Phytochemistry, Vol. 27, No. 8, pp. 2491–2500, 1988.
Printed in Great Britain.

0031-9422/88 $3.00 + 0.00
Pergamon Press plc.

STEROL REQUIREMENTS AND PACLOBUTRAZOL INHIBITION OF A CELERY CELL CULTURE

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(*Received* 22 *February* 1988)

IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—*Apium graveolens*; Umbelliferae; paclobutrazol; plant growth regulator; celery cell suspension culture; cell division; sterol biosynthesis; 14α-methylsterols; 24α-ethylsterol; stigmasterol.

Abstract—The triazole plant growth regulator paclobutrazol was an effective inhibitor of growth of a celery cell suspension culture. Exposure of the celery cells to paclobutrazol caused inhibition of sterol biosynthesis at the 14α-demethylation step and there was an accumulation of several 14α-methylsterols [obtusifoliol, cycloeucalenol, cycloartenol, 14α,24-dimethylcholest-8-en-3β-ol and 14α,24-dimethylcholesta-8,24(28)-dien-3β-ol] with an accompanying decrease in the sitosterol and stigmasterol content of the cells. There was a marked fall in the stigmasterol: sitosterol ratio. Addition of paclobutrazol during the linear phase of growth (day 6) caused a rapid cessation of cell division. Obtusifoliol, added on day six to the growing celery cultures at low concentrations (0.05 to 5 μM), also stopped cell division but at the lowest concentration an increase in dry weight continued to day 10 before starting to decline. 14α-Methylcholest-9(11)-en-3β-ol was an effective inhibitor of cell growth but other 14α-methylsterols possessing a 4β-methyl group and/or a 9β,19-cyclopropane ring (lanosterol, dihydrolanosterol, cycloartenol, cycloeucalenol) had little or no effect on cell growth. The inhibition of celery cell growth by paclobutrazol could be partially reversed by addition of 50 μM cholesterol to the growth medium. Complete restoration of growth was achieved by addition of 50 μM stigmasterol or by 50 μM cholesterol plus a low concentration (0.05 μM to 5 μM) of stigmasterol. Stigmasterol alone at these low concentrations was ineffective in promoting growth of the paclobutrazol treated cells. These results reveal that the celery cells have two requirements for sterols. One is for relatively large amounts of sterol and may be associated with membrane elaboration. The other essential role, termed 'trigger', requires trace amounts of sterol with a 24α-ethyl side chain such as stigmasterol. Thus, plants have sterol requirements for cell proliferation which resemble those demonstrated for fungi. The significance of these results in relation to the mode of action of azole plant growth regulators is discussed.

INTRODUCTION

The mode of action of synthetic plant growth regulators is currently attracting considerable attention. The triazole-type of growth retardants inhibit the *ent*-kaurene oxidase catalysed step in the biosynthetic sequence to the gibberellins [1–7]. This is a cytochrome P-450 dependent mono-oxygenase system and it is postulated that the azole compounds interact with the protohaem iron thus blocking enzyme activity [3]. This is a similar mode of action to that proposed for the antifungal imidazole drugs such as ketoconazole which prevent ergosterol biosynthesis by inhibiting the cytochrome P-450 dependent sterol 14α-demethylation system which catalyses an essential step in sterol biosynthesis [8–10]. In fungi treated with these compounds there is a marked accumulation of 14α-methylsterols, such as obtusifoliol, and a decline in ergosterol [8].

Some of the azole plant growth retardants also display additional antifungal properties [5–7, 10–13]. This has been related to their ability to inhibit sterol biosynthesis at the 14α-demethylation step in the fungi as do the imidazole antifungal drugs. Conversely some of the azole antifungal compounds such as fenarimol and triarimol cause the accumulation of 14α-methylsterols when administered to seedlings or to plant cell suspension cultures [14–17].

We have been investigating the mode of action of the plant growth regulator paclobutrazol [(2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl) pentan-3-ol] which is effective in reducing stem height, increasing yield in various crops and improving frost tolerance of fruit trees [18–23]. Paclobutrazol is believed to reduce shoot growth by inhibiting gibberellin biosynthesis at the *ent*-kaurene oxidation step [1, 4] and there is sound evidence that the (2*S*,3*S*)-enantiomer of paclobutrazol is the more effective stereoisomer in blocking this reaction [1, 24]. When applied to barley seedlings paclobutrazol causes a build-up of 14α-methylsterols [17, 24]. Paclobutrazol inhibits growth of a plant cell suspension culture [25] and in preliminary reports [26, 27], using a celery (*Apium graveolens*) cell suspension culture, we have described that this inhibition of growth is accompanied by an accumulation of 14α-methylsterols, such as obtusifoliol. Moreover, there is a corresponding decrease in the

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amounts of the 4-demethylsterols sitosterol and stigmasterol. The (2*R*,3*R*)-enantiomer of paclobutrazol exhibits the higher antifungal properties and is the most effective inhibitor of the 14α-demethylation reaction when applied to whole plants [24] or celery cell cultures [28]. This selective activity of the isomeric forms of triazole compounds towards inhibition of gibberellin biosynthesis or sterol biosynthesis has been reported also for triadimenol [29] and other related compounds [1, 6, 7].

It is considered that the inhibition of sterol biosynthesis and a loss of ordered membrane function due to accumulation of excessive amounts of 14α-methylsterols, perhaps coupled to the accompanying decline in ergosterol content in the membranes, may be an important cause of growth inhibition in fungi in response to treatment with azole drugs [8, 10, 30, 32]. The proposal that accumulated 14α-methylsterols do not maintain efficient membranes has also been advanced to explain the action of a triazole based herbicide on plant growth [33]. Pertinent to these considerations we describe in this paper the effects of paclobutrazol and 14α-methylsterols on the growth of a celery cell suspension culture. Also, we provide evidence of an essential requirement for sterol, and specifically for 24-ethylsterol, to promote cell growth.

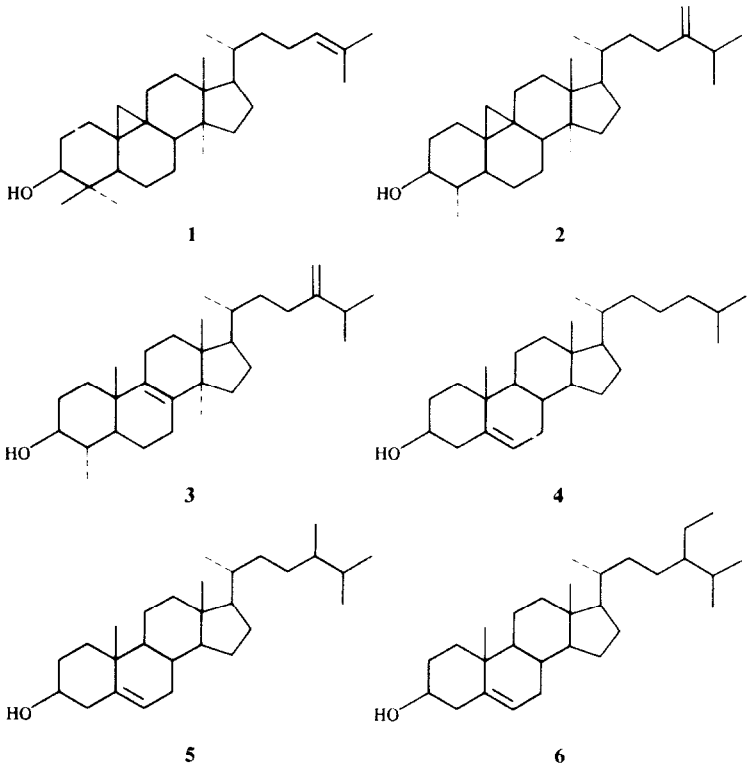
RESULTS AND DISCUSSION

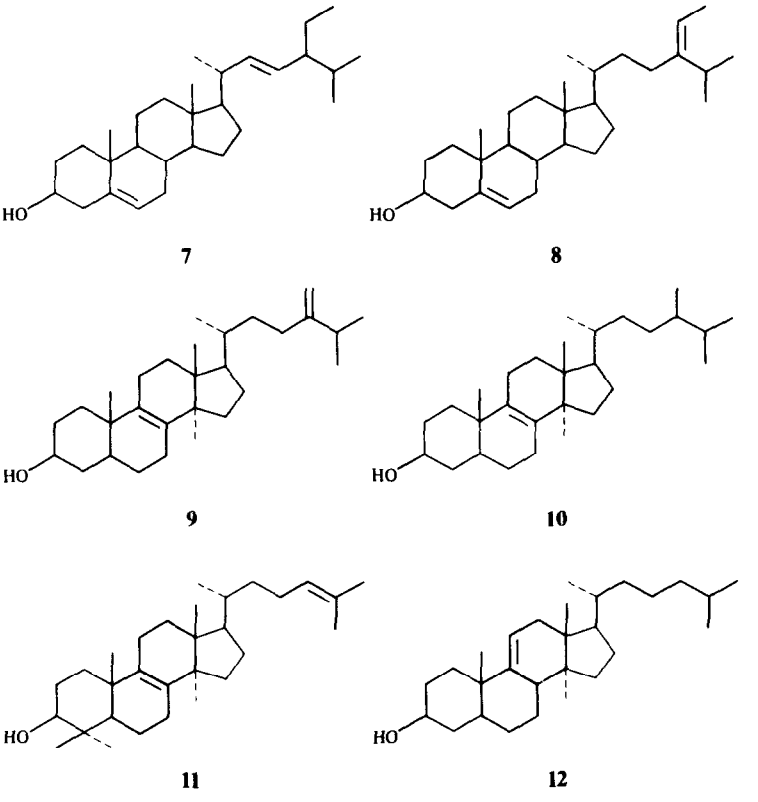
The sterols from celery suspension cells were analysed by GC-MS which showed that the major components were stigmasterol (**7**; 24*S*-ethylcholesta-5,22-dien-3β-ol, 50%), sitosterol (**6**; 24*R*-ethylcholest-5-en-3β-ol, 30%) and campesterol (**5**; probably a mixture of the 24*R*- and 24*S*-isomers of 24-methylcholest-5-en-3β-ol, 18%). A small amount of cholesterol (**4**; cholest-5-en-3β-ol, 1%) was usually present together with a variable amount of isofucosterol (**8**; 24-ethylcholesta-5,24(28)*E*-dien-3β-ol, 1-5%) which is a biosynthetic intermediate in the production of sitosterol [14, 34]. There were negligible amounts of 4,4-dimethylsterols or 4α-methylsterols.

We have previously reported that paclobutrazol inhibits celery cell growth and that this is accompanied by an inhibition of sitosterol (**6**) and stigmasterol (**7**) production due to a block of the cytochrome P-450-dependent 14α-demethylation step in sterol biosynthesis [26, 27]. Maximum inhibition of growth, based on either cell count or dry weight increases was observed with concentrations above 50 μM (Fig. 1) while 10 μM paclobutrazol reduced growth by about 50% compared to the control. Tests showed that about 70–80% of the paclobutrazol treated cells were still viable.

The inhibition of celery cell growth by the paclobutrazol treatment was accompanied by marked changes in the sterol composition resulting from the inhibition of the 14α-demethylation reaction (Table 1). There was a significant accumulation of the sterol precursor obtusifoliol (**3**; 4α,14α,24-trimethylcholesta-8,24(28)-dien-3β-ol) and to a lesser extent of cycloartenol (**1**; 4,4,14α-trimethyl-9β,19-cyclocholest-24-en-3β-ol). Cycloeucalenol (**2**; 4α,14α,24-trimethyl-9β,19-cyclocholesta-24(28)-en-3β-ol) was also observed to accumulate in some cultures.

Further metabolism of the obtusifoliol by removal of the 4α-methyl group, which is an oxidative demethylation reaction not requiring the participation of cytochrome P-450, and the reduction of the side chain 24-methylene group, resulted in the appearance of 14α,24-dimethylcholesta-8,24(28)-dien-3β-ol (**9**) and 14α,24-





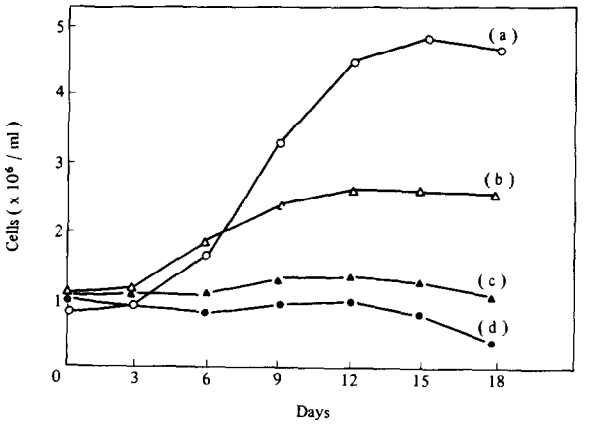


Fig. 1. Effect of paclobutrazol on the growth of celery cell cultures. The concentrations of paclobutrazol added to the cultures were (a) zero (control); (b) 10 μM; (c) 50 μM; (d) 100 μM. Growth is expressed as cell counts; similar curves were obtained if dry weight was used as a measure of growth.

dimethylcholest-8-en-3β-ol (**10**). These compounds co-chromatographed with campesterol (**5**) on the GC column employed in this work but they were readily identified by their mass spectra (see Experimental). In some incubations with paclobutrazol these 14α-methylsterols were present at high concentrations and they largely replaced campesterol. However, although trace amounts of 14α-methylsterols with a 24-ethyl or 24-ethylidine group were occasionally identified, these compounds have never been observed in high amounts in paclobutrazol treated celery cells. This could be a consequence of the specificity of the 24-methylenesterol-*S*-adenosylmethionine transmethylase which shows a preference for 24-methylenelophenol [4α,24-dimethylcholesta-7,24 (28)-dien-3β-ol] as substrate [14, 35]. Production of this latter sterol is blocked by paclobutrazol and the accumulating 14α-methyl-24-methylene sterols are presumably not suitable substrates for the transmethylation enzyme to produce 14α-methyl-24-ethylidenesterols.

The accumulation of 14α-methylsterols was accompanied by a corresponding decrease (Table 1) in the amounts of campesterol (**5**), stigmasterol (**7**) and sitosterol (**6**). The disappearance of isofucosterol, which is regarded as a sitosterol precursor, from the paclobutrazol treated cells indicates that the inhibition of sterol production at the 14α-demethylation step is very effective and that there must be a considerable decrease in formation of new sitosterol and stigmasterol in these cells. Much of the sitosterol and stigmasterol remaining in the paclobutrazol treated cells may thus represent material derived from the original innoculum of cells. A striking feature of the sterol composition of the paclobutrazol treated cells was the change in the relative proportions of stigmasterol and sitosterol compared to the control cells. In the control cells stigmasterol was the major sterol but the stigmasterol:sitosterol ratio declined markedly in the treated cells (Tables 1 and 2). The fact that the stigmasterol content decreased more rapidly than the sitosterol may reveal a more active metabolic turnover of the former sterol. However, although there is some speculation [36] about the role of sitosterol as the immediate precursor of stigmasterol, it is clear that inhibition of this conversion by paclobutrazol would lower the

Table 1. Sterol content (μg per culture) of celery cell cultures and the effect on the sterol composition during growth in the presence of paclobutrazol for 21 days

	Total lipid (mg)	Chol*	Camp plus 14α-MS	Stig	Sito	Isof	Obt	Cya	Total sterol	Stig: Sito ratio
Control	2.7	37	91†	493	233	71	...	...	925	2.12
Plus 10 μM paclobutrazol	2.5	21	143	159	201	...	31	...	555	0.79
Plus 50 μM paclobutrazol	1.5	9	165	75	181	...	156	21	607	0.41

*Chol = cholesterol (**4**); Camp = campesterol (**5**); 14α-MS = 14α,24-dimethylcholest-8-en-3β-ol(**10**) plus 14α,24-dimethylcholesta-8,24(28)-dien-3β-ol (**9**); Stig = stigmasterol (**7**); Sito = sitosterol (**6**); Isof = isofucosterol (**8**); Obt = obtusifoliol (**3**); Cya = cycloartenol (**1**).
†In the control culture GC-MS analysis revealed only campesterol (**5**) and no 14α-methylsterol (**9** or **10**).

Table 2. The sterol content (ng/10^6 cells) of celery cells treated with paclobutrazol (10 μM or 50 μM) at day zero or day six (cells were harvested at day 14 and the sterols analysed by GC-MS)

	Chol*	Camp plus 14α-MS	Stig	Sito	Obt	Cya	Cye	Stig: Sito ratio
Control	35	2590†	8530	3860	...	...	...	2.21
10 μM Paclobutrazol added at day zero	120	3090	2770	3140	2930	330	910	0.88
50 μM Paclobutrazol added at day zero	330	2830	2550	2060	1110	...	...	1.24
10 μM Paclobutrazol added at day six	60	2110	2450	2460	1120	...	...	0.99
50 μM Paclobutrazol added at day six	60	1580	1540	1210	600	60	...	1.27

*Abbreviations as for Table 1; Cye = cycloeucalenol (**2**).
†In the control culture GC-MS revealed only campesterol.

stigmasterol:sitosterol ratio. Alternatively, if stigmasterol is derived from some earlier precursor, such as stigmast-7-en-3β-ol, which also acts as a common precursor to sitosterol, then a similar inhibition of introduction of the Δ^{22}-bond into the side chain would result in the lowered stigmasterol:sitosterol proportions. It has recently been reported [37] that the formation of the Δ^{22}-bond in ergosterol by yeast requires the participation of a cytochrome P-450 dependent mono-oxygenase. If the sterol Δ^{22}-desaturase in higher plants is also a cytochrome P-450 requiring system, it can be predicted that this might be susceptible to inhibition by triazoles such as paclobutrazol and this will explain the changed stigmasterol:sitosterol ratio now observed in the celery cells.

It is speculated [8, 30] that the inhibition of fungal growth by imidazole antifungal drugs results from the disruption of membrane integrity and function due to the accumulating 14α-methylsterols. Similarly the inhibition of plant growth by a triazole herbicide has been ascribed to the damaging effects of excessive amounts of 14α-methylsterols in the plant cell membranes [33]. Clearly the inhibition of celery cell growth by paclobutrazol treatment could also be due to this cause in view of the relatively large amount of 14α-methylsterol which accumulated during a 21 day growth period (Table 1). It was therefore of interest to test if celery cell growth was sensitive to the addition of 14α-methylsterol in the absence of paclobutrazol. The results presented in Fig. 2 show that celery cell growth is retarded by added obtusifoliol. When obtusifoliol (**3**) was added to the culture media at day six to give concentrations of 0.5, 5 or 50 μM, respectively, there was a rapid cessation of cell division in all cases (Fig. 2A) followed by a steady decrease in dry weight (Fig. 2B). However, when 0.05 μM obtusifoliol was administered at day six, although cell division was arrested within two days, dry weight increased at a rate similar to the control cells until day 10 and only then showed a decline. It has previously been noted that in yeast cultures cell division is more sensitive than dry weight increase following treatment with sterol synthesis inhibitors [38, 39].

It is apparent that even if all the obtusifoliol in the culture medium at the lowest concentrations (1.3 and 13 μg, respectively) was absorbed by the celery cultures, the amount present in the cells would still be significantly below that accumulated (31 μg) in the presence of 10 μM paclobutrazol (Table 1). Moreover, in the latter case there was also a considerable amount (100–140 μg) of the other 14α-methylsterols (**9** and **10**) present in the cells but nevertheless this culture had still grown to *ca* 40% of the control (Fig. 1). It therefore appears that the inhibition of

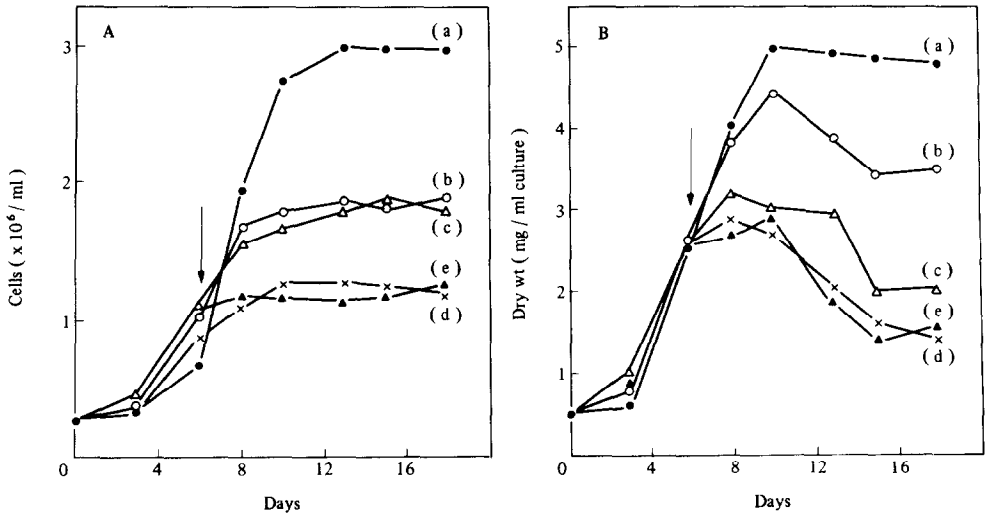


Fig. 2. Effect of addition of obtusifoliol on the growth of celery cell cultures. A: growth measured by cell counting; B: growth measured by dry weight. Obtusifoliol was added in acetone (0.1 ml) to the growth medium at day six (arrow) to give the following concentrations: (a) zero (control); (b) 0.05 μM; (c) 0.5 μM; (d) 5 μM; (e) 50 μM.

growth which follows uptake of obtusifoliol from the medium results from some mechanism which is not dependent upon the accumulation of a large amount of intracellular 14α-methylsterol. Consequently, it is possible that the large amount of 14α-methylsterol present in the paclobutrazol treated cells (Table 1) was not the primary cause of inhibition of growth but it had accumulated in the cells after some other factor had brought about the reduction in growth rate.

The inhibition of celery cell growth by other sterols with a 14α-methyl group was tested and the results presented in Fig. 3. Cholesterol was used as a control to ascertain that sterol lacking a 14α-methyl group when added in acetone to the growth medium had no adverse

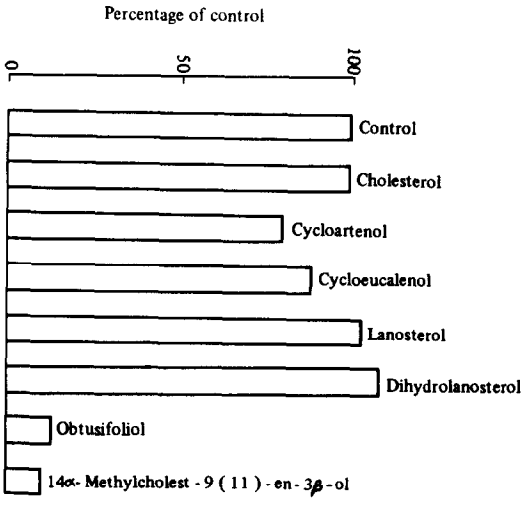


Fig. 3. Effects of 14α-methylsterols on the growth of celery cell cultures. The sterols were added in acetone to the growth media of 60 ml celery cell cultures at day six to give a final sterol concentration of 50 μM. Cell growth was measured at day 12 and is expressed as a percentage of the control.

or stimulatory effects on cell growth. Lanosterol (**11**) and 24,25-dihydrolanosterol had no effect on growth of the celery cultures while the presence of 9β,19-cyclopropane compounds, cycloartenol(**1**) and cycloeucalenol (**2**), resulted in only small reductions in growth. 14α-Methylcholest-9(11)-en-3β-ol (**12**) was strongly inhibitory to growth and, as with obtusifoliol (**3**), cell division was arrested soon after the addition of the sterol to the growth medium. However, while obtusifoliol was inhibitory to cell growth at concentrations as low as 0.05 μM (Fig. 2) somewhat higher concentrations (>5 μM) of 14α-methylcholest-9(11)-en-3β-ol were required to inhibit cell growth. It appears therefore that the efficacy of 14α-methylsterols for inhibiting celery cell growth is dependent upon the other structural features in the sterol molecule and in particular the presence of a 4β-methyl group and/or 9β,19-cyclopropane ring may vitiate the adverse effect of the 14α-methyl group. These results with celery cells compare with the observation that the growth rate of *Mycoplasma capricolum* was somewhat reduced by lanosterol added to the growth medium [30, 40].

The inhibition of cell division followed rapidly after addition of paclobutrazol at day six to a growing culture of celery cells. This treatment also prevented the increase in dry weight of the cells compared to the control (Fig. 4). Analysis of the sterols in cells harvested at day 14 showed (Table 2) that 14α-methylsterols, particularly obtusifoliol (**3**), had accumulated and that the amounts of stigmasterol (**7**) and sitosterol (**6**) had declined as observed previously (Table 1) with cells treated with paclobutrazol from the start of the culture period. The amount of obtusifoliol present in the cells treated with paclobutrazol at day six was approximately half the amount present in cells treated at day zero. This suggests that the *de novo* sterol biosynthetic pathway remains functional in the treated cells and that there may be a steady accumulation of 14α-methylsterols following inhibition of the 14α-demethylation reaction and growth by paclobutrazol as already discussed above. Clearly this is an important point which needs to be established by measurements of absolute rates of sterol synthesis at various times after

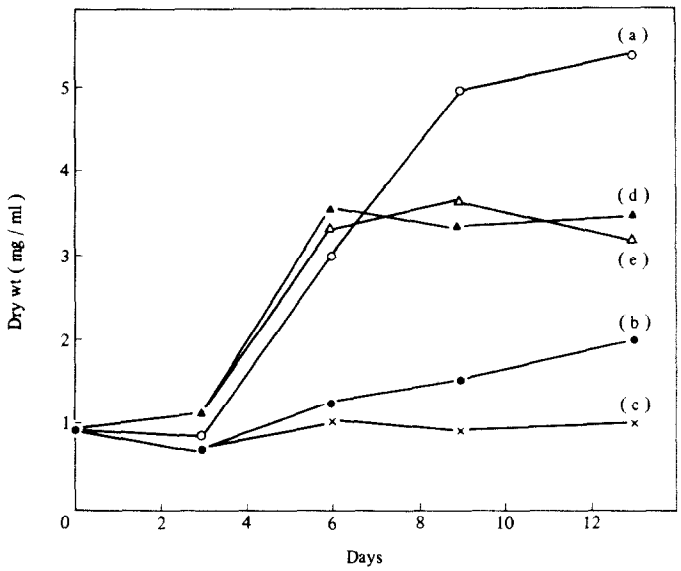


Fig. 4. Inhibition of celery cell growth by addition of paclobutrazol added either at the start of the culture or at day six. (a) Control culture without paclobutrazol; (b) 10 μM paclobutrazol added at day zero; (c) 50 μM paclobutrazol added at day zero; (d) 10 μM paclobutrazol added at day six; (e) 50 μM paclobutrazol added at day six.

paclobutrazol treatment. However, the results imply that the rapid cessation of cell division which follows paclobutrazol treatment occurs before an appreciable amount of 14α-methylsterol can have accumulated in the cells. Reductions in growth rates of some fungi have also been noted to occur relatively soon after exposure to sterol biosynthesis inhibitors [8].

As already noted the appearance of 14α-methylsterols in the cells after paclobutrazol treatment is accompanied by a corresponding decline in the amount of 4-demethylsterols such as sitosterol (**6**) and stigmasterol (**7**). The failure of the cells to produce new 4-demethylsterol could therefore also be an important factor in the mechanism of paclobutrazol inhibition of cell division. To test this hypothesis we have examined the effects of adding 4-demethylsterol to celery cells pre-treated with paclobutrazol. We have previously reported [26, 27] that addition of 50 μM cholesterol restored growth to about 40–50% of the control value but full restoration of growth required addition of 50 μM stigmasterol. We have now investigated in more detail the effects of adding various amounts of these sterols to celery cells inhibited with 30 μM paclobutrazol.

Increasing the concentration of cholesterol added to the medium to 100 μM failed to produce any marked further stimulation of growth compared to that obtained with 50 μM cholesterol (Fig. 5). When the concentration was increased to 150 μM cholesterol it actually became inhibitory. It is possible that this may be due to the manner of presentation of the sterol to the cells, as solubility is a serious problem at this concentration. Also, it is conceivable that at the high cholesterol concentration autoxidation products of cholesterol produced in the media may reach a level which is in some way inhibitory to cell growth.

The ability of stigmasterol (**7**) to reverse paclobutrazol inhibition of growth was also tested employing a range of stigmasterol concentrations. Addition of 50 μM stigmasterol at day six restored growth to the control value (Fig. 6). However, low concentrations of stigmasterol (0.05 to 5 μM) were ineffective. We have previously shown [27] that when 50 μM stigmasterol was added at day 12 to cells treated with paclobutrazol alone or to cells in the presence of paclobutrazol and 50 μM cholesterol, growth was restored to the control value. This established an essential requirement for a 24-ethylsterol for celery cell growth. The results presented in Fig. 7 confirm this point and now show that growth of paclobutrazol treated cells supplemented with 50 μM cholesterol in the medium can be reinitiated by the addition of low concentrations (0.05 to 5 μM) of stigmasterol. It is emphasised that stigmasterol at the low concentrations in the absence of 50 μM cholesterol cannot sustain cell division in the paclobutrazol treated cells (Fig. 6). Neither cholesterol nor stigmasterol added to the media in the absence of paclobutrazol had any stimulatory or inhibitory effects on celery cell growth (results not shown). In a separate study we have shown that a 24α-ethylsterol (e.g. stigmasterol) is considerably more effective than 24β-ethyl, 24α-methyl or 24β-methyl sterols in restoring growth to inhibited cells (unpublished results).

The possibility of multiple functions of sterols in plants has been discussed [41]. Douglas and Paleg [42, 43] reported that cholesterol, sitosterol or stigmasterol apparently promote growth of tobacco seedlings previously retarded by treatment with CCC or AMO-1618 which are inhibitors of gibberellin production. The growth of plant cell cultures inhibited by the plant growth regulator tetcyclasis, which also blocks gibberellin biosynthesis, was restored by sterol addition and it was suggested that sterol production may play some role in plant cell division [44, 45]. Recently, Tal and Nes [46] have indicated that 24-alkylsterols are important for growth of a sunflower cell culture. Our results

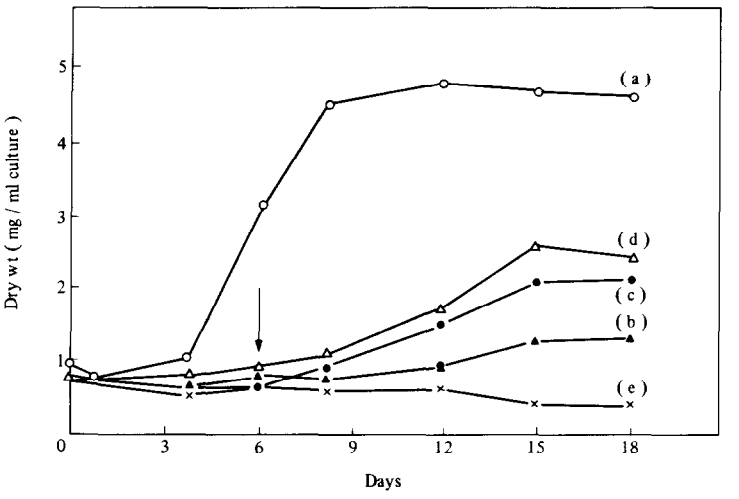


Fig. 5. Effects of increasing concentrations of cholesterol added at day six (arrow) to the media of celery cell cultures inhibited with paclobutrazol. The paclobutrazol was added in all cases at the start of the culture period. (a) Control with no paclobutrazol; (b) 30 μM paclobutrazol; (c) 30 μM paclobutrazol plus 50 μM cholesterol; (d) 30 μM paclobutrazol plus 100 μM cholesterol; (e) 30 μM paclobutrazol plus 150 μM cholesterol.

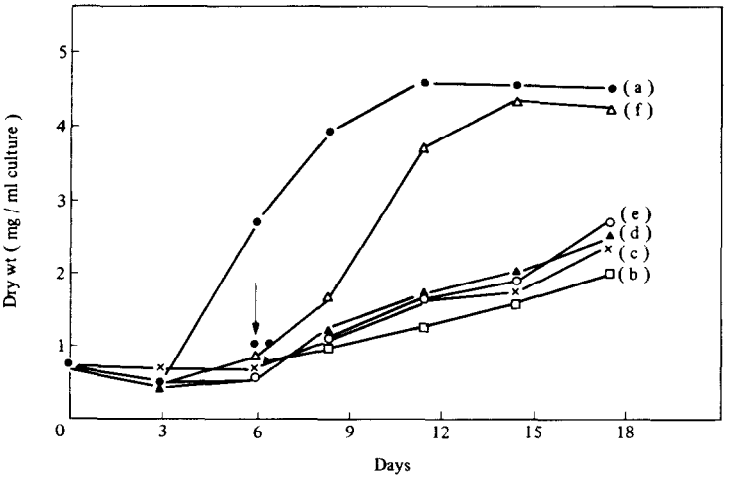


Fig. 6. Effects of increasing concentrations of stigmasterol added at day six (arrow) to the media of celery cell cultures inhibited with paclobutrazol. The paclobutrazol was added in all cases at the start of the culture period. (a) Control with no paclobutrazol; (b) 30 μM paclobutrazol; (c) 30 μM paclobutrazol plus 0.05 μM stigmasterol; (d) 30 μM paclobutrazol plus 0.5 μM stigmasterol; (e) 30 μM paclobutrazol plus 5 μM stigmasterol; (f) 30 μM paclobutrazol plus 50 μM stigmasterol.

reveal that celery cells have specific sterol requirements for two distinct purposes. One may be connected with a role in new membrane production which will be a feature of actively dividing and enlarging cells. This can be envisaged to require relatively large amounts of sterol but in the paclobutrazol treated cells there is often a substantial decrease in the 4-demethylsterol content (Tables 1 and 2). However, this need for sterol can apparently be fulfilled by the uptake of either cholesterol or stigmasterol added to the growth medium which, as we have shown previously [26, 27], can restore the total sterol content of the cells to a value comparable to the control cultures. It seems that in paclobutrazol-treated cells the 14α-methylsterols (**1–3**, **9**, **10**) which accumulate in response to paclobutrazol treatment cannot meet the large scale sterol requirement of the cells even in the presence of added trace stigmasterol. This may be because either the 14α-methyl group hinders their efficient transport from the site of synthesis or the integration of these sterols into particular membranes may be defective and the membrane could become sterol deficient in the absence of normal 4-demethylsterol production. Alternatively the insertion of the 14α-methylsterols produces membranes which do not function effectively [10, 30–32]. Cholesterol added to the inhibited celery cells can be envisaged to integrate into a sterol-deficient membrane or to displace or adequately dilute any 14α-methylsterols which may be present and this restores the membrane properties. However, in some organisms a high 14α-methylsterol content can be tolerated. For example 14α-methylcholest-9(11)-

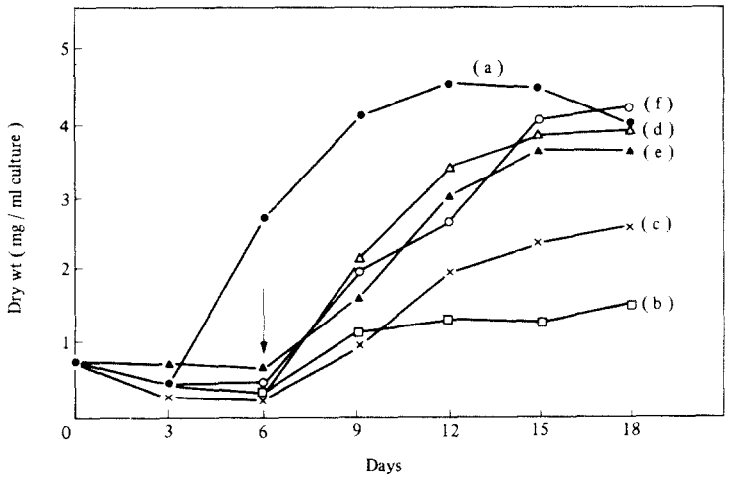


Fig. 7. The stimulation of growth of paclobutrazol-treated celery cells by the addition at day six (arrow) of cholesterol together with stigmasterol. (a) Control with no paclobutrazol; (b) 30 μM paclobutrazol alone; (c) 30 μM paclobutrazol plus 50 μM cholesterol; (d) 30 μM paclobutrazol plus 50 μM cholesterol and 5 μM stigmasterol; (e) 30 μM paclobutrazol plus 50 μM cholesterol and 0.5 μM stigmasterol; (f) 30 μM paclobutrazol plus 50 μM cholesterol and 0.05 μM stigmasterol. The paclobutrazol was added in all cases at the start of the culture period.

en-3β-ol is a major sterol constituent in some sea-cucumbers [47], tobacco cell cultures continue to grow in the presence of buthiobate which causes obtusifoliol accumulation [48] and yeast mutants with a defective 14α-demethylation mechanism are able to grow without added sterol although they are accumulating 14α-methylsterols [49, 50].

The second sterol requirement of celery cells revealed by the present work is for a trace or 'trigger' amount of stigmasterol but this is only effective in restoring growth of paclobutrazol-treated cells when there is sufficient of another sterol, such as cholesterol, also available to the cells. To act as a trigger sterol, the side chain structure is important and the most effective is a 24α-ethylsterol (Haughan, P. A., Lenton, J. R. and Goad, L. J., unpublished results). In normally growing cells the supply of trigger sterol may be met, and regulated, by the synthesis of new sterol and it may be significant that in maize 24-ethylsterol is synthesized at *ca* 3–4 times the rate of 24-methylsterol [51]. The ability of 50 μM cholesterol alone to partially restore growth (Fig. 5) may be because some of the sitosterol or stigmasterol remaining in the inhibited cells (Tables 1 and 2) is in a pool which can be mobilised to act as a source of trigger sterol. However, if 4-demethylsterol biosynthesis is blocked, the cell proliferation will cease when this supply is exhausted and the trigger 24-ethylsterol concentration falls below a cellular threshold level. When an exogenous supply of stigmasterol is provided in the medium this can be absorbed by the cells and it will enable the trigger sterol concentration to again exceed the threshold level required for resumed cell division. The observation that a low concentration of added obtusifoliol can stop celery cell division suggests that it may perhaps interfere in some way with the action of the trigger 24-ethylsterol. The rapid cessation of growth in response to paclobutrazol addition may also result from interference with trigger sterol action by the initially accumulating small amount of 14α-methylsterol. However, in this case the sudden disappearance of newly synthesised trigger 24α-ethylsterol from the cell, coupled to the concomitant fall in total sterol production for membrane elaboration, may be an equally or more important factor in halting cell division. Similar considerations may apply to explain the mode of action of other plant growth regulators [29] and antifungal compounds [32] which have as their primary site of action the inhibition of sterol biosynthesis.

Our results show that the requirements for sterols by growing plant cells are closely analogous to the situation reported for mutant yeast sterol auxotrophs [8, 52 56]. The growth of these cells can be supported by 'bulk' sterol such as cholesterol or 5α-cholestanol when in the presence of a trace of ergosterol which is referred to as sparking [54] or sterol synergism [56]. It is generally considered that the bulk sterol in yeast fulfills a membrane function and the sparking ergosterol satisfies an essential high specificity function. Multiple, essential functions for 24β-methylsterols have also been reported for another fungus, *Gibberella fujikuroi* [57]. Although the mode of action of the sparking sterol in yeast is not yet understood ergosterol has been shown to enhance phospholipid synthesis [58], stimulate polyphosphoinositide metabolism [59] and to reactivate yeast cell budding and proliferation with an associated regulation of protein kinase activity [60].

EXPERIMENTAL

Materials. Paclobutrazol [(2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl) pentan-3-ol] was a gift from ICI, Plant Protection Division, Jealotts Hill, U.K. Cholesterol (B.D.H, U.K.) and stigmasterol (Sigma, U.K.) were purified before use by alumina CC [61], crystallized from MeOH and analysed by GC, GC-MS and ^{1}H and ^{13}C NMR.

Cultures. The celery cell suspension culture was obtained from Dr Hamish Collin, (Department of Botany, University of Liverpool). Murashige and Skoog medium (Flow Laboratories) was

supplemented with sucrose (30 g/l), 2,4-dichlorophenoxyacetic acid (0.5 mg/l) and kinetin (0.6 mg/l) and adjusted to pH 5.5. The medium (50 ml) was dispensed into 250 ml conical flasks, autoclaved, and 10 ml of a celery cell suspension (6×10^6 cells/ml) were added. Cultures were grown for 15 to 21 days at 18° with a 6 hr light–6 hr dark cycle and with continuous shaking (50 rpm).

Paclobutrazol and sterols were added in solution in Me_2CO. The final concn of Me_2CO in the medium was always less than 1% v/v; an equivalent volume of Me_2CO was added to the control cultures. Experiments established that this amount of Me_2CO had no effect on growth rate. Cell growth was determined at intervals by cell counting [62]. Chromium trioxide solution (8% w/v, 0.2 ml) was added to an aliquot (0.1 ml) of the cell suspension, heated to 70° for 15 min, and passed 10 times through a No. 16 syringe needle before counting on a haemocytometer. Growth was also monitored by dry wt determination after removal of 2 ml of the culture, filtration through a preweighed glass filter (Whatman GF/A, 2.5 cm) and washing the cells with water. Cell viability was determined by a dye exclusion test using Evans' Blue [63]. Cultures, cell counts and dry wt determinations were usually performed in duplicate.

Analysis of sterols. Cells were harvested by centrifugation and 100 μg of 26,27-dinorcholesta-5,22-dien-3β-ol was added to act as an int. standard for sterol quantification. This synthetic sterol was chosen since it is well separated by GC from the endogenous celery sterols. The cells were resuspended in 10 ml water, homogenized (Potter–Elvehjem) and then centrifuged at 3000 rpm for 5 min. The aqueous supernatant was retained and the cell residue was homogenized after addition of 10 ml Me_2CO. After centrifugation the cell residue was extracted for a second time with 10 ml Me_2CO. The combined aq. and Me_2CO extracts were reduced in vol. by rotary evapn until cloudy and then extracted twice with 100 ml petrol (b.p. 40–60°). The petrol extract was washed with H_2O, dried over sodium sulphate and finally evapd to dryness to yield a lipid fraction. The sterol types (4,4-dimethyl, 4α-methyl, and 4-demethyl sterols) were isolated from this lipid by prep. TLC on silica gel with $CHCl_3$–EtOH (49:1) [61].

The sterols were identified and quantitated by GC using a Dani 3800 instrument operated in split-splitless mode. A 25 m × 0.22 mm BP-1 or 12 m × 0.22 mm BP-5 (0.25 μm film thickness) flexible fused silica column [SGE (UK) Ltd] was employed. The initial oven temp. was 240° held for 2 min and then programmed at 5°/min to 290°. Injection and detector (FID) temperatures were 290 and 300°, respectively. The carrier gas (Ar or He) flow rate was 1.5 ml/min. The GC was connected to an integrator (Spectra Physics, Model SP 4270) and individual sterols were quantitated by reference to the added int. standard.

Sterol identities were confirmed by GC-MS using a VG 70-70H mass spectrometer coupled to a Finnigan Incos data system. A BP-1 capillary column (25 m × 0.22 mm) was used; samples were injected on-column at ambient temp. and the oven temp. was then raised by 12°/min to 280°. The mass spectra of the sterols identified in the celery cells are cited below:

Cycloartenol (**1**) *m/z* (rel. int.): 426 $[M]^+$ (7), 408 (9), 393 (12), 339 (4), 286 (6), 271 (10), 205 (11), 203 (12), 175 (16).

Cycloecalenol (**2**) *m/z* (rel. int.): 426 $[M]^+$ (7), 411 (16), 393 (9), 327 (6), 285 (8), 269 (10), 218 (22), 203 (96).

Obtusifoliol (**3**) *m/z* (rel. int.): 426 $[M]^+$ (14), 411 (47), 393 (12), 327 (6), 285 (4), 245 (14), 227 (10), 201 (10).

Cholesterol (**4**) *m/z* (rel. int.): 386 $[M]^+$ (11), 368 (9), 353 (6), 301 (6), 275 (11), 255 (8), 231 (5), 213 (9), 199 (4).

Campesterol (**5**) *m/z* (rel. int.): 400 $[M]^+$ (43), 382 (25), 367 (17), 315 (16), 289 (23), 255 (15), 213 (22).

Sitosterol (**6**) *m/z* (rel. int.): 414 $[M]^+$ (37), 396 (24), 381 (13), 329 (16), 303 (20), 273 (11), 255 (16), 273 (11), 213 (20).

Stigmasterol (**7**) *m/z* (rel. int.): 412 $[M]^+$ (20), 394 (5), 351 (8), 300 (10), 271 (12), 255 (22), 213 (9).

Isofucosterol (**8**) *m/z* (rel. int.): 412 $[M]^+$ (4), 354 (4), 314 (40), 299 (10), 296 (10), 281 (12), 255 (9), 229 (11), 213 (13).

14α,24-Dimethylcholesta-8,24(28)-dien-3β-ol(**9**) *m/z* (rel. int.): 412$[M]^+$ (19), 397 (83), 379 (11), 313 (14), 281 (10), 245 (12), 231 (36).

14α,24-Dimethylcholest-8-en-3β-ol (**10**) *m/z* (rel. int.): 414 $[M]^+$ (13), 399 (100), 381 (14), 287 (7), 245 (8), 219 (15).

Acknowledgements—We thank the Science and Engineering Research Council for a CASE studentship (P.A.H.), ICI Plant Protection Division, Jealotts Hill U.K. for a gift of paclobutrazol and Dr Richard Evershed and Mark Prescot for the determination of mass spectra. Long Ashton Research Station is financed through the Agricultural and Food Research Council.

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