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EFFECT OF FENARIMOL ON STEROL BIOSYNTHESIS IN SUSPENSION CULTURES OF BRAMBLE CELLS

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Key Word Index—*Rubus fruticosus*; Rosaceae; sterol biosynthesis inhibitor; fenarimol; 14α-methyl demethylation; ^{1}H NMR 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 fenarimol, 14α-methyl sterols accumulated. Eight 14α-methyl sterols, including two new compounds, 4α,14α-dimethyl-stigmasta-8,*Z*-24(28)-dien-3β-ol and 14α-methyl-stigmasta-8,*Z*-24(28)-dien-3β-ol, were identified. Fenarimol probably inhibited the 14α-methyl demethylation. Cell lines growing permanently in a fenarimol-supplemented medium were obtained.

INTRODUCTION

Fenarimol, α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidine methanol, has been studied in relation to its antifungal effects. It is structurally very similar to triarimol, α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidine methanol, a well-known compound that interferes with the biosynthesis of sterols in rat liver [1], in *Ustilago maydis* [2] and in three species of *Chlorella* [3]. In each of these materials, triarimol strongly inhibits the 14α-demethylase involved in sterol biosynthesis, resulting in the accumulation of 14α-methyl sterols: lanosterol and dihydrolanosterol in rat liver [1]; 24-methylene dihydrolanosterol, obtusifoliol, and 14α-methyl-5α-ergosta-8,24(28)-dien-3β-ol in *U. maydis* [2] and a mixture of 24-methyl and 24-ethyl-14α-methyl sterols in the three species of *Chlorella* [3].

Triarimol and its analogue ancymidol [4] retard the growth of *Phaseolus vulgaris* but do not noticeably affect the qualitative and quantitative distribution of the main sterols present [4]. Since whole plants are often not well suited for the study of the action of xenobiotes because of compartmentation and permeability problems, we used suspension cultures of bramble to study the action of fenarimol on sterol biosynthesis. As spectacular effects have been observed in sterol biosynthesis [5] when AY 9944 (another fungicide) was fed to bramble suspension cultures, we expected that fenarimol would also interfere with sterol biosynthesis in this material. We now report strains that grow permanently on a medium supplemented with fenarimol and which contain as much of the unusual 14α-methyl Δ^8-sterols as the normally occurring Δ^5-sterols.

RESULTS

Sterol composition of control suspension cultures of bramble cells

As described previously [5], the following triterpenes and sterols were identified in bramble cells: cycloartenol (**1**), 24-methylene cycloartanol (**2**), α- and β-amyrins, cycloeucalenol (**3**), obtusifoliol (**4**), 24-methylene lophenol (**13**), 24-ethylidene lophenol (**14**), 5α-stigmasta-7,24(28)-dien-3β-ol (**15**), isofucosterol (**16**), sitosterol (**17**), 24-methylene cholesterol (**19**) and campesterol (**20**).

Strain growing on fenarimol

The culture medium was supplemented with fenarimol (7 mg/l.). Cells cultivated on this medium grew very slowly at first. After 2 months, cells were subcultured in a fresh medium supplemented with fenarimol; although their growth rate increased, it remained noticeably lower than that of control cells even after five passages. The composition of the sterol fraction was profoundly changed qualitatively and quantitatively starting with the first treatment; in contrast to our experience with cells cultivated in the presence of AY 9944 [5], it was not possible to obtain a stable cell line. The results presented here correspond to cells having undergone four passages on a fenarimol-supplemented medium. The physiological properties of the strains obtained following permanent treatment of bramble suspension cultures by xenobiotes such as AY 9944, fenarimol and others will be described in more detail elsewhere.

Sterol composition of cells growing on fenarimol

The total sterol content of the cells growing on fenarimol (9.0 mg/g dry wt) was much higher than that of the control cells (3.4 mg/g dry wt). The amount of 4α-methyl sterols increased dramatically to 3.1 mg/g dry wt in fenarimol cells and 0.3 mg/g dry wt in control cells; a lower increase was observed in the 4-desmethyl sterol fraction, to 5.5 mg/g dry wt in fenarimol cells and 3.0 mg/g dry wt in control cells. The composition and relative proportions of the sterols in fenarimol cells are given in Table 1. More than 50% of the sterols were the following 14α-methyl sterols: obtusifoliol (**4**); 4α,14α-dimethyl-5α-stigmasta-8,*Z*-24(28)-dien-3β-ol (**5**); 4α,14α-dimethyl-5α-(24*R*)-ergost-8-en-3β-ol (**6**); 4α,14α-dimethyl-5α-(24ξ)-stigmast-8-en-3β-ol (**7**); 14α-methyl-5α-ergosta-8,24(28)-dien-3β-ol (**8**); 14α-methyl-5α-

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

	Control*	Treated*
Cycloartenol (**1**)	0.5	2
24-Methylene cycloartanol (**2**)	0.15	4
Cycloeucalenol (**3**)	0.1	0.1
Obtusifoliol (**4**)	0.1	17
4α,14α-Dimethyl-5α-stigmasta-8,*Z*-24(28)-dien-3β-ol (**5**)	0	6
4α,14α-Dimethyl-5α-(24ξ)-ergost-8-en-3β-ol (**6**)	0	2
4α,14α-Dimethyl-5α-(24ξ)-stigmast-8-en-3β-ol (**7**)†	0	tr
14α-Methyl-5α-ergosta-8,24(28)-dien-3β-ol (**8**)	0	12.5
14α-Methyl-5α-stigmasta-8,*Z*-24(28)-dien-3β-ol (**9**)	0	9
14α-Methyl-5α-(24*R*)-ergost-8-en-3β-ol (**10**)	0	3
14α-Methyl-5α-(24ξ)-stigmast-8-en-3β-ol (**11**)	0	2
24-Methylene lophenol (**13**)	0.1	0
24-Ethylidene lophenol (**14**)	0.1	0
5α-Stigmasta-7,*Z*-24(28)-dien-3β-ol (**15**)	tr	0
Isofucosterol (**16**)	12	16
Sitosterol (**17**)	70	13
24-Methylene cholesterol (**19**)	2	3
Campesterol (**20**)	14	6

* As percentage of total sterol.
† Detected in only one experiment.

Table 2. MS of the steryl acetates of fenarimol-treated bramble cells

Steryl acetate	M^+	$M^+ - 15$	$M^+ - 43$	$M^+ - 60$	$M^+ - 60 - 15$	*a* − 15*	*a* − 60 − 15	*c* − 15	*c* − 60 − 15	*b*	*b* − 60
4	468	453	423	408	393	369	309	329	269	301	241
	(34)†	(100)	(6)	(20)	(96)	(10)	(20)	(3)	(25)	(11)	(29)
5	482	467	439	422	407	369	309	329	269	—	241
	(21)	(52)	(5)	(29)	(100)	(12)	(30)	(3)	(18)	—	(26)
9	468	453	425	408	393	355	295	315	255	287	227
	(28)	(100)	(5)	(14)	(62)	(22)	(28)	(4)	(28)	(15)	(34)
11	470	455	—	410	395	—	—	315‡	255	287	227
	(10)	(100)	—	(7)	(50)	—	—	—	(20)	(14)	(15)
10	456	441	—	396	381	—	—	315‡	255	287	227
	(18)	(100)	—	(5)	(50)	—	—	—	(10)	(15)	(16)
8	454	429	411	394	379	355	295	315	255	287	227
	(30)	(100)	(5)	(14)	(71)	(16)	(25)	(2)	(18)	(17)	(31)
6	470	455	—	410	395	—	—	329‡	269	301	241
	(12)	(61)	—	(16)	(100)	—	—	—	(7)	(7)	(14)

* Peaks *a*, *b* and *c* are present together with their −15 derivatives. Only the more intense of the two is represented.
† Relative intensity (only fragments with masses heavier than *m/e* 200 have been considered).
‡ Very low relative intensity.

Table 3. Chemical shifts (δ, ppm) of the proton signals of **4**-, **5**-, **8**-, **9**- and **10**-acetates in ^1H NMR spectroscopy

	C-18	C-19	C-21	C-26 and C-27		C-30	C-29	C-31	C-3Hα	C-28H		C-25H
										pro-Z	*pro-E*	
Obtusifolyil (**4**)-acetate	0.708	0.983	0.928	1.031	1.025	0.889	—	0.858	4.379	4.714	4.666	2.234
	s	*s*	*d*	*d*	*d*	*s*		*d*	*dt*	*s*	*d*	*m*(*septet*)
			J = 6.5	*J* = 7.0	*J* = 7.0			*J* = 7.0	$J_1 = 10; J_2 = 5$		*J* = 1.5	*J* = 7
14α-Methyl-ergosta-8,24-dien-3β-yl (**8**)-acetate	0.710	0.960	0.928	1.031	1.025	0.890	—	—	~ 4.7	4.716	4.665	2.233
	s	*s*	*d*	*d*	*d*	*s*			*m*	*s*	*d*	*m*(*septet*)
			J = 6.5	*J* = 7.0	*J* = 7.0						*J* = 1.5	*J* = 7
4α,14α-Dimethyl-stigmasta-8-*Z*-24(28)-dien-3β-yl (**5**)-acetate	0.708	0.983	0.925	0.980		0.886	1.590	0.859	4.380	5.114		2.837
	s	*s*	*d*	*d*		*s*	*d*	*d*	*dt*	*m*(*quartet*)		*m*(*septet*)
			J = 6.5	*J* = 7.0			*J* = 6	*J* = 6.5	$J_1 = 10; J_2 = 5$	*J* = 7		*J* = 7
14α-Methyl-stigmasta-8,*Z*-24(28)-dien-3β-yl (**9**)-acetate	0.709	0.960	0.926	0.978		0.888	1.592	—	4.772	5.115		2.834
	s	*s*	*d*	*d*		*s*	*d*	—	*m*	*m*(*quartet*)		*m*(*septet*)
			J = 6.5	*J* = 6.5			*J* = 6	—		*J* = 7		*J* = 7
14α-Methyl-ergost-8-en-3β-yl (**10**)-acetate	0.705	0.958	0.898	0.857	0.805	0.886	—	—	4.700	0.774		—
	s	*s*	*d*	*d*	*d*	*s*			*m*	*d*		
			J = 7	*J* = 7	*J* = 6.5					*J* = 6.5		

stigmasta-8,Z-24(28)-dien-3β-ol (**9**); 14α-methyl-5α-(24*R*)-ergost-8-en-3β-ol (**10**); 14α-methyl-5α-(24ζ)-stigmast-8-en-3β-ol (**11**). Sterols **5** and **9** are new compounds, and **8**, **10** and **11** were found for the first time in a higher plant tissue.

4-Desmethyl sterols. The compounds of this fraction were separated after acetylation using argentation chromatography. The resulting five bands contained, in order of increasing polarity, (band 1) 24-methylene cholesterol as in control cells plus traces of **21**-acetate; (band 2) isofucosteryl acetate, traces of stigmasta-8,Z-24(28)-dien-3β-yl (**22**)-acetate and **8**-acetate, the major 4-desmethyl sterol; (band 3) **9**-acetate; (band 4) campesteryl and sitosteryl acetates; and (band 5) **10**- and **11**-acetates. MS data for **8**, **9**, **10** and **11**-acetates are reported in Table 2. *RR*$_t$s (OV17) are reported in Experimental. The molecular ions of the **8**- and **10**-acetates corresponded to C_{29} sterols, whereas the molecular ions of the **9**- and **11**-acetates corresponded to C_{30} sterols. The fragmentation patterns were quite similar for the four sterols and suggested that all were Δ^8-sterols with a 14α-methyl group. The base peak was M − 15 in all cases, and peaks corresponding to the loss of the lateral chain (*c*) and to M − ring D (*b*) were present, together with the (*c* − 15) and (*b* − 15) peaks. In addition to the above fragmentation, **8**- and **9**-acetates had a characteristic $M^+ - 43$ peak and a McLafferty fragmentation (*a*) which strongly suggested the presence of a double bond at position C-24. Here again the (*a* − 15) peaks were much more intense than the (*a*) peaks. Similar patterns have been described for 14α-methyl Δ^8-sterols [2, 6, 7]. ^{1}H NMR data (Table 3) gave most of the structural information; **8**-, **9**-, **10**- and **11**-acetates exhibited signals at δ 0.705–710 and 0.960 that corresponded, respectively, to C-18 and C-19 methyls in a Δ^8-sterol with a 14α-methyl and no methyl at C-4 [5, 8–10]. The absence of any signal corresponding to intracyclic olefinic protons also supported the presence of the Δ^8-double bond.

4α-Methyl sterols. The 4α-methyl steryl acetates were resolved using argentation chromatography. Four bands were obtained, in order of increasing polarity: band 1 contained unknown products and was not further analysed; band 2 was by far the largest and contained mostly obtusifoliyl (**4**)-acetate with, in addition, low amounts of cycloeucalenyl (**3**)-acetate; band 3 contained **5**-acetate; and band 4 contained **6**- and traces of **7**-acetates. **7**-acetate was detected only once. The commonly occurring 24-methylene and 24-ethylidene lophenyl acetates were not detected. GC–MS data for **5**- and **6**-acetates (Table 2) showed that these two products were closely related to obtusifoliyl-(**4**)-acetate and to the 4-desmethyl steryl acetates studied above. The fragmentation patterns were in agreement with 4α,14α-dimethyl structures; fragments (*b*) and (*c*) of **4**-, **5**- and **6**-acetates were 14 mu heavier than the corresponding ones of **8**-, **9**-, **10** and **11**-acetates. As in the case of **8**- and **9**-acetates, the presence of a double bond at C-24 in **4**- and **5**-acetates was suggested by the $M^+ - 43$ peak and the McLafferty fragmentation (*a*). ^{1}H NMR data (Table 3) unambiguously demonstrated the chemical structures of **4**- and **5**-acetates. These two compounds exhibited signals at δ 0.708 and 0.983 that corresponded, respectively, to C-18 and C-19 methyls in a 4α,14α-dimethyl-Δ^8-sterol skeleton [5, 10; Gonzalez-Gonzalez, A., personal communication].

4,4-Dimethyl sterols. The 4,4-dimethyl steryl acetates of fenarimol cells were identical to those (**1** and **2**) of the control cells but were present in greater amounts (Table 1). In addition to **1** and **2**, two other 4,4-dimethyl sterols were detected in amounts too small to permit a complete identification.

*Obtusifoliyl (**4**)-acetate*

The chemical shifts of the proton signals for **4**-acetate are reported in Table 3. They are essentially identical to those obtained previously (Gonzalez-Gonzalez, A., personal communication). The use of ^{1}H NMR spectroscopy at 250 MHz allowed us to monitor fingerprints for this molecule and to assign most of its protons unambiguously. Methyls C-26 and C-27 showed magnetic nonequivalence and gave two well-resoved doublets corresponding to coupling of the C-26 and C-27 protons with the proton at C-25. Moreover, the two olefinic C-28 protons showed a typical feature: the *pro-Z* proton gave a singlet, whereas the *pro-E* proton gave a doublet (J = 1.5) due to allylic coupling of the *pro-E* C-28H with the C-25H [11]. This spectrum constituted a very useful basis for the determination of the following unknown structure.

*14α-Methyl-5α-ergosta-8,24-dien-3β-yl (**8**)-acetate.* The MS of **8**-acetate (Table 2) was consistent with the suggested structure. MS data of the free alcohol were identical with published data [2, 7, 12]. The ^{1}H NMR spectrum of **8**-acetate (Table 3) was almost identical to the spectrum of **4**-acetate except that the C-19 proton of **8**-acetate resonated at δ 0.960 instead of 0.983, in agreement with data published for chemical shifts of C-19 H of 4-desmethyl Δ^8-sterols [5, 9, 10], and that the C-3α proton gave an unresolved multiplet at 4·7 instead of the well-resolved doublet of triplet obtained in the case of **4**-acetate. The C-28 olefinic protons and the C-26 and C-27 protons gave identical features as for **4**-acetate. Thus the structure of **8** was established without ambiguity.

*14α-Methyl-5α-stigmasta-8,Z-24(28)-dien-3-β-yl (**9**)-acetate.* Most of the protons of this molecule could be assigned without ambiguity. The ^{1}H NMR spectrum was closely related to that of **8**-acetate. The only differences were the presence of a doublet resonating at δ 1.592 characteristic of the C-28 vinylic methyl; the presence of a well-resolved quartet (5.115) that corresponded to the C-28 vinylic proton, and the presence of a typical septet that corresponded to the C-25 proton and whose chemical shift (2.834) was characteristic of a C-24, C-28 olefinic bond of *Z*-configuration [5, 13–15].

*4α,14α - Dimethyl - 5α - stigmasta - 8,Z - 24(28) - dien - 3β-yl (**5**)-acetate.* The ^{1}H NMR spectrum (Table 3) was closely related to that of **9**-acetate. The presence of a doublet resonating at δ 0.859 was characteristic of a 4α-methyl. The C-3α proton resonated at 4.380 and gave a well-resolved doublet of triplet in place of the unresolved multiplet (4.722) obtained in the case of **9**-acetate. As in **9**-acetate, a C-24,C-28 olefinic bond of *Z*-configuration was unambiguously recognized.

*14α-Methyl-5α-(24R)-ergost-8-en-3β-yl (**10**)- and 14α-methyl-5α(24ζ)-stigmast-8-en-3β-yl (**11**)-acetates.* These compounds were not separated by TLC in our experimental conditions. GLC analysis showed that the mixture contained 60% of **10**-acetate and 40% of **11**-acetate. The ^{1}H NMR spectrum of this mixture allowed us to assign without ambiguity the structure of the tetracyclic nucleus which was common to **10**- and **11**-acetates. Interpretation was more complicated for the

lateral chain. Following recent ^{1}H NMR studies [16], it became possible to assign the signals of the protons of the lateral chain even if there was a mixture of 24-methyl and 24-ethyl sterols [17]. In our case, the signals corresponding to the 24-methyl compound were clearly recognizable: the two terminal isopropyl methyl groups (C-26 and C-27) showed nonequivalence and gave two well-resolved doublets and the three protons at C-28 gave a doublet. The chemical shifts measured for the C-26, C-27 and C-28 methyls were very close to the chemical shifts given for campesteryl acetate in the literature [16, 17], suggesting that the configuration at C-24 of **10**-acetate was *R*. This would be a reasonable assumption since the configuration of the 24-alkyl substituent is *R* in sterols from control bramble cells.

The signals corresponding to the 24-ethyl compound were difficult to identify unambiguously. The structure of **11**-acetate was based mainly on GC–MS data (Table 2). In addition, the GC–MS data of the alcohol (**11**) obobtained following saponification of the mixture of **10** and **11**-acetates were identical with published data [6].

4α,14α-Dimethyl-5α-(24ξ)-ergost-8-en-3β-yl (**6**)*-acetate*

This compound was obtained in amounts too small to permit ^{1}H NMR spectroscopy. Its structure was based on GC–MS (Table 2). In addition, MS data for the alcohol (**6**) were identical with published data [6].

The analyses described above were reproduced four times with qualitatively identical results. However, in one of these four analyses, three other products were detected in trace amounts: 4α,14α-dimethyl-5α-stigmast-8-en-3β-yl (**7**)-acetate, 5α-stigmasta-8,*Z*-24(28)-dien-3β-yl (**22**)-acetate and fecosteryl (**21**)-acetate: **21**- and **22**-acetate were identified by comparison with authentic samples isolated from cells treated with AY 9944 [5].

DISCUSSION

As shown in Table 1, bramble cells grown in the presence of fenarimol accumulated 14α-methyl sterols. In the experiment described in this work, 14α-methyl sterols represented about 50% of the total sterols in fenarimol-treated cells. These sterols were not present in control cells. Of the 14α-methyl sterols, obtusifoliol (**4**) predominated greatly in treated cells. In one experiment not reported here, obtusifoliol represented more than 50% of the total sterols. The other most abundant sterols were 14α-methyl-5α-ergosta-8,24(28)-dien-3β-ol (**8**), 14-methyl-5α-stigmasta-8,*Z*-24(28)-dien-3β-ol (**9**) and 4α,14α-dimethyl-5α-stigmasta-8,*Z*-24(28)-dien-3β-ol (**5**). Sterols **9** and **5** were new to the best of our knowledge. Sterol **8**, 14α-methyl-5α-(24R)-ergost-8-en-3β-ol (**10**) and 14α-methyl-5α-(24ξ)-stigmast-8-en-3β-ol (**11**) have not previously been reported in higher plants; **8** was first discovered in *C. emersonii* treated with triparanol [12] and was later found in *U. maydis* treated with triarimol [2] and in a yeast mutant [7]. The 24*S* epimer of **10** has been synthesized chemically [12]. 4α,14α-Dimethyl-5α-(24ξ)-ergost-8-en-3β-ol (**6**), better known as dihydro-obtusifoliol, is a minor compound reported in *C. emersonii* treated with triparanol [12] and in *C. ellipsoideae* and *C. emersonii* treated with triarimol [3], and is probably present in higher plants as a derivative of obtusifoliol. Results obtained with our material treated with fenarimol are very similar to those obtained by Patterson in the case of *C. ellipsoideae* treated with triarimol [3]. In this latter case, triarimol treatment led to a mixture of 14α-methyl sterols with C_9 and C_{10} side chains. Thus, as for triarimol [1–3], fenarimol acted in blocking the removal of the 14α-methyl group. The first step involved during the removal of the 14α-methyl group is a C-30 hydroxylation. Recent experimental data suggest strongly that the C-30 hydroxylation step is catalysed by an enzyme containing cytochrome P-450 [1, 18–20]. As triarimol inhibits 14-demethylation in rat-liver homogenate [1], it could be suggested that fenarimol and triarimol may act as specific inhibitors of the P-450 enzyme involved in the 14-demethylation reaction.

As shown in Table 1, fenarimol led to a strong decrease in the amount of campesterol (**20**) and sitosterol (**17**). In contrast, the concentrations of 24-methylene cholesterol (**19**) and isofucosterol (**16**), both generally considered to be precursors of **20** and **17**, increase significantly, leading to a higher relative percentage of $\Delta^{5,24(28)}$-sterols over total Δ^5-sterols in treated cells than in control cells (Table 4). This increase suggests that fenarimol blocks the reduction of the 24(28)-double bond of $\Delta^{5,24(28)}$-sterols. Fenarimol inhibition of the $\Delta^{24(28)}$-reductase was even more evident in the case of 14α-methyl sterols (Table 4).

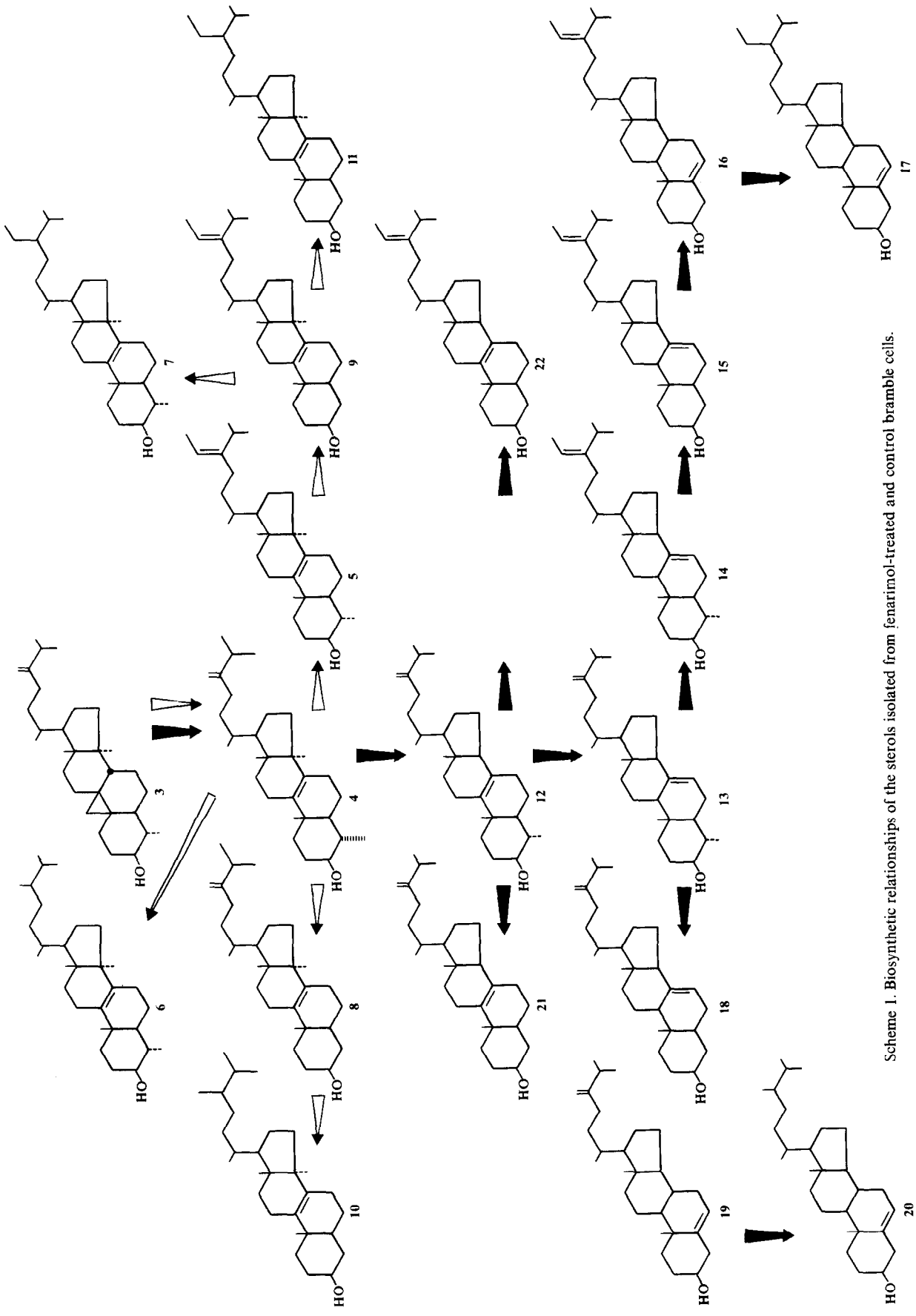
The percentage by which C_{10} exceeded C_9 side-chain sterols was not significantly changed following fenarimol treatment in the case of Δ^5-sterols. However, this percentage was much lower in the case of 14α-methyl sterols showing that the presence of the 14α-methyl inhibited the C-28 methylation reaction. This conclusion agreed well with the results of recent studies performed in our laboratory [21]. These studies had shown that bramble microsomes incubated in the presence of S-adenosyl methionine (SAM) can introduce *in vitro* a second carbon unit into the lateral chain of 24-methylene sterols. It was shown in particular that **13** is by far the best substrate for the *in vitro* SAM-C-28-methyl transferase assay and that such 14α-methyl sterols as **3** and **4** are not methylated at all [21], suggesting that 14α-methyl sterols are poorly methylated *in vivo*. The biosynthetic relationships of the various sterols isolated are shown in Scheme 1.

As pointed out above, cells grown in the presence of fenarimol contained about twice the amount of sterols as control cells. It has been shown recently [22, 23] that 14α-methyl sterols are less effective in reducing glucose permeability or increasing microviscosity than is cholesterol. These results support the hypothesis that the

Table 4. Sterol features occurring in control and fenarimol-treated bramble cells

	Control*	Treated*
Total 14α-methyl-Δ^8	—	51.5
14α-Methyl-$\Delta^{8,24(28)}$	—	44.5 (86)†
14α-Methyl-Δ^8 C_{10} side chain	—	17 (33)†
Total Δ^5	98	38
$\Delta^{5,24(28)}$	14 (15)‡	19 (50)‡
Δ^5 C_{10} side chain	82 (83)‡	29 (76)‡

* As % of total sterols.
† As % of total 14α-methyl-Δ^8-sterols.
‡ As % of total Δ^5-sterols.



Scheme 1. Biosynthetic relationships of the sterols isolated from fenarimol-treated and control bramble cells.

presence of methyl substituents on the α-face of the sterol nucleus decreases the ability of the sterol molecule to condense the lipid phase of the membrane layers and that the removal of the 14α-methyl group would be an essential step in the biosynthetic pathway that confers on the sterol molecule the ability to interact with phospholipids in eukaryotic membranes [24]. If it is assumed that 14α-methyl-Δ^8-sterols do not have the vital functions attributed to Δ^5-sterols, then, since 14α-methyl-Δ^8-sterols constituted more than 50% of the total sterols in fenarimol-treated cells, the concentration of the biologically active Δ^5-sterols would not be very different from that in control cells. The likelihood of this hypothesis could be verified by preparing plasma membranes from fenarimol-treated cells, identifying and titrating the sterols present, and comparing these sterols with those in plasma membranes from control cells. In this context, it has been shown recently in our laboratory that plasma membranes from etiolated maize coleoptiles contain a much larger amount of sterols than the other membrane fractions of the cell [25].

EXPERIMENTAL

Most of the techniques used in the present work have been previously described in detail [5]. The RR_ts (OV-17, cholesterol, RR_t 1.0) on GLC for the acetate of the sterols isolated in this study were; cycloeucalenyl (**3**)-acetate, 2.27; obtusifoliyl (**4**)-acetate, 1.90; 4α,14α-dimethyl-stigmasta-8,*Z*-24(28)-dien-3β-yl (**5**)-acetate, 2.49; 4α,14α-dimethyl-5α-(24ξ)-ergost-8-en-3β-yl (**6**)-acetate, 1.84; 4α,14α-dimethyl-5α-(24ξ)-stigmast-8-en-3β-yl (**7**)-acetate, 2.23; 14α-methyl-5α-ergosta-8,24(28)-dien-3β-yl (**8**)-acetate, 1.73; 14α-methyl-5α-(24*R*)-ergost-8-en-3β-yl (**10**)-acetate, 1.70; 14α-methyl-5α-stigmasta-8,*Z*-24(28)-dien-3β-yl (**9**)-acetate, 2.31; 14α-methyl-5α-(24ξ)-stigmast-8-en-3β-yl (**11**)-acetate, 2.09; 5α-stigmasta-8,*Z*-24(28)-dien-3β-yl (**22**)-acetate, 2.43; fecosteryl (**21**)-acetate, 1.83; isofucosteryl (**16**)-acetate, 2.30; campesteryl (**20**)-acetate, 1.69; sitosteryl (**17**)-acetate, 2.07; and 24-methylene cholesteryl (**19**)-acetate, 1.78.

Plant material. Suspension cultures of bramble cells (*Rubus fructicosus*) were grown under continuous white light at 25° on a synthetic sterile medium as described previously [21]. Fenarimol (7 mg/l.) was added in soln in EtOH to the culture medium. The drug was sterilized before use by filtration through Millipore (0.45 μm) filters.

Analytical procedure. The isolation of 4,4-dimethyl-, 4α-methyl- and 4-desmethylsteryl acetates has been described previously [5]. Each of 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) was the developing solvent and migration was for 15 hr, was performed on each class of steryl acetate and the bands obtained were analysed by GLC. There were 3 bands of 4,4-dimethylsterol acetates in the case of both control bramble cells and treated 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. The identification of α- and β-amyrin has been described previously [26]. There were 3 bands of 4α-methylsteryl acetates from control bramble cells, corresponding in order of increasing polarity to 24-methylene-lophenyl (**13**)-acetate, a mixture of cycloeucalenyl (**3**)-acetate and obtusifoliyl (**4**)-acetate, and 24-ethylidene lophenyl (**14**)-acetate: and there were 4 bands for fenarimol cells, the first 3 bands at the same R_fs as in the control cells. The most polar band did not contain detectable amounts of **13**-acetate and was not further analysed: the second band in order of increasing polarity contained mostly **4**-acetate, the most abundant sterol occurring in treated cells, and little **3**-acetate; the third band contained 4α,14α-dimethyl-5α-stigmasta-8,*Z*-24(28)-dien-3β-yl (**5**)-acetate; and the less polar band contained mostly 4α,14α-dimethyl-5α-(24ξ)-ergost-8-en-3β-yl (**6**)-acetate with in one batch of cells, traces of 4α,14α-dimethyl-5α-(24ξ)-stigmast-8-en-3β-yl (**7**)-acetate. 4- and **5**-acetates were practically pure and were crystallized from CH_2Cl_2–MeOH. **4**-Acetate (50 mg from 2 batches of fenarimol cells), mp 106–107°; **4**-alcohol, mp 145–146° (lit. 144°), $[\alpha]_D^{25}$ 69° ($CHCl_3$, *c* 1); **5**-acetate (7 mg from 2 batches of fenarimol cells), mp 121–122°; **5**-alcohol, mp 139–140°, $[\alpha]_D^{25}$ 42° (*c* 0.3). There were 3 bands of 4-desmethylsteryl acetates from control bramble cells, corresponding in order of increasing polarity to 24-methylene-cholesteryl (**19**)-acetate, isofucosteryl (**16**)-acetate, and a mixture of campesteryl (**20**)- and sitosteryl (**17**)-acetates. From fenarimol cells there were 5 bands, the first, second and fourth (in order of increasing polarity) corresponded to products having the same R_fs as **19**-,**16**- and **20**- plus **17**- acetates, respectively. The first band contained **19**-acetate and occasionally traces of fecosteryl (**21**)-acetate; the second, a mixture of **16**- and 14α-methyl-5α-ergosta-8,24(28)-dien-3β-yl (**8**)-acetates; the third, 14α-methyl-5α-stigmasta-8,*Z*-24(18)-dien-3β-yl (**9**)-acetate; the fourth, a mixture of **20**- and **17**-acetates; and the fifth and least polar band, a mixture of 14α-methyl-5α-(24ξ)-stigmast-8-en-3β-yl (**11**)-acetate and 14α-methyl-5α-(24*R*)-ergost-8-en-3β-yl (**10**)-acetate. **16**- and **8**-acetates were separated by argentation TLC using commercial unwashed $CHCl_3$ as the developing solvent. The **10**- and **11**-acetates could not be separated by TLC in our experimental conditions. The **8**- and **9**-acetates were almost pure and were crystallized with CH_2Cl_2–MeOH. **9**-Acetate (10 mg from 2 batches of fenarimol cells) mp 102–104°; **9**-alcohol, mp 128–130°, $[\alpha]_D^{25}$ 17° ($CHCl_3$, *c* 0.3); **8**-acetate (12 mg from 3 batches of fenarimol cells), mp 79–80°. For MS and ^{1}H NMR 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 = 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (**3**); obtusifoliol = 4α,14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol (**4**); 24-methylene lophenol = 4α-methyl-5α-ergosta-7,24(28)-dien-3β-ol (**13**); 24-ethylidene lophenol = 4α-methyl-5α-stigmasta-7,*Z*-24(28)-dien-3β-ol (**14**); Δ^7 avenasterol = 5α-stigmasta-7,*Z*-24(28)-dien-3β-ol (**15**); isofucosterol = stigmasta-5,*Z*-24(28)-dien-3β-ol (**16**); sitosterol = stigmast-5-en-3β-ol (**17**); episterol = 5α-ergosta-7,24(28)-dien-3β-ol (**18**); 24-methylene cholesterol = ergosta-5,24(28)-dien-3β-ol (**19**); campesterol = (24*R*)-ergost-5-en-3β-ol (**20**); fecosterol = 5α-ergosta-8,24(28)-dien-3β-ol (**21**).

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