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EFFECT OF AY-9944 ON STEROL BIOSYNTHESIS IN SUSPENSION CULTURES OF BRAMBLE CELLS

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Key Word Index—*Rubus fruticosus*; Rosaceae; sterol biosynthesis inhibitor; $\Delta^8 \rightarrow \Delta^7$ isomerase; PMR of Δ^8 sterols.

Abstract—Bramble suspension cultures normally contain Δ^5 sterols (sitosterol, campesterol, and isofucosterol). When the cells were grown in a medium supplemented with AY-9944, their content of Δ^5 sterols was greatly decreased and Δ^8 sterols accumulated. Six Δ^8 sterols, including three new compounds, (24*R*)-24-ethyl-5α-cholest-8-en-3β-ol, stigmasta-8,*Z*-24(28)-dien-3β-ol, and 4α-methyl-stigmasta-8,*Z*-24(28)-dien-3β-ol, were identified. AY-9944 probably inhibited the $\Delta^8 \rightarrow \Delta^7$ isomerase. A stable cell line growing permanently in an AY-supplemented medium was obtained.

INTRODUCTION

The fungicide AY-9944 profoundly alters the biosynthesis of sterols in animals and in algae. In rat liver, it depresses cholesterol biosynthesis, and cholesta-5,7-dien-3β-ol accumulates [1, 2]. In *Chlorella emersonii*, it depresses the formation of chondrillasterol, the main sterol of this algae, and causes the accumulation of 14α-methyl-5α-(24*S*)-stigmast-8-en-3β-ol, whereas in *C. ellipsoidae*, it blocks the synthesis of poriferasterol and ergost-5-en-3β-ol and causes the accumulation of 8,14-dienes (77%) and Δ^8 sterols (20%) [3, 4]. We wished to know whether AY-9944 also altered the biosynthesis of sitosterol in higher plants and to identify any new products formed. Other fungicides such as triarimol have been shown to depress growth in *Phaseolus aureus* but no quantitative and qualitative changes were observed on sterol biosynthesis [5]. Whole plants are not well suited for such work because of permeability problems; we therefore used suspension cultures to study the effects of AY-9944 upon sterol biosynthesis. We report having obtained a stable strain that grows well on a medium supplemented with AY-9944 and whose sterol content is less than 5% of the normally occurring Δ^5 sterols and more than 80% unusual Δ^8 sterols.

RESULTS

Sterol composition of control suspension cultures of bramble cells

The following sterols were identified in bramble cells: cycloartenol (**1**), 24-methylene cycloartanol (**2**), α- and β-amyrins, cycloeucalenol (**3**), obtusifoliol (**4**), 24-methylene lophenol (**6**), 24-ethylidene lophenol (**8**), stigmasta-7,*Z*-24(28)-3β-ol (**10**), isofucosterol (**11**), sitosterol (**13**), 24-methylene cholesterol (**15**), and campesterol (**17**). Because all these compounds are common in the plant kingdom, details of their identification are not given.

Strain growing on AY-9944

The culture medium was supplemented with AY-9944 (8 mg/l.). Cells cultivated on this medium grew very slowly. After 2 months, cells were subcultured in a fresh medium supplemented with AY-9944; their growth rate increased and after four passages became even higher than that of control cells. The concentration of 4-des-

Table 1. Sterols of control and AY-9944-treated bramble cells

	Control	Treated
Cycloartenol (**1**)	0.5*	3
24-Methylene cycloartanol (**2**)	0.15	4
Cycloeucalenol (**3**)	0.1	2
Obtusifoliol (**4**)	0.1	0.5
4α-Methyl-ergosta-8,24(28)-dien-3β-ol (**5**)	tr	3
24-Methylene lophenol (**6**)	0.1	0
4α-Methyl-stigmasta-8,*Z*-24(28)-dien-3β-ol (**7**)	tr	7
24-Ethylidene lophenol (**8**)	0.1	0
Stigmasta-8,*Z*-24(28)-dien-3β-ol (**9**)	0	28
Stigmasta-7.*Z*-24(28)-dien-3β-ol (**10**)	tr	0
Isofucosterol (**11**)	12	tr
(24*R*)-24-Ethyl-5α-cholest-8-en-3β-ol (**12**)	0	41
Sitosterol (**13**)	70	2
Ergosta-8,24(28)-dien-3β-ol (**14**)	0	1
24-Methylene cholesterol (**15**)	2	tr
(24ξ)-24-Methyl-5α-cholest-8-en-3β-ol (**16**)	0	3
Campesterol (**17**)	14	1
α- and β-amyrins	0.5	0.5
X_1 and X_2	0.0	2
Unknown sterols	tr	1.5
Total Δ^8 sterols	tr	83.5
Total Δ^5 sterols	98	3

* As percentage of total sterol.

methyl sterols remain nearly unchanged during this process, but their composition gradually changed; after two passages profound differences were observed, after four passages some stabilization occurred and a stable cell line was finally obtained. We describe here the sterol composition of this cell line. Its physiological properties will be described elsewhere.

Sterol composition of the stable cell line growing on AY-9944 (AY cells)

The total sterol content of the AY cells (4.8 mg/g dry wt) is significantly higher than that of the control cells (3.4 mg/g dry wt). However, the amount of 4-desmethyl sterols did not change significantly: 3.1 mg/g in AY cells, 3.0 mg/g in control cells. The composition and relative proportions of the sterols in AY cells are given in Table 1. Some of the sterols were already known: their identification is detailed in the experimental section. The most abundant sterols (more than 80% of the total) were (24*R*)-24-ethyl-5α-cholest-8-en-3β-ol (**12**), (24ξ)-24-methyl-5α-cholest-8-en-3β-ol (**16**), stigmasta-8,*Z*-24(28)-dien-3β-ol (**9**), ergosta-8,24(28)-diene-3β-ol (**14**), 4α-methyl-stigmasta-8,*Z*-24(28)-dien-3β-ol (**7**), and 4α-methyl-ergosta-8,24(28)-dien-3β-ol (**5**). Sterols **5** and **14** were found for the first time in a higher plant; **7**, **9**, **12**, and **16** are new compounds.

(a) *4-Desmethyl sterols.* Components of this fraction were separated using argentation chromatography. Three bands, for acetates of **14**, **9**, and **12** + **16**, were observed at the same R_fs, as respectively 24-methylene cholesteryl, isofucosteryl, and sitosteryl + campesteryl acetates (in order of increasing polarity). However, the RR_fs for **14**, **9**, and **12** + **16** acetates were higher than for the Δ^5 sterols. The MS were also very different in the two groups (Table 2), with large molecular ions appearing in all four new sterols. MS data also showed the presence of one intracyclic double bond in all four new products, the two more polar ones (**14** and **9**)

Table 2. MS of the steryl acetates of control bramble cells and cells treated with AY-9944

Steryl acetate	M^+	M^+-60	a	a−60	M^+−l.c.*	M^+−l.c. −60	c	c−60	b	b−60
(7)	468	408	370	310	329	269	327	267	287	227
	(100)†	(24)	(35)	(ε)‡	(5)	(24)	(50)	(17)	(15)	(94)
(8)	468	408	370	310	329	269	327	267	287	227
	(5)	(3)	(60)	(11)	(ε)‡	(20)	(100)	(17)	(3)	(27)
(5)	454	394	370	310	329	269	327	267	287	227
	(100)	(25)	(3)	(ε)	(2)	(20)	(35)	(15)	(10)	(78)
(6)	454	394	370	310	329	269	327	267	287	227
	(26)	(14)	(52)	(10)	(ε)	(45)	(100)	(21)	(16)	(59)
(9)	454	394	356	296	—	255	313	253	273	213
	(100)	(40)	(59)	(13)		(45)	(90)	(35)	(25)	(100)
(10)	454	394	356	296	315	255	313	253	273	213
	(5)	(5)	(40)	(45)	(ε)	(18)	(100)	(45)	(ε)	(25)
(11)	454	394	356	296	—	255	—	253	—	213
	(ε)	(15)	(ε)	(100)		(5)		(10)		(16)
(14)	440	380	356	296	315	255	313	253	273	213
	(100)	(64)	(7)	(3)	(10)	(31)	(49)	(30)	(32)	(100)
(15)	440	380	356	296	—	255	—	253	—	213
	(ε)	(100)	(ε)	(46)		(15)		(30)		(25)
(12)	456	396	—	—	315	255	313	253	273	213
	(100)	(15)			(17)	(40)	(ε)	(3)	(15)	(65)
(13)	456	396	—	—	—	255	—	253	—	213
	(ε)	(100)				(31)		(3)		(28)
(16)	442	382	—	—	315	255	313	253	273	213
	(100)	(27)			(20)	(46)	(3)	(8)	(15)	(65)
(17)	442	382	—	—	—	255	—	253	—	213
	(ε)	(100)				(34)		(ε)		(27)

* l.c. = Lateral chain.
† Relative intensity (only fragments with mass heavier than *m/e* 200 have been considered).
‡ ε = Very low relative intensity.

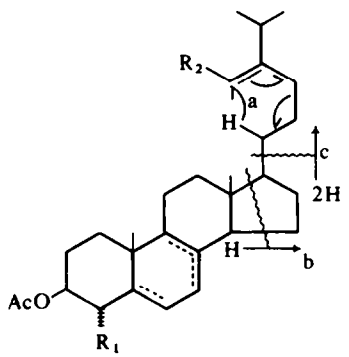


Table 3. Chemical shifts (δ, ppm) of the proton signals of 12-, 9-, 7- and 5-acetates in PMR spectroscopy (250 MHz)

	C–18	C–19	C–21	C–26	C–27	C–29	C–30	C–3Hα	C–28H	C–25H
(24*R*)-24-Ethyl-5α-cholest-8-en 3β-yl acetate (12-acetate)	0.605 *s*	0.962 *s*	0.929 *d* J = 6.0*	0.812 *d* J = 6.5	0.835 *d* J = 6.5	0.843 *t* J = 7.2	—	4.698 *m*	—	—
Stigmasta-8,*Z*-24(28)-dien-3β-yl acetate (9-acetate)	0.608 *s*	0.961 *s*	0.951 *d* J = 5.5	0.974 *d* J = 6.5		1.589 *d* J = 7.0	—	4.699 *m*	5.105 *m* (*quartet*) J = 7.0	2.831 *m* (*septet*) J = 6.5
4α-Methyl-stigmast-8,*Z*-24(28)-dien-3β-yl acetate (7-acetate)	0.607 *s*	0.985 *s*	0.951 *d* J = 5.5	0.975 *d* J = 7.0		1.588 *d* J = 6.8	0.852 *m* J = 6.0	4.333 *m*	5.072 *m* (*quartet*) J = 7.0	2.822 *m* (*septet*) J = 7.0
4α-Methyl-ergosta-8,24(28)-dien-3β-yl acetate (5-acetate)	0.611 *s*	0.987 *s*	†	1.025 *d* J = 7.0		—	0.853 *d* J = 7.0	4.333 *m*	4.670, 4.720 *dd*	—

* Coupling constants in Hz.
† Not resolved.

having one more double bond in the lateral chain. These MS were not characteristic of Δ^7 sterols, which have a typical fragmentation pattern [6, 7]; in particular, Δ^7 sterols with a double bond in the lateral chain generally show a very intense fragmentation (base peak) corresponding to the loss of the lateral chain and of two H (c) (Table 2, compounds **6**, **8**, and **10**). A McLafferty fragmentation (a, Table 2) was observed in **14** and **9**, suggesting a double bond at position C-24. However these McLafferty fragmentations were much less intense than for Δ^7,24-dienes and $\Delta^{5,24}$-dienes (Table 2, **6**, **8**, **10**, **11**, and **15**). PMR data are given in Table 3. Sterol **9** and **12** acetates exhibited signals at 0.605–608 and 0.961 ppm corresponding respectively to C-18 and C-19 methyls in a Δ^8 compound [8]. No traces of signals corresponding to Δ^5, Δ^7, and $\Delta^{8(14)}$ compounds were detected in the PMR spectra of **9** and **12** acetates [9]. The absence of any signal corresponding to intracyclic vinylic protons supported also the presence of the Δ^8 double bond.

(24R)-24-Ethyl-5α-cholest-8-en-3β-yl acetate (**12-acetate**)

The identification of this compound was greatly facilited following the article of Rubinstein *et al.* [9]. The two terminal isopropyl methyl groups (C-26 and C-27) showed nonequivalence, and gave two well resolved doublets. Also, the C-29 methyl gave a typical triplet (Table 3). The measured chemical shifts for the C-26, C-27, and C-29 methyls were almost identical with those of the sitosteryl acetate originating from normal bramble cells (data given in the experimental section) and with those chemical shifts given for sitosteryl acetate in the literature [9], suggesting strongly that the configuration at C-24 of 24-ethyl-5α-cholest-8-en-3β-yl acetate (**12** acetate) was *R*.* **12**-acetate contained small amounts of (24ξ)-24-methyl-5α-cholest-8-en-3β-yl acetate (**16** acetate). **12**- and **16**-acetates could not be separated in our experimental conditions. The chemical structure of **16**-acetate was clearly demonstrated by MS (Table 2).

5α-Stigmasta-8,Z-24(28)-dien-3β-yl acetate (**9-acetate**)

The presence of an ethylidene group at C-24 was demonstrated. Firstly, the chemical shift of the C-29 methyl was typical of a vinylic methyl. Second, a quartet typical of a proton at C-28 and a very well resolved septet characteristic of a proton at C-25 were obtained; the chemical shift of the latter was characteristic of a *Z* configuration (Table 3) [10, 11].

5α-Ergosta-8,24(28)-diene-3β-yl acetate (**14-acetate**)

14-acetate was chromatographically (TLC and GC) identical with an authentic sample. MS data (Table 2) were also identical with published data [12].

(b) *4α-Methyl sterols.* 4α-Methyl steryl acetates were resolved using argentation chromatography. Three bands were observed, having the same R_fs as 24-ethylidene lophenyl (band 1), cycloeucalenyl + obtusifoliyl (band 2), and 24-methylene lophenyl (band 3) acetates respectively, in order of increasing polarity. The RR_f/cholesterol ratios of components of band 2 were identical with those of cycloeucalenyl and obtusifoliyl acetates; however the RR_fs of components of band 1 (**5**)-acetate and band 3 (**7**)-acetate were shorter than those of 24-methylene lophenyl acetates and 24-ethylidene lophenyl respectively. The latter were not detectable in the chromatograms. MS (Table 2) were also very different from those of Δ^7 sterols: the fragmentation (c) which is the base peak in 24-methylene and 24-ethylidene lophenyl acetates, was much less intense in **5**- and **7**-acetates. A McLafferty fragmentation (Table 2) was observed in each case, suggesting a double bond at position C-24. However those fragmentations were much less intense than in the case of the lophenyl acetates. PMR data (Table 3) show the presence in the two products of a signal at *ca* 0.607–0.611 ppm, characteristic of a C-18 methyl in a Δ^8 compound. No traces of Δ^7 and $\Delta^{8(14)}$ impurities were detectable in the two products. The signal corresponding to the C-19 methyl was shifted to 0.985 ppm, in agreement with published data [13, 14]. This shift is caused by the 4α-methyl group. No intracyclic vinylic proton was detected in **5** and **7**.

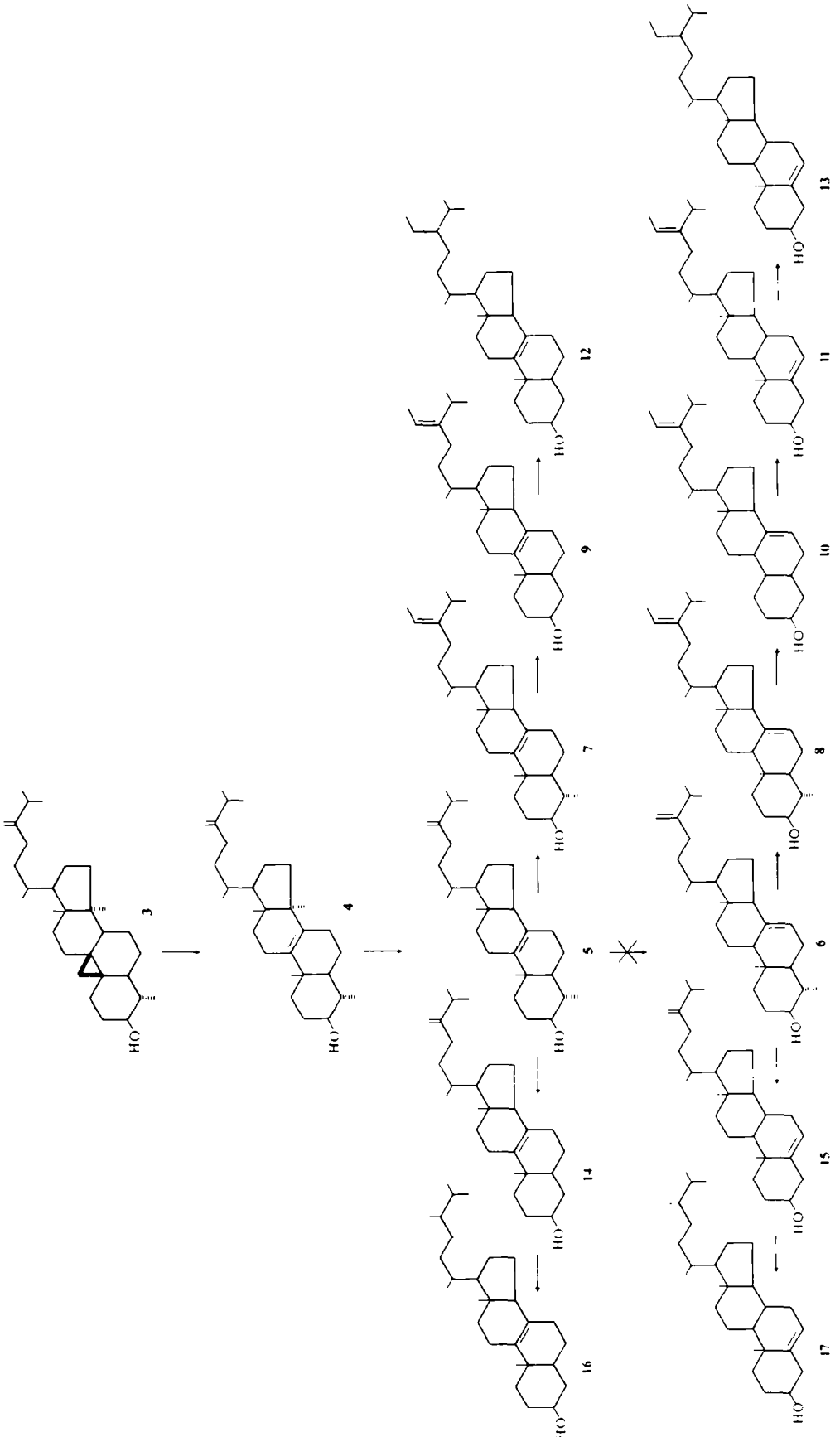
4α-Methyl-5α-stigmasta-8,Z-24(28)-dien-3β-yl (**7-acetate**)

The PMR spectrum of this compound was very similar to that of **9**-acetate (ethylidene of *Z* configuration at C-24). In addition, the 4α-methyl group gave a doublet at a typical position.

4α-Methyl-5α-ergosta-8,24(28)-dien-3β-yl acetate (**5-acetate**)

Sterol **5** has already been identified in yeast [15]. Our compound was identical (GC–MS) to an authentic sample of **5**-acetate. The PMR spectrum (Table 3) was in complete agreement with the chemical structure (two vinylic protons at C-28, 4α-methyl group, etc.).

* (24*S*)-24-Ethyl-5α-cholest-8-en-3β-ol has been reported previously [4] in *Chlorella ellipsoidae* poisoned with AY-9944.



Scheme 1

(c) 4,4-*Dimethyl sterols.* 4,4-Dimethyl sterols of AY cells were identical with those (**1** and **2**) of control cells except that a new band containing two 4,4-dimethyl sterols (X_1 and X_2) was detected in chromatograms. X_1 and X_2 have not yet been identified.

DISCUSSION

Our results show that in a bramble cell line growing on AY-9944, only a small amount (5%) of the sterols present were Δ^5 sterols, and most (80%) were Δ^8 sterols. The following substances were new compounds: (24*R*)-24-ethyl-5α-cholest-8-en-3β-ol (**12**), stigmasta-8,*Z*-24(28)-dien-3β-ol (**9**), and 4α-methyl-stigmasta-8,*Z*-24(28)-dien-3β-ol (**7**). 4α-Methyl-ergosta-8,24(28)-dien-3β-ol (**5**) and ergosta-8,24(28)-dien-3β-ol (**14**) have been found previously in yeast [12, 15] but not in a higher plant. It is generally accepted [16] that in higher plants, obtusifoliol (**4**) is the precursor of 24-methylene lophenol (**6**). By analogy with pathways established in rat liver and in yeast for the removal of the 14-methyl group [17–19], it can be expected that **4** is first demethylated in position C-14 to give 4α-methyl-ergosta-8,24(28)-dien-3β-ol (**5**) and that the Δ^8 double bond of **5** is isomerized to yield **6** (Scheme 1); in these conditions, the accumulation of **5** and 4α-methyl-stigmasta-8,*Z*-24(28)-dien-3β-ol (**7**) in AY cells may result from an inhibition of the $\Delta^8 \rightarrow \Delta^7$ isomerase which converts **5** into **6** in control cells. Thus our results suggest that **5** and **7** could be intermediates of sterol biosynthesis in control bramble cells. The detection and unambiguous identification of trace amounts of **5** and **7** in some batches of control cells supported the above hypothesis. Definitive proof of the intermediacy of **5** and **7** will be attained by incubation of these compounds and other Δ^8 sterols in an *in vitro* assay of $\Delta^8 \rightarrow \Delta^7$ isomerase.

The accumulation of stigmasta-8,*Z*-24(28)-dien-3β-ol (**9**) and (24*R*)-24 ethyl-5α-cholest-8-en-3β-ol (**12**) in AY cells showed that all the enzymes after the $\Delta^8 \rightarrow \Delta^7$ isomerase step (the C-28 methyl transferase, the C-4α methyl demethylase, and the enzymatic system which converts the 24-ethylidene group into a 24*R*-ethyl group) accepted Δ^8 compounds as substrates in place of the normal Δ^7 or Δ^5 compounds. However, these enzymes may act more slowly in AY cells than in control cells, since **5**, **7**, and **9** accumulated (Table 1). Recent studies performed with the same material (control cells) have shown that **6** was by far the best substrate for an *in vitro* *S*-adenosyl methionine-C-28 methyl transferase assay [20], and suggested that the C-28 methylation, the enzymatic step which introduced a second carbon unit into the lateral chain of plant sterols, acted *in vivo* mostly on **6** and very little on such other 24-methylene sterols as 24-methylene cholesterol. The accumulation of **7** in AY cells suggested that in this material, **5** would also be a good substrate for the C-28 methylation. In addition, we found that in the context of C-28 methylation, the ratio of 24-ethyl/24-methyl sterols was significantly higher in AY cells (7.2) than in control cells (5.0).

The results obtained in our material differed from those obtained with other organisms (rat liver, algae). In particular, treatment of *Chlorella emersonii* with AY [4] inhibited the removal of the 14-methyl group, the C-28 transmethylation, and the introduction of the Δ^{22} bond. Also 8,14-dienes (70% If the total sterols) and 8-enes (20%) accumulated dramatically in *C. ellipsoidea* treated with AY [4], showing that the fungicide inhibited mainly the Δ^{14} reductase and to a lesser extent the $\Delta^8 \rightarrow \Delta^7$ isomerase. No explanation can be suggested now for this complete lack of specificity of the drug.

As we pointed out above, a stable cell line containing mostly Δ^8 sterols and growing faster than control cells has been obtained. As AY is probably not a mutagenic compound, two alternative hypotheses could be formulated to explain our results: (1) AY may act by blocking $\Delta^8 \rightarrow \Delta^7$ isomerase, resulting in the accumulation of Δ^8 sterols which would then replace sitosterol in the vital functions attributed to Δ^5 sterols [21] in eukaryotic cells (particularly in membranes). If this hypothesis is correct, the normal pathway would gradually be restored after AY was removed from the culture medium. (2) AY may inhibit the $\Delta^8 \rightarrow \Delta^7$ isomerase, but with lethal effect if Δ^8 sterols could not replace Δ^5 sterols in membranes. However, if the cells were maintained long enough in the presence of the drug, a selection of cell lines capable of incorporating Δ^8 sterols into their membranes could occur; in that case the normal phenotype would not reappear following the removal of AY from the medium. Experiments are in progress to find out which hypothesis is correct.

EXPERIMENTAL

TLC was carried out on Merck HF 254 plates (0.2 mm). For argentation TLC, plates were immersed in a 10% soln of $AgNO_3$ in EtOH–H_2O (3:1), dried for 12 hr at room temp. and activated for 30 min at 110°. After being sprayed with a 0.1% soln of berberin hydrochloride in EtOH, the products were observed under UV (340 nm). For GLC, we used a GC fitted with two FID and two glass columns (1.50 m × 3 mm) packed with either 1% SE-30 or 1% OV-17, GC–MS was carried out at an ionizing energy of 70 eV. The separation was carried out at 270° on a glass column packed with 1% Dexsil. PMR spectra were measured in $CDCl_3$; the chemical shifts of signals are given in δ with TMS as the internal standard. The RR_ts (SE-30) on GLC for the acetates of the triterpenoids isolated in this study were cholesterol, RR_t 1.0; α-amyrin acetate, 1.70; β-amyrin acetate, 2.02; cycloartenyl (**1**)-acetate, 2.24; X_1-acetate, 2.02; X_2-acetate, 2.20; 24-methylene cycloartanyl (**2**)-acetate, 2.57; 4α-methyl-stigmasta-8,*Z*-24(28)-dien-3β-yl (**7**)-acetate, 2.54; 24-ethylidene lophenyl (**8**)-acetate, 2.65; 4α-methyl-ergosta-8,24(28)-dien-3β-yl (**5**)-acetate, 1.96; 24-methylene lophenyl (**6**)-acetate, 2.10; cycloeucalenyl (**3**)-acetate, 2.16; obtusifoliyl (**4**)-acetate, 1.92. The RR_ts (OV 17) on GLC for the acetates of the 4-desmethyl sterols isolated in this study were cholesterol, RR_t 1.0; (24*R*)-24-ethyl-5α-cholest-8-en-3β-yl (**12**)-acetate, 2.18; sitosteryl (**13**)-acetate, 2.07; (24ξ)-24-methyl-5α-cholest-8-en-3β-yl (**16**)-acetate, 1.78; campesteryl (**17**)-acetate, 1.59; stigmasta-8,*Z*-24(28)-dien-3β-yl (**9**)-acetate, 2.43; stigmasta-7,*Z*-24(28)-dien-3β-yl (**10**)-acetate, 2.64; isofucosteryl (**11**)-acetate, 2.30; ergosta-8,24(28)-dien-3β-yl (**14**)-acetate, 1.83; 24-methylene cholesteryl (**15**)-acetate, 1.65.

Plant material. Suspension cultures of bramble cells were grown under continuous white light at 25° on a synthetic sterile medium as described previously [20]. AY-9944 (*trans*-1,4-bis(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride), 8 mg/l., was added in soln in EtOH to the culture medium. The drug was not sterilized before use, because the contamination of the cultures by micro-organisms in the drug was negligible.

Analytical procedure. Bramble cells were harvested by filtration on a nylon cloth (50 μm); the cells were frozen and then lyophilized. The dried cells (*ca* 3 g) were extracted with petrol in a Soxhlet apparatus. After evapn of the solvent, the residue was saponified using KOH (5%) in MeOH. The non-saponifiable matter was extracted 3 × with petrol (50 ml). The combined extracts were dried over Na_2SO_4, evapd under red. pres. and separated by TLC with CH_2Cl_2 as the solvent (2 runs). The

bands of 4,4-dimethyl sterols, 4α-methyl sterols, and 4-demethyl sterol were scraped off and each type was eluted. The 3 classes of compounds were acetylated at room temp. for 14 hr using a mixture of C_5H_5N (50 μl) and Ac_2O (100 μl). Excess reagents were evapd under red. pres. and the crude acetates purified on TLC using CH_2Cl_2. Each of 3 classes of acetates was analysed by GLC and the total amount of sterols present in each class was quantified. Analytical argentation TLC, using cyclohexane–toluene (3:2) as the developing solvent, 15 hr of migration, was performed on each class of steryl acetates, and the obtained bands were analysed by GLC. 4,4-Dimethyl steryl acetates gave 3 bands in the case of control bramble cells, corresponding in order of increasing polarity to 24-methylene cycloartanyl (**2**)-acetate, cycloartenyl (**1**)-acetate, and a mixture of α- and β-amyrin acetate; 4 bands were obtained for AY cells, three the same as in the control cells and the fourth between bands 1 and 2. GC–MS analysis of the components of this fourth band showed the presence of two 4,4-dimethyl sterols, which were not investigated further. There were 3 bands of 4α-methyl steryl acetates for control bramble cells, corresponding in order of increasing polarity to 24-methylene-lophenyl (**6**)-acetate, a mixture of cycloeucalenyl (**3**)-acetate and obtusifoliyl (**4**)-acetates, and 24-ethylidene lophenyl (**8**)-acetate; and there were 3 bands for AY cells at the same R_fs as in the control cells, the most polar and least polar bands containing respectively 4α-methyl-ergosta-8,24(28)-dien-3β-yl (**5**)- and 4α-methyl-stigmasta-8,*Z*-24(28)-dien-3β-yl (**7**)-acetates, and the intermediary band containing **3**- and **4**-acetates. **5**- and **7**-acetates were practically pure and were crystallized with CH_2Cl_2–MeOH. 7-Acetate (4 mg from 3 batches of AY cells) mp 129–132°. **5**-acetate (1 mg from 3 batches of AY cells) mp 149–153°. For MS and PMR spectra, see Tables 2 and 3. There were 3 bands of 4-desmethyl steryl acetates for control bramble cells, corresponding in order of increasing polarity to 24-methylene cholesteryl (**15**)-acetate, isofucosteryl (**11**)-acetate and a mixture of campesteryl (**17**)- and sitosteryl (**13**)-acetates; **13**-acetate (10 mg from 3 batches of control cells) PMR (250 MHz, $CDCl_3$): δ 0.678 (3H, *s*, C-18), 0.813 (3H, *d*, *J* = 6.5 Hz, C-26), 0.834 (3H, *d*, *J* = 6.5 Hz, C-27), 0.846 (3H, *t*, *J* = 8Hz, C-29), 0.921 (3H, *d*, *J* = 6.5 Hz), 1.019 (3H, *s*, C-19), 4.623 (1H, *m*, C-3), 5.385 (1H, *m*, C-6). For AY cells there were also 3 bands, at the same R_fs as for the control cells, the most polar containing ergosta-8,24(28)-dien-3β-yl (**14**)-acetate with traces of other unknown steryl acetates, the intermediate containing mainly stigmasta-8,*Z*-24(28)-dien-3β-yl (**9**)-acetate with traces of **12**-acetate, and the least polar containing a mixture of (24ξ)-24-methyl-5α-cholest-8-en-3β-yl (**16**)-acetate, (24*R*)-24-ethyl-5α-cholest-8-en-3β-yl (**12**)-acetates, and small amounts on **13**- and **17**-acetates. Complete resolution was achieved by one more argentation TLC, using H_2O-free $CHCl_3$ in the case of **12**- and **16**-acetates, and commercial $CHCl_3$ in the case of **9**- and **14**-acetates. In these conditions, **12**- and **16**-acetates were totally separated from **13**- and **17**-acetates, **9**-acetate was separated from **11**-acetate, and **14**-acetate from **15**-acetate. **9**- and **12**-acetates were crystallized with CH_2Cl_2–MeOH. **12**-acetate (10 mg from 3 batches of AY cells) mp 131–134°, $[\alpha]_D$ +21° (c 0.5). **9**-Acetate (10 mg from 4 batches of AY cells) mp 127–130°, $[\alpha]_D$ +5° (c 0.5). For MS and PMR spectra, see Tables 2 and 3.

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NOMENCLATURE

Cycloartenol = 4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol (**1**); 24-methylene cycloartanol = 4,4,14α-trimethyl-9β,19-cyclo-5α-ergost-24 (28)-en-3β-ol (**2**); cycloeucalenol = 4,14α-dimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (**3**); obtusifoliol = 4,14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol (**4**); 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol (**5**); 24-methylene lophenol = 4α-methyl-5α-ergosta-7,24(28)-dien-3β-ol (**6**); 4α-methyl-5α-stigmasta-8,*Z*-24(28)-dien-3β-ol (**7**); 24-ethylidene lophenol = 4α-methyl-5α-stigmasta-7,*Z*-24(28)-dien-3β-ol (**8**); 5α-stigmasta-8,*Z*-24(28)-dien-3β-ol (**9**); 5α-stigmasta-7,*Z*24(28)-dien-3β-ol (**10**); isofucosterol = stigmasta-5,*Z*-24(28)-dien-3β-ol (**11**); (24*R*)-24-ethyl-5α-cholest-8-en-3β-ol (**12**); sitosterol = (24*R*)-24-ethyl-cholest-5-en-3β-ol (**13**); 5α-ergosta-8,24(28)-dien-3β-ol (**14**); 24-methylene cholesterol = ergosta-5,24(28)-dien-3β-ol (**15**); (24ξ)-24-methyl-5α-cholest-8-en-3β-ol (**16**); (24ξ)-24-methyl-cholest-5-en-3β-ol (**17**).

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