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ACCUMULATION OF $\Delta^{8,14}$-STEROLS IN SUSPENSION CULTURES OF BRAMBLE CELLS CULTURED WITH AN AZASTEROL ANTIMYCOTIC AGENT (A25822B)

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Key Word Index—*Rubus fruticosus*; Rosaceae; sterol biosynthesis inhibitor; Δ^{14}-reduction; ^{1}H NMR of $\Delta^{8,14}$-sterols.

Abstract—Bramble suspension cultures normally contain Δ^5-sterols (sitosterol, campesterol and isofucosterol). When the cells were grown in a medium supplemented with 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol, Δ^5-sterols disappeared almost completely whereas $\Delta^{8,14}$-sterols accumulated strongly. Five $\Delta^{8,14}$-sterols, including two new compounds, (24*R*)-24-ethyl-5α-cholesta-8,14-dien-3β-ol and 4α-methyl-5α-stigmasta-8,14, *Z*-24(28)-trien-3β-ol, were identified. The 15-azasterol probably inhibited the reduction of the Δ^{14}-bond. Cell lines growing permanently in an azasterol-supplemented medium were obtained.

INTRODUCTION

The antimycotic agent 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**1**) strongly inhibits ergosterol biosynthesis in *Saccharomyces cerevisiae* [1–3]. A new sterol, ergosta-8,14-dien-3β-ol, has been shown to accumulate in *S. cerevisiae* grown in the presence of **1** [3]. $\Delta^{8,14}$-Sterols have been shown to accumulate dramatically in *Chlorella ellipsoidea* treated with AY 9944 [4, 5]. $\Delta^{8,14}$-Sterols are considered to be intermediates in the biosynthesis of cholesterol in rat liver [6, 7] and of ergosterol in yeast [8], although they may not be so in higher plants [9]. The isolation of 5α-stigmasta-8,14,*Z*-24(28)-trien-3β-ol as the major sterol of *Vernonia anthelmintica* [10] and recent biosynthetic data [11] suggest strongly that $\Delta^{8,14}$-sterols are also intermediates in the biosynthesis of higher plant sterols.

Recent studies have shown that bramble cells cultured *in vitro* are good material in which to study the action of inhibitors of sterol biosynthesis. Spectacular results have been obtained with this material treated with AY 9944 [12] or with fenarimol [13]. We report here that **1** powerfully inhibits Δ^5-sterol biosynthesis in bramble cells and that treated cells synthesize mainly $\Delta^{8,14}$-sterols which constitute more than 65% of the total sterols.

RESULTS

Bramble-cell strain growing on 15-*aza*-24-*methylene*-D-*homocholesta*-8,14-*dien*-3β-*ol* (**1**)

The culture medium was supplemented with **1** (0.5 mg/l. ~ 10^{-6} M). Bramble cells cultivated on this medium grow very slowly at first. After two months the cells were subcultured in a fresh medium supplemented with **1**; although their growth rate increased, it remained lower than that of control cells even after seven passages. The composition of the sterol fraction was profoundly changed qualitatively and quantitatively starting with the first passage; as in the case of cells cultivated in the presence of AY 9944 [12], but in contrast with our experience with cells cultivated in the presence of fenarimol [13], a stable cell line was obtained after three passages. The physiological properties of the strains obtained following long-term treatment of bramble suspension cultures by xenobiotics such as AY 9944, fenarimol, **1**, and others will be described in more detail elsewhere.

Sterol composition of cells growing on **1**

The total sterol content of the cells growing on **1** (4.5 mg/g dry wt) was not very different from that of the control cells (4.8 mg/g dry wt). However, the amount of 4,4-dimethyl sterols and of 4α-methyl sterols increased noticeably, whereas that of 4-desmethyl sterols decreased slightly to 2.2 mg/g dry wt (3.4 mg/g dry wt in control cells). The proportions of the various sterols in **1**-cells and in control cells are given in Table 1. More than 65% of the sterols in **1**-cells were the following $\Delta^{8,14}$-sterols; 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol (**5**); 4α-methyl-5α-stigmasta-8,14,*Z*-24(28)-trien-3β-ol (**6**); 5α-stigmasta-8,14,*Z*-24(28)-trien-3β-ol (**8**); (24ξ)-24-methyl-5α-cholesta-8,14-dien-3β-ol (**9**) and (24*R*)-24-ethyl-5α-cholesta-8,14-dien-3β-ol (**10**), which was the major sterol. In addition to $\Delta^{8,14}$-sterols, two new 4,4-dimethyl sterols (X_1 and X_2) that are isomers of cycloartenol accumulated strongly; their structures cannot

Table 1. Sterols of control and **1**-treated bramble cells

	Control	Treated
Cycloartenol	0.5*	5
24-Methylene cycloartanol	0.15	2
X_1 and X_2	0	15
α- and β-amyrins	0.5	2.5
Cycloeucalenol (**3**)	0.1	1
Obtusifoliol (**4**)	0.1	0.5
24-Methylene lophenol (**17**)	0.1	0
24-Ethylidene lophenol (**18**)	0.1	0
4α-Methyl-5α-ergosta-8,14,24(28)-trien-3β-ol (**5**)	0	3.5
4α-Methyl-5α-stigmasta-8,14,*Z*-24(28)-trien-3β-ol (**6**)	0	12
5α-Stigmasta-8,14,*Z*-24(28)-trien-3β-ol (**8**)	0	8.5
(24*R*)-24-Ethyl-5α-cholesta-8,14-dien-3β-ol (**10**)	0	40
(24ξ)-24-Methyl-5α-cholesta-8,14-dien-3β-ol (**9**)	0	2.5
5α-Stigmasta-8,*Z*-24(28)-dien-3β-ol (**14**)	0	2.5
Isofucosterol (**20**)	12	tr
24-Methylene cholesterol (**19**)	2	tr
(24*R*)-24-Ethyl-5α-cholest-8-en-3β-ol (**16**)	0	5
(24ξ)-24-Methyl-5α-cholest-8-en-3β-ol (**15**)	0	tr
Sitosterol (**22**)	70	1.5
Campesterol (**21**)	14	tr

*As percentage of total sterols.

be correlated with that of any known 4,4-dimethyl sterol. In contrast to $\Delta^{8,14}$-sterols, whose concentrations were constant, the concentrations of X_1 and X_2 varied greatly from one bramble culture to another.

(a) 4-*Desmethyl sterols.* The components of this fraction were separated by argentation chromatography. Four bands for acetates of **8** (band 1), **19**+**9**+**10** (band 2), **14**+**20** (band 3), and **15**+**16**+**21**+**22** (band 4), were found. The components of bands 3 and 4 were easily identified as Δ^8-sterols previously found in bramble cells treated with AY 9944 [12] and the Δ^5-sterols present in control cells; band 3 contained a mixture of isofucosterol (**20**) and 5α-stigmasta-8,*Z*-24(28)-dien-3β-ol (**14**). Band 4 contained campesterol (**21**), sitosterol (**22**), (24ξ)-24-methyl-5α-cholest-8-en-3β-ol (**15**), and (24*R*)-24-ethyl-5α-cholest-8-en-3β-ol (**16**). The $\Delta^{8,14}$-sterols (bands 1 and 2) were immediately recognized by their strong UV absorption and their greater polarity. Their MS were also typical (Table 2), showing the presence of two cyclic double bonds (fragmentations b and b−60). One additional double bond in the lateral chain of **8**-acetate was clearly recognized. Another characteristic feature was the presence of a fragment (c) corresponding to M^+ − lateral chain − 1H [2]. This peak was high in the case of **8** but rather small in the case of acetates **9** and **10**. In addition, a fragment (c−60−14) found in **8**-, **9**- and **10**-acetates could be a result of aromatization of the C-cycle (Table 2). ^{1}H NMR data are given in Table 3. The **8**- and **10**-acetates exhibited signals at δ 0.817–0.820 and 1.005 and corresponded, respectively, to the C-18 and C-19 methyls in a $\Delta^{8,14}$-compound [10, 14]. Very typical was the presence in the three sterols of a narrow complex at 5.37 corresponding to one olefinic proton. No trace of signals corresponding to Δ^5-, Δ^7-, and $\Delta^{8(14)}$-compounds was detected in the ^{1}H NMR spectra of **8**- and **10**-acetates.

(b) 4α-*Methyl sterols.* They were resolved using argentation chromatography. Three bands were observed for acetates of **5** (band 1), **6** (band 2), and **3** and **4** (band 3). Band 3 contained the cycloeucalenyl (**3**)- and obtusifoliyl (**4**)-acetates identified previously in control cells [12]. $\Delta^{8,14}$-Sterols (band 1 and 2) were immediately recognized by their strong UV absorption and their much greater polarity. Their MS were typical (Table 2), showing the presence of two nuclear double bonds (fragmentation b) and of one additional double bond in the lateral chain (fragmentation a−15). The characteristic fragment (c) corresponding to M^+ − lateral chain − 1H [2] was present in the MS of **5**- and **6**-acetates, as was c−60−14; the presence of these fragments could indicate aromatization of the C-cycle. ^{1}H NMR data for **6**-acetate showed the presence of a signal at δ 0.821 characteristic of a C-18 methyl in a $\Delta^{8,14}$-compound [3, 10, 14]. The signal corresponding to the C-19 methyl was shifted to 1.027, a shift caused by the 4α-methyl group. An intracyclic vinylic proton was present in **6**-acetate at a characteristic chemical shift (5.376).

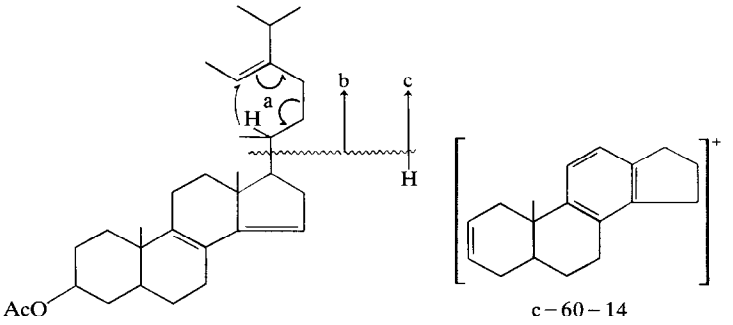
4α-Methyl-5α-stigmasta-8,14,Z-24(28)-3β-yl-acetate (**6**-*acetate*)

To our knowledge, this compound is new. The use of ^{1}H NMR spectroscopy at 250 MHz allowed us to obtain fingerprints for the molecule and to assign most of its protons unambiguously. The two terminal isopropyl methyl groups (C-26 and C-27) showed non-equivalence and gave two resolved doublets corresponding to coupling of the C-26 and C-27 protons with the proton at C-25. The presence of an ethylidene group at C-24 was demonstrated. Firstly, the chemical shift of the C-29 methyl was typical of a

Table 2. Mass spectra of the steryl acetates of cells treated with 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol

Steryl Acetate	M^+	M^+-15	M^+-43	M^+-60	$M^+-60-15$	a−15	b	c	b−60	c−60	b−60 −14	a−60 −15	c−60 −14		
5	452	437	409	392	377	353	327	326	267	266	253	293	252	341	281
	(100)	(61)	(35)	(18)	(68)	(81)	(23)	(48)	(22)	(15)	(38)	(25)	(36)	(10)	(32)
6	466	451	423	406	391	353	327	326	267	266	253	293	252	341	281
	(100)	(41)	(40)	(19)	(49)	(76)	(28)	(34)	(21)	(12)	(34)	(22)	(26)	(22)	(21)
8	452	437	409	392	377	339	313	312	253	252	239	279	238	327	267
	(100)*	(50)	(41)	(17)	(38)	(91)	(35)	(47)	(30)	(21)	(47)	(31)	(47)	(21)	(20)
9	440	425	—	380	365	—	313	312	253	—	239	—	238	—	—
	(100)	(28)		(23)	(98)		(27)	(7)	(23)		(28)		(36)		
10	454	439	—	394	379	—	313	312	253	—	239	—	238	—	—
	(100)	(26)		(15)	(95)		(26)	(4)	(20)		(22)		(26)		

*Relative intensity (only fragments with mass heavier than *m/e* 200 have been considered).



c−60−14

vinylic methyl. Second, a quartet typical of a proton at C-28 and a well-resolved septet characteristic of a proton at C-25 were obtained: the chemical shift of the latter indicated that the $\Delta^{24(28)}$ had a *Z*-configuration [15, 16].

5α-Stigmasta-8,14,Z-24(28)-trien-3β-yl-acetate (**8**-*acetate*)

This compound was identified previously in *V. anthelmintica* [10]. As reported earlier [10], **8**-acetate appeared to be very unstable and degraded rapidly upon storage. In order to obtain a proper ^{1}H NMR spectrum, it was necessary to isolate and purify the compound just before monitoring the spectrum. Our ^{1}H NMR data are essentially identical with published data [10] but give additional information. First, the methyl groups C-21, C-26 and C-27 were unambiguously resolved. Second, as for **6**-acetate, the two terminal isopropyl methyl groups (C-26 and C-27) showed nonequivalence and gave two resolved doublets.

4α-Methyl-5α-ergosta-8,14,24(28)-trien-3β-yl-acetate (**5**-*acetate*)

This compound was identified previously in a yeast mutant [14]. Not enough material was available to obtain a ^{1}H NMR spectrum; however, **5**-acetate was identified unambiguously by its MS. As shown in Table 2, the MS of **5**-acetate showed the fragmentation characteristic (c and c−60−14) of $\Delta^{8,14}$-sterols. In addition, this compound had an olefinic bond in the lateral chain (fragment a−15) as well as R_fs on TLC and RR_ts on GLC typical of a 24(28)-methylene sterol. Finally, **5**-acetate exhibited a UV spectrum (max

Table 3. ^{1}H NMR chemical shifts (δ) of the proton signals of **6**-, **8**- and **10**-acetates

	C-18	C-19	C-21	C-26	C-27	C-29	C-30	C-3αH	C-28H	C-25H	C-15H
4α-Methyl-5α-stigmasta-8,14,Z-24(28)-trien-3β-yl-acetate (**6**-acetate)	0.821 *s*	1.027 *s*	0.970 *d* $J=6$*	0.983 *d* $J=7$	0.980 *d* $J=7$	1.598 *d* $J=7$	0.880 *d* $J=6$	4.388 *dt* $J=10$, $J=5$	5.126 *m*(*quartet*) $J=7.5$	2.844 *m*(*septet*) $J=7$	5.376 *m*
5α-Stigmasta-8,14,Z-24(28)-trien-3β-yl-acetate (**8**-acetate)	0.820 *s*	1.005 *s*	0.971 *d* $J=6$	0.982 *d* $J=7$	0.978 *d* $J=7$	1.595 *d* $J=7$	—	4.728 *m*	5.124 *m*(*quartet*) $J=7$	2.840 *m*(*septet*) $J=7$	5.369 *m*
(24*R*)-24-Ethyl-5α-cholest-8-en-3β-yl acetate (**10**-acetate)	0.817 *s*	1.005 *s*	0.944 *d* $J=6$	0.842 *d* $J=6.5$	0.816 *d* $J=6.5$	0.847 *t* $J=7$	—	4.709 *m*	—	—	5.370 *m*

*Coupling constants in Hz.

249 nm) characteristic of a sterol containing a $\Delta^{8,14}$-heteroannular conjugated diene [2, 3].

(24R)-24-*Ethyl*-5α-*cholesta*-8,14-*dien*-3β-*yl-acetate* (**10**-*acetate*)

The structure of this compound was ascertained by ^{1}H NMR spectroscopy. Whereas the methyls C-19 and C-20 and the olefinic proton C-15H could be assigned easily, some uncertainity remained for the methyls C-18, C-26, C-27 and C-29, since their signals overlapped. Consequently, **10**-acetate was saponified and ^{1}H NMR spectroscopy was performed in the presence of Eu(fod)$_3$. The evolution of chemical shifts with increasing concentrations of the lanthanide shift reagent is given in Fig. 1. The signals of the methyls were resolved clearly. In particular the C-18 methyl was easily separated from the C-26 and C-27 methyls which showed nonequivalence and gave two well-resolved doublets whereas the C-29 methyl gave a triplet [17, 18]. The measured chemical shifts for C-26, C-27, and C-29 methyls were close to those of the sitosteryl acetate originating from normal bramble cells [12] and of authentic sitosteryl acetate [17], suggesting that the configuration at C-24 of **10**-acetate was *R*. The **10**-acetate contained small amounts of (24ξ)-24-methyl-5α-cholesta-8,14-dien-3β-yl-acetate (**9**-acetate). The **9**- and **10**-acetates were not separated in our experimental conditions on TLC but were easily separated on GLC. The chemical structure of **9**-acetate was clearly demonstrated by MS (Table 2).

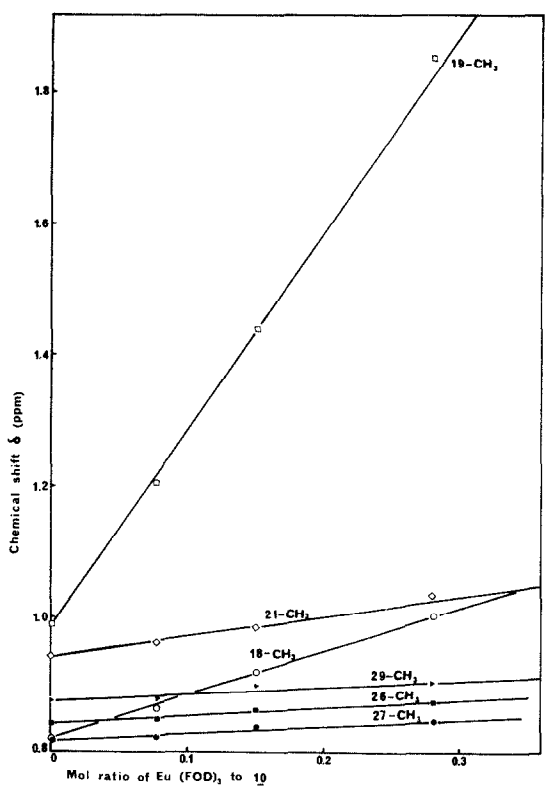


Fig 1. Effect of increasing concentrations of Eu(fod)$_3$ on the ^{1}H NMR chemical shifts of the methyl groups of (24*R*)-24-ethyl-5α-cholesta-8,14-dien-3β-yl-acetate (**10**-acetate).

DISCUSSION

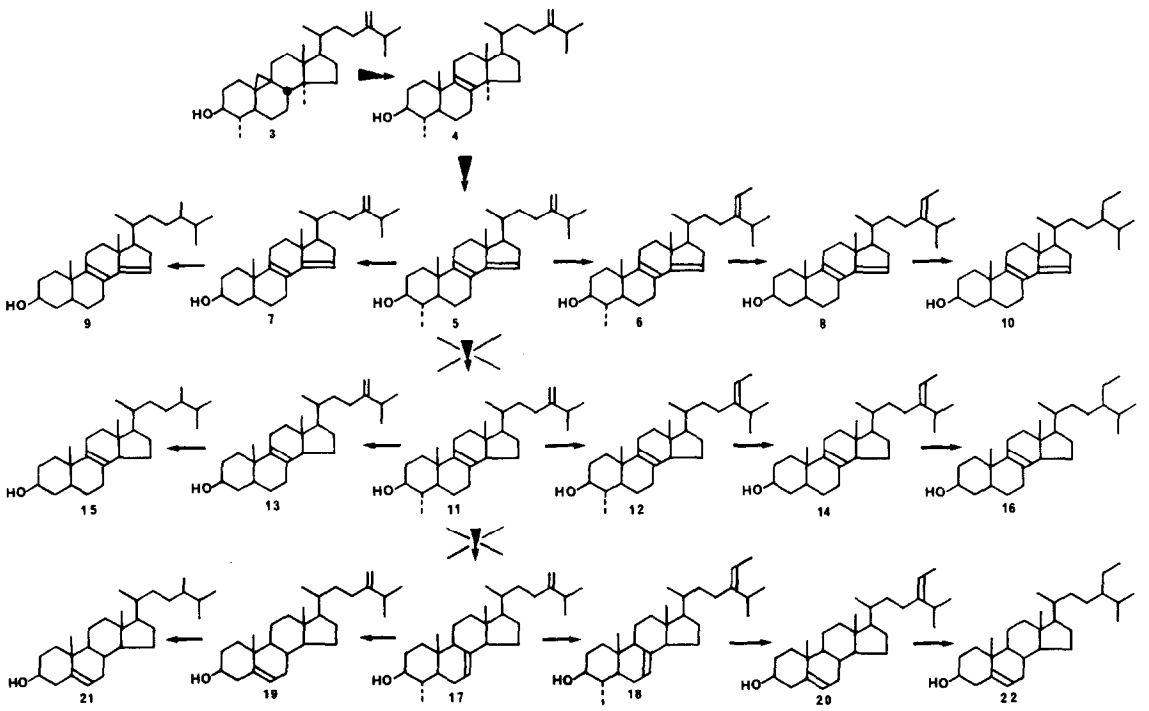
As shown in Table 1, bramble cells grown in the presence of **1** accumulated $\Delta^{8,14}$-sterols. In most of the experiments, $\Delta^{8,14}$-sterols represented about 65% of the total sterols in the **1**-treated cells and were not present in control cells. Of the $\Delta^{8,14}$-sterols, (24*R*)-24-ethyl-5α-cholesta-8,14-dien-3β-ol (**10**) predominated greatly in the treated cells. The other most abundant $\Delta^{8,14}$-sterols were 4α-methyl-5α-stigmasta-8,14,*Z*-24(28)-trien-3β-ol (**6**), 5α-stigmasta-8,14,*Z*-24(28)-trien-3β-ol (**8**), and 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol (**5**). Sterol **6** was new, as far as we know. Compound **10** had been reported in very low amounts in *Brassica napus* seed [19], but was not completely identified. This has been done in the present work by ^{1}H NMR spectroscopy (250 MHz) in the presence of Eu(fod)$_3$. The 24*S* epimer of **10** had been identified in *C. ellipsoidea* treated with AY 9944 [4]. Sterol **8** had been found to be the major sterol of *V. anthelmintica* seeds [10] but had not been reported in any other organism. Sterol **5** had been found in a yeast mutant [14].

Results obtained in the present work with higher plant cells closely resemble results obtained previously with **1**-treated yeast [2, 3], and those obtained in AY 9944-treated *C. ellipsoidea* [4, 5]. As in these two systems, $\Delta^{8,14}$-sterols accumulated, suggesting that the $\Delta^{8,14}$-reductase, i.e. the enzyme that hydrogenates the Δ^{14}-double bond of $\Delta^{8,14}$-sterols, is specifically inhibited. As shown in Tables 1 and 4, Δ^5-sterols were found in very low concentrations in **1**-treated bramble cells and were mostly replaced by $\Delta^{8,14}$-sterols. Moreover, Δ^8-sterols are also present in treated cells, generally at higher concentrations than Δ^5-sterols. This indicated that **1** could also interfere with the $\Delta^8 \rightarrow \Delta^7$-isomerase. Interestingly, the percentage by which C_{10} exceeded C_9 side-chain sterols was significantly higher in $\Delta^{8,14}$-sterols of treated cells than in Δ^5-sterols of control cells (Table 4). This could indicate that $\Delta^{8,14}$-sterols could be very efficient substrates for the C-28 methylation reaction [20] and this observation would mean that bramble cells are different from yeast in this respect, since in the latter, **1** has been found to be a competitive inhibitor of the Δ^{24}-sterol methyl transferase both *in vivo* and *in vitro* [21]. Another secondary effect of **1** consisted in an accumulation of $\Delta^{24(28)}$-sterols (Table 4), suggesting that **1**

Table 4. Percentage of sterol features in control and **1**-treated bramble cells

	Control	Treated
Total $\Delta^{8,14}$	—	66.5*
$\Delta^{8,14}$ C_{10} side chain	—	60.5 (90†)
$\Delta^{8,14,24(28)}$	—	24 (36†)
Total Δ^5	98*	<2*
Δ^5 C_{10} side chain	82 (82‡)	<2
$\Delta^{5,24(28)}$	14 (15‡)	—
Total Δ^8	—	8*

*As % of total sterols.
†As % of total $\Delta^{8,14}$-sterols.
‡As % of total Δ^5-sterols.



Scheme 1. Possible biosynthetic relationships of the sterols isolated from normal and **1**-treated bramble cells.

would also inhibit the $\Delta^{24(28)}$-hydrogenase. A similar effect was observed in fenarimol-treated [13] and AY 9944-treated [12] bramble cells and seemed to be nonspecific, occurring in injured bramble cells.

The main biosynthetic routes leading to 4-desmethyl sterols in **1**-treated bramble cells are presented in Scheme 1. Sterols **7**, **13**, **17**, and **18** were not detected in our material, possibly because they were present in too small amounts (**13**, **17**, **18**) or were too unstable (**7**); however, their involvement in the various pathways was highly probable.

$\Delta^{8,14}$-Sterols are generally not present in higher plants, nor were they detected in our control bramble cells. The question of their involvement in plant sterol biosynthesis has been debated following the isolation of 5α-stigmasta-8(14),22-dien-3β-ol from *Aplopappus heterophyllus* [22] and results obtained with *Calendula officinalis* showing that two hydrogen atoms derived from C-2 of mevalonic acid may be retained at C-15 in sitosterol [9]. However, recent findings demonstrating that the 2-*pro S* hydrogen atom of mevalonic acid is removed from C-15 during the biosynthesis of sitosterol [11], suggest that in fact $\Delta^{8,14}$-sterols enter into sterol biosynthesis in higher plants, as well as in animals and yeast. Our results strongly agree with that conclusion.

A strain growing permanently on **1** has been obtained; the strain is characterized by a high $\Delta^{8,14}$-and quite a low Δ^5-sterol content. This feature appears to be stable. The **1**-strain does not seem to differ morphologically from the control strain, but it grows slightly slower. The physiological consequences of the presence of $\Delta^{8,14}$-sterols into cells are being studied in our laboratory. In particular it will be checked whether $\Delta^{8,14}$-sterols are present in the membranes in place of the normally occurring Δ^5-sterols [23].

EXPERIMENTAL

Most of the techniques used in the present work have been previously described in detail [12]. The RR_ts (SE-30) on GLC for the acetates of the 4α-methyl sterols isolated in this study were cholesterol, RR_t 1.0; cycloeucalenyl (**3**)-acetate, 2.16; obtusifoliyl (**4**)-acetate, 1.92; 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-yl (**5**)-acetate, 1.94 and 4α-methyl-5α-stigmasta-8,14,*Z*-24(28)-trien-3β-yl (**6**)-acetate, 2.42. The RR_ts (OV-17) on GLC for the 4-desmethyl sterols isolated in this study were cholesterol, RR_t 1.0; 5α-stigmasta-8,14,*Z*-24(28)-trien-3β-yl (**8**)-acetate, 2.42; (24ξ)-24-methyl-5α-cholesta-8,14-dien-3β-yl (**9**)-acetate, 1.80; (24*R*)-24-ethyl-5α-cholesta-8,14-dien-3β-yl (**10**)-acetate, 2.23; 5α-stigmasta-8,*Z*-24(28)-dien-3β-yl (**14**)-acetate, 2.23; (24ξ)-24-methyl-5α-cholest-8-en-3β-yl (**15**)-acetate, 1.80; (24*R*)-24-ethyl-5α-cholest-8-en-3β-yl (**16**)-acetate, 2.20; 24-methylene cholesteryl (**19**)-acetate, 1.71; isofucosteryl (**20**)-acetate, 2.25; campesteryl (**21**)-acetate, 1.68, and sitosteryl (**22**)-acetate, 2.07.

Plant materials. Suspension cultures of bramble cells (*Rubus fruticosus*) were grown under continous white light at 25° on a synthetic sterile medium as described previously [20]. **1**, 0.5 mg/l., was added in soln in EtOH to the culture medium. The drug was sterilized by filtration through Millipore (ϕ 0.45 μm) filters.

Analytical procedure. The isolation of 4,4-dimethyl, 4α-methyl, and 4-desmethyl steryl acetates has been described previously [12]. Each of the three classes of acetates was analysed by GLC, and the total amount of sterols present in each class was quantified. Analytical argentation TLC, in which cyclohexane–toluene (3:2 in the case of 4,4-dimethyl steryl acetates, 1:1 in the case of 4α-methyl and 4-desmethyl acetates) was the developing solvent and migration was for 15 hr, was performed on each class of steryl acetates, and the bands obtained were analysed by GLC. There were four

bands of 4,4-dimethyl steryl acetates in the case of treated cells, corresponding in order of increasing polarity to 24-methylene cycloartanyl (**2**)-acetate, a mixture of X_1 and X_2 acetates, cycloartenyl (**1**)-acetate, and a mixture of α- and β-amyrin acetates. There were four bands of 4α-methyl steryl acetates for treated cells: the most polar band contained only **5**-acetate; the second band in order of decreasing polarity contained only 6-acetate; the third, cycloeucalenyl (**3**)- and obtusifolyil (**4**)-acetate; and the least polar band, very low amounts of **12**-acetate. **5**-acetate, UV λ_{max} nm: 249 (heptane). **6**-acetate, UV λ_{max} nm: 251, 11000 (heptane), mp 127–129°. There were four bands of 4-desmethyl steryl acetates in treated cells: the first band contained only **8**-acetate; the second, a mixture of **9**-, **10**-, and traces of **19**-acetates; the third, a mixture of **14**- and **20**-acetates; and the fourth, a mixture of **15**-, **16**-, **21**-, and **22**-acetates. **19**-acetate was separated from **9**+**10**-acetates by argentation TLC using commercial unwashed $CHCl_3$ as the developing solvent. **15**+**16**-acetates, were separated from **21**+**22**-acetates by argentation TLC using EtOH-free $CHCl_3$ as the developing solvent. The **9**- and **10**-acetates were not separated by TLC in our experimental conditions but were unambiguously separated by GLC (SE-30, OV-17). **8**-acetate, UV λ_{max} nm: 249 (heptane). **10**-acetate (16 mg from 12 g of treated cells, dry wt) UV λ_{max} nm: 251, ε23500 (heptane), $[\alpha]_D$ −22° (*c* 0.5), mp 102–105°; **10**-alcohol, mp 124–125° (two crystallizations from CH_2Cl_2–MeOH).

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