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INHIBITION OF 2,3-OXIDOSQUALENE CYCLASE AND STEROL BIOSYNTHESIS BY 10- AND 19-AZASQUALENE DERIVATIVES

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Abstract—The inhibition of 2,3-oxidosqualene-lanosterol cyclase (EC 5.4.99.7) (OSC) by new azasqualene derivatives, mimicking the proC-8 and proC-20 carbocationic high-energy intermediates of the cyclization of 2,3-oxidosqualene to lanosterol, was studied using pig liver microsomes, partially purified preparations of OSC, and yeast microsomes. The azasqualene derivatives tested were: 6E- and 6Z-10aza-10,11-dihydrosqualene-2,3-epoxide 17 and 18, 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide 19 and its corresponding N-oxide 20, and 19-aza-18,19,22,23-tetrahydrosqualene 21. The compounds 17 and 19 (i.e. the derivatives bearing the 2,3-epoxide ring and the same geometrical configuration as the OSC substrate) were effective inhibitors, as shown by the K_i obtained using partially purified OSC: 2.67 μM and 2.14 μM, respectively. Compound 18, having an incorrect configuration and the 19-aza derivative 21, lacking the 2,3-epoxide ring, were poor inhibitors, with IC_{50} of 44 μM and 70 μM, respectively. Compound 21 was a competitive inhibitor of OSC, whereas 17 and 19 were noncompetitive inhibitors, and showed a biphasic time-dependent inactivation of OSC, their apparent binding constants being 250 μM and 213 μM, respectively. The inhibition of sterol biosynthesis was studied using human hepatoma HepG2 cells. The incorporation of [^{14}C] acetate in the C_{27} sterols was reduced by 50% by 0.55 μM 17, 0.22 μM 19, and 0.45 μM 21, whereas 2 μM 18 did not affect sterol biosynthesis. In the presence of 17, 19 and 21, only the intermediate metabolites 2,3-oxidosqualene and 2,3,22,23-dioxidosqualene accumulated, demonstrating a very specific inhibition of OSC.

Key words: 2,3-oxidosqualene cyclase; sterol biosynthesis inhibition; azasqualenes; hepatoma cells; time-dependent inhibition

OSC§ (EC 5.4.99.7) is a widely distributed enzyme catalyzing the cyclization of 3(S)-2,3-oxidosqualene 1 to lanosterol in vertebrates and fungi, and to cycloartenol or a variety of tetracyclic and pentacyclic triterpenes in higher plants [1–6]. It has been suggested that the enzymatic cyclization of all-trans 1 starts with an acid-catalyzed opening of the oxirane ring, and proceeds through the discrete, conformationally rigid carbonium ion intermediates 2, 3, 4, 5, 6, or 7, called high-energy intermediates (HEI) (Fig. 1) [1, 5–10]. The last HEI 6 or 7, also called protosterol ion [11], undergoes further rearrangement to form lanosterol 8 in animals and yeasts or cycloartenol in higher plants.

We have previously shown that very potent inhibitors of OSC can be obtained by mimicking HEIs using squalene derived structures (9 and 10; Fig. 2) in which the positively charged carbocation is replaced by a nitrogen that is protonated at physiological pHs [12–14]. Mono- and bicyclic aza-derivatives possessing a nitrogen at C-10, as in compounds 11 and 12 (Fig. 2), or at C-8 as in the azadecalines 13 and 14, are also potent inhibitors of OSC [15–21], while some tricyclic or tetracyclic aza-derivatives, for example 15 or 16 (Fig. 2), are practically inactive *in vitro* [12, 16]. It has been suggested that 15 or 16 are poor inhibitors due to the relative slowness of OSC in reaching a conformation complementary to that of HEIs occurring late in the cyclization process [16]. Moreover, some of the bicyclic aza- derivatives, such as 13 or 14, in addition to showing a good activity toward OSC, are also good inhibitors of Δ^{7-8}-sterol isomerase [18, 22] and $\Delta^{8,14}$-sterol-Δ^{14}-reductase [23, 24].

Considering the above results, we have postulated that acyclic azasqualene derivatives, such as the proC-8 HEI analogue 10-aza-10,11-dihydrosqualene-2,3-epoxide 17 or the proC-20 HEI analogue 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide 19 (Fig. 3), could both be more specific and more active inhibitors of OSC than their corresponding cyclic azasteroids (e.g., 14 and 16). These acyclic, "internal" azasqualenes, which are conformationally more flexible than the cyclic azaderivatives, should be more adaptable to the OSC active site and should not, lacking a cyclic structure, interfere with other sterol biosynthesis enzymes such as the Δ^{7-8}-isomerase or sterol-Δ^{14}-reductase.

In this work, we have studied the OSC inhibition kinetics of isomers 6E- and 6Z of 10-aza-10,11-dihydrosqualene-2,3-epoxide 17 and 18, of 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide 19 and its corresponding N-oxide 20, and of 19-aza-18,19,22,23-tetrahydrosqualene 21. The effects of these acyclic aza derivatives on the biosynthesis of sterols, as well as their specificity, were also evaluated in human hepatoma HepG2 cells.

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§ Abbreviations: OSC, 2,3-oxidosqualene cyclase; HEI, high-energy intermediate.

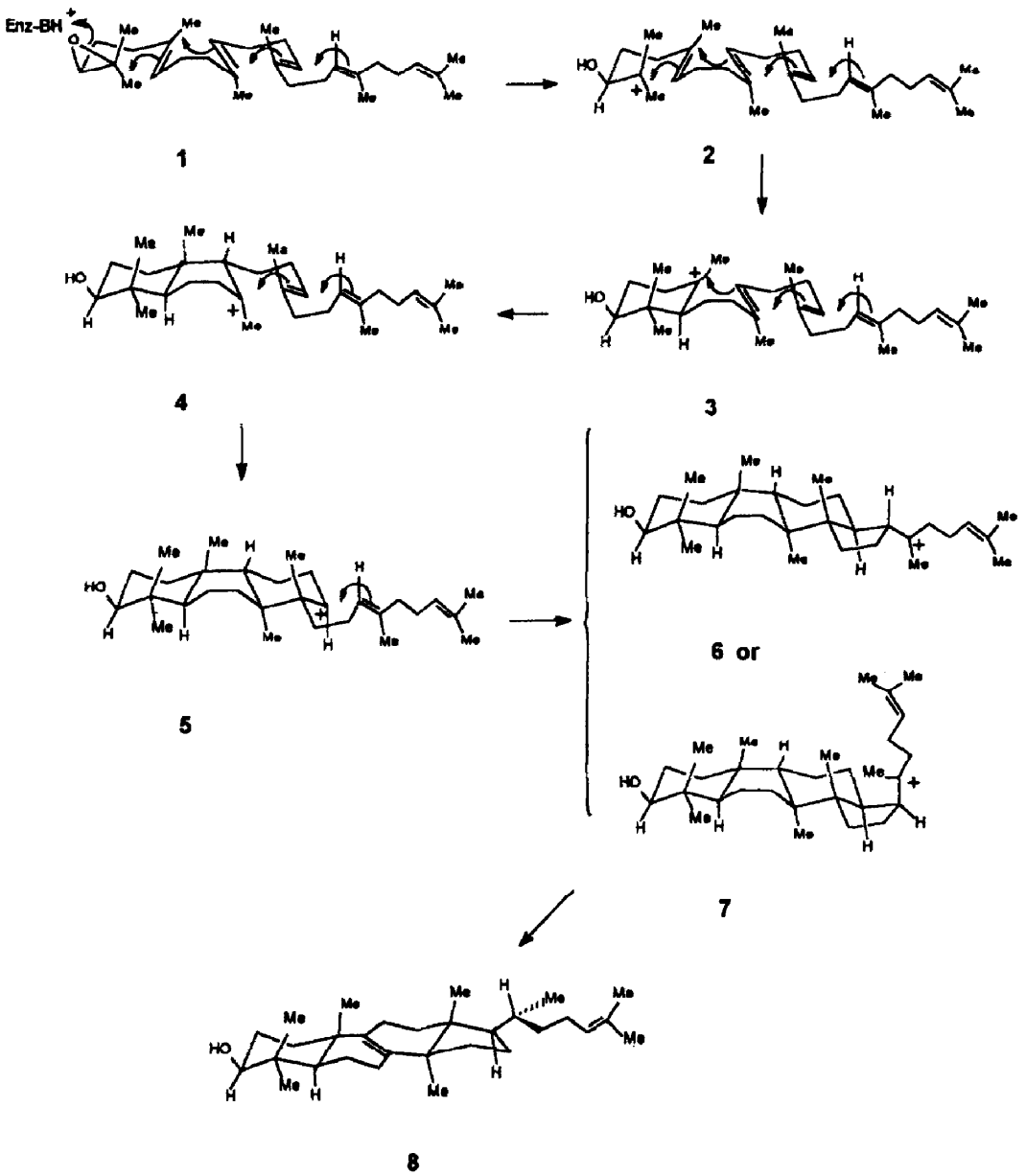

Fig. 1. Detailed mechanism of cyclization of 2,3-oxidosqualene to lanosterol.

MATERIAL AND METHODS

Chemicals

Synthesis of compound 21. A solution of N-methyl-4-methyl-pentylamine [26] (×2, 103 mg, 0.9 mmol) in anhydrous methanol (10 ml) was cooled to 0°C and $NaBH_3CN$ (×1.2, 33.9 mg, 0.54 mmol) was added with stirring. C_{22} squalenoid aldehyde [25] (0.45 mmol, 142 mg) dissolved in the minimum of methanol (2 ml) was then added, brought to room temperature, and stirred for 4 hr.

The reaction mixture was then extracted with dichloromethane (100 ml × 3) after addition of brine, dried, and evaporated to dryness *in vacuo*. The resulting oil was purified by flash chromatography using light petroleum/diethyl ether, 98:2, then 96:4, finally 92:6 to give 135 mg (72% yield) of 19-aza-18,19,22,23-tetrahydrosqualene 21 as a colourless oil.

Anal. C,H,N. IR (liq. film) ν_{max} 2950, 2920, 2860, 2780, 1450, 1380, 1360 cm^{-1}. H NMR ($CDCl_3$) δ 0.89 [d, 6H, $(C\underline{H}_3)_2CH$], 1.18 [m, 2H, $(CH_3)_2CHC\underline{H}_2$], 1.43–1.69 [m, 20H, allylic CH_3, and $[(CH_3)_2C\underline{H}CH_2C\underline{H}_2NCH_2C\underline{H}_2]$], 1.96–2.11 (m, 14H, allylic CH_2), 2.21 (s, 3H, $C\underline{H}_3N$), 2.29 (t, 4H, $C\underline{H}_2NC\underline{H}_2$), 5.10–5.15 (m, 4H, vinylic CH). HRMS: found M^+, 415.4180. $C_{29}H_{53}N$ requires M, 415.4178. EIMS: m/z 415 (20), 344 (38), 278 (33), 210 (42), 128 (100). ^1H NMR spectra were recorded on a Jeol EX-400 in $CDCl_3$ solution at room temperature, with $SiMe_4$ as internal standard. Mass spectra were obtained on a VG Analytical 7070 EQ-HF spectrometer. IR spectra were recorded on a Perkin-Elmer

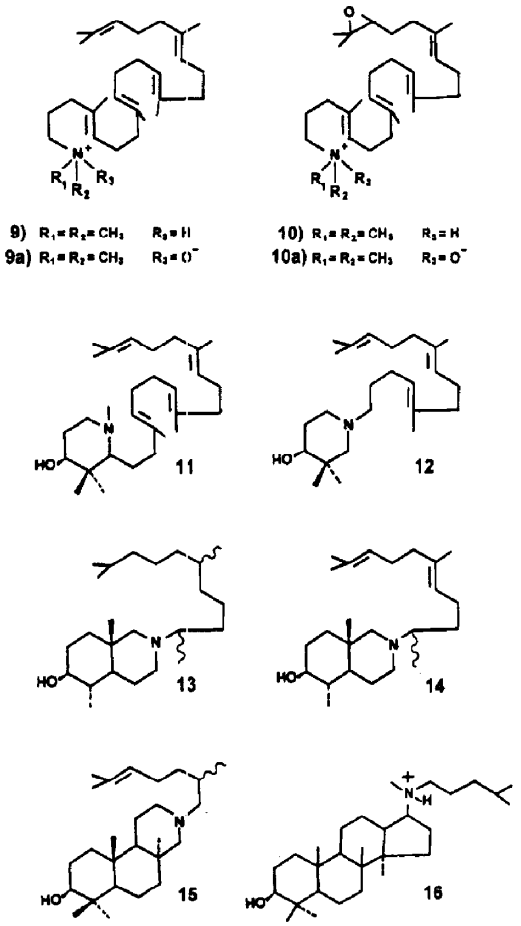

Fig. 2. Structures of different inhibitors of oxidosqualene cyclase, acting as analogues of carbocationic C2, pro-C8, pro-C10, and pro-C20 HEI.

781. Microanalyses for C, H, and N performed on an elemental analyser 1106 (Carlo Erba Strumentazione, Milan, Italy) were within ±0.4% of theoretical values.

The reactions were checked on F_{254} silica gel-precoated sheets obtained from Merck (Darmstadt, Germany); after development, the sheets were exposed to iodine vapour. Purifications were done using column "flash chromatography" on 230–400 mesh silica gel (Merck). Light petroleum refers to the fraction of bp: 40–60°C. The synthesis of compounds 17, 18, 19, 20 has been reported previously [25, 26].

Sodium [2-^{14}C]acetate (50 μCi:mmol) was obtained from NEN Dupont de Nemours (Les Ulis, France), Cholesterol, lanosterol, and squalene were obtained from Merck, polyoxyethylene 9 laurylether (Polidocanol) from Sigma Chimica (Milan, Italy), while cold and labelled 2.3-oxidosqualene 1 were synthesized as previously described [27].

Biological

Microsomes. Rat and pig liver microsomes were prepared according to the method previously described [13].

Solubilisation and purification of pig liver OSC. The enzyme was purified according to the method previously described with minor modifications [13]. Briefly, microsomal pellets were solubilized by adding a 1% (w/v) solution of polidocanol to the microsomes, to a final detergent/protein ratio of 1. After centrifugation at 100,000 g, the supernatant containing the enzymatic activity was applied to a column of DEAE Bio-gel A (from Bio-Rad laboratories, Milan, Italy), equilibrated with 0.01 M potassium phosphate buffer pH 8.0, containing 1 mM EDTA and 0.1% polidocanol (buffer A). The OSC activity was eluted by a gradient of buffer A and buffer A containing 0.5 M NaCl. Specific activity of purified OSC was 233 nmol/h/mg of proteins, and the purification factor was 50. After addition of NaN_3, this enzymatic preparation was stable at 4°C for several months. Proteins were determined by the method of Lowry modified by Peterson [28].

Assay of OSC activity and kinetic determinations. Enzyme activity was determined by incubating the microsomal suspension (1 mg of proteins) or the partially purified enzyme (20 μg of proteins) for 30 min at 45°C with [^{3}H][R,S]-2,3-oxidosqualene (50,000 cpm), as previously described [13]. IC_{50} values (the concentration of inhibitor that reduced by 50% the enzymatic conversion of 2,3-oxidosqualene to lanosterol) were determined at 25 μM substrate concentration, in the presence of different concentrations of inhibitors. The kinetics of inhibition were analyzed by Lineweaver-Burk and Dixon graphical methods or by using a computer program to analyse the values of $Km_{(app)}$ and $Vmax_{(app)}$, obtained in the absence and in the presence of inhibitors, by fitting the values of v and [S], by a nonlinear regression method, directly to the Michaelis-Menten equation [29, 30].

Time-dependent inactivation of the OSC. Time-dependent inactivation was determined at 37°C by adding the inhibitors to the enzyme solution (0.8 mg of proteins/ml)

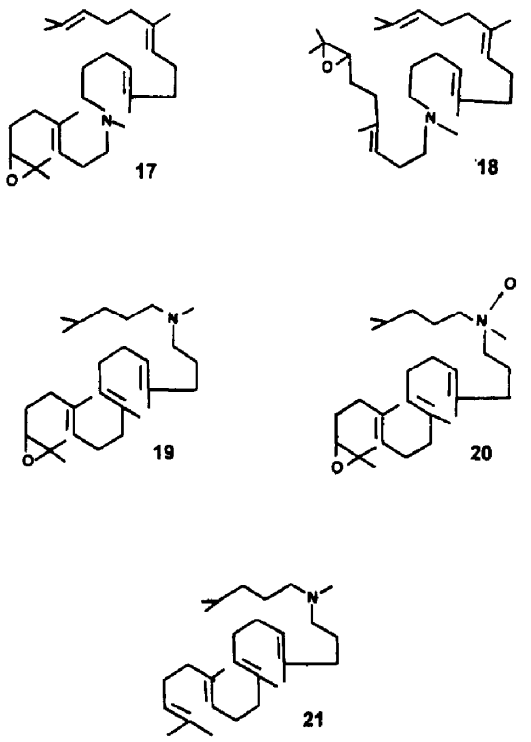

Fig. 3. Structures of "internal" 10- and 19-azasqualene derivatives.

in the absence of the substrate. Aliquots of 25 µl (20 µg of proteins) were withdrawn at time intervals from 30 sec to 45 min and diluted 40-fold by transfer to test tubes containing 2,3-oxidosqualene and Tween-80 (0.5 mg/ml) in 975 µl of potassium phosphate buffer 0.01 M, pH 8.0. Residual activity was then determined under the same conditions as above. To remove the inhibitor and check recovery of enzyme activity after preincubation, the enzyme solution was passed through a membrane anion exchange DEAE MemSep cartridge (Millipore, Bedford, MA, U.S.A.) applied to an HPLC L-6200 Merck-Hitachi and equilibrated with 0.01 M phosphate buffer, pH 8. After washing with the same buffer, the enzyme activity was eluted by a gradient (0–1 M) of NaCl in the above buffer.

Hepatoma cell cultures. HepG2 hepatoma cells obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) were routinely maintained as monolayers in Dulbecco-modified Eagle medium (DMEM) supplemented with 10% foetal calf serum. The cells were grown in a humidified atmosphere (95% air/5% CO2) at 37°C.

Inhibition of [^{14}C] acetate incorporation into sterols. Method A: Cells derived from stock cultures were seeded at approximately 1×10^6 cells in 60 mm diameter plastic Petri dishes in 5 ml DMEM containing 10% (v/v) delipidated serum [31]. After 36 hr, the cells were preincubated for 8 hr in the presence of drugs 17 and 19 given as solutions in absolute ethanol. Final concentrations of solvent in the culture medium did not exceed 0.4%, and an equivalent amount was added to the control culture. Labelled acetate (20 µCi per dish) was then added. After 2 hr of incubation, the medium was removed and the dishes rinsed three times with phosphate-buffered saline (PBS), pH 7.4. The cells were then treated twice for 30 min with 2 ml of 0.1 N NaOH. Aliquots were removed for protein determination [32], and the remainder saponified with 1 vol of 6% (w/v) potassium hydroxide in methanol for 1 hr at 55°C or overnight at room temperature. The non-saponifiable lipids were extracted with dichloromethane, an aliquot was counted, and the remainder analyzed by TLC on Silica Gel F_{254} plates (Merck) as detailed elsewhere [33] using n-hexane/ethyl acetate (85/15) as solvent. The radioactivity associated with the different compounds was read with a Berthold automatic TLC-analyzer (LB 284/285), and the peaks integrated with a data acquisition system. The radioactive band corresponding to the C_{27}-sterols was scraped off into scintillation vials and counted. Alternatively, the C_{27}-sterols were eluted and transformed into acetates (pyridine/acetic anhydride) and analyzed by GC-MS. The analysis was performed on a 30 m 0.32 mm i.d. DB5 JW (Folsom, CA, U.S.A.) capillary column coupled to a Fisons Instruments MD mass spectrometer operated at 70 eV.

Method B: Cells derived from stock cultures were seeded at approximately 3×10^5 cells in 24-well plates in a final volume of 1 ml of DMEM containing 10% (v/v) delipidated serum [31]. After 3 hr, inhibitors 17, 18, 19, and 21 were added to the monolayers and incubated for 16 hr. Final concentration of solvent (ethanol) in the wells did not exceed 0.5%, and an equivalent amount was added to the control cultures. Labelled acetate (2 µCi/well) was then added to the cells. After 2 hr of incubation, the medium was removed and the wells washed 3 times with 0.1 M PBS, pH 7.4. The cells were then treated for 30 min with 0.5 ml of 0.1 N NaOH and transferred to test tubes containing 0.5 ml of PBS. Unsaponifiable lipids were extracted with 1 ml of petroleum ether after saponification in the presence of 10% KOH in methanol. The samples are analysed as previously described [34]. For the chase experiments, the cells, after the 2-hr pulse of labelled acetate, were washed and incubated for a further 2 hr in the presence of unlabeled acetate 20 mM. The cells were then collected and treated as above.

RESULTS

IC_{50} determinations and inhibition kinetics with microsomal and partially purified mammalian OSC

Inhibition activity of the "internal" azasqualene derivatives 17–21, mimicking the proC-8 and proC-20 HEIs, was determined using 2,3-oxidosqualene cyclase associated with rat and pig liver microsomes, partially purified pig enzyme, and yeast (*Candida albicans* and *Saccharomyces cerevisiae*) microsomes [25, 26, 35]. Table 1 shows the IC_{50}s obtained in comparison with the previously determined IC_{50} values of the corresponding "external" aza squalenes 9, 9a, 10, and 10a [13, 36]. The inhibition of mammalian OSC by (6E)-10-aza-10,11-dihydrosqualene-2,3-epoxide 17 and 18,19,22,23-19-aza-tetrahydrosqualene-2,3-epoxide 19 and its N-oxide derivative 20 was generally similar to that obtained by 2-aza-2,3-dihydrosqualene derivatives. As expected, only the E-isomer, corresponding to the natural (all-E) 2,3-oxidosqualene 1, was active in the different biological systems tested. The presence of an epoxide ring was also important for activity since 19-aza-18,19,22,23-tetrahydrosqualene 21 showed poorer inhibitory activity. The "internal" azasqualenes 17 and 19 at 50 µM, the highest concentration tested, were completely inactive on pig liver microsomal squalene epoxidase, thus differing markedly from 2-aza-2,3-dihydrosqualene derivatives, which were also active on squalene epoxidase (IC_{50} = 4.5 µM in rat liver [37]). The different activities

Table 1. Inhibition (IC_{50}, µM) of 2,3-oxidosqualene cyclases by azasqualene derivatives

	Compound								
Enzyme preparation	9	9a	10	10a	17	18	19	20	21
Rat liver microsomes	7.5	3.7	4	1.5	4.8	>20	7.5	ND	ND
Pig liver microsomes	2.3	7	3.3	6	5.5	ND	1.5	ND	ND
Purified pig liver OSC	0.15	3.3	0.2	2.1	2.4	44	1.7	8.6	70
Saccharomyces cerevisiae microsomes	10	16	28	120	5	>100	35	100	130
Candida albicans microsomes	ND	ND	8.5	19	3	>100	22	50	200

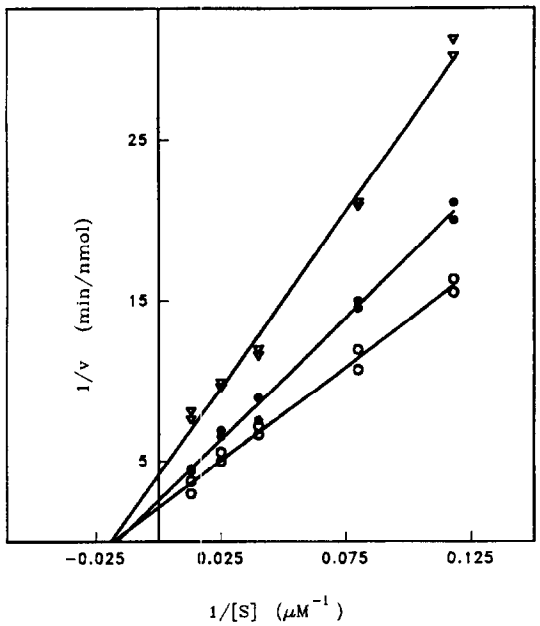

Fig. 4. Lineweaver-Burk plots of inhibition of partially purified pig liver OSC in the presence of (6E)-10-aza-10,11-dihydrosqualene-2,3-epoxide 17. Inhibitor concentrations were: none (○—○); 1 μM (●—●); 3 μM (▽—▽). Lines were obtained by linear regression analysis. Conditions: pH 8, 45°C. Protein concentration: 20 μg/ml.

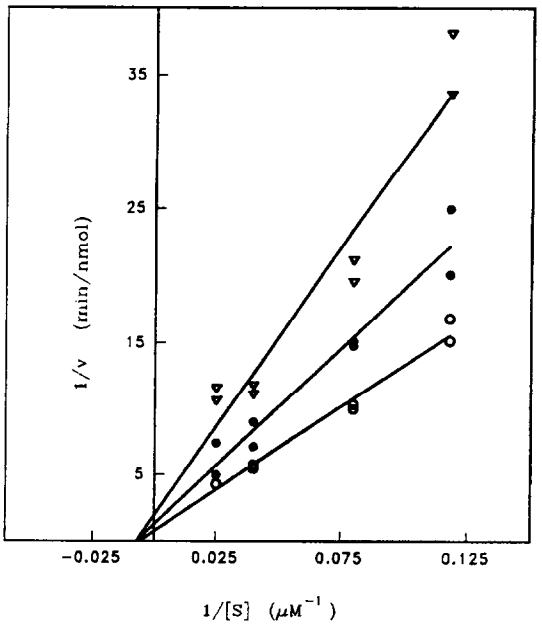

Fig. 5. Lineweaver-Burk plots of inhibition of partially purified pig liver OSC in the presence of 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide 19. Inhibitor concentrations were: none (○—○); 1 μM (●—●); 2.5 μM (▽—▽). Lines were obtained by linear regression analysis. Conditions: pH 8, 45°C. Protein concentration: 20 μg/ml.

observed in the enzyme systems studied showed a progressive target selectivity in the inhibition of OSC, going from C-2 (9) to proC-8 (17) and proC-20 (19) HEI mimics. 2-Aza-2,3-dihydrosqualene 9 indiscriminately inhibited mammalian, yeast, and higher plant OSCs, as well as squalene epoxidase and bacterial cyclase [14]. In contrast, (6E)-10-aza-10,11-dihydrosqualene-2,3 epoxide 17 inhibited yeast and mammalian OSCs. Finally, the 19-aza- derivative 19 was very selective for the mammalian enzymes: In yeast it was 20–30 times less active than in pig liver OSC. The kinetics of the inhibition of partially purified pig liver OSC by compounds 17 and 19 were apparently noncompetitive, as shown by Lineweaver-Burk plots (Figs. 4 and 5) and Dixon plots (not shown). Statistical analysis of the data was consistent with this interpretation [29, 30]. The K_i values calculated with the graphical method of Dixon were 2.67 μM for 17 and 2.14 μM for 19, respectively. As expected for this type of inhibition, these values are very similar to the corresponding IC_{50} values. The kinetic results obtained with the solubilized pig liver OSC were comparable to those previously reported for the 2-aza-2,3-dihydrosqualene derivatives [13, 17]. By contrast, the kinetics of inhibition of OSC by compound 21, lacking the epoxide function, was found to be competitive, as shown in Fig. 6 by the comparison of Dixon and Cornish-Bowden plots [38]. This compound, a poorer inhibitor of OSC, showed an affinity toward the enzyme (K_i = 74 μM) comparable to the K_m of the substrate (about 50 μM).

Time dependent inactivation of OSC

The ability of 17 and 19 to inactivate mammalian oxidosqualene cyclase in a time-dependent manner was evaluated using the partially purified pig liver enzyme. After preincubation of different concentrations of the inhibitor with the enzyme for various times (from 30 sec to 40 min) followed by 40-fold dilution, a biphasic time-dependent inactivation was obtained in all cases (Figs. 7 and 8). At a concentration of 10 μM, the azasqualene derivatives 17 and 19 were able to inactivate the enzyme very rapidly: After 1 min of pre-incubation, the enzyme activity was reduced by 50%, and after 5 minutes, by 75%. The overall kinetics showed a two-step mechanism: An initial fast inactivation phase was followed by a slower inactivation process. Apparent binding constants K_i and inactivation rate constants $k_{(app)max}$, calculated from the slope and intercept of double reciprocal plots of the pseudo-first-order inactivation rate in the fast inactivation phase, vs inhibitor concentrations, (Figs. 7 and 8, insets), were 213 μM and 4 min^{-1} for compound 19 and 250 μM and 2.3 min^{-1} for compound 17, respectively. The incorrect geometrical isomer (6Z)-10aza-10,11-dihydrosqualene-2,3-epoxide 18 and 19-aza-18,19-tetrahydrosqualene 21 lacking the oxirane ring, were unable to inactivate the enzyme in a time-dependent manner, even at the highest concentrations tested (100 μM and 40 min of preincubation). In contrast to the "internal" azasqualenes 17 and 19, 2-aza-2,3-dihydrosqualene 9 showed no time-dependent inactivation, even after a pre-incubation period of up to 60 min. When the enzyme, preincubated for 30 min with 12 μM 19, was passed after the preincubation through a membrane anion exchange cartridge, the activity was not restored, whereas full activity was recovered with the same treatment after preincubation with 500 μM 21 or 15 μM 9.

Effect of 10-aza and 19-azasqualene derivatives on sterol biosynthesis in HepG2 cells

The results obtained by examining the activity of the azasqualenes 17–19 and 21 in human HepG2 hepato-

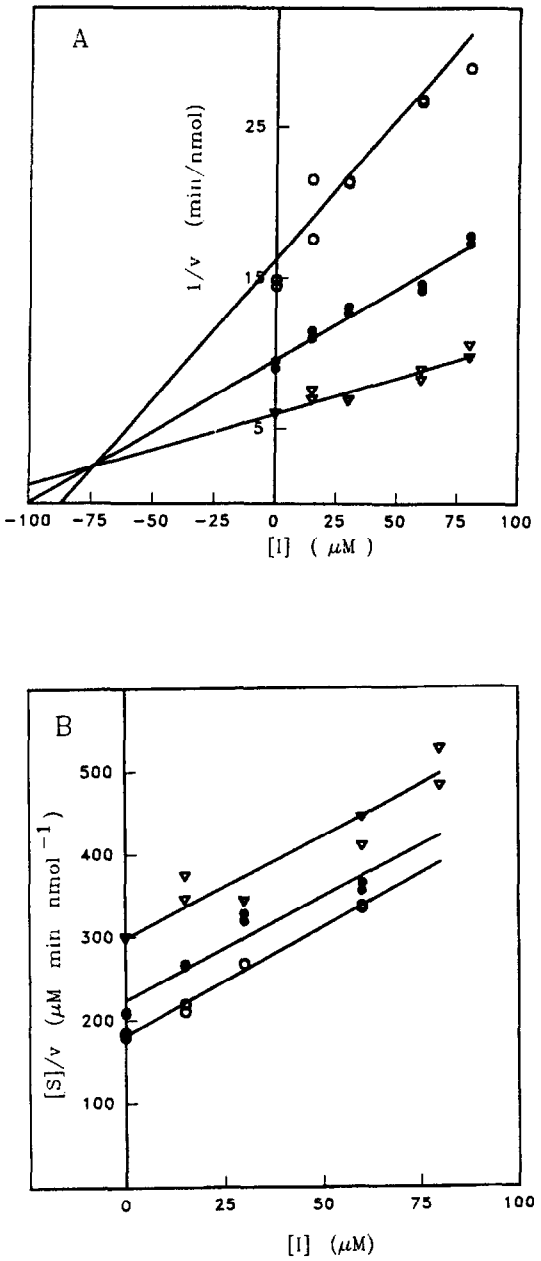

Fig. 6. Dixon (A) and Cornish-Bowden (B) plots of the inhibition of partially purified pig liver OSC in the presence of 19-aza-18,19,22,23-tetrahydrosqualene 21. Substrate concentrations were: 12.5 μM (○—○); 25 μM (●—●); 50 μM (▽—▽). Lines were obtained by linear regression analysis. Conditions: pH 8, 45°C. Protein concentration 20 μg/ml.

blastoma cells confirmed those obtained with microsomal and solubilized OSC preparations. Table 2 shows the incorporation of radioactivity from [2-^{14}C]-acetate into the different sterol fractions after treatment with the azasqualene derivatives (method B, Materials and Methods). Results are expressed as percent of total counts in non-saponifiable lipids. Of the two geometrical isomers 6E and 6Z, only 6E 17 strongly reduced incorporation in the C_{27}-sterol fraction in a dose-dependent manner. The IC_{50} (0.55 μM) was approximately ten times lower than with rat and pig liver microsomal OSCs (approximately 5 μM). This difference in potency of the inhibitor when comparing cells and microsomes is similar to that already noted for 2-aza-2,3-dihydrosqualene [34]. In the absence of inhibitor, the radioactivity was incorporated into C_{27}-sterols and a minor unidentified fraction (R_f 0.69), migrating slightly more slowly than 2,3-oxidosqualene (R_f 0.78). Treatment of the cells with 0.5 μM 17 reduced the incorporation of the label into the C_{27}-sterol fraction by 50% and caused, in parallel, increased incorporation in the region of the chromatogram corresponding to 2,3-oxidosqualene 1 and 2,3,22,23-dioxidosqualene 22. Incorporation into compounds 1 and 22 increased from 3 to 24% and from 0 to 10%, respectively. Labelled oxidosqualene and dioxidosqualene were identified as already described [34] on the basis of co-migration with authentic references. The 6Z isomer 18 at the highest concentration tested (2 μM) had no marked effect on C_{27}-sterols, but caused a small increase of label in a fraction co-migrating with lanosterol. When HepG2 cells were treated with 1 μM 17, (method A), decrease in cholesterol was accompanied by an accumulation of compound 22 and the appearance of a minor metabolite that had the chromatographic behaviour of 24,25-epoxycholesterol [33]. In contrast, the TLC revealed no 4-dimethyl or 4α-methyl sterols. The C_{27}-sterol band was eluted and after acetylation, the compounds were analysed by GC-MS. A major compound accounting for 94% of the analysed material was found; mass spectra taken across the peak revealed it to be composed exclusively of cholesteryl acetate (not shown). The minor peak had no resemblance to any known sterol. The "internal" 10-aza-10,11-dihydrosqualene-2,3-epoxide 17 therefore seems very specific for 2,3-oxidosqualene-lanosterol cyclase in HepG2 cells. This contrasts with the behaviour of both the cyclized aza analogues 13 and 14, shown to also inhibit Δ^8-isomerase [18, 22] or Δ^{14}-reductase [23, 24], and of 9, found to inhibit Δ^{24}-sterol reductase [34].

Compound 19 specifically inhibited OSC in HepG2 cell cultures, as indicated by the increase in radioactivity, larger than that caused by 17, in both 2,3-oxidosqualene 1 and 2,3,22,23-dioxidosqualene 22 fractions. The IC_{50} was 0.22 μM and the incorporation of label in the C_{27} sterols at a 0.1 μM concentration was decreased by 37%, whereas at the same concentration the 10E-azasqualene 17 lowered cholesterol synthesis by 26%. At a concentration above 5 μM there was a marked decrease in incorporation into the non-saponifiable fraction, which paralleled the decrease in label in the C_{27} fraction. This indicates that 19 at this high concentration has an effect on cellular metabolism unrelated to cholesterol biosynthesis and/or blocks the biosynthesis upstream of squalene.

The 19-aza-18,19,22,23-tetrahydrosqualene 21, which lacks the epoxide function and did not inhibit the enzyme *in vitro*, did inhibit sterol biosynthesis in hepatoma cell cultures, giving an IC_{50} of 0.45 μM, similar to that of compound 17 and twice the IC_{50} of 19. The step inhibited was again the formation of lanosterol by OSC, as shown by the accumulation of label in the fractions corresponding to 1 and 22. The 10-aza and 19-azasqualene derivatives 17, 19, and 21 failed to inhibit the squalene epoxidase in HepG2 cell cultures, since no accumulation of labelled squalene was observed.

To demonstrate a precursor-product relationship between the C_{27}-sterols and the labelled 1 and 22 formed in the presence of 0.1 and 0.5 μM 17 and 19, chase experiments were performed in the presence of unlabeled ac-

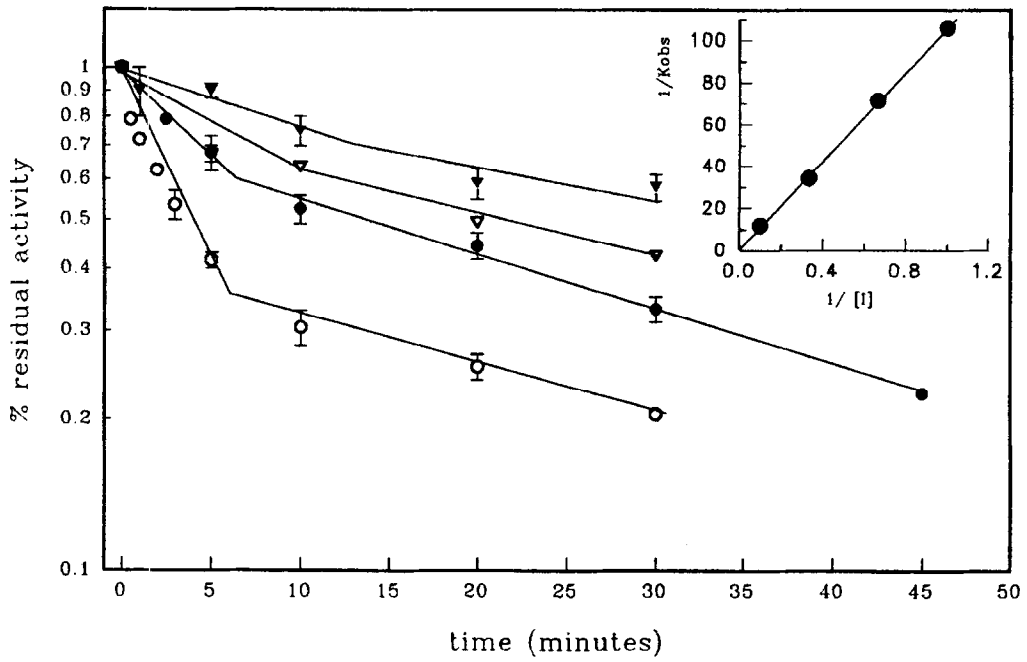

Fig. 7. Time-dependent inhibition of OSC by (6E)-10-aza-10,11-dihydrosqualene-2,3-epoxide 17. Partially purified OSC (0.8 mg of proteins/ml) was preincubated at 37°C in the presence of the inhibitor (▼—▼) 1 μM, (▽—▽) 1.5 μM, (●—●) 3 μM, (○—○) 10 μM. Residual activity (% of the control not preincubated with the inhibitor at the same time) was determined by withdrawing aliquots of 25 μl at time intervals and diluting to a final volume of 1 ml with substrate. Standard error bars are indicated when the standard error exceeds the size of the symbols. Inset: Double-reciprocal plots of the apparent inactivation pseudo-first-order rate constant (k_{obs}) vs concentration of inhibitor. (k_{obs}) were obtained by linear regression analysis of time points from 0 minutes up to deviation from linearity.

etate. After the chase, labelled 1 and 22 disappeared, and increased incorporation into the C_{27}-sterol fraction was observed. The final incorporation of label in this fraction, however, was 80% of the controls at an inhibitor concentration of 0.1 μM and 65% at an inhibitor concentration of 0.5 μM, probably indicating a certain degree of cell damage caused by the inhibitors.

DISCUSSION

It has been shown that the designed acyclic azasqualenes (6E)-10-aza-10,11-dihydrosqualene-2,3-epoxide 17 and 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide 19 are excellent inhibitors of mammalian and yeast OSCs. Moreover, all the azasqualenes studied in this work except 18 possess potent hypocholesterolemic activity in the human hepatoblastoma cell line HepG2; the IC_{50} varied from 0.2 to 0.5 μM. The inhibition of sterol biosynthesis was principally due to a specific inhibition of OSC, without affecting other important steps in sterol biosynthesis. 19-aza-18,19,22,23-tetrahydrosqualene 21 was active in HepG2 cells, whereas it was not with the enzyme *in vitro*. Compound 21 probably behaved as a pro-inhibitor, undergoing epoxidation in these cells.

The most striking difference between "internal" 17 and 19 and "external" azasqualenes, such as 9, was the progressive target selectivity gained when going from the C-2 (compounds 9, 9a, 10, 10a) to the pro-C8 (17) and to the pro-C20 (19) HEI analogues. The 2-aza-2,3-dihydrosqualene derivatives were inhibitors of OSCs from all eukaryotic sources tested: mammals, fungi, and higher plants [14]. Moreover, they also inhibited some other enzymes involved in cholesterol biosynthesis, such as squalene epoxidase and desmosterol reductase [17, 37]. Δ^{24}-reductase is also a target of compounds possessing an N-dialkyl or alkyl side chain as in the U1866A and 25-azasterol series [39, 40].

The pro-C8 HEI analogue 17 appeared to be more selective than the 2-aza-2,3-dihydro-squalene derivatives, since its inhibition was restricted to mammalian and yeast OSCs. Compound 19, mimicking the pro-C20 HEI, proved to be active only toward the mammalian OSC. The selectivity shown by the pro-C8 and pro-C20 HEI analogue inhibitors 17 and 19 suggests that the active conformation of the enzyme, able to stabilize the intermediate carbocations 4, 6, or 7, differs considerably in the mammalian, yeast, and higher plant enzymes.

The "internal" azasqualenoids must possess two fundamental features to become potent inhibitors of OSC: a 2,3-epoxide function and a geometrical configuration overlapping that of the natural substrate 1. In fact, both 21, lacking the oxirane ring, and 18, characterized by incorrect Z geometry, were poorly active on the microsomal and purified oxidosqualene cyclase. These data suggest that the "internal" azasqualenoids, bearing a structure similar to squalene-2,3-oxide 1, bind to OSC in the ground state and, because of their conformational adaptability, mimic in the active site a structure generated downstream in the cyclization process. Compounds 17 and 19 followed noncompetitive inhibition kinetic, identical to that observed for 2-aza-2,3-dihydrosqualene and derivatives [13, 17], whereas 19-azaderivative 21, lacking the epoxidic function, acted as a competitive inhib-

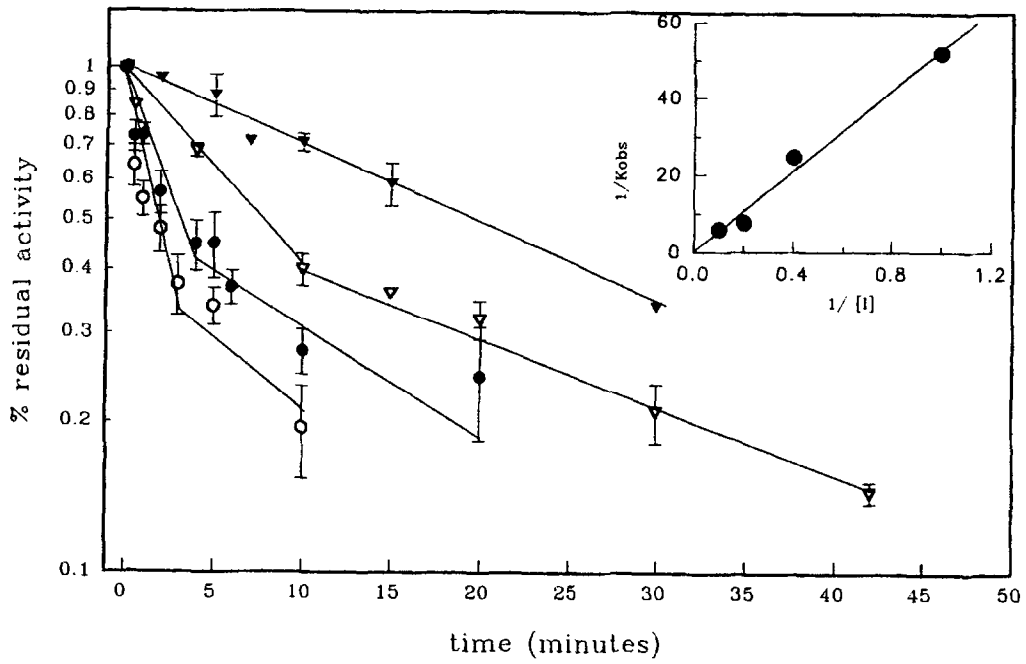

Fig. 8. Time-dependent inhibition of OSC by 19-aza-18,19,22,23-tetrahydrosqualene 2,3-epoxide 19. Partially purified OSC (0.8 mg of proteins/ml) was preincubated at 37°C in the presence of the inhibitor (▼—▼) 1 μM, (▽—▽) 2.5 μM, (●—●) 5 μM, (○—○) 10 μM. Residual activity (% of the control not preincubated with the inhibitor at the same time) was determined by withdrawing aliquots of 25 μl at time intervals and diluting to a final volume of 1 ml with substrate. Standard error bars are indicated when the standard error exceeds the size of the symbols. Inset: Double-reciprocal plots of the apparent inactivation pseudo-first-order rate constant (k_{obs}) vs concentration of inhibitor. (k_{obs}) were obtained by linear regression analysis of time points from 0 minutes up to deviation from linearity.

Table 2. Analysis by thin layer chromatography of the effect of inhibitors on incorporation of [^{14}C]acetate in non-saponifiable lipids in Hep G2 cell cultures

	Lipids (% of total counts)				
Inhibitors	C_{27} sterols	Lanosterol	2,3-22,23-dioxido-squalene	2,3-oxido-squalene	Unidentified metabolite
None	77.8	5.8	–	3.2	13.8
(6E)-azasqualene 17 0.1 μM	58.1	11.5	3	6.2	20.5
(6E)-azasqualene 17 0.5 μM	37.2	7.3	10.5	24.5	20.5
(6Z)-azasqualene 18 2 μM	62.8	17.2	–	3.4	17
19-azasqualene-2,3-oxide 19 0.1 μM	49.7	6	10.3	15.6	18.2
19-azasqualene-2,3-oxide 19 0.5 μM	26	5	25.4	26.5	17
19-azasqualene 21 0.5 μM	35.8	4.5	21	20.7	18.5
19-azasqualene 21 1 μM	22.3	5.5	26.3	24.3	21.6

itor of OSC. Moreover, 17 and 19, but not 21 and 18, were able to inactivate OSC from pig liver in a time-dependent manner. To explain this time-dependent inhibition, we propose two alternative hypotheses:

1. Compounds 17 and 19 act as "slow-binding" inhibitors [41], as suggested by Taton *et al.* [16], explaining the poor activity shown with OSC by some tricyclic or tetracyclic derivatives, such as compounds 15 and 16. For some transition-state analogues, a biphasic slow-binding inhibition has been observed in which rapid initial binding is followed by slow transformation to a stable enzyme-inhibitor complex (which could also be diffusion-limited), from which the inhibitor is released only slowly [42, 43]. Sen and Prestwich [44] suggested a reversible "tight binding" inhibition to explain the time-dependent inhibition of squalene epoxidase by squalene cyclopropylamine. In this case, the inhibition was only apparently irreversible, since the labeled inhibitor could be removed from the enzyme by anion exchange chromatography and enzymatic activity restored.

2. The azasqualene derivatives 17 and 19 could behave as suicide inhibitors that, after binding to OSC, could be cyclized to reactive intermediates able to bind the active site of OSC covalently. The cyclization to a reactive intermediate has been shown for 24-methylidene-2,3-oxidosqualene, a potent mechanism-based irreversible inactivator of OSC [45]. The irreversible inactivation of OSC caused by this inhibitor involves an

initial cyclization to the 21-methylidene protosterol cation, followed by trapping of this allylic cation by a nucleophilic group of the enzyme's active site.

The biphasic inhibition pattern observed for the inactivation of OSC by 17 and 19 could be due to the formation of a cyclized product competing with the parent compound, and resulting in a second slower phase of enzyme inactivation. Such biphasic kinetics have been found for inhibitors of cytochrome P-450 [46–48]. This hypothesis is also in agreement with the apparent noncompetitive inhibition kinetics shown by 17 and 19, and with the competitive kinetics observed with 21. After the binding of 21 to the enzyme in the ground state, both the cyclization and the slow step of conformational change may be prevented by the absence of the epoxide ring.

In conclusion, the preparation of acyclic conformationally flexible "internal" azasqualene derivatives seems very promising in designing "late" HEI analogues (i.e., from pro-C8 to pro-C20) able to act as potent and specific hypocholesterolemic drugs.

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