Phytochemistry, Vol. 20, No. 9, pp. 2153–2159, 1981.
Printed in Great Britain.

0031-9422/81/092153-07 $02.00/0
Pergamon Press Ltd.

ACCUMULATION OF 9β,19-CYCLOPROPYL STEROLS IN SUSPENSION CULTURES OF BRAMBLE CELLS CULTURED WITH TRIDEMORPH

PAULETTE SCHMITT*, PIERRE BENVENISTE* and PIERRE LEROUX†

* Institut de Botanique, Laboratoire de Botanique, Laboratoire de Biochimie Végétale, E.R.A. du C.N.R.S. No. 487, 28, rue Goethe, 67083-Strasbourg Cédex, France; † C.N.R.A., Laboratoire de Phytopharmacie, 78000-Versailles, France

(*Received* 28 *January* 1981)

Key Word Index—*Rubus fruticosus*; Rosaceae; sterol biosynthesis; accumulation of 9β,19-cyclopropyl sterols; tridemorph; ^{1}H NMR of 9β,19-cyclopropyl sterols.

Abstract—The addition of tridemorph, a systemic fungicide, to the medium of suspension cultures of bramble cells resulted after 4 weeks of growth in a strong accumulation of 9β,19-cyclopropyl sterols (90% of total sterols in treated cells) and in the disappearance of Δ^5-sterols (98% of total sterols in control cells). Cycloeucalenol and 24-methylene pollinastanol (both together constitute 70% of total sterols) are the major sterols of treated cells. Tridemorph probably inhibits the cycloeucalenol–obtusifoliol isomerase. As the fungicide impairs only slightly the growth of the cells, the possibility that 9β,19-cyclopropyl sterols substitute for Δ^5-sterols in the membranes of the treated cells is considered.

INTRODUCTION

Tridemorph (2,6-dimethyl-*N*-tridecyl-morpholine) (**1**) is a systemic fungicide used in the control of powdery mildews [1, 2]. The antifungal mode of action of **1** has not been fully explained but some authors presumed an interference with lipid biosynthesis [3] and more precisely with ergosterol biosynthesis [4]. In a recent article, Kato *et al.* [5] have given evidence that the target of tridemorph inhibition in *Botrytis cinerea* could be the $\Delta^8 \rightarrow \Delta^7$-isomerization step, i.e. the conversion of fecosterol to episterol, involved during ergosterol biosynthesis [6, 7]. Another feature characteristic of the tridemorph action was a strong accumulation (× 5) of the total amount of sterols in *Ustilago maydis* [8].

We have shown [9–11] that drugs known to inhibit cholesterol biosynthesis in animal cells, such as AY 9944 [9], or ergosterol biosynthesis in fungi, such as fenarimol [10] or 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol [11], were extremely efficient inhibitors of Δ^5-sterol biosynthesis in plant cell suspensions growing in a liquid medium. Thus we planned to use tridemorph in our system in order to check whether the dramatic increase in sterol biosynthesis observed in *U. maydis* could be observed in a plant system. We report here that **1** interfered strongly with sterol biosynthesis in bramble (*Rubus fruticosus*) cell suspensions leading to (a) a strong accumulation of 9β,19-cyclopropyl sterols which replace the Δ^5-sterols of the control and (b) an increase of the total amount of sterols in agreement with previous results [8].

RESULTS

The culture medium was supplemented with **1** (from 1 to 10 mg/l.). Growth of the cells was not significantly modified at any concentration of **1** except for the highest which was slightly inhibitory. When the cells reached the stationary phase (4 weeks after inoculation), they were harvested and the sterols extracted. As shown in Table 1, the composition of the sterol fraction from cells grown in the presence of 10 mg/l. of **1** was profoundly changed qualitatively and quantitatively. The total sterol content of the cells growing on **1** (13 mg/g dry wt) was higher than that of the control cells (4.8 mg/g dry wt). This strong increase was partially due to the increased amounts of 4,4-dimethyl sterols and of 4α-methyl sterols. As shown in Table 1, more than 90% of the sterols in **1**-treated cells were the following 9β,19-cyclopropyl sterols: cycloartenol (**2**)*; 24-methylenecycloartanol (**3**); cycloeucalenol (**4**); 24-dihydrocycloeucalenol (**8**); cyclofontumienol (**6**); 24-methylenepollinastanol (**18**); 24-methylpollinastanol (**19**); 24-ethylidenepollinastanol (**20**); 24-ethylpollinastanol (**22**). Moreover, **4** and **18** together constituted 70% of the total sterols. In addition to the 9β,19-cyclopropyl sterols, $\Delta^{8,14}$ (**9** and **10**) and Δ^8 (**11**, **12** and **15**) have been identified in minor amounts. Finally Δ^5-sterols are barely detectable in **1**-treated cells.

4-*Desmethyl sterols*

The components of this fraction were separated by argentation chromatography. Six bands for acetates of **9** (band 1), **10** + **15** (band 2), **18** (band 3), **11** (band 4), **20** + **21** (band 5), **19** + **22** + **12** (band 6), were found. The

* Nomenclature: Cycloartenol = 4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol (**2**); 24-methylenecycloartanol = 4,4,14α-trimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (**3**); cycloeucalenol = 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (**4**); obtusifoliol = 4α,14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol (**5**); cyclofontumienol = 4α,14α-dimethyl-9β,19-cyclo-5α-stigmast-Z-24(28)-en-3β-ol (**6**); 4α,14α-dimethyl-9β,19-cyclo-5α-stigmast-E-24(28)-en-3β-ol (**7**); 4α,14α-dimethyl-9β,19-cyclo-5α-ergostan-3β-ol (**8**); 24-methylenepollinastanol =14α-methyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (**18**); 24-methylpollinastanol = 14α(24*R*)-24-dimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**19**); 14α-methyl-9β,19-cyclo-5α-stigmast-Z-24(28)-en-3β-ol (**20**); 14α-methyl-9β,19-cyclo-5α-stigmast-E-24(28)-en-3β-ol (**21**); 24-ethylpollinastanol = 14α-methyl-(24ξ)-24-ethyl-9β,19-cyclo-5α-cholestan-3β-ol (**22**).

Table 1. Sterols of control and 1-treated suspension cultures of bramble cells

	Control	Treated
Cycloartenol (**2**)	0.5*	0.5
24-Methylenecycloartanol (**3**)	0.15	3.5
X_1 and X_2	0	2
α- and β-amyrins	0.5	1
Cycloeucalenol (**4**)	0.1	35
Obtusifoliol (**5**)	0.1	0
Cyclofontumienol (**6**)	0	3
4α,14α-Dimethyl-9β,19-cyclo-5α-stigmast-*E*-24(28)-en-3β-ol (**7**)	0	0.5
24(28)-Dihydrocycloeucalenol (**8**)	0	1
5α-Stigmasta-8,14,*Z*-24(28)-trien-3β-ol (**9**)	0	0.5
(24*R*)-24-Ethyl-5α-cholesta-8,14-dien-3β-ol (**10**)	0	0.5
5α-Stigmasta-8,*Z*-24(28)-dien-3β-ol (**11**)	0	0.5
(24*R*)-24-ethyl-5α-cholest-8-en-3β-ol (**12**)	0	0.5
Sitosterol (**13**)	70	tr
Campesterol (**14**)	14	tr
5α-Ergosta-8,24(28)-dien-3β-ol (**15**)	0	tr
24-Methylenecholesterol (**16**)	2	0
Isofucosterol (**17**)	12	0
24-Methylenepollinastanol (**18**)	0	35
(24*R*)-24-Methylpollinastanol (**19**)	0	7.5
24-Ethylidenepollinastanol (**20**)	0	4.5
14α-Methyl-9β,19-cyclo-5α-stigmast-*E*-24(8)-en-3β-ol (**21**)	0	0.5
(24ξ)-24-Ethylpollinastanol (**22**)	0	0.5
Unknown sterols	0.5	3

* As a percentage of total sterols
tr = trace.

components of bands 1, 2 and 4 were easily identified as $\Delta^{8,14}$-sterols (**9**, **10**) and Δ^{8}-sterols (**11**, **15**) previously found in bramble cells treated respectively with A 25822 B [11] and with AY 9944 [9]. 9β,19-Cyclopropyl sterols were essentially concentrated in bands 3, 5 and 6. Their MS were typical (Table 2) showing a fragmentation (**c**) characteristic of a cyclopropane ring [12]. Another characteristic feature of 9β,19-cyclopropyl sterols was the low relative intensity of the molecular ion peak in respect to the $M^+ - 60$ peak. A reverse situation was shown in Δ^{8}- or Δ^{7}-sterols [13]. One additional double bond in the side chain of **18**-, **20**- and **21**-acetates was clearly recognized. The existence of a McLafferty fragmentation (**b** − 60) in these products confirmed the presence of a Δ^{24}-double bond. ^{1}H NMR spectra are given in Table 3; **18**-, **19**- and **20**-acetates exhibited signals at very high field characteristic of two cyclopropyl protons. Typical also was the signal at δ 0.895–0.900 which corresponds to the C-18 methyl. This signal appears at relatively low field and was easily distinguishable from the C-18 methyl signals of Δ^{7}, Δ^{8}, $\Delta^{8,14}$ and Δ^{5}-sterols which appear at higher field.

4α-Methyl sterols

These were resolved using argentation chromatography. Three bands were observed for acetates of **4** + **5** (band 1), **6** + **7** (band 2), and **8** (band 3). Band 1 contained the cycloeucalenyl (**4**)- and obtusifoliyl (**5**)-acetates identified previously in control cells [9], but the amount of **4** was enormous (35% of total sterols) whereas **5** was barely detectable (Table 1). The acetates of **6**, **7** and **8** (bands 2 and 3) were easily recognized as cyclopropyl sterols by their mass spectra (Table 2), which showed the presence of the fragmentation (**c**) characteristic of a cyclopropane ring [12] as well as very low intensity molecular ion peaks. ^{1}H NMR spectral data for **4** and **8**-acetates showed the presence of the high field signals characteristic of the C-19 cyclopropyl protons (Table 3). These signals were shifted with respect to those obtained in the case of 4-desmethyl-9β,19-cyclopropyl sterols **18**- and **19**-acetates). This shift was caused by the 4α-methyl group.

Cycloeucalenyl acetate (**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 [14, 15]. 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 non-equivalence and gave two well-resolved 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 Hz) due to allylic coupling of the *pro-E* C-28 H with the C-25 H [16]. This spectrum constituted a useful basis for the determination of the following unknown structure.

24-Methylenepollinastanyl acetate (**18**-*acetate*)

This compound has been identified previously in various materials [17–19] but no detailed ^{1}H NMR spectroscopy data have been reported to our knowledge. The ^{1}H NMR spectrum of **18**-acetate (Table 3) was almost identical to the spectrum of **4**-acetate except that the two doublets of the two hydrogens of the cyclopropane ring (C-19) of **18**-acetate were separated by 0.36 ppm whereas those belonging to **4**-acetate were separated only by 0.24 ppm in agreement with published data [19], 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 proton and the C-26 and C-27 protons gave identical features as for **4**-acetate. As the mass spectrum (Table 2) was consistent with the suggested structure, thus the structure of **18**-acetate was established without ambiguity.

14α-Methyl-9β,19-cyclo-5α-stigmast-Z-24(28)-en-3β-yl acetate (**20**-*acetate*)

This compound was new to our knowledge. Most of the protons of this molecule could be assigned without

Table 2. Mass spectra of the 9β,19-cyclopropyl steryl acetates of cells treated with tridemorph

Acetate of	M^+	M^+-15	M^+-43	M^+-60	$M^+-60-15$	a*−60	b	b − 60	c	d
18	454	439	411	394	379	269	370	310	300	227
	(8)	(6)	(4)	(100)	(98)	(60)	(1)	(10)	(11)	(20)
20	468	453	425	408	393	269	370	310	314	227
	(11)	(6)	(3)	(100)	(88)	(63)	(8)	(58)	(5)	(29)
21	468	453	—	408	393	269	—	310	314	227
	(9)	(6)	—	(100)	(71)	(67)	—	(6)	(8)	(15)
4	468	453	425	408	393	283	384	324	300	241
	(6)	(4)	(1)	(100)	(81)	(31)	(1)	(6)	(9)	(15)
6	482	467	439	422	407	283	384	324	314	241
	(6)	(5)	(1)	(100)	(87)	(30)	(5)	(25)	(4)	(16)
7	482	467	—	422	407	283	—	324	314	241
	(7)	(4)	—	(100)	(75)	(42)	—	(4)	(11)	(11)
8	470	455	—	410	395	283	—	—	302	241
	(6)	(6)	—	(89)	(100)	(52)	—	—	(15)	(11)
19	456	441	—	396	381	269	—	—	302	227
	(7)	(6)	—	(86)	(100)	(89)	—	—	(9)	(11)
22	470	455	—	410	395	269	—	—	316	227
	(6)	(6)	—	(100)	(94)	(93)	—	—	(10)	(16)

*a: M^+ − lateral chain.

ambiguity. The ^{1}H NMR spectrum was closely related to that of **18**-acetate. The only differences were the presence of a doublet resonating at δ 1.591 characteristic of the C-28 vinylic methyl, the presence of a quartet (5.116) 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 [20, 21]. The mass spectrum (Table 2) was in complete agreement with the suggested structure with a fragment (**c**) typical of a cyclopropane ring and a fragment (**b** − 60) corresponding to a McLafferty fragmentation characteristic of the C-29 vinylic methyl.

14α-Methyl-9β,19-cyclo-5α-stigmast-E-24(28)-*en-3β-yl acetate* (**21**-*acetate*)

This compound was not separable from **20**-acetate by argentation chromatography but its RR_t (OV-17) was shorter than that of **20**-acetate. Its molecular weight was identical to that of **20**-acetate and the mass spectrum, almost identical to that of **20**-acetate, presented a fragment (**c**) characteristic of the cyclopropane ring. However, the McLafferty fragmentation (**b** − 60) characteristic of the C-29 vinylic methyl was much less intense in **21**-acetate than in **20**-acetate, suggesting that the configuration of the C-29 vinylic methyl was *E* [22]. To assess this point definitely, ^{1}H NMR data for the C-25 H would be necessary. Unfortunately **21**-acetate was present in too minor an amount to obtain a ^{1}H NMR spectrum. Thus the proposed structure remains only tentative, although very probable.

Cyclofontumienyl-acetate (**6**-*acetate*)

Too little product was available to obtain a ^{1}H NMR spectrum. However, the compound showed the same chromatographic (TLC, GLC) properties as authentic cyclofontumienyl acetate [23]. Moreover, its mass spectrum (Table 2) was identical to published data for **6**-acetate and in full agreement with the structure showing the presence of a fragment (**c**) characteristic of a cyclopropane ring [12], and of a fragment (**b** − 60) corresponding to a McLafferty fragmentation. As in **20**-acetate, **6**-acetate contained a minor constituent (**7**-acetate) which was not separated from **6**-acetate by argentation TLC but showed a shorter RR_t than **6**-acetate on GLC (OV-17). Its mass spectrum was almost identical to that of **6**-acetate except that the McLafferty fragmentation (**b** − 60) was much less intense than for **6**-acetate. Thus it appeared that **7**-acetate could be the *E*-24(28) isomer of cyclofontumienyl-acetate, i.e. 4α,14α-dimethyl-9β,19-cyclo-5α-stigmast-*E*-24(28)-en-3β-yl-acetate.

(24*R*)-24-*Methylpollinastanyl acetate* (**19**-*acetate*)

This compound was rigorously identified by its mass spectrum (Table 2) and ^{1}H NMR spectrum (Table 3). The latter exhibited the major characteristic features of 9β,19-cyclopropyl sterols: the two doublets of the two hydrogens of the cyclopropane ring (C-19) were separated by 0.35 ppm and were typical of 4-desmethyl 9β,19-cyclopropyl sterols; methyls C-26 and C-27 showed magnetic non-equivalence and gave two well-resolved doublets corresponding to coupling of the C-26 and C-27 protons with the proton at C-25. Finally, the chemical shift of the signals corresponding to the methyl C-28 suggested strongly that its configuration was *R* [24]. The fraction containing **19**-acetate contained small amounts (~7%) of a compound (**22**-acetate) not separable from **19**-acetate by argentation TLC but having a longer RR_t on GLC than **19**-acetate. The mass spectrum (Table 2) showed without ambiguity that **21**-acetate was (24ξ)-24-ethylpollinastanyl-acetate.

DISCUSSION

The present work demonstrates that treatment of suspension cultures of bramble cells with tridemorph (**1**) caused a dramatic accumulation of 9β,19-cyclopropyl

Table 3. ^{1}H NMR chemical shifts (δ) of the proton signals of **4**, **18**-, **19**- and **20**-acetates

	C-18	C-19		C-21	C-26	C-27	C-29	C-30	C-32	C-28 H		C-3αH	C-25 H
		endo	exo							*pro-Z*	*pro-E*		
Cycloeucalenyl acetate (**4**-acetate)	0.901 *s*	0.152 *d* $J = 4$*	0.388 *d* $J = 4$	0.897 *d* $J = 6.5$	1.031 *d* $J = 7$	1.025 *d* $J = 7$	—	0.844 *d* $J = 6.5$	0.969 *s*	4.716 *s*	4.661 *d* $J = 1.5$	4.459 *dt* $J_1 = 10$ $J_2 = 5$	2.234 *m*(*septet*) $J = 7$
24-Methylenepollinastanyl acetate (**18**-acetate)	0.896 *s*	0.080 *d* $J = 4$	0.438 *d* $J = 4$	0.895 *d* $J = 6.5$	1.029 *d* $J = 6.5$	1.025 *d* $J = 6.5$	—	—	0.960 *s*	4.711 *s*	4.660 *s*	4.754 *m*	2.232 *m*(*septet*) $J = 7$
14α-Methyl-9β,19-cyclo-5α-stigmast-*Z*-24(28)-en-3β-yl acetate (**20**-acetate)	0.893 *s*	0.078 *d* $J = 4$	0.446 *d* $J = 4$	0.889 *d* $J = 6.5$	0.979 *d* $J = 8$		1.591 *d* $J = 7$	—	0.965 *s*	5.116 *m*(*quartet*) $J = 7$		4.799 *m*	2.834 *m*(*septet*) $J = 7$
(24ξ)-24-Methylpollinastanyl acetate (**19**-acetate)	0.892 *s*	0.075 *d* $J = 3.5$	0.428 *d* $J = 3.5$	0.864 *d* $J = 6.5$	0.856 *d* $J = 7$	0.779 *d* $J = 7$	—	—	0.954 *s*	0.806 *d* $J = 6.5$		4.795 *m*	—

* Coupling constants in Hz.

sterols. Some accumulation of cyclopropyl sterols (essentially 24-methylenepollinastanol) has been observed previously in triparanol-treated cells of *Chlorella emersonii* [25]. However, in that case the relative percentage of cyclopropyl sterols was less than 14% whereas in **1**-treated bramble cells, 9β,19-cyclopropyl sterols were more than 90% of the total sterols while Δ^5-sterols were barely detectable (Tables 1 and 4). Among cyclopropyl sterols, cycloeucalenol (35%) and 24-methylenepollinastanol (35%) were by far the major sterols. Another characteristic feature of the cyclopropyl sterols of treated cells concerned the relative percentage of 9β,19-cyclopropyl C_{10}-side chain sterols. The latter (9%) was much lower than the relative percentage of Δ^5-C_{10} side chain sterols (82%) (Table 4). This confirms that 24-methylene 9β,19-cyclopropyl sterols would be very poor substrates for the sterol C-28-methyltransferase activity as suggested by a previous study with a cell-free extract [26]. Among 9β,19-cyclopropyl C_{10}-side chain sterols, cyclofontumienol (**6**) and 24-ethylidenepollinastanol (**20**) are present in appreciable amounts. Cyclofontumienol has been reported previously in *Fontumia latifolia* [23] whereas 24-ethylidenepollinastanol was isolated in our material for the first time, to the best of our knowledge. These two com, nds possessed a 24-ethylidene group of *Z*-configuration. Interestingly, they contain minor amounts of compounds which we have suggested would be the *E*-24(28)-ethylidene isomers of **6** and **20**, i.e. **7** and **21**. The biosynthetic relationships of the sterols isolated from **1**-treated cells are shown in Scheme 1. From the

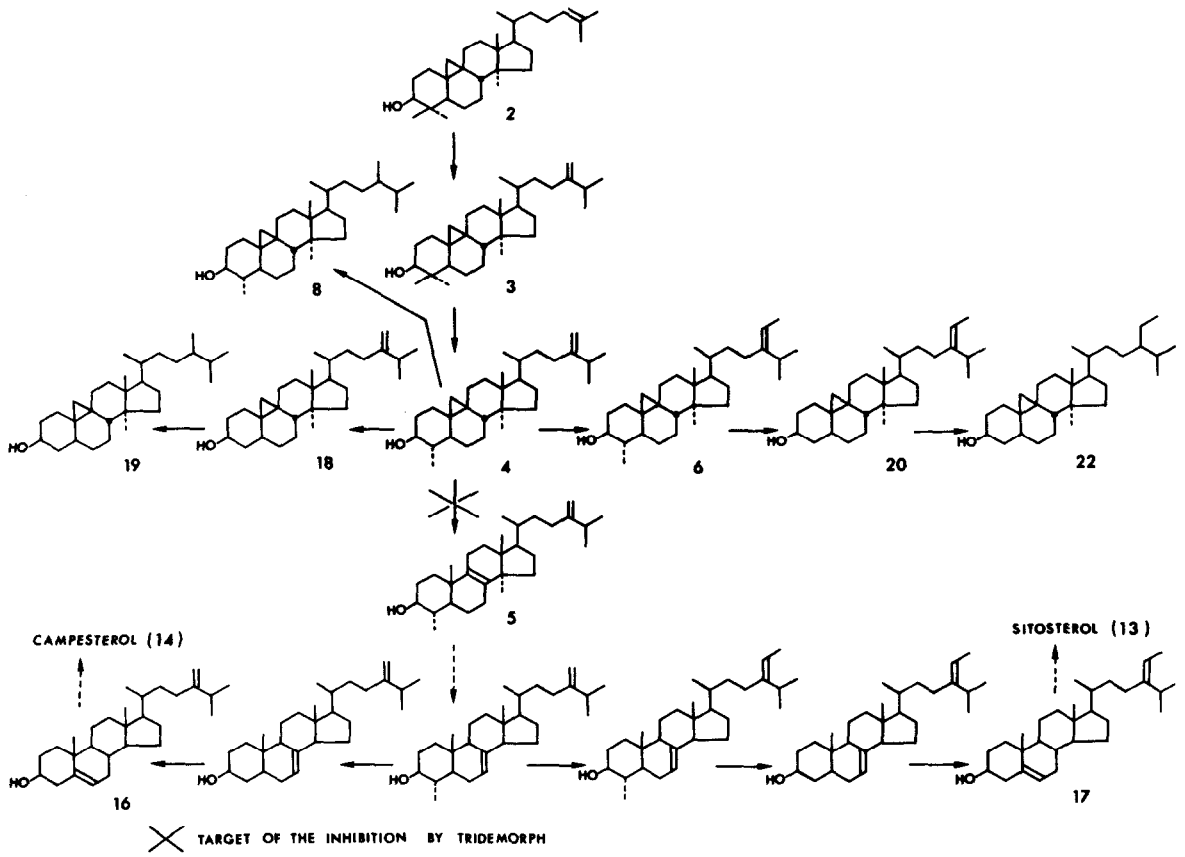
Table 4. Sterol features occurring in control and tridemorph-treated bramble cells

	Control	Treated
Total 9β,19-cyclopropyl sterols	0.7*	91*
Total Δ^5-sterols	98	tr
Total Δ^8-sterols	0	1
Total $\Delta^{8,14}$-sterols	0	1
9β,19-Cyclopropyl C_{10}-side chain sterols	—	8(9†)
Δ^5 C_{10}-side chain sterols	82(82‡)	—
9β,19-Cyclopropyl $\Delta^{24(28)}$-sterols	—	82(90†)
$\Delta^{5,24(28)}$-sterols	14(15‡)	—

* As % of total sterols.
† As % of total 9β,19-cyclopropyl sterols.
‡ As % of total Δ^5-sterols.

chemical structure of all the compounds identified, it is clear that the target of tridemorph is the cycloeucalenol–obtusifoliol isomerase. In addition, tridemorph has recently been shown to inhibit *in vitro* the cycloeucalenol–obtusifoliol isomerase (A. Rahier, unpublished results).

It has been shown that the growth of bramble cells was only slightly reduced in the presence of amounts of **1** producing the largest accumulation of cyclopropyl



Scheme 1.

sterols. This result suggests that 9β,19-cyclopropyl sterols could meet, at least partially, the functions normally ascribed to Δ^5-sterols in control cells. Such a conclusion could have important physiological and biochemical consequences; 9β,19-cyclopropyl sterols have chemical structures very remote from those of Δ^5-sterols, in particular their conformation was bent and not flat as in the case of Δ^5-sterols. Studies performed in other laboratories with model membranes have shown that in order to interact with phospholipids, the sterol molecule must be flat, have an intact isooctyl side chain and possess a free 3β-hydroxyl group [27]. Apparently cyclopropyl sterols do not meet these conditions; however, the situation in living membranes may be quite different from that in model membranes. It has been recently reported that cycloartenol, cyclolaudenol and other 9β,19-cyclopropyl sterols could partially or even totally substitute for cholesterol to support growth of *Mycoplasma capricolum* [28] and of the yeast mutant strain GL 7 [29], both organisms being auxotrophic for sterols. Our results showing that the almost complete replacement of Δ^5-sterols by 9β,19-cyclopropyl sterols in suspension cultures of bramble cells only slightly impaired the growth of the culture are in agreement with results obtained in the case of *M. capricolum* and of the yeast mutant strain and suggest that even in cells from higher organisms, cyclopropyl sterols seem to be able to substitute for Δ^5-sterols. Two explanations can be suggested to explain these results. Firstly, the bent structure of cyclopropyl sterols could fit with some kind of molecular organization of bramble cell membranes. As 3–4 weeks are required by bramble cells to reach the stationary phase, some adaptive changes could occur in the phospholipids (or other constituents) present in the membranes. These changes, concerning either the polar or the fatty acid moieties, could make these phospholipids able to interact with cyclopropyl sterols. Another explanation suggested by Bloch *et al.* [28, 29] in the case of *M. capricolum* and of the yeast mutant GL 7 stipulates that the non-planar conformation imposed by the 9β,19-cyclopropane ring of cyclopropyl sterols moderated the adverse membrane effects of the nuclear methyl group at C-14 which has been shown to be deleterious for membranes [30–32]. Such an interpretation could equally apply to suspension cultures of bramble cells treated by **1**.

The likelihood of these two hypotheses could be verified by preparing plasma membranes from tridemorph-treated suspension cultures of bramble cells, identifying and titrating the 9β,19-cyclopropyl sterols and the phospholipids present and comparing these components 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 [33, 34].

Finally our results showing that 9β,19-cyclopropyl sterols can accumulate strongly in bramble cells without impairing the growth of the culture is in agreement with several reports describing the accumulation of 9β,19-cyclopropyl sterols in photosynthetic eukaryotes under various physiological conditions or in particular organs. An increase in cycloartenol concentration and a corresponding decrease in 24-alkylated 4-desmethyl sterol content have been shown to occur in potato tubers stored at low temperature [35]; 9β,19-cyclopropyl sterols (mainly pollinastanol and 24-methylenepollinastanol) are present in large amounts in pollen from various sources [25, 36, 37] and finally cycloartenone accumulated dramatically in the latex from the fruit of *Artocarpus integrifolia* [38] and cyclolaudenol in *Papaver somniferum* [39].

EXPERIMENTAL

Most of the techniques used in the present work have been described in detail previously [9]. The RR_ts (OV-17, cholesterol, RR_t 1.0) on GLC for the acetates of the 4-desmethyl sterols and 4α-methyl sterols isolated in this study were: sitosteryl (**13**)-acetate, 2.05; isofucosteryl (**17**)-acetate, 2.24; campesteryl (**14**)-acetate, 1.67; 24-methylenecholesteryl (**16**)-acetate, 1.73; 5α-stigmasta-8,14,*Z*-24(28)-trien-3β-yl (**9**)-acetate, 2.45; (24*R*)-24-ethyl-5α-cholesta-8,14-dien-3β-yl (**10**)-acetate, 2.22; 5α-stigmasta-8,*Z*-24(28)-dien-3β-yl (**11**)-acetate, 2.43; (24*R*)-24-ethyl-5α-cholest-8-en-3β-yl (**12**)-acetate, 2.21; 5α-ergosta-8,24(28)-dien-3β-yl (**15**)-acetate, 1.81; 24-methylenepollinastanyl (**18**)-acetate, 2.06; (24*R*)-24-methylpollinastanyl (**19**)-acetate, 1.99; 24-ethylidenepollinastanyl (**20**)-acetate, 2.70; 14α-methyl-9β,19-cyclo-5α-stigmast-*E*-24(28)-en-3β-yl (**21**)-acetate, 2.44; (24ξ)-24-ethylpollinastanyl (**22**)-acetate 2.46; cycloeucalenyl (**4**)-acetate, 2.21; cyclofontumienyl (**6**)-acetate, 2.89; 4α,14α-dimethyl-9β,19-cyclo-5α-stigmast-*E*-24(28)-en-3β-yl (**7**)-acetate, 2.55; 24(28)-dihydrocycloeucalenyl (**8**)-acetate, 2.14. The RR_ts (SE-30, cholesterol, RR_t 1.0) on GLC for the acetates of the 4,4-dimethyl sterols isolated in this study were: cycloartenyl (**2**)-acetate, 2.21; 24-methylenecycloartanyl (**3**)-acetate, 2.52; X_1-acetate, 2.02; X_2-acetate, 2.20. The RR_ts (OV-17, cholesterol, RR_t 1.0) on GLC for the acetates of pentacyclic triterpenes present in bramble cells were: α-amyrin acetate, 2.37; β-amyrin acetate, 2.10.

Plant material. Suspension cultures of bramble cells were grown under continuous white light at 25° on a synthetic sterile medium as described previously [26]. Tridemorph (1–10 mg/l.) was added in soln in EtOH to the culture medium. The drug was sterilized before use by filtration through a Millipore (0.45 μm) filter.

Analytical procedure. The isolation of 4,4-dimethyl-, 4α-methyl- and 4-desmethylsteryl acetates has been described previously [9]. 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 (7:3) 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 three bands of 4,4-dimethylsteryl acetates in the case of both control bramble cells and treated cells, corresponding in order of decreasing polarity to **3**-acetate, **2**-acetate and a mixture of α- and β-amyrin acetates. In the case of treated cells, there was one additional band at an R_f intermediate between the R_fs of **2**- and **3**-acetates. This band contained the acetates of two tetracyclic triterpenes, X_1 and X_2 [11]. There were three bands of 4α-methylsteryl acetates from control bramble cells, corresponding in order of decreasing polarity to 24-methylenelophenyl acetate, a mixture of **4**- and **5**-acetates, and 24-ethylidene lophenyl acetate; and there were three bands also for **1**-treated cells. The first band at the same R_f as **4**- or **5**-acetate contained a large amount of **4**-acetate, the second band at the same R_f as 24-ethylidenelophenyl acetate did not contain this compound but instead a mixture of **6**- and **7**-acetate, the third band corresponding to a very non-polar compound was not present in the control and contained **8**-acetate. There were three bands of 4-desmethylsteryl acetates from control bramble cells, corresponding in order of decreasing polarity to **16**-acetate, **17**-acetate

and a mixture of **13**- and **14**-acetates. From **1**-treated cells there were seven bands. The first band corresponding to a very polar compound contained **9**-acetate, the second band at the same R_f as **16**-acetate did not contain **16**-acetate but a mixture of **10**- and **15**-acetate, the third band was by far the major one and contained only **18**-acetate, the fourth band at the same R_f as **17**-acetate did not contain **17**-acetate but **11**-acetate, the fifth band contained a mixture of **20**- and **21**-acetates, the sixth band at the same R_f as **13**- and **14**-acetates contained only traces of these sterols and in addition **12**-acetate and finally the seventh band contained a mixture of **19**- and **22**-acetates. All the sterols isolated from **1**-treated cells were identified by their mass spectra and their ^{1}H NMR spectra (Tables 2 and 3).

Acknowledgements—We are indebted to B.A.S.F. (Ludwigshafen-am-Rhein, R.F.A.) for a generous gift of **1**. We also warmly thank Mr. Roland Graff (Institut de Chimie, Strasbourg) for ^{1}H NMR and discussions, and Dr. P. Bouvier for GC/MS measurements. The investigation was supported by grant 79-7-0784 from the Délégation Générale à la Recherche Scientifique et Technique.

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