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INHIBITORS OF STEROL BIOSYNTHESIS AND GROWTH IN PLANTS AND FUNGI

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Abstract—Sterols and their derivatives promote and maintain growth and development in plants and fungi by acting as membrane constituents and probably also as hormones, engaged in control of metabolism. Inhibitors of sterol biosynthesis, operating at various stages in the pathway, are useful probes for investigating these functions. Some of the inhibitors have assumed considerable commercial importance as agricultural fungicides and antimycotic drugs in medicine. There is also potential for using similar compounds to regulate plant growth.

INTRODUCTION

Sterols and steroids, whether possessing architectural, metabolic or hormonal functions, have long been considered important targets for control and manipulation in mammals [1]. More recently, there has been considerable success in the discovery and deployment of fungicides and antimycotics which interfere both with the production and efficient functioning of fungal sterols [2–4]. In common with most eukaryotic organisms, plants also possess sterols and here too there is considerable potential for developing growth regulators and herbicides based on similar modes of action [5].

This review compares chemical inhibition of the sterol biosynthetic pathways in plants and fungi. As far as possible this is also considered in relation to the growth of the organisms. The reasons for this approach are threefold. Firstly, it is intended to evaluate critically the use of such inhibitors as molecular probes for assessing sterol structure in relation to growth and development. In this respect inhibitors can complement the use of sterol auxotrophs and genetic mutants, blocked at specific biochemical stages [6–8]. Secondly, as some of the inhibitors discussed are agricultural fungicides which have, of necessity, to be applied to plants, it is important to understand the mechanisms by which they are selectively toxic towards the fungus and relatively safe to the plant and, if necessary, to optimise these characteristics. Thirdly, by adopting a comparative approach it may be possible to utilize leads from studies with one group of organisms to develop selective toxicants against another.

A summary is presented of the chemical nature of the plant and fungal sterols followed by a critical assessment of the present understanding of their role in growth and development. An outline of the biosynthetic pathways leading to plant and fungal sterols is then given with some examples of inhibitors that affect key enzymes. Because of the large number and structural variations of the sterol biosynthesis inhibitors (SBIs), it is not possible to provide an exhaustive coverage of this topic. Therefore, examples are chosen of the most representative, active or otherwise interesting compounds.

Some of the more recently discovered inhibitors have been designed rationally, for example, those bearing a close resemblance to the normal enzyme substrate or to the postulated high energy intermediate (HEI) of the enzyme catalysed reactions [9, 10]. Others have been discovered from extensive screening programmes of synthetic compounds or antibiotics [11].

A number of methods have been described for the study of enzyme inhibitors of sterol biosynthesis [12]. One commonly employed is to grow the plant or fungus in the presence or absence of a sub-lethal dose of the inhibitors and then to analyse the sterols after a suitable time period. Because of the lack of absolute substrate specificity by many enzymes in the sterol biosynthetic pathway, the compounds then found to accumulate usually include not only those preceding the stages inhibited but also those derived from them by 'abnormal pathways' [13, 14]. Another commonly used technique is to apply a pulse of radioactive precursor, frequently acetate or mevalonate, and then to assess the rate of incorporation into various sterol fractions. These approaches using whole organisms can be supplemented with studies on *in vitro* enzyme systems using subcellular preparations. Isolated enzyme studies can be particularly valuable where an inhibitor has the potential to inhibit more than one enzyme in the pathway for, with whole-organism experiments, an interference with an early stage may well obscure effects on later ones [15, 16]. Both microsomal

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and reconstituted enzyme preparations have been described and a useful and frequently quoted parameter is the I_{50} (concentration of inhibitor producing 50% inhibition at a given substrate concentration).

The accumulation of precursors of normal terminal sterols might be expected to have important consequences for growth. It can be argued [10] that the further upstream the inhibition from the terminal sterol(s) the greater likelihood there is for detrimental effects on growth since accumulating intermediates, structurally similar to the final sterols, may at least partially fulfil growth requirements. It is important to assess how far the available evidence supports this contention. It must, of course, be considered that a given sterol inhibitor may affect overall growth through biochemical mechanisms other than, or in addition to, those involved in sterol biosynthesis. This is more likely to occur, for example, with cytochrome P-450 inhibitors than with specifically designed high energy intermediate analogues, but it cannot be discounted in any instance.

The experimental conditions described for growing plants and fungi in the presence of sterol inhibitors differ widely. With whole plants, effects on growth are frequently expressed simply in terms of changes in height or dry weight, together with a description of morphological disturbances. Growth of plant tissues in culture is normally assessed on a dry weight or cell count basis [16]. Inhibition studies using fungicides are normally done in nutrient-rich cultures where fungal growth follows a typical sigmoid pattern with a lag phase followed by a period of logarithmic growth which then levels off [17, 18]. The ED_{50} (concentration of inhibitor required to reduce growth by 50%) is a useful parameter for inhibition studies because at this concentration biochemical changes responsible for the principal mode of action should be very clearly expressed. However, as fungal growth can be highly sensitive to culture conditions [19] great care has to be taken in selectivity studies when comparing results from different laboratories. There is often, but not always, a positive correlation between inhibition of fungal growth in culture and control of fungal diseases [20] and thus a good *in vitro* performance by an SBI should, at least, suggest a potential practical fungicide.

STEROLS OF PLANTS AND FUNGI

With very few exceptions, sterols are present in all eukaryotic organisms [21]. The sterols of plants and fungi may display considerable structural diversity although this has, in practice, proved to be of limited taxonomic value. Nonetheless certain generalizations may be made with some confidence.

In most fungi the major sterol, both in distribution and quantity, is 24β-methylcholesta-5,7,22*E*-trien-3β-ol (ergosterol, **1**) [22] while vascular plants possess predominantly 24α-ethylcholest-5-en-3β-ol (sitosterol, **2**), 24α-ethylcholesta-5,22*E*-dien-3β-ol (stigmasterol, **3**) and 24-methylcholest-5-en-3β-ol, (**4**) present either as the 24α-(campesterol) or 24β-(dihydrobrassicasterol) epimer [14]. Cholesterol, originally associated only with animal tissues, is now known to occur widely in higher plants, usually in trace amounts [23], and also in fungi of several taxonomic groups [22].

Notable exceptions to the above pattern of sterol distribution are certain taxa of the *Ascomycotina* which have 24β-methylcholesta-5,22*E*-dien-3β-ol (brassicasterol) as the dominant sterol [24], the *Uredinales* (rusts) which have mainly 24-ethylidenesterols with double bonds [22], some powdery mildews in which ergosta-5,24(28)-dien-3β-ol predominates [25] and certain members of the oomycetes which have as their major sterols cholesterol, 24-methylcholesterol, cholesta-5,24-dien-3β-ol(desmosterol) and stigmasta-5,24(28)*E*-dien-3β-ol (fucosterol) [26]. The Pythiaceae (*Phytophthora* and *Pythium* species) are unusual in not synthesizing sterols but they will readily acquire and utilize them from the growing medium [26, 27]. Some higher plants, including members of the *Cucurbitaceae*, possess not the usual Δ^5 but have instead Δ^7-sterols [28], while the cactus *Lophocereus schottii* is anomalous in having both Δ^7 and $\Delta^{8,14}$-sterols [29]. The isolation of the biologically active brassinosteroids, originally from rape pollen but more recently from other sources, has provided a challenging new impetus in the discovery of plant steroids [30–33].

Sterols may be present not only in the free state but also as various derivatives including long chain fatty acid esters, glycosides and acylated glycosides. In plants any one of these classes may be dominant depending on the species or type of tissue [34, 35]. Fungi may also possess steryl esters, usually in cytosolic lipid droplets [36]. In some species such as *Saccharomyces cerevisiae* the amount of steryl ester may increase with the age of the culture [37] while in others such as *Aspergillus nidulans* [38] the sterols may exist predominantly in the free state throughout the whole growth period. Steryl glycosides and esterified steryl glycosides are also known in fungi but are probably not of widespread occurrence [39].

THE FUNCTIONS OF PLANT AND FUNGAL STEROLS

Quite how the structural features present in plant and fungal sterols and their derivatives relate to the role of these compounds in growth and development is an intriguing but, at the present time, a largely unanswered question. However, from the evidence outlined below, it does now appear that the structure of a sterol should be viewed with regard to a possible function either as a membrane constituent, where the sterol aligns with the bilayer phospholipid and hence regulates fluidity, or else as a cellular metabolite or hormone.

An organism which has proved especially useful for probing sterol structure/function relationships is the yeast *Saccharomyces cerevisiae*. This grows well in culture, becomes auxotrophic for sterols when grown under strictly anaerobic conditions and provides a range of genetically well-defined mutants, blocked at various stages of sterol biosynthesis [40, 41]. It has also been used extensively in enzyme inhibitor-growth studies as outlined below. Independent research groups, notably those led by Bloch, Parks and Nes, are beginning to provide a complex appraisal of sterol structure in relation to growth. Using the auxotrophic strain GL7, blocked at the stage of cyclization of 2,3-oxidosqualene, Buttke and Bloch [42] have demonstrated the necessity of 4-desmethylsterols for growth and, surprisingly perhaps in view of the efficacy of 14α-demethylation inhibitors as fungicides, they found that 14α-methyl 4-desmethylsterols were effective growth supplements. Work with the RD5-R auxotroph by Rodriguez and Parks [43] has emphasized the necessity of the Δ^5-bond in sterols to

ensure optimum growth, while Pinto and Nes [44] have stressed the requirement for some sterol with a 24β-methyl group as a micronutrient. All three groups agree that a distinction should be made between a bulk membrane function, requiring large quantities of sterol, but with a relatively low structure specificity, and a metabolic or hormonal function, termed variously 'regulatory' [45], 'sparking' [46] or 'synergistic' [47], where the quantities involved may be much smaller but the structural requirements more demanding. In normal wild-type yeast grown under aerobic conditions ergosterol appears able to satisfy both bulk and regulatory requirements. However, another fungus *Gibberella fujikuroi* was found to possess three sterols—ergosterol, brassicasterol and 22(23)-dihydrobrassicasterol—and all appear necessary for optimum growth [48]. Pythiaceous species are capable of vegetative growth without sterols, apparently keeping cellular leakage at a sufficiently low level and maintaining the operation of the plasma membrane, but they will readily take them up from the environment with an enhancement of growth [26, 27]. In at least some oomycetes there is an absolute requirement for sterols in reproduction [27, 28] and steroid sex hormones of *Achlya* have been isolated and characterized [49].

In plants, work with celery cell suspension cultures using paclobutrazol as a sterol biosynthesis inhibitor as described below, has provided evidence for multi-roles for sterols in this tissue [16, 50]. When cell growth was inhibited by the azole, it could be partially restored by addition of cholesterol to the medium but only stigmasterol was fully effective in this respect. Bulk cholesterol (50 μM) and trace amounts of stigmasterol (0.05 μM) were as effective as bulk stigmasterol (50 μM) alone in restoring growth, suggesting an essential growth requirement for at least some 24-ethylsterol. The 14α-methylsterol obtusifoliol (**5**), but not other 14α-methylsterols such as lanosterol (**6**) or cycloartenol (**7**) stopped cell division when added to the medium at dosages as low as 0.05 μM. This most interesting result suggests that obtusifoliol might be interfering in some way with the 'triggering' function in cell division ascribed to stigmasterol [50].

The brassinolides, known to elicit powerful growth responses [30–33], are obvious candidates for the role of plant steroid hormones, although much remains to be discovered about their distribution and function. Little is known about which metabolic process in plants and fungi may be controlled by sterols in 'hormonal' amounts. However, four suggestions, all relating to the growth of yeast, are that ergosterol might control the ratio of saturated to unsaturated fatty acids [40], control the conversion of phosphatidylethanolamine to phosphatidylcholine [51], stimulate polyphosphoinositide metabolism [52] or else stimulate a protein kinase involved in cell cycle control [53]. It remains to be established whether it is the sterol molecule *per se* or an, as yet, unidentified metabolite [54] which may be responsible for these functions.

The dominant sterols, ergosterol in fungi, campesterol, stigmasterol and sitosterol in plants, must constitute the majority of membrane sterols in these organisms. Work on model [55] and biological [56–58] membranes has demonstrated that these sterols are less effective than cholesterol in regulating membrane fluidity and permeability, an observation that has been rationalized in terms of rotation of the bulky side chain at C-17 [59]. It is not known why plants and fungi should expend energy—it has been calculated that 15 ATP equivalents are involved in a single methylation [41]—to produce what appears to be structurally inefficient sterols for membrane regulation. One possibility is that the extra fluidity conferred by the presence of such sterols may enable the organisms to grow under a wide range of environmental conditions [60] and, at least in the case of plants, to allow a more flexible alteration of membrane composition in response to stress than if cholesterol alone was the membrane sterol. Thus, there are reports [61–66] that adverse conditions such as low temperatures, short day-length or high salinity may induce an increase in the more effective sterols such as cholesterol and campesterol at the expense of the less effective stigmasterol and sitosterol. However, as there is much inconclusive or even contradictory evidence [67–69], this may not be a widespread mechanism by which plants adapt to environment. To be effective it would also necessitate a fairly rapid turnover of sterols in the membranes—a subject of which little is known.

Few convincing explanations of a role for steryl esters and glycosides have been forthcoming but, in yeast, it has been suggested that steryl esters may serve as a storage form of sterol and unsaturated fatty acids [41].

INHIBITION OF PLANT AND FUNGAL STEROL BIOSYNTHESIS

Biosynthetic pathways

There have been several detailed reviews of sterol biosynthesis in plants [14, 70–72] and fungi [73, 74] and hence only an outline will be given here.

The formation of mevalonic acid from hydroxymethylglutaric acid is the first reaction unique to polyisoprenoid biosynthesis and successive phosphorylations and a decarboxylation of mevalonic acid then occur to provide isopentenyl pyrophosphate, the basic C-5 unit of sterol biosynthesis. Condensation of isoprenoid units leads eventually to the formation of squalene which, after epoxidation of the Δ^2-double bond, undergoes cyclization. It is here that a clear bifurcation exists between plants and fungi, for cycloartenol (**7**) is the first cyclic product in photosynthetic eukayotes, while lanosterol (**6**) is formed in non-photosynthetic organisms [60]. Other important differences are the early removal of a C-4 methyl group in plants, believed to assist the subsequent opening of the cyclopropyl ring [14, 60], and differences in the stereochemistry of the methylation at C-24 [14, 74].

The post cycloartenol/lanosterol steps are shown in Fig. 1. The sequences given are believed to be 'typical' but differences in the precise order of the stages may occur with different species. A notable example of this is in yeast where C-24 methylation occurs later in the sequence with cholesta-8,24-dien-3β-ol (zymosterol) the preferred substrate [74]. Details of the biosynthesis of the brassinolides are not yet available but, on structural grounds, sterols such as 24-methylenecholesterol, brassicasterol or 22-dehydrocampesterol are likely precursors [31].

3-Hydroxy-3-methylglutaryl coenzyme A reductase

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyses the biosynthesis of mevalonic acid and is now thought to be a key regulatory enzyme of

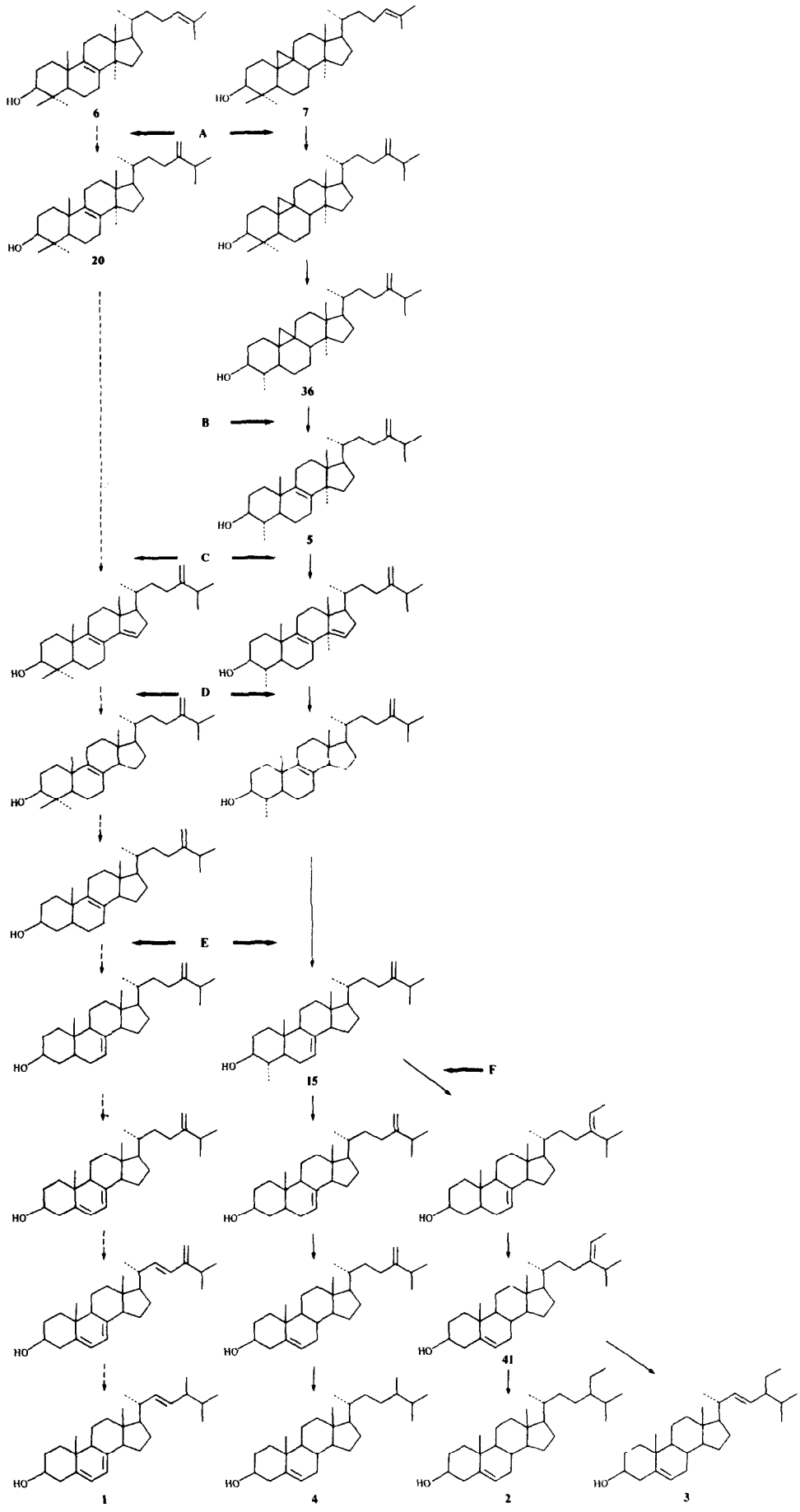
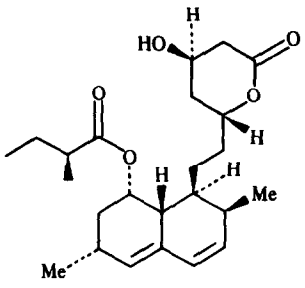


Fig. 1. Plant (→) and fungal (- - - ->) sterol biosynthesis after the lanosterol (**6**) cycloartenol (**7**) bifurcation. Sites of inhibition are indicated by A–F.



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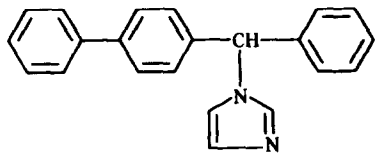

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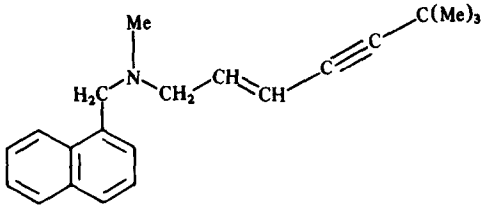

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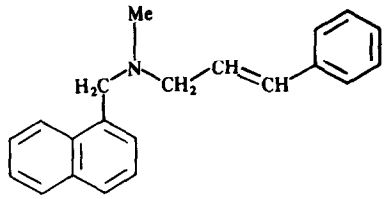

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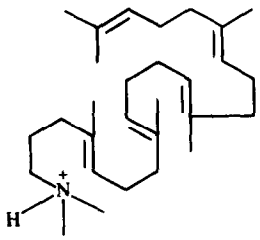

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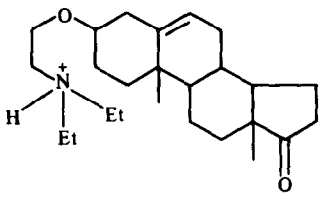

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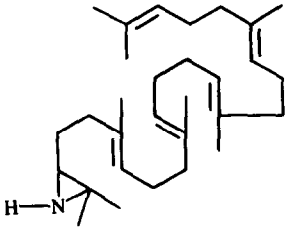

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sterol biosynthesis in plants [75] and fungi [76] as well as in mammals [77]. Two fungal metabolites, compactin and its methyl analogue mevalonin (**8**), are highly specific competitive inhibitors of HMG-CoA reductase. Mevalonin has K_i values of 3.5 and 2.2 nM with the enzymes from yeast and radish seedlings preparations respectively [78]. Although compactin was originally isolated as an antibiotic [79], its antifungal activity is not substantial [2] and neither compound appears markedly to depress fungal sterol biosynthesis [80]. By contrast mevalonin strongly inhibited the root growth of radish and wheat seedlings at concentrations between 25 and 250 nM [81]. Sterol biosynthesis in the roots was also markedly reduced by these low concentrations but the biosynthesis of other polyisoprenoids such as carotenoids, chlorophylls or ubiquinones was less or not affected [81]. A similar correlation between growth inhibition and sterol accumulation was observed with cell suspension cultures of *Silybum marianum* [81].

The azole bifonazole (**9**) was found [80] to inhibit directly HMG-CoA reductase in microsomes from the dermatophyte *Trichophyton mentagrophytes* and this may contribute to its antifungal activity. HMG-CoA reductase is also subject to feed-back inhibition by terminal sterols or sterol intermediates [82], an important factor in its regulatory properties [75].

Squalene epoxidase

Squalene epoxidase is a membrane-bound enzyme catalysing the formation of 2,3-oxidosqualene from squalene and molecular oxygen with an additional requirement for NAD(P)H and FAD [83]. The allylamines terbinafine (**10**) and naftifine (**11**), used in medicine against a range of fungi, are powerful inhibitors of fungal squalene epoxidase. In *Candida albicans*, the compounds are specific non-competitive reversible inhibitors with K_i values of 0.03 μM and 1 μM respectively [83]. In *C. albicans* the fungistatic effect of allylamines correlated well with ergosterol deficiency [84]. By contrast, in dermatophytes such as *Trichophyton mentagrophytes*, the mycelia were killed at concentrations where some sterol biosynthesis was still occurring. It has, therefore, been inferred that in these species an accumulation of squalene may have a direct toxic effect [84].

Application of naftifine to radish roots showed only a low-to-moderate inhibition of growth [85]. Data for inhibition of the isolated plant enzyme are not available,

but the *in vivo* result described above suggests that plant squalene epoxidase, rather like that from rat liver [84], may be poorly inhibited by these compounds.

2,3-*Oxidosqualene cyclase*

The first committed step to a sterol-ring structure is through the operation of 2,3-oxidosqualene cyclase. At this stage there is a clear bifurcation between photosynthetic and non-photosynthetic eukaryotes with cycloartenol (**7**) and lanosterol (**6**) the respective products of the reactions. Inhibitors of the enzyme have been designed which, after protonation, show similarities to the high energy C-2 carbocationic intermediates or the transition-state analogues [9, 10]. 2-Aza-2,3-dihydrosqualene (**12**) and its *N*-oxide were shown [86] to be active inhibitors of the microsomal enzyme from pea seedlings (I_{50} 1.3 and 0.3 μM, respectively), but less so for the equivalent enzyme preparation from *S. cerevisiae* (I_{50} 10 and 16 μM). However, compound U18666A (**13**) was very active against the enzymes from both pea (I_{50} 0.2 μM) and yeast (I_{50} 0.1 μM) sources. Included in the same study [86] was 2,3-iminosqualene (**14**, I_{50} 0.2 μM pea enzyme, 10 μM yeast enzyme), a compound used to block sterol biosynthesis in *S. cerevisiae* [44] and *Gibberella fujikuroi* [48] in a study of the sterol requirements of these organisms.

Application of 2-aza-2,3-dihydrosqualene (**12**) to bramble cell suspension cultures inhibited 4-desmethylsterol biosynthesis as measured by incorporation of [2-^{14}C]acetate (IC_{50} 7.3 μM) [87]. This correlated well with the observed reduction of cell growth (IC_{50} 11 μM) strongly suggesting a causal relationship [87].

The plant growth retardant AMO-1618 applied to tobacco seedlings was shown to inhibit the incorporation of [2-^{14}C]MVA into 4-desmethylsterols and to cause the accumulation of 2,3-oxidosqualene [88, 89]. However, it showed no activity against the squalene cyclase from pea microsomes [86].

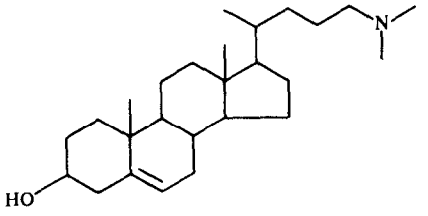
C-24 and C-28 Sterol methyltransferases (Fig. 1, reactions A and F)

The sterol C-24 methyltransferases catalyse the insertion of a methyl group into a Δ^{24}-acceptor sterol. It has been shown that the plant substrate is cycloartenol [14], in most fungi it is lanosterol [74], while in yeast the best substrate is zymosterol [74]. In plants further methylation of 24-methylenelophenol (**15**) occurs to afford the characteristic C-24 ethyl phytosterols [14]. In all cases the donor of the methyl group is *S*-adenosyl-L-methionine.

Inhibitors of the enzymes have been designed and synthesized rationally using the concept of mimicing the HEIs involved in the reactions [9, 10]. The most efficient inhibitors were found to possess a sterol-type structure with a heteroatom, usually nitrogen but sometimes sulphur or arsenic, at C-25 in the sterol side-chain [10].

Effective inhibitors in fungi include 25-azacholesterol (**16**) which as the hydrochloride salt was found to have an I_{50} of 0.05 μM in a *S. cerevisiae* assay [90]. Another HEI-type inhibitor, 24-epiiminolanosterol (**17**), has been shown to strongly inhibit mycelial growth and 24β-methylsterol production in *Gibberella fujikuroi* [91]. However, in the oomycete *Saprolegnia ferax*, which normally produces a mixture of Δ^5-24-desalkyl and 24-alkylidene sterols, administration of 10 ppm 25-azacholesterol did not inhibit mycelial growth even though sterol C-24 methyltransferase activity was blocked [92]. Oosporogenesis, however, was completely prevented suggesting a possible interference with putative sex hormone biosynthesis.

Using microsomal preparations from maize seedlings, 25-azacycloartenol (**18**) blocked *S*-adenosyl-L-methionine-sterol-C-24-methyltransferase and *S*-adenosyl-L-



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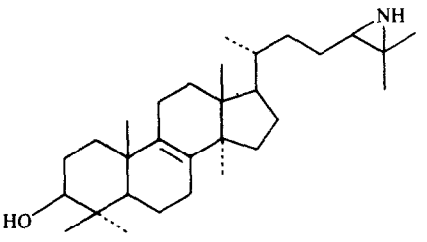

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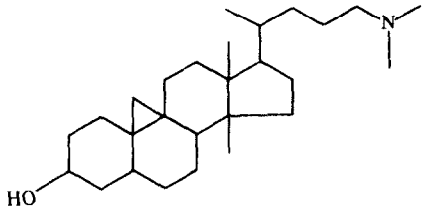

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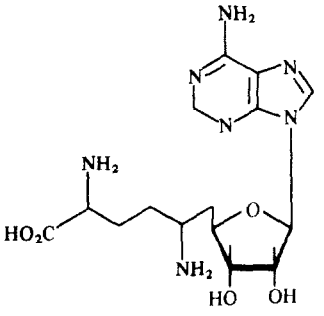

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methionine-sterol-C-28-methyltransferase with K_is of 30 and 100 nM, respectively [10]. When this compound was applied *in vivo* to bramble cell cultures at 1 μM, a dramatic reduction of C_{10}-side chain sterols (83% in control, 9% in treated cells) and an increase in C_8-side chain sterols (1% in control, 53% in treated cells) was demonstrated [93]. In this experiment cell culture growth was severely inhibited at 1 μM and higher concentrations stopped growth completely.

Suspension cultures of *Helianthus annuus*, supplied with increasing concentrations (0–2.5 ppm) of 24-epiiminolanosterol (**17**) showed a diminution of 24-alkylsterols and an accumulation of cycloartenol. This was followed by a progressive inhibition of growth [94]. Thus in both plants and fungi the use of azasteroid inhibitors has provided powerful evidence for the necessity of C-24 alkylsterols to ensure optimum growth.

Sinefungin (**19**), an antibiotic analogue of *S*-adenosyl-L-methionine, blocked the C-24 sterol methylation in yeast but, as growth was inhibited at lower concentrations than those required for sterol inhibition, it was inferred that inhibition of other essential methylation sites might be involved [2].

The norbornenodiazetine PGR tetcyclacis, when applied to oat, produced a large increase in the shoot cholesterol content at the expense of other phytosterols [95]. This could be interpreted as the result of an interference with C-24 methylation but as tetcyclacis did not produce a similar response in maize [96] or rye [Cooke, D. T. and Burden, R. S., unpublished results], and furthermore did not inhibit C-24-sterol methyltransferase *in vitro* [97], the mechanism involved is likely to be indirect and may be species specific.

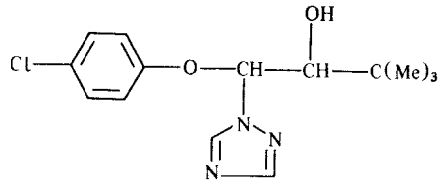
14α-*Methylsterol demethylase* (*Fig.* 1, *reaction C*)

Substrates for the sterol 14α-demethylation reaction in many fungi and plants are 24-methylenedihydrolanosterol (**20**) and obtusifoliol (**5**), respectively (Fig. 1). In *Saccharomyces* species, because the C-24-transmethylation reaction takes place later, the normal substrate is lanosterol. In all cases the reaction is mediated by a specific form of cytochrome P-450 dependent monooxygenase [14, 74]. Inhibitors of the fungal enzyme include the DMI group of agricultural fungicides and medical antimycotics and the large number of reviews in which they are discussed [2, 3, 12, 98–104] is commensurate with their economic importance. Notable examples of the fungicides are the triazoles triadimenol (**21**), diniconazole (**22**), propiconazole (**23**) and fenetrazole (**24**); the pyrimidines fenarimol (**25**) and nuarimol (**26**); and the imidazoles imazalil (**27**) and prochloraz (**28**).

DMIs are believed to operate through the binding of a heterocyclic nitrogen atom in the fungicide to the prohaem iron of the enzyme and the resulting inhibition leads to a depletion of terminal sterols and an accumulation of sterols containing 14α-methyl groups. These normally comprise 24-methylenedihydrolanosterol and the sterols obtusifoliol and 14α-methylfecosterol; subsequently formed by further removal of the C-4 methyl groups, but other 14α-methylsterols have been isolated following DMI treatment, depending mainly on the fungal species [3, 103, 104]. Because of the protrusion of the axial 14α-methyl group from the α-face of these sterols, it has been suggested, with supporting evidence [60], that such sterols are structurally ill-equipped to stabilize membrane phospholipids and that this is the underlying reason for the success of the DMI fungicides [103, 104]. The Pythiaceae, lacking sterols in their membranes, are generally not affected by DMIs except at high dosages [100, 102]. Evidence of hyperfluid and leaky membranes resulting from DMI treatment has been provided [105, 106]. Nonetheless, there are examples where the replacement of normal terminal sterols by 14α-methylsterols does not seem to correspond with growth inhibition [107, 108] and for this and other reasons, alternative explanations for the fungitoxicity have been proposed. These include direct insertion of the fungicide into the membrane [100], effects on membrane desaturases [103], the build-up of free fatty acids [103] and interference with the performance of membrane-based enzymes including chitin synthetase [109]. This last proposed mode of action may explain many of the observed morphological effects which include cell wall thickening, incomplete septum formation, excessive branching and an irregular deposition of chitin [3, 97, 102, 103, 109]. Interference with the formation or function of sterols engaged in a 'hormone-type' role is also a distinct possibility [50, 110].

In vitro studies on 14α-methylsterol demethylase have been largely confined to *Saccharomyces* and *Candida* species, the lack of reports of comparable studies with phytopathogenic fungi probably reflecting the unstable nature of the membrane-bound enzymes rather than lack of interest or effort! Experiments with microsomal [109, 111] and, more recently, reconstituted [112, 113] enzyme preparations have demonstrated that the inhibitor binds to the enzyme at two sites—one involving the iron atom and a second at a nearby lipophilic site. In contrast to initial reports [114], the group led by Yoshida and Aoyama have now provided evidence in yeast that not only the first but all three oxygen-requiring stages in the removal of the methyl group are catalysed by a cytochrome P-450 dependent enzyme which is, therefore, a true lanosterol C-14/C-32 lyase [115]. However, no C-32 oxygenated intermediates have yet been reported in DMI-treated fungi. Computer modelling has been employed in an attempt to rationalise and predict azole structures which will fit on to the active enzyme site [116].

Despite these studies it is still puzzling how such a large range of widely differing chemical structures can effectively fit the topography of an enzyme which can, however, be extremely sensitive to small changes in fungicide stereochemistry. Thus, the enantiomers of triadimenol (**21**) show a rapidly diminishing fungitoxicity towards many pathogens in the order (1*S*,2*R*) (most active) > (1*R*, 2*R*) > (1*R*,2*S*) > (1*S*,2*S*) [117] and in vinyl azoles such as diniconazole (**22**) [118] and triapenthenol (**29**) [119] the (*R*,*Z*)-isomer is much more fungitoxic than the (*S*,*Z*)-isomer. The very marked differences which can occur in the sensitivity of various fungi towards the different DMIs [100, 101] is largely unexplained and the difficulties of working with 14α-demethylases in cell-free systems has clearly hampered direct comparison between the enzymes of different species. It is possible that differences in fungicide uptake, distribution and metabolism may contribute to the varying selectivity observed and, in some cases at least, lack of sensitivity to DMIs may be attributed to a low sterol requirement or to a capacity to tolerate 14α-methylsterols [108]. These considerations



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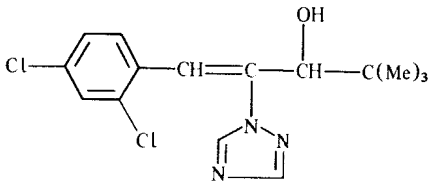

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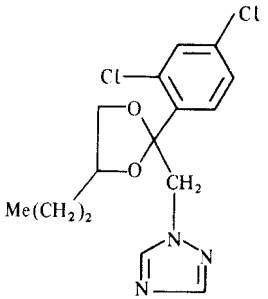

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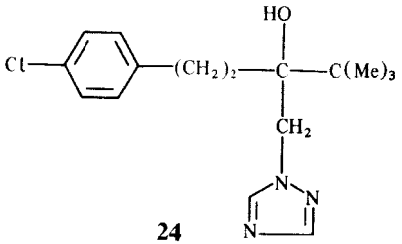

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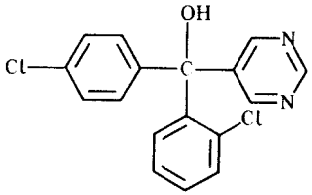

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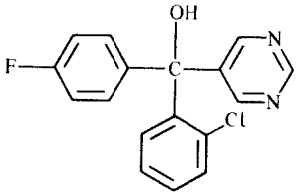

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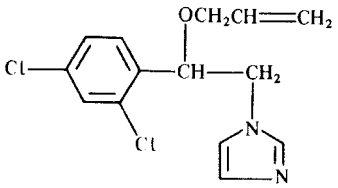

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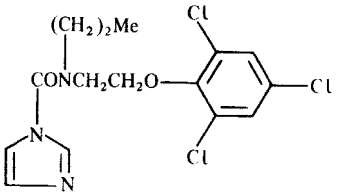

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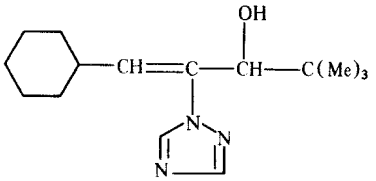

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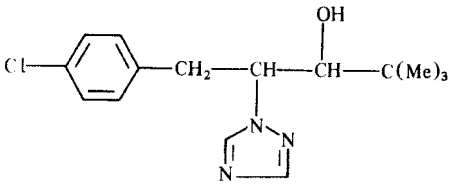

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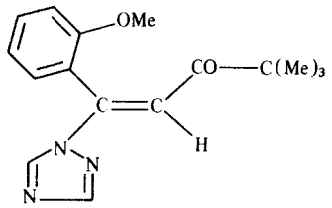

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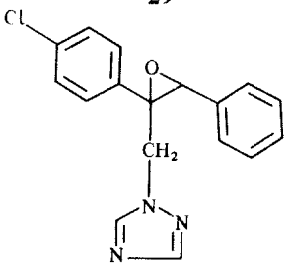

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may be very important with regard to the increasing problem of resistance to DMIs [120].

Triazoles, imidazoles, pyrimidines and pyridines of the type used as DMI fungicides can retard plant growth [100, 102] and some analogues such as paclobutrazol (**30**) and triapenthenol (**29**) are now marketed or are being developed as plant growth regulators [5, 119, 121]. Initial work [122] suggested that the principal reason for the growth retardation is unconnected with plant sterol 14α-demethylation but is in fact due to inhibition of another cytochrome P-450 dependent biochemical site—that involved in the oxidation of *ent*-kaurene to *ent*-kaurenoic acid in the gibberellic acid (GA) biosynthetic pathway. However, further studies indicated that DMI fungicides such as triadimenol (**21**), nuarimol (**26**) and triarimol (**25**) can inhibit plant sterol biosynthesis in both whole plants [123, 124] and cell cultures [125, 126] but in general, high concentrations were found necessary to achieve this effect.

The separation of some chiral azole fungicides and plant growth regulators into their constituent enantiomers [117, 119, 121, 127] has provided useful probes for assessing the relative importance of sterols and GAs to plant growth. With triadimenol (**21**), the extent of shoot inhibition observed in etiolated wheat seedlings after enantiomer application paralleled the capacity of the enantiomers to inhibit sterol biosynthesis in the order (1*S*, 2*R*)>(1*R*, 2*S*)>(1*S*, 2*S*)>(1*R*, 2*R*) [128]. The growth inhibition was not relieved by GA indicating that, at least under the conditions employed, triadimenol was retarding growth through an inhibition of sterols rather than GA biosynthesis. However, (1*R*,2*S*)-triadimenol has subsequently been shown [127] to be a relatively potent inhibitor of *ent*-kaurene oxidase from pumpkin endosperm (I_{50} 0.17 μM) and hence some inhibition of growth might be expected to result from GA inhibition by this enantiomer.

The (2*S*,3*S*)-enantiomer of the PGR paclobutrazol was far more effective than the (2*R*,3*R*)-enantiomer in reducing the growth of pot-grown apple seedlings [121] and light grown wheat shoots [Lenton, J. R. personal communication]. This correlated well with the performance of these enantiomers in a pumpkin endosperm *ent*-kaurene oxidase assay (I_{50} 0.023 μM and 1.6 μM respectively) [127] and hence suggested that a reduction in GAs was responsible for the growth inhibition. However, paclobutrazol also inhibited growth of celery cell suspension cultures with an I_{50} of *ca* 10 μM [16, 50] and here studies with separated enantiomers revealed a different pattern [129]. The (2*R*,3*R*)- and (2*R*,3*S*)-forms were potent inhibitors of both growth and sterol biosynthesis while the (2*S*,3*S*)- and (2*S*,3*R*)- enantiomers were only effective at much higher dosages. Therefore, it appears that in cell cultures, which grow mainly by cell division, inhibition of sterol biosynthesis is the determinant for growth inhibition.

With the PGR triapenthenol (**29**), again a distinction could be made between anti-GA [(*S*,*Z*)-enantiomer] and anti-sterol [(*R*,*Z*)-enantiomer] effects [119]. In greenhouse-grown rape plants (*S*,*Z*)-triapenthenol was found to be responsible for the reduced elongation of the internodes [119]. Thus, it seems clear that the nature of the growth response in azole-treated plants will depend on the inherent capacity of the compound to inhibit GA or sterol biosynthesis, the concentration applied, and the relative dependence of the tissues on GAs and sterols for growth under the prevailing conditions [129].

The (*R*,*Z*)-enantiomer of triapenthenol (**29**) inhibited both fungal and phytosterol biosynthesis [119]. It reduced root growth, caused a complete cessation of new leaf formation and induced necrosis when applied at 10 × the normal field rate [130]. This result implies that a reduction in normal phytosterols and/or an accumulation of 14α-methylsterols can lead to phytotoxicity. This view was strengthened by the demonstration that the experimental ICI γ-keto triazole herbicide (**31**) applied to barley and oat seedlings at 10, 1 and 0.1 μM was very effective in inducing accumulation of 14α-methylsterols (obtusifoliol, dihydroobtusifoliol and 14α-methyl-Δ^8-ergostenol in barley; 14α-methyl-Δ^8-cholestenol in oat) at the expense of the normal phytosterols [131]. This effect was correlated with shoot inhibition at all dosages and eventual death of the plants at the higher rates. It is, therefore, tempting to conclude that the herbicide inhibits plant growth, sometimes lethally, through a mechanism analogous to that shown in fungi by the DMI fungicides. The γ-keto triazole showed no *ent*-kaurene oxidase inhibition when tested in cell free preparations at concentrations up to 10 μM (Hedden, P., personal communication), and inhibited neither growth nor sterol biosynthesis in the fungus *Cladosporium cucumerinum* (Carter, G. A. and Burden, R. S., unpublished results).

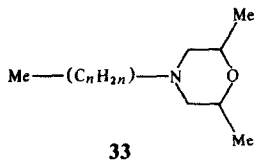
A logical explanation of the above observations is that there are slightly different forms of cytochrome P-450 involved in fungal sterol demethylation, plant sterol demethylation and plant *ent*-kaurene oxidation, and that they may vary in their capabilities to bind to different azole-type inhibitors. A microsomal obtusifoliol 14α-methyl demethylase assay has now been described [97, 132] with I_{50} values for different triazoles in the range of 0.04–10 μM. The most active compound in the assay was the experimental fungicide LAB 170250F (**32**) and the *in vitro* performance paralleled its capability to induce 14α-methylsterols in intact maize seedlings [97].

It cannot be discounted that in some cases the effects of azoles on plant growth may arise from interference with brassinolide biosynthesis. This could occur at the 14α-demethylation stage or else later in the pathway at some other putative cytochrome P-450 dependent step.

Cycloeucalenol—obtusifoliol isomerase (COI), Δ^{14}-sterol reductase and $\Delta^8 \rightarrow \Delta^7$ sterol isomerase (Fig. 1, reactions B, D and E)

These enzymes although operating at different stages in the sterol biosynthetic pathway (Fig. 1) catalyse reactions which proceed through a similar mechanism. The first step in each case [14] consists of the addition of a proton to the sterol substrate leading to the formation of a carbonium ion HEI with the positive charge sited at C-9 for the COI, C-14 for the reductase and C-8 for the $\Delta^8 \rightarrow \Delta^7$ isomerase. These carbonium ion sites are spatially close in the sterol skeleton, thereby explaining why a given inhibitor may be effective against more than one of these enzymes.

Known inhibitors include the *N*-alkylated morpholines, tridemorph (**33**) and fenpropimorph (**34**), and the *N*-alkylated piperidine, fenpropidin (**35**), all of which are important agricultural fungicides [98–101, 133, 134].



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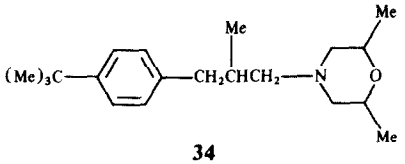

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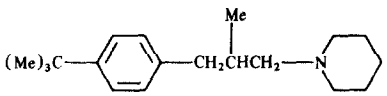

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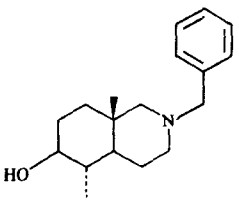

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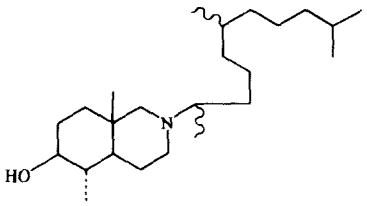

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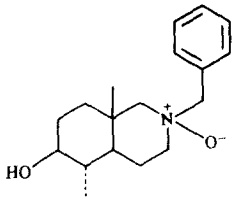

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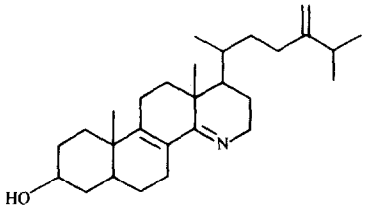

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These compounds which are likely to be protonated at physiological pH have some structural and electronic similarity to the sterol carbocationic HEIs [135]. It has now been established, from an analysis of sterols of fungi grown in culture, that in a wide range of species including *Botrytis cinerea*, *Ustilago maydis*, *Saccharomyces cerevisiae*, *Pencillium expansum* and *Pyricularia oryzae* tridemorph is mostly effective in the inhibition of the Δ^8–Δ^7 isomerase [13, 134]. By contrast, fenpropidin applied to a similar range of fungi inhibited the Δ^{14}-reductase more effectively while fenpropimorph appeared able to inhibit both enzymes [13, 134].

The above conclusions have been fully substantiated by *in vitro* studies using microsomal preparations. Thus, an enzyme preparation from yeast using [^{14}C]ergosta-8,14,24(28)-trien-3β-ol as substrate for the Δ^{14}-reductase reaction gave I_{50} values of 98.0, 2.3 and 1.8 μM for tridemorph, fenpropimorph and fenpropidin, respectively [136]. However, assay of the $\Delta^8 \rightarrow \Delta^7$-isomerase using a powdered acetone extract from a yeast cell homogenate with fecosterol as the substrate gave I_{50} values of 0.033 μM for tridemorph, 0.013 μM for fenpropimorph and 0.30 mM for fenpropidin [136].

The morpholine and piperidine compounds are powerful and effective systemic fungicides with particularly good activity against powdery mildews and an absence of resistance problems [133]. As with the DMI fungicides the precise cause of the growth inhibition is unclear, but it has been suggested [137] that the Δ^8- and particularly the $\Delta^{8,14}$-sterols cannot effectively substitute for ergosterol or an equivalent terminal sterol in fungal membranes. Morphological effects observed with the morpholines include swollen, branched or distorted conidia, incomplete septum formation and an irregular deposition of chitin [134].

The effect of the morpholine fungicides on plant sterols has been the subject of extensive studies by Benveniste and his group [14, 135, 138–141]. Both tridemorph [138] and fenpropimorph [139] applied to maize seedlings at 5 ppm induced the formation in the leaves and shoots of 9β,19-cyclopropylsterols, particularly cycloeucalenol (**36**) and 24-methylpollinastanol at the expense of the normal Δ^5-phytosterols. However, at lower concentrations, Δ^8-sterols were detected in addition to the cyclopropylsterols. A similar replacement of normal phytosterols by cyclopropylsterols was noted in barley shoots following treatment with fenpropimorph and tridemorph [124] and in cell cultures treated with tridemorph [126, 140].

A microsomal preparation prepared from maize embryos converted cycloeucalenol (**36**) to obtusifoliol (**5**) [135]. From the inhibition curves obtained with tridemorph and fenpropimorph it was concluded that both inhibitors had an I_{50} for the COI of 0.4 μM and that the inhibition was non-competitive. I_{50} values of 0.4 μM and 0.6 μM were reported for fenpropimorph and tridemorph respectively in a similar microsomal assay for the $\Delta^8 \rightarrow \Delta^7$-isomerase [141]. Thus, the morpholines had comparable activity against both COI and the isomerase *in vitro*, although a notable difference was that the I_{50}s for the $\Delta^8 \rightarrow \Delta^7$-isomerase were strongly pH dependent, while those for the COI were not [141]. The (*S*)-enantiomer of fenpropimorph, which displays the greater fungicidal activity, was also shown to be more effective than the (*R*)-enantiomer for the inhibition of COI *in vitro* [135].

Some 8-azadecalins have proved to be even more effective than the morpholine fungicides as inhibitors of the plant enzymes. A particularly potent compound was *N*-benzyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol (**37**) [142] which had I_{50} values of 0.1 and 0.13 μM for the COI and $\Delta^8 \rightarrow \Delta^7$-isomerase, respectively [142]. A very

potent inhibitor of the COI was *N*-(1,5,9-trimethyldecyl)-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol (**38**) which, when applied at 1 ppm, led to the virtually complete replacement of Δ^5 by cyclopropylsterols in bramble cells and maize roots [143].

In contrast to their performance in fungi, tridemorph and fenpropimorph do not appear to inhibit the Δ^{14}-sterol reductase in plants [135]. However, in bramble cells the *N*-oxide (**39**) appeared to be a good inhibitor of the plant sterol Δ^{14}-reductase as $\Delta^{8,14}$-4-desmethyl sterols accumulated following its application [143].

An important observation of the Benveniste group is the general lack of phytotoxicity shown after application of these inhibitors of COI, Δ^{14}-reductase and $\Delta^8 \rightarrow \Delta^7$-isomerase to plants [138, 139, 141, 143]. Such phytotoxicity which has been observed following morpholine treatment [133] appears, at least from results with wheat [144], to arise from action at sites unconnected with sterol biosynthesis. Thus, both whole plants and tissue cultures appear capable of good growth, even though their normal Δ^5-phytosterols have been almost totally replaced by cyclopropyl and/or Δ^8-sterols. These sterols which are incorporated into cellular membranes [139], may, therefore, largely fulfil the function ascribed to the usual phytosterols. There is some experimental and theoretical explanation for this—cycloartenol was found to be considerably more efficient than lanosterol in stabilizing membranes, an observation rationalized by suggesting that the axial 14α-methyl group is shielded within a 'bent' structure [60]. However, the 4-desmethyl-Δ^8-sterols which accumulate in plants following morpholine treatment, often at lower dosages than those required to produce cyclopropylsterols [138, 139], seem very comparable in structure to those found in morpholine-treated fungi. Thus, the tolerance of plants to the abnormal sterols induced by morpholines should re-focus attention on the precise cause of the toxicity of morpholines to fungi.

A potentially useful consequence of the tolerance of plants to morpholines was demonstrated when adult females of the insect *Locusta migratoria*, reared on wheat seedlings containing cyclopropylsterols as a result of fenpropimorph treatment, laid eggs with the ecdysteroid levels reduced by up to 80% [145]. It was concluded that the cyclopropylsterols could not replace the Δ^5-phytosterols as dietary precursors of the ecdysone insect moulting hormone [145].

The antibiotic 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**40**) is a potent non-competitive inhibitor of the Δ^{14}-reductase in yeast with a K_i of 2 nM [146]. At 5 ppm it was highly toxic to wild-type yeast but not to a strain doubly defective in genes for the removal of the 14α-methyl group and for the introduction of the Δ^5-double bond, or to a sterol auxotrophic strain growing on an exogenous supply of sterol [147]. This is compelling evidence that the fungitoxicity of this azasterol is a result of the inhibition of ergosterol biosynthesis, with the concomitant accumulation of the $\Delta^{8,14}$-sterol ignosterol [147].

In plants 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**40**) was reported as a good growth inhibitor in the radish root test system [85]. Bramble cells cultured on 5 ppm of the azasterol had their Δ^5-phytosterols almost entirely replaced by $\Delta^{8,14}$-sterols, but the growth continued although slower than in untreated controls [148]. A number of hypocholesteremic compounds appear to inhibit the $\Delta^8 \rightarrow \Delta^7$-isomerase in fungi as reported in previous reviews [3, 100].

Other enzymes

The reduction of the $\Delta^{24(28)}$-bond and the formation of the Δ^{22}-double bond occur late in the biosynthetic pathways (Fig. 1). Current evidence suggests that the phytosterol stigmasterol (**3**) is more likely to arise from a common intermediate such as isofucosterol (**41**) than from sitosterol (**2**) [149].

23-Azacholesterol at 1 μM inhibited the 24-methylene-sterol $\Delta^{24(28)}$-reductase in *S. cerevisiae* with some inhibition of growth [3, 150]. Evidence from the structures of sterols accumulating in plant and fungal tissues [3, 99, 129] suggests that both the reductase and the desaturase can be inhibited by DMI-type fungicides and PGRs. However, these effects are generally regarded as secondary or side effects of this type of inhibitor. The formation of the Δ^{22}-double bond appears to be cytochrome P-450 dependent [151] but the form of the cytochrome P-450 involved is likely to be different from that responsible for the 14α-demethylation because Δ^{22}-sterol defficient mutants of fungi are known to produce 14α-demethylated sterols [3].

Because of their late involvement in the biosynthetic pathway, the $\Delta^{24\ (28)}$-reductase and Δ^{22}-desaturase are not regarded as prime targets for growth inhibitors. More promising are the enzymes involved in the removal of the C-4 methyl groups, for work with the yeast mutant GL7 showed that 4-desmethyl sterols are obligatory for growth in this organism [42]. The enzymes involved in the removal of the C-4 methyl groups require molecular oxygen but are not cytochrome P-450 dependent [14, 74]. No specific inhibitors have yet been discovered.

Inhibition of a putative cytochrome P-450 dependent Δ^7-desaturase, present in *Pyricularia oryzae* and other fungi, has been proposed as a secondary mode of action for fenarimol (**25**) [80] and the new Bayer DMI fenetrazole (**24**) [152]. However, the evidence for this was based only on the accumulation of Δ^5, C-24 alkylated sterols and other explanations of the data are possible. The numerous hydroxylation stages which must be involved in brassinolide biosynthesis are potential sites for cytochrome P-450 type inhibitors.

SUMMARY

The work reviewed here, much of it done within the last few years, points unmistakably to a fundamental role for sterols in promoting growth and development in both plants and fungi.

A clear target enzyme in the biosynthetic pathway for growth inhibition in both plants and fungi is the 14α-methylsterol demethylase. Work with triazoles, particularly with closely related analogues and separated enantiomers, has provided an insight into the affinity of these inhibitors for the different forms of cytochrome P-450 which catalyse fungal sterol 14α-demethylation, plant sterol 14α-demethylation and plant *ent*-kaurene oxidation. It may become increasingly necessary to evaluate the relative contribution of these processes to optimise performance of fungicides, herbicides and PGRs, both with regard to their activity at the primary site of action and with a view to controlling side effects. There is clear

scope here for extending and improving *in vitro* enzyme assay methods.

The effectiveness of the morpholines as agricultural fungicides contrasts with the high tolerance of plants to cyclopropyl and Δ^8-sterols which are readily induced by application of these compounds. Further study of the basic reason for morpholine toxicity is, therefore, an area well worthy of further investigation.

Inhibition of squalene epoxidase by allylamines has proved successful in the development of medical antimycotics but no agricultural fungicides, based on this mode of action, have been forthcoming. Other enzymes of the pathway have yet to be commercially exploited but the C-24 alkylation process seems clearly linked to growth in both plants and fungi and hence would appear to be a particularly promising site for new inhibitors. The pre-squalene enzyme HMG-CoA reductase seems to offer potential for regulating or stopping plant growth.

Above all there is a need to evaluate precisely how sterols and their derivatives control growth and developmental processes. The judicious use of inhibitors of the type described here may help in this undertaking which, in turn, could generate new ideas for the design and discovery of more active and specific compounds.

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