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Ergosterol biosynthesis inhibition: a target for antifungal agents

Keith Barrett-Bee and Graham Dixon

Infection Research Department, ZENECA Pharmaceuticals, Alderley Park, Macclesfield, Cheshire SK10 4TG, United Kingdom

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The isoprenoid sterols play a crucial role in the viability of all fungi; those unable to synthesise ergosterol because of inhibition, growth conditions or mutation must take it up from the environment. A range of compound types have been discovered which interfere with the biosynthetic pathway from acetate to ergosterol and these compounds have antifungal actions. Inhibition of several of the steps has yielded agents which have been used with great success as medical and agrochemical agents. The most important biosynthetic steps that have been exploited are inhibition of squalene epoxidase, (the allylamines and tolnaftate) C14 demethylation (the azoles), Δ7,8 isomerase and Δ14 reductase which are inhibited by the morpholines. Recent research has shown that inhibition of C24 methyltransferase and C4 demethylation also yield antifungal agents. Combination studies demonstrate that synergy between agents of different types can be measured. Fungicidal effects were observed when a combination of two fungistatic agents was used.

The major product of sterol biosynthesis in fungi and some trypanosomes is ergosterol, unlike mammalian systems which synthesise cholesterol as a major membrane lipid [1, 2]. The two sterols differ in a few minor ways: cholesterol has a second double bond (Δ5-6) in the B ring (see Fig. 1) and has a fully saturated side chain without a methyl group at C24. These small differences are clearly very important as ergosterol has been shown to be essential for the aerobic growth of most fungi. This requirement is demonstrated by the sparking phenomenon discussed by Nes *et al.* [3] who described the essential structural parts of the sterol molecule needed for growth. In a series of experiments, the groups of Nes and Parks supplied anaerobicaly grown *Saccharomyces cerevisiae* with different types of sterols to "spark" growth [3, 4]. The degree of growth was assessed and the structures of the sterols were compared. The following parts of the sterol molecule were found to be important (Fig. 1). In the tetracyclic nucleus, the 3β-OH was obligatory for growth, whereas the presence of methyl groups at C14 or C4 did not allow growth. There appeared to be little advantage in the Δ5-7 conjugated diene over the Δ5 olefin or even fully saturated ring sterols. In the side chain, the β-methyl at position 24 was obliga-

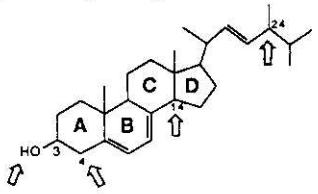

Fig. 1. The structure of ergosterol.
The arrows indicate the parts of the molecule which have been shown to be essential for the growth of fungi.

Abbreviations: APB, 6-amino-2-n-pentylthiobenzothiazole; GC-MS, gas chromatography-mass spectrometry method; MIC, minimum inhibitory concentration; SAM, *S*-adenosyl methionine.

tory for growth, with better growth observed with 24 methyl Δ22 side chains. A *trans* double bond at 22 was essential since *cis* isomers did not support growth. Clearly, if these features that are essential for growth could be exploited as antifungal drug targets, then it should be possible to obtain an effective antifungal agent. Different functions for ergosterol have been described by Parks *et al.* [5] together with the amounts of sterol required for growth, e.g. the sparking (1–10 ng/ml), critical domain (100 ng/ml), and domain (1 μg/ml) functions, whilst the bulk membrane function requires about 15 μg/ml ergosterol involving a quantitatively variable pool of free sterol [5]. The sparking effect has the most stringent requirements whilst the bulk domain has the least.

Many of the most successful antifungal agents available today in the medical and agrochemical fields interfere in some way with sterol biosynthesis or function. The polyenes are thought to act by complexing directly with membrane ergosterol and have been reviewed by Hamilton-Miller [6]. The most common group of compounds used as antifungal agents are the azoles which interfere at the stage of demethylation of the sterol nucleus [7]. The allylamine and thiocarbamate types of compounds interfere at the stage of squalene epoxidation [8]. However, there are potentially more antifungal targets within the sterol biosynthetic pathway.

Figure 2 shows the synthetic scheme for sterols with the possible interrelationships of the metabolic steps from lanosterol to ergosterol, together with the preferred route in *Candida albicans*. The sequence of these inter-conversions varies among fungi and the biosynthetic route differences may account for the claimed different modes of inhibition reported for some antifungal agents. The bars on these charts show the points of inhibition of known antifungal agents which we will discuss in relation to our own and published work.

Table 1 taken from Lees *et al.* [9] shows a summary of work carried out to clone genes from the sterol biosynthetic pathway in *S. cerevisiae*. The genes denoted *ERG* have been cloned for 9 of the steps, two notable exceptions being the genes for C4 demethylation and for Δ22 reduction. The table shows the essentiality of the gene in *S. cerevisiae* under normal growth conditions.

SQUALENE SYNTHASE

The biosynthesis of squalene from farnesyl pyrophosphate, catalysed by the enzyme squalene synthase, is the first committed step in sterol biosynthesis. The reaction proceeds in two distinct steps, both catalysed by squalene synthase [10]. The first step, requiring Mg^{2+} or Mn^{2+} is a 1′-2-3 prenyl transfer which results in the formation of the chiral intermediate presqualene pyrophosphate. The second step is the NADPH-dependent reductive rearrangement of the presqualene pyrophosphate to form squalene. The reactions catalysed by squalene synthase connect the cytoplasmic and microsomal segments of the sterol biosynthetic path-

Table 1
Cloned genes in the ergosterol biosynthesis pathway

Gene	Enzyme encoded	Essential?	Inhibitors
ERG 9	Squalene synthase	yes	squalestatin
ERG 1	Squalene epoxidase	yes	allylamines
ERG 7	Squalene cyclase	yes	U1866A
ERG 11	C14 Demethylase	yes*	azoles
ERG 24	Δ14 Reductase	yes	morpholines
ERG 6	C24 Methyltransferase	no	tomatidine
ERG 2	Δ7-8 Isomerase	no	morpholines
ERG 3	Δ5 Desaturase	no	
ERG 4	Δ24 Reductase	no	

*Suppressed by *ERG* 3

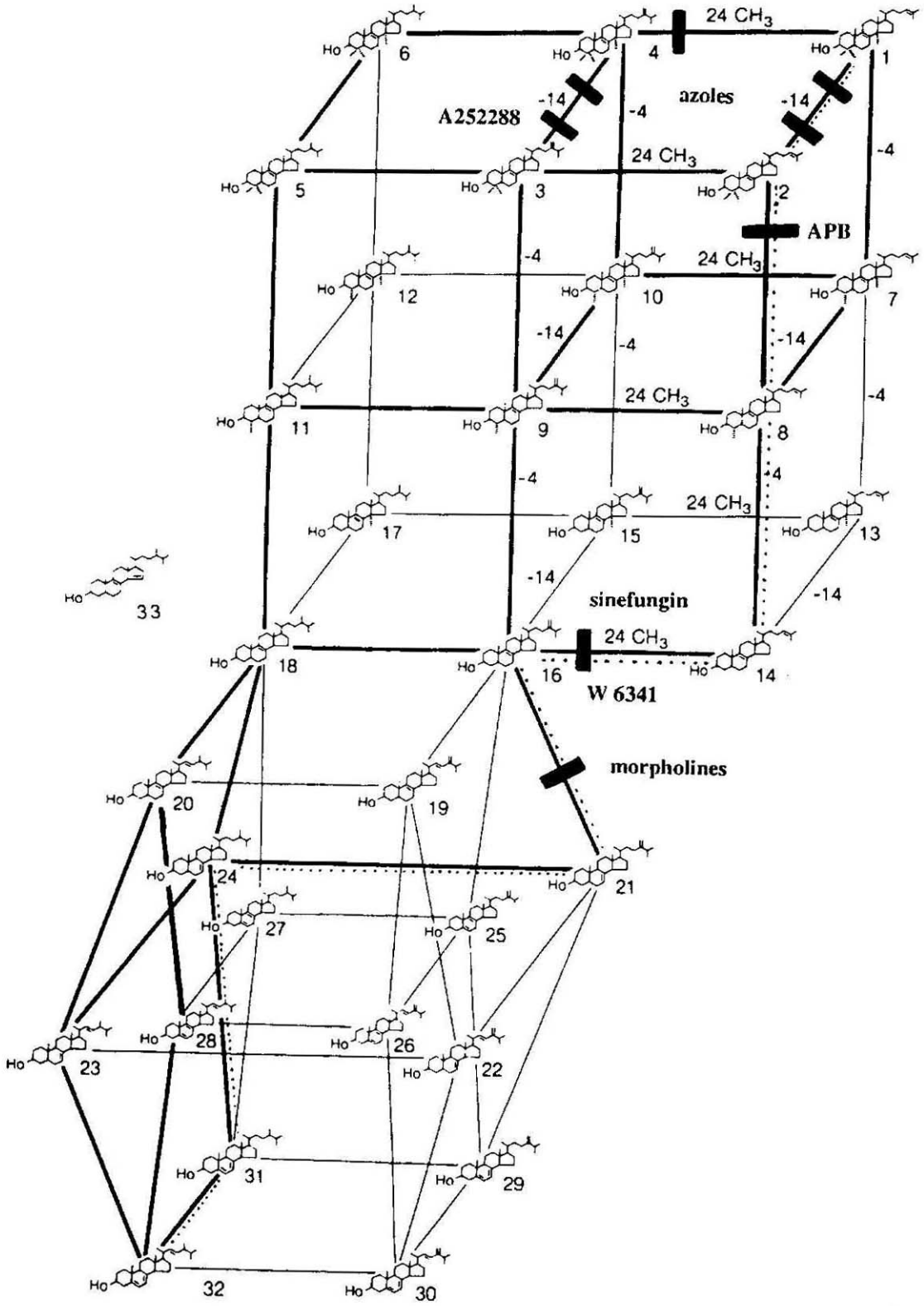

Fig. 2. A generalized biosynthetic pathway from lanosterol to ergosterol.
The figure shows the alternative pathways that exist. The darker lines represent the more preferred transformations, the dotted line is the main route followed in *C. albicans*. Transformations where antifungal agents are known to inhibit are shown by bars, these are explained in the text. -4, -14, 24, etc. are the chemical reactions, i.e. C4 demethylation, C14 demethylation, and C24 methyltransferase. The numbers represent individual sterols: 1, lanosterol; 4, 24-methylene lanosterol; 14, zymosterol; 16, fecosterol; 32, ergosterol; 33,: the product of Δ14 reductase inhibition ignosterol (modified from Pierce *et al.* 1977, *Can. J. Biochem.* **56**, 135–142.).

way. Several reaction mechanisms have been proposed which have been reviewed by Oehlschlager & Czyzewska [11].

Inhibition of squalene synthase has been achieved using substrate analogues and intermediate mimics [10, 12]. Many squalene synthase inhibitors have been patented recently, most of which are claimed to possess both antilipaemic and antifungal activity. The biphosphanates and quinuclidines among synthetic compounds and the squalestatins, zaragosic acids [13], nonadrides and viridiofungins among the natural products [14]. The most studied of these are the squalestatins (squalestatin 1 is the same as zaragosic acid A). These are potent competitive inhibitors of the fungal and mammalian squalene synthase, and are believed to mimic the reaction intermediate presqualene pyrophosphate [13]. Most of these compounds have good *in vitro* antifungal activity although data on their *in vivo* activity is sparse. Squalene synthase appears therefore to be a valid target and the recent cloning and expression of a truncated solubilised form of the fungal enzyme may result in major advances in this area in the near future. However, selectivity will remain a serious issue for antifungal drugs for use in both clinical and agrochemical areas. As shown in Table 1 the *ERG9* gene is essential, mutants being auxotrophic for ergosterol.

SQUALENE EPOXIDASE

Epoxidation at the 2,3-position is the first step in the conversion of the 30-carbon chain squalene to the tetracyclic sterol skeleton. This reaction is performed by a microsomal enzyme complex consisting of a flavoprotein with NAD(P)H cytochrome *c* reductase activity, and a terminal oxidase which is not of the cytochrome P-450 type. Squalene epoxidase is the first enzyme in the pathway to require molecular oxygen and is of particular interest in fungi as the step at which ergosterol biosynthesis is modulated by availability of oxygen. The enzymology of the fungal and mammalian epoxidases has recently been reviewed [15].

With the development of the allylamine antifungals over the last decade, squalene epoxidase has emerged as a major target enzyme for antifungals. Naftifine, the first of these synthetic compounds to be found, has activity against a wide range of pathogenic fungi [16] and is used clinically as a topical antifungal. Terbinafine is a more potent derivative of this class and shows both oral and topical efficacy in the clinic. Both naftifine and terbinafine are potent inhibitors of fungal ergosterol biosynthesis [17], acting by direct specific inhibition of squalene epoxidase [18]. This inhibition is reversible and non-competitive with respect to squalene, NAD(P)H and FAD. Although fungal and mammalian squalene epoxidases are quite similar in properties [14], the rat liver enzyme is several orders of magnitude less sensitive than the *C. albicans* enzyme to inhibition by allylamines [18, 19]. This has important implications for chemotherapeutic target enzymes in general, by demonstrating the possibility of obtaining a highly selective inhibitor for a fungal enzyme, without adverse effects on the equivalent mammalian enzyme.

It seems clear that squalene epoxidase inhibition provides the biochemical basis for the antifungal activity of the allylamines, since these two parameters correlate quite well with each other in different fungi [20, 21]. The primary fungicidal action and an important characteristic for the clinical efficacy of these compounds [22], is the intracellular accumulation of squalene resulting from epoxidase inhibition [21].

Subsequent to the discovery of the mechanism of action of the allylamines, an old class of antifungals, the thiocarbamates, was also found to act by inhibition of squalene epoxidase. This mechanism has now been demonstrated for the topical compounds tolnaftate [8, 22, 23], tolciclate [23] and piritetrate [24]. These compounds are primarily active against dermatophytes and have little effect on *C. albicans*, apparently due to poor penetration of the Candida cell envelope [8, 23]. As with the allylamines, the thiocarbamates are selective for the fungal rather than rat liver epoxidase [23].

A further approach to inhibition of squalene epoxidase is provided by modification of the substrate, squalene. One such compound, 2-aza-2,3-dihydrosqualene was originally designed as a cyclase inhibitor [24], but found also to inhibit squalene epoxidase [25]. It is, however, more effective against rat liver epoxidase than the fungal enzyme. A similar degree of activity against mammalian squalene epoxi-

dase has been reported for trisnorsqualene cyclopropylamine [26], and a number of other squalene derivatives with weaker epoxidase inhibitory activity have recently been described [27–29]. This approach seems unlikely to deliver compounds with a high degree of selectivity and therefore appears unpromising as a potential source of antifungal drugs.

2,3-OXIDOSQUALENE CYCLASE

2,3-Oxidosqualene is cyclised to lanosterol, the initial precursor of the vast array of steroid structures formed by fungi and mammals. This cyclisation, which has been described as the most complex enzyme-catalysed reaction known, is carried out by a single protein without requirement for any organic cofactors. The key position of the cyclase and the complexity of its action have encouraged a large amount of work on the mechanism of cyclisation and its inhibition. The structure and activity of various cyclase inhibitors have been reviewed by Cattel *et al.* [27]. Many of these are derived from structures related to squalene, such as 2,3-iminosqualene [30], reported to inhibit liver microsomal cyclase by 90% at 1.4 μM. Various azasqualene derivatives have been synthesised as mimics of a carbocationic intermediate of cyclisation [31]. These compounds inhibit cyclases from animals and higher plants [32] and also from fungi [33, 25]. Related structures such as *N,N*-diethylazasqualene were reported to inhibit ergosterol biosynthesis in yeast and to have antifungal activity *in vitro* [34], although at relatively high concentrations (10–100 μM). Further cyclase inhibitors described in the literature include 4,4,10β-trimethyl-*trans*-decal-3β-ol, and azadecaline derivatives which are postulated to act as analogues of a high-energy intermediate in the reaction [32]. The first cyclase inhibitor to be identified was 1-dodecylimidazole, which inhibited sterol biosynthesis in rat liver [35] and in the fungus, *Ustilago maydis* [36]. In contrast, Mercer *et al.* [37] reported that this compound and related heterocycles inhibited only the rat liver cyclase but had no effect on the enzyme from yeast. These authors suggest that the heterocycle blocks the active site, occupied by the incipient sterol ring A, while the side-chain binds to a nearby lipophilic site, which could vary between the fungal and mammalian enzymes, thus explaining the selective inhibition. Evidence obtained with the currently available inhibitors suggest that the cyclase may be a very promising target enzyme for development of novel antifungals. As in the case of squalene epoxidase, there appears to be potential for highly selective inhibition of the fungal enzyme.

Once lanosterol has been formed by the cyclisation of squalene epoxide, it undergoes several sequential transformations to form ergosterol. The exact route that the sterol skeleton follows depends on the availability of the substrate and the specific interaction between the individual enzyme and that substrate in a given fungus (Fig. 2). This is true for many of the steps, but some must always preceed others e.g., demethylation always occurs before the isomerisation of double bonds in the B ring of the nucleus. Thus different genera of fungi may follow different biosynthetic routes.

C14 DEMETHYLATION

One of the earliest steps in the pathway after the cyclisation to form the true sterols is the demethylation of the ring system at the C14 position. The demethylation step utilises a cytochrome P-450-containing monooxygenase enzyme [38]. Removal of the methyl group is a two-stage oxidative reaction. The first stage is a hydroxylation to the hydroxymethyl derivative followed by a second hydroxylation, leading to loss of a water molecule and the methyl group as formaldehyde which is then converted to formic acid. The loss of water produces a double bond by withdrawing a proton from the C15 carbon atom. The Δ14 double bond produced is subsequently reduced in a NADPH dependent reaction to give the demethylated sterol. The Δ14 sterol is a transient intermediate since it is rapidly reduced to the next intermediate in the pathway. The mechanism is shown in Fig. 6 of Oehlschleger & Czyzewska [11].

The major antifungal class, the azoles, interfere at the demethylation stage of the pathway. Some of these compounds are important clinical agents (miconazole, ketoconazole, fluconazole) and others as agrochemicals (propiconazole, tebuconazole, hexaconazole) [7].

The lone-pair electrons which exist on the azole nitrogen atoms of the antifungal azoles interact with the haem group of the cytochrome P-450 in a tight binding type II manner. This interaction is stabilised at the binding site by the hydrophobic parts of the molecule, preventing the oxidation of the methyl group and its subsequent removal. The inhibition has been shown to be non-competitive with regard to the substrate [39]. This type of inhibition is important because it leads to a greater reduction in flow through a metabolic pathway than does a competitive inhibitor. The reason for this is that the accumulation of intermediates will not compete for the binding site of the drug and reduce its inhibition of the reaction.

If fungi are grown for some time in the presence of azoles and the sterols extracted and examined by Gas Chromatography-Mass Spectrometry (GC-MS), there is an accumulation of trimethylated intermediates. In *Ustilago maydis* the precursor is 24-methylene lanosterol whilst in *C. albicans* it is lanosterol [40] (see also Fig. 2). The different intermediates that accumulate also demonstrate that different sterol biosynthetic pathways exist among fungi. Broken-cell systems utilising radiolabelled precursors have been used to demonstrate inhibition of demethylation. The fact that the fungus is unable to remove the methyl groups at the C4 position and to further metabolise lanosterol indicates the strict structural requirements of the C4 demethylase enzyme.

The trimethylated sterols that accumulate after inhibition by the azole antifungals are bulkier than, and cannot pack into the membranes as well as, ergosterol and it is believed that these membranes function less efficiently [41]. There is an interference with the function of membrane-bound enzymes. Vanden Bossche has suggested that one effect is the alteration of the activity of membrane-bound enzymes such as chitin synthase and the inappropriate synthesis of chitin [42]. This has been demonstrated in *C. albicans* incubated with ketoconazole and also in *U. maydis* incubated with imazalil [43].

It has also been shown that when yeasts are grown in the presence of azole antifungals there is an inhibition of the uptake of compounds such as amino acids and nucleotides [44]. This inhibition has been shown to be non-competitive with respect to the substrate, i.e. there is a reduction in flow through the permeation system rather than an interference with the affinity of the substrate for the receptor [40].

The mycelial form of *C. albicans* is very important in the pathogenic process [45] and this form is particularly susceptible to the azoles [46]. This increased susceptibility may be due to the difference in the cellular content of ergosterol, the mycelial form contains ten times more than the yeast form [47]. Possibly, if there is a greater requirement for ergosterol biosynthesis, then a smaller perturbation in its rate of synthesis would have a greater impact on the cell growth by disturbing the steady state. Also, the greater amounts of chitin and chitin synthase required in mycelia may be important if perturbation of enzymes such as chitin synthase are important. Germ-tube formation, the initial step in mycelial growth, is particularly susceptible to azoles; this stage of hyphal growth requires a highly co-ordinated biosynthetic switching and synthesis of chitin which may be very sensitive to membrane disturbance [48]. In addition, mycelial forms have also been shown to take up more radiolabelled azole than yeasts and this may be a factor in the increased susceptibility to azoles [47].

The extensive use of the azole antifungals both in the clinic and in agriculture has lead to the selection of resistant isolates of various fungi. The reason for the resistance has been determined and comes from three causes. The most common cause of resistance is the lack of accumulation of the compound, this has been demonstrated in *C. albicans, Drechslera halodes* and *Aspergillus nidulans* [49]. Unpublished studies have also shown that variation in pH of the medium alters the amount of drug accumulation and this parallels changes in sterols and antifungal sensitivity (Barrett-Bee, unpublished observations). Other causes of resistance have been changes in target sensitivity of the P450 enzyme [50] and mutations in *ERG3* of the Δ5 desaturase [51].

Although new azoles have been described recently with potential as medical and agricultural antifungal agents, there has been some interest in inhibitors of different types. Within the last few years the mode of action of the restricticins, compounds which exhibit antifungal activity against pathogenic fungi of medical importance, has been confirmed as inhibition of C14 demethylase [52]. This dis-

covery will surely provide the impetus to further exploit the target.

C4 DEMETHYLATION

C4 demethylase removes both of the C4 methyls in two steps [53]. The initial β-methyl group is epimerised during this process and the cycle repeats. Each demethylation step occurs by an oxidation of the methyl group to the corresponding keto group *via* the hydroxyl. The keto group is further oxidised to a carboxyl group. All these steps take place in the presence of molecular oxygen and pyridine nucleotides *via* the mixed-function oxidase. The subsequent decarboxylation is an anaerobic process, with CO_2 released and a 3-keto group formed. The latter group is reduced by 3-keto reductase which inverts the existing methyl group stereochemistry so that the cycle can repeat in an exactly analogous way. The overall reaction is sensitive to KCN but not to CO. Inhibitors of this pathway would be expected not to suffer from the potential toxicity seen with the C14 demethylase inhibitors which have the potential to inhibit mammalian cytochrome P-450 containing enzymes. It has been reported that the compound 6-amino-2-n-pentylthiobenzothiazole (APB) is an inhibitor of C4 demethylation in *S. cerevisiae*. GC-MS studies in *C. albicans* treated with the compound have suggested that the primary target is the C4 enzyme with some inhibition at C14 and squalene epoxidase. In *S. cerevisiae* the results suggested that the compound was more selective for the first demethylation step than the second in that some C4 methyl sterols were seen [54]. APB has been shown to have activity against model *C. albicans* infections in mice [55]. The potential importance of the C4-demethylase is underlined by the fact that no yeast mutant defective in this reaction step has been isolated to date.

Δ14 REDUCTASE

In the C14 demethylation step described above, the sterol goes through an intermediate state with a double bond at the C14 position; this olefin is then reduced to the C4 dimethyl derivative [56]. Antifungal compounds have been described which inhibit this reductase; i.e., inhibition of the growth of fungi will induce an accumulation of sterols possessing a Δ14 bond. Subsequent enzymes in the pathway, however, will not metabolise this unnatural sterol normally. It is metabolised in *C. albicans* by the C4 demethylase enzymes and Δ24 methyl transferase to form Δ14-fecosterol. Under normal conditions fecosterol would undergo ring isomerisation at the Δ7 position. However, this unnatural sterol is not able to be further metabolised by the Δ7-8 isomerase. The consequence is that the sterol acts as a substrate for the Δ22 reductase and is reduced to the unnatural saturated side chain sterol, ignosterol. This sterol (ignosterol, shown as no. 33 in Fig. 2) is a Δ14 derivative of sterol 18. Ignosterol is not able to be further metabolised and accumulates in the organism to become the predominant sterol. All fungi normally follow a sterol biosynthetic route that passes through fecosterol; hence, this type of inhibitor induces the accumulation of ignosterol in all species of fungi [57].

At higher concentrations of this type of inhibitor, the Δ22 reductase enzyme is also inhibited, and the triene, Δ14 fecosterol, is accumulated as well. An example of this type of inhibitor is the nitrogen-containing sterol A25822B [58], that is active against many species of fungi. The morpholine antifungal agent fenpropidine also acts exclusively at this stage in *C. albicans* [59]. In many species of fungi, the morpholine antifungal compounds act at this stage, but many of this class have multiple points of inhibition [57]. The morpholines are used extensively in agriculture but recently amorolfine has been introduced into the clinic as a topical agent for dermatophyte infections. The demonstration that the *ERG24* gene is essential for viability of *S. cerevisiae* indicates the importance of this enzymic step [60].

Δ7-8 ISOMERASE

Following the removal of the C4 and C14 methyl groups and the methenylation of the side chain, the next reaction in the sequence is the isomerisation of the B-ring double bonds of fecosterol (16 in Fig. 2). Δ7-8 Isomerisation has been shown to be a reversible reaction by tritium-transfer experiments and does not require

any cofactors such as NADH. The morpholine antifungal agents are able to inhibit this enzyme in plant pathogens and are used as crop protectants. Many of the compounds have a mixed mode of inhibition, also inhibiting the Δ14 reductase [57].

Tridemorph is claimed to be a selective Δ7-8 isomerase inhibitor in *U. maydis*, whereas fenpropimorph inhibits both Δ14 reduction and 7-8 isomerisation [61]. The anti-psychotic drug, trifluperidol, has been shown to inhibit production of Δ7 sterols in cultures of *S. cerevisiae* and to reduce fungal growth [62]. When *C. albicans* was grown in the presence of this agent there was no inhibition of growth despite an accumulation of Δ7 sterols shown by GC-MS [63]. This must mean that, in this species of fungus there is not as strict a structural requirement for the sterols in the cell membranes as in *S. cerevisiae*. GC-MS analysis of sterols from *C. albicans* grown in the presence of tridemorph indicated that the drug was a mixed inhibitor. Since A25822B has been shown to be fungitoxic, then either ignosterol (the product of inhibition of Δ14 reduction) is toxic *per se* or incapable of being metabolised by the Δ7-8 isomerase. If the 5,7 diene is not necessary for fungal growth as suggested by Nes *et al.* [3], then the Δ14 inhibition is the key step in fungitoxicity. Disruption of the *ERG2* gene has suggested that the 7-8 isomerase is non essential in *S. cerevisiae* supporting the idea that the Δ14 reductase is the important target for the morpholines at least in this organism [64].

C24 METHYLTRANSFERASE

One stage in the biosynthesis of ergosterol that does not exist in the biosynthesis of cholesterol is the addition of a methyl group at the C24 position in the sterol side chain. This occurs early in many fungi and before the C4 demethylation. However, in the yeasts *C. albicans*, and *S. cerevisiae* it occurs at the level of zymosterol (14 in Fig. 2) [40]. Inhibitors of this step would be selective and not expected to inhibit any mammalian enzymes and so there would be less potential for toxicity.

The cellular localisation and kinetic properties of the C24-sterol methyltransferase have been studied [65]. Mechanistically, the methyl donor for the 24-methenylation is *S*-adenosyl methionine (SAM). A positively charged sulphonium ion in SAM renders the methyl group electrophilic and susceptible to the nucleophilic Δ24 double bond. The ensuing reaction results in S-CH_3 cleavage, formation of a C24--CH_3 bond and generation of a transient carbonium ion at C25. A hydride shift followed by proton loss from the newly introduced methyl group gives rise to the new sterol, (fecosterol in *C. albicans* and *S. cerevisiae*, 24-methylene dihydrolanosterol in filamentous fungi).

High concentrations of *S*-adenosyl-homocysteine, the product of the reaction, inhibit 24-methenylation. Analogues of this compound have been sought and sinefungin was discovered; this natural product had been known to have antifungal properties for some time [66]. When its potency against the enzyme and growing cells is compared, however, it is a more potent antifungal than a 24-methenylation inhibitor. This result indicates that other fungal pathways are inhibited at lower concentrations of the agent. This compound also inhibits methylation reactions in mammalian systems that depend on SAM, leading to toxicity.

Some 25 years ago it was shown that a series of nitrogen-containing side chain analogues of cholesterol were able to inhibit the production of cholesterol leading to rises in desmosterol, consistent with inhibition of Δ24-28 reductase [67]. Later it was shown that the mechanism of 28-reduction in mammals and 24-transmethylation in fungi were very similar and involved attack of an electrophile on the electron-rich centre at C24 [68]. This result suggested to us that an alternative approach to analogues of SAM was to look for nitrogen-containing substrate-derived competitive inhibitors. We have studied the natural product tomatidine and natural and synthetic analogues as potential inhibitors of this enzyme reaction [63].

When *C. albicans* was grown in the presence of tomatidine and the sterols extracted and examined by GC-MS there was an accumulation of zymosterol (14 in Fig. 2). When a similar experiment was performed using the filamentous fungi, *Trichophyton quinckeanum*, or *Aspergillus fumigatus*, the product was lanosterol (1 in Fig. 2). This result indicates that these filamentous organisms will not demethylate lanosterol and the natural substrate for the C14-demethylase stage is 24-methylene dihydrolanosterol (4 in Fig. 2). This gives us information

on the biosynthetic pathway in this organism. This experiment also indicated that in *C. albicans*, the natural route for sterol biosynthesis is methenylation at the stage of zymosterol, the same is true for *S. cerevisiae*.

When a microsomal preparation of *C. albicans* or *A. fumigatus* was incubated with radiolabelled SAM, the methenylation of sterol pathway intermediates could be measured. Compounds were added to these systems and structure-activity relationships could be determined from relative inhibition potencies of the compounds. Since SAM is able to enter intact cells, a similar study could be performed and the comparative IC_{50} values give a measure of penetrability of the compounds. The substrate specificity of the two enzymes, i.e. zymosterol for *C. albicans* and lanosterol for *A. fumigatus*, confirmed the different biosynthetic pathways observed in the GC-MS studies with these two organisms.

A series of sterol-like and other known inhibitors were tested against enzymically active microsomal preparations of *C. albicans* and *A. fumigatus* and inhibition of C24-methyltransferase measured (Table 2). In general the Candida enzyme was far more sensitive to inhibition by these compounds than that of Aspergillus. The most potent compound against Aspergillus (ZM308973), which was equipotent against the Candida enzyme, was methylated at the C4 position. This therefore more closely mimics lanosterol the substrate of the reaction in Aspergillus, than the other sterol-like inhibitors tested. It is particularly notable that this lanosterol analogue (ZM308973) was a potent inhibitor of the Candida enzyme in spite of the inability of the parent sterol to act as a substrate for the enzyme in agreement with the results of Ator *et al.* [69]. The non-methylated analogue ZM310866 was a much weaker inhibitor of the Aspergillus enzyme whilst maintaining its potency against the Candida enzyme. These data indicate that the strong interactions between the pharmacophore and the Candida enzyme must overwhelm the negative attributes of the methylated sterol nucleus. In nearly all cases the high MICs observed with these compounds against Aspergillus were the result of poor penetration into the cell. This was shown by whole cell methylenation assays (results not shown).

Suboptimal potential suicide substrates: aromatic olefinic or acetylenic, C22-23 side chain analogues, all gave poor inhibition. Secondary and tertiary C23 amino derivatives in general were the best inhibitors of the enzyme. The analogue of tomatidine where the nitrogen was replaced with an oxygen atom, solacidine, had no activity [63].

To further explore this chemical series ZM59620 was chosen as an example. The degree of inhibition of ergosterol biosynthesis and zymosterol accumulation in *C. albicans* grown in various concentrations was measured. Table 2 shows that for significant inhibition of growth over a 16-hour period there must be around 90% inhibition of ergosterol biosynthesis, with subsequent accumulation of zymosterol.

An *ERG6* mutant of *S. cerevisiae* has been shown to be viable suggesting that it is a non essential gene. Why such an energetically costly reaction should be conserved is not clear, however the data of Parks [5] has indicated that the *ERG6* gene is required for several membrane functions and cells possessing the wild type gene have a definite selective advantage [70].

There are no known inhibitors of the later stages of the pathway that are antifungals. This

Table 2
Inhibition of C24-sterol methyltransferase

Compound	IC_{50} enzyme (μg/ml)		MIC (μg/ml)	
	Aspergillus	Candida	Aspergillus	Candida
ZM59620	3.5	0.0007	128	2
ZM308973	0.0058	0.0058	128	16
ZM310866	2.11	0.048	128	64
W6341	1.10	0.0008	16	32
PD.UK50-98	6.20	0.127	128	64

may be because the organisms are more tolerant to the small changes in the structure of the ring, as exemplified by our results with trifluperidol in *C. albicans*. These observations are in agreement with the work of Nes *et al.* with his sparking experiments [3].

SYNERGY

Mixtures of sulphonamides and dihydrofolate reductase inhibitors have been shown to be synergistic in the treatment of bacterial infections. These two agents inhibit different steps in the folic acid biosynthetic pathway in bacteria and are clinically synergistic (e.g., Septrin). In the same way, it would be expected that different types of inhibitors of the sterol biosynthetic pathway may act in concert. Combinations of naftifine, 15 azasterol, triarimol, and mevinolin with ketoconazole have been reported to reduce the MIC for *C. albicans* by a factor of 4 [71]. We have extended these studies in *C. albicans* to combinations of various types of inhibitors. Both growth of the organism and the sterols produced were measured.

Table 4 shows a summary of the changes in sterols in the presence of different ergosterol biosynthesis inhibitors. These results were obtained by growing the organism (*C. albicans* in Table 3, *T. quinckeanum* in Table 5) in the presence and absence of the compounds under test. After various growth times the fungi were harvested and the sterols extracted and analysed by GC-MS. When there were mixtures of compounds present i.e. two points of inhibition

Table 3

The effect of ZM 59620 on growth and sterol accumulation by C. albicans

Concentration	Growth inhibition (%)	Ergosterol (%)	Zymosterol (%)
1.0 nM	0	100	0
10 nM	10	94	7
100 nM	12	83	17
1 µM	20	78	18
10 µM	50	5	95
100 µM	80	1	99

C. albicans yeasts were grown in a shake flask for 16 h at 37°C and the A_{650} measured. Sterols were extracted and quantitated by Gas Chromatography; identity was confirmed by GC-MS.

Table 4

The accumulation of sterols extracted from C. albicans when grown in the presence of mixtures of ergosterol biosynthesis inhibitors

	Major Sterols Accumulated			
Compounds	Terbinafine	62965	153066	Fenprop
Terbinafine	squal++			
ZM 62965	squal ++ zymo--	zymo++		
ZM 153066	squal++ lano--	zymo-- lano++	lano++	
Fenpropidine	squal++ igno--	zymo-- lano-- D-14-zymo++	lano++ igno--	igno++

C. albicans B2630 was grown in shake flasks at 37°C in the presence of the ergosterol biosynthesis inhibitors above. Cells were harvested, the sterols extracted and analysed by GC-MS. The predominant sterols are, lanosterol (lano), zymosterol (zymo), squalene (squal), ignosterol (igno), ergosterol (ergo), ergosta-8, 14, 24-triene (D-14-zymo). ++ Represent an increase, -- represent a decrease.

Table 5

The accumulation of sterols extracted from T. quinckeanum when grown in the presence of mixtures of ergosterol biosynthesis inhibitors

	Major Sterols Accumulated			
Compounds	Terbinafine	62965	153066	Fenprop
Terbinafine	squal++			
ZM 62965	squal ++ lano--	lano++		
ZM 153066	squal++ lano--	lano++	lano++	
Fenpropidine	squal++ igno--	lano++ igno--	lano++ igno--	igno++

T. quinckeanum was grown in shake flasks at 30°C in the presence of the ergosterol biosynthesis inhibitors above. Cells were harvested, the sterols extracted and analysed by GC-MS. The predominant sterols are lanosterol (lano) squalene (squal), ignosterol (igno), ergosterol (ergo); ++ represents an increase, -- represent a decrease.

Table 6

The effect of mixtures of ergosterol biosynthesis inhibitors on the growth of C. albicans

Compounds	Minimum inhibitory concentration (μg/ml)				
	ZM 59620	Naftifine	ZM 153066	Trifluperidol	Fenproidine
ZM 59620	25				
Naftifine	2.5 + 1.6	200			
ZM 153066	NS	0.1 + 6.2	25		
Trifluperidol	6.2 + 6.2	25 + 6.2	100 + 0.006	>400	
Fenpropidine	3.1 + 3.1	6.2 + 6.2	NS	6.2 + 100	100

C. albicans B2630 was incubated in Yeast Nitrogen broth for 24 h at 37°C in microtitre plates. Compounds were serially dilute across the plates for each of a pair of inhibitors. The growth was assessed and the minimum inhibitory concentration determined. The values quoted are the minimum synergistic concentrations. The first value is for the compound in the vertical column and the second value is for the compound in the horizontal row. NS, no synergy.

Table 7

The candicidal effects of mixtures of ergosterol biosynthesis inhibitors

	Minimum fungicidal concentration (μg/ml)				
Compounds	59620	Terbinafine	153066	Triflu	Fenprop
ZM 59620	100				
Terbinafine	25 + 12.5	100			
ZM 153066	12.5 + 50	3.1 + 3.1	NC		
Trifluperidol	400 + 25	50 + 6.2		400 + 0.4	NC
Fenpropidine	6.2 + 3.1	6.2 + 6.2	NS	25 + 400	NC

C. albicans was grown in microtitre plates, after 24 h growth period, 0.010 ml of incubation fluid was transferred to a microtitre plate of fresh medium and growth continued for 48 h. The plates were then visually scored for regrowth of the organism. The values are for the minimum concentration of the mixture which in the initial plate prevented regrowth in the second incubation. The values given are for the compound in the vertical column plus the compound in the horizontal row. NC, no killing; NS, no synergy.

in the pathway, the balance of sterols was changed with an accumulation of the first blockage product in the pathway. In the case of inhibitors of 24-methenylation and Δ14 reduction, however, the product was a novel sterol, Δ14-zymosterol in the case of *C. albicans* but lanosterol in the case of filamentous fungi such as *A. fumigatus* or *T. quinckeanum*. These data again indicate that the sterol biosynthetic pathway is different in the yeasts and in filamentous fungi. The accumulation of lanosterol by inhibition of C24 methyltransferase and C14 demethylase indicates that the pathway in the filamentous organisms does not pass through zymosterol i.e. lanosterol is methenylated.

Combinations of sterol biosynthesis inhibitors were examined for effects on the growth of *C. albicans* (see Table 6). After 24 h the cultures were sampled and diluted into fresh broth diluting the drugs at least 100-fold. The culture dishes were incubated for another 24 h for growth assessment. When no growth was observed, it was interpreted to mean that the original treatment had been fungicidal. It can be seen from Table 7 that fungicidal effects are seen with all combinations of inhibitors except Δ24-methyltransferase inhibitors and C14 demethylase inhibitors. This observation indicates that whilst most ergosterol biosynthesis inhibitors are fungistatic alone, combinations can be fungicidal. The implication of this type of treatment for immunocompromised patients is considerable.

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