Biochemical Pharmacology, Vol. 34, No. 15, pp. 2765–2777, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

IN VITRO INHIBITION OF ANIMAL AND HIGHER PLANTS 2,3-OXIDOSQUALENE-STEROL CYCLASES BY 2-AZA-2,3-DIHYDROSQUALENE AND DERIVATIVES, AND BY OTHER AMMONIUM-CONTAINING MOLECULES

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(*Received* 19 *December* 1984; *accepted* 8 *March* 1985)

Abstract—2-Aza-2,3-dihydrosqualene and related molecules, a series of new compounds designed as analogues of the transient carbocationic high energy intermediate, occurring in the oxirane ring opening during the cyclization of 2,3-oxidosqualene, were tested *in vitro* as inhibitors of the microsomal 2,3-oxidosqualene cyclase of animals (rat liver) and of higher plants (maize, pea). These molecules proved to be good and specific inhibitors for the cyclases of both phyla. The inhibition is due to positively charged species and is sensitive to the steric hindrance around the nitrogen-atom. 4,4,10β-Trimethyl-*trans*-decal-3β-ol and 4,10β-dimethyl-*trans*-decal-3β-ol, which have previously been described (J. A. Nelson *et al.*, *J. Am. chem. Soc.* **100**, 4900 (1978)) as inhibitors of the 2,3-oxidosqualene cyclase of chinese hamster ovary cells, were found to be non-competitive inhibitors of the rat liver microsomal enzyme and presented no activity towards the higher plants cyclases. Aza derivatives of these decalines (A. Rahier *et al.*, *Phytochemistry*, in press), which were aimed to mimic the C-8 carbocationic intermediate occurring during later steps of the 2,3-oxidosqualene cyclization did not inhibit the cyclases. This result underlines the theoretical limitations of the high energy analogues concept in designing enzyme inhibitors. Amongst other molecules tested, 2,3-epiminosqualene was found to be a reversible, non-competitive inhibitor of the cyclases; similarly U18666A was a very potent inhibitor of the microsomal cyclases. In contrast AMO 1618, a known anticholesterolemic agent reported previously to act at the level of the 2,3-oxidosqualene cyclization step, was not found *per se* to act on the cyclases.

2,3-Oxidosqualene cyclase (EC 5.4.99.7) is a key enzyme in the biosynthesis of sterols [1]. Due to subtle changes in its catalytic pathway this enzyme catalyses the cyclization of (3*S*)-2,3-oxidosqualene into lanosterol in non-photosynthetic organisms and into cycloartenol and β-amyrin in photosynthetic organisms [2]. Our laboratory is interested in the mechanistic differences underlying the catalytic activity of this unique enzyme. Besides the challenge of its intricate mechanism, the 2,3-oxidosqualene cyclase is also an interesting target for manipulating sterol contents in animal and plant cells, and several of its inhibitors have been described in literature [3, 4].

Powerful enzyme inhibitors can be rationally designed considering that molecules which bear stereoelectronic analogies with transition states or high-energy intermediates occurring along the reaction pathway present high affinities for the active site [5, 6]. Enzymatic cyclization of the all-*trans* squalene 2,3-epoxide is believed to be triggered by a general-acid catalysed epoxide-ring opening assisted by the neighbouring π-bond [7]. The concertedness of the ensuing overall annulation and backbone rearrangement is a matter of debate [8]. However, for entropic reasons and experimental evidence the reaction is more likely to proceed through a series of discrete conformationally rigid carbocationic intermediates [9, 10]. We have previously shown that carbocationic high energy intermediates can be successfully mimicked by compounds bearing a suitable positive charge, e.g. an ammonium group [11].

The oxirane-ring opening of 2,3-oxidosqualene implies the polarization of the C-2-oxygen bond leading to a charge deficiency at C-2 of the molecule (Fig. 1). In an attempt to mimic this first transient carbocationic high energy intermediate (1) we have synthesized 2-aza-2,3-dihydrosqualene (2) and a series of related aza-compounds (3)–(11) (Fig. 2) and tested their effect *in vitro* on 2,3-oxidosqualene-lanosterol cyclase (rat liver microsomes), 2,3-oxidosqualene-β-amyrin cyclase (pea seedlings microsomes) and on 2,3-oxidosqualene-cycloartenol cyclase (maize seedlings microsomes). At physiological pH, the amine group of (2) is protonated and therefore this molecule should present some structural and electronic similarities with the postulated intermediate (1). The results obtained indicate that such compounds are good inhibitors of the cyclases of both phyla. Preliminary results have been presented elsewhere [12, 13].

The cyclization of the chair-chair-boat conformation of 2,3-oxidosqualene involves a transient bicyclic carbocationic intermediate at position C-8

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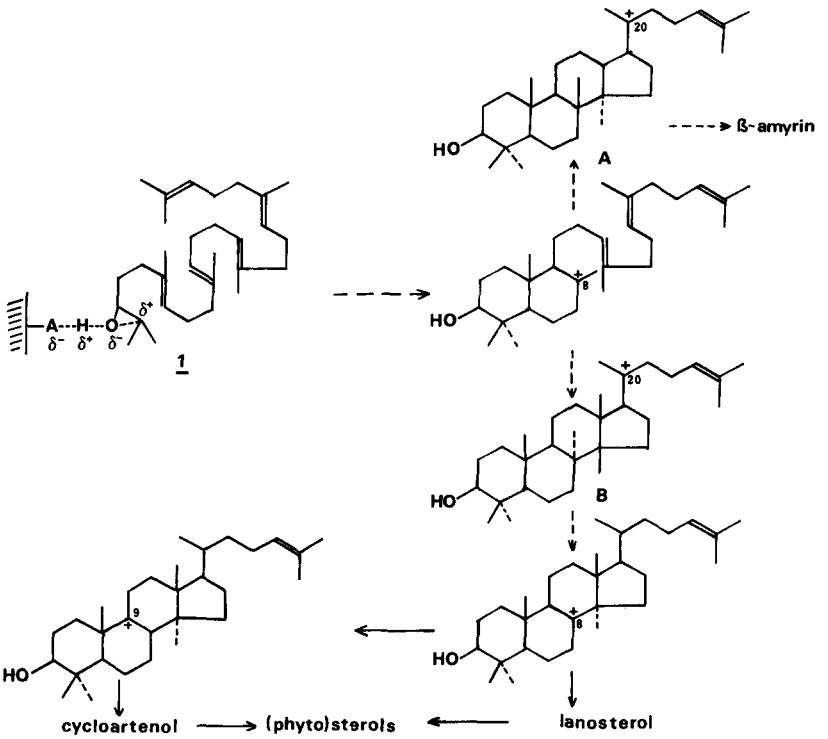


Fig. 1. Cyclization of 2,3-oxidosqualene. Hypothetical mechanism for the general-acid catalyzed oxirane-ring opening (1) and occurrence of carbocationic intermediates at position C-8, C-9 and C-20 (A = dammarenyl carbocation; B = protosteryl carbocation) during the cyclization and sterol synthesis in animals and higher plants.

(Fig. 1), which apparently can be trapped besides the formation of triterpenoids in certain plants [10]. Similarly the last step in the biosynthetic pathway leading to lanosterol or cycloartenol involves a transient carbocationic species respectively at C-8 and C-9 (Fig. 1). Recently it was shown that similar carbocationic species occurring in the reactions catalyzed by $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and by cycloeucalenol-obtusifoliol isomerase could be efficiently mimicked by azadecalines 20–23 [14]. We undertook therefore to mimic these intermediates occurring in the cyclization of 2,3-oxidosqualene by azadecalines, compounds deriving also from the observation that decalines such as (18) were good inhibitors of the animal cyclase [4, 15]. In both animal and plants systems tested, the aza-decalines failed to appreciably inhibit the cyclases.

Besides the molecules described above, which were specifically designed to inhibit the 2,3-oxidosqualene cyclases, we have also tested in our *in vitro* systems, other compounds such as AMO 1618 and U18666A which were known from literature [16–18] to affect, *in vivo* and *in vitro*, the biosynthesis of sterols at the cyclization step. This allowed us to get a better insight on the mode of action of such compounds and we have found, for example, that AMO 1618 *per se* was not an inhibitor of the 2,3-oxidosqualene cyclase.

MATERIALS AND METHODS

Materials

AMO 1618, NDI, U18666A* were kind gifts from respectively BASF, Rhône-Poulenc and Dr. Cenedella. *N,N*-Dimethyldodecylamine *N*-oxide was purchased from Serva and *N,N*-dimethyldodecylamine from Fluka. The decalines (18) and (19) were synthesized following Nelson *et al.* [4] and were provided by Rhône-Poulenc (Agrochimie). The azadecalines (20–23) were prepared by Rhône-Poulenc (Agrochimie) according to a synthesis described by Rahier *et al.* [14]. Lanosterol was separated from dihydrolanosterol by conversion to the acetates followed by thin layer chromatography on 10% silver nitrate plates with cyclohexane/toluene (60/40 v/v) as the developing solvent. Authentic lanosterol was recovered after saponification and recrystallization in methanol. The synthesis of the other compounds used in the study, including [3-^{3}H]*R,S*-2,3-oxidosqualene are given in the Appendix.

Microsomes

(a) *Rat liver.* Male rats (Wistar) weighing about 250 g, having fasted overnight, were anesthetized with ether. After sacrifice the liver was perfused with cold (4°) 0.1 M potassium phosphate buffer (pH 7.5), removed and homogenized with 5 vol. of the same medium in a *loose-fitting* (0.4 mm clearance) Teflon–

* Abbreviations used: AMO 1618, 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine carboxylate methyl chloride; U18666A, 3β-[2-(diethylamino) ethoxy] androst-5-en-17-one hydrochloride; NDI, *N*-dodecylimidazole.

glass Potter. The post-mitochondrial fraction supernatant was centrifuged for 60 min at 105,000 *g*. The supernatant (S_{105}) was saved, the pellet resuspended in 5 vol. of the buffer and recentrifuged under the same conditions. The washed microsomes were then resuspended in the phosphate buffer (10 ml/liver) to yield a suspension containing 5–8 mg protein/ml.

(b) *Pea cotyledons*. The cotyledons (100 g) of 72-hr-old etiolated pea seedlings (*Pisum sativum* var. Douce Provence) were ground in the presence of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.45 M sucrose, 10 mM glutathion, 10 mM $MgCl_2$ and 1 mM EDTA (200 ml). The post-mitochondrial supernatant was centrifuged for 90 min at 105,000 *g*. The microsomal pellets were resuspended in 0.1 M Tris–HCl buffer (pH 7.4) containing 10 mM mercaptoethanol and 1 mM EDTA (50 ml) to yield a concentration of 9–12 mg protein/ml.

(c) *Maize embryos*. The embryos (60 g) of 60-hr-old etiolated corn seedlings (*Zea mays* var. LG-11) were ground in the presence of 0.1 M Tris–HCl buffer (pH 7.5) containing 0.5 M sucrose, 3.0 g/l bovine serum albumin, 10 mM mercaptoethanol and 4 mM $MgCl_2$ (180 ml). The post-mitochondrial supernatant was centrifuged as above. The microsomal pellets were resuspended in 0.12 M sodium phosphate buffer (pH 7.5) containing 2 mM mercaptoethanol and 2 mM $MgCl_2$ (20 ml) (final concentration 7.5 mg protein/ml).

2,3-Oxidosqualene-sterol cyclases assays

(a) *2,3-oxidosqualene-lanosterol cyclase*. The assay procedure was adapted from a protocol previously published by Saat and Bloch [19]. The substrate, insoluble inhibitors and detergent were added to test tubes as organic solutions; after evaporation of the solvent under a stream of nitrogen, the products were emulsified in the presence of a minimum of buffer. The reaction mixtures contained in a volume of 1 ml: [3-^{3}H]*R,S*-2,3-oxidosqualene (50,000 dpm) diluted with *R,S*-2,3-oxidosqualene (final concentration 40 μM), Tween-80 (final concentration 0.15% w/v), 0.1 M potassium phosphate buffer (pH 7.5), 200 μl of rat liver microsomal suspension and 400 μl supernatant fraction S_{105}. The reaction mixture was flushed with nitrogen and incubated anaerobically for 60 min at 37°. A boiled enzyme preparation served as control. The reaction was terminated by addition of 1 ml of 6% potassium hydroxide in methanol (w/v). The mixture was left overnight in the cold and the sterols were then extracted 3 times with an equivalent volume of hexane. The combined extracts were dried over anhydrous sodium sulfate. After evaporation to dryness, authentic lanosterol in methylene chloride was added as a carrier to the extracts which were then applied to silica TLC. After development in methylene chloride, the plates were sprayed with a berberine solution and the compounds visualized under u.v. light. The area corresponding to lanosterol was scraped off and counted for radioactivity. Under these assay conditions, lanosterol was the only radioactive product formed, as judged by autoradiography. Under our work-up conditions extraction of [^{3}H]lanosterol added to control experiments was in the order of 93 ± 6% (N = 4). Calculation of lanosterol formed, assuming that one isomer of *R,S*-2,3-oxidosqualene is used by the enzyme, gave an average conversion, under these experimental conditions, of 4.0 ± 0.5 nmol/hr/mg protein (N = 10).

(b) *2,3-oxidosqualene-β amyrin and 2,3-oxidosqualene-cycloartenol cyclases*. The assays were very similar to the one described above. The final composition of the incubation mixtures (1 ml) was: [3-^{3}H]-2,3-oxidosqualene (2.5×10^5 dpm), 2,3-oxidosqualene (100 μM) and about 10 mg of microsomal proteins (from pea cotyledons and maize coleoptiles respectively) in their resuspension buffers. The incubations were run at 30° for 1 hr. After the work-up, radioactive β-amyrin or cycloartenol were purified by TLC as described above and counted. The specific activities for the 2,3-oxidosqualene β-amyrin and cycloartenol cyclases, assuming that one isomer of 2,3-oxidosqualene is used, were respectively 1.2 ± 0.3 nmoles/hr/mg protein (N = 7) and 1.4 nmol/hr/mg protein.

Inhibition constants determination

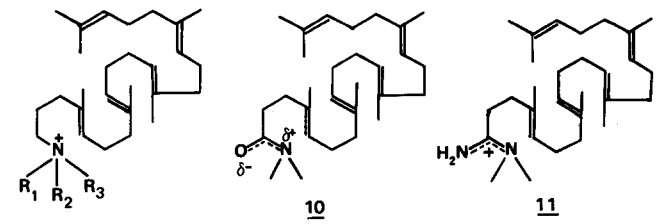
The rates of 2,3-oxidosqualene cyclization were found to be linear with time, at least up to 60 min under the assay conditions described above. Initial velocities were determined for <10% reaction and were repeated twice for each substrate or inhibitor concentration. For most inhibitors, I_{50} values were determined [20] at substrate concentrations of respectively 0.5 K_m and 0.4 K_m for the animal and plant cyclases (I_{50} corresponds to the inhibitor concentration which reduces the observed reaction rate by 50%); the inhibitor concentration range used for such determination was $0.1\,I_{50} \leqslant I \leqslant 10\,I_{50}$. The values given are the means of at least two experiments. For several compounds, the inhibition constant was calculated. The kinetic parameters obtained from experiments using at least two different inhibitor concentrations, were determined from initial rates by the procedure of Wilkinson [21] or Duggleby [22] with the use of a Basic program for a microcomputer. The substrate concentrations used for the calculation of the kinetic parameters were half of that of *R,S*-2,3-oxidosqualene given to the incubation medium. K_i values were then determined from replots of slopes and intercepts of Lineweaver–Burk double-reciprocal plots [23]. When applicable, competitive and non-competitive inhibition types were distinguished using Cornish-Bowden plots [24], or using a statistical method according to Cleland [25].

RESULTS

The inhibitory activities of a series of compounds were determined on the activity of 2,3-oxidosqualene cyclase associated with microsomes of rat liver, germinating pea cotyledons and maize coleoptiles. From the inhibition curves we have calculated I_{50} values (inhibitor concentrations required to reduce the reaction velocity by half). In several cases the kinetics of the inhibition were determined.

Inhibition of the 2,3-oxidosqualene cyclases by 2-aza-2,3-dihydrosqualene (2) *and related compounds* (3)–(11)

In an attempt to mimic the first intermediate (1)



2 $R_1 = R_2 = Me$; $R_3 = H$
3 $R_1 = Me$; $R_2 = R_3 = H$
4 $R_1 = R_2 = Et$; $R_3 = H$
5 $R_1 = Et$; $R_2 = R_3 = H$
6 $R_1 = R_2 = iPr$; $R_3 = H$
7 $R_1 = iPr$; $R_2 = R_3 = H$
8 $R_1 = R_2 = R_3 = H$
9 $R_1 = R_2 = R_3 = Me$

Fig. 2. Structure of 2-aza-2,3-dihydrosqualene and related compounds.

(Fig. 1), of the cyclization of 2,3-oxidosqualene we have synthesized 2-aza-2,3-dihydrosqualene (2) and parent compounds (3)–(11) (Fig. 2) and tested their effect on the cyclases. As shown in Table 1, these compounds were indeed good inhibitors. Among the molecules tested, the *N*-dimethyl (2) and the *N*-diethyl (4) compounds were the most powerful, the inhibition being particularly strong for the pea enzyme. The I_{50} values compared favorably with the apparent K_m determined with the 2,3-oxidosqualene cyclase from rat liver (40 ± 12.5 μM, N = 6) and from pea seedlings (125 ± 25 μM, N = 5). When (2) was tested with the maize enzyme (i.e. formation of cycloartenol) a similar result was observed (I_{50} = 2 μM). In order to check whether the neutral or the protonated form of the amine was the inhibitory species, the derivative (9), containing a quaternary amine, was tested. For both enzymes studied (9) was as strongly inhibitory as the tertiary amine (2). Moreover a compound such as 1,1′,2-trisnorsqualene-3-carboxylic acid was not inhibitory of the cyclase (pea seedlings). When the *N*-substituents were bulkier than ethyl, e.g. *N*-diisopropyl (6), the inhibition sharply decreased (indicating the incidence of steric problems). Similarly when the primary amine (8) was tested, the lack of the methyl substituents resulted also in a decreased affinity for the enzyme. These results show that the inhibition of the 2,3-oxidosqualene cyclase is controlled by steric factors at the N-2 center. The amide derivative (10) because of its electron-delocalization and bond polarization (Fig. 2) was thought to ideally mimic the postulated intermediate (1); it was a poor inhibitor of the cyclase from pea seedlings and a fairly good one for the liver enzyme, although less powerful than (2). In contrast, the squalene dimethylamidine (11), a more basic compound, was an excellent inhibitor of the pea seedlings cyclase. When the kinetics of the inhibition of 2,3-oxidosqualene cyclase by (2) were determined, surprisingly both the rat liver and pea seedlings enzymes, were found to be non-competitively inhibited. This non-competitive behaviour of (2) was assessed by Lineweaver Burk plots (Fig. 3) and by replots of Dixon plots [23, 24] (not shown). Statistical analysis of the data obtained in 4 experiments [25] was consistent with this interpretation. The K_i values obtained were respectively 9.8 μM and 1.0 μM.

Inhibition by phenylpropylamines, phenoxy (-o-)-ethylamines (12)–(15) *and by alkylamines* (16), (17) *series*

In order to determine whether the 1,1′,2-trisnorsqualene chain was important for an inhibition of the 2,3-oxidosqualene cyclase by aza-compounds such as (2) we have tested the activity of molecules which possess a dimethyl- (or diethyl-) amino group linked to substituents fully saturated such as *n*-alkyl chains (16, 17) or bearing an aryl residue which mimics the B-cycle of the cyclization process (12–15) (Fig. 4). The results (Table 2) show that some of these compounds are good inhibitors of the cyclase. Interestingly they all possess an *N*-diethyl substituent, this effect being particularly pronounced in the aryl series. For the *N*-dimethyl series of compounds the inhibition of the cyclases shows a marked selectivity in favor of the 1,1′,2-trisnorsqualene chain.

Inhibition by 2,3-dihydro-2,3-epiminosqualene

This compound bearing an aziridine in place of

Table 1. Inhibition of 2,3-oxidosqualene cyclases by 2-aza-2,3-dihydrosqualene (2) and derivatives (3)–(11)

	I_{50} (μM)									
Compound	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Rat liver	8.8	ND	7.7	ND	100	ND	62	5.1	40	ND
Pea seedlings	1.3	3.0	0.55	3.0	120	48	32	1.1	>100(75)*	1.5

The microsomal cyclases from rat liver and pea seedlings were tested in the presence of varying concentrations of inhibitors and the I_{50} values determined as described under Methods. The values represent the means of at least two independent determinations.

* The number in brackets represents the residual activity (%) at I = 0.1 mM (ND = Not determined).

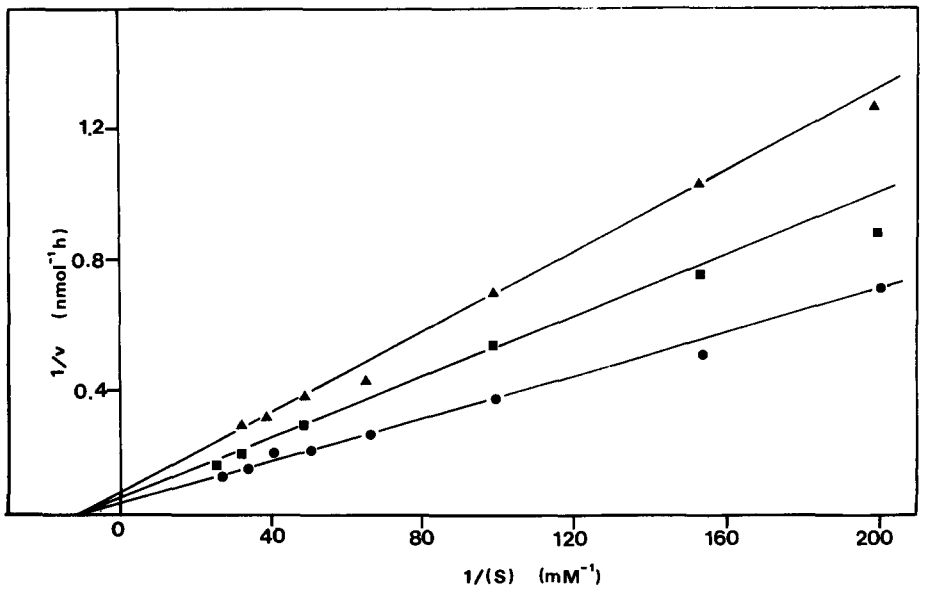
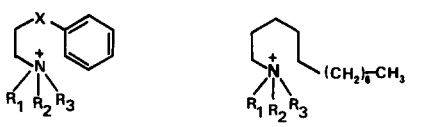


Fig. 3. Inhibition of rat liver 2,3-oxidosqualene-lanosterol cyclase by 2-aza-2,3-dihydrosqualene (2). Conditions: pH 7.4, 37°. The concentrations of (2) are (—●—) none, (—■—) 5 μM and (—▲—) 10 μM. Protein concentration: 1.95 mg microsomal protein/ml.

the oxirane ring has been shown by Corey *et al.* [26] to be a potent inhibitor of liver 2,3-oxidosqualene-lanosterol cyclase. However, to our best knowledge the kinetic parameters of this inhibition were not determined. This molecule has interesting features, i.e. it could either act as a reversible inhibitor or, because of the reactivity of aziridinium derivatives towards nucleophiles, as a potential affinity label of the enzyme. In the latter case the protonated form of the aziridine could react directly with a nucleophilic residue of the active site or the aziridine could first be protonated, in the active site, by the acidic group which catalyzes the oxirane ring opening. Such an active site directed irreversible inhibition, due to protonation, has been documented before [27].



12 X = CH_2 ; R_1 = R_2 = Me ; R_3 = H
13 X = CH_2 ; R_1 = R_2 = Et ; R_3 = H
14 X = O ; R_1 = R_2 = Me ; R_3 = H
15 X = O ; R_1 = R_2 = Et ; R_3 = H

16 R_1 = R_2 = Me ; R_3 = H
17 R_1 = R_2 = Et ; R_3 = H

Fig. 4. Structure of the *N*-dimethyl and *N*-diethyl compounds.

When the 2,3-oxidosqualene cyclases from rat liver, or pea seedlings were preincubated with the 2,3-iminosqualene in a concentration range up to 10 μM, no time dependent loss of activity was measurable over a period up to 120 min, suggesting that this compound did not readily act as an irreversible inhibitor. Under similar experimental conditions, but in the absence of enzyme, there was no noticeable aziridinium ring opening (measured by the appearance of free amino- groups using fluorescamine according to Böhlen *et al.* [28]). Instead, 2,3-iminosqualene behaved as a powerful reversible inhibitor for both enzymes tested (I_{50} = 0.3 μM). The potency of this compound can be explained by two factors: (i) as originally suggested by Corey *et al.* [26], 2,3-iminosqualene mimics the substrate, 2,3-oxidosqualene in the active site of the cyclase and because of its basicity could interact with the acidic subsite responsible for the proton transfer in the oxirane ring opening; (ii) the protonated form of 2,3-iminosqualene could also mimic the postulated high energy intermediate (1), since it is known that the positive charge of ammonium residues is fairly dis-

Table 2. Inhibition of 2,3-oxidosqualene cyclases by the *N*-dimethyl and *N*-diethyl compounds.

	I_{50} (μM)					
Compound	(12)	(13)	(14)	(15)	(16)	(17)
Rat liver	>100(66)*	ND	—†	ND	>100(60)	2.0
Pea seedlings	>100(90)	13	—	40	32	0.8

* The number in brackets represents the residual activity (%) at I = 0.1 mM.
† No inhibition at I = 0.1 mM (ND = not determined).

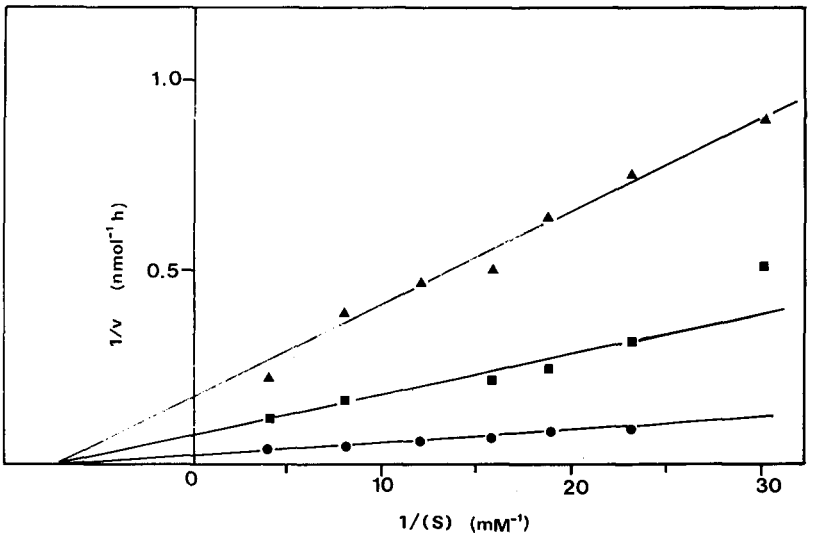


Fig. 5. Inhibition of 2,3-oxidosqualene-β-amyrin cyclase (pea seedlings) by 2,3-dihydro-2,3-epiminosqualene. Conditions: pH 7.4, 30°. The concentrations of the inhibitor are (—●—) none, (—■—) 0.3 μM and (—▲—) 0.9 μM. Protein concentration: 12.2 mg microsomal protein/ml.

tributed over the adjacent carbon-hydrogen bonds [29]. For both cyclases the kinetics were surprisingly compatible with a non-competitive inhibition type (Fig. 5), the K_i being 0.2 μM and 0.4 μM respectively for the pea seedlings and the rat liver enzymes (see Discussion).

Inhibition by decalines (18), (19) *and azadecalines* (20)–(23)

4,4,10 β-Trimethyl-*trans*-decal-3β-ol (18) and 4,10 β-dimethyl-*trans*-decal-3β-ol (19) (Fig. 6) have previously been described by Nelson *et al.* [4] and Chang *et al.* [15] to be specific inhibitors of the 2,3-oxidosqualene-lanosterol cyclase from chinese hamster ovary cells. This finding was very interesting since in principle it would allow the design of inhibitors of the cyclases presenting simplified molecular structures. When tested with the enzyme of rat liver microsomes, the decalines (18) and (19) were found to be good inhibitors and, as observed with the CHO cells [15], 4,4-dimethyl-compound (18) was more effective than the 4α-methyl compound (19) (see Table 3). Since in the original work the inhibition type was not determined, we have studied the kinetics of the inhibition of the 2,3-oxidosqualene-lanosterol cyclase by (18). This compound proved to be a non-competitive inhibitor (K_i = 13 μM) (Fig. 7). Surprisingly when tested with the cyclases from higher plants both decalines (18) and (19) had virtually no effect, indicating a marked difference between the two classes of enzymes with respect to this type of inhibitor.

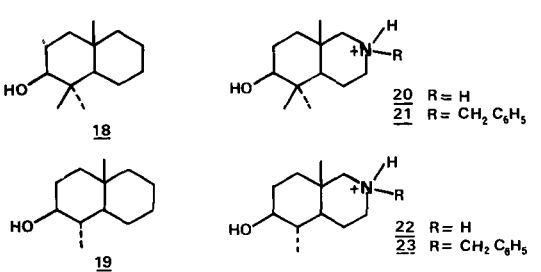


Fig. 6. Structure of the decalines and azadecalines.

We have also tested azadecalines (20)–(23) [14] (Fig. 6) whose structure is based on the known inhibitory activity of the decalines (18, 19) and which bear a stereoelectronic resemblance with the postulated C-8 carbocationic intermediates (see the Introduction and Fig. 1). These compounds did not inhibit the cyclases of higher plants and, to our surprise, neither did they inhibit the rat liver cyclase indicating that the presence of an amino-group in the decaline structure abolished their inhibitory properties for the mammalian 2,3-epoxysqualene cyclase.

Inhibition by imidazole and imidazoline derivatives (24)–(27), *U18666A* (28) *and by AMO 1618* (29)

Evidence exists in literature that the biosynthesis of sterols can be inhibited *in vivo* and *in vitro* at the 2,3-oxidosqualene cyclization step by a variety of hypocholesteremic compounds. However, few examples are known where kinetic measurements were made with the enzyme. Since such molecules might prove valuable tools in manipulating intracellular cholesterol contents and bring some insights into the mechanisms controlling the cyclase activity we have studied some representative molecules.

Imidazole derivatives, such as *N*-dodecylimidazole (NDI) (24) (Fig. 8) are well known for their potency in inhibiting cholesterol biosynthesis, leading e.g. to an accumulation of 2,3-oxidosqualene in rat livers [30]. In rat brain extracts Dennick and Dean [31] have shown that the cyclase is a target of (24). When tested in our *in vitro* assay systems, *N*-dodecyl imidazole proved to be an excellent inhibitor of both the rat liver and pea seedlings cyclases (Table 4). Kinetic determinations showed that (24) is a non-

Table 3. Inhibition of 2,3-oxidosqualene cyclases by the decalines (18), (19) and by the aza decalines (20)–(23)

Compound	I_{50} (μM)					
	(18)	(19)	(20)	(21)	(22)	(23)
Rat liver	9	20	>100(95)*	—†	ND	—
Pea seedlings	>100(90)	ND	ND	—	ND	ND
Maize coleoptiles‡	>100(80)	>100(75)	—	ND	—	—

* The number in brackets represents the residual activity (%) at I = 0.1 mM.
† No inhibition at I = 0.1 mM.
‡ Data taken from [74].
(ND = not determined).

competitive inhibitor of the pea seedlings enzyme, with a K_i = 0.2 μM (not shown). In contrast, when the alkyl chain is shifted at position 2 of the imidazole ring the compound (25) lacks most of the properties of (24) in the case of the plant enzyme and is not inhibitory for the mammalian enzyme. Similarly imidazoline derivatives possessing an alkyl chain or a "squalenoid" substituent at position 2 (respectively 26 and 27) were not powerful inhibitors.

Compounds such as 3β-(β-dimethylaminoethoxy)-androst-5-en-17-one and its diethylamino analog U18666A (28) are known hypocholesteremics in mammalian and yeast cells [32, 18, 33]. Their main target was thought to be the squalene epoxide cyclization step [34–36, 18]. Recently U18666A was unambiguously shown to block the conversion of 2,3-oxidosqualene into lanosterol in rat intestinal epithelial cell cultures [37, 38], leading to intracellular accumulation of 2,3: 22,23-dioxidosqualene. However to our best knowledge, no studies have been performed *in vitro* on the inhibition of the 2,3-oxidosqualene cyclase by U18666A. The compound (28) was indeed an excellent inhibitor of both the mammalian and plant cyclases (Table 4).

AMO 1618 (29) a plant growth retardant, besides its known effect on the biosynthesis of gibberelins has been reported to affect the biosynthesis of sterols by interfering with the cyclization of 2,3-oxidosqualene in plant systems and in animals [16, 39, 17 and references therein]. However, when tested in our *in vitro* systems AMO 1618 failed to inhibit the cyclization of 2,3-oxidosqualene both in animal and plants.

DISCUSSION

The aims of the present study on the inhibition of microsomal 2,3-oxidosqualene cyclase in animals and higher plants were twofold: (i) design rationally new inhibitors for this enzyme, based on the concept of high energy intermediates analogues; (ii) assess with a specific *in vitro* assay the inhibitory properties of compounds known to interfere with the cyclization of 2,3-oxidosqualene. Such a study was an obligatory step in our program because of the limited kinetic data available for this enzyme.

New compounds, i.e. the azasqualene series, were developed in order to mimic the carbocationic high

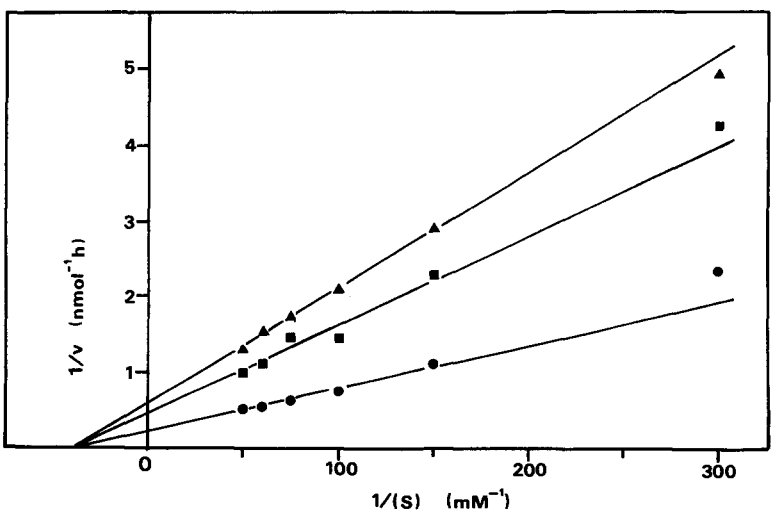


Fig. 7. Inhibition of rat liver 2,3-oxidosqualene-lanosterol cyclase by 4,4,10 β-trimethyl-*trans*-decal-3β-ol (18). Conditions: pH 7.4, 37°. The concentrations of (18) are (—●—) none, (—■—) 10 μM and (—▲—) 25 μM. Protein concentration: 1.065 mg microsomal protein/ml.

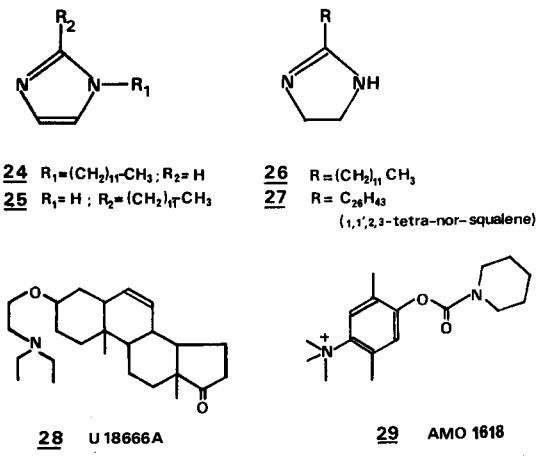


Fig. 8. Structure of the imidazole derivatives, of U18666A and of AMO 1618.

energy intermediates occurring during the cyclization of 2,3-oxidosqualene (Fig. 1). We were previously very successful in mimicking such intermediates involved in the biosynthesis of sterols [11, 13, 14] by the use of molecules bearing positively charged atoms (e.g. nitrogen) at appropriate positions. In this particular work we were aware of stretching the concept of transition-states analogues to its very limits. Even if the cyclization process is not fully concerted [8–10], it is difficult to predict the depth of the potential wells in the energy curve corresponding to the carbocationic intermediates occurring along the reaction pathway and hence to predict the need and extent of stabilization of these transient intermediates by the active site of the enzyme.

The azasqualene series (2)–(11) which were designed to mimic the high energy intermediate (1) occurring during the oxirane ring opening, were found to be good inhibitors of the cyclases of both animals and higher plants.* When comparing the K_i/K_m ratio of (2) for the liver and pea seedlings enzymes, respectively 0.24 and 0.008, it appears that this compound is particularly powerful in inhibiting the plant enzyme. This seems to be a general trend in this series, except for (10). This latter molecule, because of the polarization of its amide group, was thought to be close to the postulated high energy intermediate (1); however, it did not prove to be the most powerful inhibitor. This could be due in part to the fact that the electron delocalization of the amide linkage and therefore its electronic resemblance to (1), is very dependent on the polarity of its microenvironment [40], which in this particular case might not be favorable.

A point we already alluded to is the transient existence of the high energy intermediate (1). According to van Tamelen [7] the oxirane ring opening of 2,3-oxidosqualene can be viewed as a SN 2-like reaction with a high degree of the neighbouring π-bond participation. However, the paramount importance of a trisubstituted center at C-2 of the molecule for its enzymic transformation suggests also an appreciable degree of SN 1-character in the A-ring closure (see [41]). The fact that a stabilization of the high energy intermediate (1) would not seem an absolute requirement in this multistep (multicentre) cyclization mechanism might explain why, in comparison to other examples where an enzyme-stabilized carbocationic intermediate is part of the reaction process [11, 13, 14], the inhibitory power of compounds such as (2) is relatively modest at least in the liver system. Nevertheless, as expected from our hypothesis, the inhibition of the cyclases tested in this study is due to positively charged species (compare (2) and (9)) and is sensitive to the nature of the substituents at the nitrogen atom in analogy to the substrate specificity of the mammalian cyclase [26, 41]. More work is clearly needed to answer the question whether molecules such as (2) take advantage of the favourable binding interaction occurring in a transition-state complex, or whether their inhibitory properties are due to opportunistic binding to the active site of the squalene cyclases. A perturbation of the microenvironment of the membrane-associated cyclases by molecules such as (2) cannot be totally excluded; such an effect has indeed been suggested to occur with polyisoprenoid amphiphilic compounds, albeit at much higher concentrations, in the case of the squalene synthase [42]. Nevertheless, it is known that the activity of 2,3-oxidosqualene cyclase is unaffected by detergents [43]. Related to that point is the perplexing finding that (2) appears to be a non-competitive inhibitor; however, a similar result was found for all the molecules for which we have tested the inhibition type.

Besides the azasqualene series, we have tested for their inhibitory properties several tertiary amines whose structures were unrelated to squalene (Table 2); compounds of this type have been known to affect the biosynthesis of sterols [32, 44]. It resulted from our study that only the molecules possessing an *N*-

* Similarly in *Saccharomyces cerevisiae* (2) was found to be a good inhibitor of the 2,3-oxidosqualene-lanosterol cyclase (G. Balliano, to be published).

Table 4. Inhibition of 2,3-oxidosqualene cyclases by imidazole derivatives (24)–(27), U18666A (28) and by AMO 1618 (29)

	I_{50} (μM)					
Compound	(24)	(25)	(26)	(27)	(28)	(29)
Rat liver	3.9	—*	ND	ND	0.8	—
Pea seedlings	0.4	>100(60)†	—	100	0.25	>100(95)

* No inhibition at I = 0.1 mM.
† The number in brackets represents the residual activity (%) at I = 0.1 mM.
(ND = not determined).

diethyl substituent proved to be effective inhibitors of the 2,3-oxidosqualene cyclases tested, their corresponding *N*-dimethyl analogues being much less potent. This is in strong contrast with the specificity of the azasqualenes where such a difference between (2) and (4) was inapparent with the rat liver cyclase and little marked for the pea seedlings enzyme (Table 1). For the moment such observations are only phenomenological, and no rational explanation can be provided to explain why such molecules are inhibitors and why a change from a methyl to an ethyl substituent on their amino-centre results in such a dramatic effect on the inhibition of the oxidosqualene cyclases. This observation seems nevertheless quite general, for example Sipe and Holmlund [32] already noted the importance of the diethyl-amino substituent and e.g. U18666A (28) is a much more potent inhibitor than its *N*-dimethyl analogue. Interestingly, most recently chloroquine, an *N*-diethyl tertiary amino-containing molecule, was also found to inhibit the 2,3-oxidosqualene-lanosterol cyclase in mouse L-cells [45].

The results obtained with the decalines and azadecalines (18)–(23) are of interest. The decalines (18, 19) which were described by Nelson *et al.* [4] and by Chang *et al.* [15] as inhibitors of the 2,3-oxidosqualene-lanosterol cyclase in CHO cells (intact cells or cell free extracts) were also found to be good inhibitors of the rat liver enzyme. In marked contrast these molecules were ineffective to block the higher plants (pea seedlings, maize) cyclases. These are the only examples we have found among the molecules tested in this study which gave such a clear-cut difference between the 2,3-oxidosqualene cyclases of both phyla. This result underlines subtle differences between the cyclases, but because the decaline (18) appears to be a non-competitive inhibitor, we do not yet know if this reflects differences in the active site binding properties. When in the decalines the C-8 was replaced by an amino-group in order to mimic the transient carbocationic high energy intermediates occurring after the B-ring formation and the C-8 and C-9 carbocations leading respectively to lanosterol and cycloartenol (Fig. 1), all inhibitory activity was abolished in the case of the rat liver cyclase and none appeared in the case of the plant cyclase. Such a result is in sharp contrast with the potent inhibition ($K_i/K_m < 10^{-3}$) of the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and of cycloeucalenol-obtusifoliol isomerase in maize-seedlings by *N*-benzyl-8-aza-4,10-dimethyl-*trans*-decal-3β-ol (23) [14]. The reactions catalyzed by these latter enzymes imply the transient formation of carbocations respectively at C-8 and C-9 of the intermediates involved in the reaction mechanism. The different activity shown by the azadecalines towards the squalene epoxide cyclases as compared with the isomerases raises interesting questions on the application of the high energy intermediate analogue concept. Along these lines it is important to mention that 17(βH)- and 17(αH)-azadammaran-3β-ol, compounds designed to mimic the transient C-20 dammarenyl carbocation occurring during the cyclization of 2,3-oxidosqualene into β-amyrin (Fig. 1), also failed to inhibit the cyclase in pea seedlings [46]. A fundamental difference resides in the nature of the reaction catalyzed by these enzymes. In the case of the isomerases, the carbocationic intermediates are fairly localized and most probably, because of stereochemical constraints, transiently stabilized by an appropriate subsite of the active site. Moreover in these particular reactions the geometries of the substrate in its ground-state and the carbocationic intermediates are not fundamentally different, therefore one would not expect a large conformational change of the enzyme in order to stabilize the reaction intermediates. The situation is fairly different and more complex in the case of the 2,3-oxidosqualene cyclization. Although the cyclization is not completely concerted and most probably involves a series of discrete conformationally frozen carbocationic intermediates including at C-8 and C-20 (see the Introduction), we are faced with a multistep and multicentre reaction which implies for the enzyme a large conformational flexibility. One can predict that the form of the cyclase which binds squalene epoxide in its ground state must be fairly different from the conformational state of the enzyme which stabilizes, e.g. conformationally rigid polycyclic intermediates. This takes into account the fact that the molecular features which determine the affinity of an enzyme for an intermediate are different from those affecting the recognition of the substrate in its ground-state [5]. Therefore, besides questioning the occurrence of localized carbocationic intermediates at C-8 (or C-9 and C-20), the negative results obtained with the aza-decalines and the aza-dammaranols in the case of the squalene epoxide cyclases, might also indicate that the active site of this enzyme reaches certain conformations, complementary to particular intermediates, e.g. carbocationic C-8 or C-20, only after undergoing a specific sequence of catalytic events and hence conformational changes, which in the absence of catalysis cannot be reached spontaneously or only slowly [47]. Such factors have to be taken into account in the present study in explaining the lack of inhibition of the cyclases by the azadecalines.

Among the compounds known in literature to inhibit the 2,3-oxidosqualene cyclase, we have demonstrated that 2,3-dihydro-2,3-epimino squalene [26] is a potent reversible inhibitor of animal and higher plant cyclases. Similarly *N*-dodecylimidazole and U18666A were excellent inhibitors when assayed in our *in vitro* assay systems of the cyclases. This contrasts with the lack of activity of AMO 1618, when tested *in vitro*, on the conversion of 2,3-oxidosqualene into lanosterol or β-amyrin, although this compound was known for its inhibitory activity when tested in several systems [16, 17]. In fact, the inhibitory activity of AMO 1618 was only documented under experimental conditions which favored oxidative processes, i.e. whole cell systems or cell free extracts converting precursors, e.g. mevalonate, squalene, into 2,3-oxidosqualene whereas in our experimental systems, the *in vitro* conversion of 2,3-oxidosqualene into sterols was performed under conditions avoiding oxidative processes. Therefore one cannot exclude that AMO 1618 is an active precursor (prodrug) of a metabolite which inhibits the cyclases. Ultimately it is possible that AMO 1618 cannot block the activity of a cyclase population which is active on a substrate given exogenously as opposed to cyclases

active on the 2,3-oxidosqualene diffusing in the membrane phase where it has been formed.

The last point we wish to comment on is the limitations of the kinetics observed in such a study. We are dealing with complex anisotropic systems and with substrates, and inhibitors, which are hydrophobic. Some problems encountered with such systems have been examined in literature [48–51]. In microsomal preparations containing the cyclase the pool of kinetically active substrate, or inhibitor, which distribute randomly, and according to their partition coefficient, might represent only a variable fraction of the total number of molecules. It is therefore difficult to compare, e.g. the values of I_{50} obtained for the different families of compounds. A similar reasoning might apply to explain why molecules such as 2-aza-2,3-dihydrosqualene (2), 2,3-dihydro-2,3-epiminosqualene, N-dodecyl imidazole (24) and 4,4,10α-trimethyl-trans-decal-3β-ol (18) were consistently found to be non-competitive inhibitors.

CONCLUSION

This work represents the most comprehensive study to date on inhibitors of the 2,3-oxidosqualene cyclases from animal and higher plants. About 29 compounds have been tested for their inhibitory properties, under conditions which *in vitro* assayed the cyclization of 2,3-oxidosqualene into lanosterol, β-amyrin and cycloartenol. Among these compounds 12 were new and we have developed potent new inhibitors of structural types different from the known inhibitors [52]. Work is in progress on the application of such molecules in animal and plant cultured cells.

Acknowledgements—We thank S. Schmitt for expert technical assistance, N. Angele for her participation in the early stage of this work and B. Bastian for secretarial assistance. The gift of the decalines and azadecalines by Drs C. Anding and P. Place (Rhône-Poulenc, Agrochimie) is gratefully acknowledged. We thank Dr. Cenedella for his generous gift of U16888A and Dr. F. Durst for the use of the programme of statistical analysis of inhibitors. We are also indebted to the researchers of the "Laboratorio di Gascromatografia-Spettrometria di Massa della Provincia-Universita di Torino" for the Mass Spectra and to the researchers of the NMR laboratory of the "Istituto di Chimica Generale ed Inorganica della Facolta di Scienze dell Universita di Torino", for the 270 MHz spectra.

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APPENDIX: SYNTHESIS OF THE INHIBITORS (by L. Delprino, G. Balliano and L. Cattel)

General procedures

Preparative TLC were performed on Merck F 254 plates, coated with silica gel; after development the plates were sprayed with a 0.1% solution of berberine hydrochloride in ethanol and the separated compounds were observed under u.v. light (340 nm). ^{1}H-NMR spectra were recorded on a Jeol GX 270 or on a Varian T-60 spectrometer, in $CDCl_3$ solution; the chemical shifts are given in δ units, with TMS as internal standard. Mass spectra were done on a Kratos MS 80 spectrometer by electron impact, in high resolution (7500), electron energy 70 eV, trap current 100 μA. I.r. spectra were recorded on a Perkin Elmer 267.

The phrase "the product was worked up in the usual way" summarizes the following procedure: the reaction mixture was diluted with water and extracted with ether, the organic layer was washed with saturated brine, dried over anhydrous sodium sulphate and evaporated to dryness *in vacuo*.

Synthesis

The synthesis of *N,N*-diethyl-3-phenylpropylamine (13) or *N,N*-diethyldodecylamine (17) was realized treating the parent primary amine with an excess of diethyl sulphate, under reflux. *N,N*-Dimethyl-3-phenylpropylamine (12) was prepared by reductive alkylation of the corresponding primary amine with CH_2O–$NaBH_3CN$ [53–55]. 2,3-Epiminosqualene was synthesized directly by addition of iodine azide to a saturated solution of squalene in THF/CH_3CN followed by $LiAlH_4$ reduction of the squalene-iodine azide adducts [56].

The synthesis of 1,1',2-*tris-nor*-squalene aldehyde was realized from squalene, following a described method [57].

[3^3-H]-*R,S*-2,3-oxidosqualene was prepared starting from 1-[^{3}H] labelled 1,1',2-*tris-nor*-squalene aldehyde, which was made by reduction of the aldehyde with sodium borotritide (22 Ci/mmole) in methanol, followed by oxidation with CrO_3–pyridine complex [58]. Reaction of the labelled aldehyde with diphenylsulfonium isopropylide led to the labelled [^{3}H]-oxidosqualene. Its specific activity was 44×10^6 dpm/μmole.

*Reductive amination of 1,1',2-*tris-nor-*squalene aldehyde*

The general procedure for synthesizing the azasqualene derivatives consisted in the reductive amination of 1,1',2-*tris-nor*-squalene aldehyde [57], using $NaBH_3CN$ at pH 6 [53, 54, 59]. The alternative method using an alkaline solution of $Fe(CO)_5$, which was reported to give better results under mild conditions [60, 61], was not suitable for the synthesis of azasqualene derivatives, giving very poor yields.

The following procedures were developed.

Squalene dimethylamine: (4*E*,8*E*,12*E*,16*E*)-*N,N*-dimethyl - 4,8,13,17,21 - pentamethyl - 4,8,12,16,20 - docosapentaenylamine (2). To 1,1',2-*tris-nor*-squalene aldehyde [57] (1 g, 2.6 mmoles), dissolved in ethanol, we added a solution of dimethylamine (715 mg, 15.9 moles) in absolute ethanol (3 ml). A 5 N HCl–methanol solution was added until pH 6, followed by $NaBH_3CN$ (100 mg, 1.59 mmoles). The reaction mixture was stirred at room temperature, under argon, for 16 hr. After the usual work up, the crude product was purified by preparative TLC (0.5 mm, methanol), to give (2) (215 mg, 20% yield), as a colourless oil. (*Found*: M^+, 413.4039. $C_{29}H_{51}N$ requires M, 413.4021). *m/z*: 413 (M^+, 25), 344 (15), 276 (15), 208 (15), 140 (15), 113 (7), 95 (12), 71 (28), 58 (100). δ($CDCl_3$, 270 MHz): 1.55 (15 H,s) and 1.63 (3 H, s) (allyl methyls), 1.96 (20 H, broad peak, envelope methylenes); 2.26 (8 H, broad s, —CH_2—N(CH_3)$_2$), 5.08 (5 H, broad peak, vinylic hydrogens).

Squalene methylamine: (4*E*,8*E*,12*E*,16*E*)-*N*-methyl-4,8,13,17,21 - pentamethyl - 4,8,12,16,20 - docosapentaenylamine (3). The aldehyde (500 mg), methylamine (320 mg) and $NaBH_3CN$ (100 mg) gave in the same way (3) (104 mg, 20% yield). (*Found*: M^+, 399.3869. $C_{28}H_{49}N$ requires M, 399.3865). δ($CDCl_3$, 60 MHz): 1.58 (18 H, s, allyl methyls), 1.98 (20 H, broad peak, envelope methylenes), 2.29 (5 H, broad peak, —CH_2—NH—CH_3), 5.10 (5 H, broad peak, vinylic hydrogens).

Squalene diethylamine: (4*E*,8*E*,12*E*,16*E*)-*N,N*-diethyl-4,8,13,17,21 - pentamethyl - 4,8,12,16,20 - docosapentaenylamine (4). The aldehyde (150 mg), diethylamine (100 mg) and $NaBH_3CN$ (15 mg) afforded in the same way (4) (8 mg, 5% yield). (*Found*: M^+, 441.4356. $C_{31}H_{55}N$ requires M, 441.4334). *m/z*: 441 (M^+, 12), 372 (8), 304 (10), 236 (12), 168 (40), 99 (35), 86 (100). δ($CDCl_3$,

270 MHz): 0.96 (6 H, t, J = 7.2 Hz, —$N(CH_2CH_3)_2$), 1.52 (15 H,s), and 1.59 (3 H, s) (allyl methyls), 1.93 (20 H, broad peak, envelope methylenes), 2.33 (2 H, t, J = 8 Hz, —CH_2—CH_2—N), 2.47 (4 H, q, J = 7 Hz, —$N(CH_2CH_3)_2$), 5.04 (5 H, broad peak, vinylic hydrogens).

Squalene ethylamine: (4*E*,8*E*,12*E*,16*E*)-*N*-ethyl-4,8,12,17,21 - pentamethyl - 4,8,12,16,20 - docosapentaenylamine (5). The aldehyde (250 mg), ethylamine (160 mg) and $NaBH_3CN$ (30 mg) afforded (5) (52 mg, 20% yield). (*Found*: M^+, 413.4030. $C_{29}H_{51}N$ requires M, 413.4021). $\delta(CDCl_3$, 60 MHz): 0.96 (3 H, t, J = 7.1 Hz, —NH—CH_2—CH_3), 1.58 (18 H, s, allyl methyls), 1.98 (20 H, broad peak, envelope methylenes), 2.40 (4 H, broad peak —CH_2—NH—CH_2CH_3), 5.10 (5 H, broad peak, vinylic hydrogens).

Squalene diisopropylamine: (4*E*,8*E*,12*E*,16*E*)-*N*,*N*-diisopropyl - 4,8,13,17,21 - pentamethyl - 4,8,12,16,20-docosapentaenylamine (6). The aldehyde (200 mg), diisopropylamine (420 mg) and $NaBH_3CN$ (30 mg) gave in the same way (6) (10 mg, 4% yield). (*Found*: M^+, 469.4652. $C_{33}H_{59}N$ requires N, 469.4647). $\delta(CDCl_3$, 60 MHz): 1.10 (12 H, d, J = 7.5 Hz, —$N(CH(CH_3)_2)_2$,), 1.58 (18 H, s, allyl methyls), 1.98 (20 H, broad peak, envelope methylenes), 2.40 (2 H, m, —CH_2—N), 2.98 (2 H, m, —$N(CH(CH_3)_2)_2$), 5.10 (5 H, m, vinylic hydrogens).

Squalene isopropylamine: (4*E*,8*E*,12*E*,16*E*)-*N*-isopropyl - 4,8,13,17,21 - pentamethyl - 4,8,12,16,20 - docosapentaenylamine (7).

The aldehyde (60 mg), isopropylamine (50 mg) and $NaBH_3CN$ (10 mg) yielded (7) (13.5 mg, 20% yield). (*Found*: M^+, 427.4170 $C_{30}H_{53}N$ requires M, 427.4178). $\delta(CDCl_3$, 60 MHz): 1.06 (6 H, d, J = 7.5 Hz, NH—$CH(CH_3)_2$), 1.58 (18 H, s, allyl methyls), 1.98 (20 H, broad peak, envelope methylenes), 2.38 (2 H, m, —CH_2—N—), 2.62 (1 H, m, —NH—$CH(CH_3)_2$), 5.10 (5 H, m, vinylic hydrogens).

Squalene amine: (4*E*,8*E*,12*E*,16*E*)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenylamine (8). The aldehyde (100 mg), CH_3COONH_4 (120 mg) and $NaBH_3CN$ (30 mg) afforded with the same method the primary amine (8) (15 mg, 15% yield). (*Found*: M^+, 385.3695, $C_{27}H_{47}N$ requires M, 385.3708). $\delta(CDCl_3$, 60 MHz): 1.58 (18 H, s, allyl methyls), 1.98 (20 H, broad peak, envelope metilenes), 2.93 (2 H, m, —CH_2—N—), 5.10 (5 H, m, vinylic hydrogens).

Squalene trimethylammonium iodide: (4*E*,8*E*,12*E*,16*E*)-trimethyl - 4,8,13,17,21 - pentamethyl - 4,8,12,16,20-docosapentaenylammonium iodide (9). This compound was prepared by a conventional method [62] from (2) (50 mg), K_2CO_3 (830 mg) and CH_3I (150 mg) in ethanol, heating under reflux for 15 hr (67 mg, quantitative yield). $\delta(CDCl_3$, 60 MHz): 1.60 (18 H, s, allyl methyls) 2.0 (20 H, broad peak, envelope methylenes), 3.50 (11 H, broad peak, —$N(CH_3)_3$ and —CH_2—N), 5.10 (5 H, broad peak, vinylic hydrogens).

Squalene dimethylamide: (4*E*,8*E*,12*E*,16*E*)-*N*,*N*-dimethyl - 4,8,13,17,21 - pentamethyl - 4,8,12,16,20 - docosapentaenamide (10). The synthesis of this compound was accomplished in two steps: (i) the oxidation of 1,1',2-*tris*-*nor*-squalene aldehyde, in ether with the Brown reagent [63] at room temperature, yielded the corresponding, 1,1',2-*tris*-*nor*-squalene-carboxylic acid. This two-phase system allows the slow oxidation of the aldehyde under mild conditions, since the organic phase extracts the acid, protecting it from further degradation; (ii) the synthesis of the squalene dimethylamide (10) was carried out coupling directly the acid and dimethylamine, in the presence of dicyclohexylcarbodiimide (DDC) and $ZnCl_2$ [64, 65]. The following procedure was developed: to a stirred and cooled (0°) solution of 1,1',2-*tris*-*nor*-squalene aldehyde (500 mg) in diethyl ether (10 ml), a solution of chromic acid (5 g $Na_2Cr_2O_7$ and 3.75 ml H_2SO_4 (0.6% v/v, in 25 ml of water) was added dropwise, under N_2, till a brown color persisted: 2.5 ml). After stirring further the solution at room temperature for 14 hr, the reaction mixture was worked up in the usual way and the crude product was characterized.

λ_{max} cm^{-1} ($CHCl_3$): 3500 (—OH), 1720 (—CO). ($CDCl_3$, 60 MHz): 9.2 (1 H, s, —COO*H*). The crude acid was treated with dimethylamine (210 mg), DCC (290 mg), $ZnCl_2$ (150 mg) in THF (3 ml). After this mixture was stirred at room temperature for 14 hr, acetic acid was added in order to decompose the reagent in excess. The insoluble urea was removed, the solvent was replaced by ethyl acetate and the organic layer was washed with diluted HCl (0.5 N), aqueous bicarbonate (10% solution), treated with anhydrous sodium sulphate and evaporated under vacuum. The residue was purified by TLC (0.5 mm, cyclohexane/ethyl acetate 70:30), to give the amide (10) (68 mg, 12% yield from the aldehyde). (*Found*: M^+, 427.3830, $C_{29}H_{49}NO$ requires M, 427.3814). $\delta(CDCl_3$, 60 MHz): 1.58 (18 H, s, allyl methyls), 1.98 (20 H, broad peak, envelope methylenes), 3.00 (6 H, s, —$CON(CH_3)_2$), 5.1 (5 H, m, vinylic hydrogens).

Squalene dimethylamidine: (4*E*,8*E*,12*E*,16*E*)-N^1,N^1-dimethyl - 4,8,13,17,21 - pentamethyl - 4,8,12,16,20-docosapentaenamidine (11). The squalene dimethylamidine was conveniently prepared from the parent amide using triethyloxonium fluoborate [67], followed by the conversion of the amidine salt by treatment with dimethylamine [68]. The following procedure was used: to a solution of triethyloxonium fluoborate (13.3 mg, 0.07 mmoles) in 1 ml of dry CH_2Cl_2, the squalene dimethylamide (10) (30 mg), dissolved in dry CH_2Cl_2 (3 ml), was added under dry argon. The solution was stirred for 14 hr, until the squalene imidate fluoborate was completely formed. IR: λ_{max} cm^{-1}: 1650, 1100. The crude imidate fluoborate was then stirred at room temperature with a 4% solution of dimethylamine in absolute ethanol for 12 hr. After removal of the solvents, the residue was treated with water, basified with 5 N aqueous NaOH and extracted with ether. After the usual work up, the TLC purification (0.25 mm, CH_2Cl_2/CH_3OH/NH_4OH 25% 94:4:2), yielded the squalene dimethylamidine.

(*Found*: M^+, 426.3966. $C_{29}H_{50}N_2$ requires M, 426.3974). λ_{max} cm^{-1} ($CHCl_3$): 3350 (—C=N—H), 1660 (—C=N—). $\delta(CDCl_3$, 60 MHz): 1.58 (18 H, s, allyl methyls, 1.98 (20 H, broad peak, envelope methylenes), 2.90 and 2.93 (6 H, s, —$N(CH_3)_2$), 5.11 (5 H, m, vinylic hydrogens).

The synthesis of 2-substituted imidazoline (26) *and* (27)

The 2-dodecyl-2-imidazoline (26) or the squalene 2-imidazoline (27) were prepared by reaction of the corresponding ester with an excess of ethylenediamine to produce the *N*-acyl derivative, which was not isolated, but directly cyclized by heating under reflux for several hours [66].

2-Dodecyl-2-imidazoline (26). Ethyl tridecanoate (6.3 g) was heated under reflux with ethylenediamine (2.34 g), for 14 hr. After cooling, the precipitated 2-dodecyl-2-imidazoline (26) was filtered and crystallized from methanol, m.p. 156° (2.17 g, 35% yield, based on the ester). λ_{max} cm^{-1} (KBr): 3340 (—NH), 1650 (—C=N), 1570 (—NH). $\delta(CDCl_3$, 60 MHz): 0.95 (3 H, t, J = 7 Hz, —CH_3), 1.30 (20 H, broad peak —CH_2—$(CH_2)_{10}$—), 2.15 (2 H, t, —CH_2—C=N—), 3.30 (2 H, t, J = 6 Hz, —CH_2—NH—), 3.95 (2 H, t, J = 6 Hz, —CH_2—N=C). *m/z*: 238 (M^+, 22), 140 (100).

Squalene-2-imidazoline: (3'*E*,7'*E*,11'*E*,15'*E*)-2- (3',7',12',16',20' - pentamethyl - 3',7',11',15',19' - henicosapentaenyl)-2-imidazoline (27). The 1,1',2-*tris*-*nor*-squalene carboxylic acid was treated with diazomethane, giving the corresponding methyl ester. λ_{max} cm^{-1} (liquid film): 1730 (—C=O). $\delta(CDCl_3$, 60 MHz): 1.58 (18 H, s, allyl

methyls), 1.98 (18 H, broad peak, envelope methylenes), 2.35 (2 H, s, —CH_2—COO), 3.65 (3 H, s, —O—CH_3) 5.10 (5 H, m, vinylic hydrogens). To the methyl ester (200 mg) were added 1.35 g of ethylenediamine and the mixture was heated under reflux for 7 hr. After the excess of reagent was removed *in vacuo*, the residue was purified by TLC (0.5 mm, $CHCl_3$/CH_3OH/NH_4OH 25% 91:6:3), to give (27) (115 mg, 56% yield). (*Found*: M^+, 424.3820. $C_{29}H_{48}N_2$ requires M, 424.3817) λ_{max} cm^{-1} (liquid film): 3207 (—NH—), 1636 (—C=N—), 1562 (—NH—). δ($CDCl_3$, 60 MHz): 1.58 (18 H, s, allyl methyls), 1.98 (18 H, broad peak, envelope methylenes), 2.28 (2 H, t, —CH_2—C=N—), 2.75 (2 H, t, J = 6 Hz, —CH_2—NH—), 3.30 (2 H, t, J = 6 Hz, —CH_2—N=C), 5.10 (5 H, m, vinylic hydrogens).

2-Dodecylimidazole (25)

A number of methods have been described to prepare 2-alkyl imidazoles by dehydrogenation of the corresponding imidazolines. Negative results were obtained heating 2-dodecylimidazoline (26) with a nickel hydrogenation catalyst in the liquid phase [66], with active MnO_2 [69] or with the more recent barium manganate method [70]. The use of selenium as hydrogen acceptor [71, 72] gave also very poor results (2.5% yield). Here we describe a more efficient method for the synthesis of 2-dodecylimidazole, characterized by the use of palladium on charcoal (10% Pd), spread on the melted imidazoline at 250° (17% yield). 2-Dodecyl-2-imidazoline 26 (30 mg) was mixed with palladium on charcoal (10% Pd) (10 mg) and the reaction mixture was gradually heated till 280°, for 2 hr. After cooling, hot ethanol was added, the catalyst removed by filtration and the solvent evaporated in vacuo. The residue was purified by TLC (0.25 mm, $CHCl_3$/MeOH/NH_4OH 25% 97:2:1), to give the dehydrogenated product (25) (5.1 g, 17% yield). *m/z*: 236 (M^+, 0.2), 94 (100), 81 (15). λ_{max} cm^{-1} ($CHCl_3$): 1665 (—C=N—), 1600 and 1500. δ($CDCl_3$, 60 MHz): 0.95 (3 H, t, J = 7 Hz, —CH_3), 1.30 (20 H, broad peak, —CH_2—(CH_2)$_{10}$—), 2.01 (2 H, t, J = 7 Hz, —CH_2—C=NH—), 7.25 (2 H, m, —CH=CH—).

N,N-*Dimethyl-2-phenoxyethylamine* (14) *and* N,N-*diethyl-2-phenoxyethylamine* (15)

These compounds were synthesized by Kuroda [73], treating sodium phenolate with the corresponding *N,N*-dialkylethylamine. In order to improve the yield of these compounds, we utilized two different procedures (a) and (b).

(a) 2-Phenoxyethanol (2.7 g) dissolved in pyridine (35 ml) reacted under stirring with tosyl chloride (6 g) for 60 hr. After the usual work up, the crude residue was purified by column chromatography (silica gel, 3 × 21 cm, $CHCl_3$), giving the pure tosyl ester (4.0 g, 71% yield). δ($CDCl_3$, 60 MHz): 2.45 (3 H, s, CH_3—C), 4.12 (2 H, t, —O—CH_2—), 4.30 (2 H, t, —CH_2—O—SO_2—), 7.30 (9 H, m, aromatic hydrogens). The tosylate (730 mg) in dry acetone (10 ml) was heated under reflux with NaI (750 mg) for 2 hr. After the usual procedure, the purification by TLC (2 mm, $CHCl_3$) gave 1-iodo-2-phenoxyethane (489 mg, 79% yield). δ($CDCl_3$, 60 MHz): 3.70 (2 H, t, J = 6 Hz, —CH_2—I), 4.15 (2 H, t, J = 6 Hz, —O—CH_2—), 7.10 (5 H, m, aromatic hydrogens). 1-Iodo-2-phenoxyethane (100 mg) was then treated with diethylamine (400 mg), under stirring at 50°, for 2 hr. The reaction mixture was left for 60 hr more at room temperature. The usual work up and the TLC purification (0.5 mm, CH_2Cl_2/NH_4OH 25% 98:2) yielded the *N,N*-diethyl derivative (15) (23.4 mg), 30% yield). δ($CDCl_3$, 60 MHz): 1.11 (6 H, t, J = 7 Hz, —CH_3), 2.62 (4 H, t, J = 7 Hz, —N(CH_2CH_3)$_2$, 2.90 (2 H, t, J = 7 Hz, —O—CH_2—CH_2—N), 4.12 (2 H, t, J = 7 Hz, —O—CH_2—), 7.11 (5 H, m, aromatic hydrogens). Under the above conditions, using dimethylamine, we prepared the *N,N*-dimethyl-2-phenoxyethylamine (14) (40% yield).

(b) 2-Phenoxyethanol (2 g) was chlorurated with $SOCl_2$ (1.77 g), giving 1-chloro-2-phenoxyethane (1.60 g, 71% yield). This compound (500 mg) was added in a sealed tube to a solution of dimethylamine (600 mg) or diethylamine (600 mg) in absolute ethanol (2 ml). The tube was gradually heated in an oil bath at 130° for 2–3 hr, then the reaction mixture was cooled, filtered and evaporated to dryness *in vacuo*. The residue, after TLC purification, yielded the expected compounds (14) (164 mg, 31% yield) or (15) (216 mg, 35% yield).