Phytochemistry, Vol. 26, No. 10, pp. 2709–2714, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00
Pergamon Journals Ltd.

INHIBITION OF STEROL BIOSYNTHESIS AND ACCUMULATION OF 2,3-OXIDOSQUALENE IN BRAMBLE CELL SUSPENSION CULTURES TREATED WITH 2-AZA-2,3-DIHYDRO-SQUALENE AND 2-AZA-2,3-DIHYDROSQUALENE-*N*-OXIDE

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(*Received* 21 *November* 1986)

Key Word Index—*Rubus fruticosus*; Rosaceae; oxidosqualene cyclase; 2-aza-2,3-dihydrosqualene-*N*-oxide.

Abstract—2-Aza-2,3-dihydrosqualene (**1**), 2-aza-2,3-dihydro-squalene-*N*-oxide (**2**) and derivatives are new compounds designed to inhibit the 2,3-oxidosqualene-cycloartenol (lanosterol) cyclase. The effects of these compounds were studied on sterol biosynthesis in suspension cultures of bramble cells. Both **1** and **2** inhibited the growth of cells with an IC_{50} of 11 μM for **1** and 21 μM for **2**. When the cells grown in the presence of the two drugs were analysed, accumulation of squalene and 2,3-oxidosqualene was observed but no significant decrease of the total sterol content per g of dry weight of cells was noticed. Pulse experiments with [2-^{14}C]acetate on 15-day-old cells treated with **1** resulted in a strong decrease of the incorporation of radioactivity into the 4-desmethyl sterol fraction. An IC_{50} of 7.5 μM was determined when the cells were preincubated for a period of two hr with **1** or **2**. This inhibition was correlated with an accumulation of [^{14}C]-2,3-oxidosqualene and of [^{14}C]-squalene. No [^{14}C]-2,3:22(23)-dioxidosqualene was detectable in these conditions. Derivatives of **1** and **2** or similar compounds were also assayed; *N*-lauryl-dimethylamino-*N*-oxide (LDAO) was shown to be particularly effective and produced accumulation of enormous amounts of [^{14}C]-2,3-oxidosqualene. Compound **1** (but not **2** or LDAO) leads also to the accumulation of a red pigment identified as lycopene. Our work confirms studies with enzymatic systems in demonstrating that **1**, **2** and LDAO inhibit the 2,3-oxidosqualene-cycloartenol cyclase and provides evidence that the squalene synthetase and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase are also inhibited.

INTRODUCTION

Recent work performed in this laboratory showed that 2-aza-2,3-dihydrosqualene (**1**) [1–3] and 2-aza-2,3-dihydrosqualene-*N*-oxide (**2**) [4], two rationally designed 2,3-oxidosqualene (OS) cyclase inhibitors, strongly inhibited OS-β-amyrin- [1–4], OS-cycloartenol- [3], and OS-lanosterol-cyclases [3, 4]. It has been suggested that **1** and **2** could be high energy intermediate (HEI) and transition state (TS) analogue inhibitors, respectively (Fig. 1) [1–4]. Although this point has still not been firmly established, it would be compatible with the very high affinity of **1**, **2** and related compounds **3**, **5** and **6** for the plant enzymes ($K_i/K_m = 10^{-3}$ in the case of the most potent inhibitor of the series). As discussed in a previous article [5], a large array of molecules already exist which inhibit sterol biosynthesis in higher plant cells. Most of these sterol biosynthesis inhibitors (SBI) when given to plant cells lead to the accumulation of compounds possessing the tetracyclic sterolic skeleton which, as discussed elsewhere [6], could at least partly meet some of the sterol requirements of plant membranes. It could be of interest to consider a target enzyme such as the OS cyclase whose inhibition would lead to accumulation of OS, an aliphatic flexible compound, which obviously would not be able to replace sterols in membranes. Thus it could be expected that OS cyclase inhibitors may lead to profound physiological effects in treated cells. For all these reasons we have submitted bramble cell suspension cultures to the action of **1**, **2** and derivatives. The results obtained show that the inhibitors are also very active *in vivo* and lead to sterol biosynthesis inhibition as well as OS accumulation. Growth of treated cells is also strongly inhibited.

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Abbreviations: U 18666 A, 3β-[2-diethylamino ethoxy] androst-5-en-17-one; CPTA, 2-(4-chlorophenylthio) triethylamine; OS, 2,3-oxidosqualene; DOS, 2,3:22,23-dioxidosqualene; SBI, sterol biosynthesis inhibitors; LDAO, *N*-lauryl-*N*-dimethyl-amino-*N*-oxide.

RESULTS

Sterol profile of bramble cell suspension cultures treated with 2-aza-2,3-dihydrosqualene and its N-oxide derivative

When bramble cell suspension cultures were grown in the presence of increased amounts of 2-aza-2,3-dihydrosqualene (**1**), an inhibition of cell growth was observed for concentrations of **1** higher than 2 mg/l. The concentration of **1** for which the growth was reduced by 50% (IC_{50}) was shown to be 4.5 ± 1.5 mg/l (11 μM). No growth was observed for concentrations of **1** higher than 7.5 mg/l. At concentrations of **1** lower than 2 mg/l, the growth was only slightly inhibited but the cells were shown to develop first a pink and then a reddish colour

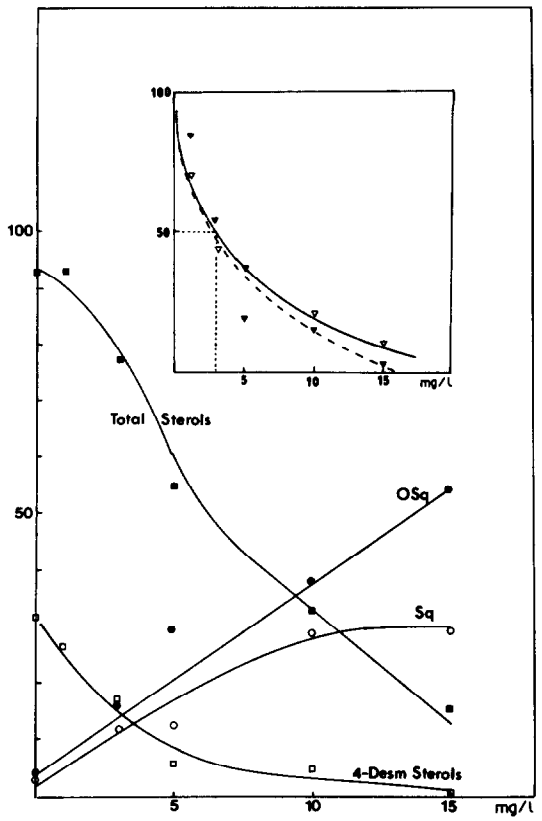

Fig. 1. Influence of 2-aza-2,3-dihydrosqualene-*N*-oxide concentration on sterol biosynthesis. Bramble cells, two weeks old, were incubated for 2 hr, in the presence of the indicated concentration of the drug. [2-^{14}C]-acetate (100 μCi) was then given to the medium and after 4 hr the incorporation in squalene (-○—○-), oxido-squalene (-●-●-), 4-desmethyl sterols (—□—□—), and the mixture of 4-desmethyl-, 4α,-methyl- and 4,4-dimethylsterols (—■—■—) was determined. The results are expressed as percentages of the total radioactivity incorporated in squalene, oxido-squalene and total sterols. Insert: Influence of 2-aza-2,3-dihydrosqualene (—▽—) and its N-oxide (—▼—) on 4-desmethyl sterol biosynthesis. The results are expressed as percentage of [^{14}C]-4-desmethyl sterol measured in the absence of drug.

which contrasted strongly with the pale yellow colour of control cells. 2-Aza-2,3-dihydrosqualene *N*-oxide (**2**) inhibited cell growth but the measured IC_{50} 9 ± 3 mg/l. (21 μM) was significantly higher. *N*-Lauryl-dimethylamine-oxide (LDAO) (**3**) which was shown previously to inhibit strongly the OS cyclases [4] was less inhibitory with a IC_{50} of about 15 mg/l (66 μM). In this latter case no pink or red colouration was observed.

Table 1 gives the triterpenoid content of control bramble cells and of cells treated with **1** and **2**. A strong accumulation of Δ^8-sterols (mainly (24ξ)-24-ethyl-5α-cholest-8-en-3β-ol and 5α-stigmasta-8,*Z*-24(28)-dien-3β-ol) was observed while the Δ^5-sterol content decreased dramatically. In addition, oxidosqualene and squalene, which are detectable in trace amounts in the control, accumulate in treated cells, OS and squalene were further characterized by TLC purification, GC analysis and comparison with authentic OS and squalene. During the purification by TLC of squalene (see Experimental), a dark red band appeared at lower R_f than squalene. The eluted compounds presented chromatographic and spectral characteristics identical to lycopene. All these results suggest that the OS-cycloartenol cyclase and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase are probably major enzymatic targets of **1** and **2**. Although the growth reduction was important at the concentration of inhibitor (5 mg/l) used in this experiment (Table 1), the sterol content of treated cells (2 mg/g dry weight) was not significantly different from that of the control.

Pulse experiments

The results presented in Table 1 suggest that the OS-cycloartenol cyclase is probably a major enzymatic target of **1** and **2**. In order to test this hypothesis, we performed pulse experiments with [^{14}C]-acetic acid. Two-week-old bramble cells were treated with [2-^{14}C]-acetic acid (100 μCi, 200 μM) for 4 hr in the presence of various azasqualene derivatives (**1–6**). The different classes of triterpenoids were extracted, purified and their radioactivity determined. As shown in Tables 2 and 3 and Fig. 1, a feature common to most of the inhibitors tested was a dramatic accumulation of radioactivity in the fractions containing squalene and OS and a strong decrease in the radioactivity incorporated in 4-desmethylsterols. However, there were differences between the effects of 2-aza-2-dihydrosqualene (**1**) and the *N*-oxides (**2** and **3**). Whereas in the case of **1** (Experiment B), the accumulation of squalene was always greater than OS, in the case of the tertiary amine *N*-oxides, OS accumulated more strongly than squalene. In Table 3, the effects of the inhibitors (**4–6**) have been compared. It is clear that the primary amine (**4**) lead to a low inhibition of 4-desmethylsterol synthesis and to a moderate accumulation of OS. By contrast the *N*-diethyl derivative of squalene (**5**) and especially the hypocholesterolemic compound U 18666 A (**6**) lead to a very strong accumulation of OS and a marked inhibition of 4-desmethylsterol biosynthesis. As shown in Tables 2 and 3, there were often very important differences in the sterol biosynthetic capabilities of the various bramble cell batches used in the present study. To explain such a difference we suggest that some heterogeneity could appear in the different batches of cells which are growing independently. This could be avoided if a single batch of cells cultivated in a fermentor is used to perform a series of parallel pulses. However, in spite of these differences in the sterol biosynthetic capabilities, the relative percentages of the radioactivity incorporated into each category of triterpenoid were shown to be reproducible. Indeed, five pulse experiments performed with inhibitors **1** and **2** gave essentially identical distributions of radioactivity in triterpenoids.

The inhibition of 4-desmethylsterol biosynthesis by **1** and **2** was shown to be concentration dependent. The half-maximal inhibition of 4-desmethylsterol biosynthesis was determined using increasing concentrations of **1** and **2**. Under these conditions the measured IC_{50} values were 3 mg/l (7.3 μM) for both inhibitors (Fig. 1A). At concentrations of **1** and **2** higher than 15 mg/l (36 μM) the block after OS was nearly total resulting in a dramatic accumulation of squalene and OS (85% of total radioactivity) and negligible synthesis of 4-desmethylsterols (1.5% of total radioactivity) (Fig. 1).

2,3:22,23-Dioxidosqualene (DOS) has been shown to accumulate in 3T3 fibroblasts, rat intestine epithelial cell

Table 1. Sterol composition of bramble cell suspension cultures treated with 2-aza-2-dihydrosqualene (1) and 2-aza-2-dihydrosqualene-*N*-oxide (2)

	Percent of total sterols			*RR*†
	A*	B	C	
Ergosta-5,24(28)-dien-3β-ol‡	3	0	0	1.28
(24ξ)-24-Methyl-cholest-5-en-3β-ol	3	0	0	1.29
(24ξ)-24-Methyl-5α-cholest-8-en-3β-ol	0	1.5	0.5	1.32
(24*R*)-24-Ethyl-cholest-5-en-3β-ol	40	tr	tr	1.43
(24 ξ)-24-Ethyl-5α-cholest-8-en-3β-ol	0	19	33	1.45
Stigmasta-5,*Z*-24(28)-dien-3β-ol	37	tr	tr	1.44
5α-Stigmasta-8,*Z*-24(28)-dien-3β-ol	0	38	21	1.45
4α-Methyl-5α-ergosta-8,24(28)-dien-3β-ol	0	1	1	
4α,14α-Dimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol	0.7	3.5	2	1.44
4α-Methyl-5α-stigmasta-8,*Z*-24(28)-dien-3β-ol	0	1.5	2.5	
4,4,14α-Trimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol	2	7	12	1.50
4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol	5	9	5	1.45
2,3-Oxido-2,3-dihydrosqualene	tr	8.5	5	
Squalene	tr	2	2.5	
Other triterpenoids	9	11.5	14	

*A: control cells; B: cells grown in the presence of 2-aza-2-dihydrosqualene (5 mg/l); C: cells grown in the presence of 2-aza-2-dihydrosqualene-*N*-oxide (5 mg/l). In B and C the cell suspension cultures were allowed to grow in the presence of the inhibitor for four weeks. The dry weight of the cells after harvest were 2.4, 1.5 and 1.4 g for A, B and C respectively.

†*RR*$_t$: relative retention time to cholesterol.

‡Trivial names: (24 ξ)-24-methyl-cholest-5-en-3β-ol: campesterol + 22-dihydrobrassicasterol; (24 *R*)-24-ethyl-cholest-5-en-3β-ol: sitosterol; stigmasta-5,*Z*-24(28)-dien-3β-ol: isofucosterol; 4α,14α-dimethyl-9β,19-cyclo-5α-ergosta-24(28)-en-3β-ol: cycloeucalenol; 4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol: cycloartenol.

Table 2. Effect of 2-aza-2-dihydrosqualene (1), its *N*-oxide derivative (2) and *N-lauryl-N*-dimethylamine-*N*-oxide (3) on the incorporation of [2-^{14}C]-acetic acid into triterpenoid of control (A) and experimental (B,C . . .) bramble cell suspension cultures

	Experiment I		Experiment II			Experiment III	
	A†	B	A	B	C	A	D
	(10^3 cpm/g dry weight)						
Squalene	2.1 [0.5]‡	119 [30]	45.5 [3]	166 [20]	94 [13]	17 [7]	137 [21]
2,3-Oxidosqualene	1.5 [0.3]	29.5 [7.5]	56.1 [4]	132 [16]	216 [31]	7.5 [3]	228 [36]
4,4-Dimethylsterols	96.9 [27]	74.8 [19]	619.5 [42]	322.5 [38]	236 [33]	105 [43]	115 [18]
4α-Methyl-sterols	57.5 [16]	114.7 [29]	275 [19]	118 [14]	123 [17]	34 [14]	114 [18]
4-Desmethylsterols	203 [56]	56.4 [14]	464 [32]	97.5 [12]	39 [6]	78.5 [33]	42 [7]
Total	361	395	1460	836	708	242	636

*The sterol biosynthetic capability of bramble cells was measured by incubation of the cells with [2-^{14}C]-acetic acid (100 μCi = 200 μM) in the presence (B, C, D) or absence (A) of the inhibitor. The inhibitor was solubilized in ethanol and the solution was introduced into the incubation medium to give a final concentration of 5 mg/l. A preincubation of 2 hr of the inhibitor was performed in the absence of [^{14}C]-acetic acid then the incubation was initiated by addition of the [^{14}C]-acetic acid and was stopped after 4 hr.

†A: Control; B: 2-aza-2-dihydrosqualene; C: 2-aza-2-dihydrosqualene-*N*-oxide; D: *N*-lauryl-*N*-dimethyl-amine-*N*-oxide (LDAO).

‡Percent of the total radioactivity.

cultures, or yeast treated with **1**, U18666A, or related molecules [7–9]. Using the same experimental conditions as those described in the literature (see material and methods) DOS was not detectable in bramble cell suspension cultures whatever the concentration of **1**, **2** or **3** used.

The IC_{50} for 4-desmethylsterol biosynthesis inhibition and for cell growth were in the same range when **1** was used as inhibitor, therefore it could be suggested that a defect in 4-desmethylsterol production could be responsible of the growth inhibition. Some trials have been done to reverse the growth inhibition caused by **1** by supplementing the medium with exogenous sterols. Sitosterol or cholesterol (1–10 mg/l) were added to the medium culture in the presence of tergitol (1 mg/l, a non

Table 3. Effect of squalene amine (4), squalene diethylamine (5) and of U18666A (6) on the incorporation of [2-^{14}C]acetic acid into triterpenoids of bramble cell suspension cultures

	A†	B	C
	(10^3 cpm/mg dry weight)		
Squalene	7.5 [3]‡	95.5 [21]	410 [32]
2,3-Oxidosqualene	21.5 [9]	150 [33.5]	674 [51.5]
4,4-Dimethylsterols	90.5 [33]	102 [23]	96.5 [7.5]
4α-Methylsterols	68.75 [25]	83 [18]	89.5 [7]
4-Desmethylsterols	82.5 [30]	20 [4.5]	27.5 [2]
Total	270.75	450.5	1300

*The conditions of incubations are described in the Table 2.
†A: squalene amine; B: squalene diethylamine; C: U18666A.
‡% of the total radioactivity.

toxic concentration), a detergent used to help solubilization of sterols. No reversion of the growth inhibition could be observed under these conditions.

DISCUSSION

The results obtained show unequivocally that 2-aza-2-dihydro-squalene (1) and its *N*-oxide derivative (2) which have been designed to be high energy intermediates or transition state analogue inhibitors [1–4] of the 2,3-oxidosqualene-β-amyrin-, cycloartenol and lanosterol cyclases, inhibit the OS-cycloartenol cyclase *in vivo* resulting in a strong accumulation of OS and in a decrease of 4-desmethylsterol biosynthesis. Similar 2,3-oxidosqualene accumulation has been reported in other materials with other cyclase inhibitors [8–14]. The strong accumulation of squalene which is observed in our case could be ascribed to an inhibition by 1 and 2 of the squalene-2,3-epoxidase. Recent work [15] performed on cell free extracts from yeast and rat liver has shown that this was indeed the case since 1 inhibited both squalene epoxidase and OS-lanosterol cyclase. An alternative explanation is that the epoxidase is inhibited by OS itself which is the product of squalene epoxidation and which accumulates strongly following inhibition of the OS cyclase by 1 or 2. However, it has to be pointed out that the *N*-oxide derivatives (2, 3) lead to less accumulation of squalene and much more accumulation of OS than 1 (Table 2). Similar observations have been made in 3T3 fibroblasts treated with the same molecules [7].

The accumulation of squalene and of OS is correlated with a decrease in 4-desmethylsterol synthesis (Fig. 1). The IC_{50} for the inhibition of sterol synthesis (3 mg/l, 7.3 μM) is in the same range as the IC_{50} for growth inhibition (4.5 mg/l, 11 μM) suggesting strongly that a lack in 4-desmethylsterol content is responsible for the growth inhibition by 1 and 2. Such a conclusion has been recently drawn in a study where 1 and 2 were used on 3T3 cells [7]. Exogenously added sitosterol or cholesterol do not allow reversal growth inhibition in contrast to studies performed on animal cells [7, 9]. This could result from the inability of exogenous sterols to penetrate into the bramble cells. The IC_{50} measured for sterol biosynthesis inhibition (7.3 μM) is much higher than the K_i value (1 μM) for OS-cycloartenol cyclase inhibition *in vitro* [3]. To explain this rather poor correlation, it could be suggested that a fraction of 1, 2 or 3 would be adsorbed on the cell walls, a likely event in the case of 1, which being totally protonated at the pH of the culture medium, could interact strongly with the free galacturonate residues of the wall. In conclusion the potency of 1, and the *N*-oxide derivatives 2 and 3 (IC_{50} for inhibition of sterol synthesis 7.3 μM) compares favorably with other known cyclase inhibitors which have been tested in mammalian tissue culture cells e.g. 4,4,10β-trimethyl-trans-decal-3β-ol [10] and chloroquine [11], or in yeast e.g. 2,3-epiminosqualene [16]. By contrast 1, 2 and 3 are less efficient than U 18666 A which appears to be one of the best sterol biosynthesis inhibitors acting on OS-lanosterol cyclase [9, 17] and which is the strongest inhibitor in our tests (Table 3).

Compounds 1 and 2 lead to a strong accumulation of Δ^8-sterols (Table 1) suggesting that they inhibit the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase. An important accumulation of Δ^8-sterols was still observed for lower concentrations of 1 (1 mg/l) while no OS was shown to accumulate (results not shown). Such a result suggests that the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase would be an even more sensitive target of 1 than the OS cyclase. For concentrations of 1 and 2 (1 to 5 mg/l) which allow cell growth, no significant decrease in the total sterol amount was noticed. This contrasted with the strong inhibition in sterol biosynthesis capabilities observed after incorporation of [2-^{14}C]-acetate. To explain this discrepancy it must be recalled that in order to obtain enough material to proceed to complete identification of sterols, cell cultures were cultivated for four weeks in the presence of the inhibitor resulting possibly in some destruction of the inhibitor. This could reduce the level of inhibitor in the culture medium to a concentration which does not inhibit OS-cyclase but which would still interfere with the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase. Such an hypothesis would be consistent with previous findings showing that Δ^5-sterols were replaced by Δ^8-sterols in cells treated with inhibitors of sterol biosynthesis such as AY 9944 [18] and fenpropimorph [19] which did not affect cell growth. For concentrations of 1 and 2 (more than 5 mg/l) inhibiting strongly cell growth it can be noticed that the block of the biosynthetic flow after OS leads to OS accumulation which is not acceptable as a substitute for sterols [6, 20].

Finally, the present work has also revealed that 1 but not 2 and 3 interferes with carotenoid biosynthesis resulting in an accumulation of lycopene. This suggests that the cyclase (s) involved during the conversion of lycopene to β-carotene were inhibited. Such an hypothesis is corroborated by recent findings showing that 1 inhibits lycopène cyclization in *Capsicum annuum* chromoplasts [21]. Another explanation would be that 1 stimulated the enzymatic complex involved in lycopene synthesis. Such a conclusion has been reached in *Citrus paradisicum* treated with CPTA (7) and related compounds [22], a class of molecules possessing some common features with the inhibitors used in the present work.

In conclusion 2-aza-2,3-dihydrosqualene (1), its *N*-oxide derivative (2) and related molecules (3–6) proved to be interesting and potent new SBI in plant cell suspension cultures. Their main targets are the cyclization of OS into cycloartenol and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase. In addition the inhibition of OS cyclase results probably in cell growth inhibition.

EXPERIMENTAL

Chemicals. Sodium[2-^{14}C]-acetate (48 mCi/mmol) was purchased from the Commissariat á l'Energy Atomique (Saclay,

France). The synthesis of 2,3-oxidosqualene, [3-^{3}H]-(*R, S*)-2,3-oxidosqualene, 2-aza-2,3-dihydrosqualene (**1**), its *N*-oxide derivative (**2**), as well as derivatives **4**, **5**, **6** have been reported previously [1, 3, 4]. A sample of 2,3:22,23-dioxido squalene was provided by N. Gerst. LDAO (**3**) was purchased from Serva (Heidelberg, West Germany), U 16888 A (**6**) was kindly provided by Dr Cenedella (Kirksville, Missouri, U.S.A.). 24-methylpollinastanol was extracted from bramble cell suspension cultures treated with fenpropimorph [23]. (24*R*)-24-ethyl-5α-cholest-8-en-3β-ol and 5α-stigmasta-8,*Z*-24(28)-dien-3β-ol were isolated from bramble cells treated with AY 9944 [18].

Plant material. Suspension cultures of bramble cells were grown under continuous white light at 25° on a synthetic sterile medium. Compounds **1–6** (1–20 mg/l) were added in soln in EtOH to the culture medium. The drug was sterilized before use by filtration through a Millipore (0.45 μm) filter. At the end of the growth period (4 weeks in general) the cells were harvested, filtered, lyophilized and their dry wt determined.

Incorporation of radiolabelled precursor into nonsaponifiable sterols. Suspension cultures of bramble cells (2 weeks old) were incubated with [2-^{14}C] acetic acid (100 μCi:200 μM) in the presence or absence (control) of the inhibitor. The inhibitor was solubilized in EtOH and the ethanolic soln was sterilized by filtration through a Millipore (0.45 μm) filter and introduced in the incubation medium to reach a final concentration of 5 mg/l. A preincubation of 2 hr of the cells in the presence of the inhibitor was performed, then the incubation was initiated by addition of the [^{14}C]-acetic acid and was stopped after 4 hr. The same operations were done in the case of control cells but in the absence of the inhibitor. When the incubation was terminated, the cells were filtered, rinsed with cold culture medium and lyophilized.

Analytical procedure. The techniques used for sterol extraction, purification and identification have been detailed elsewhere [18, 19, 23, 24]. Bramble cells were treated as described previously [18, 19, 23, 24]. This procedure led to the isolation of squalene (R_f 0.9), 2,3-oxidosqualene (R_f 0.75), 4,4-dimethyl-, 4α-methyl- and 4-desmethylsterols (R_f 0.50, 0.45, 0.30, respectively). The sterols were acetylated [23]. Each of the three classes of steryl acetates was analysed by GC with a GC equipped with a FID and a silica fused capillary column (WCOT, 25 m × 0.25 mm i.d.) coated with OV1 (H_2 flow: 2 ml/min). The temp. programme used included a fast rise from 60° to 230° (30°/min), then a slow rise from 230° to 280° (2°/min). The total amount of sterols present in each class was quantified using an integrator. Analytical argentation TLC (10% $AgNO_3$ impregnated silica gel; cyclohexane–toluene (7:3); migration for 15 hr) was performed on each class of steryl acetate and the bands obtained were analysed by GC. The details relative to the detection, separation and identification of steryl acetates (essentially Δ^8-steryl acetates obtained after inhibitor treatment have been published elsewhere [23, 24]. The sterols are listed in the Table 1. All the sterols isolated from inhibitor treated bramble cells were identified by their ^{1}H NMR and mass spectra as described previously [18, 23, 24]. These data, being identical to those already published have not been detailed here. In the case of pulse experiments with [2-^{14}C] acetate, the radioactivity associated with purified steryl acetates was measured using a scintillation counter.

Identification of squalene and 2,3-oxidosqualene. The nonsaponifiable lipid fraction was submitted to TLC (silica gel; CH_2Cl_2; 2 migrations). Two bands were detected at R_f 0.9, 0.75 corresponding to authentic squalene and 2,3-oxidosqualene (OS), respectively. The components comigrating with squalene were submitted to TLC (silica gel; hexane–toluene (95:5); one migration); the bulk of the compounds did not migrate but some compound was detectable at R_f 0.45 identical to that of authentic squalene. After elution from the silica gel, the components were submitted to GC. The temp. programme used included a fast rise from 60 to 200° (30°/min), then a slow rise from 200 to 250° (2°/min). The total amount of squalene was determined according to a standard curve. In the case of pulse experiments with [2-^{14}C]-acetate, the radioactivity associated with squalene was determined using a scintillation counter. The compounds comigrating with OS were submitted to TLC (silica gel; CH_2Cl_2; one migration); the bulk of the compounds present in this fraction co-chromatographed at R_f 0.55 identical to that of authentic OS. After elution from the silica gel, the recovered compounds were submitted to GC with the same temperature programme as above. The OS present was clearly identified at RR_t 1.30 relative to cholesterol. The radioactivity associated to pure OS was determined using a scintillation counter. In order to check for the presence of 2,3:22,23-dioxidosqualene (DOS), the nonsaponifiable extract was submitted to TLC using hexane–EtOAc (85:15), one migration. In these conditions authentic DOS migrates at R_f 0.55 and is clearly separated from 4,4-dimethylsterols [25]. No trace of radioactivity was shown to occur at this R_f in our material. Biosynthetically labelled OS was identified chemically according to the procedure of Willett *et al.* [26]. The band migrating with authentic OS was eluted and hydrolysed in acidic medium (100 μl of $HClO_4$ in EtOH–H_2O (2:1, v/v). The material obtained after extraction co-migrated on TLC (hexane–EtOAc 75:25 with authentic squalene-2,3-glycol (R_f 0.15). This compound was in turn converted into 1,1',2-trisnorsqualene aldehyde by treatment with periodic acid in THF on TLC (CH_2Cl_2, one migration), the labelled metabolite showed the same R_f (0.55) as an authentic sample.

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