Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 315 (2004) 1–9
BBRC
www.elsevier.com/locate/ybbrc

Photoaffinity labeling identifies the substrate-binding site of mammalian squalene epoxidase☆

Hee-Kyoung Lee,[a,b] Yi Feng Zheng,[b] Xiao-Yi Xiao,[c] Mei Bai,[c,1] Jun Sakakibara,[d] Teruo Ono,[d] and Glenn D. Prestwich[a,b,c,*]

[a] *Department of Biochemistry and Cell Biology, The University at Stony Brook, Stony Brook, NY 11794-5215, USA*
[b] *Department of Medicinal Chemistry, The University of Utah, 419 Wakara Way, Suite 205, Salt Lake City, UT 84108, USA*
[c] *Department of Chemistry, The University at Stony Brook, Stony Brook, NY 11794-3400, USA*
[d] *Department of Biochemistry, Niigata University, School of Medicine, Niigata 951, Japan*

Received 4 November 2003

Abstract

Squalene epoxidase (SE) catalyzes the conversion of squalene to (3*S*)-2,3-oxidosqualene. Photolabeling and site-directed mutagenesis were performed on recombinant rat SE (rrSE) in order to identify the location of the substrate-binding site and the roles of key residues in catalysis. Truncated 50-kDa rrSE was purified and photoaffinity labeled by competitive SE inhibitor ($K_i = 18.4\,\mu M$), [^{3}H]TNSA-Dza. An 8-kDa CNBr/BNPS-skatole peptide was purified and the first 24 amino acids were sequenced by Edman degradation. The sequence PASFLPPSSVNKRGVLLLGDAYNL corresponded to residues 388–411 of the full-length rat SE. Three nucleophilic residues (Lys-399, Arg-400, and Asp-407) were labeled by [^{3}H]TNSA-Dza. Triple mutants were prepared in which bulky groups were used to replace the labeled charged residues. Purified mutant enzymes showed lower enzymatic activity and reduced photoaffinity labeling by [^{3}H]TNSA-Dza. This constitutes the first evidence as to the identity of the substrate-binding site of SE.

Keywords: Diazoacetate; Trisnorsqualene; Competitive inhibitor; Active site mapping; Site-directed mutagenesis

Squalene epoxidase (SE) (EC 1.14.99.7) catalyzes the conversion of squalene to (3*S*)-2,3-oxidosqualene [1], which together with the cyclization of (3*S*)-2,3-oxidosqualene to sterols, is a key step in the conversion of acyclic lipids to sterols in plants, fungi, and vertebrates [2]. SE is a membrane-bound protein, which requires oxygen, FAD, NADPH–cytochrome P-450 reductase, NADPH, and a soluble protein factor (SPF) for catalysis [1]. SPF, a 47-kDa protein, has been purified and shown to be involved in intermembrane squalene transport [3] and acts as a cytosolic squalene transfer protein [4]; it may be replaced by 0.1% Triton X-100 (TX-100) in enzyme assays. Recently, the rat and human cDNAs encoding SPF have been cloned and expressed in *Escherichia coli* [4]. The crystal structure of human SPF at a resolution of 1.9 Å revealed a two-domain topology [5]. Phosphorylation of SPF enhances its ability to stimulate microsomal squalene monooxygenase [6]. SE has been purified from rat [7,8] and pig [7,9] liver microsomes. A cDNA for the 63-kDa rat SE has been cloned and truncated, and an enzymatically active 50-kDa His-tagged construct has been overexpressed in bacteria [10,11]. The expression of SE is highly regulated transcriptionally by cholesterol [12] and its localization on human chromosome turned out to be on region 8q24.1 [13]. Most recently, human SE has been cloned, expressed, and characterized enzymatically using a variety of inhibitors [14–18].

Inhibition of SE has become an important pharmaceutical target for the regulation of cholesterol

☆ This work was supported by the National Institutes of Health (Grant GM 44836 to G.D.P.) and The University of Utah.
* Corresponding author. Fax: 1-801-585-9053.
E-mail address: gprestwich@pharm.utah.edu (G.D. Prestwich).
[1] Present address: Endocrine-Hypertension Division, Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115, USA.

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.01.012

biogenesis in humans and in the design of therapeutically important anti-fungal agents [2,19,20]. However, a detailed understanding of the protein–protein and protein–ligand interactions relevant to the catalytic mechanism remains elusive. Pharmaceutical interest has therefore stimulated the design, synthesis, and evaluation of a wide variety of inhibitors based on squalenoid [21–27] and nonsqualenoid [19,20,28–33] structural templates. Recently, several natural products from common foods, e.g., garlic-derived selenocystine and S-allylcysteine [17], resveratrol from grape skins [15], gallate esters from green tea and rhubarb (as well as synthetic gallates) [34–37], and ellagitannins from various plant sources [38] have shown micromolar inhibition of purified rat or human SE. In addition, time-dependent inhibitors [39] and photoaffinity labels [40–43] have been synthesized and evaluated in vitro. SE catalyzes the transfer of molecular oxygen to both squalene and 2,3-oxidosqualene [44], and this process occurs with retention of the C-3 olefinic hydrogen [45].

The substrate requirements for SE have been deduced from studies with substrate analogs and inhibitors [21–24,40–42], which, when considered together, suggest that SE possesses extremely strict steric and electronic requirements for epoxidation. For example, trisnorsqualene alcohol (TNSA) is an inhibitor but not a substrate [22]. The intact polyene chain appears to be required for the substrate to be accepted, since even a single dihydro feature in place of a trisubstituted alkene resulted in lower yields of oxidosqualene analogs [46]. In a more recent work, substrates with even modest conformational alterations, which were induced by heteroatom substitutions (e.g., dimethylsilyl [47], trimethylsilyl, and fluorine [48]) in the polyene backbone or pendant methyl groups, were also inefficiently processed, if at all (M. Bai, X.-Y. Xiao, C. Wawrzenczyk, S.E. Sen, and G.D. Prestwich, unpublished results). Of the many substrate analogs examined, 26-hydroxysqualene and 2,3-oxidosqualene were among the few that were readily converted to epoxide products [27,44]. Even minor structural changes, such as those in 26-acetoxysqualene or 26-aminosqualene, led to compounds that were not accepted as substrates [49]. In order to obtain a substrate-like photoaffinity analog for active site modification, additional design, synthesis, and evaluation were required.

TNSA [22], trisnorsqualene cyclopropylamine (TNS-CPA) [21], and the corresponding hydroxylamine [26] were shown to be potent inhibitors of vertebrate SE ($IC_{50} = 2$–$4\,\mu M$ for native rat SE). Terminal difluoroolefin analogs of squalene ($IC_{50} = 5.4\,\mu M$ for rat) were described as the first mechanism-based inactivators of rat (but not pig) SE [39]. Synthetically prepared labeled [^{3}H]-1,1-difluorovinyltrisnorsqualene and [^{3}H]-bis(1,1-difluorovinyl)hexanorsqualene failed to result in covalent modification of either rat or pig SE [50], resulting in our questioning the mechanism of the time-dependent inactivation observed for this substrate [39]. After examining a large number of substrate-like inhibitors that also possessed photoactivatable moieties, we found that the combination of the diazoacetate extension of the isopropylidene function at one terminus and the TNSA moiety for inhibition at the other terminus provided a competitive inhibitor that was also an effective photoaffinity label. That is, TNSA-Dza showed modest inhibition of rat SE and was also able to photocovalently modify the native protein [49].

In the current investigation, we have photoaffinity-labeled homogeneous recombinant rat SE (rrSE) with [^{3}H]TNSA-Dza, double-digested the covalently modified protein with CNBr/BNPS-skatole, and purified the peptide fragment that contained the covalently attached radioligand by tricine SDS–PAGE. Amino acid sequencing identified one labeled peptide fragment, PASF LPPSSVNKRGVLLLGDAYNL, which corresponded to residues 388–411 of the rat SE. Three nucleophilic residues (Lys-399, Arg-400, and Asp-407 of full-length rat SE) were radioactively labeled by the carbene derived from TNSA-Dza. Mutagenesis, enzyme assay, and photoaffinity labeling of mutant enzymes have provided the first direct evidence for the location of the substrate-binding site of SE.

Materials and methods

Purification of rrSE. Luria broth (LB) media were inoculated with *E. coli* transformed with wild-type His-tagged Δ^{99}-rat SE-encoding plasmid [11], incubated for 3 h, and protein expression was induced by adding isopropylthio-β-D-galactoside (IPTG) to a final concentration of 0.4 mM. After an additional 3 h, the cells were pelleted by centrifugation and washed. Cell pellets were resuspended in 10 mL binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl, pH 8.0) with additives [1 mM *p*-hydroxybenzoate hydroxylase (PMSF), 1 mg/mL lysozyme, 0.5% TX-100, and 5% glycerol] and stirred for 1 h on ice. This suspension was frozen, thawed, and sonicated. The lysate was clarified by centrifugation at 32,000*g* for 30 min and dialyzed against binding buffers including 0.5% TX-100 and 5% glycerol. The supernatant was applied to a Ni–NTA agarose column (bed volume, 3 mL), which had been pre-equilibrated with binding buffer that contained 0.5% TX-100 and 5% glycerol. The column was then rinsed with wash buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl, pH 8.0) and the protein was eluted with 3 mL elution buffer (1 M imidazole, 0.5 M NaCl, and 20 mM Tris–HCl, pH 8.0). The protein sample was applied to Blue-Sepharose column previously equilibrated with buffer A (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, and 0.5% TX-100). After extensive washing of the column with 0.2 M KCl in buffer A, the active fraction was eluted with 1.0 M KCl in buffer A. The fraction that contained SE activity was dialyzed against buffer C (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 5% glycerol, 0.5% TX-100, and 0.5 mM DTT). The purified rrSE was analyzed under standard conditions using SDS–PAGE gel (10%) in Tris–glycine buffer, followed by silver nitrate staining (Fig. 1).

Chemical synthesis of [^{3}H]TNSA-Dza. The diazoester **3** was prepared (Fig. 2) as described for related allylic isoprenoid insect hormone photoaffinity labels [51]. Thus, to a solution of aldehyde **2** (33 mg, 0.082 mmol) in CH_2Cl_2 (8 mL) at 0 °C under argon was

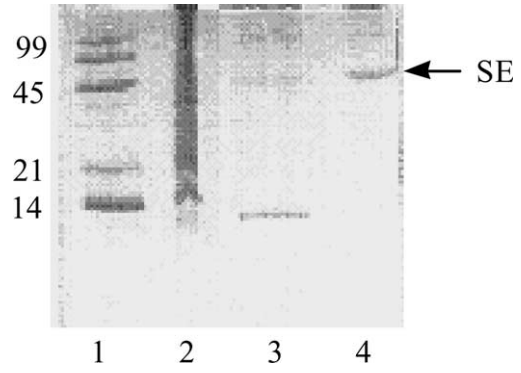

Fig. 1. Silver-stained, denaturing polyacrylamide gel (10% SDS–PAGE) separation of purified SE. rrSE was expressed in BL21(DE3) and purified using Ni–NTA and Blue-Sepharose columns. Equal amounts of protein were loaded in each lane. Lane 1, low range protein marker; lane 2, cellular extract; lane 3, Ni–NTA column; and lane 4, Blue-Sepharose column.

added glyoxylic acid chloride *p*-toluenesulfonylhydrazone (40 mg, 0.153 mmol). Dimethylaniline (19 μL, 0.15 mmol) was added [52] and the mixture was stirred at rt for 30 min. Triethylamine (62 μL, 0.432 mmol) was then added and the mixture was stirred at rt for 30 min. Water (10 mL) was added followed by saturated aqueous citric acid (10 mL). The aqueous layer was extracted with 10% ethyl acetate–hexane (3 × 50 mL). The combined organic phases were washed (saturated NaCl) and dried ($MgSO_4$). The organic solvent was removed and the residue was purified by flash column chromatography using 15% ethyl acetate–hexane to give diazoester **3** (12 mg, 31% yield). ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 9.75 (m, 1H); 5.2–5.0 (m, 6H); 3.89 (s, 2H); 2.48 (m, 2H); 2.31 (m, 2H); 2.10–2.01 (m, 16H); 1.68 (s, 3H); 1.60 (s, 9H); and 1.55 (s, 3H).

*Preparation of TNSA-Dza (**4a**).* To a solution of **3** (11 mg, 23.4 μmol) in MeOH (1 mL) was added $NaBH_4$ (2 mg, 52 μmol). The mixture was stirred at rt for 30 min. Water (0.2 mL) was added and the MeOH was removed by rotary evaporation. The residue was purified by flash column chromatography using 30% ethyl acetate–hexane to give **4a** (7 mg, 65% yield). ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 5.2–5.0 (m, 6H); 3.89 (s, 2H); 3.62 ($t, J = 7.5$ Hz, 2H); 2.20–2.01 (m, 20H); 1.68 (s, 3H); 1.60 (s, 9H); and 1.57 (s, 3H).

*Preparation of [^{3}H]TNSA-Dza (**4b**).* To a solution of **3** (1 mg, 2.1 μmol) in MeOH (0.1 mL) was added NaB_3H_4 (25 mCi, 75 Ci/mmol). The mixture was stirred at rt for 10 h. Water (0.1 mL) was added and MeOH was removed by evaporation under a stream of nitrogen. The residue was purified by flash column chromatography using 30% ethyl acetate–hexane to give [^{3}H]**4b** (1.5 mCi, 18.75 Ci/mmol).

Kinetic studies. Three different concentrations of inhibitor were added to give final concentrations as follows: [TNSA-Dza] = 0, 5, and 20 μM. The SE assay mixtures (see below) that contained an inhibitor were pre-incubated at 37 °C for 10 min. Then, [^{14}C]squalene was added to give final substrate concentrations of 7.5, 10, 14, and 25 μM, and the mixture was incubated for an additional 50 min.

Assay method for SE at pH 7.4. Purified rrSE was reconstituted as follows: rrSE (50 μL, 0.55 mg/mL), 0.06 U NADPH–cytochrome P-450 reductase, 1 mM NADPH, and 0.1 mM FAD in a total volume of 200 μL of 20 mM Tris–HCl, pH 7.4. This reconstituted enzyme solution was pre-incubated at 37 °C for 10 min. Then, 15,000 cpm of squalene in 1 μL of 2-propanol was added to the mixture (final substrate concentration, 6.75 μM). Incubation was continued at 37 °C for 50 min. The enzymatic reaction was quenched by addition of 200 μL of 10% KOH in methanol and incubated at 37 °C for 30 min. The nonsaponifiable lipids were extracted with 1 mL CH_2Cl_2 by vortexing thoroughly and then centrifuged at 1000*g* for 5 min. The organic extracts were evaporated in a Savant Speed-Vac for 15 min. The residue was dissolved in 50 μL CH_2Cl_2 and applied to the pre-adsorbent layer of a silica gel thin layer plate (Whatman silica gel 60 Å, 20 × 20 cm, 250 μm layer). The plate was developed with 5% ethyl acetate–hexane. Radioactive regions in each lane were visualized using Bioscan Imaging Scanner System 200 IBM with Autoexchanger 3000. R_f values were 0.84 for squalene and 0.45 for oxidosqualene. All assays were carried out in duplicate.

*Photoaffinity labeling of SE with TNSA-Dza [^{3}H]**4b**.* A solution of 5 μg rrSE was incubated on ice for 1 h in quartz tubes with a 100-fold molar excess of competitor. The competitor was 1 mM of either TNSA, TNS-CPA, NB-598, squalene, or 26-hydroxysqualene. The mixture was then incubated for an additional 1 h on ice with 10 μM of [^{3}H]**4b** (18 Ci/mmol). Next, the samples were irradiated at 254 nm (six 8-W bulbs) for 45 s. The samples were transferred to 1.5-mL Eppendorf tubes and proteins were precipitated at −20 °C with two volumes of ethanol. This simultaneously removed most unbound radioligand and unlabeled competitors. After ethanol precipitation, samples were dissolved in the SDS sample buffer for electrophoresis. After Coomassie-blue staining, the gels were processed for fluorography [53] by impregnation with a scintillant fluor, shrunk in 50% poly(ethylene glycol), dried in vacuo, and exposed to Kodak XAR-5 X-ray film at −80 °C for 10 days.

Active site mapping. A preparative scale photoaffinity labeling of purified rrSE (50 μg in a total volume of 200 μL) was conducted by

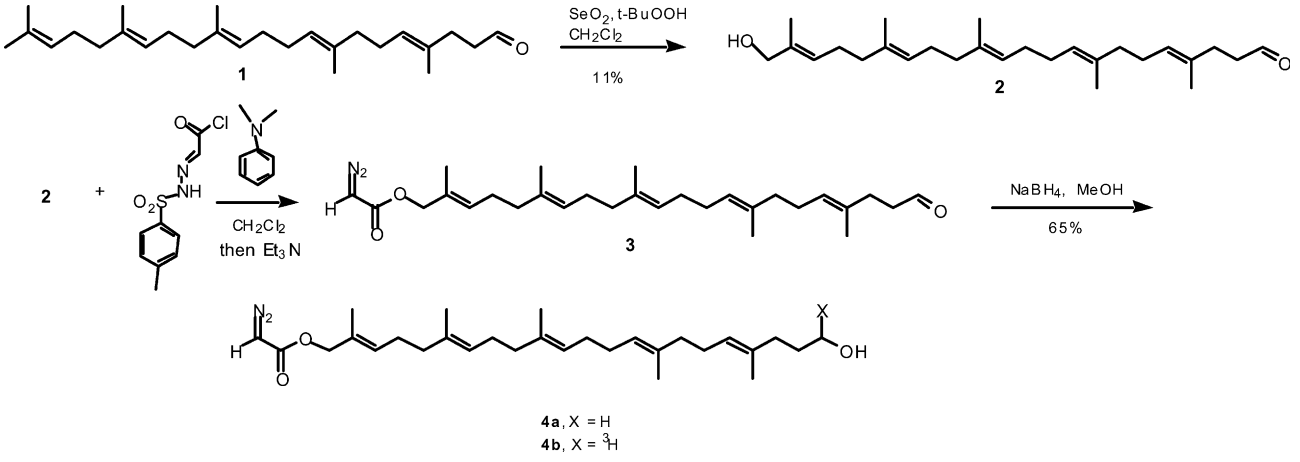

Fig. 2. Synthesis of TNSA-Dza (**4**). See text for experimental details.

incubation of 10 μM [^{3}H]**4b** in the presence of 0.1% TX-100 for 15 min at 4 °C, followed by irradiation at 254 nm for 45 s. The labeled enzyme was chemically digested with 1000-fold molar excess of CNBr (Sigma) in 70% formic acid for 24 h at rt under nitrogen in a foil-wrapped container. After lyophilization, the digested peptides were resuspended in 50% acetic acid. Subsequently, the labeled protein was digested for 24 h at rt under nitrogen gas in darkness with 50-fold molar excess of BNPS-skatole. Peptides were separated using the tricine SDS–PAGE system (16% T, 3% C, 10 mA constant). The radioactivity was monitored on a separate gel by fluorography. Peptides were transferred electrophoretically in 10 mM of 3-(cyclohexylamino)-1-propanesulfonic acid, 10% MeOH (pH 11.0) to a polyvinylidene difluoride (PVDF) membrane. The membrane was then stained briefly in 0.1% Coomassie Blue R-250 in 50% MeOH, destained in 50% MeOH, washed with water, and air-dried. The radiolabeled 8-kDa fragment (4.6×10^4 dpm) was excised and sequenced by Edman degradation using a pulsed liquid phase sequenator (Applied Biosystems Model 475 A). Radiosequencing was carried out separately with an independent preparation of the 8-kDa peptide. Radioactivity was detected in the 12th, 13th, and 20th cycles, representing 20%, 24%, and 35% of the total radioactivity, respectively. After 24 cycles of Edman degradation, no detectable radioactivity remained on the recovered PVDF membrane.

Site-directed mutagenesis of rrSE. Mutagenesis of rrSE was performed as described [54]. Each of the three photoaffinity-labeled residues was mutated to either Pro, Phe, or Trp, and the sequences of the mutated plasmids were confirmed. The single mutants K399F, R400F, and D407F were expressed in the BL21(DE3) strain of *E. coli*, purified, and assayed for activity. No reduction in efficiency of expression was observed for any of the mutants. Next, triple mutants ablating all three sites were prepared. Each triple mutant (K399P/R400P/D407P, K399F/R400F/D407F, and K399W/R400W/D407W) was expressed as above. Triple mutant constructs were purified as described above assayed for SE activity, and subjected to photoaffinity labeling by [^{3}H]**4b**.

Results

Truncated His-tagged rrSE [10,11] was expressed in BL21(DE3) [54] and purified in two steps to give a homogeneous protein as illustrated in Fig. 1. The presence of multiple bands is due to the formation of SDS-stable oligomers of rrSE, as has been previously observed for this recombinant protein [54].

The preparation of 22-hydroxytrisnorsqualene aldehyde (**2**) from the trisnorsqualene aldehyde **1** (Fig. 2) was accomplished by selective oxidation of the terminal (*E*)-methyl group with selenium dioxide as described [55]. Reaction of **2** with glyoxylic acid chloride *p*-toluenesulfonylhydrazone and then with dimethylaniline (1.8 eq.) followed by triethylamine (5 eq.) [52] gave diazoester aldehyde (**3**). Reduction of **3** with $NaBH_4$ gave diazoester **4a** for inhibition studies. Reduction with [^{3}H]$NaBH_4$ (25 mCi, 75 Ci/mmol) provided [^{3}H]**4b** (1.5 mCi, 18.75 Ci/mmol) for use in photoaffinity labeling studies.

We first investigated the kinetics of inhibition of rrSE by TNSA-Dza (**4a**) at pH 7.4 (Fig. 3). For each assay, 5 μg of recombinant SE was used. The Lineweaver–Burk analysis of the data obtained at three concentrations of the diazoester indicated that **4a** was a competitive

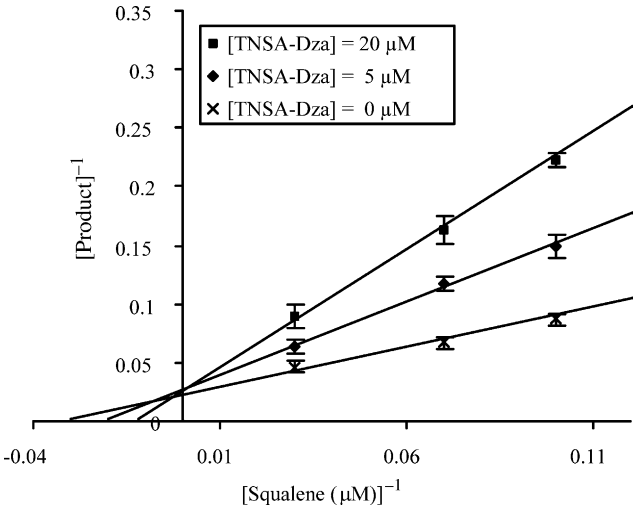

Fig. 3. Double reciprocal plot for determination of K_i value for TNSA-Dza (**4a**). This plot showed that **4a** was a competitive inhibitor with a K_i value of 18.4 μM.

inhibitor with a K_i value of 18.4 μM. Incorporation of radioactivity into the enzyme was investigated by photoaffinity labeling rrSE with [^{3}H]TNSA-Dza at concentrations of 2.5, 5, 7.5, 10, and 15 μM with irradiation at 254 nm (4 °C, 45 s). A plateau was reached at 10 μM and this concentration was employed in further labeling and mapping studies. Importantly, irradiation of the enzyme solution in the absence of **4a** did not result in a measurable loss of SE activity, demonstrating that SE activity survives the photolysis conditions employed for photoaffinity labeling.

In any photoaffinity labeling experiment, it is essential to address the specificity of the labeling reaction [56,57]. Thus, competition experiments (Fig. 4) were carried out with compounds possessing either squalenoid or nonsqualenoid structures. The squalenoid noncompetitive inhibitors TNSA, TNS-CPA, and allylamine inhibitor NB-598 were used; in addition, a competitive inhibitor (26-hydroxysqualene) and the substrate (squalene) were used as competitors. For each labeling experiment, 5 μg rrSE and a 100-fold molar excess (1 mM) of each competitor were pre-incubated for 10 min; then, 10 μM [^{3}H]TNSA-Dza (**4b**) was added and the solution was irradiated. Bovine serum albumin (BSA), a protein capable of nonspecific binding of many lipophilic ligands, was employed as a negative control. Two rrSE protein bands (50 kDa and the dimer at 100 kDa) were labeled and both bands showed the same competition result. Excess TNSA, TNS-CPA, and NB-598, nonsubstrates that exhibit noncompetitive or mixed inhibition, showed significant competitive displacement. Most importantly, excess 26-hydroxysqualene, a competitive inhibitor and substrate, showed complete displacement of labeling of rrSE by [^{3}H]TNSA-Dza (**4b**). No irradiation-dependent labeling by **4b** was observed

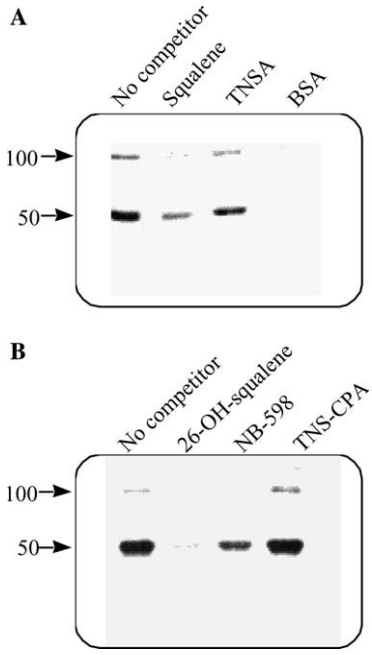

Fig. 4. (A) Photoaffinity labeling of rrSE by [^{3}H]TNSA-Dza (**4b**) and competition with substrate. Five micrograms of rrSE and 10 μM [^{3}H]TNSA-Dza was employed for each experiment (lanes 1–3). Lane 1, no competitor; lane 2, 1 mM squalene; lane 3, 1 mM TNSA; and lane 4, BSA, a protein capable of nonspecific-binding of many lipophilic ligands, was employed instead of SE as a negative control. (B) Photoaffinity labeling of rrSE by [^{3}H]TNSA-Dza and competition with various inhibitors. Lane 1, no competitor; lane 2, 1 mM of 26-hydroxysqualene; lane 3, 1 mM NB-598; and lane 4, 1 mM TNS-CPA.

for BSA, a protein capable of nonspecific binding of many lipophilic ligands.

The CNBr/BNPS-skatole digestion of the ^{3}H-labeled rat liver SE gave two radioactive fragments (less than 10 kDa) and these peptides could be efficiently resolved by a tricine SDS–PAGE (Fig. 5). After electrophoretic transfer to a PVDF membrane, the labeled 8-kDa peptide was subjected to sequential Edman degradation to identify the labeled residues (Fig. 5). The first 10 cycles of sequencing showed an 8 kDa fragment, which corresponded to the amino acids 388–397 of rat SE (PASFLPPSSV). The radioactivity associated with the covalently attached irreversible inhibitor was detected in the 12th, 13th, and 20th cycles, corresponding to three nucleophilic amino acid residues (Lys-399, Arg-400, and Asp-407) located within the amino acid residues 388–411 (PASFLPPSSVNKRGVLLLGDAYNL) of the full-length rat SE. According to the Kyte–Doolittle analysis, this fragment was moderately hydrophobic and proximal to the second FAD-binding motif known to interact with the ribose moiety of FAD [58]. This was not surprising, as we predicted that the active site of SE would be located in the hydrophobic region at the C-terminus of the protein and close to the cofactor-binding site.

Based on the sites of labeling, we selected three sites (Lys-399, Arg-400, and Asp-407) for point mutation to test the hypothesis that photoaffinity labeling had occurred in the active site. From previous inhibitor studies [21–24,40–42], it was apparent that SE had strict steric and electronic requirements for substrate epoxidation. Accordingly, we selected mutations within the labeling site to introduce bulkier side chains that should alter both catalytic activity and photoaffinity labeling. First, single point mutants were prepared: K399F, R400F, and D407F. These three mutants showed 28%, 24%, and 8% of wild-type rrSE activity, respectively (data not shown). In addition, all three point mutants were readily

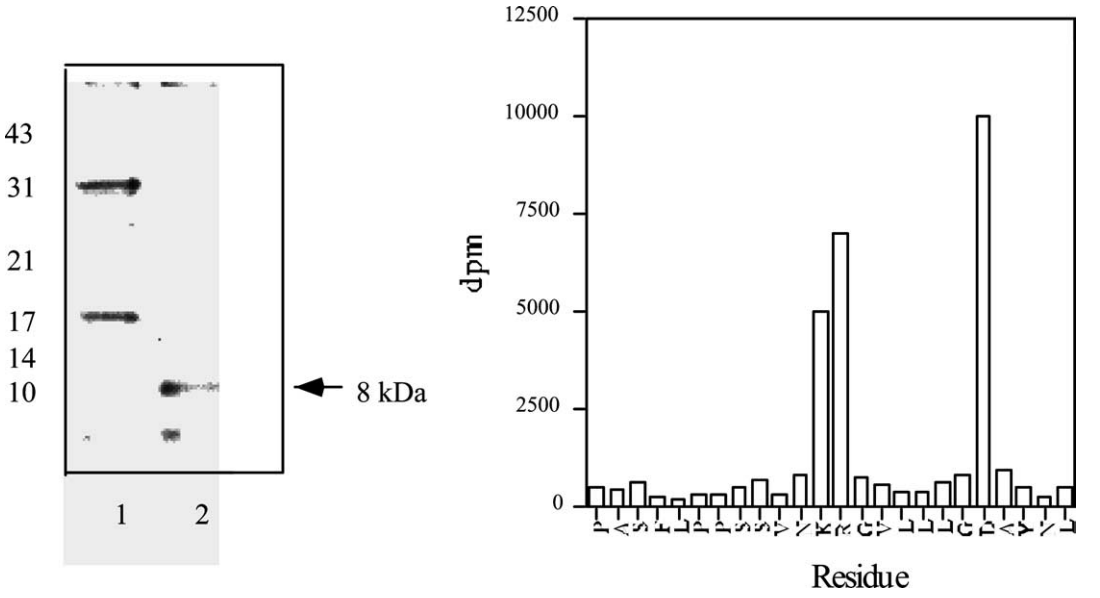

Fig. 5. Fluorogram of the labeled peptides (left) and radiosequencing of the 8-kDa radiolabeled peptide from CNBr/BNPS-skatole digest. The radiolabeled fragment (4.6×10^4 dpm) was excised from the PVDF membrane and sequenced by Edman degradation (right). Lane 1, CNBr digestion and lane 2, CNBr digestion followed by BNPS-skatole digestion.

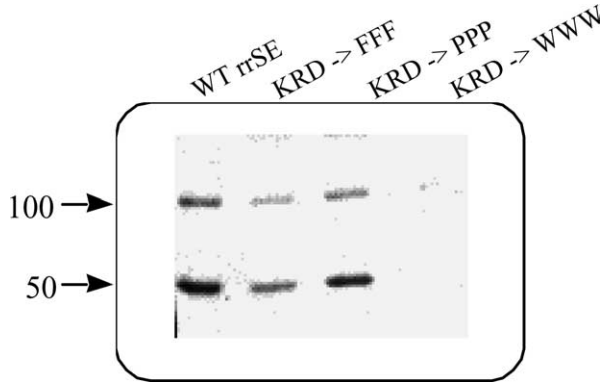

Fig. 6. Photoaffinity labeling of triple mutants by [^{3}H]TNSA-Dza (**4b**). Five micrograms of protein and 10 μM [^{3}H]TNSA-Dza were employed for each experiment. Lane 1, wild-type rrSE; lane 2, K399F/R400F/D407F; lane 3, K399P/R400P/D407P; and lane 4, K399W/R400W/D407W.

photoaffinity labeled with [^{3}H]TNSA-Dza. No change in labeling intensity was observed for K399F, while R400F and D407F showed only minimal reduction in labeling efficiency (data not shown). Next, the three sites were triply mutated to either Pro, Phe, or Trp to give K399P/R400P/D407P, K399F/R400F/D407F, and K399W/R400W/D407W (data not shown). The activity of Pro and Phe triple mutant constructs was $10 \pm 2\%$ of the wild-type rrSE. Interestingly, the Trp triple mutant showed undetectable SE activity. Each of the triple mutants was then subjected to photoaffinity labeling with [^{3}H]TNSA-Dza (Fig. 6). Labeling by **4b** was reduced for each of the three mutant constructs relative to the wild-type rrSE, with the Trp triple mutant showing undetectable labeling. Taken together, the labeling and mutagenesis data provide the first direct evidence regarding the location of the substrate-binding domain of SE.

Discussion

Identification of the active site domains and the specific amino acid contacts important in the binding of the substrate and relevant cofactors is important to achieve a full understanding of the structure and function of an enzyme. For SE, rather limited data exist on residues directly involved in binding the substrates, inhibitors, and cofactors. Furthermore, the tendency of SE to form oligomeric complexes and aggregates, even in the presence of detergents, has thus far prevented crystals suitable for high-resolution structural studies from being obtained. Only recently have inhibitor-based affinity labeling [40] and site-directed mutagenesis [54] been successful in defining some of the cofactor-binding regions. We now demonstrate that TNSA-Dza (**4**) is a useful photoactivatable probe for identification of a putative substrate-binding site of SE by photoaffinity labeling. Moreover, the activity and labeling of triple mutants in the TNSA-Dza-labeled region support the importance of this region for substrate binding.

In addition to the rat SE, the cDNA-derived sequences of SE are now known from human [14], mouse [59], *Saccharomyces cerevisiae* [60], *Candida albicans* [61], the plants *Arabidopsis thaliana* and *Brassica napus* [62], and pig (H.-K. Lee, unpublished results). These proteins show very high sequence homology of 30.2–35.0% between fungal and mammalian enzymes and 87–93% among the mammalian sequences. Significant structural homology exists among SE and the other flavoprotein hydroxylases, in spite of the low sequence similarity [58]. The flavoprotein hydroxylases are monooxygenases that catalyze the insertion of one oxygen atom into the substrate, using pyridine nucleotides as the external donor [63]. These enzymes use an exogenously added reducing agent (NADPH) to provide electrons for the reduction of oxygen to the level of hydrogen peroxide, which is then responsible for the insertion of oxygen into the substrate. FAD is the most important cofactor of those flavin-dependent monooxygenases [63]. However, until now only a few flavin-containing monooxygenases have been described that epoxidize an alkene as the substrate, e.g., the SE from pig liver, the zeaxanthin epoxidase of plants [64], and the alkene monooxygenase from a *Mycobacterium* species [65]. The most thoroughly studied member of this group of flavoproteins is *p*-hydroxybenzoate hydroxylase (PHBH), an enzyme that carries out hydroxylation of activated aromatic compounds.

Even though the flavoprotein monooxygenases lack a known fingerprint sequence for NADPH binding, two characteristic motifs for FAD-binding are present. The first FAD fingerprint sequence is the well-known Rossman fold or βαβ-fold (containing the GXGXXG sequence), which binds the ADP moiety of FAD. The second FAD-binding motif contains the GD sequence with a highly conserved Asp residue that contacts O-3′ of the ribose moiety of FAD [58]. In SE, mutation of either Gly or Asp to Ala increased the K_M of FAD for SE activity [54]. In addition, a newly defined DG amino acid sequence between two FAD-binding domains is highly conserved among all flavoprotein hydroxylases studied [58]. In PHBH, this short sequence motif is situated near a cleft leading toward the active site and faces the putative NADPH-binding pocket [66,67].

Photoaffinity labeling is an important approach used for the identification and purification of novel enzymes, as well as for the identification of the specific residues in the active site. The technique is particularly important in mapping active sites in drug discovery when three-dimensional structures of the target protein are unavailable [68]. Carbenes, which can be formed photochemically from diazoesters, are highly reactive

entities that most commonly undergo trapping by an active site nucleophilic residue [69,70]. We have successfully utilized diazoacetates for labeling of insect hormone and pheromone binding proteins [56,57] and odorant binding protein of the Asian elephant [71], including identification of an active site Thr residue in a moth pheromone binding protein [72]. In this study, the labeling of multiple sites suggests that the carbene has mobility in the binding site and "scans" nearby nucleophiles accessible for direct insertion, or rearranges to a ketene and is then trapped by reaction with an O–H or N–H group.

Previously, we described the preparation of two new photoaffinity analogs [40] of the allylamine SE inhibitor NB-598, a nanomolar inhibitor of rat SE [28] with in vivo cholesterol-lowering activity in dogs and in vitro activity in Hep G2 cells [73,74]. Labeling of SE with one of these analogs, followed by digestion with Lys-C, HPLC purification, and MALDI-TOF mass spectrometry, indicated covalent adducts between *N*-(6,6-dimethyl-2-hepten-4-ynyl)-*N*-ethyl-3-(aminomethyl)benzophenone (PDA-I) and a tripeptide, Asp^{426}–Ile–Lys. Site-directed mutagenesis studies confirmed that this region was involved in the FAD binding [54].

Squalene epoxidation is carried out by a complex consisting of SE and a reductase that supplies reducing equivalents from NADPH, embedded in a lipid membrane. In mammalian systems, this complex also appears to require a SPF [3]. Such a complex system provides many possible mechanisms for inhibition [75]. SE is known to interact with various detergents and lipids such as phospholipids and polyunsaturated fatty acids, but with significant differences between the fungal and mammalian enzymes. One possible mechanism of action of the allylamines would therefore be interaction with either a specific lipid-binding domain of the epoxidase or with the lipids themselves; this could change the conformation of the enzyme and render it catalytically inactive.

One model [76] suggested for SE inhibition postulates that the high affinity of the allylamines for the fungal SE may result from entropically favored binding of the molecule to two separate sites on the enzyme, namely the squalene-binding site (naphthalene ring of the allylamine terbinafine) and an adjacent lipophilic pocket (enyne side-chain). This model is compatible with the available evidence that naphthalene and 1-methylnaphthalene are weak inhibitors of the fungal SE, while the terbinafine side-chain itself has no inhibitory effect at concentrations up to 1 mM. There appears to be a specific lipid-binding domain on SE, while the fungal and rat liver SE enzymes display quite different interactions with respect to detergents, phospholipids, and fatty acids [32]. Slight differences in alignment of the two binding sites could also provide the molecular basis for the selectivity of the allylamines. It is interesting to note that SPF is a member of a family of cytosolic lipid-binding protein that includes Sec14P, α-tocopherol transfer protein, and cellular retinal-binding protein [4]. The recent identification of SPF as α-tocopherol-associated protein (TAP) has called into question its long-standing association with cholesterol biosynthesis [77].

In conclusion, we have prepared TNSA-Dza (**4a**), a novel competitive inhibitor of mammalian SE that contains a photoactivatable diazoacetate moiety. The tritium-labeled isotopomer **4b** was employed to photocovalently modify rrSE. This photolabeling could be displaced by pre-incubation with either an excess of substrate or substrate analogs. The labeled SE was digested by two selective chemical reagents to give a major radiolabeled 24-amino acid peptide fragment, 388-PASFLPPSSVNKRGVLLLGDAYNL-411, in which Lys-399, Arg-400, and Asp-407 were significantly labeled by the [^{3}H]TNSA-Dza. This covalently modified hydrophobic region is located adjacent in the primary SE sequence to the putative FAD-binding region and near the region identified by a benzophenone-containing allylamine photoaffinity label, PDA-1, discussed above. SE activity and photolabeling by **4b** of three triple mutants supported the importance of the three modified residues in substrate recognition and turnover. This result provides the first direct evidence regarding the location of a substrate-binding site for a mammalian SE.

Acknowledgments

The authors are indebted to Dr. I. Abe (The University of Utah, Utah) for the preparation of reductase. Drs. B.A. Madden (The University at Stony Brook, Stony Brook, NY) and C.M. Amann (Chemical Synthesis Facility, UUtah) provided [^{14}C]squalene employed for SE activity assays. This work was supported by NIH GM 44836 to G.D.P.

References

[1] S. Yamamoto, K. Bloch, Studies on squalene epoxidase of rat liver, J. Biol. Chem. 245 (1970) 1670–1674.
[2] I. Abe, G.D. Prestwich, Squalene epoxidase and oxidosqualene: lanosterol cyclase key enzymes in cholesterol biosynthesis, in: D.H.R. Barton, K. Nakanishi (Eds.), Comprehensive Natural Products, Elsevier Science, London, 1999, pp. 267–298.
[3] I.W. Caras, K. Bloch, Effects of a supernatant protein activator on microsomal squalene-2,3-oxide-lanosterol cyclase, J. Biol. Chem. 254 (1979) 11816–11821.
[4] N. Shibata, M. Aritai, Y. Misaki, N. Dohmae, K. Takio, T. Ono, K. Inoue, H. Arai, Supernatant protein factor, which stimulates the conversion of squalene to lanosterol, is a cytosolic squalene transfer protein and enhances cholesterol biosynthesis, Proc. Natl. Acad. Sci. USA 98 (2001) 2244–2249.
[5] A. Stocker, T. Tomizaki, C. Schulze-Briese, U. Baumann, Crystal structure of the human supernatant protein factor, Structure 10 (2002) 1533–1540.
[6] D.K. Singh, V. Mokashi, C.L. Elmore, T.D. Porter, Phosphorylation of supernatant protein factor enhances its ability to

stimulate microsomal squalene monooxygenase, J. Biol. Chem. 278 (2003) 5646–5651.
[7] P. Denner-Ancona, M. Bai, H.-K. Lee, I. Abe, G.D. Prestwich, Purification of pig and rat liver squalene epoxidase by affinity chromatography, Bioorg. Med. Chem. Lett. 5 (1995) 481–486.
[8] T. Ono, Y. Imai, Squalene epoxidase from rat liver microsomes, Methods Enzymol. 110 (1985) 375–380.
[9] M. Bai, G.D. Prestwich, Inhibition and activation of porcine squalene epoxidase, Arch. Biochem. Biophys. 293 (1992) 305–313.
[10] J. Sakakibara, R. Watanabe, Y. Kanai, T. Ono, Molecular cloning and expression of rat squalene epoxidase, J. Biol. Chem. 270 (1995) 17–20.
[11] A. Nagumo, T. Kamei, J. Sakakibara, T. Ono, Purification and characterization of recombinant squalene epoxidase, J. Lipid Res. 36 (1995) 1489–1497.
[12] M. Nagai, J. Sakakibara, Y. Nakamura, F. Gejyo, T. Ono, SREBP-2 and NF-Y are involved in the transcriptional regulation of squalene epoxidase, Biochem. Biophys. Res. Commun. 295 (2002) 74–80.
[13] M. Nagai, J. Sakakibara, K. Wakui, Y. Fukushima, S. Igarashi, S. Tsuji, M. Arakawa, T. Ono, Localization of the squalene epoxidase gene (SQLE) to human chromosome region 8q24.1, Genomics 44 (1997) 141–143.
[14] B.P. Laden, Y. Tang, T.D. Porter, Cloning, heterologous expression, and enzymological characterization of human squalene monooxygenase, Arch. Biochem. Biophys. 374 (2000) 381–388.
[15] B.P. Laden, T.D. Porter, Resveratrol inhibits human squalene monooxygenase, Nutr. Res. 21 (2001) 747–753.
[16] B.P. Laden, T.D. Porter, Inhibition of human squalene monooxygenase by tellurium compounds: evidence of interaction with vicinal sulfhydryls, J. Lipid Res. 42 (2001) 235–240.
[17] N. Gupta, T.D. Porter, Garlic and garlic-derived compounds inhibit human squalene epoxidase, J. Nutr. 131 (2001) 1662–1667.
[18] N. Gupta, T.D. Porter, Inhibition of human squalene monooxygenase by selenium compounds, J. Biochem. Mol. Toxicol. 16 (2002) 18–23.
[19] S. Halazy, J.-P. Gotteland, Squalene epoxidase inhibitors, Expert. Opin. Ther. Patents 6 (1996) 1165–1173.
[20] L. Cattel, M. Ceruti, G. Balliano, F. Viola, G. Grosa, F. Schuber, Drug design based on biosynthetic studies: synthesis, biological activity, and kinetics of new inhibitors of 2,3-oxidosqualene cyclase and squalene epoxidase, Steroids 53 (1989) 363–391.
[21] S.E. Sen, G.D. Prestwich, Trisnorsqualene cyclopropylamine: a reversible, tight-binding inhibitor of squalene epoxidase, J. Am. Chem. Soc. 111 (1989) 8761–8762.
[22] S.E. Sen, G.D. Prestwich, Trisnorsqualene alcohol, a potent inhibitor of vertebrate oxidase, J. Am. Chem. Soc. 111 (1989) 1508–1510.
[23] S.E. Sen, G.D. Prestwich, Squalene analogs containing isopropylidene mimics as potential inhibitors of pig liver squalene epoxidase and oxidosqualene cyclase, J. Med. Chem. 32 (1989) 2152–2158.
[24] S.E. Sen, C. Wawrzenczyk, G.D. Prestwich, Inhibition of vertebrate squalene epoxidase by extended and truncated analogs of trisnorsqualene alcohol, J. Med. Chem. 33 (1990) 1698–1701.
[25] M. Ceruti, S. Amisano, P. Milla, F. Viola, F. Rocco, M. Jung, L. Cattel, Stereocontrolled synthesis of fluorosqualenes and fluoroepoxysqualenes as inhibitors of squalene epoxidase and 2,3-oxidosqualene cyclase, J. Chem. Soc. Perkin Trans. 1 (1995) 889–893.
[26] G.M. Anstead, H.-K. Lin, G.D. Prestwich, Trisnorsqualene methyl hydroxylamine: a potential dual inhibitor of mammalian squalene epoxidase and oxidosqualene cyclase, Bioorg. Med. Chem. Lett. 3 (1993) 1319–1322.
[27] M. Bai, X.-Y. Xiao, G.D. Prestwich, 26-Hydroxysqualene and derivatives: substrate and inhibitor of squalene epoxidase, Bioorg. Med. Chem. Lett. 1 (1991) 227–232.
[28] M. Horie, Y. Tsuchiya, M. Hayashi, Y. Iida, Y. Iwasawa, Y. Nagata, Y. Sawasaki, H. Fukuzumi, K. Kitani, T. Kamei, NB-598: a potent competitive inhibitor of squalene epoxidase, J. Biol. Chem. 265 (1990) 18075–18078.
[29] J.-P. Gotteland, I. Brunel, F. Gendre, J. Désiré, A. Delhon, D. Junquéro, P. Oms, S. Halazy, (Aryloxy)methylsilane derivatives as new cholesterol biosynthesis inhibitors: Synthesis and hypocholesterolemic activity of a new class of squalene epoxidase inhibitors, J. Med. Chem. 38 (1995) 3207–3216.
[30] J.-P. Gotteland, A. Delhon, D. Junquéro, P. Oms, S. Halazy, Design and synthesis of new hypocholesterolemic organosilanes with antioxidant properties, Bioorg. Med. Chem. Lett. 6 (1996) 533–538.
[31] J.-P. Gotteland, D. Junquéro, P. Oms, A. Delhon, S. Halazy, Comparative study of new and known thienyl derivatives of ENE-YNE benzylamine as mammalian squalene epoxidase inhibitors, Med. Chem. Res. 6 (1996) 333–342.
[32] B. Favre, N.S. Ryder, Differential inhibition of fungal and mammalian squalene epoxidases by the benzylamine SDZ SBA 586 in comparison with the allylamine terbinafine, Arch. Biochem. Biophys. 340 (1997) 265–269.
[33] J.-P. Gotteland, C. Loubat, B. Planty, D. Junquéro, A. Delhon, S. Halazy, Sulfonamide derivatives of benzylamine block cholesterol biosynthesis in HepG2 cells: a new type of potent squalene epoxidase inhibitors, Bioorg. Med. Chem. Lett. 8 (1998) 1337–1342.
[34] I. Abe, T. Seki, H. Noguchi, Potent and selective inhibition of squalene epoxidase by synthetic galloyl esters, Biochem. Biophys. Res. Commun. 270 (2000) 137–140.
[35] I. Abe, T. Seki, H. Noguchi, Y. Kashiwada, Galloyl esters from rhubarb are potent inhibitors of squalene epoxidase, a key enzyme in cholesterol biosynthesis, Planta Med. 66 (2000) 753–756.
[36] I. Abe, T. Seki, K. Umehara, T. Miyase, H. Noguchi, J. Sakakibara, T. Ono, Green tea polyphenols: novel and potent inhibitors of squalene epoxidase, Biochem. Biophys. Res. Commun. 268 (2000) 767–771.
[37] I. Abe, Y. Kashiwagi, H. Noguchi, Inhibition of vertebrate squalene epoxidase by isoprenyl gallates and phenylalkyl gallates, Bioorg. Med. Chem. Lett. 10 (2000) 2525–2528.
[38] I. Abe, Y. Kashiwagi, H. Noguchi, T. Tanaka, Y. Ikeshiro, Y. Kashiwada, Ellagitannins and hexahydroxydiphenoyl esters as inhibitors of vertebrate squalene epoxidase, J. Nat. Prod. 64 (2001) 1010–1014.
[39] W.R. Moore, G.L. Schatzman, E.T. Jarvi, R.S. Gross, J.R. McCarthy, Terminal difluoro olefin analogues of squalene are time-dependent inhibitors of squalene epoxidase, J. Am. Chem. Soc. 114 (1992) 360–361.
[40] P.A. Denner-Ancona, Purification and Characterization of Vertebrate Squalene Epoxidase and Oxidosqualene Cyclase, State University of New York, Stony Brook, NY, 1995.
[41] G.M. Anstead, S.E. Sen, G.D. Prestwich, Evaluation of squalene analogs bearing photoreactive groups as photoaffinity labels for squalene epoxidase and oxidosqualene cyclase, Bioorg. Chem. 19 (1991) 300–313.
[42] M. Ceruso, G.D. Prestwich, Novel non-squalenoid photoaffinity labels for mammalian squalene epoxidase, Bioorg. Med. Chem. Lett. 4 (1994) 2179–2184.
[43] J.-P. Gotteland, C. Dax, S. Halazy, Design and synthesis of new potential photoaffinity labels for mammalian squalene epoxidase, Bioorg. Med. Chem. Lett. 7 (1997) 1153–1156.
[44] M. Bai, X.-Y. Xiao, G.D. Prestwich, Epoxidation of 2,3-oxidosqualene to 2,3,22,23-squalene dioxide by squalene epoxidase, Biochem. Biophys. Res. Commun. 185 (1992) 323–329.
[45] O. Boutaud, M. Ceruti, L. Cattel, F. Schuber, Retention of the label during the conversion of [3-^{3}H]squalene into (3S)-2,3-oxidosqualene catalyzed by mammalian squalene oxidase, Biochem. Biophys. Res. Commun. 208 (1995) 42–47.

[46] E.E. van Tamelen, J.R. Heys, Enzymatic epoxidation of squalene variants, J. Am. Chem. Soc. 97 (1975) 1252–1253.
[47] G.D. Prestwich, C. Wawrzenczyk, Synthesis of allyl vinyl silanes: preparation of 12-(dimethylsila)squalene, Tetrahedron Lett. 30 (1989) 403–406.
[48] S.E. Sen, Squalene Analogs and Inhibitors of Pig Liver Squalene Epoxidase and Oxidosqualene Cyclase, State University of New York, Stony Brook, NY, 1989.
[49] M. Bai, Purification and Characterization of Vertebrate Squalene Epoxidase, State University of New York, Stony Brook, NY, 1991.
[50] B. Madden, Synthesis of Rat Squalene Epoxidase and Oxidosqualene Cyclase Inhibitors, State University of New York, Stony Brook, NY, 1997.
[51] I. Ujváry, W.-S. Eng, G.D. Prestwich, Syntheses of homologous, tritium-labeled analogs of the natural juvenile hormones JH I, JH II, and JH III, J. Labelled Compd. Radiopharm. 28 (1990) 65–72.
[52] E.J. Corey, A.G. Myers, Efficient synthesis and intramolecular cyclopropanation of unsaturated diazoacetic esters, Tetrahedron Lett. 25 (1984) 3559–3562.
[53] M.A. Mohamed, K.A. Lerro, G.D. Prestwich, Polyacrylamide gel miniaturization improves protein visualization and autoradiographic detection, Anal. Biochem. 177 (1989) 287–290.
[54] H.-K. Lee, P. Denner-Ancona, J. Sakakibara, T. Ono, G.D. Prestwich, Photoaffinity labeling and site-directed mutagenesis of rat squalene epoxidase, Arch. Biochem. Biophys. 381 (2000) 43–52.
[55] X.-Y. Xiao, G.D. Prestwich, Regio- and stereo-controlled synthesis of [24,30-^{14}C]-labeled 2,3-epoxysqualene, J. Labelled Compd. Radiopharm. 29 (1991) 883–890.
[56] G.D. Prestwich, K. Touhara, L.M. Riddiford, B.D. Hammock, Larva lights: a decade of photoaffinity labeling with juvenile hormone analogues, Insect Biochem. Mol. Biol. 24 (1994) 747–761.
[57] G.D. Prestwich, Chemistry of pheromone and hormone metabolism in insects, Science 237 (1987) 999–1006.
[58] M.H.M. Eppink, H.A. Schreuder, W.J.H. van Berkel, Identification of a novel conserved sequence motif in flavoprotein hydroxylases with a putative dual function in FAD/NAD(P)H binding, Protein Sci. 6 (1997) 2454–2458.
[59] K. Kosuga, S. Hata, T. Osumi, J. Sakakibara, T. Ono, Nucleotide sequence of a cDNA for mouse squalene epoxidase, Biochim. Biophys. Acta: Gene Struct. Expr. 1260 (1995) 345–348.
[60] A. Jandrositz, F. Turnowsky, G. Högenauer, The gene encoding squalene epoxidase from *Saccharomyces cerevisiae*: cloning and characterization, Gene 107 (1991) 155–160.
[61] B. Favre, N.S. Ryder, Cloning and expression of squalene epoxidase from the pathogenic yeast *Candida albicans*, Gene 189 (1997) 119–126.
[62] U.A. Schafer, D.W. Reed, D.G. Hunter, K. Yao, A.M. Weninger, E.W.T. Tsang, M.J.T. Reaney, S.L. MacKenzie, P.S. Covello, An example of intron junctional sliding in the gene families encoding squalene monooxygenase homologous in *Arabidopsis thaliana* and *Brassica napus*, Plant Mol. Biol. 39 (1999) 721–728.
[63] K. Büch, H. Stransky, A. Hager, FAD is a further essential cofactor of the NAD(P)H and O_2-dependent zeaxanthin-epoxidase, FEBS Lett. 376 (1995) 45–48.
[64] E. Marin, L. Nussaume, A. Quesada, M. Gonneau, B. Sotta, P. Hugueney, A. Frey, A. Marion-Poll, Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*, EMBO J. 15 (1996) 2331–2342.
[65] F.J. Weber, W.J.H. van Berkel, S. Hartmans, J.A.M. de Bont, Purification and properties of the NADH reductase component of alkene monooxygenase from *Mycobaterium* Strain E3, J. Bacteriol. 174 (1992) 3275–3281.
[66] M.H.M. Eppink, H.A. Schreuder, W.J.H. van Berkel, Interdomain binding of NADPH in *p*-hydroxybenzoate hydrxoylase as suggested by kinetic, crystallographic and modeling studies of histidine 162 and arginine 269 variants, J. Biol. Chem. 273 (1998) 21031–21039.
[67] M.S. Lah, B.A. Palfey, H.A. Schreuder, M.L. Ludwig, Crystal structures of mutant *Pseudomonas aeruginosa p*-hydroxybenzoate hydroxylases: the Tyr201Phe, Tyr385Phe, and Asn300Asp variants, Biochemistry 33 (1994) 1555–1564.
[68] G. Dormán, G.D. Prestwich, Using photolabile ligands in drug discovery and development, Trends. Biotechnol. 18 (2000) 64–77.
[69] S.A. Fleming, Chemical reagents in photoaffinity labeling, Tetrahedron 51 (1995) 12479–12520.
[70] H. Bayley, Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier, New York, 1983.
[71] J. Lazar, D.R. Greenwood, L.E. Rasmussen, G.D. Prestwich, Molecular and functional characterization of an odorant binding protein of the Asian elephant, *Elephas maximus*: implications for the role of lipocalins in mammalian olfaction, Biochemistry 41 (2002) 11786–11794.
[72] G. Du, C.-S. Ng, G.D. Prestwich, Odorant binding by a pheromone binding protein: active site mapping by photoaffinity labeling, Biochemistry 33 (1994) 4812–4819.
[73] Y. Hidaka, H. Hotta, Y. Nagata, Y. Iwasawa, M. Horie, T. Kamei, Effect of a novel squalene epoxidase inhibitor, NB-598, on the regulation of cholesterol metabolism in HepG2 cells, J. Biol. Chem. 266 (1991) 13171–13177.
[74] Y. Hidaka, T. Satoh, T. Kamei, Regulation of squalene epoxidase in HepG2 cells, J. Lipid Res. 31 (1990) 2087–2094.
[75] N.S. Ryder, Post-squalene inhibition of sterol biosynthesis, Biochem. Soc. Trans. 18 (1990) 45–46.
[76] N.S. Ryder, Squalene epoxidase as a target for the allylamines, Biochem. Soc. Trans. 19 (1991) 774–777.
[77] T.D. Porter, Supernatant protein factor and tocopherol-associated protein: an unexpected link between cholesterol synthesis and vitamin E (Review), J. Nutr. Biochem. 14 (2003) 3–6.