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25-AZACYCLOARTANOL, A POTENT INHIBITOR OF S-ADENOSYL-L-METHIONINE-STEROL-C-24 AND C-28 METHYLTRANSFERASES IN HIGHER PLANT CELLS

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SUMMARY : 25-azacycloartanol (*1*) is a potent inhibitor of S-adenosyl-L-methionine-cycloartenol-C-24-methyltransferase and of S-adenosyl-L-methionine-24-methylene lophenol-C-28-methyltransferase, two microsomal enzymes of maize (*Zea mays*) embryos. Addition of *1* to a suspension of bramble (*Rubus fruticosus*) cells grown *in vitro* resulted in a strong decrease of the 24-ethylsterol content and in a dramatic increase of sterols lacking an alkyl group at C-24.

S-adenosyl-L-methionine (SAM)-C-methyltransferases play an important role in the biosynthesis of sterols in higher plants for these enzymes catalyze the introduction of two extra carbon atoms at position C-24 of higher plant sterols (1). Experiments have shown that some plant cell-free extracts when incubated in the presence of SAM are capable of introducing one methyl group at C-24 in various Δ^{24} sterols (2-4). These studies have established also that cycloartenol is the preferred Δ^{24} substrate suggesting that *in vivo* C-24 methylation occurs mainly at the cycloartenol level (3,4).

It was recently found (4) that bramble cell-free extracts can transfer a second methyl group to position C-28 of a suitable sterol bearing a methylene group at C-24. Of the substrates assayed, only 24-methylene lophenol was methylated, giving 24-ethylidene lophenol, a ubiquitous 4α-methyl sterol of plants. This finding suggested that 24-methylene lophenol could be the target of C-28 methylation *in vivo* (4).

Avruch *et al.* (5) showed recently that treating yeast cells with 25-azadihydrozymosterol resulted in the accumulation of 27-C sterols such as cholesta-5,7,24-trien-3β-ol instead of ergosterol. This suggested

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ABBREVIATIONS : I_{50} : concentration of inhibitor producing 50% inhibition ; cycloartenol : 4,4,14α-trimethyl-9β,19β-cyclo-5α-cholest-24-en-3β-ol ; 24-methylene lophenol : 4α-methyl-5α-ergosta-7,24(28)-dien-3β-ol ; cycloeucalenol : 4α,14α-dimethyl-9β,19β-cyclo-5α-ergost-24(28)-en-3β-ol ; obtusifoliol : 4α,14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol ; isofucosterol : stigmasta-5,Z-24(28)-dien-3β-ol.

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that C-24 methylation in this material was blocked probably at the level of zymosterol (5). As cycloartenol appears to be the preferred substrate of C-24 methylation in higher plant cells (3,4), we have synthesized 25-azacycloartanol (*1*) and studied its action on a cell-free extract from maize embryos containing a microsomal C-24 methyltransferase. Bramble cells were also grown in the presence of *1*. The results presented here show that *1* inhibits the methyltransferases both *in vitro* and *in vivo*.

MATERIAL AND METHODS

Bramble cells were grown in a liquid medium as described previously (4). 25-azacycloartanol was synthesized by a method derived from that used in the synthesis of 25-azadihydrozymosterol (5). Four weeks after the drug (10^{-6}M) was added to the cells, these latter were harvested and the 4,4-dimethylsterols, the 4α-methylsterols, and the 4-desmethylsterols were isolated, purified, and identified as described earlier (6). The SAM-sterol-C-24 and C-28 methyltransferases were assayed in microsomes prepared from maize embryos as before (4). The incubation mixture contained 0,5 ml of the membrane fraction, 100 µM (0,1 µCi) of [methyl-^{14}C] -SAM (C.E.A., Gif sur Yvette, France), the sterol substrate (50 µM), and various concentrations of *1*, in a total volume of 0,6 ml (final pH 7.4). Before the incubation, the sterol substrate and *1* were emulsified in aqueous 0.5% (v/v) Tween 80. The incubations were carried out at 30°C for 1 hr and were stopped by the addition of 1 vol KOH in ethanol. The neutral lipids were extracted and analyzed as described earlier (4,6). The cycloeucalenol-obtusifoliol isomerase was assayed as before (7).

RESULTS

1. Effect of 25-azacycloartanol on SAM-cycloartenol-C-24-methyltransferase and on SAM-24-methylene lophenol-C-28-methyltransferase.

After incubating microsomes from maize embryos in the presence of SAM-^{14}C and cycloartenol (50 µM) and extracting the incubate with light petroleum, the extract was chromatographed as described in Material and Methods. Fig. 1A shows the distribution of radioactivity on a thin-layer plate. A peak (c) appeared at the level of 4,4-dimethyl sterols and a smaller peak (b) at the level of 4α-methyl sterols. No radioactivity was associated with the 4-desmethyl fraction (a). The radioactivity associated with c corresponded to 24-methylene cycloartanol only, a product resulting from the methylation of the added cycloartenol (4). The radioactivity associated with b corresponded to 24-ethylidene lophenol, the product resulting from the methylation of the endogenously present 24-methylene lophenol (4). Fig. 1B represents the effect of 25-azacycloartanol (0.5 µM) on the enzymatic activity

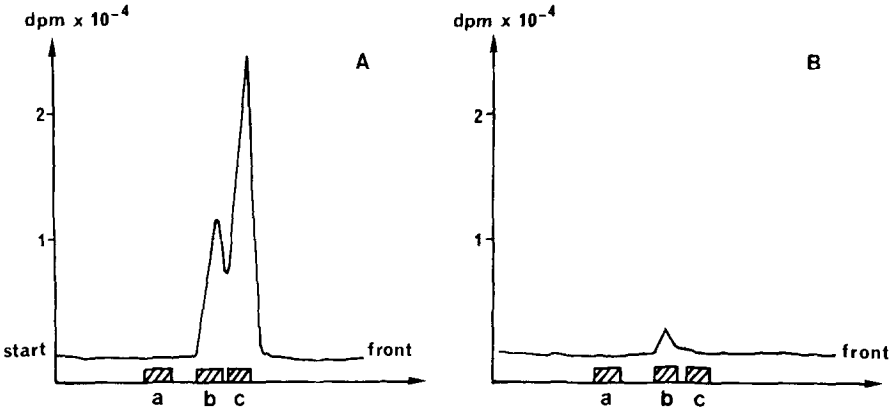


Fig 1 : Radiochromatogram of light petroleum extract of microsomes from maize embryos incubated with [methyl-^{14}C] -SAM (100 µM) and cycloartenol (50 µM) in the absence (A) or in the presence (B) of 25-azacycloartanol (0.5 µM). a, 4-desmethyl sterols ; b, 4α-methyl sterols ; c, 4,4-dimethyl sterols.

measured using cycloartenol (50 µM) as substrate. The peak associated with the 4,4-dimethyl sterols (c) has disappeared, and the radioactivity associated with the 4α-methyl sterols (b) decreased strongly. Quantitative data of the inhibition of the two methyltransferase activities are given in Fig. 2. From curve i, it is possible to determine the concentration of *1* that produced a 50% inhibition of the cycloartenol-C-24-methyltransferase (I_{50}) in the presence of cycloartenol (50 µM). This value was reproducibly 1.5 x 10^{-7}M (3 determinations). From curve j, one can evaluate the concentration of *1* that produced a 50% inhibition of the 24-methylene lophenol-C-28-methyltransferase (concentration of endogenous 24-methylene lophenol = 10 µM). This value was approximately 4 x 10^{-7}M. From curve k, one can conclude that *1* is completely inactive on cycloeucalenol-obtusifoliol isomerase (7), another microsomal enzyme involved in plant-sterol biosynthesis.

2. Effect of 25-azacycloartanol on the biosynthesis of sterols in bramble cells.

Sterols extracted from bramble cells treated with *1* (1 µM) were analyzed and compared with the sterols extracted from control cells, which were not treated with *1*; the results (Table 1) showed that the amount of 24-ethyl sterols (sitosterol + isofucosterol), decreased dramatically in the treated cells, whereas the amount of sterols with an unalkylated lateral chain (cycloartenol, cholesterol, desmosterol), which were present in trace amounts in the control cells, increased strongly in the treated cells. Cycloartenol became the major sterol of

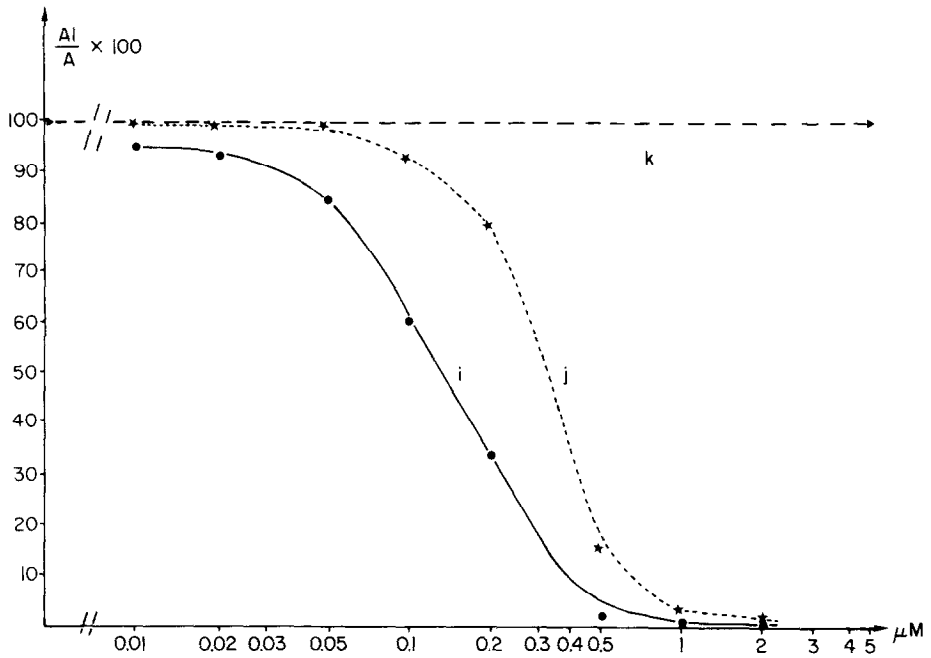


Fig 2 : Inhibition by 25-azacycloartanol (1) of the SAM-cycloartenol-C-24-methyltransferase (i) ●—●—● , of the SAM-24-methylene lophenol-C-28-methyltransferase (j) ★- - -★- - -★ and of the cycloeucalenol-obtusifoliol isomerase (k) ▲—▲ A_I = activity in the presence of the inhibitor ; A = activity of the control.

TABLE 1

Sterols of Control and 25-azacycloartanol-treated bramble cells.

	Control	Treated
Cycloartenol	0.5*	36*
Cholesterol	0	4.5
Desmosterol	0	5
24-methylene cholesterol	2	32
Campesterol	14	4
Isofucosterol	12	5.5
Sitosterol	70	3
Other sterols	2	10**
Sterols with a nonalkylated lateral chain	tr.	53
24-methyl sterols	16	37
24-ethyls sterols	83	9
Total sterols	3.4***	2.6***

* As percentage of total sterols

** 11 minor sterols identified (unpublished results)

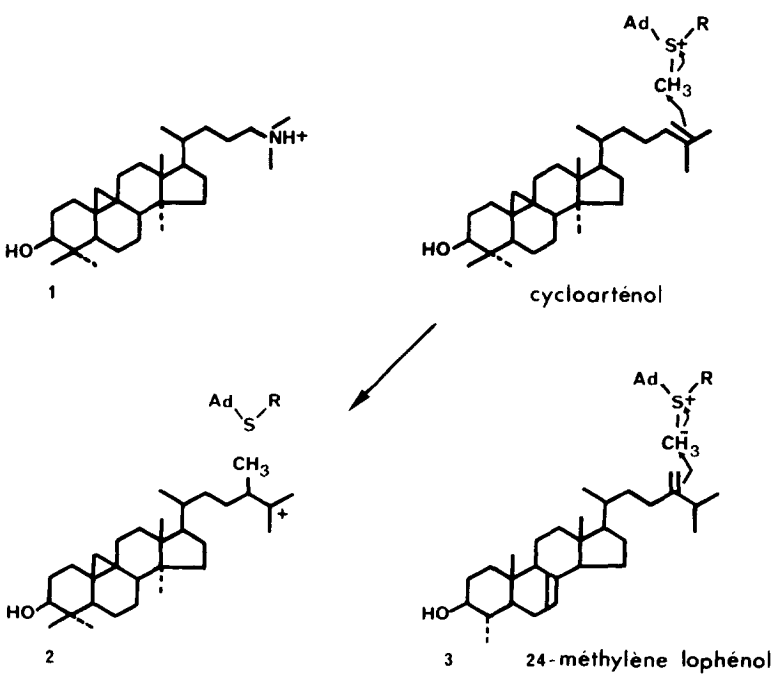
*** in mg/g dry wt.

the treated cells. Thus the results obtained confirm that 1 strongly inhibits the cycloartenol-C-24-methyltransferase (accumulation of cycloartenol) but also inhibits the 24-methylene lophenol-C-28-methyltransferase (accumulation of 24-methylene cholesterol). A side effect of 1 would be the inhibition of the $\Delta^{24(25)}$ or $\Delta^{24(28)}$ reduction of sterols as attested by the accumulation of desmosterol, 24-methylene cholesterol, and isofucosterol.

DISCUSSION

The results shown in this paper clearly show that 1 is an inhibitor of cycloartenol-C-24-methyltransferase and of 24-methylene lophenol-C-28-methyltransferase. A low I_{50} value ($\sim 10^{-7}$M) was found for the former. Comparison of this value with the Km of the enzyme for cycloartenol ($\sim 7.5 \times 10^{-5}$M) suggests that 1 would be a very potent inhibitor. Moreover, the bramble cells treated with 1 accumulated sterols with unalkylated lateral chain, while the concentration of 24-ethylsterols decreased strongly confirming that the methyltransferases are also inhibited *in vivo*.

The molecular basis of the inhibition of cycloartenol-C-24-methyltransferase by 1 are debatable. At pH 7.5, 1 is present in its protonated



SCHEME I

form and present structural similarities with a potential high energy intermediate (2) in the methylation reaction resulting from the nucleophilic attack of the methyl group of the SAM by the Δ^{24} of cycloartenol and which would possess a carbonium ion at C-25 (Scheme 1) (1). According to considerations developed by Wolfenden concerning inhibitors that structurally resemble chemically activated intermediates in catalysis (8), this could explain the strong inhibitory properties of 1 for the C-24 methyltransferase. In contrast, the enzymic methylation of 24-methylene lophenol (3) could involve the formation of a carbonium ion at C-24 rather than at C-25. This and considerations relative to the structural differences between 1 and 3 could explain why 1 inhibited the C-28 methyltransferase less efficiently.

Another interpretation of our results would be that 1 behaves as a substrate for an N-methylation catalyzed by the C-24 methyltransferase leading possibly to quaternization of the nitrogen atom. A similar interpretation was proposed earlier in the case of the inhibition of C-24 methyltransferase by triparanol (9). A more thorough kinetic study is now being made in order to find out which hypothesis is valid.

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