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3-(4-Chlorophenyl)-2-(4-diethylaminoethoxyphenyl)-A-pentenonitrile Monohydrogen Citrate and Related Analogs

REVERSIBLE, COMPETITIVE, FIRST HALF-REACTION SQUALENE SYNTHETASE INHIBITORS

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ABSTRACT. Squalene synthetase (SQS) catalyzes the head-to-head condensation of two molecules of farnesyl pyrophosphate (FPP) to form squalene. The reaction is unique when compared with those of other FPP-utilizing enzymes, and proceeds in two distinct steps, both of which involve carbocationic reaction intermediates. In this report, we describe the mechanism of action of, and structure–activity relationships within, a series of substituted diethylaminoethoxystilbenes that mimic these reaction intermediates, through characterization of the biochemical properties of 3-(4-chlorophenyl)-2-(4-diethylaminoethoxyphenyl)-A-pentenonitrile monohydrogen citrate (P-3622) and related analogs. As a representative member of this series, P-3622 inhibited SQS reversibly and competitively with respect to FPP (K_i = 0.7 μM), inhibited the enzymatic first half-reaction to the same extent as the overall reaction, exhibited a 300-fold specificity for SQS inhibition relative to protein farnesyltransferase inhibition, inhibited cholesterol synthesis in rat primary hepatocytes (IC_{50} = 0.8 μM), in cultured human cells (Hep-G2, CaCo-2, and IM-9; IC_{50} = 0.2, 1.2, and 1.0 μM), and in chow-fed hamsters (62% at 100 mg/kg) without accumulation of post-squalene sterol precursors, and reduced plasma cholesterol in experimental animals. Structure–activity relationships among 72 related analogs suggest that the phenyl residues and central *trans*-olefin of the stilbene moiety serve as mimics of the three isoprene units of the donor FPP, that substitutions across the central olefin and *para*-substitutions on the terminal phenyl residue mimic the branching methyl groups of the donor FPP, and that the diethylaminoethoxy moiety of these molecules mimics the various carbocations that develop in the C1–C3 region of the acceptor FPP during reaction. Members of this series of reversible, competitive, first half-reaction SQS inhibitors that show a high degree of specificity for SQS inhibition relative to inhibition of other FPP-utilizing enzymes and other cholesterol synthesis pathway enzymes may serve as useful tools for probing the unique catalytic mechanisms of this important enzyme. BIOCHEM PHARMACOL 53;6:839–864, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. squalene synthetase; cholesterol synthesis; enzyme kinetics; enzyme inhibition; diethylaminoethoxystilbenes; structure–activity relationships

SQS† (farnesyl diphosphate: farnesyl diphosphate farnesyltransferase; EC 2.5.1.21) catalyzes the head-to-head condensation of two molecules of FPP to form squalene [1–4]. The reaction is unique when compared with those of other FPP-utilizing enzymes [1, 5], and proceeds in two distinct steps, both of which involve the formation of carbocationic reaction intermediates [1–4, 6–12]. In the first half-reaction, two molecules of FPP react to form the stable cyclopropylcarbinyl diphosphate intermediate PSQPP [1–4], with concomitant release of a proton and a molecule of PP_i [1–4], through a mechanism in which the C2–C3 double bond of one FPP molecule serves as the prenyl acceptor for the farnesyl residue of the other [1–4]. In the second half-reaction, thought to occur at a second catalytic site within the enzyme active center [1, 11, 13–15], PSQPP undergoes heterolysis, isomerization, and reduction with NADPH to form squalene [1–4]. Translocation of PSQPP to the second reaction center occurs without its release from the enzyme [14, 15].

Since FPP is situated at the final branch-point in the isoprenoid biosynthetic pathway [16–18], its conversion to squalene through the action of SQS represents both the first committed step in the formation of cholesterol and related sterols [16, 17] and the point of transition from

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† *Abbreviations:* SQS, squalene synthetase; FPP, farnesyl pyrophosphate; PFT, protein farnesyltransferase; PSQPP, presqualene pyrophosphate; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; DMEM, Dulbecco's minimal essential medium; HI-FBS, heat-inactivated fetal bovine serum; HBSS, Hanks' balanced salt solution; and PMSF, phenylmethylsulfonyl fluoride.

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hydrophilic to hydrophobic pathway intermediates [18], and therefore is thought to represent a major control point of sterol and isoprenoid biosynthesis in eukaryotes [5, 19, 20]. Conversion of FPP to squalene thus represents an ideal site for selectively inhibiting sterol formation without interfering with the production of key nonsterol polyisoprenoids such as dolichol, ubiquinone, and prenylated proteins that play important roles in the regulation of cellular growth and metabolism [16, 17, 21–23]. Inhibitors that interfere with various aspects of the reactions catalyzed by SQS not only could serve to further our understanding of the unique reaction mechanisms catalyzed by this enzyme, but also could serve to further our understanding of the regulatory mechanisms controlling mevalonate metabolism and the differential utilization of FPP by the various prenyltransferases catalyzing key reactions in this pathway.

In this regard, various studies using either FPP analogs as alternative substrates [3, 4, 24–26], or reaction inhibitors that act either as ground-state FPP [27–34] or PSQPP mimics [4, 13], or mimics of the putative carbocationic intermediates of either the first or second half-reactions [1, 7–12, 35], have been designed to probe the substrate binding sites, reaction centers, and catalytic mechanisms of the two half-reactions catalyzed by SQS. Based on these and other studies, plausible reaction mechanisms for the conversion of FPP to PSQPP and for the conversion of PSQPP to squalene have been advanced [1–3, 6, 11, 14]. In this regard, it has been postulated that the SQS catalytic machinery consists of two non-identical FPP binding sites with different substrate [3, 4, 24, 26] and inhibitor [33] binding affinities, one site that binds FPP in a conformation that facilitates its heterolytic cleavage to yield an allylic carbocation that acts as a farnesyl donor, and one site that binds FPP in an orientation that facilitates insertion of the developing carbocation from the donor FPP into its C2–C3 double bond to form PSQPP [1, 3]. Such studies have also suggested that the two FPP molecules may bind sequentially, with the donor FPP binding first [14, 26], that the acceptor FPP binding site is able to accommodate a wider variation in the structure of FPP analogs than is the donor FPP binding site [3, 4, 24], and that FPP analogs may bind selectively to either FPP binding site [33]. Finally, studies with PSQPP mimics [13] and with carbocationic reaction intermediate mimics [11] have suggested that the SQS first and second half-reactions are catalyzed by distinct reaction centers within the SQS active center that are either spatially overlapping or are nonoverlapping but are strongly interacting [1, 11, 13, 15].

Use of inhibitors for evaluating the SQS reaction mechanism has led to identification of two distinct classes of SQS inhibitors. The first class is composed of polyanionic FPP and PSQPP mimetics whose multiple acidic groups serve as stable mimetics of the PP_i functionalities of FPP and/or PSQPP, and whose hydrophobic side-chain moieties serve as mimetics of the farnesyl residues of these molecules. Representatives of this class of inhibitors include substituted and/or partially saturated farnesyl-based alternate substrates or inhibitors [3, 24–27], farnesyl-based bisphosphonates [33, 36], (phosphinylmethyl)phosphonates [28, 29, 32, 34], phosphinato(dialkylmethyl)phosphonates [31], methylphosphonophosphates [13], phosphinylformates and acetates [28], and presqualene-based methylphosphonophosphates [13]. In addition, diverse natural products have been identified that contain acidic cores that possess multiple carboxylate functions linked to one or two long chain hydrophobic residues that mimic both PP_i and farnesyl moieties of FPP and/or PSQPP (e.g. the zaragozic acids (a.k.a. squalestatins; [37–42]) and semi-synthetic analogs [43–48], the grotonic acids [49–51], and others reported in the patent literature [52a–c]. Structure–function studies have also indicated that inhibitory activity is maintained in certain mono- and dicarboxylate analogs of these natural products [44, 45, 47, 48], and reports of unrelated synthetic mono and dicarboxylate SQS inhibitors have appeared in the patent literature [53a–c].

The second class of SQS inhibitors is composed of hydrophobic cationic farnesyl or difarnesyl mimetics that mimic carbocationic intermediates of either (or both) of the SQS-catalyzed half-reactions. Representatives of this class of inhibitors include farnesylamines [10, 35], *N*-(alkylaryl)farnesylamines [10], *N,N*-difarnesylamines [10], amidinium analogs of *N,N*-difarnesylamine [35], ammonium [8, 9, 11], and sulfonium [7] analogs of PSQPP, phenoxypropylamines [54], phenylbenzyloxyquinuclidines [42], and other hydrophobic amine-containing compounds that have appeared in the patent literature [55a–c]. Many of these compounds demonstrate significantly improved activity when presented to the enzyme active center as an inhibitor/PP_i ion pair [7–11, 35], suggesting that the SQS-catalyzed reactions proceed through carbocation-diphosphate ion pairs in which the developing carbocationic intermediates remain associated with released PP_i anions [1, 3], an observation that has prompted evaluation of amphiphilic polyisoprenoid compounds containing both cationic ammonium functionalities and covalently linked methylphosphonophosphate moieties in close proximity [1, 8, 11, 12].

Studies using the farnesyl-based anionic substrates and inhibitors described above have demonstrated a critical dependence of inhibitory or alternative substrate activity on the presence of the unsaturations in the farnesyl chains [24–27, 43], particularly that of the central isoprene unit of the farnesyl residue [25], an observation that is consistent with structure–activity relationships observed within the zaragozic acid series with respect to the terminal aryl ring of the C6 side-chain [43]. In this regard, Biller and co-workers [56] evaluated the utility of replacing the isoprene units of such farnesyl-containing anionic FPP and PSQPP mimetics with conformationally restricted aromatic isosteres, and found that while a variety of heterocyclic aromatic isoprene replacements were tolerated [56], the most potent aromatic isostere was the *para*-disubstituted phenyl replacement for

these isoprene units [56]. Biller and co-workers [32] also noted that insertion of an ether oxygen between the farnesyl residue and the (phosphinylmethyl)phosphonate residue of farnesyl (phosphinylmethyl)phosphonate, as a hydrogen bond acceptor from a putative active site acid residue, markedly improved inhibitory potency over its carbon isostere.

In exploring application of the above replacement theories of Biller *et al.* [32, 56] to the farnesyl-based cationic SQS inhibitors, and with a consideration of the importance of the unsaturation in the central isoprene unit of all-*trans*-FPP to its utility as a substrate [25], we evaluated a series of substituted diethylaminoethoxystilbenes, previously shown to inhibit cholesterol synthesis and lower plasma cholesterol levels in experimental animals [57, 58], for their ability to act as SQS inhibitors. We regarded these compounds as potential reaction intermediate mimetics based on a consideration that the terminal and diethylaminoethoxy-proximal phenyl residues and central *trans*-olefin of the stilbene moiety could serve as mimics of the three isoprene units of the farnesyl moiety of the donor FPP and that the diethylaminoethoxy moiety could serve as a mimic of the various cyclopropane carbocations that develop in the C1–C3 region of the first isoprene unit of the acceptor FPP during the course of the SQS-catalyzed reaction.

In this report, we describe the mechanism of action of, and the structure–activity relationships within, a series of substituted diethylaminoethoxystilbenes that mimic proposed cationic intermediates of the SQS-catalyzed reaction, through characterization of the biochemical properties of 3-(4-chlorophenyl)-2-(4-diethylaminoethoxyphenyl)-4-pentenonitrile monohydrogen citrate, P-3622, and related analogs.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, squalene, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, PMSF, trypsin, soybean trypsin inhibitor, collagenase, bovine serum albumin, insulin, ketoconazole, and EDTA were from the Sigma Chemical Co. (St. Louis, MO). Dithiothreitol was from U.S. Biochemicals (Cleveland, OH). Ascorbate oxidase was from Boehringer Mannheim Biochemicals (Indianapolis, IN). [1-^{3}H]Farnesyl pyrophosphate ([^{3}H]FPP; 22.5 Ci/mmol), *R,S*-[2-^{14}C]mevalonolactone (54.1 mCi/mmol), sodium [2-^{14}C]acetate (56 mCi/mmol), and Aquasol-2 were from New England Nuclear (Boston, MA). Ready-Safe was from Beckman Instruments (Fullerton, CA). MicroScint-20, solvent-resistant UniFilter GF/B glass fiber 96-well filter plates, and TopSeal-P solvent-resistant protective cover films were from the Packard Instrument Co. (Meriden, CT). Non-radiolabeled FPP was from either American Radiolabeled Chemicals Inc. (St. Louis, MO) or R. K. Keller (University of South Florida Research Foundation, Tampa, FL). NaF, $MgCl_2$, $ZnCl_2$, $CaCl_2$, $MgSO_4$, HCl, NaOH, K_xPO_4, KCl, KBr, Tris base, Tri–HCl, toluene, chloroform, ethyl acetate, petroleum ether, hexane, diethyl ether, acetic acid, glycerol and DMSO were from the Fisher Scientific Co. (Philadelphia, PA). Absolute EtOH was from Pharmco Products Inc. (Bayonne, NJ). Sodium pentobarbital was from The Butler Co. (Columbus, OH). Sterile saline was from Kendall–McGaw Laboratories (Irvine, CA). RPMI-1640, DMEM, DMEM/F12, L-glutamine, gentamicin, trypsin-EDTA solution, PBS, HBSS, and penicillin/streptomycin antibiotic solution were from GIBCO Laboratories (Grand Island, NY). HI-FBS for growth of IM-9 cells and Hep-G2 cells was from Intergen (Purchase, NY). HI-FBS for growth of CaCo-2 cells and for maintenance of rat primary hepatocytes was from HyClone Laboratories (Logan, UT). Silica gel TLC plates were from Eastman Kodak (Rochester, NY). BCA protein assay reagent was from Pierce (Rockford, IL). Zaragozic acid A was prepared from the unidentified fungal species (American Type Culture Collection No. 20986) previously described by Bergstrom *et al.* [41]. Recombinant H-ras was from Oncogene Sciences (Uniondale, NY). Hep-G2 cells, IM-9 cells, and CaCo-2 cells were obtained from the American Type Culture Collection (Rockville, MD).

Buffers and Solutions

PMED Buffer contained 50 mM K_xPO_4 (pH 7.4), 5 mM $MgCl_2$, 1.0 mM EDTA, and 5.0 mM dithiothreitol. *TEDK Buffer* contained 50 mM Tris (pH 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol, and 70 mM KCl. *TEDG Buffer* contained 50 mM Tris (pH 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol, and 50% (v/v) glycerol. *TEDK$_{1000}$ Buffer* contained 50 mM Tris (pH 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol, and 1.0 M KCl. *Trypsin/HCl Solution* contained 7.6 mg/mL trypsin in 1.0 mM HCl. *Soybean Trypsin Inhibitor Solution* contained 15.2 mg/mL soybean trypsin inhibitor in 1.0 mM HCl. *Rat Brain Homogenization Buffer* contained 50 mM Tris–HCl (pH 7.5), 5 mM dithiothreitol, 200 mM KCl, 20 μM $ZnCl_2$, and 1 mM PMSF. *SQS Assay Buffer* contained 50 mM K_xPO_4 (pH 7.4) and 5 mM $MgCl_2$. *SQS Cofactor/Substrate Solution I* was prepared in a quantity sufficient for 160 SQS activity determinations by adding 4000 μL of double-strength SQS Assay Buffer, 2400 μL of 50 mM NaF, 800 μL of 200 U/mL glucose-6-phosphate dehydrogenase, 735 μL of deionized water, and 65 μL of 1 mM [^{3}H]FPP (sp. act. 252 dpm/pmol) to a lyophilized tube containing 2.52 mg $NADP^+$ and 8.19 mg glucose-6-phosphate. *SQS Cofactor/Substrate Solution II* was prepared in a quantity sufficient for 160 SQS activity determinations by adding 2000 μL of double-strength SQS Assay Buffer, 2000 μL of double-strength SQS assay buffer containing 125 U/mL ascorbate oxidase, 2400 μL of 50 mM NaF, 800 μL of 200 U/mL glucose-6-phosphate dehydrogenase, 500 μL of 1.25 M sodium ascorbate, 235 μL of deionized water, and 65 μL of 1 mM [^{3}H]FPP (sp. act. 252 dpm/pmol) to a lyophilized tube containing 2.52 mg $NADP^+$ and 8.19 mg glucose-6-phosphate. *PFT Assay Buffer* contained 50 mM Tris–HCl (pH 7.5), 5 mM dithiothreitol, and 20 μM

$ZnCl_2$. *PFT Cofactor/Substrate Solution* was prepared in sufficient quantity for 80 PFT activity determinations by mixing 50 μL of 20 μM [^{3}H]FPP (sp. act. 12,800 dpm/pmol), 100 μL of 100 mM $MgCl_2$, 400 μL of double-strength PFT Assay Buffer, and 250 μL of deionized water. *Supplemented DMEM-A* contained 10% HI-FBS, 2 mM L-glutamine, and 40 μg/mL gentamicin in DMEM. *Supplemented DMEM-B* contained 20% HI-FBS, 2 mM L-glutamine, and 40 μg/mL gentamicin in DMEM. *Density 1.125 Saline Solution* contained 335 g/L KBr in saline. *Dialysis Buffer A* contained 0.24 mM EDTA (pH 7.4) and 150 mM NaCl.

Preparation of Lipoprotein-Deficient Serum

HI-FBS was adjusted to a density of 1.25 g/mL with solid KBr. Aliquots, 25 mL, of the density-adjusted serum were layered under 15 mL of Density 1.215 Saline Solution and centrifuged at 10° for 48 hr at 504,000 *g*. The upper fraction containing all the lipoproteins was removed and discarded. The lower fraction containing delipidated serum was dialyzed extensively against Dialysis Buffer A with a minimum of five buffer changes, each of 100 mL buffer/mL delipidated serum. After dialysis, the delipidated serum was adjusted with Buffer A to the original serum volume and filter-sterilized, and 5-mL aliquots were apportioned into 50-mL sterile plastic tubes and stored frozen at −20°.

Growth of Hep-G2 Cells

Hepatoma cells from the Hep-G2 cell line were seeded in T-75 flasks at a density of 2.0×10^6 cells/flask in 12 mL of Supplemented DMEM-A and were maintained in culture in a 37°, 5% CO_2 incubator for 7 days with medium changes on days 3 and 5.* At this time, cultures reached approximately 80–90% confluency (late-log phase) and maintained a >90% cell viability as judged by Trypan blue dye exclusion. Late-log phase cultures were passaged by removing the medium, rinsing the cells with 5 mL of trypsin-EDTA solution, and then adding 5 mL of fresh trypsin-EDTA solution to each flask. Following incubation for 2–5 min at 37°, 5 mL of Supplemented DMEM-A was added, and the trypsin-released cells were transferred to a 15-mL conical plastic tissue culture tube, sedimented by centrifugation at 2000 *g* for 5 min at 4°, and resuspended in 12 mL of fresh Supplemented DMEM-A.

Growth of CaCo-2 Cells

Colonic adenocarcinoma cells from the CaCo-2 cell line were seeded in T-75 flasks at a density of 5.0×10^6 cells/flask in 12 mL of Supplemented DMEM-B and were maintained in culture in a 37°, 10% CO_2 incubator for 6 days with medium changes on days 2 and 4. At this time, cultures reached 80% confluency and maintained a >90% cell viability (Trypan blue dye exclusion). Late-log phase cultures were passaged essentially as described above for Hep-G2 cells except that following addition of the second 5-mL aliquot of Trypsin-EDTA solution, incubation was for 5–10 min at 37°, and after centrifugation, sedimented cells were resuspended in 12 mL of fresh Supplemented DMEM-B.

Growth of IM-9 Cells

Lymphoid cells from the IM-9 cell line were maintained in suspension culture at 37° in 1000-mL spinner flasks containing 500 mL of RPMI-1640 medium supplemented with 10% HI-FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin [59]. Every 3 days, resulting late-log phase cultures whose cell viability was >90% (Trypan blue dye exclusion) were separated into two equal portions. One portion was diluted to a concentration of 5×10^5 cells/mL with fresh medium, and maintained at 37° for continuation of the culture. The second portion was utilized as described below.

Isolation of Primary Rat Hepatocytes

Isolated, morphologically intact, rat hepatic parenchymal cells were obtained from anesthetized rats as follows. Livers were perfused *in situ* at 37° with oxygenated HBSS for 20 min at a flow rate of 7.0 mL/min, followed by perfusion for 15 min at 37° with HBSS containing 1 mg/mL collagenase and 2 mM $CaCl_2$. After perfusion, cells were separated from debris by passage through cheesecloth, and then were washed extensively with DMEM containing 1% bovine serum albumin by repetitive cycles of resuspension and sedimentation by centrifugation at 2000 *g* for 5 min at 4°. Washed cells were filtered through a 100-μm mesh screen and resuspended in DMEM/F12 containing 10% fetal bovine serum, 2 mM L-glutamine, 40 μg/mL gentamicin, and 1 μg/mL insulin. At least 90% of the final cell preparation consisted of intact parenchymal cells that excluded Trypan blue dye.

Isolation of Hepatic Microsomes

Hepatic microsomes were prepared as previously described [42, 60]. Liver tissue from male Sprague–Dawley rats (approx. 150 g), male CD1 mice (approx. 30 g), and male SEA (Susceptible to Experimental Atherosclerosis) quail (approx. 100 g), administered food and water *ad lib.*, was collected after sodium pentobarbital injection and immediately rinsed in PBS. Human liver tissue from a brain dead organ donor, stored frozen in liquid N_2, was thawed rapidly to room temperature and rinsed in PMED Buffer. Liver tissues were homogenized at 4° in PMED Buffer at a ratio of 2 mL PMED Buffer/g liver, using 15 strokes of a Dounce tissue homogenizer. Homogenates were centrifuged at 10,000 *g* for 20 min at 4°, and the resultant supernatants were centrifuged at 178,000 *g* for 90 min at 4°. For microsomal preparations to be used for SQS measurement, microsomal pellets were resuspended in 1.0 mL PMED

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Buffer/g liver by 5 strokes of a Potter–Elvehjem pestle and stored frozen in liquid N_2. There was no notable loss in SQS activity following 6 months of storage in liquid N_2. Just prior to use, microsomal suspensions were thawed and diluted with PMED Buffer to a protein concentration of 1.0 mg/mL. For microsomal preparations to be used for SQS solubilization, microsomes were isolated as described above using TEDK Buffer in place of PMED Buffer, and microsomal pellets were stored frozen in liquid N_2 without resuspension.

Isolation of Hep-G2 Cell Microsomes

Hep-G2 cell microsomes were prepared as described for isolation of IM-9 cell microsomes [59] with the following modifications. Hep-G2 cells isolated from T-75 flasks as described above were sedimented by centrifugation at 2000 *g* for 5 min at 4°, resuspended in 10 mL per flask of Supplemented DMEM-A containing 0.33 mg/mL soybean trypsin inhibitor, transferred to 50-mL conical plastic centrifuge tubes, and resedimented. Resulting Hep-G2 cell pellets were resuspended in Supplemented DMEM-A, combined, and washed twice with Supplemented DMEM-A and once with PBS. The resulting cell pellet was frozen in liquid N_2 (for a minimum of 30 min), then rapidly thawed at 37° and homogenized in PMED buffer at a ratio of 1.0 mL/g of cells, first using 15 strokes of a ground glass pestle in a 2-mL Potter–Elvehjem tissue homogenizer and subsequently in a 5-mL Potter–Elvehjem tissue homogenizer, using 5 strokes of a motor-driven teflon pestle. The homogenate was then centrifuged at 10,000 *g* for 15 min at 4°, and the resulting supernatant was centrifuged at 178,000 *g* for 60 min at 4°. The resulting microsomal pellet was resuspended in PMED Buffer at a ratio of 0.5 mL/g cells and stored frozen in liquid N_2. Just prior to use, microsomal suspensions were thawed and diluted with PMED Buffer to a protein concentration of 1.0 mg/mL.

Preparation of Trypsin-Solubilized Rat Liver SQS

SQS was solubilized by limited trypsinolysis [42]. A 10-g aliquot of rat hepatic microsomes, isolated and frozen in liquid N_2 as described above, was thawed slowly at room temperature, and then homogenized in 7.4 mL of TEDG Buffer with 3 strokes of a Potter–Elvehjem tissue homogenizer using a motor-driven teflon pestle. To the resulting homogenate was added 357 μL of Trypsin/HCl Solution. After rehomogenization, the mixture was incubated for 60 min at room temperature with an additional homogenization midway through incubation. After incubation, 889 μL of Soybean Trypsin Inhibitor/HCl solution was added to the homogenate to terminate the proteolysis reaction, and, after rehomogenization, the volume was adjusted to 50 mL with $TEDK_{1000}$ Buffer and the diluted homogenate was centrifuged at 178,000 *g* for 60 min at 4°. The resulting supernatant (solubilized SQS) was then stored frozen in liquid N_2 in 5-mL aliquots.

Isolation of Rat Brain Cytosol

Rat brain cytosol was prepared as previously described [60]. Brain tissue from seven, 150–200 g male Sprague–Dawley rats was collected after sodium pentobarbital injection and immediately homogenized in 14 mL of 4° Rat Brain Homogenization Buffer, using 15 strokes of a Dounce tissue homogenizer. Homogenates were centrifuged at 178,000 *g* for 90 min at 4°, and the supernatants (cytosol) were removed and stored frozen in 2-mL aliquots in liquid N_2. There was no notable loss in enzyme activity following 3 months of storage in liquid N_2. Just prior to use, microsomal suspensions were thawed and diluted with PFT Assay Buffer to a protein concentration of 3.0 mg/mL.

Measurement of SQS Activity

SQS activity was evaluated by measuring the conversion of [^{3}H]FPP to [^{3}H]squalene under anaerobic conditions [42, 60] to prevent conversion of nascent squalene to squalene epoxide. In the majority of studies, anaerobic conditions were achieved through deoxygenation of reagents by repetitive cycles of evacuation and reaeration with nitrogen and by maintenance of reaction mixtures in a nitrogen atmosphere during incubation [60]. However, in some studies, anaerobic conditions were achieved by inclusion of an ascorbate/ascorbate oxidase oxygen consumption system [42].

For studies in which anaerobic conditions were achieved through mechanical deoxygenation, immediately prior to measurement, all reagent solutions were subjected to three, 5- to 10-min cycles of evacuation and re-equilibration to atmospheric pressure with nitrogen. SQS activity was then measured as follows. To a 50-μL mixture containing 3.0 μL of deoxygenated DMSO ± effector compounds and 47 μL of deoxygenated SQS Cofactor/Substrate Solution I were added 25 μL of a deoxygenated 1.0 mg/mL suspension of hepatic microsomes in PMED Buffer [final assay concentrations: 48 mM K_xPO_4 (pH 7.4), 4.8 mM $MgCl_2$, 0.33 mM EDTA, 1.67 mM dithiothreitol, 258 μM $NADP^+$, 2.1 mM glucose-6-phosphate, 0.94 U glucose-6-phosphate dehydrogenase, 9.4 mM NaF, 4% DMSO, 5.1 μM [^{3}H]FPP (sp. act. 253 dpm/pmol), 0.33 mg/mL microsomal protein, and the indicated concentrations of effector compounds]. Enzyme blanks received 25 μL of deoxygenated PMED Buffer in place of deoxygenated hepatic microsomal suspension. After completing all additions, reaction vessels were flushed with nitrogen and capped, and the contents were mixed by gentle vortexing. After incubation at 37° for 30 min, enzymatic reactions were terminated by the addition of 40 μL of 10 M NaOH, followed sequentially by the addition of 40 μL of absolute EtOH and 10 μL of 2 mg/mL squalene in chloroform. Reaction mixtures were then capped, vortexed, and saponified for 90 min at 37°. After saponification, mixtures were mixed vigorously, 25-μL aliquots were applied to 1.2 × 10 cm channels of a silica gel TLC, and newly formed squalene was separated from unreacted substrate by chro-

matography in toluene:EtOAc::9:1. The region of each chromatogram from 2 cm below the squalene band (visualized with iodine vapors; R_f = 0.74) to the top of the chromatogram was removed, immersed in Aquasol-2 liquid scintillation fluid, and assessed for radioactivity using a Beckman LS6500 liquid scintillation counter.

For studies in which anaerobic conditions were achieved by inclusion of an ascorbate/ascorbate oxidase oxygen consumption system, SQS activity was measured as described above except that mechanical deoxygenation of reagent solutions and maintenance of reaction mixtures in a nitrogen atmosphere were omitted, and the 47 µl addition of deoxygenated SQS Cofactor/Substrate Solution I was replaced by 47 µL of SQS Cofactor/Substrate Solution II (final assay concentrations in addition to those listed above: 50 mM sodium ascorbate and 1.5 U ascorbate oxidase).

Under either assay variation, squalene production from radiolabeled FPP was a linear function both of the duration of incubation at 37°, for a minimum of 120 min, and of the concentration of microsomal protein present during incubation, up to 100 µg microsomal protein. In addition, little or no farnesol (R_f = 0.23) was formed as the result of FPP dephosphorylation and no further metabolism of newly-formed squalene to downstream intermediates of the cholesterolgenesis pathway (e.g. cholesterol R_f = 0.15; lanosterol R_f = 0.28) was noted. SQS activity is expressed as picomoles of squalene formed from FPP per minute of incubation at 37° per milligram of microsomal (or solubilized microsomal) protein, based on the stoichiometry of the reaction whereby 2 moles of [^{3}H]FPP react to form 1 mole of [^{3}H]squalene and half of the radiolabel is lost from the C-1 position of the prenylated [^{3}H]FPP due to 1-*pro*-S hydrogen release. Variations in SQS activity measured using either version of this procedure averaged 3–5%.

Measurement of the SQS First Half-Reaction

The first half-reaction catalyzed by SQS, in which 2 molecules of [1-^{3}H]FPP react to form [^{3}H]PSQPP with concomitant release of a proton from the C-1 position of the prenylating FPP molecule, was evaluated by measuring the rate of proton release through equilibration of the expelled tritium with the primary hydroxyl of a fixed volume of added MeOH and then isolation of the methanol by distillation and quantitation of the specific activity of the distilled MeOH [15]. The initial steps of the proton release reaction were conducted exactly as described above for measurement of the overall reaction (either version) except that the volumes of all reagent additions were doubled (final assay volume 150 µL), and reactions were terminated by the addition of 80 µL of 10 M NaOH. A volume of 20 µL of water was added to each mixture to bring the total volume to 250 µL, and mixtures were transferred to 12-mL conical glass-stoppered centrifuge tubes and washed three times, each with 2.0 mL of petroleum ether. Residual solvent was removed from the remaining aqueous phases with a gentle stream of nitrogen; then 2 mL of anhydrous MeOH was added to each aqueous phase. Tubes were capped and samples were incubated for 30 min at 50° with gentle shaking. After incubation, samples were cooled to room temperature, 425 mg of anhydrous (oven-dried at 120° for 3 hr) $MgSO_4$ was added to each sample, and the resulting mixtures were sealed and stored at room temperature overnight. Mixtures were then vortexed to break up the resulting caked precipitates and centrifuged briefly in a desk-top centrifuge to facilitate separation. Resulting supernatant fractions were transferred to 10-mL round bottom flasks and approximately 0.7 to 1.0 mL of MeOH was distilled (distillation temperature 60–62°) using a microdistillation apparatus (Lab Glass Cat. No. ML-905A). Aliquots, 0.5 mL, of each distillate were mixed with 15 mL of Aquasol-2 liquid scintillation fluid and assessed for radioactivity using a Beckman LS6500 liquid scintillation counter. The rate of proton release is expressed as picomoles released per minute of incubation at 37° per milligram of microsomal protein based on the relationship whereby dpm tritium released is equal to 6.25 times the dpm contained in the assessed 0.5 mL of MeOH for the sample and MeOH volumes utilized in these studies, as outlined by Agnew [15].

Using this configuration, both the overall SQS catalyzed reaction, and the SQS first half-reaction can be assessed simultaneously by tripling the volumes of all reagent additions described above for measurement of the overall reaction (final assay volume 225 µL), terminating reactions by the addition of 120 µL of 10 M NaOH, and then apportioning 115 µL of terminated reaction mixtures to 500-µL microfuge tubes for overall reaction assessment, and processing the remaining 230 µL of each reaction mixture for proton release measurement as outlined above. Under these conditions, in the absence of inhibitor, the rate of proton release averaged 120 ± 5 pmol/min/mg rat liver microsomal protein (SD; N = 6) and was approximately equal to the rate of squalene formation of 144 ± 17 pmol/min/mg (SD; N = 8).

Measurement of PFT Activity

PFT activity was evaluated by measuring the covalent incorporation of the farnesyl moiety of [^{3}H]FPP into H-ras as previously described [60]. Briefly, to either 0.3 µL DMSO (control incubations) or 0.3 µL of DMSO containing effector compounds were added 10 µL of PFT Cofactor Substrate Solution, and then 5 µL of a 20 µM solution of H-ras in PFT Assay Buffer. Reactions were initiated by the addition of 9.7 µL of a 3 mg/mL solution of rat brain cytosol [final assay concentrations: 0.5 µM [^{3}H]FPP (sp. act. 12770 dpm/pmol), 5 mM $MgCl_2$, 20 µM $ZnCl_2$, 4 µM H-ras, 50 mM Tris–HCl (pH = 7.5), 5 mM dithiothreitol, 20 mM KCl, 1.2% DMSO, 30 µg (1.2 mg/mL) rat brain cytosolic protein]. Enzyme blanks received 10 µL of PFT Assay Buffer in place of rat brain cytosol, whereas endogenous cytosolic protein farnesylation blanks received 5 µL of PFT Assay Buffer in place of H-ras solution addition. After a

60-min incubation at 37°, reactions were terminated by the addition of 200 μL of 10% (v/v) concentrated HCl in absolute EtOH. After an additional 15-min incubation at 37°, to allow protein precipitation and FPP hydrolysis to occur, 300 μL absolute EtOH was added to each sample, and then the entire sample was applied to a filtration well of a Packard UniFilter GF/B 96-well plate under gentle (house) vacuum using a Millipore Millititer Vacuum Filtration apparatus. After complete sample addition, each well was washed with 500 μL volumes of absolute EtOH and allowed to dry. The bottom of the UniFilter GF/B plate was then sealed, 50 μL MicroScint-20 liquid scintillation fluid was added to each well, and the plate was heat-sealed with TopSeal-P, using a Packard MicroMate 496 Microplate Heat Sealer, and then counted in a Packard Top-Count Microplate Scintillation Counter. PFT activity is expressed as picomoles H-ras farnesylated per minute of incubation at 37° per milligram of cytosolic protein.

Measurement of Cholesterol Synthesis in Cultured Cells

The rate of cholesterol formation in primary rat hepatocytes and in cultured Hep-G2, IM-9 and CaCo-2 cells was assessed by measuring incorporation of [2-^{14}C]acetate into nonsaponifiable lipids as outlined below. For measurement of cholesterol synthesis in Hep-G2 cells, cells isolated as described above were seeded in 24-well tissue culture plates at a density of 1.2×10^5 cells/well and maintained in culture in 1.0 mL of Supplemented DMEM-A for 7 days at 37° in a 5% CO_2 atmosphere with medium changes on days 3 and 5. On day 8, at a time when cultures reached approximately 80–90% confluency (late-log phase) and maintained a >90% cell viability (Trypan blue dye exclusion), the medium was removed and replaced with fresh Supplemented DMEM-A containing 1% DMSO ± effector compounds. For measurement of cholesterol synthesis in CaCo-2 cells, cells isolated as described above were seeded in 24-well tissue culture plates at a density of 1×10^5 cells/well and maintained in culture in 1.0 mL of Supplemented DMEM-B for 6 days at 37° in a 10% CO_2 atmosphere with medium changes on days 2 and 4. On day 6, at a time when cultures reached 80% confluency (late-log phase) and maintained >90% cell viability, the medium was removed and replaced with 1.0 mL of fresh Supplemented DMEM-B that contained 20% lipoprotein-deficient HI-FBS in place of 20% HI-FBS. After 18 hr of incubation in this medium to maximize rates of cholesterol synthesis, the medium was removed and replaced with 1.0 mL of fresh medium containing 0.4% DMSO ± effector compounds. For measurement of cholesterol synthesis in IM-9 cells, cells isolated as described above were apportioned in 2.5-mL aliquots to 12 × 75 mm plastic culture tubes, sedimented at 2000 *g* for 5 min, and resuspended to a density of 2.5×10^6 cells/mL with 1.0 mL of RPMI-1640 medium containing 10% HI-FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.4% DMSO ± effector compounds. For measurement of cholesterol synthesis in primary rat hepatocytes, hepatocytes prepared as described above, were plated in 6-well culture plates at a density of 5×10^5 cells/well in 1.0 mL DMEM/F12 containing 10% HI-FBS, 2 mM L-glutamine, 40 μg/mL gentamicin, and 1 μg/mL insulin, and incubated for 3 hr at 37° in a 5% CO_2 atmosphere to allow cell attachment. The medium was then removed, 1.0 mL of fresh DMEM/F12 containing 10% lipoprotein-deficient fetal bovine serum, 2 mM L-glutamine, 40 μg/mL gentamicin, and 1 μg/mL insulin was added, and cells were incubated for an additional 24 hr at 37° in a 5% CO_2 atmosphere. After incubation in this medium to maximize rates of cholesterol synthesis, the medium was removed and replaced with 1.0 mL of fresh medium containing 1% DMSO ± effector compounds.

Immediately following the addition of the respective medium containing DMSO ± effector compounds, 25 μL of medium containing 4 μCi of [2-^{14}C]acetate was added to each well or culture tube. Plates were then sealed with parafilm, culture tubes were capped, to avoid evaporation, and cells were incubated at 37° for 6 hr with gentle shaking. After incubation, samples were saponified by the addition of 1 mL of 5 N KOH in MeOH, followed first by incubation for 2 hr at 70° and then by overnight incubation at room temperature. Following saponification, mixtures were transferred to glass conical tubes and extracted three times with 4.5 mL hexane. The pooled organic fractions were dried under nitrogen, resuspended in 25 μL chloroform, and applied to 1 × 20 cm channels of Silica Gel 60C TLC plates. Channels containing non-radioactive cholesterol, lanosterol, and squalene were included on selected TLC plates as separation markers. TLC plates were developed in hexane:diethyl ether:acetic acid (70:30:2), air dried, and assessed for radioactive nonsaponified lipids by analysis using a Berthold Linear Radioactivity Analyzer that reports peak location and integrated peak area. A reduction of total radioactivity incorporated into post-squalene cholesterol synthesis intermediates is indicative of inhibition in the isoprene portion of the cholesterol synthesis pathway (acetate to squalene). Disproportionation of sterols among post-squalene cholesterol synthesis intermediates is indicative of inhibition in the sterol portion of the pathway (squalene to cholesterol). Cholesterol synthesis from [2-^{14}C]acetate is expressed as dpm incorporated into either post-squalene cholesterolgenic intermediates or into cholesterol during the 6-hr incubation at 37°.

Measurement of Cholesterol Synthesis in Chow-Fed Hamsters

Hepatic incorporation of radiolabeled mevalonate into cholesterol in hamsters was measured as previously described for hepatic incorporation of radiolabeled acetate into cholesterol in rats [61] with the following modifications. Male golden Syrian hamsters weighing approximately 150 g were given food and water *ad lib.*, and administered a 1.0-mL i.p. injection of either water (control animals) or water containing the indicated concentration of effector

compounds. One hour following compound administration, animals received an i.p. injection of 0.5 mL of *R,S*-[2-^{14}C]mevalonolactone (2 μCi/mL; 54.1 mCi/mmol) per 150 g of body weight. Two hours after administration of the radiolabeled mevalonolactone, animals were euthanized by pentobarbital injection and two, 1-g liver pieces were removed. Tissue samples were saponified at 70° for 120 min in 2.5 mL of 2.5 M NaOH, then 5 mL of absolute EtOH was added to each sample, and the solutions were mixed. Petroleum ether (10 mL) was added to each sample, and the mixtures were first shaken vigorously for 2 min and then centrifuged at 2000 *g* in a bench-top Sorvall for 10 min. The resultant petroleum ether layers were removed, and 3.0-mL aliquots were mixed with Ready-Safe liquid scintillation fluid and assessed for radioactivity as a measure of total nonsaponifiable lipid synthesis (approximately 66% of which results from cholesterol and post-squalene cholesterolgenic intermediates; [62]) using a Beckman LS6500. To determine the formation of cholesterol relative to other post-squalene cholesterolgenic intermediates, an additional 5.0-mL aliquot of each petroleum ether extract was dried under nitrogen, resuspended in 25 μL chloroform, applied to 1 × 20 cm channels of Silica Gel 60C TLC plates, developed in hexane:diethyl ether:acetic acid (70:30:2), and visualized for radioactive nonsaponified lipids using a Berthold Linear Radioactivity Analyzer as described above. Cholesterolgenesis from mevalonate is expressed as either dpm radiolabeled mevalonate incorporated into nonsaponifiable lipids per gram liver (based on direct counting of petroleum ether extract) or dpm radiolabeled mevalonate incorporated into cholesterol per gram liver (based on TLC separation of cholesterol from other post-squalene intermediates of the cholesterolgenic pathway) during the 2-hr interval between radiolabeled mevalonate injection and pentobarbital administration.

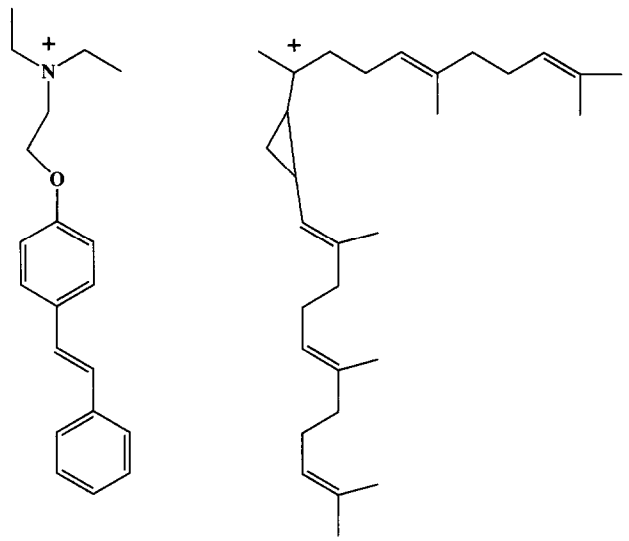
Measurement of Protein Concentration

Protein concentration was measured using BCA (bicinchoninic acid) Protein Assay Reagent and bovine serum albumin as standard.

RESULTS

Similarities Between Substituted Diethylaminoethoxystilbenes and Proposed Cationic Intermediates of the SQS-Catalyzed Reaction

As shown in Fig. 1, 4-(diethylaminoethoxy)stilbene bears a close resemblance to proposed cationic intermediates for the SQS-catalyzed reaction (e.g. the terminal and diethylaminoethoxy-proximal phenyl residues and central *trans*-olefin of the stilbene moiety mimic the three isoprene units of the donor FPP and the diethylaminoethoxy moiety mimics the various cyclopropane carbocations that develop in the C1–C3 region of the first isoprene unit of the acceptor FPP after condensation). Substitutions across the central double bond of the stilbene moiety as well as *para*-substitutions on the terminal phenyl moiety and substitutions *meta* to the central olefin on the diethylaminoethoxy-proximal phenyl moiety could potentially mimic the three branching methyl groups of the farnesyl residue of the donor FPP molecule. Interaction of these molecules with the SQS active center as ion pairs with P_i from solution serving as PP_i counter ion replacements could allow these molecules to function both as first half-reaction intermediate mimetics, with P_i from solution serving as replacements for both the attached PP_i from the acceptor FPP and the released PP_i from the donor FPP, as well as second half-reaction intermediate mimetics, with P_i from solution serving as replacements for released PP_i molecules. Indeed, when evaluated using K_m FPP concentrations (5.1 μM; [42]) in buffer containing 50 mM P_i, diethylaminoethoxystilbene inhibited rat liver microsomal SQS with an IC_{50} of 6 μM.



4-(diethylamino-ethoxy)stilbene **Proposed transition-state intermediate**

FIG. 1. Structures of 4-(B-diethylaminoethoxy)stilbene and proposed carbocationic intermediates of the SQS-catalyzed reaction.

Inhibition of SQS by P-3622

Of the substituted diethylaminoethoxystilbenes evaluated for SQS inhibitory activity (see below), 3-(4-chlorophenyl)-2-(4-diethylaminoethoxyphenyl)-A-pentenonitrile monohydrogen citrate (P-3622; Fig. 2, inset) was among the most potent, exhibiting an IC_{50} for inhibition of rat liver microsomal SQS in the presence of 5.1 μM FPP and 50 mM P_i of 0.75 ± 0.2 μM (SD; N = 4) (Fig. 2), and was thus the subject of further mechanistic characterization. P-3622 also inhibited SQS in microsomes from a variety of sources including rat, mouse, SEA quail, and human liver, and Hep-G2 cells, exhibiting IC_{50} values that were similar for all species evaluated (Table 1).

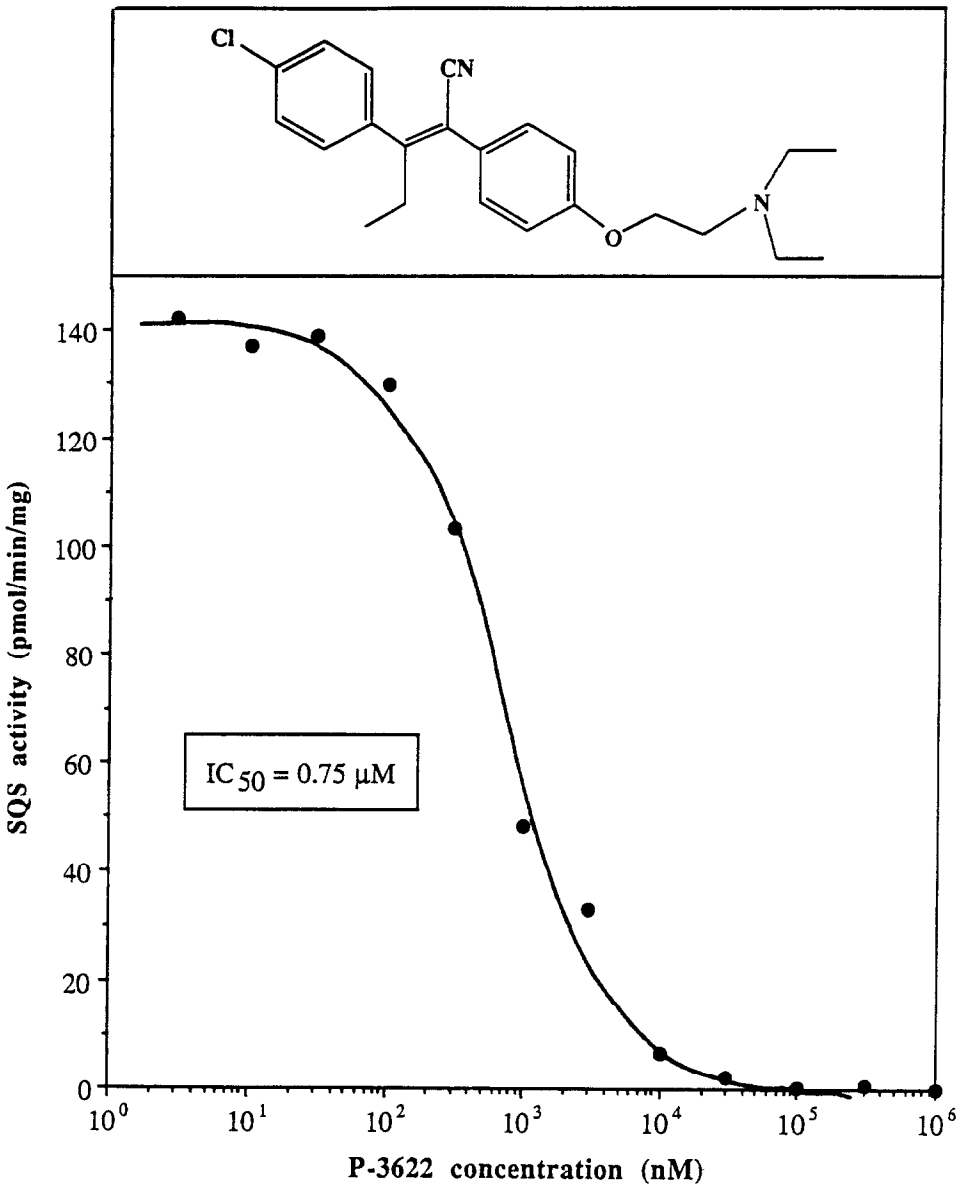


FIG. 2. Concentration-dependent inhibition of rat liver microsomal SQS activity by P-3622. Rat liver microsomes (25 μg microsomal protein, 142 pmol/min/mg) were incubated in a closed reaction vessel under anaerobic conditions as described in Materials and Methods for 30 min at 37° in a final volume of 75 μL of PMED buffer containing 5.1 μM [^{3}H]FPP (sp. act. 253 dpm/pmol), 258 μM NADP$^+$, 2.1 mM glucose-6-phosphate, 0.95 U glucose-6-phosphate dehydrogenase, 9.4 mM NaF, 4% DMSO, and the indicated concentrations of P-3622. Following incubation, squalene production was assessed and SQS activity calculated as described in Materials and Methods. Shown is the activity of SQS as a function of P-3622 concentration. Inset: the structure of P-3622.

P-3622 also inhibited rat liver SQS that had been solubilized from the microsomal membrane by limited trypsinolysis, exhibiting an IC_{50} for inhibition of the solubilized enzyme that was comparable to that noted for inhibition of the microsomal enzyme (Table 1), indicating that inhibition of SQS by P-3622 was a consequence of direct interference with the SQS catalytic machinery rather than a consequence of membrane modification leading to reduced enzyme specific activity.

In the presence of added PP_i (10 mM), the inhibitory activity of P-3622-related analogs evaluated was increased by less than 2-fold over inhibition in its absence, suggesting that P_i from solution can serve as a PP_i replacement counter ion in generating inhibitor–P_i ion pairs in the enzyme active center, but that PP_i is a more efficient counter ion, an observation consistent with previous observations noted for various ammonium ion and sulfonium ion reaction intermediate mimetics [7–11, 35].

TABLE 1. Concentration-dependent inhibition of SQS activity by P-3622

Species/enzyme source	Control SQS activity (pmol/min/mg)	IC_{50} (μM)
Rat liver microsomes	130 ± 10	4
Rat liver solubilized SQS	239 ± 11	10
Human liver microsomes	173 ± 15	11
Hep-G2 cell microsomes	196 ± 13	11
Mouse liver microsomes	19 ± 2	3
SEA quail liver microsomes	124 ± 2	0.5

Rat, mouse, SEA quail, and human liver microsomes, Hep-G2 cell microsomes, and a preparation of trypsin-solubilized rat liver microsomes (25 μg microsomal protein; specific activities as indicated ± SD N = 3) were incubated in a closed reaction vessel under anaerobic conditions as described in Materials and Methods for 30 min at 37° in a final volume of 75 μL of PMED buffer containing 20.4 μM [^{3}H]FPP (sp. act. 264 dpm/pmol), 258 μM NADP$^+$, 2.1 mM glucose-6-phosphate, 0.95 U glucose-6-phosphate dehydrogenase, 9.4 mM NaF, 4% DMSO, and concentrations of P-3622 ranging between 0.03 and 30 μM, at half-log intervals. Following incubation, squalene production was assessed, and the concentration of P-3622 required to inhibit SQS by 50% was calculated as described in Materials and Methods.

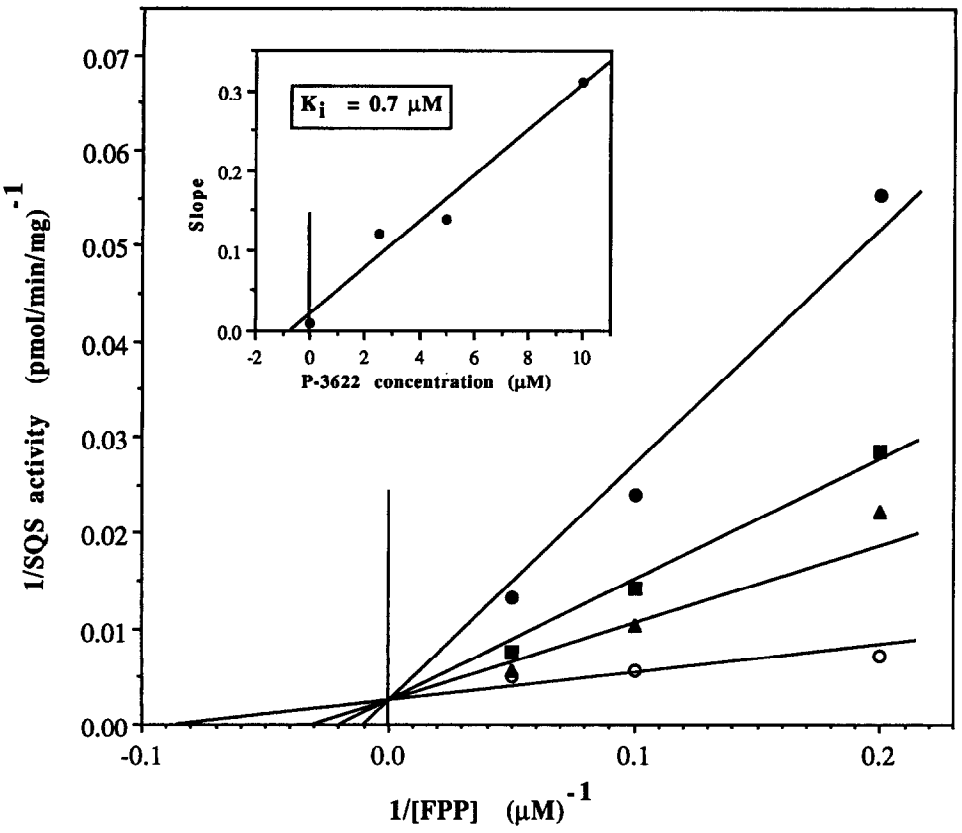


FIG. 3. Competitive inhibition of SQS activity by P-3622 with respect to FPP. Rat liver microsomes (25 μg microsomal protein, 199 pmol/min/mg) were incubated in a closed reaction vessel under anaerobic conditions as described in Materials and Methods for 30 min at 37° in a final volume of 75 μL of PMED buffer containing either 0 μM (○), 2.5 μM (▲), 5 μM (■), or 10 μM (●) P-3622, 4% DMSO, the indicated concentrations of [^{3}H]FPP (sp. act. 253 dpm/pmol), and the remaining SQS cofactors as described in Materials and Methods. Following incubation, squalene production was assessed and SQS activity calculated as described in Materials and Methods. Data are the averages of duplicate determinations of SQS activity and are expressed as reciprocal SQS activity as a function of reciprocal FPP concentration. Inset: Secondary plot of slope as a function of P-3622 concentration.

Reversible, Competitive Inhibition of SQS by P-3622

In the presence of 5.1 μM FPP and 50 mM P_i, inhibition of rat liver SQS was log linear between 0.1 and 10 μM (Fig. 2). Within this concentration range, P-3622 inhibited SQS competitively with respect to the substrate FPP (Fig. 3), exhibiting a K_i of 0.7 μM (Fig. 3, inset). SQS inhibition by P-3622 was reversible, as judged by the intersection at the origin of a series of lines generated for various inhibitor concentrations in plots of remaining enzymatic activity versus enzyme concentration (Fig. 4), and also by the lack of effect of enzyme concentration or 37° incubation duration on enzyme inhibition (data not shown), as previously shown for the reversible biphenylquinuclidine SQS inhibitor, 3-(4-phenyl)benzyloxy-1-azabicyclo[2.2.2]octane, CP-210172 [42]. By contrast, irreversible inactivation of SQS by zaragozic acid A is characterized by inhibition that is both enzyme concentration dependent and dependent upon duration of incubation at 37° [42] and also by intersection of such lines at various points on the x-axis whose distance along the x-axis is proportional to the amount of enzyme titrated by the irreversible inactivator ([42]; Fig. 4, inset).

First Half-Reaction Inhibition of SQS by P-3622

Consistent with its action as a competitive inhibitor with respect to the first half-reaction substrate FPP (Fig. 3), and its proposed mimicry of both the farnesyl moiety of the donor FPP and the carbocation formed in the first isoprene unit of the acceptor FPP following condensation (see above), P-3622 inhibited the SQS-catalyzed first half-reaction to the same extent as it inhibited the overall reaction leading to squalene production (Fig. 5). In the absence of inhibitor, proton release from the C1 position of the donor FPP, which is a measure of PSQPP formation in the enzymatic first half-reaction [15], closely paralleled squalene production, which is a measure of both the formation of PSQPP from FPP and also its reduction to squalene (Fig. 5, left). When evaluated in the presence of P-3622, proton release was inhibited concentration dependently exhibiting log-linear inhibition between 0.1 and 10 μM (Fig. 5, right). Inhibition of proton release paralleled inhibition of squalene formation measured in the same reaction vessel (Fig. 5, right), with inhibition of both exhibiting identical IC_{50} values (Fig. 5, right). That no additional inhibition of the second half-reaction by P-3622 was noted (independent inhibition of both half-reactions would result in greater inhibition of the overall reaction relative to the enzymatic first half-reaction) suggests that inhibition of the first half-reaction is sufficient to inhibit the overall reaction and that PSQPP formed in the first half-reaction cannot be prevented, by P-3622, from continuing its conversion to squalene, an observation consistent with previous findings that both half-reactions occur at catalytic sites within the enzyme active center that are either spatially overlapping or highly interactive [1, 11, 13, 15]. P-3622 is thus not able to interact only with the second half-reaction center without at the same time interacting with the first half-reaction center.

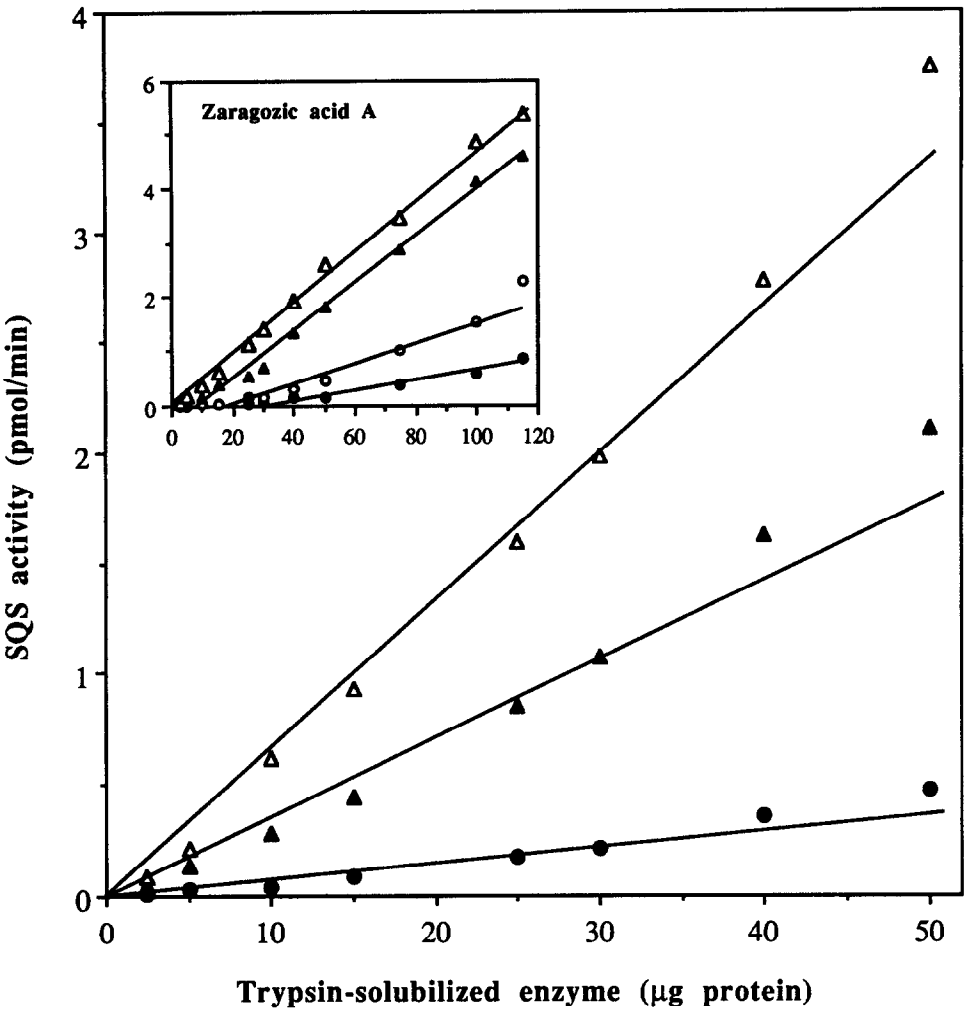


FIG. 4. **Reversible inhibition of SQS activity by P-3622. Aliquots of a preparation of trypsin-solubilized rat liver SQS (5 mg/mL; 47 pmol/min/mg) containing the indicated amounts of protein were incubated for 30 min at 37° in a final volume of 75 μL of PMED buffer containing 4% DMSO, either 0 μM (△), 10 μM (▲), or 100 μM (●) P-3622, or 0 nM (△), 1 nM (▲), 5 nM (○), or 10 nM (●) zaragozic acid A (inset), 20.4 μM [^{3}H]FPP (sp. act. 380 dpm/pmol), and the remaining SQS cofactors as described in Materials and Methods. Following incubation, squalene production was assessed and SQS activity calculated as described in Materials and Methods. Shown is the activity of SQS as a function of solubilized microsomal protein concentration in the presence or absence of various concentrations of either P-3622 or the irreversible inactivator zaragozic acid A (inset).**

Specificity of P-3622 for SQS Inhibition Versus PFT Inhibition

The ability of the stilbene moiety of P-3622 to function as a farnesyl mimic, together with the competitive nature of the inhibition by P-3622 with respect to the substrate FPP and its enzymatic first half-reaction inhibition, suggested that P-3622 and related analogs might also inhibit other FPP-utilizing enzymes such as the protein prenyltransferases [23, 60, 63, 64] and the *cis* and *trans* prenyltransferases involved in dolichol and ubiquinone production [23, 65–68]. However, the protein prenylation reactions [63, 64, 69] and the 1′-4 head-to-tail prenylation reactions of dolichol and ubiquinone synthesis [65–68] undergo mechanisms of catalysis that are distinct from the 1′-2,3 head-to-head condensation reaction catalyzed by SQS [1–4, 14]. In addition, P-3622 and related analogs also possess the diethylaminoethoxy moiety and accompanying positive charge that mimics the cyclopropylcarbocation moiety that develops within the second farnesyl residue following condensation that has no parallel in either the 1′-4 head-to-tail prenylation reaction mechanisms or the protein prenylation reaction mechanisms. Thus, similar inhibition of SQS and these prenyltransferases would suggest an FPP mimetic action of P-3622 and related analogs, whereas specificity for SQS relative to these prenyltransferases would substantiate the suggestion that the cationic nature of P-3622 together with its mimicry of both the farnesyl residue of the donor FPP and cyclopropylcarbocation that develops within the first isoprene of the acceptor FPP following condensation allows P-3622 and related analogs to act as reaction intermediate mimetics of the SQS-catalyzed reaction.

As shown in Fig. 6, P-3622 did inhibit PFT activity, as assessed by measuring incorporation of farnesyl residues from FPP into the prenylatable substrate, H-ras. Using rat brain cytosol as the source of PFT, and in the presence of saturating concentrations of H-ras (4 μM; [60]) and K_m concentrations of FPP (0.5 μM; [60]), incorporation of radiolabeled farnesyl residues into H-ras was inhibited concentration dependently by P-3622 with an IC_{50} of 150 μM (Fig. 6). However, in studies comparing SQS inhibitory activity with PFT inhibitory activity, in which both enzymes were evaluated at their respective K_m FPP concentrations, to eliminate potential differences in the degree of inhibition induced by P-3622 and related analogs as a result of differing degrees of saturation of the enzymes with FPP, P-3622 inhibited SQS with an IC_{50} of 0.5 μM (Fig. 6), and inhibited PFT with an IC_{50} of 150 μM (Fig. 6), indicating

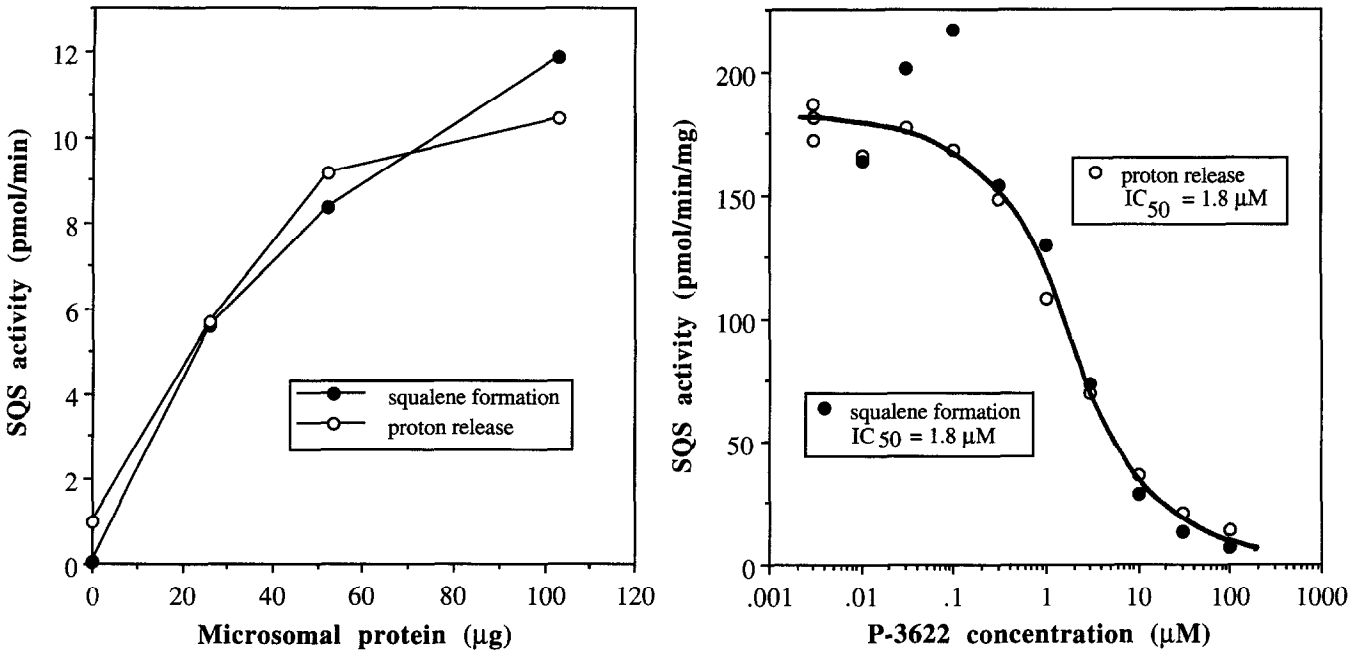


FIG. 5. Inhibition of the SQS-catalyzed first half-reaction by P-3622. Rat liver microsomes [75 μg microsomal protein (right panel) or as indicated (left panel); 180 pmol/min/mg] were incubated for 30 min at 37° in a final volume of 225 μL PMED buffer containing 20.4 μM [^{3}H]FPP (sp. act. 270 dpm/pmol), 258 μM $NADP^+$, 2.1 mM glucose-6-phosphate, 0.95 U glucose-6-phosphate dehydrogenase, 9.4 mM NaF, 50 mM sodium ascorbate, 1.5 U ascorbate oxidase, 4% DMSO, and the indicated concentrations of P-3622. Following incubation, reactions were terminated by addition of 120 μL of 10 M NaOH. Aliquots of 115 μL of the terminated reaction mixtures were apportioned to 500-μL microfuge tubes for assessment of squalene formation (overall reaction) as described in Materials and Methods. The remaining 230 μL of each reaction mixture was diluted to 250 μL by the addition of 20 μL water and assessed for tritium release (first half-reaction) by equilibration of the released tritium with the primary hydroxyl of MeOH, and subsequent isolation of the resulting radiolabeled MeOH by distillation as described in Materials and Methods. Shown are the first half-reaction and overall reaction activities of SQS expressed as picomoles tritium released (first half-reaction) and pmol squalene formed (overall reaction) from [1-^{3}H]FPP per minute of incubation at 37° as a function of rat liver microsomal protein concentration (left panel) and as a function of P-3622 concentration (right panel).

an approximately 300-fold specificity for SQS inhibition versus PFT inhibition.

In similar experiments, two related analogs, 3-phenyl-2-(4-diethylaminoethoxyphenyl)-acrylonitrile, P-2294, and 4-(B-diethylaminoethoxy)stilbene, P-2042, also showed a high degree of specificity for SQS inhibition relative to PFT inhibition, with P-2042 exhibiting an approximately 100-fold specificity (SQS IC_{50} = 5 μM; PFT IC_{50} = 500 μM) and P-2294 exhibiting a greater than 1000-fold specificity (SQS IC_{50} = 0.8 μM; PFT IC_{50} > 1000 μM). Thus, the high degree of specificity for SQS inhibition relative to PFT inhibition within the series of substituted diethylaminoethoxystilbenes, together with their donor farnesyl and condensation product carbocation mimicry, is consistent with their role as SQS reaction intermediate mimetics, and suggests a high degree of specificity for SQS inhibition relative to other FPP utilizing enzymes such as those involved in dolichol and ubiquinone production.

Inhibition of Cholesterolgenesis in Primary Rat Hepatocytesand and in Cultured Human Liver, Intestinal and Hemopoietic Cells Through SQS Inhibition by P-3622

As a consequence of SQS inhibition, P-3622 inhibited cholesterol synthesis in a variety of human cell lines including Hep-G2 cells, CaCo-2 cells, and IM-9 cells, and in primary rat hepatocytes, exhibiting IC_{50} values for inhibition of cholesterol formation from [^{14}C]acetate that closely parallel inhibitory activity against both the rat and human liver enzymes (Fig. 7). Inhibition of cholesterol synthesis that paralleled intrinsic potency against the isolated microsomal enzyme was also noted for a variety of related analogs of P-3622 (see below).

In all cell lines evaluated, cholesterol synthesis inhibition of up to 90% by P-3622 and related analogs evaluated occurred without corresponding accumulation of post-squalene cholesterol precursors, indicating that all inhibition of cholesterol synthesis occurred prior to the formation of squalene. As shown in Fig. 8, as a representative example, inhibition of cholesterol synthesis in near-confluent Hep-G2 cells by an IC_{75} concentration of P-3622 inhibited cholesterolgenesis from [^{14}C]acetate by 75% relative to untreated cells (Fig. 8, left panels) without inducing accumulation of post-squalene cholesterolgenic intermediates (Fig. 8, left panels). The approximately 30 count peaks corresponding to lanosterol (R_f = 0.34), squalene dioxide (R_f = 0.42), and squalene oxide (R_f = 0.65) were present on the chromatograms of both the control (Fig. 8, top left) and P-3622-treated (Fig. 8, bottom left) cells but are only observable on the P-3622 chromatogram as a consequence of

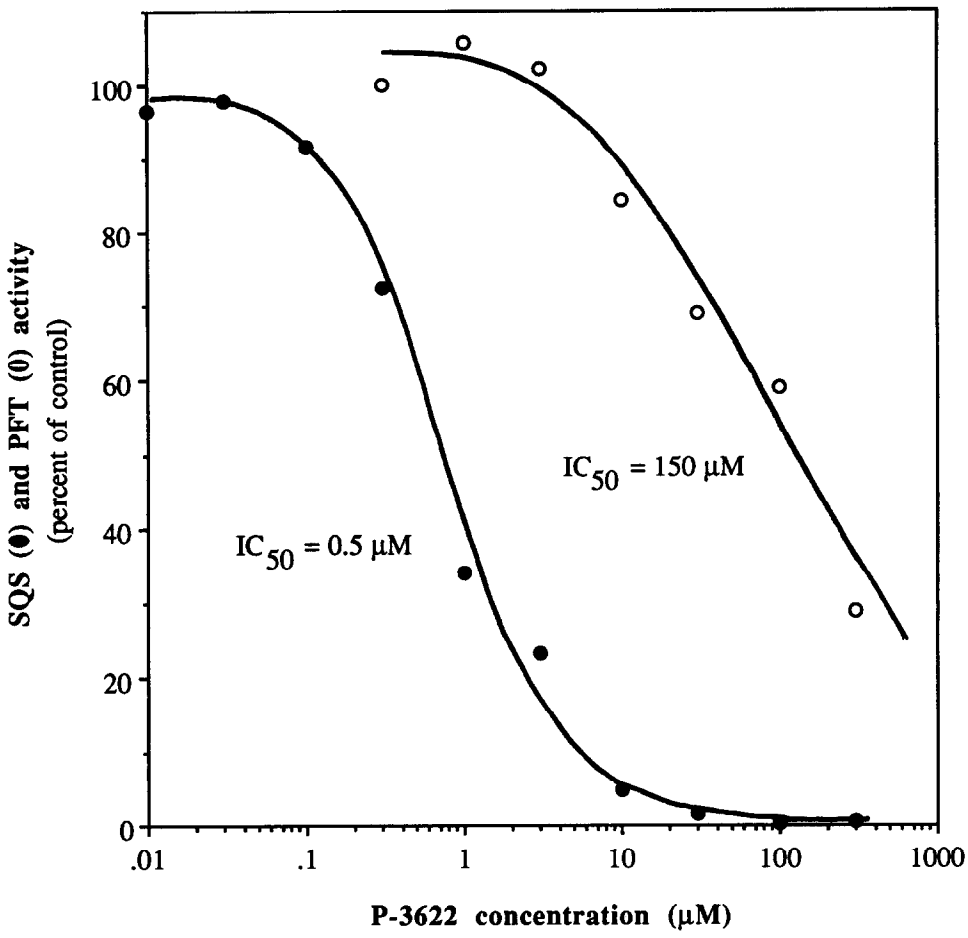


FIG. 6. Specificity of P-3622 for SQS inhibition relative to PFT inhibition. For assessing SQS inhibition by P-3622, 25 μg of rat liver microsomal protein was incubated in a closed vessel under anaerobic conditions as described in Materials and Methods for 30 min at 37° in a final volume of 75 μL of deoxygenated PMED Buffer containing 5.1 μM [^{3}H]FPP (sp. act. 253 dpm/pmol), 258 μM $NADP^+$, 2.1 mM glucose-6-phosphate, 0.94 U glucose-6-phosphate dehydrogenase, 9.4 mM NaF, and 4% DMSO ± the indicated concentrations of P-3622. Following incubation, squalene production was assessed and SQS activity calculated as described in Materials and Methods. For assessing PFT inhibition by P-3622, 30.5 μg of rat brain cytosolic protein was incubated for 30 min at 37° in a final volume of 25 μL of PFT Assay Buffer containing 0.5 μM [^{3}H]FPP (sp. act. 12,770 dpm/pmol), 5 mM $MgCl_2$, 4 μM H-ras, 20 mM KCl, and 1.2% DMSO ± the indicated concentrations of P-3622. After incubation, FPP incorporated into H-ras was assessed, and PFT activity was calculated as described in Materials and Methods. Shown are the percentages of control SQS (●) and PFT (○) activities as a function of P-3622 concentration. Control SQS activity averaged 142 ± 21 pmol FPP incorporated into squalene per min per mg microsomal protein (SD; N = 3). Control PFT activity averaged 0.31 ± 0.20 pmol FPP incorporated into H-ras per min per mg cytosolic protein (SD; N = 4).

scale expansion. Inhibition of post-squalene cholesterolgenic enzymes by P-3622 would have resulted in an accumulation of cholesterol precursors corresponding to pathway intermediates prior to the site of pathway inhibition equal to the amount of cholesterol not formed as a result of the blockade. For example, an IC_{75} concentration of ketoconazole, a lanosterol 14α-demethylase inhibitor [70], inhibited cholesterolgenesis from [^{14}C]acetate by approximately 85% relative to untreated cells (Fig. 8, right panels) and induced a concomitant accumulation of lanosterol that was equal to the amount of cholesterol not formed as a consequence of its lanosterol 14α-demethylase inhibition, such that the total amount of sterol formation was unaltered (Fig. 8, right panels). Similar results were also noted for the oxidosqualene cyclase inhibitor, UK-124,617, except that squalene oxide and squalene dioxide accumulated rather than lanosterol (data not shown).

In addition, since P-3622 and related analogs do not induce compensatory increases in HMG-CoA reductase activity at concentrations that inhibit cholesterol synthesis by up to 95% (see below), it is unlikely that P-3622 and related analogs significantly inhibit any steps involved in the formation of FPP. Thus, cholesterol synthesis inhibition by P-3622 and related analogs is a consequence of inhibition

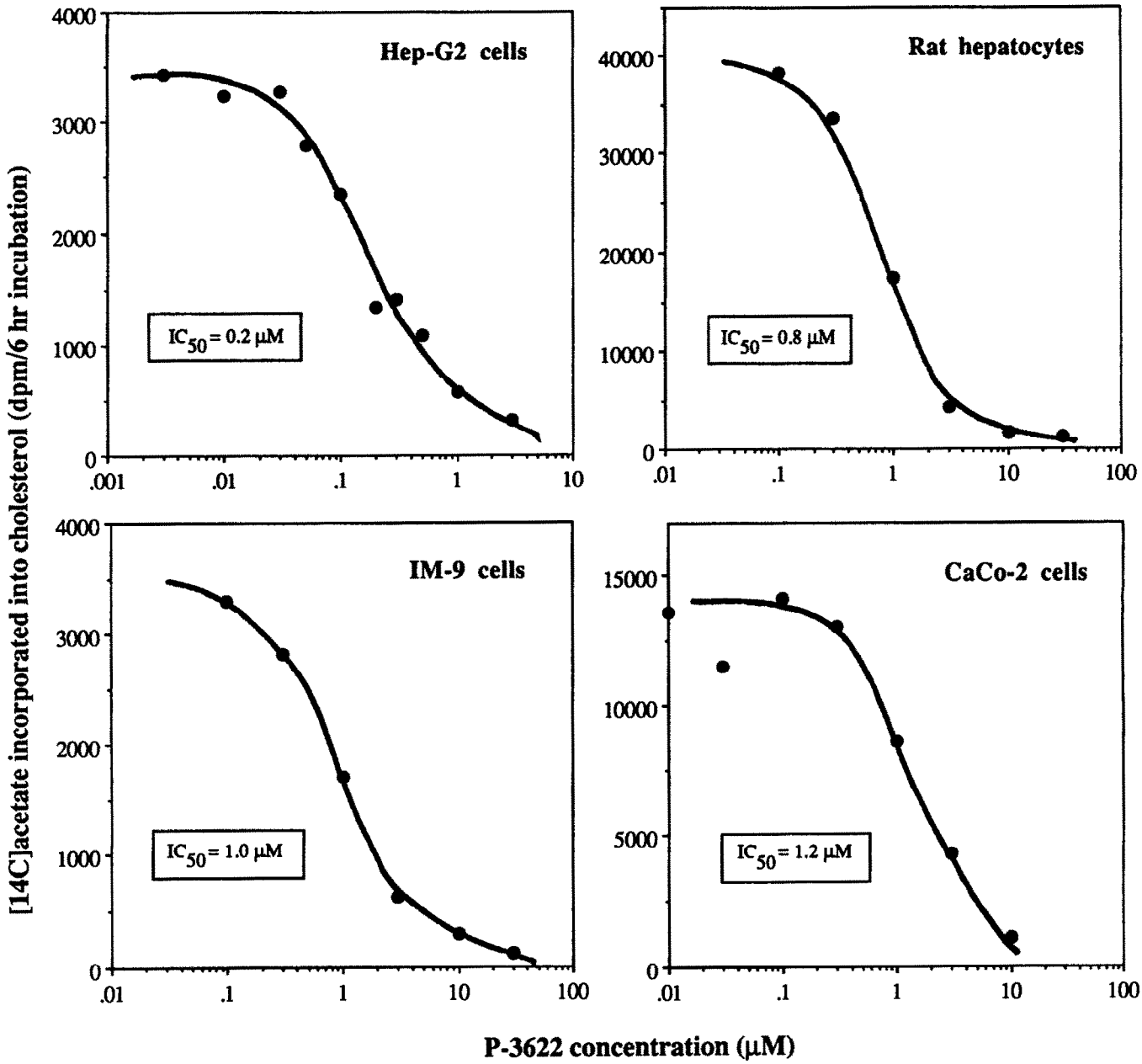


FIG. 7. Inhibition of cholesterol biosynthesis in primary rat hepatocytes and in cultured Hep-G2, CaCo-2 and IM-9 cells. Hep-G2 cells, CaCo-2 cells, IM-9 cells, and rat primary hepatocytes, seeded and maintained in culture as described in Materials and Methods, were administered 1.0 mL of their respective fresh medium containing either 1% DMSO (Hep-G2 cells, rat hepatocytes) or 0.4% DMSO (IM-9 cells, CaCo-2 cells) ± the indicated concentrations of P-3622. Immediately following addition of the respective medium containing DMSO ± effector compounds, 25 μL of the respective medium containing 4 μCi of [2-^{14}C]acetate was added to each well or culture tube. Plates were then sealed with parafilm, culture tubes were capped, and cells were incubated at 37° for 6 hr with gentle shaking. After incubation, samples were saponified, the nonsaponifiable lipids were extracted with hexane and separated by silica gel TLC, and the cholesterol and cholesterol precursor peaks were quantitated using a Berthold Linear Radioactivity Analyzer as described in Materials and Methods. Shown is the radiolabeled acetate incorporated into cholesterol by Hep-G2 cells (top left), IM-9 cells (bottom left), primary rat hepatocytes (top right), and CaCo-2 cells (bottom right) during the 6-hr incubation as a function of P-3622 concentration.

of SQS alone, rather than of inhibition of SQS together with concomitant inhibition at additional pre-FPP or post-squalene sites along the cholesterol synthetic pathway.

The high degree of specificity for inhibition of SQS relative to other enzymes in the cholesterol synthesis pathway may be, in part, a consequence of the ability of P-3622 and related analogs to mimic carbocationic intermediates of the SQS-catalyzed reaction in addition to resembling a polyisoprenoid. In this regard, it is noteworthy that at increased concentrations of P-3622 between 1 and 5 μM (i.e. those that induce greater than 90% inhibition of cholesterol synthesis in cultured cells), P-3622 begins to show secondary inhibition at the site of oxidosqualene cyclase, as evidenced by a modest accumulation of squalene oxide and squalene

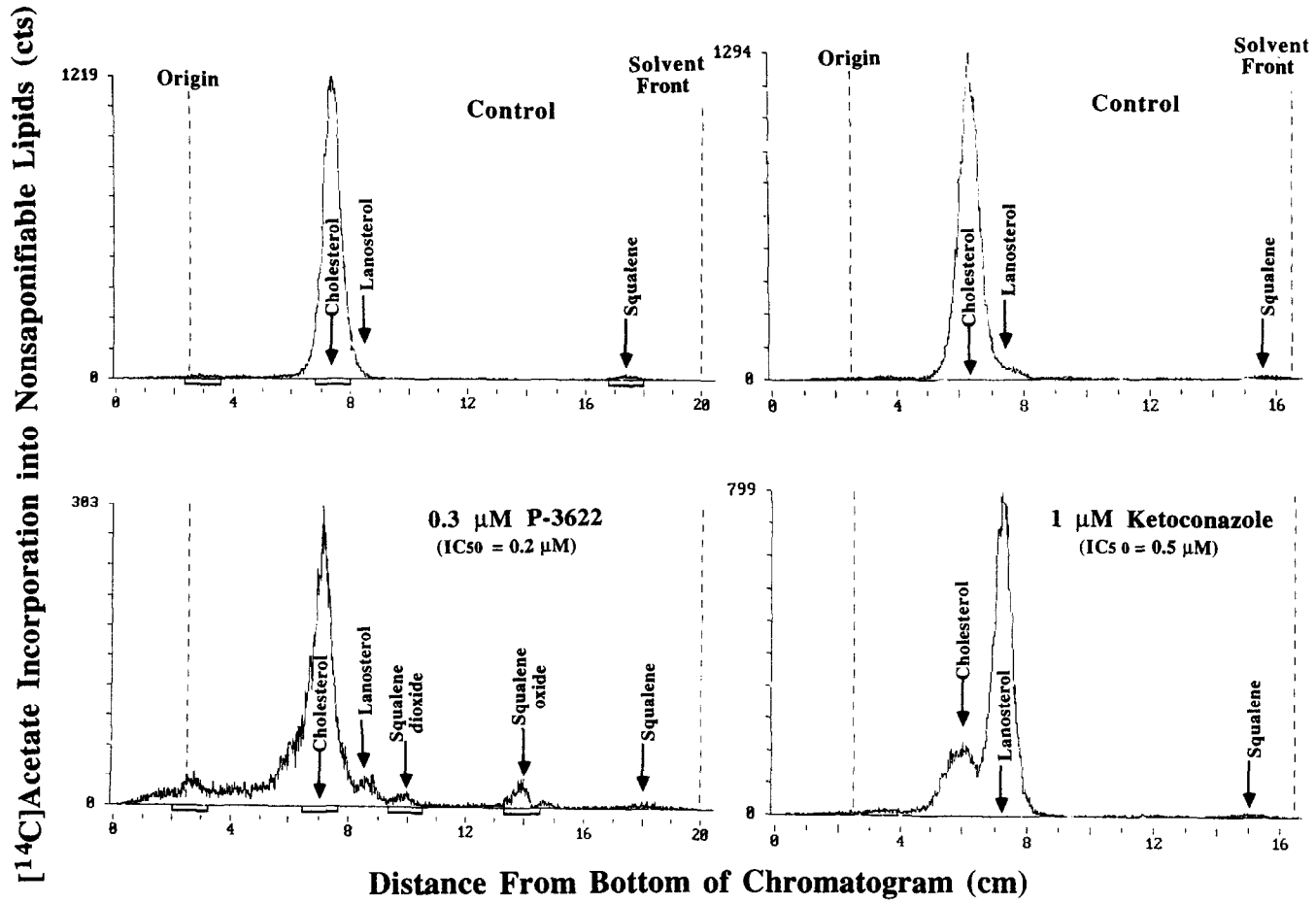


FIG. 8. Inhibition of cholesterol synthesis in Hep-G2 cells by P-3622 without concomitant accumulation of post-squalene cholesterol precursors. Hep-G2 cells, seeded and maintained in culture as described in Materials and Methods, were administered 1.0 mL of fresh medium containing either 1% DMSO (control incubations; top panels), 1% DMSO plus 0.3 μM P-3622 (bottom left), or 1% DMSO plus 1 μM ketoconazole (bottom right). Immediately following the addition of medium containing either DMSO or DMSO plus effector compounds, 25 μL of medium containing 4 μCi of [2-^{14}C]acetate was added to each well, and plates were sealed with parafilm and incubated at 37° for 6 hr with gentle shaking. After incubation, samples were saponified, the nonsaponifiable lipids were extracted with hexane and separated by silica gel TLC, and the cholesterol and cholesterol precursor peaks were quantitated using a Berthold Linear Radioactivity Analyzer as described in Materials and Methods. Shown are the radioactivity tracings from the Berthold Linear Radioactivity Analyzer for control cells (top left) and cells exposed to 0.3 μM P-3622 (bottom left) and for control cells (top right) and cells exposed to a 1 μM concentration of the lanosterol 14α-demethylase inhibitor, ketoconazole (bottom right). Radiolabeled acetate incorporated into specific nonsaponifiable lipids is expressed as counts per pixel (y-axis graduations are 1/10th of the height of the largest peak of the chromatogram) as a function of distance from the bottom of the chromatogram. The location of arrows indicating migration of cholesterol (R_f = 0.27), lanosterol (R_f = 0.34), and squalene (R_f = 0.88) were determined from parallel lanes of the chromatogram containing non-radioactive cholesterol, lanosterol, and squalene, visualized with iodine vapors. The location of arrows indicating migration of squalene oxide (R_f = 0.65) and squalene dioxide (R_f = 0.42) were determined from the location of radiolabeled cholesterol precursor peaks accumulating in response to incubation of Hep-G2 cells with the squalene epoxide cyclase inhibitor UK-124,617.

dioxide (approximately 100 counts over background relative to 100 counts remaining in cholesterol from an initial 1200 counts; see Fig. 8, left panels for reference), an enzyme whose catalytic mechanism also proceeds through carbocationic reaction intermediates [71].

Inhibition of Cholesterol Synthesis in Hamsters by P-3622

Consistent with inhibition of cholesterol synthesis in cultured cells without accumulation of post-squalene cholesterol precursors, P-3622, P-2294, and P-2042 also inhibited cholesterol synthesis from [^{14}C]mevalonate in chow-fed hamsters following either a single oral dose [P-2042; 33% (P = 0.031) inhibition at 100 mg/kg] or following a single i.p. injection (P-3622 and P-2294; 62 and 76% inhibition at 100 mg/kg; Table 2). Nonsaponifiable lipid synthesis (sterols + polyprenols + prenoic acids) was also reduced by both P-3622 and P-2294 (Table 2), but to a lesser extent than cholesterol synthesis as a consequence of the presence of polyprenols and prenoic acids in the fraction (only approximately 66% of nonsaponifiable lipids in this fraction are

TABLE 2. Inhibition of hepatic cholesterol biosynthesis in hamsters

Treatment (i.p. injection)	[^{14}C]Mevalonate incorporation into nonsaponifiable lipids (dpm/hr/g liver)	Percent inhibition	Integrated cholesterol peak from linear radioactivity analyzer (counts)	Percent inhibition
Control	12,600 ± 4,890*		1,820 ± 630*	
20 mg/kg Ketoconazole	13,900 ± 4,550 (NS)	-10	1,390 ± 530	24
100 mg/kg P-3622	7,970 ± 3,140 (P = .013)	37	690 ± 340	62
100 mg/kg P-2294	7,250 ± 3,800 (P = .006)	43	430 ± 110	76

Twenty-four male golden Syrian hamsters weighing approximately 150 g and housed in groups of six animals each were given food and water *ad lib.*, and administered a 1.0-mL i.p. injection of either water (control animals) or water containing the indicated concentration of effector compounds. One hour following compound administration, animals received an i.p. injection of 0.5 mL of *R,S*-[2-^{14}C]mevalonolactone (2 μCi/mL; 54.1 mCi/mmol) per 150 g of body weight. Two hours after administration of the radiolabeled mevalonolactone, animals were euthanized by pentobarbital injection and two 1-*g* liver pieces were removed. Tissue samples were saponified and the nonsaponifiable lipids extracted with 10 mL of petroleum ether as described in Materials and Methods. Aliquots, 3.0 mL, of each petroleum ether extract were mixed with Ready-Safe liquid scintillation fluid and assessed for radioactivity as a measure of total nonsaponifiable lipid synthesis as described in Materials and Methods. An additional 5.0-mL aliquot of each petroleum ether extract was dried under nitrogen, resuspended in 25 μL chloroform, applied to 1 × 20 cm channels of Silica Gel 60C TLC plates, developed in hexane:diethyl ether:acetic acid (70:30:2), and assessed for radioactive cholesterol and other nonsaponifiable lipids by analysis using a Berthold Linear Radioactivity Analyzer as described Materials and Methods.

* Values are means ± SD, N = 6.

sterols; [62]). That nonsaponifiable lipid synthesis was reduced in a proportion consistent with the reduction in cholesterol synthesis (Table 2) is consistent with inhibition of cholesterol synthesis through SQS inhibition alone, since inhibition of cholesterol formation at a site subsequent to squalene formation would result in accumulation of cholesterol precursors that would also appear in the nonsaponifiable lipid fraction and mask reductions in cholesterol synthesis in that fraction. For example, administration of an ED_{50} dose of ketoconazole (20 mg/kg) by i.p. injection resulted in a 24% inhibition of cholesterol synthesis in this study with no reduction in nonsaponifiable lipid production (Table 2).

Indeed, the 72% reduction in cholesterolgenesis following P-3622 administration as estimated from peak height following TLC and radioactivity assessment via a linear radioactivity analyzer was not accompanied by corresponding increases in the levels of any post-squalene cholesterol precursors (Fig. 9), whereas the 64% inhibition of cholesterolgenesis by ketoconazole was accompanied by a corresponding accumulation of lanosterol such that the sums of the lanosterol plus cholesterol peaks in the control and ketoconazole-treated animals were the same. These observations, together with those in cultured cells, indicate that P-3622 and related analogs evaluated inhibit cholesterol synthesis through inhibition of SQS activity without any significant additional effects on other enzymes in the pathway.

Structure–Activity Relationships within the Diethylaminoethoxystilbene Series of SQS Inhibitors

P-3622 can thus be characterized as a reversible, competitive, first half-reaction SQS inhibitor that shows a high degree of specificity for SQS inhibition relative to inhibition of other FPP-utilizing enzymes as well as relative to inhibition of other enzymes in the cholesterolgenic pathway, and inhibits cholesterol synthesis due to its action as an SQS inhibitor in both cultured human cells and in experimental animals.

In examining structure–activity relationships within this series of SQS inhibitors, 72 analogs of P-3622 were evaluated for their ability to inhibit rat liver SQS *in vitro* using a saturating FPP concentration of 20.4 μM and an analog concentration of 100 μM. Under these conditions P-3622, P-2294, and P-2042 exhibited IC_{50} values of 5.6 ± 1.4 μM (SD; N = 4), 5.5 μM, and 25 μM, respectively, relative to 0.75 ± 0.2 μM (SD; N = 4), 0.8 μM, and 6 μM, respectively, using a K_m FPP concentration of 5.1 μM. Analogs showing greater than 25% SQS inhibition under these conditions were subjected to IC_{50} determination using 5.1 μM FPP, and those analogs showing IC_{50} values of less than 15 μM were evaluated for their ability to inhibit cholesterol synthesis from [^{14}C]acetate in Hep-G2 cells. The SQS inhibition noted at 100 μM inhibitor concentration using 20.4 μM FPP, IC_{50} values for SQS inhibition determined using 5.1 μM FPP, and IC_{50} values for cholesterolgenesis inhibition in Hep-G2 cells for the analogs evaluated are listed in Tables 3–5, together with the plasma cholesterol reductions previously reported in rats [57] following 6 days of oral administration at 40 mg/kg/day.

As shown in Table 3, modifications at either the R1 or R2 position, or both, of the central olefin of the diethylaminoethoxystilbene moiety had only modest effects on inhibitory activity (e.g. **1** vs **4** vs. **17** vs **38–40**), presumably due to the presence of a pocket in the enzyme active center that accommodates the branching methyl of the central isoprene unit of the donor FPP. Substitutions in the R2 position generally lead to greater inhibitory activity than substitutions in the R1 position (e.g. **4** vs. **17**; **40** vs **17**; **42** vs **18**), an observation that is also consistent with a space in the enzyme active center that accommodates this methyl

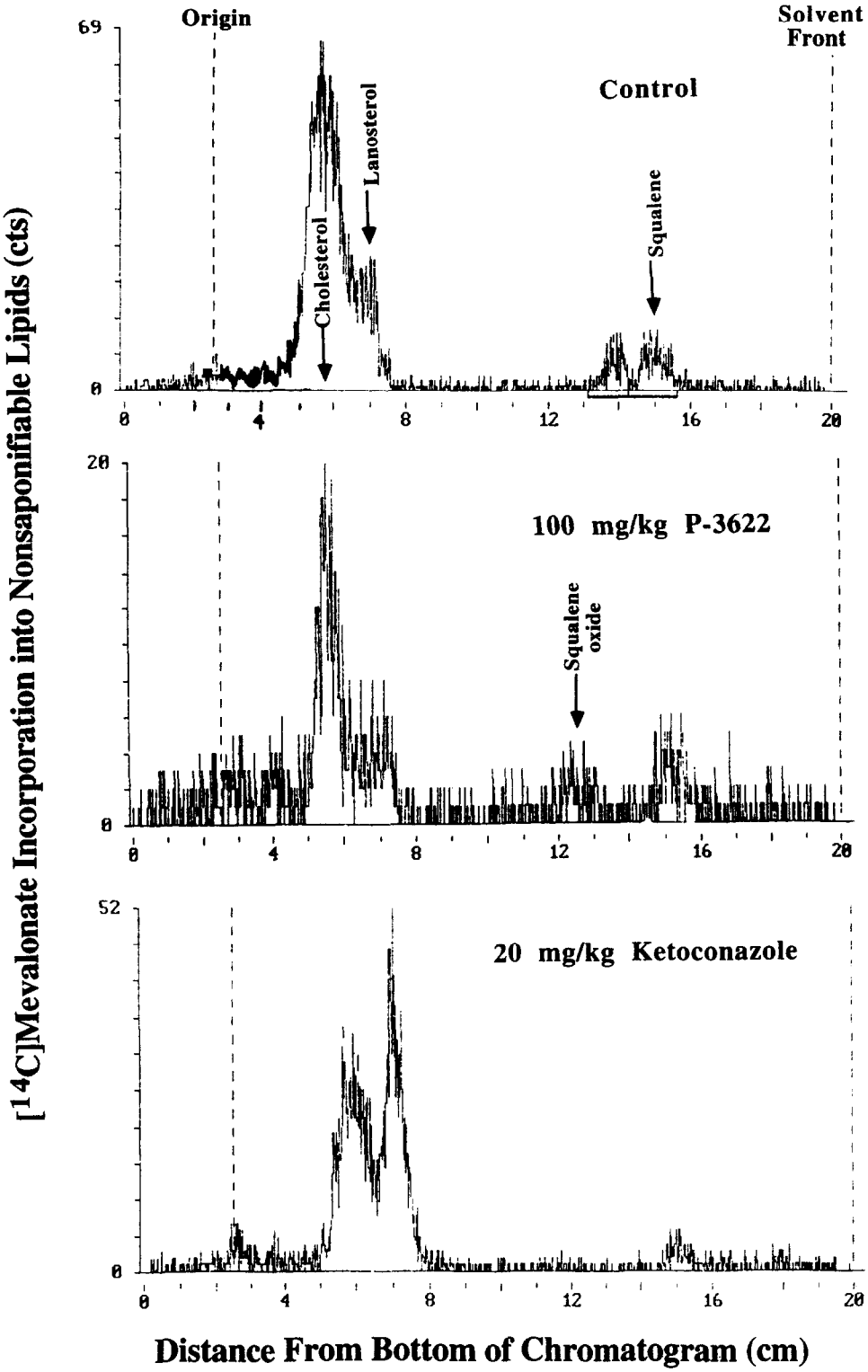
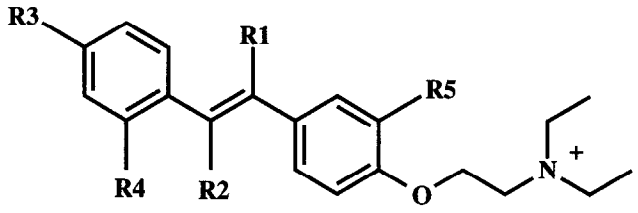


FIG. 9. Inhibition of cholesterol synthesis in hamsters by P-3622 without concomitant accumulation of post-squalene cholesterol precursors. Shown are radioactivity tracings from the Berthold Linear Radioactivity Analyzer of the TLC separation of extracted nonsaponifiable lipids for representative animals from the control group (top), the 100 mg/kg P-3622-treated group (center), and the 20 mg/kg ketoconazole-treated group (bottom) of hamsters from the experiment outlined in Table 2. Radiolabeled mevalonate incorporated into specific nonsaponifiable lipids is expressed in terms of counts per pixel (y-axis graduations are 1/10th of the height of the largest peak of the chromatogram) as a function of distance from the bottom of the chromatogram. The location of arrows indicating migration of cholesterol, lanosterol, squalene, and squalene oxide were determined as outlined in the legend to Fig. 8.

group. However, reductions in inhibitory activity were noted with all but the most modest substitutions at the R3 position in the terminal phenyl residue of the stilbene moiety (e.g. **4** vs **11–15**; **39**, **41** vs **45**), suggestive of a more highly restrictive pocket in the enzyme active center that accommodates the branching methyl of the terminal isoprene unit of the donor FPP. Additions at the R3 position, however, appeared to be better tolerated when concomitant with R1 rather than R2 substitutions, suggesting that molecules with R1 substitutions may reside within the enzyme active center in a slightly different orientation than those with R2 substitutions, possibly due to steric interference with amino acid side-chain residues that interact with inhibitors on the R1 face of the stilbene moiety, allowing for greater flexibility in R3 substitutions when the inhibitor is in this orientation.

TABLE 3. Structure–activity relationships among members of the diethylaminoethoxystilbene series of SQS inhibitors



Compound No.	R1	R2	R3	R4	R5	SQS inhibition (rat liver microsomes) (% at 100 μM*)	SQS inhibition (rat liver microsomes) IC_{50} (μM)†	Cholesterol synthesis inhibition (Hep-G2 cells) IC_{50} (μM)	Plasma cholesterol lowering (6-day rat)‡ (% at 40 mg/kg)
1§	-H	-H	-H	-H	-H	79 ± 5 (4)‖	6	0.4	57
2	-H	-COOEt	-H	-H	-H	71 ± 3 (3)	20 ± 3 (2)	—¶	—
3	-H	-COMe	-H	-H	-H	47	81	—	—
4§	-H	-CN	-H	-H	-H	81 ± 9 (3)	0.8	0.4	37
5	-H	-CN	-OMe	-H	-H	17	—	—	22
6	-H	-CN	-Cl	-H	-H	10	—	—	27
7	-H	-CN	$-NH_2$	-H	-H	45	43	—	22
8	-H	-CN	-F	-H	-H	21	—	—	13
9	-H	-H	-Cl	-Cl	-H	31	90	—	—
10	-H	-CN	-H	-H	-OMe	38	8	0.7	—
11	-H	-CN	$-NHCOCH_2N(Et)_2$	-H	-H	16	—	—	27
12	-H	-CN	$-NHCONH_2$	-H	-H	11	—	—	15
13	-H	-CN	$-O(CH_2)_2$-N⟨⟩N-Et	-H	-H	<10	—	—	42
14	-H	-CN	$-O(CH_2)_2N(Et)_2$	-H	-H	<10	—	—	42
15	-H	-CN	$-O(CH_2)_2N(Et)_2$	-H	-OMe	<10	—	—	—
16	-Et	-H	-H	-H	-H	54	42	—	—
17	-CN	-H	-H	-H	-H	52	12	—	33
18	-CN	-H	-Cl	-H	-H	30	—	—	28
19	-CN	-H	-Me	-H	-H	32	—	—	13
20	-CN	-H	-OMe	-H	-H	38	>100	—	15
21	-CN	-H	-CN	-H	-H	<10	—	—	30
22	-CN	-H	$-CF_3$	-H	-H	9	—	—	37
23	-CN	-H	$-N(CH_3)_2$	-H	-H	54	4.2	1.0	35

TABLE 3. Continued

Compound No.	R1	R2	R3	R4	R5	SQS inhibition (rat liver microsomes) (% at 100 μM*)	SQS inhibition (rat liver microsomes) IC_{50} (μM)†	Cholesterol synthesis inhibition (Hep-G2 cells) IC_{50} (μM)	Plasma cholesterol lowering (6-day rat)‡ (% at 40 mg/kg)
24	-CN	-H	-SMe	-H	-H	49	3.9	0.3	42
25	-CN	-H	-SEt	-H	-H	45	5.8	0.5	33
26	-CN	-H	$-SCH(CH_3)_2$	-H	-H	20	—	—	50
27	-CN	-H	$-SCH_2CH(CH_3)_2$	-H	-H	28	>100	—	42
28	-CN	-H	$-SC_6H_{11}$	-H	-H	37	>100	—	17
29	-CN	-H	$-S(CH_2)_6CH_3$	-H	-H	<10	—	—	20
30	-CN	-H	$-CH(CH_3)_2$	-H	-H	14	—	—	8
31	-CN	-H	$-NHCOCH_3$	-H	-H	24	—	—	17
32	-CN	-H	$-O(CH_2)_2OH$	-H	-H	<10	—	—	25
33	-CN	-H	$-OCH_2CH(CH_3)CH_2N(CH_3)_2$	-H	-H	<10	—	—	13
34	-CN	-H	$-O(CH_2)_2$-N⬡	-H	-H	35	>100	—	—
35	-CN	-H	-H	-Cl	-H	40	20	—	22
36	-CN	-H	-H	-OMe	-H	<10	—	—	20
37	-CN	-H	-H	$-O(CH_2)_2N(Et)_2$	-H	19	—	—	2
38	-CN	-Ph	-H	-H	-H	49	4.3	2.6	—
39	-Et	-CN	-H	-H	-H	48	8.9	0.9	53
40	-CN	-Et	-H	-H	-H	83 ± 6 (3)	2.2	2.5	78
41	-Et	-CN	-Cl	-H	-H	78 ± 2 (2)	5.5 ± 0.5 (2)	0.4	73
42§	-CN	-Et	-Cl	-H	-H	94 ± 2 (4)	0.75 ± 0.2 (4)	0.3 ± 0.1 (4)	75
43	-CN	-Me	-SMe	-H	-H	43	1.8	0.4	—
44	-Et	-CN	-F	-H	-H	16	—	—	18
45	-Et	-CN	$-O(CH_2)_2N(Et)_2$	-H	-H	14	—	—	2
46	-Et	-Et	$-O(CH_2)_2N(Et)_2$	-H	-H	14	—	—	—

* Measurement conducted using 20.4 μM FPP.

† Measurement conducted using 5.1 μM FPP.

‡ Data from Hughes *et al.* [574].

§ P-3622, P-2042, and P-2294 are compounds 42, 1, and 4, respectively.

‖ Mean ± SD for N ≥ 3 or mean ± difference from the mean for N = 2 (replicate determinations indicated in parentheses).

¶ — denotes an unevaluated parameter.

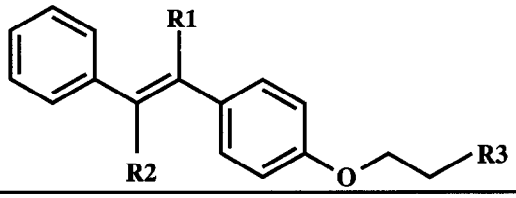
In addition, restricting the mobility of the N-ethyl groups, through cyclization, had in most cases only minimal effects on inhibitor activity (Table 4), and cyclizations around the central olefin of the diethylaminoethoxy moiety that included either of the phenyl rings were tolerated, with in most cases only modest reductions in inhibitory activity (Table 5), suggesting that additional conformational restrictions around the central olefin of the stilbene moiety through cyclization is either of minimal or of little consequence until the size of the group becomes considerable (e.g. **58**).

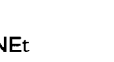
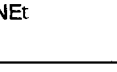
A high correlation between SQS inhibition at 100 μM using 20.4 μM FPP and IC_{50} values determined at 5.1 μM FPP was noted (R^2 = 0.86) as anticipated, as well as a reasonable correlation between intrinsic inhibitory activity and inhibition of cholesterol synthesis in Hep-G2 cells (R^2 = 0.43), considering that only a small subset of compounds evaluated for intrinsic activity (those with IC_{50} values <15 μM) were evaluated for their ability to inhibit cholesterol synthesis in Hep-G2 cells and that absolute differences of both intrinsic activity and cholesterolgenesis inhibition within this subset were relatively small. The decrease in plasma cholesterol levels in rats administered analogs for 6 days at 40 mg/kg/day was also highly correlated with intrinsic SQS inhibitory activity (R^2 = 0.53), considering that differences in pharmacokinetic properties among members of this series of SQS inhibitors undoubtedly existed and played contributing roles in the degree of cholesterol lowering observed that were independent of the intrinsic potency of the inhibitors.

DISCUSSION

In this report we have evaluated the mechanism of action of, and structure–activity relationships within, a series of substituted diethylaminoethoxystilbenes that resemble carbocationic intermediates of the SQS-catalyzed half-reactions, such that the terminal and diethylaminoethoxy-proximal phenyl residues and central *trans*-olefin of the stilbene moiety mimic the three isoprene units of the farnesyl moiety of the donor FPP and the cationic diethylaminoethoxy moiety of these molecules mimics the various carbocations that develop in the C1–C3 region of the first isoprene unit of the acceptor FPP during the course of the SQS-catalyzed reaction. Based on a detailed mechanistic evaluation of 3-(4-chlorophenyl)-2-(4-diethylaminoethoxyphenyl)-A-pentenonitrile monohydrogen citrate (P-3622), a representative member of this series, this series can be characterized as that of reversible, competitive, first half-reaction SQS inhibitors that show a high degree of specificity for SQS inhibition relative to inhibition of other FPP-utilizing enzymes as well as relative to inhibition of

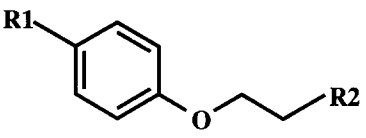
TABLE 4. Structure–activity relationships among members of the diethylaminoethoxystilbene series of SQS inhibitors

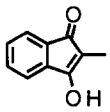
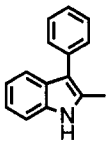
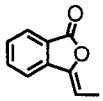
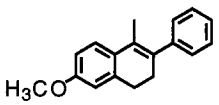
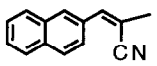
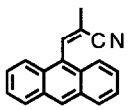
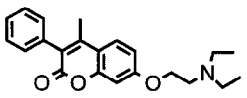


Compound No.	R1	R2	R3	SQS inhibition (rat liver microsomes) (% at 100 μM)*	SQS inhibition (rat liver microsomes) IC_{50} (μM)†	Cholesterol synthesis inhibition (Hep-G2 cells) IC_{50} (μM)	Plasma cholesterol lowering (6-day rat)‡ (% at 40 mg/kg)
1	-H	-H	$-N(Et)_2$	79 ± 5(4)§	6	0.4	57
47	-H	-H	$-CH_2N(Et)_2$	52	15	—‖	22
48	-H	-H		<10	<100	—	0
49	-H	-H		23	—	—	—
50	-H	-H		44	12	3.0	—
51	-H	-CN		54	6	0.6	—
17	-CN	-H	$-N(Et)_2$	52	12	—	33
52	-CN	-H		<10	—	—	18

* Measurement conducted using 20.4 μM FPP.
† Measurement conducted using 5.1 μM FPP.
‡ Data of Hughes *et al.* [57].
§ Mean ± SD for the number of replicate determinations indicted in parentheses.
‖ — denotes an unevaluated parameter.

TABLE 5. Structure–activity relationships among members of the diethylaminoethoxystilbene series of SQS inhibitors



Compound No.	R1	R2	SQS inhibition (rat liver microsomes) (% at 100 μM)*	IC_{50} (μM)†
53		$-N(Et)_2$	<10	—‡
54		$-N(Et)_2$	49	42
55		$-N(Et)_2$	57	20
56			44	23
57		$-N(Et)_2$	27	—
58		$-N(Et)_2$	<10	—
59			63 ± 3 (3)§	17

* Measurement conducted using 20.4 μM FPP.
† Measurement conducted using 5.1 μM FPP.
‡ — denotes an unevaluated parameter.
§ Mean ± SD for the number of replicate determinations indicated in parentheses.

other enzymes in the cholesterolgenic pathway, and that inhibit cholesterol synthesis in cultured human cells and inhibit cholesterol synthesis and reduce plasma cholesterol levels in experimental animals, due to their action as SQS inhibitors.

These studies also demonstrate a number of structural features within the series of SQS inhibitors and mechanistic features of the SQS-catalyzed reactions. First, the ability to replace the terminal and PP_i-proximal isoprene residues of FPP with conformationally restricted aromatic isosteres, as previously described by Biller *et al.* [56] for farnesyl-based polyanionic FPP mimetics, can be translated directly to cationic SQS inhibitors, such that the *para*-disubstituted phenyl residues that showed optimal potency as isoprene unit replacements in the polyanionic series [56] also showed activity when utilized as isoprene unit replacements in these cationic inhibitors. Furthermore, structure–activity relationships within this series demonstrated that the most potent analogs of diethylaminoethoxystilbene possessed *trans*-substitutions across the central olefin of the stilbene moiety and also *para*-substitutions on the terminal phenyl residue of the stilbene moiety, consistent with such substi-

tutions mimicking the branching methyl groups of the central and terminal isoprene residues of FPP. Substitutions *meta* to the central olefin at the PP_i-proximal phenyl residue of the stilbene moiety were also tolerated, suggesting that such substitutions mimic the branching methyl of the PP_i-proximal isoprene residue of FPP.

Second, the ability of P_i from solution to act as a surrogate for PP_i in the SQS/inhibitor complex allows inhibitory activity of these compounds to be observed even in the absence of added PP_i. Previous evaluations of cationic reaction intermediate mimetics [7–11, 35] have demonstrated that the SQS catalyzed half-reactions proceed through cation-diphosphate ion pairs in which developing carbocationic intermediates remain associated with released PP_i during the course of the various cationic rearrangements leading to PSQPP and squalene production. That activity noted in the presence of 50 mM P_i was only increased by approximately 2-fold upon addition of 10 mM PP_i indicates that P_i can act as a surrogate for PP_i, albeit not as efficiently.

Third, studies reported by Biller *et al.* [32] for farnesyl (phosphinylmethyl)phosphonates demonstrated improved inhibitory activity of analogs in which an ether oxygen was inserted between the farnesyl and (phosphinylmethyl)phosphonate residues, reportedly as a result of the ability of the ether functionality to accept a hydrogen bond from an acid residue within the enzyme active center. The phenolic ether oxygen of the substituted diethylaminoethoxystilbenes is positioned in the same location, with respect to the farnesyl surrogate, as is the aliphatic ether oxygen of the previously described (phosphinylmethyl)phosphonates [32], suggestive of a similar hydrogen bond formation in the substituted diethylaminoethoxystilbene SQS inhibitor series.

Fourth, based on consideration of the various carbocations that form during the first and second enzyme half-reactions, the location of the ammonium ion in the substituted diethylaminoethoxystilbenes, the farnesyl mimicry of the stilbene residue, and the proposed function of the phenolic ether moiety, it is proposed that the substituted diethylaminoethoxystilbenes mimic intermediates of the first and second SQS-catalyzed half-reactions in such a manner that the stilbene moiety mimics the farnesyl residue of the donor FPP, whereas the diethylaminoethoxy residue mimics the various carbocations developing within the first isoprene residue of the acceptor FPP. Such mimicry is also supported by the competitive nature of the inhibition of SQS by the substituted diethylaminoethoxystilbenes with respect to FPP, since the donor and acceptor FPP molecules are thought to interact with the enzyme in a sequential fashion [14] with the donor FPP binding first [26], such that primary mimicry of the acceptor FPP by the substituted diethylaminoethoxystilbenes would presumably lead to either an uncompetitive or a mixed-noncompetitive pattern of inhibition kinetics.

It is noteworthy that the substituted diethylaminoethoxystilbenes mimic the farnesyl residue of the donor FPP in its entirety, but mimic only the first isoprene residue of the acceptor FPP, in which the carbocationic rearrangements of both the first and second half-reactions occur. Modifications of one of the N-ethyl groups of the diethylaminoethoxy residue of these inhibitors could thus potentially lead to analogs that more closely resemble transition states of the SQS-catalyzed first and second half-reactions.

Fifth, that the substituted diethylaminoethoxystilbenes inhibit the SQS-catalyzed first half-reaction to the same extend as they inhibit the overall reaction indicates that these compounds interact with FPP binding sites rather than with a distinct PSQPP binding site within the enzyme active center. However, since these inhibitors could presumably mimic reaction intermediates of both the first and second half-reactions, that no additional inhibition of the second half-reaction was noted, suggests that inhibition of the first half-reaction is sufficient to inhibit the overall reaction such that PSQPP formed in the first half-reaction cannot be prevented from continuing its conversion to squalene. This observation is consistent with previous observations [1, 11, 13, 15] indicating that both half-reactions occur at catalytic sites within the enzyme active center that are either spatially overlapping or highly interactive, such that the substituted diethylaminoethoxystilbenes are not able to interact only with the second half-reaction center without at the same time interacting with the first half-reaction center. Thus, the substituted diethylaminoethoxystilbenes could potentially function both as first half-reaction intermediate mimetics, with P_i from solution serving as replacements for both the attached PP_i of the acceptor FPP molecule and the released PP_i from the donor FPP, as well as second half-reaction intermediate mimetics, with P_i from solution serving as replacements for the released PP_i molecules.

Finally, that the substituted diethylaminoethoxystilbenes resemble carbocationic intermediates of the SQS-catalyzed half-reactions rather than strictly resembling the first half-reaction substrate, FPP, is further evidenced by the high, 300- to 1000-fold, specificity of these inhibitors for SQS inhibition relative to other FPP-utilizing enzymes such as PFT. This specificity occurs presumably since reactions catalyzed by other FPP-utilizing enzymes, such as protein prenylation reactions [63, 64, 69] and the 1′-4 head-to-tail prenylation reactions of dolichol and ubiquinone synthesis [65–68], undergo mechanisms of catalysis that are distinct from the 1′-2,3 head-to-head condensation reaction catalyzed by SQS [1–4, 14], such that the diethylaminoethoxy moiety and accompanying positive charge that mimics the cyclopropylcarbocation moiety that develops within the first isoprene unit of the second farnesyl residue following condensation has no parallel in either the 1′-4 head-to-tail prenylation reaction mechanisms of dolichol and ubiquinone synthesis [65–68] or the protein prenylation reaction mechanisms [63, 64, 69].

In previous studies, a number of other dialkylamino-

ethoxy-containing compounds have been shown to inhibit hepatic cholesterolgenesis at a diversity of sites along the synthetic pathway [72–74], primarily at or prior to squalene cyclization [62], although many of their sites of inhibition along the pathway were never fully delineated [62, 73, 74]. While many of these compounds lack key determinants thought to afford SQS inhibitory activity to the substituted diethylaminoethoxystilbene series of SQS inhibitors (e.g. the central *trans* olefin of the stilbene series and spatial requirements for farnesyl residue mimicry), an in-depth evaluation of the specificity of the substituted diethylaminoethoxystilbenes for SQS inhibition relative to inhibition of other sites along the cholesterolgenesis pathway was undertaken both in cultured cells and in experimental animals. The results of these studies demonstrate that inhibition of cholesterol synthesis by representative members of this series was primarily, if not exclusively a consequence of SQS inhibition. The high degree of specificity for inhibition of SQS relative to post-squalene enzymes in the cholesterol synthesis pathway may be, in part, a consequence of the ability of these inhibitors to mimic the carbocationic intermediates of the SQS-catalyzed reaction in addition to resembling a polyisoprenoid. In this regard, it is noteworthy that at concentrations that induce greater than 90% inhibition of cholesterol synthesis in cultured cells, these inhibitors also show modest secondary inhibition at the site of oxidosqualene cyclase, an enzyme whose catalytic mechanism also proceeds through carbocationic reaction intermediates [71].

Consistent with the high degree of specificity of the substituted diethylaminoethoxystilbenes for SQS inhibition relative to inhibition of other enzymes in the cholesterolgenic pathway, members of this series exhibited reductions in plasma cholesterol levels in rats that were correlated with intrinsic SQS inhibitory activity, further indicating that inhibition of squalene production was the primary, if not sole mechanism through which these compounds inhibit cholesterolgenesis and reduce plasma cholesterol levels in experimental animals. Representative members of this series also lowered plasma cholesterol in a variety of other species (e.g. chow-fed hamsters, dogs, and monkeys).

Interestingly, the ability of these compounds to reduce plasma cholesterol in species that do not respond to the HMG-CoA reductase inhibitors with reductions in plasma cholesterol (e.g. rats and hamsters) due to compensatory increases in HMG-CoA reductase transcription following treatment [17], suggests a potential mechanistic advantage of SQS inhibition versus HMG-CoA reductase inhibition. Indeed, other structurally distinct classes of SQS inhibitors have also demonstrated plasma cholesterol reduction in species considered to be resistant to actions of the HMG-CoA reductase inhibitors [37, 75].

In this regard, in studies to be reported elsewhere,* we have demonstrated that in cultured human (IM-9) cells HMG-CoA reductase activity was not increased by either P-3622 and related analogs or by representative members of three other distinct structural classes of SQS inhibitors (quinuclidines, bisphosphonates, and benzoxazepinones) following incubation for 24 hr at concentrations that inhibit cholesterol synthesis by up to 90% [76]. By contrast, under these experimental conditions, the HMG-CoA reductase inhibitor, mevinolin, produced the predicted increases in HMG-CoA reductase activity that were dose-dependently related to the degree of reduction in cholesterol synthesis, such that a 4-fold increase in HMG-CoA reductase activity was noted at concentrations of mevinolin that produced 50% inhibition of cholesterol synthesis, with a 17-fold increase in enzymatic activity noted following 90% inhibition of cholesterolgenesis [76].†

These observations are consistent with the proposed ability of SQS inhibitors to maintain regulatory levels of key nonsterol FPP-derived [78] modulators of HMG-CoA reductase translation and/or degradation, thereby maintaining post-transcriptional control of HMG-CoA reductase activity, even in the face of transcriptional derepression due to reduced cholesterol production, and thus preventing the compensatory increases in mevalonate production that limit efficacy of the HMG-CoA reductase inhibitors. Indeed, such mechanistic advantages, together with a more favorable site of inhibition along the sterol and polyisoprenoid pathway, that of the first committed step in the formation of cholesterol and related sterols [16, 17], suggest that cholesterol synthesis inhibition via SQS inhibition may have distinct advantages over similar cholesterol synthesis inhibition via HMG-CoA reductase inhibition that could be of therapeutic relevance.

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* Petras SF, Lindsey S and Harwood HJ Jr, manuscript in preparation.

† Consistent with previous studies in experimental animals [77], in the studies mentioned in the previous footnote, zaragozic acid A exhibited even greater induction of HMG-CoA reductase activity than mevinolin at concentrations that produced similar degrees of cholesterol synthetase inhibition [76], suggesting that zaragozic acid A may directly prevent the production of a key nonsterol regulator of HMG-CoA reductase translation and/or degradation, possibly through direct inhibition of a key reaction involved in its production.

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