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# Synthesis and biological activity of a squalenoid maleimide and other classes of squalene derivatives as irreversible inhibitors of 2,3-oxidosqualene cyclase

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**Summary** — New classes of squalene derivatives were rationally designed and synthesized as irreversible inhibitors of 2,3-oxidosqualene cyclase (OSC), a key enzyme in sterol biosynthesis. The derivatives synthesized were maleimide **5**, disulfides **8–9**, α,β-unsaturated nitriles **10–11** and oxirane **12**. The inhibitor activities of these derivatives were determined *in vitro* on OSC associated with pig liver microsomes. Squalene and dodecyl maleimide were the best inhibitors of OSC, showing a time-dependent enzyme inactivation. Moreover 2,3-oxidosqualene (OS), the natural substrate of OSC, partially protected the enzyme from squalene maleimide inactivation whereas the dodecyl derivative did not. This fact and the complex kinetics shown by squalene maleimide suggest the presence of different classes of thiolic groups essential to the activity of OSC.

**2,3-oxidosqualene cyclase / squalene maleimide / irreversible inhibitor**

## Introduction

The 2,3-oxidosqualene cyclases (OSC) (EC 5.4.99.7) form a group of enzymes that catalyze both the conversion of (3*S*)-2,3-oxidosqualene (OS) into lanosterol, the first precursor of cholesterol in animal tissues, and ergosterol in yeast and fungi [1]. The enzyme is therefore a potential target for new antifungal and hypocholesterolemic drugs [2–5].

Mammalian OS-lanosterol cyclase is associated with the endoplasmic reticulum and its activity is stimulated by a soluble protein factor and anionic phospholipids [6]. Pig-liver OSC has been solubilized by treatment with several detergents [7, 8] and partially purified [9]. The purification of the pig enzyme to homogeneity was carried out by Abe *et al* [10], who found a molecular weight of 75 kDa, and by Moore and Schatzman [11], who described the native enzyme as a single subunit of 65 kDa. Yeast OSC, which was also initially considered as a soluble protein [12], was later partially purified (112–140-fold) and definitively characterized as a membrane-bound enzyme [13, 14]. Corey fully purified yeast OSC by affinity chromatography, showing a molecular weight of 26 kDa [15]. Buntel and Griffin [16] recently published the first nucleotide sequence of the OSC gene from *Candida albicans* and deduced the amino-acid sequence and a molecular weight of 83.7 kDa. Two other related cyclases, the OS-cycloartenol cyclase (EC 5.4.99, 55 kDa) and the OS-β-amyrine cyclase (EC 5.4.99, 35 kDa) were purified from pea seedlings [17]. However, despite the wide interest in the mechanism of OSC shown by many groups [3], the molecular basis of these enzymic cyclizations remains largely unknown. In particular, it is still not known which amino acids participate in the active site, the binding of the substrate and its conversion into products.

It has been suggested that OSC has an essential thiol group. Results from *Ochromonas malhamensis* [18] and hog's liver [19] indicate inhibition by the thiol reagent *p*-chloro-mercuribenzensulfonic acid or *N*-ethylmaleimide (NEM). Schuber found that the partially purified enzyme is inactivated by NEM [9], following pseudo-first-order kinetics, but he did not demonstrate that the thiol group is located at the enzymic binding site. Similar results were reported by Abe *et al* for pea seedling cyclases [17] and by Poralla *et al* for squalene hopene cyclase from *Bacillus acidocaldarius* [20]. Prestwich *et al* [21] recently synthesized a mechanism-based irreversible

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inhibitor of liver OSC, which is cyclized by the enzyme into an allylic cation intermediate which can be trapped by an active-site nucleophile. These results suggest that a thiol group is involved in the catalytic activity of the enzyme.

In order to obtain further insight into the mechanism of the mammalian OS cyclization, as well as new potential hypocholesterolemic drugs, a series of compounds were designed such that they could link covalently to an active-site thiol group. In this paper we describe the synthesis and the partial characterization of the biological activity of these potential irreversible inhibitors of OSC.

## Chemistry

All the squalene derivatives were prepared from 1,1',2-tris-norsqualene aldehyde **1**, which is easily obtained from the degradation of squalene [22]. The synthesis of squalene maleimide **5** (fig 1) required the preparation of squalene amine **4** by reduction of squalene azide **3**. The latter was synthesized in 1 step from squalene alcohol by a Mitsunobu reaction [23] using a zinc azide/bis-pyridine complex.

The reaction of **4** with maleic anhydride led to the maleamic derivative, which was cyclized *in situ* in the presence of acetic anhydride and anhydrous sodium acetate to give **5**.

Mixed disulfide squalene derivatives **8** and **9** were synthesized in good yield by reacting squalene thiol **7** in ethanol with the appropriate disulfide: 5,5'-dithiobis/2-nitrobenzoic acid or 2,2'-dipyridyl disulfide, respectively.

Squalene thiol was prepared in 2 steps from squalene alcohol using a modification of the Mitsunobu reaction [24] and was used without purification. The unsaturated nitriles **10** and **11** were synthesized by reacting **1** with diethylcyanomethylphosphonate in the presence of sodium amide (fig 2). Finally, compound **12** was prepared in a 1-step Darzen condensation from **1** and ethylmonochloroacetate. The dodecyl derivatives **13–16** (fig 2) were synthesized as described above for the squalenoid compounds.

## Biological results and discussion

The following types of potential irreversible inhibitors of OSC from pig-liver microsomes were assayed:

– Michael-type inhibitors [25], exemplified by the maleimide derivatives **5** and **13** and the α,β-unsaturated nitriles **10** and **11**.

– Calpain-type oxirane inhibitors, like **12** and **16**; these compounds should be attacked after protonation

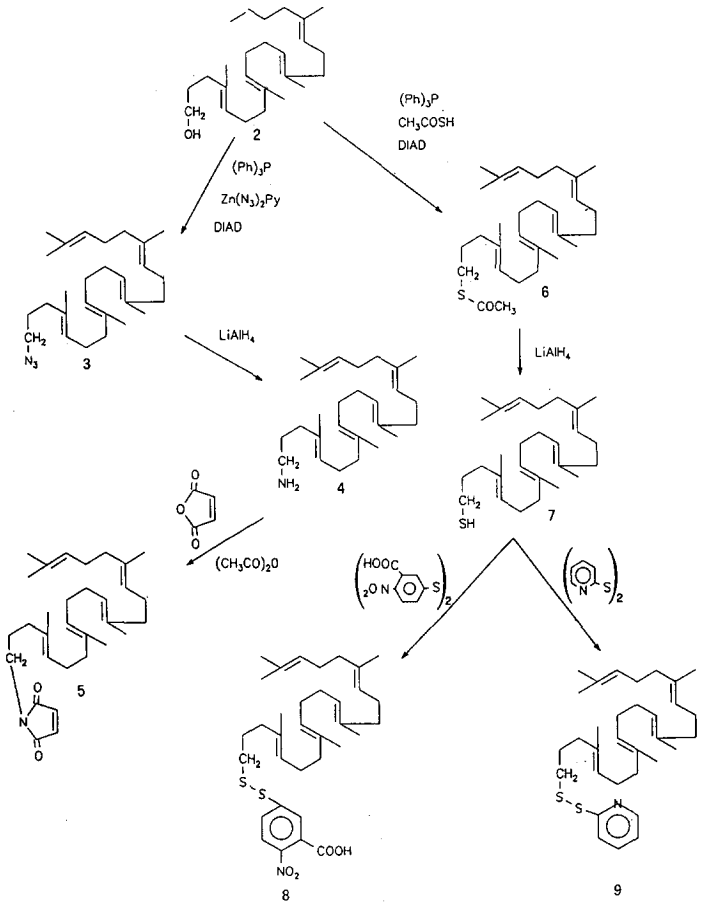


**Fig 1.** Structures and synthetic pathways to squalene derivatives **5**, **8** and **9**.

of the epoxide group by a suitable nucleophilic thiol group of the enzyme [26, 27].

– Ellman-type inhibitors [28] such as the 2-nitrobenzoic-5,5'-dithioderivatives **8** and **14** and the pyridyl-2,2'-dithioderivatives **9** and **15**.

In order to ascertain the structure–activity relationship for each type of inhibitor, we synthesized 2 different chemically related compounds: one characterized by a dodecyl substituent, which has been considered to have an optimal chain length for good inhibition of OSC [2]; and the other based on a squalenoid skeleton which is similar to the natural substrate.

Considering the ability of these compounds to inhibit the transformation of OS into lanosterol, we found that maleimido derivatives **5** and **13** were the most active, followed by the Ellman-type inhibitors **8** and **14** (table I). The α,β-unsaturated nitriles **10** and **11** and the oxirane-type derivatives **12** and **16** showed little or no activity.

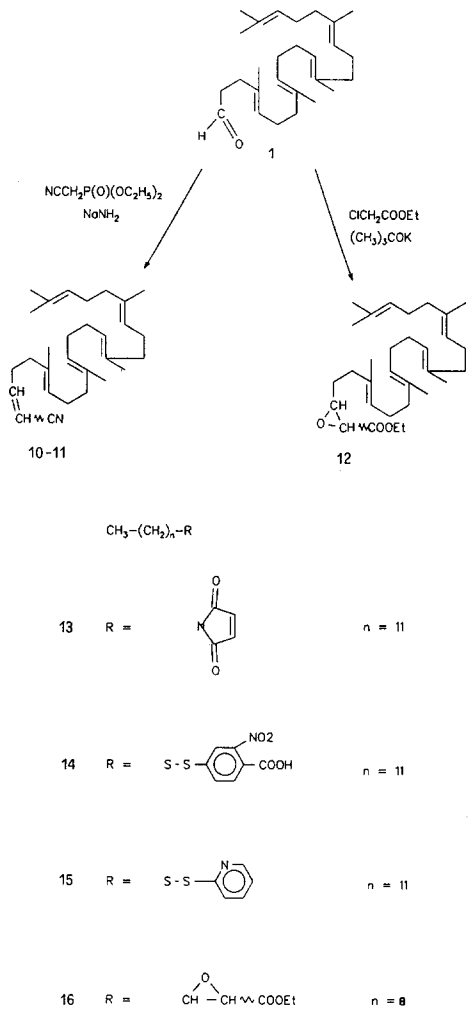


**Fig 2.** Structures and synthetic pathways to squalene derivatives **10**, **11**, **12** and related dodecyl analogues **13**, **14**, **15** and **16**.

The next step in the biological study of these inhibitors was the evaluation of a time-dependent inactivation following their incubation with the enzyme. Of all the tested compounds only the 2 maleimide derivatives **5** and **13** were effective as irreversible inhibitors (figs 3, 4).

We focussed our attention on the possible protection of the enzyme from inactivation by the maleimide derivatives by using an excess of substrate. We found that OS failed to protect the OSC from inactivation by **13** (fig 3), but partially protected the enzyme after incubation for up to 90 min with 50 μM squalene maleimide (fig 4). The same degree of substrate protection (≈ 15–25%) was also obtained in 2 other

**Table I.** Inhibition of pig-liver OSC by squalene and dodecyl derivatives.

| *Compd* | *% Inhibition*[a] |
|---|---|
| **5** | 82 |
| **8** | 65 |
| **9** | 67 |
| **10** | 26 |
| **11** | 24 |
| **12** | 37 |
| **13** | 83 |
| **14** | 73 |
| **15** | 67 |
| **16** | 0 |

[a]The concentration of inhibitors was 200 μM; values were the means of 2 independent determinations which did not differ by more than 5%.

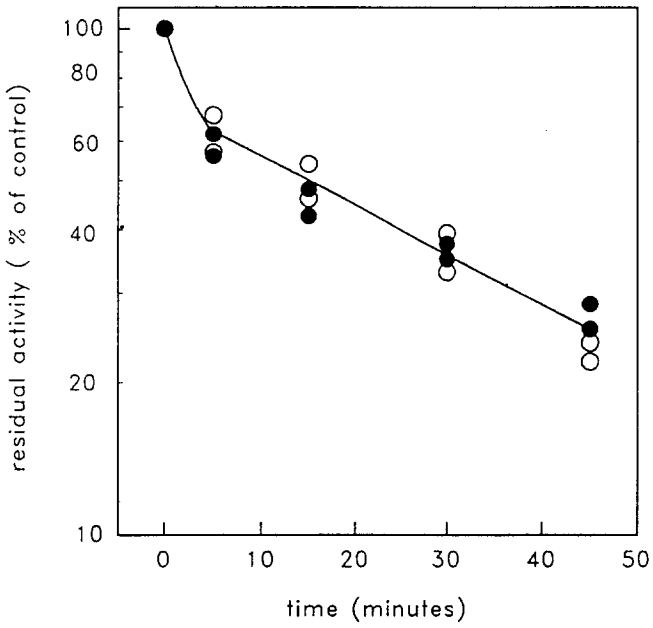


**Fig 3.** Time-dependent inhibition of OSC by 500 μM dodecyl maleimide **13**. Solubilized OSC was preincubated for 5–45 min with dodecyl maleimide, either in the presence (●—●) or the absence (○—○) of 500 μM OS substrate. Residual activity (see *Biological methods*) was expressed as percentage of the activity of a control preincubated for the same time interval in the absence of dodecyl maleimide.

experiments (data not shown) where **5** was used at 250 and 500 μM concentrations. The regression coefficients of the 2 lines obtained in the presence and the absence of substrate for 15–90 min (fig 4) were significantly different ($p < 0.01$ in a test of parallelism).

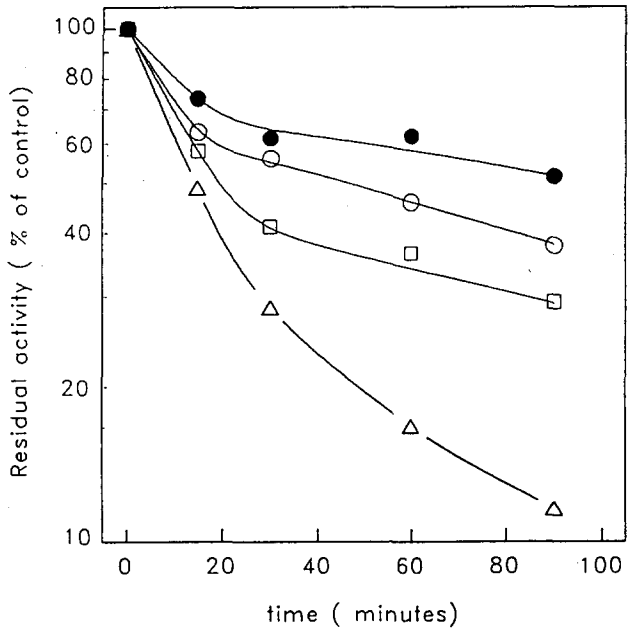


**Fig 4.** Time-dependent inhibition of OSC with different concentrations of squalene maleimide **5**. Residual activity (expressed as percentage of controls without inhibitor) was determined after preincubation of solubilized OSC for increasing times (15–90 min) with squalene maleimide, 50 μM (○—○), 100 μM (□—□) and 200 μM (△—△). The black circles indicate the residual activity after preincubation of 50 μM of the inhibitor in the presence of 500 μM OS.

Under similar conditions, Schuber [8] obtained no protection of the enzyme by NEM inactivation in the presence of the competitive inhibitor 2,3-oxido-2-*O*-noroxasqualene [29].

Surprisingly, the Ellman-type inhibitors **8** and **15**, did not behave as time-dependent irreversible inhibitors of the pig-liver OSC. On the other hand, the derivative **8** was found to be an irreversible inhibitor of OS from *Saccharomyces cerevisiae*, probably by reacting with an essential thiol group present at the binding site of the enzyme (Balliano *et al*, unpublished results). These data confirm the difference, observed by many authors [10, 30, 31], between animal and yeast OSC (*ie S cerevisiae*).

The different inhibitory activities shown by squalene maleimide and squalene Ellman derivatives could also be related to their chemical reactivity toward thiol groups.

The *N*-alkyl maleimide derivatives can react with protein thiol groups as strong Michael-type acceptors [32], whereas the disulfide-containing reagents are active thiol titrants in proteins because they participate in a disulfide-thiol exchange reaction with a suitable thiol residue [33]. At physiological pH, the reactivity of a thiol group *versus* a Michael-type acceptor (*ie* maleimide derivatives) may depend on its intrinsic $pK_a$ which, in turn, depends on the molecular environment near the thiol group [34]. It has been observed that a protonated thiol is over $5 \times 10^{10}$-fold less reactive with NEM than the corresponding thiolate anion, suggesting that only the thiolate form contributes to the reaction of NEM with protein thiols [35].

The squalene pyridine derivative **9** has been designed as an active thiol reagent existing in 2 acid-base forms, and is useful in determining a possible nucleophilic thiol group of OSC that can react in both acidic and basic forms [36, 37]. Its ineffectiveness seems to exclude an enzyme model similar to that suggested for cysteine protease [34]. A similar type of acid-base catalysis was also effective for Calpain, a known cysteine protease [27]. Indeed, the squalene oxirane derivative **12** designed to mimic a Calpain-type inhibitor was also ineffective.

While determination of the exact nature of the nucleophilic groups participating in the complex mechanism responsible for the OS cyclization await further study, the results obtained here suggest the presence of different sets of thiol groups essential for the activity of OSC. One of these groups, possibly in a thiolate form, could be present at the active site of the enzyme. Squalene maleimide could also be considered as a leading compound in the design of new potent and specific hypocholesterolemic drugs.

## Experimental protocols

*Chemistry*

Triphenylphosphine, lithium aluminum hydride, maleic anhydride, ethylmonochloroacetate and thiolacetic acid were purchased from E Merck. Diisopropyl azodicarboxylate, 5,5'-dithiobis(2-nitrobenzoic acid), 2,2'-dipyridyl disulfide, dodecylamine, 1-dodecanethiol, potassium *tert*-butoxide diethyl cyanomethyl phosphonate and polyoxyethylene 9-lauryl ether (polidocanol) were from Sigma–Aldrich Srl (Milan). $ZnN_6$•2Py was prepared according to the method described by Viaud *et al* [23]. 1,1',2-tris-norsqualene aldehyde and 1,1',2'-tris-norsqualene alcohol were obtained according to the literature method [22] starting from squalene and *via* the intermediates squalene monobromohydrine and squalene 2,3-epoxide. $^1$H-NMR spectra were recorded either on Jeol GX–400 or Jeol GX–270 spectrometer, with $SiMe_4$ as internal standard. Mass spectra were obtained on a VG–Analytical 7070 EQ-HF spectrometer by electron impact. IR and UV spectra were recorded on Perkin–Elmer 781 and Beckman DU70 spectrophotometers, respectively.

Microanalyses for C, H, N, S, were within ±0.4% of the 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 visualized

by either UV light or exposure to iodine vapor. Purifications were performed by column flash chromatography [38] on 230–400 mesh silica gel (Merck). Light petroleum refers to the fraction of bp: 40–60°C.

*(4E, 8E, 12E, 16E)-4,8,13,17,21-Pentamethyl-4,8,12,16,20-docosapentaenyl azide* ***3***

$ZnN_6$•2Py (0.907 g, 2.95 mmol) was suspended in a solution of squalene alcohol (1.45 g, 3.93 mmol) and triphenylphosphine (2.06 g, 7.86 mmol) in 30 ml anhydrous toluene. To this stirred mixture, at rt, diisopropyl azodicarboxylate (1.55 ml, 7.86 mmol) was added dropwise, causing a slightly exothermic reaction. Stirring was continued until complete consumption of squalene alcohol was observed (2 h) by TLC monitoring (eluent: light petroleum 100%). The heterogeneous mixture was filtered over a Celite pad, concentrated under reduced pressure and purified by column chromatography (eluent: light petroleum/diethylether, 98:2) to afford 1.45 g pure squalene azide (yield 90%).

MS (EI) *m/z*: 411 ($M^+$, 3); 384 (4); 368 (9); 314 (18); 300 (5); 246 (18); 192 (10); 178 (20); 137 (30); 110 (61); 95 (30); 81 (74); 69 (100). $^1$H-NMR 400 MHz ($CDCl_3$): δ 1.61–1.71 (m, 18H, allylic $CH_3$); 2.0–2.08 (m, 20H, allylic $CH_2$ and N-$CH_2$-C$H_2$); 3.23 (t, 2H, -C$H_2$-$N_3$); 5.15 (m, 5H, vinylic CH). IR (film): $\nu_{max}$ 3400; 2960; 2920; 2840; 2100; 1650; 1450 $cm^{-1}$. Anal calcd for $C_{27}H_{45}N_3$: C, 78.77; H, 11.02; N, 10.21; found: C, 78.80; H, 11.05; N, 10.19.

*(4E, 8E, 12E, 16E)-4,8,13,17,21-Pentamethyl-4,8,12,16,20-docosapentaenyl amine* ***4***

To an ice-cold solution of squalene azide (1.45 g, 3.5 mmol) in 25 ml anhydrous diethyl ether, $LiAlH_4$ (150 mg, 3.95 mmol) was added dropwise and the mixture stirred overnight at rt. Excess $LiAlH_4$ was destroyed by addition of 20 ml water, followed by extraction with diethylether (3 x 30 ml). The combined organic layers were washed with saturated brine (3 x 30 ml), dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. Crude squalene amine 0.97 g (yield 72%) was sufficiently pure for the following step. An analytical sample was obtained by preparative TLC (eluent: $CH_2Cl_2$/$CH_3OH$/$NH_3$ 32%, 96:1:3).

MS (EI) *m/z*: 385 (3); 356 (10); 288 (18); 248 (13); 220 (32); 180 (10); 152 (100); 138 (11); 112 (58); 95 (74); 69 (93). $^1$H-NMR 400 MHz ($CDCl_3$): δ 1.61–1.71 (m, 18H, allylic $CH_3$); 2.0–2.08 (m, 20H, allylic $CH_2$ and N-$CH_2$-C$H_2$); 3.23 (t, 2H, -C$H_2$-$N_3$); 5.15 (m, 5H, vinylic CH). IR (film): $\nu_{max}$ 3380; 2960; 2920; 2850; 1660; 1570; 1450 $cm^{-1}$. Anal calcd for $C_{27}H_{47}N$: C, 84.08; H, 12.29; N, 3.63; found: C, 84.01; H, 12.27; N, 3.63.

*(4E, 8E, 12E, 16E) N-[4,8,13,17,21]-Pentamethyl- 4,8,12,16, 20-docosapentaenyl maleimide* ***5***

To an ice-cold and well-stirred solution of maleic anhydride (224 mg, 2.28 mmol) in 20 ml anhydrous diethylether, a solution of **4** (881 mg, 2.28 mmol) in 30 ml anhydrous diethylether was added dropwise. After 1 h at 0°C, the mixture was raised to rt and stirred for 1 h. The reaction mixture was then evaporated *in vacuo*. The crude product was treated with sodium acetate (96 mg, 1.16 mmol) and 4 ml acetic anhydride (large excess) and heated at 100°C for 2 h, causing the formation of a white precipitate. The mixture was poured into 40 ml ice-cold water and stirred for 2 h at rt. The aqueous phase was extracted with diethylether (3 x 30 ml) and the combined organic layers were washed with 5% $NaHCO_3$ solution. After dehydration with sodium sulfate the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (eluent: $CH_2Cl_2$/$CH_3OH$, 98:2) to give 214 mg of **5** (yield 20%).

MS (EI) *m/z*: 465 ($M^+$, 7); 422 (3); 396 (9); 273 (9); 260 (12); 192 (37); 163 (15); 149 (41); 137 (26); 121 (23); 110 (39); 95 (87); 81 (73); 69 (100). $^1$H-NMR 400 MHz ($CDCl_3$): δ 1.56–1.70 (m, 18H, allylic $CH_3$); 1.96–2.07 (m, 20H, allylic $CH_2$ and N-$CH_2$-C$H_2$); 3.48 (t, 2H, -C$H_2$-N); 5.14 (m, 5H, squalene vinylic CH); 6.68 (s, 2H, CO-C*H*=CH-CO). IR (film): $\nu_{max}$ 2920; 2840; 1710; 1440; 1405 $cm^{-1}$. Anal calcd for $C_{31}H_{47}NO_2$: C, 79.94; H, 10.18; N, 3.00; found: C, 80.05; H, 10.21; N, 3.00.

*(4E, 8E, 12E, 16E) S-[4,8,13,17,21-Pentamethyl-4,8,12,16,20-docosapentaenyl] thioacetate* ***6***

Diisopropyl azodicarboxylate (1.05 g, 5.17 mmol) was added to a well-stirred solution of triphenylphosphine (1.36 g, 5.17 mmol) in 15 ml anhydrous tetrahydrofuran at 0°C. The mixture was stirred at 0°C for 30 min and gave a white precipitate. A solution of squalene alcohol (1 g, 2.58 mmol) and thiolacetic acid (0.394 g, 5.17 mmol) in 3 ml anhydrous tetrahydrofuran was added dropwise under nitrogen and the mixture stirred for 1 h at 0°C and 1 h at rt. The mixture was concentrated *in vacuo* and purified by column chromatography (eluent: light petroleum/diethylether, 99.5:0.5) to give 800 mg of **6** (yield 70%).

MS (EI) *m/z*: 444 ($M^+$, 10); 401 (5); 375 (5); 209 (5); 197 (14); 136 (19); 135 (19); 129 (70); 101 (18); 95 (32); 93 (15); 81 (62); 69 (100). $^1$H-NMR 400 MHz ($CDCl_3$): δ 1.56–1.71 (m, 18H, allylic $CH_3$); 1.97–2.11 (m, 20H, allylic $CH_2$ and S-$CH_2$-C$H_2$); 2.32 (s, 3H, C$H_3$-CO-); 2.83 (t, 2H, -C$H_2$-S); 5.13 (m, 5H, vinylic CH). IR (film): $\nu_{max}$ 2960; 2910; 2840; 1690; 1440 $cm^{-1}$. Anal calcd for $C_{29}H_{48}OS$: C, 78.32; H, 10.88; S, 7.21; found: C, 78.29; H, 10.86; S, 7.20.

*(4E, 8E, 12E, 16E)-4,8,13,17,21-Pentamethyl-4,8,12,16,20-docosapentaen-1-thiol* ***7***

Squalene thiolacetate (710 mg, 1.59 mmol) was dissolved in 15 ml anhydrous diethylether and added dropwise to a suspension of $LiAlH_4$ (90 mg, 2.4 mmol) in 20 ml anhydrous diethylether under nitrogen. The mixture was stirred 2 h at rt and the $LiAlH_4$ excess was destroyed by the careful addition of 10 ml 1 N HCl solution. The ether layer was separated and the aqueous phase extracted with diethylether (2 x 15 ml). The combined organic phases were dried over sodium sulfate and the solvent evaporated under reduced pressure. The crude product (750 mg) was used without purification for the following step.

*6-Nitro-3-[(4E, 8E, 12E, 16E)-4,8,13,17,21-pentamethyl-4,8, 12,16,20-docosapentaenyldisulfamyl] benzoic acid* ***8***

5,5'-Dithiobis(2-nitrobenzoic acid) (1.10 g, 2.79 mmol) was dissolved in 70 ml ethanol. To this solution, squalene thiol (750 mg crude product, 1.86 mmol) dissolved in 15 ml ethanol was added under nitrogen. The mixture was stirred overnight at rt, and then the solvent was evaporated under reduced pressure. The resulting yellow oil was purified by column chromatography (eluent: $CH_2Cl_2$/$CH_3OH$, 98:2) to give 550 mg of **8** (the calculated yield with respect to squalene thiolacetate was 57%).

MS (EI) *m/z*: 401 ($M^+$-198, 100); 331 (9); 263 (24); 195 (24); 182 (30); 169 (14); 149 (11); 126 (35); 113 (65); 101 (42); 81 (50); 69 (82). $^1$H-NMR 400 MHz ($CDCl_3$): δ 1.41–1.68 (m, 18H, allylic $CH_3$); 2.00–2.07 (m, 20H, allylic $CH_2$ and S-$CH_2$-C$H_2$); 2.62 (t, 2H, -C$H_2$-S); 5.12 (m, 5H,

vinylic CH); 7.3–7.7 (m, 3H, aromatic protons). IR (film): $\nu_{max}$ 2960; 2920; 2840; 1600; 1570; 1520 $cm^{-1}$. Anal calcd for $C_{34}H_{49}O_4S_2$: C, 68.08; H, 8.23; N, 2.33; S, 10.69; found: C, 68.21; H, 8.24; N, 2.33; S, 10.71.

*2-[(4E, 8E, 12E, 16E)-4,8,13,17,21-Pentamethyl-4,8,12,16,20-docosapentaenyldisulfamyl] pyridine* ***9***

Squalene thiol (750 mg, crude product, 1.86 mmol) was reacted with 2,2'-dipyridyl disulfide as above described for compound **8** to give 510 mg **9** (the calculated yield with respect to squalene thiolacetate was 63%).

MS (EI) *m/z*: 511 ($M^+$, 9); 401 (39); 368 (4); 331 (9); 263 (13); 195 (18); 165 (36); 127 (34); 112 (44); 101 (76); 95 (40); 81 (66); 69 (100). $^1$H-NMR 400 MHz ($CDCl_3$): δ 1.48–1.83 (m, 18H, allylic $CH_3$); 1.96–2.09 (m, 20H, allylic $CH_2$ and S-$CH_2$-$CH_2$); 2.75 (t, 2H, -$CH_2$-S); 5.12 (m, 5H, vinylic CH); 7.07 (m, 1H, aromatic proton); 7.64 (m, 1H, aromatic proton); 7.73 (d, 1H, aromatic proton); 8.46 (d, 1H, aromatic proton). IR (film): $\nu_{max}$ 2960; 2920; 2840; 1570; 1550; 1440; 1410 $cm^{-1}$. Anal calcd for $C_{32}H_{49}NS_2$: C, 75.08; H, 9.65; N, 2.74; S, 12.53; found: C, 75.01; H, 9.63; N, 2.75; S, 12.55.

*(2-cis, trans, 6E, 10E, 14E, 18E)-6,10,15,19,23-Pentamethyl-2,6,10,14,18,22 tetracosahexaenenitrile* ***10–11***

To an ice-cold suspension of sodium amide (173 mg, 2.21 mmol, 50% p/p) in 5 ml anhydrous tetrahydrofuran, diethylcyanomethylphosphonate (392 mg, 2.21 mmol) dissolved in 2 ml tetrahydrofuran was added under nitrogen. The resulting solution was stirred for 3 h at 15°C. The temperature was then lowered to 0°C and squalene aldehyde (393 mg, 1.02 mmol) in 2 ml anhydrous tetrahydrofuran was added dropwise. The mixture was stirred at rt overnight, and then poured into 20 ml ice-cold water. The aqueous phase was extracted with dichloromethane (3 x 30 ml) and the combined organic layers were washed with saturated brine. After dehydration over sodium sulfate, the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (eluent: light petroleum/diethylether, 99.8: 0.2) to give 240 and 138 mg of *cis*- and *trans*-isomers, **11** and **12**, respectively.

*cis–Isomer* ***11***. MS (EI) *m/z*: 407 ($M^+$, 11); 392 (1); 364 (6); 338 (18); 296 (5); 273 (5); 215 (9); 202 (15); 192 (15); 149 (44); 137 (77); 123 (46); 107 (57); 95 (61); 81 (95); 69 (100). $^1$H-NMR 400 MHz ($CDCl_3$): δ 1.54–1.67 (m, 18H, allylic $CH_3$); 1.95–2.09 (m, 18H, allylic $CH_2$); 2.30 (m, 2H, -$CH_2$-CH=CH-); 5.10 (m, 5H, squalene vinylic CH); 5.30 (dt, 1H, -CH=C*H*-CN); 6.68 (dt, 1H, -C*H*=CH-CN). IR (film): $\nu_{max}$ 2950; 2920; 2850; 2220; 1450 $cm^{-1}$. Anal calcd for $C_{29}H_{45}N$: C, 85.51; H, 11.13; N, 3.36; found: C, 85.48; H, 11.11; N, 3.36.

*trans–Isomer* ***12***. MS (EI) *m/z*: 407 ($M^+$, 7); 392 (2); 364 (4); 338 (12); 296 (4); 273 (5); 231 (8); 215 (9); 202 (27); 149 (31); 134 (52); 123 (32); 107 (40); 95 (50); 81 (90); 69 (100). $^1$H-NMR 400 MHz ($CDCl_3$): δ 1.53–1.66 (m, 18H, allylic $CH_3$); 1.96–2.14 (m, 18H, allylic $CH_2$); 2.51 (m, 2H, -$CH_2$-CH=CH-); 5.13 (m, 5H, squalene vinylic CH); 5.27 (m, 1H, -CH=C*H*-CN); 6.42 (m, 1H, -C*H*=CH-CN). IR (film): $\nu_{max}$ 2950; 2920; 2850; 2220; 1450 $cm^{-1}$. Anal calcd for $C_{29}H_{45}N$: C, 85.51; H, 11.13; N, 3.36; found: C, 85.48; H, 11.11; N, 3.36.

*(6E, 10E, 14E, 18E)-Ethyl-2,3-epoxy-6,10,15,19,23-pentamethyl-6,10,14,18,22 tetracosapentaenoate* ***12***

To a solution of squalene aldehyde (0.89 g, 2.23 mmol) and freshly distilled ethylchloroacetate (0.366 g, 2.99 mmol) in 5 ml dry *tert*-butyl alcohol, potassium *tert*-butoxide (0.335 g, 2.99 mmol) in 4.5 ml dry *tert*-butyl alcohol was added dropwise under nitrogen. The solution was stirred 3 h at rt. Saturated brine (30 ml) was then added and the mixture was extracted with $CH_2Cl_2$ (3 x 30 ml). The combined organic layers were dried over sodium sulfate and evaporated under reduced pressure. The oily liquid remaining was purified by column chromatography (eluent: light petroleum/diethylether, 99:1) to give 400 mg of **12** as a mixture of *cis*- and *trans*-isomers (yield 37%).

MS (EI) *m/z*: 470 ($M^+$, 4); 427 (3); 401 (4); 205 (5); 149 (14); 137 (25); 121 (30); 105 (24); 95 (40); 81 (90); 69 (100). $^1$H-NMR 400 MHz ($CDCl_3$): δ 1.30 (t, 3H, O-$CH_2$-$CH_3$); 1.55–1.71 (m, 18H, allylic $CH_3$); 2.01–2.14 (m, 20H, allylic $CH_2$ and -$CH_2$-HCO); 3.15 (m, 1H, H-3); 3.21 (d, 0.7H, *trans*-H-2); 3.55 (d, 0.3H, *cis*-H-2); 4.23 (m, 2H, O$CH_2CH_3$); 5.12 (m, 5H, squalene vinylic CH). IR (film): $\nu_{max}$ 2960; 2920; 2840; 1750; 1730; 1440; 1380 $cm^{-1}$. Anal calcd for $C_{31}H_{50}O_3$: C, 79.16; H, 10.71; found: C, 79.08; H, 10.69.

*N-Dodecylmaleimide* ***13***

Dodecylamine (4.5 g, 0.024 mol) was reacted as described for compound **5** to give, after purification by column chromatography (eluent 100% $CH_2Cl_2$), 1.6 g (25% yield) of **13**.

MS (EI) *m/z*: 265 ($M^+$, 80); 152 (11); 138 (16); 124 (15); 111 (62); 110 (100); 99 (42); 82 (31); 69 (41). $^1$H-NMR 270 MHz ($CDCl_3$): δ 0.88 (t, 3H, $CH_3$-$CH_2$-); 1.26 (m, 18H, (-$CH_2$-)$_9$); 1.57 (m, 2H, $CH_3$-$CH_2$-); 3.51 (t, 2H, -$CH_2$-N); 6.68 (s, 2H, -CH=CH-). UV ($CH_3OH$): $\lambda_{max}$ 296; 225 nm. IR (KBr): $\nu_{max}$ 3540; 3170; 3040; 3020; 2940; 1750; 1490; 1450; 1420 $cm^{-1}$. Anal calcd for $C_{16}H_{27}NO_2$: C, 72.41; H, 10.25; N, 5.28; found: C, 72.35; H, 10.22; N, 5.27.

*6-Nitro-3-(dodecyldisulfamyl) benzoic acid* ***14***

1-Dodecanethiol (0.5 g, 2.5 mmol) was reacted in ethanol with 5,5'-dithiobis(2-nitrobenzoic acid) as described for **8** to give 750 mg of **14** (75% yield).

MS (EI) *m/z*: 399 ($M^+$, 11); 369 (17); 313 (9); 234 (11); 213 (17); 201 (36); 168 (10); 147 (10); 111 (17); 97 (36); 85 (65); 71 (100). $^1$H-NMR 270 MHz ($CDCl_3$): δ 0.98 (t, 3H, $CH_3$-$CH_2$-); 1.36–1.48 (m, 18H, (-$CH_2$-)$_9$); 1.76 (m, 2H, $CH_3$-$CH_2$-); 2.88 (t, 2H, -$CH_2$-S-); 7.73 (dd, 1H, aromatic proton); 7.82 (d, 1H, aromatic proton); 7.99 (d, 1H, aromatic proton). UV ($CH_3OH$): $\lambda_{max}$ 210; 320 nm. IR (KBr): $\nu_{max}$ 3700; 3040; 3010; 2940; 1650; 1610; 1560; 1440 $cm^{-1}$. Anal calcd for $C_{19}H_{29}NO_4S_2$: C, 57.12; H, 7.31; N, 3.50; S, 16.05; found: C, 57.04; H, 7.30; N, 3.49; S, 16.04.

*2-(Dodecyldisulfamyl) pyridine* ***15***

Dodecylamine (0.5 g, 2.5 mmol) was reacted with 2,2'-dipyridyl disulfide (826 mg, 3.7 mmol) in ethanol as described for compound **9** to give 0.6 g of **15** (yield 78%).

MS (EI) *m/z*: 312 ($MH^+$, 64); 221 (6); 143 (17); 111 (100); 78 (12). $^1$H-NMR 270 MHz ($CDCl_3$): δ 0.88 (t, 3H, $CH_3$-$CH_2$-); 1.25–1.38 (m, 18H, (-$CH_2$-)$_9$); 1.69 (q, 2H, $CH_3$-$CH_2$-); 2.78 (t, 2H, -$CH_2$-S-); 7.07 (m, 1H, aromatic proton); 7.63 (m, 1H, aromatic proton); 7.73 (d, 1H, aromatic proton); 8.45 (d, 1H, aromatic proton). UV ($CH_3OH$): $\lambda_{max}$ 203; 237; 283 nm. IR (film): $\nu_{max}$ 3140; 3020; 2940; 1620; 1605; 1490; 1460 $cm^{-1}$. Anal calcd for $C_{17}H_{29}NS_2$: C, 65.55; H, 9.38; N, 4.49; S, 20.58; found: C, 65.60; H, 9.36; N, 4.49; S, 20.54.

*Ethyl-2,3-epoxy-dodecanoate* ***16***

Decanal (3 g, 0.0192 mol), ethylmonochloroacetate (3.05 g, 0.0248 mol) and potassium *tert*-butoxide (2.79 g, 0.0248 mol) were reacted as described for **11** to give 2.3 g of **17** (50% yield)

as a mixture of *cis*- and *trans*-isomers, which were purified by column chromatography (eluent: light petroleum/diethylether, 98:2).

MS (EI) *m/z*: 242 ($M^+$, 20); 214 (36); 185 (93); 169 (42); 143 (37); 130 (82); 115 (26); 109 (45); 96 (68); 95 (88); 83 (100); 69 (96). $^1$H-NMR 270 MHz ($CDCl_3$): δ 0.88 (t, 3H, $CH_3$-$CH_2$-); 1.26–1.33 (m, 12H, $(CH_2)_6$, $OCH_2CH_3$); 1.46–1.66 (m, 4H, $CH_3$-$CH_2$-, $CH_2$-HCO); 3.15 (m, 1H, H-3); 3.20 (d, 0.6H, *trans*-H-2); 3.50 (d, 0.4H, *cis*-H-2); 4.25 (m, 2H, $OCH_2CH_3$). IR (film): $\nu_{max}$ 2940; 2920; 2840; 1750; 1730; 1460 $cm^{-1}$. Anal calcd for $C_{14}H_{26}O_3$: C, 69.38; H, 10.81; found: C, 69.30; H, 10.84.

*Biological methods*

OSC was obtained from pig-liver microsomes solubilized with polyoxyethylene 9-lauryl ether (polidocanol) and partially purified by DEAE Biogel P ion-exchange chromatography as previously described [39], substituting the detergent emulphogene with polidocanol.

OSC activity was determined by incubation of the solubilized and partially purified enzyme (50 μl, 18 μg protein, 500 nmol/h/mg protein spec act) for 30 min at 45°C with labelled OS (50 000 cpm) diluted with cold OS (25 nmol) in 10 mM K/K phosphate buffer, pH 8, containing 1 mM EDTA and 0.1% polidocanol (final vol 1 ml). The substrate and the inhibitors were added to the test tubes as organic solutions in ethanol in the presence of 0.5 mg/ml Tween–80, and the solvent was evaporated under nitrogen, before addition of the aqueous components of the mixture. The enzymic reaction was stopped by the addition of 1 ml 10% solution of KOH in methanol and the amount of lanosterol formed was evaluated as previously described [29]. Time-dependent inhibition was determined at 37°C by adding the inhibitors to the enzymatic solution in the absence of substrate or, for substrate protection experiments, in the presence of 500 μM cold OS. Residual activity was determined in the same conditions as previously described, at 45°C for 30 min after lowering the inhibitor concentration 20-fold. Aliquots of 50 μl were withdrawn at time intervals 5–90 min and transferred to test tubes containing the labelled OS (25 μM) and Tween–80 (0.5 mg/ml) in 950 μl of phosphate buffer. In the absence of preincubation of the inhibitor, the concentration obtained after dilution caused <10% inhibition.

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