized [22-^{3}H]-**8** having the tritium label in the *pro*-sterol side chain. Unlabeled epoxymesylate **6a** was first coupled with the TBDMS ether of 4-mercaptopentan-1-ol (**9**)[13] (Scheme 2). Oxidation of **10a** (NCS, Me_2S, Et_3N)[14] gave aldehyde **11a**, which was reduced with [^{3}H]$NaBH_4$ to [^{3}H]-**10b**. The tritiated alcohol was reoxidized to **11b** and olefinated by a Wittig reaction to give [22-^{3}H]-**8** (specific activity 1.8 Ci/mmol).

When purified pig OSC or rat OSC was incubated with [17-^{3}H]- or [22-^{3}H]-**8** followed by protein separation by SDS-PAGE,

* Authors to whom correspondence should be addressed at: Department of Medicinal Chemistry, The University of Utah, 308 Skaggs Hall, Salt Lake City, UT 84112.

† University at Stony Brook.

‡ Simon Fraser University.

§ Phone: 801 581-7063. Fax: 801 581-7087. E-mail: gprestwich@deans.pharm.utah.edu.

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(11) The partition ratio of inactivation of pig liver OSC by **8** was determined as described previously[6a] for 29-MOS, i.e., by extrapolating (to infinite dilution) the linear range of a plot of remaining OSC activity vs the ratio of [**8**] to [pure OSC activity]. The data was generated by adding increasing amounts of **8** to a constant amount of enzyme solution (0.125 μM).

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(13) Compound **9** was prepared from 6-methyl-5-hepten-2-ol by a five-step synthesis as described previously.[8] Thus, reaction of the alcohol with thiolacetic acid followed by *m*-CPBA epoxidation and oxidative cleavage by HIO_4 afforded 4-thioacetoxypentanal. Finally, reduction with $NaBH_4$ and treatment with TBDMSCl gave **9**.

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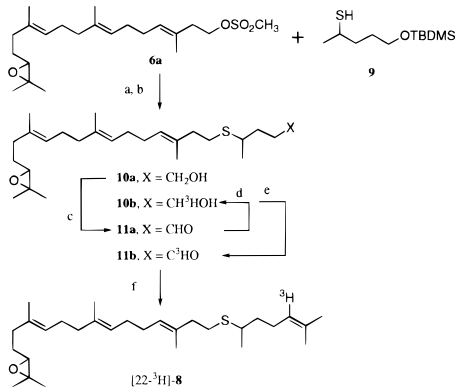
Scheme 2[a]



[a] Reagents: (a) 50% NaOH, *n*-Oct_4NBr, toluene, H_2O, 40 °C, 8 h, 47%; (b) *n*-Bu_4NF, THF, rt, 8 h, 95%; (c) NCS, DMS, Et_3N, toluene, −25 °C, 2 h, 15%; (d) NaB^3H_4, EtOH, 0 °C, 2 h, 90%; (e) NCS, DMS, Et_3N, toluene, −25 °C, 2 h, 20%; (f) $Ph_3PCH(CH_3)_2I$, *n*-BuLi, THF, −78 °C, 95%.

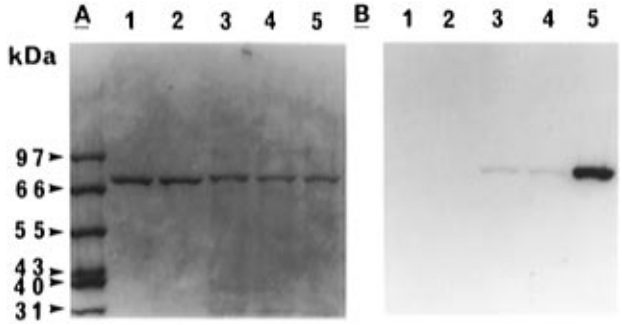
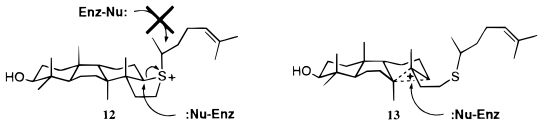


Figure 1. Affinity labeling of OSC. (A) SDS-PAGE (7.5%) gel stained with Coomassie blue. (B) Corresponding fluorogram. Lane 1, rat OSC incubated with [17-^{3}H]-**8**; lane 2, rat OSC incubated with [22-^{3}H]-**8**; lane 3, pig OSC incubated with [17-^{3}H]-**8**; lane 4, pig OSC incubated with [22-^{3}H]-**8**; lane 5, pig OSC incubated with **14** (1.8 Ci/mmol). Each reaction was carried out at 1 μM concentration at 37 °C for 1 h.

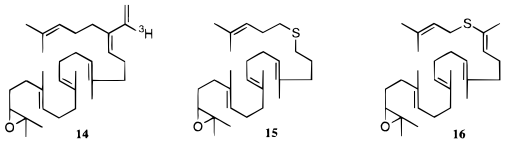
only pig OSC showed a covalently-labeled protein band (Figure 1). Both [^{3}H]-**8** regioisotopomers labeled the pig OSC, while neither covalently modified the rat OSC. This result was consistent with the observation that while pig OSC showed time-dependent, irreversible inactivation by **8**, inhibition of rat OSC was reversible.[15] The labeling efficiencies of [17-^{3}H]- and [22-^{3}H]-**8** were essentially equivalent, with about 8% incorporation of the total radioactivity used at 1 μM concentration.[16] By comparison, 29-MOS **14** (K_I = 4.4 μM, k_{inact} = 221 min^{-1}, partition ratio = 3.8 for pig OSC) labeled OSC 1,000-fold faster and with 10-fold higher efficiency than **8**. Preincubation of the pig enzyme with 80 μM 29-MOS reduced labeling 5-fold, while 100 μM of the substrate **1** only reduced labeling by 20%. Thus, both **8** and **14** require an unmodified active site for binding, and both require catalytic activation of the inhibitor to inactivate and covalently modify the enzyme. Cyclization of the 0.5 μM [^{3}H]-**8** by pig OSC was monitored by radio-TLC, and a polar product was obtained in 30% yield. However, at [**8**] > K_I, complete inactivation of the enzyme precluded isolation of sufficient product for structure determination.[17]

Scheme 3. Potential Modes of Mechanism-Based Inactivation by Analogue **8**



Analogue **8** is therefore a substrate mimic and a mechanism-based irreversible inhibitor of OSC. Covalent modification of OSC requires partial cyclization of **8** at the active site of the enzyme with trapping of a cationic intermediate by an active-site nucleophile. Earlier, we envisaged[8] the formation of a protosterol-type tetracyclic sulfonium ion **12**, which could suffer attack α to the sulfonium ion by an active-site nucleophile (Scheme 3). The retention of the tritium label for both the [17-^{3}H] and [22-^{3}H] isotopomers of **8** excluded the possibility of an attack at C-20 with transfer of the side chain to the active site (Scheme 3). Nucleophilic trapping could alternatively occur on a bicyclic or tricyclic intermediate, as shown in structure **13**. This contrasts with the proposed covalent modification of both rat and pig OSC by **14**, in which a tetracyclic cation is trapped by an active site Asp residue. This hypothesis predicts that a nucleophile elsewhere in the primary sequence should be modified by [^{3}H]-**8**. Interestingly, in the entire series of thia-substituted analogues of **1**,[8] only **8** covalently modified pig OSC, and none of the analogues irreversibly modified rat OSC.

The position of the sulfur is critical. Although 19-thia-2,3-oxidosqualene[8] (**15**) was equipotent for pig OSC with **8** (IC_{50} = 1.0 μM, K_I = 1.4 μM), inhibition was reversible and not time dependent.[8] Moreover, the inhibition of OSC shows an unexpected species difference, since **8** was a more potent but reversible inhibitor of rat OSC. Another analogue, 20-thia-2,3-oxidosqualene (**16**) was neither a substrate nor inhibitor for cyclization by yeast lanosterol synthase;[18] in our assays, **16** was 10^3 less potent than **8**.[8c] Steric requirements alone are insufficient to explain the divergent behavior of heteroatom-substituted inhibitors in the active sites of vertebrate or yeast OSCs. Just as the modification of Asp^{456} of rat OSC by **14** suggested the possible catalytic role this "negative point charge"[2e] plays in stabilization of a tetracyclic C-20 cationic species, we expect that [^{3}H]-**8** can target nucleophiles that stabilize bi- or tricyclic species involved in the enzyme-mediated cyclization process.



Acknowledgment. The authors are indebted to Ms. B. A. Madden (Stony Brook) for synthesis of the [^{14}C](3*S*)-2,3-oxidosqualene used in this study. We thank the NIH (Grant GM 44836 to G.D.P.) and the NSERC (grants to A.C.O.) for financial support and Dr. D. G. Ahern (Dupont NEN) for providing radiochemical reagents.

JA961643A

(15) The irreversibility of the enzyme inhibition was tested as follows. Homogeneous pig liver or rat liver OSC (total volume of 240 μL) was incubated (37 °C, 30 min) with **8** at concentrations of 0, 1 ×, and 2 × IC_{50} values; IC_{50} = 2.3 and 0.05 μM for pig and rat OSCs, respectively. The enzymatic mixture was then combined with 200 μL of DEAE-Sephacel that had been preequilibrated with 10 mM Tris-HCl, pH 7.4, containing 0.1% Triton X-100 (=buffer A). After incubation at 4 °C for 30 min and centrifugation at 16 000 g for 1 min, the precipitate was washed (3 × 1.5 mL of buffer A) and OSC was eluted with 200 μL of 0.2 M KCl. Enzyme activities were then measured as described.[3a] As a positive and a negative control for the irreversible inhibition, unlabeled **14** and **15** were employed at concentrations of 0, 1 ×, and 2 × IC_{50} values; IC_{50} = 1.0 and 0.26 μM for pig and rat OSCs, respectively. Both **8** and **14** dramatically reduced the enzyme activity of pig OSC to 40% at 2 × IC_{50} concentration, while **15** gave quantitative recovery of OSC activity. In addition, with rat OSC, nearly 100% activity was recovered for **8** as well as for **15**.

(16) Labeling efficiency was monitored by separating protein and ligand by ion exchange. After incubation with [17-^{3}H]- or [22-^{3}H]-**8** at 37 °C for 1 h, a 15 μL aliquot of the assay mixture was diluted with 300 μL of buffer A, added to 50 μL of preequilibrated DEAE-Sepahacel. After incubation for 1 h at rt, the 10 000*g* supernatant was removed, the DEAE resin was washed (5 × 1 mL of buffer A) and resuspended in 1 mL buffer, and bound radioactivity was assessed by LSC.

(17) [17-^{3}H]- or [22-^{3}H]-**8** was incubated with homogeneous pig liver OSC at 37 °C for 1 h, and reaction products were analyzed by TLC developed with CH_2Cl_2. R_f values for **8**, **1**, **3**, and the polar product were 0.39, 0.48, 0.26, and 0.06, respectively. As a control experiment, boiled-enzyme preparation was employed.

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