Eur J Med Chem (1993) 28, 675–682
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Synthesis and biological activity of 19-azasqualene 2,3-epoxide as inhibitor of 2,3-oxidosqualene cyclase

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(Received 4 December 1992; accepted 5 April 1993)

Summary — 19-Azasqualene 2,3-epoxide and its *N*-oxide, high-energy intermediate analogue inhibitors of 2,3-oxidosqualene (SO) cyclase, were obtained by total synthesis. These compounds were designed to mimic the C-20 carbonium ion precursor of lanosterol formed during SO cyclization. The synthesis involved the preparation of C_{22} squalenoid aldehyde epoxide through a new procedure and the reconstruction of the squalenoid chain bearing a nitrogen at C-19 (pro C-20). 19-Azasqualene 2,3-epoxide was active on SO cyclase from rat and pig liver with an IC_{50} of 1.5 μM in pig, while in SO cyclases of yeast (*S cerevisiae* and *C albicans*) microsomes it was 20–30-fold less active. It was inactive on squalene epoxidase from rat and pig liver at the highest concentrations tested (100 μM).

2,3-oxidosqualene cyclase inhibitors / epoxy azasqualenes / hypocholesterolemics / antifungals

Introduction

2,3-Oxidosqualene cyclase (EC 5.4.99.7; 2,3-epoxysqualene cyclase, SO cyclase) is an enzyme which catalyses the cyclization of 2,3-oxidosqualene (SO) **1** into polycyclic triterpenoids such as lanosterol **8** in animals and fungi, cycloartenol or a variety of tetracyclic and pentacyclic triterpenes in higher plants [1–7].

The enzymatic conversion of SO to lanosterol has been suggested to proceed through a series of discrete conformationally rigid carbocationic intermediates **2–5** giving a C-20 carbonium ion (**6** or **7**) which undergoes a series of suprafacial 1,2-shifts leading, after loss of a proton, to lanosterol **8** (scheme 1) [8–11].

An important question in this biosynthetic scheme is whether the C-20 carbonium ion intermediate **6** or **7** can form a covalent bond with a suitable nucleophile of the enzyme active site [9] or can simply be stabilized by a correctly located ion pair group [7, 12, 13] which could lower the energy necessary to carry out the rearrangement of **6** or **7** to lanosterol **8**.

We failed to obtain a suicide inhibitor of SO cyclase by synthesizing squalenoid epoxide vinyl ether **9** (fig 1) which would be able to cyclize to form an oxenium ion, yielding a stable covalent adduct with the postulated nucleophilic group of the enzyme [14]. Recently Corey [15] showed that almost the same compound **10** was converted by the yeast cyclase to a 17β-acetylsterol, suggesting an analogous protosterol intermediate for the enzymic conversion of SO to lanosterol having a 17β side chain at C-17 as **7** and not the **17α** side chain as previously assumed [9].

Prestwich succeeded in preparing an irreversible inhibitor of SO cyclase from pig liver, 29-methylidene-2,3-oxidosqualene **11**, showing that the tetracyclic intermediate arising from its biocyclization could react with a nucleophile in the active site of the enzyme [16]. By using tritiated inhibitor **11**, affinity labeling of specific proteins in rat, dog, pig and human cyclase and the absence of labeling of yeast or pea cyclase was shown [17].

Our group has applied a general strategy to prepare potent inhibitors of SO cyclase such as azasqualenes **12** and **14** and *N*-oxide **13** mimicking the first high-energy intermediate (HEI) **2** resulting from the oxirane ring opening of SO, and the C-8 HEI **4** formed during SO cyclization, respectively [13, 18–23]. This was accomplished by replacing a carbonium ion in the squalenoid molecule with a nitrogen, protonated at physiological pH, which should present structural and charge similarities with the postulated intermediate [24].

A similar approach has been followed by other groups, who found that ammonium analogues of carbocationic intermediates involved in sterol or isoprenoid biosynthesis were very effective inhibitors [25–32].

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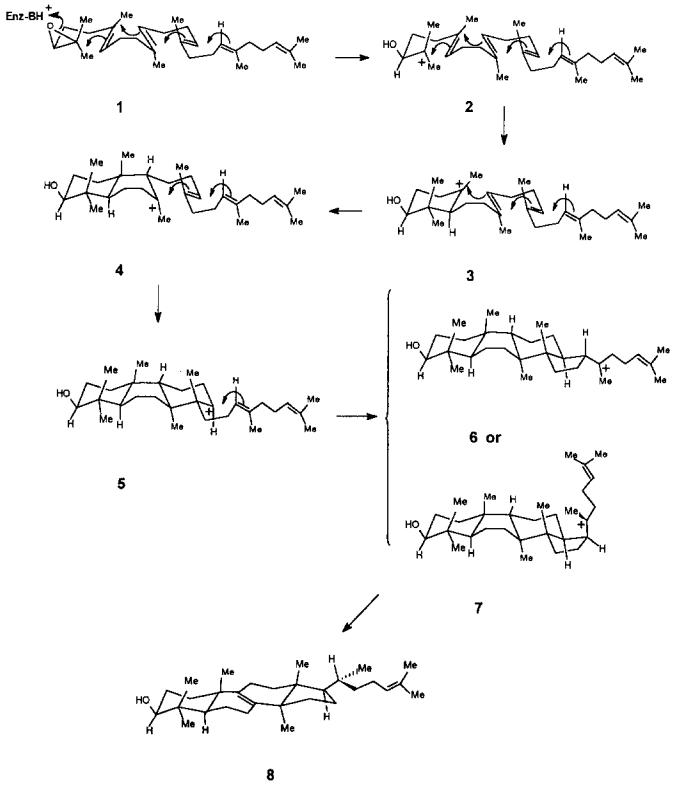


Scheme 1. Detailed mechanism of cyclization of 2,3-oxidosqualene to lanosterol.

Our aims in preparing azasqualenoid molecules were to gain deeper insight into the mechanism of action of SO and to obtain potent and specific hypocholesterolemic or antifungal drugs [11, 13, 21, 23, 33].

We have now extended the aza analogue approach by preparing with a total synthesis new compounds which mimic the C-20 carbonium ion. We synthesized 2,3-epoxy-19-aza-18,19,22,23-tetrahydrosqualene (19-azasqualene 2,3-epoxide) **15** and its *N*-oxide **16** (scheme 3), in order to obtain a substrate analogue that could be recognized more specifically by the enzyme, and then partially cyclized to give the aza analogue of **7**. The activity of the new compounds **15** and **16** was assayed in different *in vitro* systems: partially purified pig liver enzyme, microsomes from rat and pig liver, *Saccharomyces cerevisiae* and *Candida albicans*.

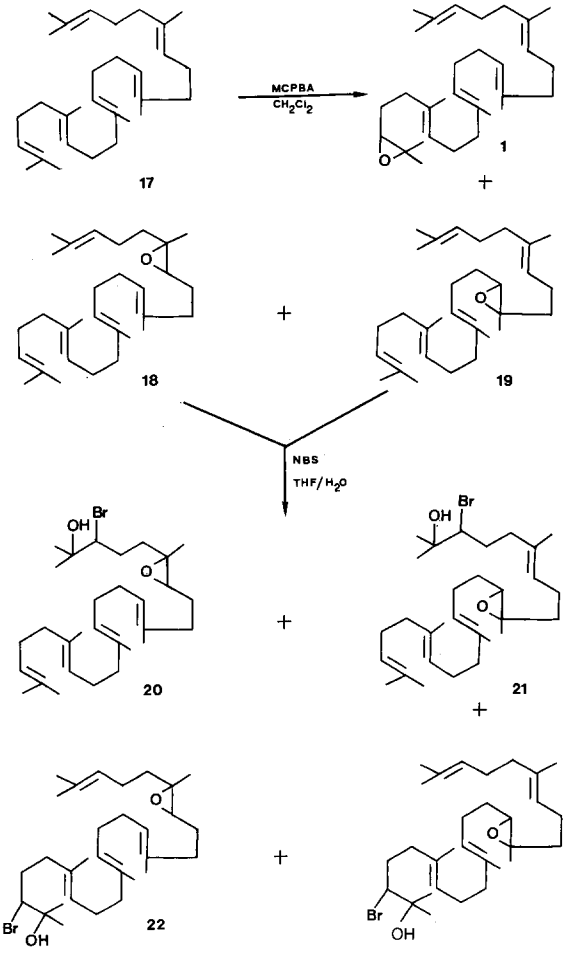
Chemistry

The overall strategy for the synthesis of 19-aza-squalene 2,3-epoxide **15** involved (a), the preparation of C_{22} squalenoid aldehyde epoxide **28** through a new procedure; and (b) the reconstruction of the squalenoid chain bearing a nitrogen atom at C-19.

Synthesis of C_{22} squalenoid aldehyde epoxide 28

Direct epoxidation of squalene **17** with *m*-chloroperbenzoic acid (MCPBA) followed by separation *via* flash chromatography gave a mixture of the 2 *trans* internal monoepoxides **18** and **19** in 29% yield and then the external monoepoxide **1** (scheme 2).

The mixture of the internal monoepoxides **18** and **19** was reacted with *N*-bromosuccinimide in aqueous tetrahydrofuran (THF) according to the procedure previously developed for squalene, suitably modified (see *Experimental protocols*). In this case, marked selectivity towards the terminal double bonds was also observed, giving a mixture of the monobromohydrin epoxides **20–23**, which was separated by flash



Scheme 2. Synthesis of squalene bromohydrin epoxides.

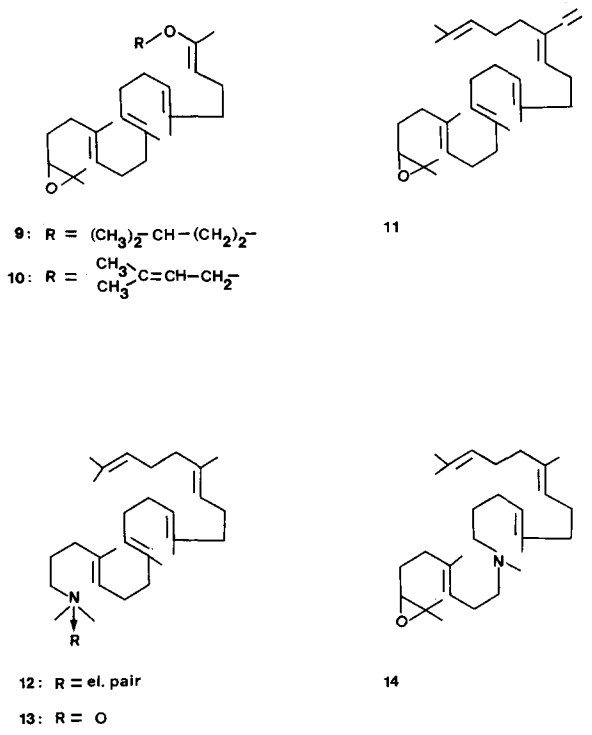
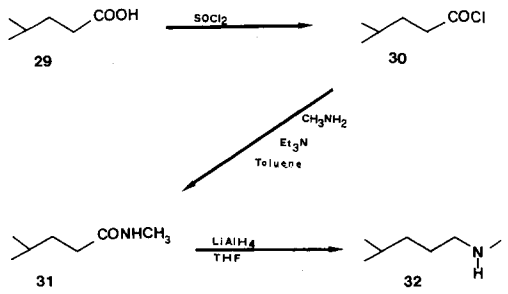


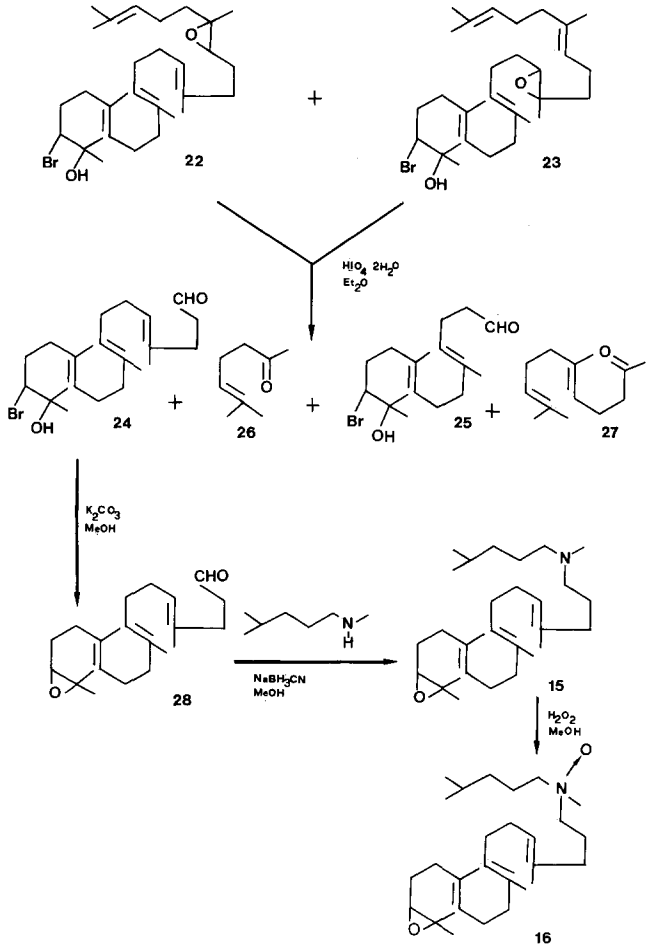
Fig 1. Structures of various 2,3-oxidosqualene cyclase inhibitors.

chromatography into 3 fractions. Fraction 1 contained the desired squalene monobromohydrin 18,19-epoxide **22** together with 14,15-epoxide **23**.

Following a general procedure developed by us [23], the mixture of compounds **22** and **23** was cleaved by using periodic acid in diethyl ether to give C_{22} and C_{17} squalenoid aldehyde external bromohydrins **24** and **25** which were separated by flash chromatography (scheme 3). Subsequent closure of bromohydrin **24** to epoxide **28** was accomplished with potassium carbonate in methanol.



Scheme 4. Synthesis of *N*-methyl-4-methylpentylamine.



Scheme 3. Synthesis of 19-azasqualene 2,3-epoxide and its *N*-oxide.

*Synthesis of 19-azasqualene 2,3-epoxide **15** and its N-oxide **16***

19-Azasqualene 2,3-epoxide **15** was obtained by reacting C_{22} squalenoid aldehyde external epoxide **28** with *N*-methyl-4-methylpentylamine **32** in the presence of sodium cyanoborohydride in anhydrous methanol (schemes 3 and 4). With our method, the addition of methanolic HCl usually employed in reductive aminations did not prove necessary and the desired product was obtained in 68% yield.

The synthesis of 19-azasqualene 2,3-epoxide *N*-oxide **16** was accomplished by reacting compound **15** with 30% aqueous H_2O_2 in methanol (1:1). The reaction proceeded smoothly at room temperature (12 h), affording *N*-oxide **16** in good yield (74%).

Biological results

We assayed the inhibition caused by 19-azasqualene 2,3-epoxide **15** and the corresponding *N*-oxide **16** on partially purified SO cyclase from pig liver and on SO cyclase associated with microsomes of rat liver, pig liver and the yeasts *Saccharomyces cerevisiae* and *Candida albicans*. From the inhibition curves we calculated the IC_{50} values (inhibitor concentrations required to reduce reaction velocity by 50%) (table I).

19-Azasqualene 2,3-epoxide **15** showed an inhibitory activity on SO cyclase from pig and rat liver which was very similar to that found in the parent compound 2-aza-2,3-dihydrosqualene **12** [12, 34]. In contrast, compounds **12** and **15** differed greatly in their behaviour towards the yeast cyclases, since only **12** was active; thus **15** presented the interesting feature of being selective for the mammalian enzyme. The *N*-oxide derivative **16** also showed an activity similar to that of some other azasqualene *N*-oxides previously synthesized [20, 33, 34], but only **16** was selective for the mammalian enzyme.

Compound **15** did not inhibit squalene epoxidase from pig or rat liver microsomes up to 100 μM. This result is in contrast with those previously obtained with 2-aza-2,3-dihydrosqualene **12** which showed good inhibitory activity on squalene epoxidase from rat liver [35].

Table I. IC_{50} values[a] (μM) of inhibition of 2,3-oxidosqualene cyclase by 19-azasqualene 2,3-epoxide **15** and 19-azasqualene 2,3-epoxide *N*-oxide **16**.

Enzymatic assay [a]	*Compounds*	
	15	***16***
Partially purified pig enzyme	1.7	7
Rat liver [b]	7.5	ND
Pig liver [b]	1.5	ND
S cerevisiae [c]	35	100
C albicans [c]	22	55

[a]The values are means of 2 different experiments; [b]microsomal protein concentration was 1 mg/ml for pig liver and 5 mg/ml for rat liver; [c]microsomal protein concentration was 3 mg/ml; ND: not determined.

Discussion

The most interesting result of the present study is that compound **15**, which has been obtained as a possible 19-aza analogue of the C-20 carbocationic intermediate **7** formed during the cyclization of SO, was found to be a specific and potent inhibitor of SO cyclase. The potent inhibitory properties of **15** and its selectivity confirmed our previous strategy of constructing acyclic azasqualenoid molecules such as 10-azasqualene 2,3-epoxide **14** in such a way that the positively charged nitrogen resulting from protonation of the amine at physiological pH would coincide, after recognition and cyclization by the enzyme, with the position occupied by the carbocationic HEI formed during the transformation of SO to lanosterol [23].

In the past we have synthesized potent SO cyclase inhibitors such as **12** and **14**, which proved able to mimic the C-2 ion intermediate **2** originating from the opening of the oxirane ring of SO [13] or the C-8 ion intermediate **4** derived from bicyclization of SO [23]. Rahier *et al* synthesized some partially cyclized aza derivatives which mimicked the HEI possessing the positive charge at carbons C-10 **3**, C-8 **4** and C-13 **5** [36]. It was shown that only the monocyclic C-10 and bicyclic C-8 aza analogues were powerful inhibitors, whereas the tricyclic C-13 aza derivatives were inactive *in vitro* [36, 37]. Bicyclic amides of similar structure showed potent competitive inhibition of SO cyclase [38].

The fact that 19-azasqualene 2,3-epoxide **15** is a potent inhibitor of SO cyclase suggests that only acyclic substrate analogues can successfully mimic the C-20 **7** carbonium ion. In fact, we previously found that 20-azadammaran-3β-ol, which may be considered a cyclized aza analogue of the C-20 ion intermediate **7**, failed to inhibit the β-amyrin cyclase from germinating peas [39].

Another attractive feature of the biological properties of 19-azasqualene 2,3-epoxide **15** was its selectivity, since it inhibited SO cyclase but was inactive against squalene epoxidase. This latter enzyme, a non-cytochrome P-450 monooxygenase [7], is inhibited when a suitable group such as an acetylenic, allenic, alcoholic, cyclopropyl amine or tertiary amine function is located at the end of the squalenoid skeleton [22, 35, 40–42].

As our interest in the study of SO cyclase inhibitors is that of the development of new hypocholesterolemic or antifungal drugs, we are seeking compounds which selectively inhibit mammalian and yeast cyclases and hence cholesterol and ergosterol biosynthesis respectively. 19-Azasqualene 2,3-epoxide **15** met this purpose, since it was ≈ 20–30 fold more active towards the mammalian rather than the *S cerevisiae* or *C albicans* enzyme (table I). These data confirm previous suggestions that mammalian (rat and pig liver) [43] and yeast cyclases [44, 45] may have different structural properties, particularly in the part of the enzyme able to interact with the C-8 **4** or C-20 **7** HEI [16, 46].

The purpose of preparing *N*-oxide **16** was based on the hypothesis that in general, the amine *N*-oxide

group containing squalenoid molecules was considered able to mimic the positively charged intermediates involved in sterol biosynthesis by charge-charge or dipole–dipole interaction [20, 24]. In this context, *N*-oxide **16** resembled other aza-squalenoid molecules in being active in pig liver SO cyclase and less active on yeast cyclases.

In conclusion, it may be possible to design new pharmacologically active compounds, taking advantage of our knowledge of the enzymatic mechanism of SO cyclization in different tissues [11]. In particular, the synthesis of 19-azasqualene 2,3-epoxide **15** as an inhibitor of mammalian SO cyclase may provide new prospects for developing novel specific hypocholesterolemic drugs, even though recognition and cyclization of **15** at the moment is at a completely speculative stage.

Experimental protocols

Chemistry

^{1}H-NMR spectra were recorded either on a Jeol EX-400 or a Jeol JNM-PMX 60, with $SiMe_4$ as internal standard. Mass spectra were obtained on a VG Analytical 7070 EQ-HF or a VG ZAB 2F spectrometer, by electron impact or by chemical ionization. IR spectra were recorded on a Perkin–Elmer 781. Microanalyses for C, H, Br, N, O were within ± 0.4% of theoretical values and were performed on an Elemental Analyser 1106 (Carlo–Erba Strumentazione).

The reactions were checked on F_{254} silica gel precoated sheets (Merck); after development, the sheets were exposed to iodine vapour. Purification was carried out using column flash chromatography on 230–400 mesh silica gel (Merck). Light petroleum refers to the fraction with bp: 40–60°C. MCPBA refers to *m*-chloroperbenzoic acid.

*Squalene epoxides (as a mixture of the 2 trans internal monoepoxides): (6E,10E,14E)-trans-18,19-epoxy-2,6,10,15,19,23-hexamethyl-2,6,10,14,22-tetracosapentaene **18** and (6E,10E,18E)-trans-14,15-epoxy-2,6,10,15,19,23-hexamethyl-2,6,10,18,22-tetracosapentaene **19***

A solution of squalene **17** (10 g, 24.3 mmol) in CH_2Cl_2 (250 ml) at 0°C was stirred, while MCPBA (85% purity; 1.5 equiv, 7.41 g, 36.5 mmol) was added over a 30-min period; it was then allowed to react for a further 30 min with continuous stirring. The reaction mixture was washed with 20% aqueous $NaHCO_3$ (100 ml x 3) and saturated brine (100 ml x 2), dried over anhydrous sodium sulfate and evaporated to dryness to give a mixture of products. The resulting oil was purified by flash chromatography (light petroleum/diethyl ether, 95:5) to give a mixture of the 2 *trans* internal monoepoxides **18** and **19** (3.00 g, 29% yield) [23] and then the external monoepoxide **1** (1.48 g, 14% yield), as colourless oils.

18 and **19**. IR (liq film) ν_{max} 2980, 2910, 2850, 1450, 1385, 1250, 1110, 985 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 1.25 (s, 3H, epoxidic CH_3), 1.58–1.67 (m, 25H, allylic CH_3 and CH_2-epoxide-CH_2), 1.97–2.05 (m, 16H, allylic CH_2), 2.70 (m, 1H, epoxidic CH), 5.06–5.15 (m, 5H, vinylic CH). HRMS: found M$^+$, 426.3867. $C_{30}H_{50}O$ requires M, 426.3861. EIMS: *m/z* 426 (4), 400 (2), 383 (2), 357 (10), 339 (4), 289 (4), 276 (4), 247 (30), 203 (20), 191 (15), 177 (17), 161 (20), 149 (43), 135 (75), 109 (100).

1. ^{1}H-NMR ($CDCl_3$) δ 1.242 and 1.283 (two s, 6H, epoxidic CH_3), 1.58–1.66 (m, 20H, allylic CH_3 and epoxide-CH_2), 1.98–2.06 (m, 18H, allylic CH_2), 2.690 (m, 1H, J = 6.2 Hz, epoxidic CH), 5.06–5.17 (m, 5H, vinylic CH).

*Squalene bromohydrin epoxides: (10E,14E,18E)-3-bromo-trans-6,7-epoxy-2,6,10,15,19,23-hexamethyl-10,14,18,22-tetracosatetraen-2-ol **20**; (6E,14E,18E)-3-bromo-trans-10,11-epoxy-2,6,10,15,19,23-hexamethyl-6,14,18,22-tetracosatetraen-2-ol **21**; (6E,10E,14E)-3-bromo-trans-18,19-epoxy-2,6,10,15,19,23-hexamethyl-6,10,14,22-tetracosatetraen-2-ol **22** and (6E,10E,18E)-3-bromo-trans-14,15-epoxy-2,6,10,15,19,23-hexamethyl-6,10,18,22-tetracosatetraen-2-ol **23***

The mixture of internal epoxides **18** and **19** (42.67 g, 0.10 mol) was dissolved in THF (250 ml), and cooled to 0°C; water was then added till the solution became opalescent. A small amount of THF was then added to clear the solution. *N*-bromosuccinimide (1.2 equiv, 21.4 g, 0.12 mol) was added over a 30–min period at 0°C, and then left for 1 h at room temperature with stirring. The reaction mixture was extracted with ether (200 ml x 3), the combined extracts were washed with 10% $NaHCO_3$ (100 ml x 1), saturated brine (100 ml x 2), dried over anhydrous sodium sulfate and evaporated *in vacuo*. The resulting oil was chromatographed with light petroleum/diethyl ether, 98:2 to remove unreacted epoxides **18** and **19**, then 90:10 to give fraction 1 (compounds **22** and **23**), then 85:15 to give fraction 2 (compound **21**) followed by fraction 3 (compound **20**), as pale yellow oils. In total 15.70 g, 30% yield; F_1:F_2:F_3 = 44:28:28.

20. Anal C, H, Br, O. IR (liq film) ν_{max} 3400–3500, 2960, 2920, 2860, 1450, 1390, 1250, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 1.20 (s, 3H, epoxidic CH_3) 1.30 [s, 6H, $(CH_3)_2COH$], 1.49–1.65 (m, 19H, allylic CH_3 and CH_2-epoxide-CH_2), 1.94–2.18 (m, 16H, allylic CH_2 and CH_2CHBr), 2.66 (t, 1H, epoxidic CH), 3.90 (m, 1H, CHBr), 4.99–5.21 (m, 4H, vinylic CH). HRMS: found M$^+$, 522.3077. $C_{30}H_{51}BrO_2$ requires M, 522.3073. EIMS: 524 (1), 522 (1), 506 (11), 504 (12), 443 (9), 426 (13), 409 (4), 407 (4), 289 (8), 71 (100).

21. Anal C, H, Br, O. IR (liq film) ν_{max} 3400–3500, 2960, 2920, 2860, 1450, 1390, 1250, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 1.19 (s, 3H, epoxidic CH_3) 1.30 [s, 6H, $(CH_3)_2COH$], 1.52–1.66 (m, 19H, allylic CH_3 and CH_2-epoxide-CH_2), 1.97–2.20 (m, 16H, allylic CH_2 and CH_2CHBr), 2.66 (t, 1H, epoxidic CH), 3.91 (m, 1H, CHBr), 5.00–5.22 (m, 4H, vinylic CH). HRMS: found M$^+$, 522.3071. $C_{30}H_{51}BrO_2$ requires M, 522.3073. EIMS: 524 (1), 522 (1), 506 (3), 504 (3), 491 (0.6), 489 (0.6), 463 (0.5), 461 (0.5), 425 (6), 407 (3), 301 (9), 299 (10), 289 (4), 271 (5), 69 (100).

22 and **23**. [14] IR (liq film) ν_{max} 3400–3500, 2960, 2920, 2860, 1450, 1390, 1250, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 1.22 (s, 3H, epoxidic CH_3) 1.29 [s, 6H, $(CH_3)_2COH$], 1.50–1.65 (m, 19H, allylic CH_3 and CH_2-epoxide-CH_2), 1.95–2.20 (m, 16H, allylic CH_2 and CH_2CHBr), 2.68 (t, 1H, epoxidic CH), 3.92 (m, 1H, CHBr), 4.98–5.25 (m, 4H, vinylic CH). HRMS: found M$^+$, 522.3068. $C_{30}H_{51}BrO_2$ requires M, 522.3073. EIMS: 524 (1), 522 (1), 506 (7), 504 (7), 491 (1), 489 (1), 463 (1), 461 (1), 425 (5), 407 (3), 271 (8), 257 (8), 81 (100).

C_{22} Squalenoid aldehyde external bromohydrin: (4E,8E,12E)-16-bromo-17-hydroxy-4,9,13,17-tetramethyl-4,8,12-octadecatrienal ***24*** *and C_{17} squalenoid aldehyde external bromohydrin: (4E,8E)-12-bromo-13-hydroxy-5,9,13-trimethyl-4,8-tetradecadienal* ***25***

$HIO_4 \cdot 2H_2O$ (1.30 equiv, 11.85 g, 52 mmol) was almost completely dissolved in diethyl ether (500 ml) under vigorous stirring. The mixture of squalene bromohydrin epoxides **22** and **23** (20.95 g, 40 mmol) was added and allowed to react for 15 min with stirring. The reaction mixture was extracted with ether (100 ml x 3), the combined extracts were washed with 10% $NaHCO_3$ (100 ml x 1), saturated brine (100 ml x 2), dried over anhydrous sodium sulfate and evaporated *in vacuo*. The resulting oil was chromatographed with light petroleum/diethyl ether, 92:8 to give 6.95 g (42% yield) of compound **24** and 5.78 g (42% yield) of compound **25** in 84% complexive yield, as pale yellow oils.

24. [14] IR (liq film) ν_{max} 3400–3500, 2960, 2920, 2860, 1725 (CO), 1450, 1390, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 1.28 [s, 6H, $(CH_3)_2COH$], 1.48–1.62 (m, 9H, allylic CH_3), 1.85–2.20 (m, 14H, allylic CH_2 and CH_2CHBr), 2.35–2.40 (m, 2H, CH_2CHO), 3.84 (m, 1H, CHBr), 4.98–5.23 (m, 3H, vinylic CH), 9.78 (m, 1H, CHO). HRMS: found M^+, 412.1962. $C_{22}H_{37}BrO_2$ requires M, 412.1977. EIMS: *m/z* 414 (0.5), 412 (0.5), 332 (3), 316 (1), 247 (1), 153 (6), 135 (15), 111 (16), 93 (38), 81 (90), 43 (100).

25. [14] IR (liq film) ν_{max} 3400–3500, 2970, 2920, 2850, 1725 (CO), 1445, 1385, 1115 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 1.31 [s, 6H, $(CH_3)_2COH$], 1.52–1.67 (m, 6H, allylic CH_3), 1.98–2.23 (m, 10H, allylic CH_2 and CH_2CHBr), 2.34–2.41 (m, 2H, CH_2CHO), 3.91 (m, 1H, CHBr), 5.05–5.23 (m, 2H, vinylic CH), 9.73 (m, 1H, CHO). HRMS: found M^+, 344.1347. $C_{17}H_{29}BrO_2$ requires M, 344.1351. EIMS: *m/z* 346 (3), 344 (3), 328 (3), 326 (3), 302 (1), 300 (1), 264 (5), 243 (4), 229 (2), 203 (1), 135 (32), 107 (15), 93 (35), 81 (100).

C_{22} Squalenoid aldehyde external epoxide: (4E,8E,12E)-16,17-epoxy-4,9,13,17-tetramethyl-4,8,12-octadecatrienal ***28***

K_2CO_3 (13.8 g, 100 mmol) was dissolved in methanol (300 ml), C_{22} squalenoid aldehyde external bromohydrin **24** (8.27 g, 20 mmol) was added and the mixture was stirred for 2 h at room temperature. The reaction mixture was extracted with ether (100 ml x 3), the combined extracts were washed with saturated brine (100 ml x 2), dried over anhydrous sodium sulfate and evaporated *in vacuo*. The resulting oil was chromatographed with light petroleum/ethyl acetate, 97:3 to give 4.84 g (73% yield) of compound **28** as a colourless oil.

28. [14] IR (liq film) ν_{max} 2950, 2920, 2840, 1730 (CO), 1460, 1360, 1280, 1170 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 1.24 and 1.28 (two peaks, 6H, epoxidic CH_3), 1.59–1.70 (m, 11H, allylic CH_3 and epoxide-CH_2, 1.96–2.08 (m, 12H, allylic CH_2), 2.40 (m, 2H, CH_2CHO), 2.70 (t, 1H, epoxidic CH), 5.05–5.15 (m, 3H, vinylic CH), 9.73 (m, 1H, CHO).

4-Methylpentanoyl chloride ***30***

4-Methylpentanoic acid **29** (6.97 g, 60 mmol) was refluxed for 2 h with $SOCl_2$ (excess, 16 ml). The excess of thionyl chloride was eliminated by distillation at 760 mm Hg, then 4-methylpentanoyl chloride **30** was distilled (4.72 g, 68% yield). It had K_p^{760} = 142–146°C (Lit 47: K_p^{760} = 140–144°C). IR (liq film) ν_{max} 2960, 2920, 2870, 1790 (CO), 1460, 910 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 0.89 (d, 6H, 2 CH_3), 1.58 (m, 3H, $CHCH_2$), 2.86 (t, 2H, CH_2CO).

N-Methyl-4-methylpentanamide ***31***

Methylamine (x 2, 149 mmol, 6.9 ml) was condensed in a 2-necked flask at –30°C according to the general method reported in [48]. Triethylamine (x 2, 149 mmol, 21 ml) was then added, followed by anhydrous toluene (300 ml) and stirred under nitrogen. 4-Methylpentanoyl chloride **30** (x 1, 74.3 mmol, 10.0 g) in toluene (10 ml) was added dropwise at –30°C with stirring. The temperature was then raised to –10°C for 2 h and then for 12 h at room temperature; the solid triethylammonium hydrochloride was filtered off and the solvent evaporated under reduced pressure. The resulting oil was extracted with diethyl ether (50 ml x 3) after addition of water (50 ml), washed with saturated brine (50 ml x 2) and evaporated to dryness *in vacuo* to give 7.7 g of the crude product (found pure by ^{1}H-NMR) that was used directly in the next step; Lit 49: K_p^{19} = 142– 145°C. IR (liq film) ν_{max} 3250–3350, 2950, 2860, 1640 (CO), 1560, 1460, 1410, 1160 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 0.84 [d, 6H, $(CH_3)_2CH$], 1.48 (m, 3H, $CHCH_2$), 2.14 (t, 2H, CH_2CO), 2.73 (d, 3H, CH_3N), 6.33 (broad peak, 1H, NH). CIMS: *m/z* 130 (100), 114 (4), 100 (3), 86 (5), 73 (11).

N-Methyl-4-methylpentylamine ***32***

$LiAlH_4$ (x 4, 294 mg, 7.76 mmol) was suspended in dry THF (20 ml) and stirred under nitrogen at 0°C. A solution of the crude *N*-methyl-4-methylpentanamide **31** (252 mg, ≈ 1.94 mmol) in dry THF (10 ml) was added dropwise. After addition was complete, the mixture was refluxed for 5 h, cooled at 0°C and then water (1 ml) was added dropwise with vigorous stirring. This was followed by the addition of aqueous 20% NaOH (0.5 ml) and stirring was continued for 1 h at room temperature. The salts were filtered off, the solvent evaporated with caution and the crude amine **32** was rapidly distilled at 0.3 mm Hg; Lit 49: K_p^{41} = 134°C (179 mg, 64% yield from compound **30**). IR (liq film) ν_{max} 2960, 2920, 2860, 2780, 1460 1380 1360 1120 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 0.74 [d, 6H, $(CH_3)_2CH$], 1.07 (m, 2H, $CHCH_2$), 1.36 (m, 3H, $CHCH_2CH_2$), 2.10 (broad peak, 1H, NH), 2.28 (broad peak, 3H, CH_3N), 2.41 (t, 2H, CH_2N). EIMS: *m/z* 115 (20), 69 (5), 44 (100).

19-Azasqualene 2,3-epoxide: (4E,8E,12E)-N-methyl-N-(4-methylpentyl)-16,17-epoxy-4,9,13,17-tetramethyl-4,8,12-octadecatrienylamine ***15***

A solution of *N*-methyl-4-methylpentylamine **32** (x 2, 103 mg, 0.90 mmol) in anhydrous methanol (10 ml) was cooled to 0°C and $NaBH_3CN$ (x 1.2, 33.9 mg, 0.54 mmol) was added with stirring. C_{22} squalenoid aldehyde external epoxide **28** (0.45 mmol, 150 mg), dissolved in the minimum of methanol (2 ml) was then added, brought to room temperature and stirred for 4 h. The reaction mixture was then extracted with dichloromethane (100 ml x 3) after addition of brine, dried and evaporated to dryness *in vacuo*. The resulting oil was purified by flash chromatography using light petroleum/methanol, 98:2, then 96:4 to give 132 mg (68% yield) of 19-azasqualene 2,3-epoxide **15** as a colourless oil. Anal C, H, N, O. IR (liq film) ν_{max} 2950, 2920, 2860, 2780, 1460, 1370, 1360, 1240, 1120 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 0.89 [d, 6H, $(CH_3)_2CH$], 1.18 [m, 2H, $(CH_3)_2CHCH_2$], 1.27 and 1.31 (two peaks, 6H, epoxidic CH_3), 1.43–1.64 [m, 16H, allylic CH_3, $(CH_3)_2CHCH_2CH_2CH_2NCH_2CH_2$ and epoxide-CH_2], 1.96–2.12 (m, 12H, allylic CH_2), 2.22 (s, 3H, CH_3N), 2.30 (t, 4H, CH_2NCH_2), 2.71 (t, 1H, epoxidic CH), 5.12–5.18 (m, 3H, vinylic CH). HRMS: found M^+, 431.4126. $C_{29}H_{53}NO$ requires M, 431.4127. EIMS: *m/z* 431 (8), 360 (35), 278 (25), 210 (42), 128 (100).

19-Azasqualene 2,3-epoxide N-oxide: (4E,8E,12E)-N-methyl-N-(4-methylpentyl)-16,17-epoxy-4,9,13,17-tetramethyl-4,8,12-octadecatrienylamine N-oxide ***16***

19-Azasqualene 2,3-epoxide **15** (70 mg, 0.162 mmol) was dissolved in methanol (0.5 ml) and 30% H_2O_2 (excess, 0.5 ml) was added. The solution immediately became turbid and frothy. The reaction mixture was left for 12 h under stirring; during this time it progressively cleared. Light petroleum (30 ml) was added, the 2-phase system was cooled to 0°C, vigorously stirred, and MnO_2 was added in catalytic amounts to decompose H_2O_2. When this decomposition was complete, the suspension was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The resulting oil was purified by flash chromatography using acetone/isopropylamine, 99:1 to remove traces of unreacted **15**, then methanol to give 54 mg (74% yield) of 19-azasqualene 2,3-epoxide *N*-oxide **16** as a colourless oil. Anal C, H, N, O. IR (liq film) ν_{max} 2950, 2920, 2880, 1450, 1380, 1240, 1120 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 0.91 [d, 6H, $(CH_3)_2CH$], 1.21 [m, 2H, $(CH_3)_2CHCH_2$], 1.26 and 1.30 (two peaks, 6H, epoxidic CH_3), 1.58–1.93 [m, 16H, allylic CH_3, $(CH_3)_2CHCH_2CH_2CH_2NCH_2CH_2$ and epoxide-CH_2], 1.98–2.11 (m, 12H, allylic CH_2), 2.70 (t, 1H, epoxidic CH), 3.22 (s, 3H, CH_3N), 3.28 (t, 4H, CH_2NCH_2), 5.13–5.20 (m, 3H, vinylic CH). HRMS: found M^+, 447.4072. $C_{29}H_{53}NO_2$ requires M, 447.4076. CIMS: *m/z* 448 (2), 432 (100), 418 (45), 364 (20), 348 (28), 278 (15), 264 (15), 128 (64), 116 (65).

Biological assays

IC_{50} (the concentration of inhibitor which reduces by 50% the enzymatic conversion of SO to lanosterol) values on SO-lanosterol cyclase activity were determined in microsomal preparations from rat and pig liver, *S cerevisiae* and *C albicans* and on partially purified SO cyclase of pig liver.

IC_{50} on squalene epoxidase activity were determined in microsomal preparations from rat and pig liver.

Microsomes of rat and pig liver and of *S cerevisiae* were prepared according to the methods previously described [14, 23, 33, 34]. *C albicans* microsomes were kindly provided by Lepetit.

Pig liver SO cyclase was purified according to the method previously described [34].

Assay of mammalian SO cyclase activity

The substrate and the insoluble inhibitors were added to the test tubes as organic solutions in $CHCl_3$, in the presence of 0.05% (w/v) Tween 80 and the solvent evaporated under nitrogen before the addition of the aqueous components of the mixture. SO cyclase activity was determined by incubating the microsomal suspension (5 mg proteins for rat or 1 mg for pig liver) or the solubilized enzyme preparation (250 µl, 0.1 mg proteins) for 30 min at 45°C with [3-^{3}H]-(*R,S*)-2,3-oxidosqualene (100 000 dpm) diluted with (*R,S*)-2,3-oxidosqualene (50 nmol), 0.1 M K/K phosphate buffer, pH 8, containing 1 mM EDTA, Tween 80 (final concentration, 0.05% w/v), the inhibitor (when present) and 0.05% Emulphogene for solubilized enzyme (final vol 1 ml). The reaction was stopped and the amount of lanosterol was determined as previously described [14].

IC_{50} values were determined at 50 µM substrate, in the presence of different concentrations of inhibitors [34].

Assay of yeast SO cyclase activity

The substrate SO, detergent and inhibitors, when present, were added to the test-tubes as organic solutions. After evaporation of the solvent under N_2, the products were emulsified by stirring vigorously with a minimum of buffer. When not otherwise indicated, the reaction mixture contained in a vol of 0.5 ml: [3-^{3}H]-(*R,S*)-2,3-oxidosqualene (100 000 dpm) diluted with (*R,S*)-2,3-oxidosqualene (25 nmol), Tween 80, 0.01% (w/v), 0.5 mM dithiothreitol, 0.1 M phosphate buffer, pH = 7.1 and 1.5 mg microsomal protein. The reaction mixture was incubated for 30 min at 35°C in a shaking water-bath. Boiled microsomes were used as a blank. The reaction was stopped by adding 1 ml of KOH 15% (w/v) in methanol. Tubes were capped and heated at 80°C for 30 min. The nonsaponifiable lipids were extracted [33] and chromatographed with authentic samples of SO and lanosterol on silica gel plates developed with CH_2Cl_2. Areas corresponding to SO and lanosterol were visualized under ultraviolet light with berberine. Plates were scraped and counted for radioactivity in a Beckman LS 5000 liquid scintillator [45].

Isotope counting and activity calculations were carried out as already described [33].

Assay of mammalian squalene epoxidase activity

Squalene epoxidase activity was determined in rat and pig liver microsomes in the presence of supernatant fraction S_{100} and of the SO cyclase inhibitor: 3β-(β-dimethylaminoethoxy)androst-5-en-17-one (U-14226A).

The substrate and the insoluble inhibitors were added to the test tubes as organic solutions in $CHCl_3$ in the presence of 0.05% (w/v) Tween 80 and the solvent evaporated under nitrogen before the addition of the aqueous components of the mixture. The reaction mixture contained in the final vol of 1 ml: [^{3}H]squalene (20 000 dpm) diluted with squalene (20 nmol), Tween-80 (final concentration, 0.05% w/v), 0.1 M K/K phosphate buffer pH 7.4 containing 1 mM EDTA, microsomes (5 mg proteins) S_{100} (10 mg proteins), U-14226A (50 µM), $NADP^+$ (2 mM), glucose-6-P (5 mM), glucose-6-P dehydrogenase (1 UI), $MgCl_2 \cdot 6H_2O$ (5 mM). Incubations lasted 30 min at 37°C. The reaction was stopped by the addition of 1 ml of 10% ethanolic KOH and saponification for 30 min at 80°C. Extraction and chromatographic procedures similar to those of the SO cyclase assay were used. After developing the TLC in CH_2Cl_2, the areas corresponding to authentic squalene and SO were scraped and counted for radioactivity in a Beckman LS 5000 liquid scintillator. The enzymatic activity was expressed as nmol of SO formed/h [23].

Acknowledgments

This work was supported by grants from the Ministero della Ricerca Scientifica e Tecnologica and from the CNR, Progetto Finalizzato Chimica Fine No 92.02421.72. We thank P Milla for her helpful suggestions, and the NMR group of the Dipartimento di Chimica Inorganica, Chimica Fisica e Chimica dei Materiali, Università di Torino, and the mass spectrometry groups of the Dipartimento di Chimica Organica e Industriale, Università di Milano and the CNR, Area della Ricerca di Padova.

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