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Original paper

Synthesis and biological activity of azasqualenes, bis-azasqualenes and derivatives

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Summary — Azasqualenes, bis-azasqualenes and derivatives, designed as inhibitors of squalene 2,3-epoxide cyclase, a key enzyme in sterol biosynthesis, were synthesized and their *in vitro* activities against a variety of yeasts, fungi, gram-positive and gram-negative bacteria were determined.

The synthesis involves a new method of squalene degradation, together with an unusual procedure for the aminative reduction of lipophilic aldehydes.

A study of the structure—activity relationship was attempted for different biological parameters: anti-bacterial and anti-fungal activities *(MIC)*, inhibition of mycelial growth *(GTT)*, surfactant activity *(CMC)* and membrane perturbation activity (induction of leakage in liposomes).

Résumé — **Synthèse et activité biologique d'azasqualènes, bis-azasqualènes et dérivés.** *Les azasqualènes, bis-azasqualènes et dérivés ont été conçus pour inhiber l'époxydo-2,3 squalène cyclase, une enzyme clé dans la biosynthèse des stérols. Ces produits ont été synthétisés et leur activité testée* in vitro *vis-4-vis d'une grande variété de champignons, de levures et de bactéries gram-positives et -négatives.*

La synthèse chimique de ces composés fait appel à une nouvelle méthode de dégradation du squalène ainsi qu'à une technique particulière pour l'amination réductrice d'aldéhydes hydrophobes.

Nous avons tenté d'effectuer une étude de relations structure—activité avec les différents paramètres mesurés: activité bactéricide et fongicide (MIC), *inhibition de la croissance du mycélium* (GTT), *activité surfactante* (CMC) *et perturbation membranaire (perméabilisation de liposomes).*

azasqualenes / squalene 2,3-epoxide cyclase inhibitors / structure—activity relationship / anti-microbial activity / membrane perturbation

Introduction

Several series of compounds have been developed within the past ten years, which are referred to as ergosterol synthesis inhibitors and which are effective fungicides against a wide variety of sterol-producing fungi.

Most of these substances, which include clotrimazole, miconazole and ketoconazole, have in common the ability to block the C-14 demethylation of lanosterol, resulting in a decrease of functional 4,14-demethyl sterols [1—9]. On the other hand, there are very few reports of new structural classes of sterol inhibitors which show anti-fungal activity [10—18]. This group of compounds includes the allylamine anti-mycotics naftifine and terbinafine [19—24], which are powerful inhibitors of squalene epoxidase (EC 1.14.99.7), a key enzyme in sterol biosynthesis.

For many years, we have been interested in the design of new inhibitors of 2,3-oxidosqualene (SO) cyclases (EC 5.4.99.7), a group of enzymes which transform SO into polycyclic triterpenoids [25, 26].

Recently, we demonstrated that 2-aza-2,3-dihydrosqualene **1** and a series of related derivatives **2**—**7** were potent inhibitors of SO cyclase in both animal and higher plant microsomes [27—30]. In a preliminary report [31], we showed that these compounds also inhibited the SO cyclase of *Saccharomyces cerevisiae*. We now describe the synthesis

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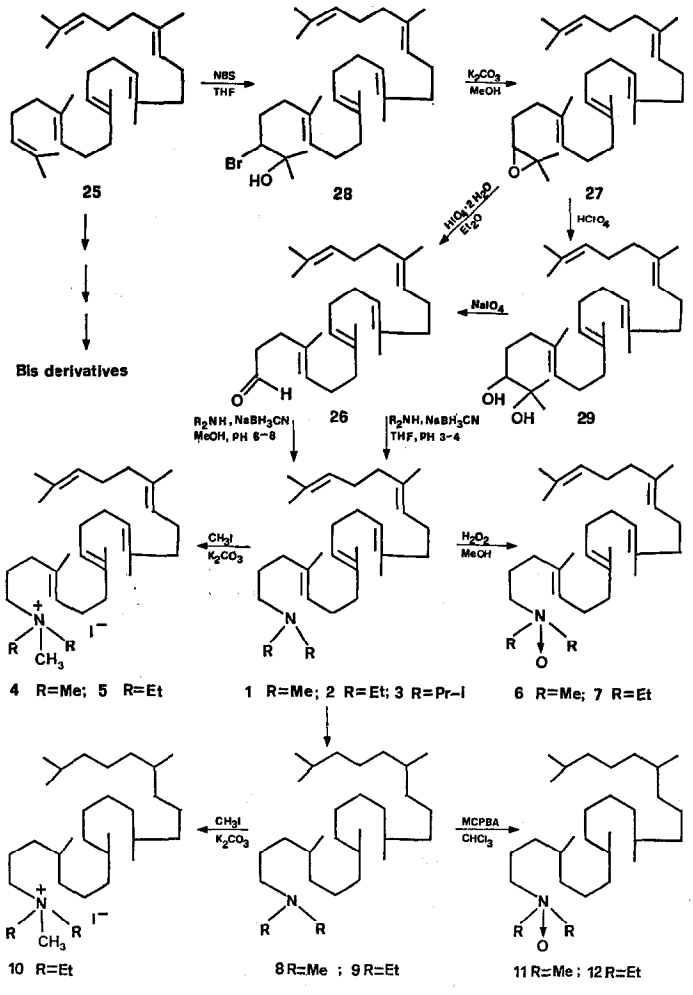
Abbreviations: **CMC:** critical micellar concentration; **DMSO:** dimethyl sulfoxide; **GTT:** germ test tube; IC_{50}: concentration of inhibitor producing 50% inhibition of SO cyclase in yeast microsomes; **MCPBA:** *meta*-chloroperbenzoic acid; ***MIC*:** the lowest concentration of the substances which inhibited visible growth of microorganisms; **NBS:** *N*-bromosuccinimide; **SO:** squalene 2,3-epoxide; **THF:** tetrahydrofuran.

and the anti-microbial activities of azasqualenes and related aza compounds **1**—**24**, including a discussion on structure—activity relationships.

Chemistry

General Remarks

The key feature of our plan for the synthesis of 2-aza-2,3-dihydrosqualene **1** and related aza compounds **1**—**12** involves the degradation of squalene **25** into the corresponding 1,1'2-*tris-nor*-squalene aldehyde **26**. This compound was then subjected to a reductive amination giving the corresponding aza derivatives **1**—**12** (Scheme 1).



Scheme 1

The synthesis of azasqualene derivatives **1**—**12** was partially reported in the experimental part, following general procedures in [29]. We have now improved the yields in the azasqualene series, by modifying two steps in the method of synthesis: degradation of squalene 2,3-epoxide **27** and reductive amination of aldehyde **26**. The purity of azasqualenes and related aza compounds was established by controls on TLC and microanalysis, except in the case of the ammonium derivatives. Yields, IR, ^{1}H NMR and mass spectra are reported in Tables I and II.

*Degradation of squalene (synthesis of 1,1',2-*tris-nor-*squalene aldehyde 26)*

The initial addition of hypobromous acid (*via* aqueous *N*-bromosuccinimide) to a terminal double bond of squalene, developed by van Tamelen [32, 33] allowed the selective formation of the terminal bromohydrin **28** (Scheme 1) [34].

Following Nadeau—Hanzlik's procedure [35], the halohydrin **28** would be transformed into epoxide **27** and then converted into 1,2-glycol **29** by treatment with aqueous perchloric acid in diglyme. Subsequent cleavage of squalene 2,3-diol **29** with sodium periodate gave 1,1'2-*tris-nor*-squalene aldehyde **26** [36]. In our hands, this method suffered from a low yield due to multistep reactions and prolonged column chromatography for the purification of **29**.

We describe herein a new method for a one step cleavage of **27** into the corresponding aldehyde **26**, using periodic acid in diethyl ether (Scheme 1). This procedure provides a new general way for the direct oxidation of lipophilic polyene epoxides to form aldehydes in organic medium.

Aldehyde **26** could be obtained from epoxide **27** in nearly quantitative yield (98%) and could be converted directly into azasqualenes without further purification. No 1,2-hydride migration [37] occurred during the reaction, as verified by ^{1}H NMR analysis.

The mechanism of this oxidation is still under investigation and will be discussed in a future publication.

*Reductive amination of 1,1',2-*tris-nor-*squalene aldehyde 26*

In our first attempt to synthesize the azasqualenes from 1,1',2-*tris-nor*-squalene aldehyde **26**, we utilized classical reductive amination as described by Borch [29, 38, 39]. This method proceeded smoothly with a variety of aldehydes and ketones by simply reacting the carbonyl compound with the appropriate amine at pH 6—8 in the presence of sodium cyanoborohydride in anhydrous methanol [40, 41] (Scheme 1). This procedure has proved satisfactory for the synthesis of 2-aza-2,3-dihydrosqualene **1** (45% yield). However, when the reductive amination was carried out with more sterically hindered amines, such as *N,N*-diethylamine or *N,N*-diisopropylamine, yields were very low (5%). This was very disappointing, since squalene diethylamine **2** and its derivative **7** were the most active members of this series as inhibitors of SO cyclase in animal and higher plant microsomes [29, 31]. Consequently, we decided to use different conditions (more acid pH, aprotic solvents).

Thus **26** was reacted with a suitable amine at pH 3—4 in the presence of sodium cyanoborohydride in an aprotic solvent (THF). Under these conditions, a dramatic increase in the yield (from 5 to 46%), together with a drastic reduction in the reaction time (from 70 h to 30 min), was observed.

Azasqualanes 8, 9, azasqualene N-*oxides 6, 7, azasqualane* N-*oxides 11, 12 and quaternary derivatives 4, 5 and 10*

Hydrogen peroxide in methanol was found to be the best

Table I. Method, yields and spectral data for azasqualenes, azasqualanes, *N*-oxides and quaternary derivatives.

Comp.	Method	Yield[a] [%]	Molec.formula or (lit.)	I.R. ν[cm^{-1}]	^{1}H-N.M.R. ($CDCl_3$/TMS int.) δ(p.p.m.)	M.S. (m/e)
1	A	45	(28)	($CHCl_3$)2965, 2925, 2870, 1730, 1670, 1450, 1380	1.54-1.63 (m, 20H, allylic CH_3 and CH_2-CH_2-N); 1.95-2.01 (m, 18H, allylic CH_2); 2.20-2.24 [m, 8H, CH_2-N(CH_3)$_2$]; 5.03-5.13 (m, 5H, vinylic CH)	found: M^+,413.4039. $C_{29}H_{51}N$ req. M, 413.4021. m/z: 413(M^+,25);344 (15);276(15);208(15);140(15);113 (7);58(100)
2	A	46	(28)	($CHCl_3$)2965, 2925, 2872, 1730, 1670, 1450, 1380	0.96 [t, 6H, J=7 Hz, (CH_3-CH_2)$_2$N]; 1.52-1.59 (m, 20H, allylic CH_3 and CH_2-CH_2-N); 1.90-1.98 (m, 18H, allylic CH_2); 2.33 (t, 2H, J=7 Hz, CH_2-CH_2-N); 2.47 [q, 4H, J=7 Hz, (CH_3-CH_2)$_2$N]; 4.98-5.07 (m, 5H, vinylic CH)	found: M^+,441.4356. $C_{31}H_{55}N$ req. M, 441.4334. m/z: 441(M^+,12);372 (8);304(10);236(12);168(40);86 (100)
3	A	42	(28)	($CHCl_3$)2965, 2925, 2870, 1730, 1670, 1450, 1380	1.10{d, 12H, J=7 Hz, [(CH_3)$_2$CH]$_2$N}; 1.58-1.61 (m, 20H, allylic CH_3 and CH_2-CH_2-N); 1.93-1.99 (m, 18H, allylic CH_2); 2.38 (t, 2H, CH_2-N); 2.95 {m, 2H, [(CH_3)$_2$CH]$_2$N}; 5.05-5.11 (m, 5H, vinylic CH)	found: M^+, 469.4652. $C_{33}H_{59}N$ req. M, 469.4647
4	G	b	(28)	($CHCl_3$)2960, 2920, 2875, 1665, 1610, 1450, 1380	1.52-1.63 (m, 20H, allylic CH_3 and CH_2-CH_2-N^+); 1.94-2.13 (m, 18H, allylic CH_2); 3.47-3.53 [m, 11H, $(CH_3)_3N^+$ and CH_2-N^+]; 5.05-5.20 (m, 5H, vinylic CH)	c
5	G	b	$C_{32}H_{58}NI$ (583.7)	($CHCl_3$)2960, 2920, 2875, 1665, 1610, 1450, 1380	1.37-1.64 [m, 26H, allylic CH_3, CH_2-CH_2-N^+ and (CH_2-CH_3)$_2$$N^+$]; 1.91-2.19 (m, 18H, allylic CH_2); 3.29 (s, 3H, CH_3-N^+); 3.58 [broad q, 6H, CH_2-N^+(CH_2-CH_3)$_2$]; 5.02-5.25 (m, 5H, vinylic CH)	c
6	D	92	$C_{29}H_{51}NO$ (429.7)	(liq. film)2970, 2920, 2860, 1700, 1670, 1450, 1385	1.55-1.67 (m, 20H, allylic CH_3 and CH_2-CH_2-N); 1.98-2.10 (m, 18H, allylic CH_2); 3.20-3.24 [m, 8H, CH_2-NO(CH_3)$_2$]; 5.09-5.19 (m, 5H, vinylic CH)	found: M^+,429.3962. $C_{29}H_{51}NO$ req. M, 429.3970. m/z: 429(M^+,2);413 (28);276(15);208(16);140(32);100 (20);58(100)
7	D	90	$C_{31}H_{55}NO$ (457.8)	(liq. film)2960, 2920, 2860, 1670, 1610, 1450, 1385	1.30 [t, 6H, (CH_3-CH_2)$_2$N]; 1.55-1.63 (m, 20H, allylic CH_3 and CH_2-CH_2N); 1.97-2.10 (m, 18H, allylic CH_2); 3.21 (t, 2H, CH_2-CH_2-N); 3.39 [q, 4H, (CH_3-CH_2)$_2$N]; 5.11-5.20 (m, 5H, vinylic CH)	found: M^+,457.4303. $C_{31}H_{55}NO$ req. M, 457.4283. m/z: 457(M^+,2);441 (2);276(4);168(30);140(29);112 (20);86(100)
8	C	94	$C_{29}H_{61}N$ (423.8)	(liq. film)2960, 2925, 2860, 1515, 1380	0.79-0.83 (m, 18H, alkylic CH_3); 0.95-1.40 (m, 35H, alkylic CH_2 and CH); 2.18-2.23 [m, 8H, CH_2-N(CH_3)$_2$]	found: M^+,423.4823. $C_{29}H_{61}N$ req. M, 423.4804. m/z: 423(M^+,28); 408(10); 281(5);219(6);169(11); 131(15);58(100)
9	C	91	$C_{31}H_{65}N$ (451.9)	($CHCl_3$)2960, 2920, 2860, 1465, 1380	0.79-0.84 (m, 18H, alkylic CH_3); 0.97-1.38 [m, 41H, alkylic CH_2, CH, and (CH_3-CH_2)$_2$N]; 2.35 (t, 2H, J=7 Hz, CH_2-CH_2-N); 2.54 [q, 4H, J=7 Hz, (CH_3-CH_2)$_2$N]	found: M^+,451.5113. $C_{31}H_{65}N$ req. M, 451.5117. m/z: 451(M^+,10); 436(8);112(5);86(100)
10	G	b	$C_{32}H_{68}NI$ (593.8)	($CHCl_3$)2960, 2920, 2875, 1460, 1375	0.78-0.85 (m, 18H, alkylic CH_3); 1.18-1.67 [m, 41H, alkylic CH_2, CH, (CH_3-CH_2)N^+ and CH_2-CH_2-N^+]; 3.28 (s, 3H, CH_3-N^+); 3.54 [broad q, 6H, CH_2-N^+(CH_2-CH_3)$_2$]	c
11	E	91	$C_{29}H_{61}NO$ (439.8)	(liq. film)2960, 2920, 2855, 1615, 1565, 1415, 1380, 1370	0.78-0.83 (m, 18H, alkylic CH_3); 1.04-1.35 (m, 35H, alkylic CH_2 and CH); 3.21-3.27 [m, 8H, CH_2-NO(CH_3)$_2$]	found: M^+,439.4760. $C_{29}H_{61}NO$ req. M, 439.4753. m/z: 439(M^+,2); 423(8);378(10);155(10);125(11); 111(12);58(100)
12	E	88	$C_{31}H_{65}NO$ (467.9)	(liq. film)2960, 2920, 2850, 1465, 1370	0.82-0.87 (m, 18H, alkylic CH_3); 1.08-1.42 [m, 41H, alkylic CH_2, CH and (CH_3-CH_2)$_2$N]; 3.15 (t, 2H, CH_2-CH_2-NO); 3.30 [q, 4H, (CH_3-CH_2)$_2$NC]	found M^+, 467.5085. $C_{31}H_{65}NO$ req. M, 467.5065. m/z: 467(M^+,2); 449(5);269(8);112(10);86(100)

[a] Yield of pure, isolated product.
[b] The crude product was not purified. It was sufficiently pure (TLC, IR and ^{1}H NMR analyses).
[c] Satisfactory electron impact mass spectrum could not be obtained.

Table II. Method, yields and spectral data for bis-azasqualenes, bis-azasqualanes, *N*-oxides and quaternary derivatives.

Comp.	Method	Yield[a] [%]	Molec. formula or (lit.)	I.R. ν [cm^{-1}]	^{1}H-N.M.R. ($CDCl_3$/TMS int.) δ(p.p.m.)	M.S. (m/e)
13	B	41	$C_{32}H_{60}N_2$ (472.8)	($CHCl_3$)2965, 2925, 2870, 1730, 1670, 1450, 1380	0.97 [t, 12H, J=7 Hz, 2(CH_3-CH_2)$_2$N]; 1.55-1.63 (m, 16H, allylic CH_3 and CH_2-CH_2-N); 1.90-2.05 (m, 16H, allylic CH_2); 2.38 (t, 4H, J=8 Hz, $2CH_2$-CH_2-N); 2.51 [q, 8H, J=7Hz, 2(CH_3-CH_2)$_2$N]; 5.00-5.19 (m, 4H, vinylic CH)	found M^+,472.4740. $C_{32}H_{60}N_2$ req. M, 472.4756. m/z: 473 (M^+,21);458(15);445(15);444 (33);387(37);374(10);305 (19);236(21);168(100);112 (45);99(95)
14	G	b	$C_{34}H_{66}N_2I_2$ (756.7)	($CHCl_3$)2960, 2920, 2870, 1660, 1620, 1450, 1380	1.36-1.70 [m, 28H, allylic CH_3, $2CH_2$-CH_2-N^+, and 2(CH_3-CH_2)$_2N^+$]; 1.88-2.20 (m, 16H, allylic CH_2); 3.30 (s, 6H, $2CH_3$-N^+); 3.58 [broad q, 12H, $2CH_2$-N^+(CH_2-CH_3)$_2$]; 5.00-5.19 (m, 4H, vinylic CH)	c
15	D	87	$C_{32}H_{60}N_2O_2$ (504.8)	($CHCl_3$)2965, 2920, 2860, 1700, 1665, 1450, 1380	1.28 [t, 12H, 2(CH_3-CH_2)$_2$N]; 1.54-1.63 (m, 16H, allylic CH_3 and $2CH_2$-CH_2-N); 1.93-2.09 (m, 16H, allylic CH_2); 3.20 (t, 4H, $2CH_2$-CH_2N); 3.40 [q, 8H, 2(CH_3-CH_2)$_2$N]; 5.06-5.20 (m, 4H, vinylic CH)	c
16	C	88	$C_{32}H_{68}N_2$ (480.9)	($CHCl_3$)2960, 2920, 2860, 1465, 1380	0.78-0.83 (m, 12H, alkylic CH_3); 0.96-1.40 [m, 44H, alkylic CH_2, CH and (CH_3-CH_2)$_2$N]; 2.35 (t, 4H, $2CH_2$-CH_2N); 2.50 [q, 8H, 2(CH_3-CH_2)$_2$N]	found M^+,480.5375. $C_{32}H_{68}N_2$ req. M, 480.5382. m/z: 480 (M^+,5);425(2);112(5);86(100)
17	G	b	$C_{34}H_{74}N_2I_2$ (764.8)	($CHCl_3$)2960, 2920, 2870, 1460, 1380	0.80-0.86 (m, 12H, alkylic CH_3); 1.16-1.63 [m, 44H, alkylic CH_2, CH, $2CH_2$-CH_2-N^+ and 2(CH_3-CH_2)$_2N^+$]; 3.25 (s, 6H, $2CH_3$-N^+); 3.52-3.59 [m, 12H, $2CH_2$-N^+(CH_2-CH_3)$_2$]	c
18	F	84	$C_{32}H_{68}N_2O_2$ (512.9)	($CHCl_3$)2960, 2920, 2860, 1550, 1470	0.80-0.85 (m, 12H, alkylic CH_3); 1.05-1.40 [m, 44H, alkylic CH_2, CH and 2(CH_3-CH_2)$_2$N]; 3.12 (t, 4H, $2CH_2$-CH_2NO); 3.34 [q, 8H, 2(CH_3-CH_2)$_2$NO]	c

[a] Yield of pure, isolated product.
[b] The crude product was not purified. It was sufficiently pure (TLC, IR and ^{1}H NMR analyses).
[c] Satisfactory electron impact mass spectrum could not be obtained.

reagent for the synthesis of azasqualene *N*-oxides **6**, **7** (Scheme 1) [42—45]. The reaction proceeded smoothly at room temperature (12 h), affording *N*-oxides **6**, **7** in high yields (90—92%).

The synthesis of azasqualanes **8**, **9** and azasqualane *N*-oxides **11**, **12** was accomplished by hydrogenation of the corresponding azasqualene derivatives with palladium on charcoal in ethanol [46], followed by oxidation of the parent tertiary amine with MCPBA [47—50] in chloroform.

Quaternary derivatives **4**, **5** and **10** were obtained by conventional procedure [51], by heating under reflux tertiary amines **1**, **2** or **9** with K_2CO_3 and CH_3I in absolute ethanol.

Bis-azasqualenes 13—15, bis-azasqualanes 16—18 and other aza compounds 19—24

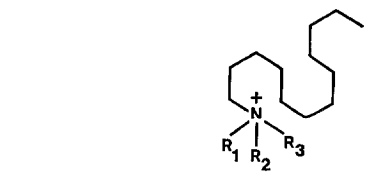
For the preparation of these compounds, we followed the same strategy utilized for the synthesis of azasqualenes **1**—**5**, azasqualanes **8**—**10** and the corresponding *N*-oxides **6**, **7** and **11**, **12**, starting from squalene dibromohydrin **30** (Schemes 1 and 2). Thus, by reacting squalene **25** with a double molar equivalent of *N*-bromosuccinimide in aqueous THF, we obtained squalene dibromohydrin **30**, which was transformed into the corresponding squalene diepoxide **31** [32, 52]. The subsequent cleavage of **31** by $HIO_4 \cdot 2H_2O$ in diethyl ether afforded C_{24} dialdehyde **32** in high yield.

Bis-azasqualene derivative **13** was obtained by reductive amination of **32** with the suitable aliphatic secondary amine in accordance with the described procedure.

Catalytic hydrogenation on Pd/C of bis-azasqualene derivative **13** afforded the corresponding bis-azasqualane derivative **16**. Bis-azasqualene *N*-oxide **15** and bis-azasqualane *N*-oxide **18** were synthesized by oxidation of the parent tertiary amines with 30% H_2O_2 in methanol or with MCPBA in $CHCl_3$, respectively. The bis-quaternary compounds **14** and **17** were prepared by conventional procedure, as described for **4**, **5** and **10**. Diethylation of dodecylamine with diethyl sulfate gave *N*,*N*-diethyldodecylamine **20**.

The corresponding *N*-oxide **24** was obtained by using MCPBA as previously described.

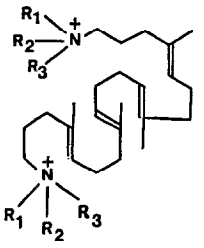
Quaternarization of **19** and **20** to give **21** and **22** was accomplished as described for squalene ammonium derivatives **4**, **5** and **10**.



19 $R_1=R_2=Me$, $R_3=H$ 22 $R_1=R_2=Et$, $R_3=Me$

20 $R_1=R_2=Et$, $R_3=H$ 23 $R_1=R_2=Me$, $R_3=O^-$

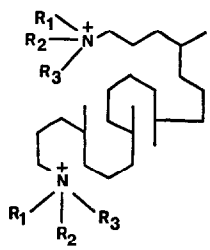
21 $R_1=R_2=R_3=Me$ 24 $R_1=R_2=Et$, $R_3=O^-$



13 $R_1=R_2=Et$, $R_3=H$

14 $R_1=R_2=Et$, $R_3=Me$

15 $R_1=R_2=Et$, $R_3=O^-$



16 $R_1=R_2=Et$, $R_3=H$

17 $R_1=R_2=Et$, $R_3=Me$

18 $R_1=R_2=Et$, $R_3=O^-$

Scheme 2

Results and Discussion

MIC values for azasqualenes and derivatives **1**, **2**, **4**, **7**, **8**, **11**, **13**—**18**, **21**, **23** against different strains of gram-positive and gram-negative bacteria, as well as against dermatophytes, moulds, biphasic fungi and yeasts are shown in Tables III and IV. The effect of azasqualenes and derivatives on *Candida albicans* mycelia formation (germ tube test) is shown in Table V. The GTT was carried out in order to verify the azasqualene ability to inhibit the *Candida albicans* mycelial phase, which could be regarded as the true infective form in microorganisms ([5] and ref. therein, [53—55]). Indeed, it seems that formation of mycelia was the major way for pathogenic fungi to escape intracellular killing by leukocytes, which become necrotic and are killed by their interaction with the fungus [55].

In a preliminary study [31], it was established that the azasqualenes and derivatives inhibited squalene 2,3-epoxide cyclase in microsomes obtained from *Saccharomyces cerevisiae* and that the activity decreased in the following order: bis-azasqualene ammonium derivative **14** and bis-azasqualene *N*-oxide **15** > bis-azasqualene **13** > azasqualene **2** and azasqualene *N*-oxide **7**.

Before attempting a discussion on the structure—activity relationship of azasqualenes and derivatives, the possible 'non specific' action of these compounds must be taken into account. Indeed, azasqualenes, at physiological pH, possess the two basic features of cationic surfactant agents: a hydrophobic alkyl chain together with a positively charged hydrophilic group, and thus could act on the fungal cytoplasmic membrane as surfactants.

For this reason, we decided to test the critical micellar concentration *(CMC)* of azasqualenes and derivatives in the concentration range used for our biological experiments. Plots of rhodamine 6G fluorescence *vs* compound concentration showed no changes up to 0.4 mM, indicating that azasqualenes and derivatives did not form micelles in this concentration range. The *CMC* values determined with a fluorescent probe, reported in the literature ([56] and ref. therein) for cationic surfactants such as CTAB (hexadecyltrimethylammonium bromide) and TTAB (tetradecyltrimethylammonium bromide), varied from 2.5—3.6 mM (TTAB) to 0.40—0.47 mM (CTAB). Alternatively, these compounds could show anti-fungal and anti-bacterial activities by adsorption onto the negatively charged cell wall, followed by interaction with hydrophobic portions of oriented phospholipids, leading to the disorganization of the membrane.

Several biophysical techniques have been used, in order to study the interaction of various membrane effectors with natural or artificial membranes [57, 58].

In the present study, we adopted as the initial model a chemically defined bilayer membrane system: the large unilamellar liposomes, prepared from phosphatidyl choline (PC)/cholesterol (1:1 ratio) [59], and we studied the increasing permeability of an encapsulated fluorescent probe (6-carboxyfluorescein). By plotting fluorescence change

Table III. *MIC* values (μg/ml) of azasqualenes and derivatives against different strains of gram-positive and gram-negative bacteria.

Compd	*Staphylococcus aureus* ATCC 10832	*Staphylococcus aureus* ATCC 23067	*Staphylococcus faecalis* NCIB 775	*Pseudomonas aeruginosa* ATCC 29511	*Escherichia coli* S 120	*Proteus mirabilis* NCIB 60
1	6.25	6.25	5	> 100	> 100	> 100
2	10	10	10	100	> 100	> 100
7	10	> 100	5	100	> 100	100
8	100	100	100	> 100	> 100	> 100
11	100	> 100	> 100	100	> 100	100
13	10	5	5	10	> 100	100
14	1.25	1.25	2.5	50	100	50
15	10	10	10	50	> 100	100
16	10	10	10	> 100	> 100	> 100
17	3.12	3.12	6.25	100	25	100
18	50	100	10	> 100	> 100	> 100
23	100	> 100	100	> 100	> 100	> 100

Table IV. *MIC* values (μg/ml) of azasqualenes and derivatives against dermatophytes, moulds, biphasic fungi and yeasts.

Compd	*Trichophyton mentagrophytes* S 158	*Microsporum canis* S 150	*Aspergillus fumigatus* S 159	*Sporotrichum schenckii* S 177	*Candida albicans* S 124	*Candida albicans* S 9	*Torulopsis glabrata* S 244
1	12.5	12.5	100	25	50	25	50
2	100	100	> 100	> 100	100	100	100
4	6.25	6.25	25	6.25	6.25	6.25	12.5
7	> 100	> 100	> 100	> 100	200	200	> 200
8	50	100	> 100	> 100	> 200	> 200	> 200
11	> 100	> 100	> 100	> 100	> 200	> 200	> 200
13	25	50	25	50	25	50	25
14	25	50	50	50	25	25	25
15	100	100	> 100	> 100	200	100	100
16	50	50	> 100	25	100	50	100
17	3.12	3.12	50	3.12	3.12	50	3.12
18	> 100	> 100	> 100	> 100	> 200	> 200	> 200
21	50	50	100	> 100	25	12.5	50
23	50	50	> 100	> 100	200	100	100

Table V. Germ tube test of azasqualenes and derivatives on *Candida albicans* (S 124) (μg/ml).

Compd	Value	Compd	Value	Compd	Value
1	12.5	**11**	> 100	**17**	3.12
2	6.25	**13**	25	**18**	50
4	3.12	**14**	< 1.56	**21**	25
7	25	**15**	12.5	**23**	12.5
8	> 100	**16**	25		

as a function of the added azasqualene concentration, we obtained an index of membrane perturbation [2, 60, 61].

Results are given in Figs. 1 and 2. These indicate that the quaternary ammonium compounds are particularly powerful in inducing leakage of 6-carboxyfluorescein from liposomes and that azasqualenes and derivatives bearing a double tertiary amine or quaternary ammonium function displayed a higher activity. The simple 2-aza-2,3-dihydrosqualene **1** and the corresponding *N*-oxide **6** affected the liposomal membrane to a much lesser extent, at least at a high phospholipid/compound ratio. As expected, when this latter ratio decreases, the compounds become more powerful. A similar cytotoxic behavior of **1** was noted with 3T3 fibroblasts, which varied, depending upon cell concentration [62]. As already seen for miconazole and derivatives [1—9], azasqualenes, in addition to their anti-fungal activities, are active against gram-positive bacteria. Since bacteria do not contain ergosterol in their cell membranes,

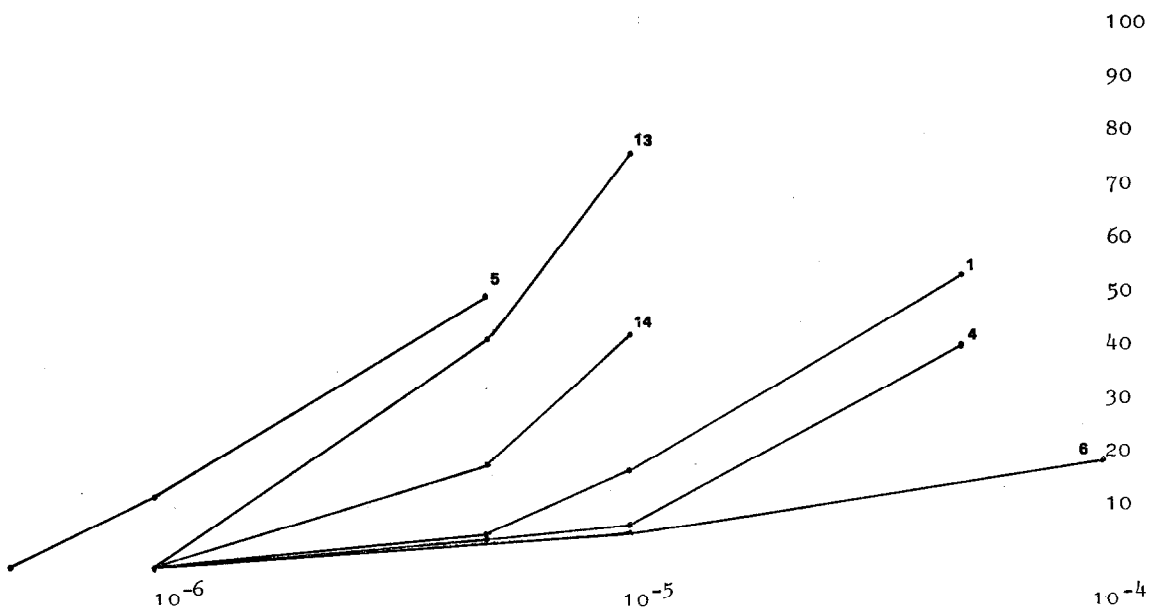


Fig. 1. Activity of azasqualenes and derivatives on liposomes. Liposome concentration = 1.6 mM; abscissa: compound concentration (M); ordinate: percent of fluorescence compared to total leakage induced by Triton X—100 (final concentration = 0.5%).

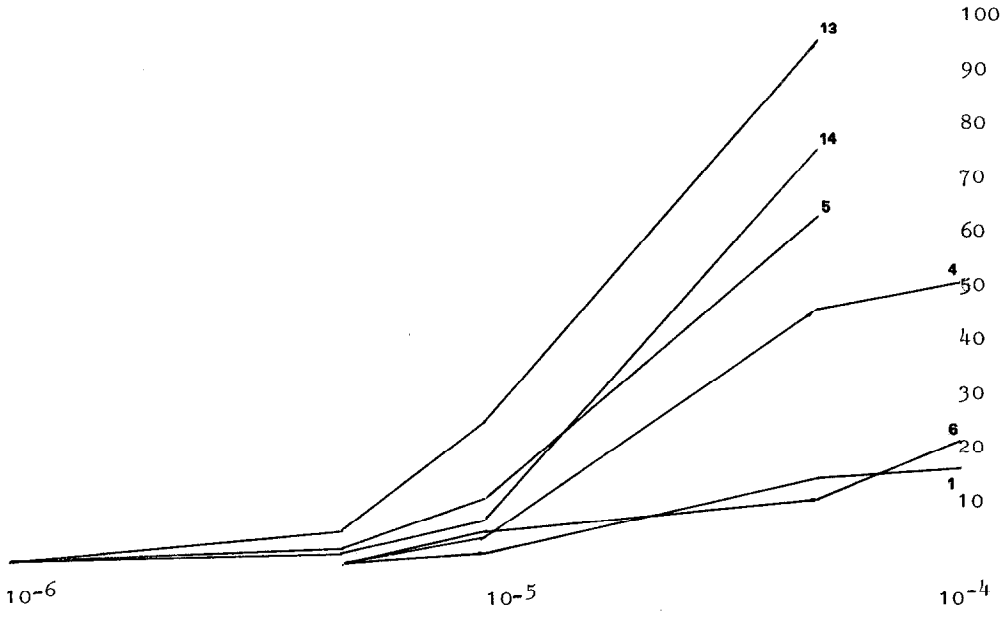


Fig. 2. Activity of azasqualenes and derivatives on liposomes. Liposome concentration = 16 mM; abscissa: compound concentration (M); ordinate: percent of fluorescence compared to total leakage induced by Triton X—100 (final concentration = 0.5%).

it is clear that azasqualenes affect other cellular functions. Gram-positive bacteria, in comparison with fungi or gram-negative bacteria, have quite a simple cell wall, composed of a rigid peptidoglycane layer, which allows foreign molecules to enter without much difficulty [63]. Thus, amphiphilic molecules such as azasqualenes, which are positively charged at physiological pH could easily interact with the negatively charged bacterial cell membrane, inducing a change in lipid organization, similar to that observed in liposomes.

In order to study the structure—activity relationships, we decided to modify the structure of azasqualenes: 1) by saturating the double bond system; 2) by oxidating the tertiary amino group to *N*-oxide; 3) by introducing an additional polar group to the basic squalene carrier. By hydrogenation of the squalene carrier in the azasqualene molecules, both anti-bacterial and anti-fungal activities were almost completely lost. The *N*-oxide derivatization, which usually resulted in higher inhibitory activities of animal and higher plant sterol biosyntheses [28, 31], greatly reduced the anti-microbial activity, without affecting the capacity to inhibit mycelium formation (Tables III, IV and V). Quaternarization, or the doubling of the tertiary amine function in the azasqualene molecules, enhanced the non specific activity against fungi, gram-positive and also gram-negative bacteria, as exemplified by squalene bis-diethylmethylammonium iodide **14**, which proved to be the most active compound of the series (Tables III, IV and VI). The modification of the structure of azasqualene led to a loss of the anti-microbial activity and to an increased 'non specific' activity, the only exception being the *N*-oxide derivatives, which showed a specific action as inhibitors of mycelium formation. Presumably, a combination of

Table VI. *MIC* values (μg/ml) of squalene diethylmethylammonium iodide **14** against different strains of gram-positive and gram-negative bacteria.

Bacterium (G^+)	*MIC*	Bacterium (G^+)	*MIC*
Staph. aureus		*Strept. pyogenes*	
S'G 511	2.5	ATCC 29218	1.563
Smith ATCC 29506	2.5	NCIB 8884	3.125
209P	2.5	*Strept. aronson*	
ATCC 10390	5	ATCC 29217	3.125
S 555	2.5	*Strept. pneumoniae*	
S 736	2.5	ATCC 6301	3.125
S 1147	2.5	*Strept. faecalis*	
S 1231	2.5	ATCC 29505	6.25
Staph. epidermis		S 872	6.25
NCIB 8853	0.781		

Bacterium (G^-)	*MIC*	Bacterium (G^-)	*MIC*
Pseudom. aeruginosa		*Enterob. cloacae*	
NCIB 950	50	ATCC 15012	> 100
S 493	100	ATCC 23355	> 100
E. coli		S 964	100
S 2	100	*Enterob. agglomerans*	
S 71	100	S 581	100
S 118	> 100	*Serratia marcescens*	
S 170	100	ATCC 9103	> 100
Salm. typhimurium		ATCC 25179	> 100
ATCC 29509	100	NCIB 1377	> 100
Klebsiella pneumoniae		*Proteus mirabilis*	
ATCC 29519	> 100	S 644	> 100
ATCC 29518	12.5	S 1438	> 100
ATCC 29516	> 100	*Proteus vulgaris*	
ATCC 10031	12.5	ATCC 29513	100
S 1133	> 100	ATCC 13315	100
		Proteus morganii	
		NCIB 232	> 100

sterol deficiency and heavy intracellular accumulation of squalene or squalene 2,3-epoxide [30] could deeply affect the formation of yeast mycelia, whereas budding cells seem to be more resistant.

In conclusion, though the effects of azasqualene derivatives on sterol biosynthesis have been amply demonstrated [29—31], their relation to the growth inhibitory action on fungi remains to be clarified.

Indeed, as previously shown by some other anti-fungal drugs [1—9], azasqualene derivatives showed a 'non specific' component in their biological actions, which appeared both in their anti-bacterial activities and in their capacities to induce leakage from liposomes. These activities increased for the bis-functional derivatives **13** and **14**, which could be similar to those found for the cationic analogue amphiphilic molecules as membrane perturbators.

Experimental protocols

^{1}H NMR spectra were recorded either 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 the internal standard. Mass spectra were performed either on a Kratos MS 80 or on a VG analytical 7070 EQ-HF spectrometer by electron impact, in high resolution (6000), electron energy 70 eV, trap current 100 μA, spring temperature 230°C. IR spectra were recorded either on a Perkin—Elmer 267 or on a Perkin—Elmer 781.

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

N,N-Dimethyldodecylamine **19** and the corresponding *N*-oxide **23** were availably commercially.

For isolation, purification and identification, the following techniques were used: 1) column 'flash chromatography' [64]: an air pressure column chromatography which has been optimized for rapid separations. A column of appropriate diameter is selected and filled with 15—30 cm of the appropriate silica gel (230—400 mesh), the sample is introduced and the column is eluted at a high flow rate. 2) Thin—layer chromatography (TLC): Merck silica gel 60 F_{254}, 0.2 mm coated plates for analytical purposes were used. After development, the plates were exposed to iodine vapors.

Chemistry

Squalene monobromohydrin: 3-bromo-2,6,10,15,19,23-hexamethyl-6,10,14,18,22-tetracosapentaen-2-ol (all E*) **28***

Squalene (41.1 g: 0.10 mol) was dissolved in THF (250 ml), upon cooling at 0°C, and then water was added until the solution became opalescent. Then a small amount of THF was added to clear the solution. NBS (21.4 g: 0.12 mol) was added, over a period of 30 min at 0°C, and then left for 1 h at room temperature, under stirring. The product was worked up in the usual way and the resulting oil was purified by 'flash chromatography' (petroleum ether to remove all the unreacted squalene, then petroleum ether/diethyl ether, 95:5) to give 17.8 g (35%) of **28** as a pale yellow oil.

Squalene 2,3-epoxide: 22,23-epoxy-2,6,10,15,19,23-hexamethyl-2,6,10,14,18-tetracosapentaene (all E*) **27***

K_2CO_3 (13.8 g: 0.10 mol) was dissolved in methanol (300 ml), squalene monobromohydrin **28** (25.4 g: 0.05 mol) was added, and the mixture was stirred for 2 h at room temperature. The product was worked up in the usual way; it was obtained in an essentially pure form and was used directly in the next step.

*1,1',2-*Tris-nor*-squalene aldehyde: 4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenal (all* E*) **26***

$HIO_4 \cdot 2H_2O$ (13.7 g: 0.06 mol) was quite completely dissolved in diethyl ether (600 ml), under vigorous stirring, the crude squalene 2,3-epoxide was added and allowed to react for 10 min, under stirring. The product was worked up in the usual way and the resulting oil was purified by 'flash chromatography' (petroleum ether/diethyl ether, 95:5), to give 19.2 g (96% yield from **28**) of **26** as a colorless oil.

Squalene dibromohydrin: 3,22-dibromo-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraen-2,23-diol (all E*) **30***

Squalene (41.1 g: 0.10 mol) was dissolved in THF (400 ml), upon cooling at 0°C and water was added until the solution became opalescent. Then a small amount of THF was added to clear the solution. NBS (35.6 g: 0.20 mol) was added, over a period of 30 min and then left for 1 h at room temperature, under stirring. The product was worked up in the usual way and the resulting oil was purified by 'flash chromatography' (petroleum ether to remove traces of unreacted squalene, then petroleum ether/diethyl ether, 95:5 for monobromohydrin, finally petroleum ether/diethyl ether, 90:10), to give 18.1 g (30%) of **30** as a pale yellow oil.

Squalene 2,3:22,23-diepoxide: 2,3:22,23-diepoxy-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraene (all E*) **31***

30 (30.2 g: 0.05 mol) was treated with K_2CO_3 (25.7 g: 0.2 mol), under the same conditions as described for **27**. The crude product was obtained in an essentially pure form and was used directly in the next step.

C_{24}-Dialdehyde: 4,8,13,17-tetramethyl-4,8,12,16-icosatetraendial (all E*) **32***

The crude squalene 2,3:22,23-diepoxide **31** was treated with $HIO_4 \cdot 2H_2O$ (27.4 g: 0.12 mol), reacted and purified under the same conditions as described for **26**, to give 16.8 g of **32** as a colorless oil (94% yield from **30**).

Method A. Squalene diethylamine: N,N*-diethyl-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenylamine (all* E*) **2***

1,1',2-*Tris-nor*-squalene aldehyde **26** (1.0 g: 2.6 mmol) was dissolved in anhydrous THF (20 ml) and diethylamine (1.9 g: 26 mmol) was added under stirring at 0°C. A solution of HCl in anhydrous THF was added dropwise up to pH 3, followed by $NaBH_3CN$ (163 mg: 2.6 mmol). After stirring at room temperature for 30 min, the product was worked up in the usual way and purified by 'flash chromatography' (methanol as eluant) to give 528 mg (46%) of **2** as a colorless oil.

Method B. Squalene bis-diethylamine: 3,24-diethyl-7,11,16,20-tetramethyl-3,24-diaza-7,11,15,19-hexacosatetraene (all E*) **13***

32 (1 g: 2.8 mmol) was treated with $NaBH_3CN$ (352 mg: 5.6 mmol), under the same conditions as described for **2** and purified in the same way to give 542 mg of **13** (41% yield).

Method C. Squalane diethylamine: N,N*-diethyl-4,8,13,17,21-pentamethyldocosylamine **9***

Pd/C (10% Pd; 50 mg) was added to absolute ethanol (100 ml), under stirring and then the suspension was put under H_2 atmosphere. Squalene diethylamine **2** (1 g: 2.26 mmol) was added and left for 24 h under H_2 atmosphere, upon stirring. The suspension was filtered to remove the catalyst and concentrated *in vacuo*. The crude product was purified by a short 'flash chromatography' (methanol as eluant), to give 929 mg of **9** (91% yield), as a colorless viscous oil.

Method D. Squalene diethylamine N*-oxide:* N,N*-diethyl-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenilamine* N*-oxide (all* E*) **7***

Squalene diethylamine **2** (300 mg: 0.679 mmol) was dissolved in methanol (1 ml) and 30% H_2O_2 was added. The solution immediately became turbid. The reaction mixture was left for 12 h at room temperature, under stirring; during this time the reaction mixture cleared. Petrol ether (50 ml) was added, the biphasic system was cooled to 0°C, vigorously stirred, and MnO_2 was added in catalytic amounts to decompose H_2O_2. When the decomposition of H_2O_2 was complete, the suspension was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by 'flash chromatography' (methanol as eluant), to give 280 mg (90% yield) of **7** as a colorless oil.

Method E. Squalane diethylamine N-*oxide:* N,N-*diethyl-4,8,13,17,21-pentamethyldocosylamine oxide* **12**
Squalane diethylamine **9** (300 mg: 0.664 mmol) was dissolved in chloroform (5 ml) and MCPBA (244 mg: 1.416 mmol) in chloroform (5 ml) was added at 0°C, under stirring. After 1 h, the reaction mixture was washed with $NaHCO_3$ 10% (50 ml × 3), saturated brine (50 ml × 2), dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by 'flash chromatography' (methanol as eluant), to give 273 mg (88% yield) of **12** as a colorless oil.

Method F. Squalane bis-diethylamine N-*oxide: 3,24-diethyl-7,11,16,20-tetramethyl-3,24-diazahexacosane-3,24-dioxide* **18**
16 (300 mg: 0.624 mmol) was treated with MCPBA (323 mg: 1.872 mmol), under the same conditions as described for **12** and purified in the same way, to give 269 mg of **18** (84% yield).

Method G. Squalene diethylmethylammonium iodide: diethylmethyl-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenylammonium iodide (all E) **5**
Squalene diethylamine **2** (100 mg: 0.226 mmol), absolute ethanol (10 ml), K_2CO_3 (625 mg: 4.52 mmol), CH_3I (321 mg: 2.26 mmol) were added sequentially and the reaction mixture was heated under reflux for 15 h. After evaporation of ethanol, water (50 ml) was added, the reaction mixture extracted with dichloromethane (50 ml × 3), dried over anhydrous Na_2SO_4 and evaporated *in vacuo*, to give 132 mg of **5**.

N,N-*Diethyldodecylamine* **20**
Diethyl sulfate (1.4 ml: 10.8 mmol) was added to dodecylamine (1g: 5.39 mmol) and heated at 100°C for 2 days. The reaction mixture was cooled, added to an aqueous solution of KOH (10%) and extracted with dichloromethane (50 ml × 3). The organic phases were washed with 10% KOH (50 ml × 2), saturated brine (50 ml × 3), dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The crude product was purified by 'flash chromatography' (methanol as eluant), to give 243 mg (21% yield) of **20**.

Microbiology

Anti-bacterial activity
The anti-bacterial activity *in vitro* of azasqualenes and derivatives **1, 2, 7, 8, 11, 13—18, 23** was investigated against gram-positive and gram-negative bacteria (Table III).

Minimal inhibitory concentrations *(MIC)* were determined in trypticase soy broth (BBL) in a microsystem (Dynatech MIC 200). Substances were dissolved in DMSO and further diluted in 2 steps in broth. Bacterial strains (Sandoz Research Institute Culture Collection) were stored in liquid nitrogen and the inoculum was prepared immediately after thawing, by adjusting the concentration to 10^5—10^6 cfu/ml in broth. After an 18 h incubation period at 37°C, the *MIC* was determined.

Test compound **14**, which showed the highest anti-bacterial activity, was subjected to the *in vitro* II screen (Table VI) and *MIC* was determined.

Anti-fungal activity
The *in vitro* anti-fungal activity of tested compounds was determined against dermatophytes, moulds, biphasic fungi and yeasts (Table IV).

Yeast blastospores were obtained from a 30 h shaken culture incubated at 30°C in Sabouraud dextrose 2% broth, whereas the filamentous fungi inoculum was scraped with a spatula from a 21 day old culture on Kimmig's agar. This macerate was then finely suspended in Sabouraud dextrose 2% broth, using a glass homogenizer.

The minimal inhibitory concentration *(MIC)* was determined by a serial dilution technique using Sabouraud dextrose broth (pH 6.8). The test compounds were dissolved in DMSO and further diluted with a dilution factor of two on autotray-microtiter plates and 0.1 ml of the corresponding dilution plus 50 μl of the fungal culture were added to 50 μl of sterile medium. The plates were incubated for 7 days (dermatophytes and dimorphic fungi), 72 h (moulds) or 48 h (yeasts), at 30°C and 60% relative humidity. The *MIC* was taken to be the lowest concentration of test substance in the nutrient medium at which, compared with growth controls, non macroscopic signs of fungal growth were detectable.

Germ tube test inhibition
The GTT was defined as the lowest concentration of the tested compounds which totally suppressed the formation of mycelia when blastospores of *Candida albicans* (500 cfu ml^{-1}) were incubated for 24 h at 37°C in a 5% CO_2 atmosphere (Table V).

The GTT was carried out by serial dilution with a microtitration technique using microtiter plates filled with MEM^{++} medium (Gibco nr 410—1100 + 0.292 g glutamine IL + 2 g $NaHCO_3$/l + 10% fetal calf serum) and inoculated with blastospores of *Candida albicans* (500 cfu ml^{-1}). The plates were read using an inverted microscope after an incubation period of 24 h at 37°C in a 5% CO_2 atmosphere. As a control, a strain of *Candida albicans* (S 124) was chosen, which upon cultivation in Eagle's medium containing fetal calf serum, was able to form mycelia from the inoculated blastospores.

Surfactant activity
Surfactant activity of azasqualene derivatives **1, 4—6, 13, 14** was assayed using a fluorimetric method, with cationic rhodamin 6G as the fluorescent probe [56]. Fluorimetric titrations were performed by several additions of small aliquots of the test compound to a solution of rhodamine 6G at 25°C. The excitation and the emission wavelengths were at 480 and 550 nm, respectively. No change in fluorescence is observed as long as only monomers are present in solution. In the presence of micelles, fluorescence is quenched with a linear dependency on the surfactant concentration. The intersection of the two straight lines gives the critical micellar concentration *(CMC)*.

Membrane perturbation
The induction of liposome leakage by azasqualenes and derivatives **1, 4—6, 13, 14**, was studied using large unilamellar vesicles [59] which had encapsulated 40 mM 6-carboxyfluorescein [60, 61]. The concentration-dependent self-quenching of 6-carboxyfluorescein permits leakage from liposomes to be monitored continuously [65]. The entrapment of the compounds in the vesicles was obtained when an aqueous phosphate buffer containing compounds to be encapsulated was introduced into a mixture of phospholipid (egg PC [66] and cholesterol 50:50 mol/mol) and organic solvent (diethyl ether). Subsequently, the two phase system was briefly sonicated and then the organic solvent evaporated under reduced pressure. The vesicles were separated from free dye by gel filtration over a Sephadex G—75 column eluted with 5 mM HEPES, 100 mM NaCl (pH 7.4). Lipid phosphate was determined according to Bartlett [67]. Leakage experiments were performed at 25°C, with suspended vesicles (1.6 or 16 mM phospholipid) in 2 ml of phosphate buffered saline (pH 7.4). Change of fluorescence, after addition of emulsions in the same buffer of azasqualenes and derivatives was recorded at λ = 520 nm (λ_{exc} = 490 nm) [60]. It was found convenient to plot the fluorescence change obtained five min after the addition of azasqualenes as a function of azasqualene concentration. The 100% fluorescence setting was obtained with a vesicle preparation containing 0.5% (v/v) Triton X—100.

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