The Mechanistic Targets of Antifungal Agents: An Overview

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Abstract

Pathogenic fungi are a major causative group for opportunistic infections (OIs). AIDS patients and other immunocompromised individuals are at risk for OIs, which if not treated appropriately, contribute to the mortality associated with their conditions. Several studies have indicated that the majority of HIV-positive patients contract fungal infections throughout the course of their disease. Similar observations have been made regarding the increased frequency of bone marrow and organ transplants, the use of antineoplastic agents, the excessive use of antibiotics, and the prolonged use of corticosteroids among others. In addition, several pathogenic fungi have developed resistance to current drugs. Together these have conspired to spur a need for developing new treatment options for OIs. To aid this effort, this article reviews the biological targets of current and emerging drugs and agents that act through these targets for the treatment of opportunistic fungal infections.

Keywords

INTRODUCTION

The continuing need to develop drugs that treat opportunistic infections (OIs) is related to several factors including the capacity of OIs to cause serious and life threatening illnesses in immunocompromised patients, emerging opportunistic pathogens, limited availability of effective antifungal drugs and the emergence of fungal strains resistant against drugs on the market [1]. The major infectious pathogens are fungi, but bacteria also contribute to opportunistic infections. The most common opportunistic pathogenic fungi include the *Candida* species, *Cryptococcus neoformans* (sometimes referred to as *Filobasidiella neoformans*), the *Aspergillus* species, *Histoplasma capsulatum, Coccidioides imitis* and the *Fusarium* species [2–4]. As a result of the increasing prevalence of these life threatening systemic mycoses, [5] there is a continuing need for novel antifungal agents not only to treat the common pathogens but also for resistant fungi associated with the immunosuppressant

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#Deceased: This Paper is Dedicated to His Memory.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

conditions used during solid organ transplants [6]. Classified as eukaryotes, fungal cells present several similarities with mammalian cells including the fact that their cell nucleus contains DNA organized into chromosomes. Fungal cells also have distinct cytoplasmic organelles and biosynthetic pathways analogous to mammalian cells. While the so called "magic-bullet concept" worked fairly well for bacteria, due to the similarities to mammalian cells, its application to fungi has been less certain [1]. These similarities have created additional problems in the design of drugs with selective toxicity to fungal cells [7, 8]. As a consequence, no therapeutic drug was available for treatment of fungal infections until the discovery of amphotericin B in 1953 [9]. This was followed by the development of flucytosine in 1957 [10], azoles in the 1960s [11] and later the triazoles.

More recently, with the advent of the AIDS epidemic, the increasing number of patients living with suppressed immune systems and the lack of effective drugs for the treatment of systemic fungal infections, there has been resurgence in the interest in the development of new antifungal agents [12, 13]. Other conditions such as the increased frequency of bone marrow and organ transplants, prolonged use of corticosteroids, the use of antineoplastic agents, abusive use of antibiotics [14] and the emergence of fungal resistance to currently available agents have made the need for new and effective antifungal agents a high priority [15–18].

An ideal antifungal agent should meet several criteria including the following: a) To have a broad spectrum of activity against a variety of yeast and filamentous fungi, b) to be fungicidal rather than fungistatic, c) to be directed at a specific fungal target and spare interference with host targets including enzymes such as Cytochrome P_{450}, which is responsible for most drug interactions, d) to have multiple delivery methods, particularly oral availability, to enable prolonged out-patient care, and e) to have minimal side effects or toxicities. It is clear that obtaining or designing a drug that satisfies the above criteria is a major challenge and requires the identification of biological targets that are unique to fungi. Thus, the primary objective of this article is to review the current biological targets in fungi, the drugs that target them and their mechanisms of action so as to aid antifungal drug development.

FUNGAL CELL MEMBRANE AS A DRUG DEVELOPMENT TARGET

Polyene Antifungal Agents

The polyene antibiotics (Fig. **1**) produced by the *Streptomyces* species are fungicidal and have the broadest spectrum of activity of any clinically useful antifungal compounds [19, 20]. Agents in this class act by forming a complex with ergosterol in the plasma membrane [21], causing membrane disruption [22], increased permeability, leakage of cytoplasmic contents and ultimately cell death [23]. Recent evidence [24] suggests that they also cause oxidative damage, which may contribute to their fungicidal activity [25]. The clinically useful polyenes including amphotericin B, nystatin, natamycin (pimaricin), rimocidin, filipin and candicin, all have a higher affinity for ergosterol than its mammalian counterpart, cholesterol, and are thus, relatively less toxic to mammalian cells [26]. The acute and chronic side effects of amphotericin B may be reduced in newer formulations, such as liposomes [20, 27, 28], lipid complexes [29] and colloidal dispersions [30]. The major goal

of developing newer formulations has been to obtain a compound with lower toxicity with at least similar efficacy compared to the parent compound. Ambisome is an amphotericin B liposomal formulation composed of high transition temperature phospholipids and cholesterol. The formulation is designed to incorporate amphotericin B securely into a liposomal bilayer such that upon binding to the cell wall, the liposome is disrupted, and the drug is released and binds to ergosterol after being transferred through the cell wall. By this mechanism of action, the integrity of the liposome in mammalian cells is maintained, and thus, resulting in minimal toxicity to the host [31].

Azole Antifungal Agents

The first azole derivatives (Fig. **2**) were discovered in the late 1960s. These are totally synthetic and currently are the most rapidly expanding group of antifungal compounds [32, 33]. They are classified as imidazoles or triazoles on the basis of whether they have two or three nitrogens in the five-membered azole ring respectively. Depending on the particular compound, azole antifungal agents have fungistatic and broad-spectrum activity against most yeasts and filamentous fungi. Inhibition of ergosterol biosynthesis (Fig. **3**) [34] at the C-14 demethylation stage is the basis of their mechanism of action [35]. Azole antifungal agents form, through their azole ring, a stoichiometric complex with the heme iron of P-450 demethylase (DM), which can be measured spectrophotometrically by the red shift of the Soret band of heme from 417 to 447 nm [36]. The resulting ergosterol depletion and the accumulation of lanosterol and other 14-methylated sterols interfere with the "bulk" functions of ergosterol as a membrane component [33, 37, 38]. In addition, the resulting disruption of the structure of the plasma membrane makes it more vulnerable to further damage, including the alteration of the activity of several membrane bound enzymes, such as those associated with nutrient transport and chitin synthesis [39], growth, and proliferation [40]. Imidazoles including, miconazole, ketoconazole, clotrimazole, econazole, bifonazole, butoconazole, fenticonazole, isoconazole, oxiconazole, sertaconazole, sulconazole and tioconazole which are used primarily as topical agents, also interact and damage the cell membrane directly at higher concentrations and are fungicidal and toxic [41]. The other class of the azoles, the triazoles, are newer, less toxic and more effective and include fluconazole, itraconazole, isavuconazole, ravuconazole, posaconazole, voriconazole and terconazole [41]. Several related agents are reported in the literature and include non-azole inhibitors of P-450DM that apparently have not been pursued further [42]. Other notable agents include SCH-56592 (posaconazole), a triazole which was first presented at the 1995 Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) meeting by Schering-Plough [43]; TAK-187, an optically active azolone triazole [1]; ER-30346, a thiazole, which was shown *in vitro* to be more potent than itraconazole against *C. albicans, C. parapsilosis, C. glabrata, Trichosporon beigelli, A. fumigatus, and C. neoformans* [44]; and T-8581, a triazole amide [45], which is highly water-soluble and active against *C. neoformans and C. albicans.*

Allylamine, Thiocarbamate and Morpholine Antifungal Agents

The mechanism of action of allylamines and thiocarbamates (Fig. **4**) is described as a reversible, noncompetitive inhibition of squalene epoxidase [46], an enzyme, which together with (2,3)-oxidosqualene cyclase, is responsible for the cyclization of squalene to lanosterol.

The resulting ergosterol depletion and squalene accumulation as indicated earlier affect membrane structure and functions, such as nutrient uptake [47, 48]. Allylamines such as naftifine and terbinafine along with tolnaftate, a thiocarbamate, have found utility as antifungal agents in the clinic [48]. The benzylamine, butenafine, has a mechanism of action similar to that of allylamines and, in addition, causes direct membrane effects in ergosterol-depleted cells [49].

The morpholines are totally synthetic and, with the exception of amorolfine, are agricultural fungicides [50]. Amorolfine, a morpholine antifungal agent, acts on the ergosterol pathway by inhibiting two enzymes, Δ^{14} reductase and Δ^{7}–Δ^{8} isomerase [50, 51]. However, the antifungal activity of morpholines is probably due solely to sterol Δ^{14} reductase inhibition [52]; Δ^{14} reductase is an essential enzyme [53] while Δ^{7}–Δ^{8} isomerase is not considered as such [54]. Interestingly, the morpholine fenpropimorph also inhibits cholesterol biosynthesis in mammalian cells, although it appears to affect the demethylation of lanosterol rather than sterol reductases or isomerases [55].

Inhibitors of Sphingolipid Biosynthesis

Sphingolipids are essential membrane components of both mammalian and fungal cells and they are localized primarily on the outer leaflet of the fungal cytoplasmic membrane [56]. The first committed step in the biosynthesis of sphingolipid is the condensation of serine and fatty acyl-Coenzyme A (acyl-CoA, usually palmitoyl-CoA), catalyzed by serine palmitoyltransferase to form the long-chain base, ketodihydrosphingosine. Serine palmitoyltransferase is the target of several known natural product inhibitors including: sphingofungins, lipoxamicin, myriocin, pramanicin and viridiofungins. The other enzyme in the sphingolipid biosynthetic pathway, the sphingamine N-acetyltransferase (ceramide synthase), is inhibited by such agents as fumonisins. All early steps of sphingolipid biosynthesis have mammalian counterparts and thus, are less attractive for the development of antifungal agents. The first fungal-specific enzyme in sphingolipid biosynthesis is inositol phosphoceramide (IPC) synthase [57]. It catalyzes the transfer of phosphatidylinositol from glycerophosphatidylinisitol to the C_1 hydroxyl of ceramide to yield inositol phosphoceramide. IPC synthase has been suggested to be an attractive fungicidal target [58] and is the target for agents such as aureobasidin A, khafrefungin, rustmicin, 21-hydroxyrustrin, galbonolide, 21-hydroxygalbonolide and pleofungin A (Fig. **5**).

Inhibitors of Proton ATPases

Proton ATPases are involved in electrochemical proton gradient maintenance and intracellular pH regulation. Plasma membrane H1 ATPase is an abundant and essential enzyme [59] and given that there are significant differences between the fungal and mammalian enzymes, plasma membrane H1 ATPase offers a great opportunity for exploitation as a rational drug design target. Fungal proton ATPase inhibitors (Fig. **6**) include: folimycin, an agent structurally related to bafilomycins [60], which acts by inhibiting the vesicular H1 ATPase (V-ATPase) and bafilomycins, which generally block acidification of intracellular organelles and thereby affect intracellular protein trafficking and translocation to the cell surface. The H^+-ATPase encoded by the *PMA1* gene in *C. neoformans* has emerged as a particularly interesting target with the discovery of the

fungicidal effects of the ATPase antagonist, ebselen [61], and a conjugated styryl ketone, NC1175 [62]. Monk and Perlin [63] have previously reported that the anti-gastric ulcer drug omeprazole (a cysteine-modifying agent which inhibits the gastric K^+ H^+-ATPase) inhibited *C. albicans* growth, although high concentrations of the drug were needed to have such an effect. They further demonstrated that the inhibition of *C. albicans* growth was correlated with the inhibition of the H^+-ATPase of the organism.

Other Antifungal Agents Interfering With the Cell Membrane

Ciclopirox (Fig. **7**) is a broad-spectrum antifungal agent used in the treatment of fungal infections of the nails and hair [64] but which also has antibacterial and anti-inflammatory properties. The mechanism of action is thought to be through the chelation of polyvalent metal cations, such as Fe^{3+} and Al^{3+}. These cations are cofactors of many enzymes, including cytochromes and their inhibition may lead to the disruption of the biosynthesis of ergosterol. Ciclopirox is also thought to act by modifying the fungal plasma membrane, resulting in the disorganization of internal structures [64]. Octenidine and pirtenidine are reported to have antifungal activity against *C. albicans* and act by inhibiting ergosterol biosynthesis through the inhibition of 14-α-demethylase, [65] (Fig. **3**). Syringomycins are members of the *Pseudomonas syringae* pv. *syringae* group [66]. Syringomycin E (SE), the abundant form, alters several membrane functions including membrane potential, protein phosphorylation, H1-ATPase activity, and cation transport fluxes. Syringomycins (Fig. **7**) also appear to bind to ergosterol in yeasts [67]. Sorenson *et al.* have published a study on fungicidal properties of several agents [68] which includes syringostatin A, SE, and syringotoxin B. Ro 09-1571 was designed as an analog of restricticin with *in vitro* activity close to that of ketoconazole against *C. albicans* and *A. fumigatus* and similar to fluconazole against *C. neoformans* [1]. Restricticin, restricticinol and lanomycin are reported to act by inhibiting C-14 α-demethylase [69]. SP-19502, isolated from the plant species *I. Alata* and *A. djalonensis* [70], has potent antifungal activity probably by inhibiting 2,3-oxidosqualene-lanosterol cyclase [71]. Zaragozic acid has been found to inhibit squalene synthase [72] while several pyrimidinium compounds were reported to have antifungal activity by acting as squalene cyclase inhibitors [73].

FUNGAL CELL WALL AS A DRUG DEVELOPMENT TARGET

Fungal cells, like several other microorganisms are enclosed in a cell wall that provides structural support and protection to the cell. Mammalian cells however lack a cell wall, making the fungal cell wall a desirable target for the development of antifungal agents [74, 75]. β-glucan, chitin and mannoproteins are components of the cell wall and disruption of their synthesis can lead to an ineffective cell wall unable to fully protect the cell [76]. In *C. albicans*, it has been suggested that β-glucan and chitin are associated with the strength and shape of the cell wall, while mannoproteins are responsible for the porosity, its antigenicity and adhesion [77]. Many of these targets and the drugs that act on them have previously been extensively reviewed [78].

Glucan Biosynthesis as a Target

Glucans are polysaccharides of D-glucose monomers and β-(1,3)-glucan synthase is the enzyme which catalyzes their synthesis. At least two functional components have been identified in the enzyme: a catalytic component and a regulatory component. Inhibition of glucan synthesis is thought to be the mode of action of glucan biosynthesis inhibitors (Figs. **8a, b**). Specifically, inhibition results in a loss of the enzymatic activity of 1,3-β-glucan synthase and this in turn compromises the structure and osmolarity of the cell wall [79, 80].

Caspofungin is the first of the approved first in-class echinocandin antifungal agents in clinical use. Its introduction was quickly followed by micafungin and anidulafungin. These agents have been shown to target the fungal cell wall by inhibiting the synthesis of β-1,3-D-glucan, the key and critical cell wall component of many pathogenic fungi including *Candida* spp. [81, 82]. Since then several additional echinocandins have been introduced into clinical use (Figs. **8a, b**).

Echinocandins as a group are fungicidal against *Candida* spp. and fungistatic against molds, such as *Aspergillus fumigatus*. While they are highly effective against most *Candida* spp., including azole-resistant strains and biofilms, the echinocandins as a group have a rather limited antifungal spectrum and are thus, limited in their clinical utility.

Echinocandins are fatty acid derivatives of cyclic hexapeptides, while the related papulacandins are fatty acid derivatives of the disaccharide β-(1,4)-galactosylglucose [83]. As a family, the echinocandins include the echinocandins, pneumocandins, aculeacins, mulundocandin and WF11899. Pneumocandin analogs including L-693,989 (a phosphate ester of pneumocandin A); L-733,560; L-743,872; L-731,373 and L-773,560, derivatives of pneumocandin B_0, have been shown to be more potent when compared to the lead compound [84].

Mulundocandin and deoxymulundocandin have been shown to be active against *A. niger* and *C. albicans* [85]. The WF11899 group containing WF11899 A, B, and C have demonstrated anti-*Candida* activities *in vivo* in a murine model of systemic infection and were more effective than cliofungin or fluconazole [86]. Papulacandin analogs include: a) corynecandin [87] isolated from cultures of *Coryneum modonium*, a glycolipid analogous to the structural variant of papulacandin named chaetiacandin, b) Mer-WF3010, isolated from the culture broth of *Phialophora cyclaminis* [88], and c) fusacandin, another structural variant of chaetiacandin isolated from *Fusarium sambucinum* [89]. Fusacandin is a trisaccharide and unlike the other analogs which are disaccharides, has demonstrated poor activity in animal models, prompting attempts to find analogs with improved activity *in vivo* using structure–activity relationship (SAR) studies [90]. Saponins, another group of natural product glycosides, have been reported to inhibit both glucan and chitin synthesis [91].

Chitin Biosynthesis as a Target

Chitin is a polymer of β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) residues [92] and serves as an essential structural component of the fungal cell wall. Its synthesis is catalyzed by chitin synthase and three such chitin synthases (Chs 1, 2 and 3) have been identified in *C. albicans* and *S. cerevisiae* [93]. Chitin synthase 1 (Chs1) plays only a nonessential repair

role in *C. albicans*. Chitin synthase 2 (Chs2) is engaged in septum formation while Ch3 participates in cell wall maturation and bud ring formation [94]. Inhibitors of Chs (Fig. **9**) include polyoxins and nikkomycins produced by streptomycetes. These have been recently reviewed [95].

The inhibitors act as analogs of the UDP-GlcNAc substrate which are transported by peptide permeases into the cell, and bind to the catalytic site of the chitin synthase [96]. HWY-289 is another potent inhibitor of the chitin synthase isozymes CaChs1 and CaChs2. Arthrichitin has a broad-spectrum of activity against *Candida* spp., *Trychophyton* spp. and a number of phytopathogens through the inhibition of fungal chitin and glucan synthases but demonstrates a greater potency against chitin synthase [97]. The low *in vitro* potency of arthrichitin however has prevented its clinical development. Structural modifications could potentially lead to an improvement of activity [98]. FR-900403 differs structurally from nikkomycins and polyoxins but was found to be active against *C. albicans* and inactive against filamentous fungi [99]. Aureobasidins, produced by *Aureobasidium pullulans* [100], differ structurally from the echinocandins and their mode of action is believed to be a disruption of the actin assembly and delocalization of chitin in cell walls leading to lysis. A different study however, has shown that aureobasidin A targets sphingolipid biosynthesis [58].

An associated enzyme in chitin biosynthesis is chitinase. Chitinase has been suggested to play an essential role in the remodeling of the fungal cell wall and hence can serve as an important target for drug design and development. Consistent with this thought, a co-crystal structure of *Af*ChiA1 with acetazolamide has been used to explore the identification of inhibitors of chitinase as new antifungal agents [101]. There has also been a report of a screening-based discovery of chitinase inhibitors as antifungal agents against *Aspergillus fumigatus* [102].

Mannoprotein Biosynthesis as a Target

Mannoproteins are interstitial components of fungal cell walls. Structurally, these are complex chains of mannose units linked to proteins through N-acetylglucosamine and asparagine residues. Mannoproteins may be involved in cell-cell recognition and reproductive processes. Available evidence suggests that prevention of N- or O-glycosylation of sensitive proteins is lethal to fungi [1]. The mechanism of action of mannoprotein biosynthesis inhibitors (Fig. **10**) apparently involves complexation of their carboxyl group with the saccharide moiety of the cell surface mannoproteins. Agents such as benanomicins, pradimicins, and benzonaphthacene quinones are among the known inhibitors of mannoprotein biosynthesis.

FUNGAL NUCLEIC ACID AND PROTEIN BIOSYNTHESIS AS A DRUG DEVELOPMENT TARGET

As a consequence of the similarities between eukaryotes and mammalian cells, DNA/RNA and protein synthesis have been difficult targets for antifungal drug development. More

recently however, as important differences between the cell types become known, attempts have been made to exploit the differences for the development of new antifungal agents.

Interference with Normal Metabolic Processes

Targeting normal metabolic processes can be a viable strategy for drug development against fungal pathogens. This will require designing compounds that structurally resemble key metabolites such that they are mistakenly utilized by fungi but not mammalian cells. The drug flucytosine or s5-fluorocytosine (5-FC) (Fig. **11**) provides an obvious example. It was originally developed in the 1950's as a potential antineoplastic agent but was found to be ineffective against tumors [15]. Subsequent testing led to its identification as an antimetabolite antifungal agent. Transported inside susceptible fungal cells by a cytosine permease, cytosine deaminase converts 5-FC into 5-fluorouracil (5-FU). Subsequent phosphorylation and incorporation into RNA, leads to miscoding and disruption of protein synthesis. Additionally, phosphorylated 5-FU is converted to its deoxynucleoside and is believed to block DNA synthesis by inhibiting thymidilate synthase [103] leading to the disruption of DNA replication.

Inhibitors of Topoisomerase

Topoisomerases (TOP) I and II are involved in the replication, transcription, repair, and chromosomal segregation [104] in cells. Topoisomerase II is an essential enzyme [105] and the mammalian topoisomerase II is the target of the known anticancer drugs anthracyclines and epipodophylotoxins. Targeting DNA gyrase, the equivalent enzyme in bacteria, has led to the identification and clinical utility of quinolones such as ciprofloxacin. While not considered an essential enzyme, fungal topoisomerase I has been successfully targeted in the development of inhibitors such as camptothecin. Fungal TOP I inhibitors apparently form complexes with the enzyme and ultimately derail the oncoming replication fork [106]. The alkaloid eupolauridine (Fig. **11**) inhibits the growth of *C. albicans* and it has been suggested that these actions may be through the stabilization of the cleavage complex formed by the *C. albicans* topoisomerase [107a]. Fortunately, it has been demonstrated that the response of the *C. albicans* TOP I to eupolauridine is greater than that of the human enzyme. Specific targeting of the fungal TOP I has also been demonstrated using the aminocatechol A-3253 which is less effective against human Top I [107b]. In contrast, camptothecin demonstrates greater activity against the human rather than fungal Top I. The combined observations suggest differences exist that could be exploited for drug design purposes. It is not entirely clear whether fungal topoisomerase II, the other topoisomerase, could serve as a selective target relative to its mammalian counterpart.

Inhibitors of Elongation Factors

Elongation factor 1 (EF-1) and elongation factor 2 (EF-2) are required for the polypeptide chain elongation reactions in the synthesis of proteins in both fungal and mammalian cells [108]. Elongation factor 3 (EF-3) required by fungi but not by mammalian cells [109, 110] is present in most fungi including *C. albicans* and *P. carinii* and is essential for cell viability [111]. EF-3 has ATPase activity and is specifically required by the yeast 40S ribosomal subunit. EF-3 may also be involved in the translocation of the growing peptide although its

exact function in the elongation cycle remains unclear. Sordarins are believed to act by inhibiting the protein synthesis elongation cycle in yeasts. The inhibition occurs without affecting the protein synthesis machinery in mammals [112]. The mechanism of action of sordarins appears to involve the inhibition of EF-2 [113]. The structure-affinity relationship for binding to EF-2 from different species is influenced by the nature of the sugar residue of the glycoside. BE31405, whose sugar is a rigid tricyclic ring system, has a broader spectrum of activity compared to sordarin. GM222712 and GM237354, analogs (Fig. **12**) of sordarins, reportedly display *in vitro* activities against several pathogenic fungi [114].

Inhibitors of Protein Farnesyltransferase (PFT)

Many small G proteins require post-translational modification to allow functional association to the cell membrane. This process often involves the enzymatic addition of hydrophobic prenyl groups to a conserved cysteine residue near the C-terminus of the protein. The enzymes that catalyze these reactions include protein farnesyltransferase and protein geranylgeranyltransferases. *Cryptococcus neoformans* requires post-translational modification with Ras and Rho proteins in order to undergo normal growth and differentiation. Inhibition of farnesyltransferase has been shown to result in dose-dependent cytostasis of *C. neoformans*, as well as prevention of hyphal differentiation [115]. FPT inhibitor III (Fig. **13**) is reported to produce antifungal activity by inhibiting the protein farnesyltransferase.

Inhibitors of *N*-myristoyltransferase (NMT)

Another post-translational modification involves the synthesis of *N*-myristoylated proteins in a process described as myristoylation. This involves the co-translational transfer of myristate from CoA to the amino-terminal glycine of proteins [116]. The reaction is essential in *C. neoformans* [117] and other fungi [118] and is catalyzed by myristoyl CoA:protein-*N*-myristoyltransferase (NMT). An analog of myristic acid with an oxygen atom replacing a methylene at position 4 had shown *in vitro* fungicidal activity. In view of the fact that there has been a demonstration of differences in substrate specificities of fungal and mammalian NMTs, fungal NMTs may serve as desirable targets for drug design against pathogenic microorganisms that require N-myristoylation.

Enactins and neoenactins are antibiotics with antifungal activity. They are reported to act through inhibition of NMT [1, 119 a–d]. Analogs of 2-bromotetradecanoic acid [120], putative inhibitors of NMT have demonstrated *in vitro* activity against yeasts including *S. cerevisiae*, *C. albicans* and *C. neoformans* and filamentous fungi such as *A. niger*. The benzofuran derivatives RO-09-4609 and RO-09-4879 have been reported to be fungal NMT inhibitors. Several of these inhibitors (Fig. **14**) have been reported to show high selectivity over human NMT and have exhibited antifungal activity *in vivo*.

Inhibitors of Nucleic Acid Biosynthesis

Several natural products including sampangine, meridine, onychine and berberine (Fig. **15**) have been reported to have antifungal activity by interfering with fungal cell nucleic acid biosynthesis [121, 122].

Inhibitors of Amino Acids Biosynthesis

Studies have suggested that cispentacin [123], an antifungal agent with *in vivo* activity against multiple targets, interferes with amino acid synthesis [124]. Cispentacin (Fig. **16**) and FR109615 [125] are unnatural cyclic β-amino acids. These compounds and the analog BAY108888, appear to have a dual mode of action. The suggested mechanism of action involves interference with amino acid transport and cellular regulation of amino acid metabolism. Other amino acid analogs with antifungal activity are: RI-331, which inhibits homoserine dehydrogenase [126], a desirable target for drug design as a mammalian counterpart has not been reported, and azoxybacilin, which inhibits the biosynthesis of sulfur-containing amino acids. Azoxybacilin interferes with the induction of gene expression by low levels of methionine in the sulfate assimilation (SA) pathway, but has weak activity *in vivo*, perhaps because of a poor pharmacokinetic profile. Compounds not antagonized by methionine, such as Ro091824, have been obtained and reportedly are antagonized to a lesser extent [127].

Inhibitors of Polyamine Biosynthesis

Ornithine decarboxylase, an enzyme involved in the biosynthesis of polyamine and a target of anticancer chemotherapeutic agents, is a potential antifungal target [128]. Eflornithine, a difluoromethyl analog of ornithine (Fig. **16**), displays some antifungal activity [129]. *P. carinii* ornithine decarboxylase is far less susceptible to eflornithine [130] than the mammalian enzyme, suggesting differences in the active sites, although it remains unclear whether targeting the enzyme for rational drug design purposes is a viable option.

Inhibitors of Folic Acid Biosynthesis

The diaminoquinazoline structures (Fig. **17**), are reported to inhibit the dihydrofolate reductase enzyme, which catalyzes the reduction of dihydrofolic acid to tetrahydrofolic acid, and thus interferes with DNA biosynthesis [131].

OTHER CELLULAR FUNCTIONS AS DRUG DEVELOPMENT TARGETS

Inhibitors of Microtubule Aggregation

Microtubules are polymers of α- and β-tubulin dimers [132]. Microtubule aggregation-disaggregation plays a key role in cell morphology and growth. Griseofulvin (Fig. **18**) inhibits microtubule aggregation by interacting with β-tubulin, a protein highly conserved in eukaryotes. Nevertheless, there appears to be differences between mammalian and fungal tubulins; for example, colchicine binds preferentially to mammalian tubulin [133].

Inhibitors of Signal Transduction Pathways

The signal transduction cascades in fungi have become very attractive targets since their components are now emerging as potential targets for the development of new antifungal agents. A review by Cardenas *et al.* [134] has provided insight on the mechanism of action of several natural products (Fig. **19**) including cyclosporin A (CsA), tacrolimus (FK506), rapamycin, wortmannin and geldanamycin.

Inhibitors of Calcineurin-dependent Signaling

Calcineurin is critical in maintaining the perfect homeostasis of the cell under stress conditions. In response to increases in intracellular calcium, calmodulin activates calcineurin which is required to control cell survival [135]. CsA and FK-506 are immunosuppressive agents that have been shown to inhibit fungal calcineurin [136]. Mechanistically, they are believed to diffuse across the plasma membrane forming complexes with cyclophilin A (CyPA) and FKBP12, respectively. Cyclophilin A (CyPA) and FKBP12 are cytosolic immunophilins that catalyze *cis-trans* prolyl isomerization, required for protein folding. When FK-506 binds to FKBP12, inhibition of prolyl-isomerase is observed. Inhibition of prolyl-isomerase is also observed when CsA binds to CyPA and forms a drug-immunophilin complex [134]. As a result of the essential role of calcineurin in fungal virulence, its inhibitors are expected to have several therapeutic uses in a variety of clinical settings. To develop CsA and FK-506 as clinically useful antifungal agents however, will require the design of analogs selective for the antimicrobial activities but that do not produce immunosuppression. Indeed, there have been indications that antifungal agents with reduced immunosuppressive properties have been identified [134b]. Because calcineurin inhibitors have also been observed to demonstrate significant synergism with azoles or echinocandins, they could provide another therapeutic window for the treatment of fungal infections and especially for the treatment of resistant fungal pathogens [136].

Inhibitors of Target of Rapamycin (TORs) Dependent Signaling

TORs regulate cell growth and are targets for the drug rapamycin [136, 137]. Rapamycin (sirolimus) has been shown to bind to the FKBP receptor to yield a rapamycin-FKBP complex that binds to the TOR protein, and blocks signal transduction. In a number of human and plant pathogens, rapamycin blocks filamentation, a conserved mechanism of action among eukaryotes [136]. With several studies suggesting a globally conserved role for the TOR protein in regulating growth and proliferation, targeting its essential functions is expected to result in an effective antifungal activity [137].

Inhibitors of Phosphoinositide 3-Kinases (PI-3-kinases)-dependent Signaling

Wortmannin (Fig. **19**) is a hydrophobic steroid-related product of the fungus *Talaromyces wortmanni* that inhibits signal-transduction pathways [138]. In the yeast *S. cerevisiae*, although the sole PI-3 kinase (VPS34) is only inhibited at very high concentrations of Wortmannin, PI-4 kinase STT4 is potently inhibited by wortmannin at much lower concentrations.

Inhibitors of HSP90 and HSP90-dependent Signaling

Geldanamycin is a benzoquinone ansamycin, natural-fermentation product that was originally thought to be a direct tyrosine-protein-kinase inhibitor. However, subsequent studies revealed that geldanamycin and two other structurally related analogues (herbimycin and macbecin) bind to and inhibit the 90 kDa heat-shock protein HSP90 instead [139]. HSP90, HSP70 and many of the associated chaperones are highly conserved between yeast and humans. Steroid-receptor and oncogenic kinase functions in yeasts are HSP90 dependent. However, geldanamycin is not toxic to wild-type yeast strains. It has been

recently discovered that yeast mutants lacking HSP90-associated chaperones become sensitive to geldanamycin, suggesting that the mechanism of geldanamycin action is conserved between yeast and human, and involves the inhibition of HSP90-dependent signaling cascades that are required for cell function. These findings will permit further genetic dissection of HSP90-dependent signaling cascades and of the mechanisms of action of geldanamycin (Fig. **19**).

Inhibitors of Electron Transport

A series of related antibiotics, including antimycin A, UK-2A and UK3A (Fig. **20**), have been shown to inhibit incorporation of nucleic acid precursors, amino acids, glucose and mannose into acid-insoluble material in a dose-dependent manner, making it difficult to identify the specific mode of antifungal action [140]. UK-2A and UK-3A however, are known to inhibit mitochondrial electron transport and interfere with *in vitro* respiration in both yeast and rat liver mitochondria [140].

MISCELLANEOUS ANTIFUNGAL AGENTS

Organic acids such as caprylic acid, salicylic acid, undecylenic acid, propionic acid, and benzoic acid (Fig. **21**) exhibit antifungal activity by interacting with non-specific components in the cell membrane. Reports over the years have indicated that short and medium-chain organic acids have antimicrobial properties [141]. In addition, common aromatic acids such as benzoic acid, have found utility as preservatives in foods on the basis of their antifungal properties [142]. Sorbic acid is an antimycotic agent which demonstrates broad spectrum activity against yeast and fungal molds [143]. Biotin, essential for the activity of many important metabolic reactions, plays a key role in maintaining healthy skin, hair, sweat glands, nerves and bone marrow. With primary activity in the mitochondria, biotin is a cofactor in at least four key carboxylase enzymes. Its activity is required for normal neuronal and hematopoietic function. In a study seeking to validate biotin biosynthesis as a potential target for the development of new antibiotics, the authors have demonstrated that *de novo* biotin synthesis is essential for *M. tuberculosis* to establish and maintain a chronic infection [144]. In another study, analogs of biotin have been synthesized and have demonstrated antimicrobial activity through the inhibition of biotin protein ligase (BPL) [145]. Taken together, there appears to be a great potential for targeting biotin biosynthesis, not only in antibacterial drug development but perhaps fungal BPL could serve as a potential drug development target for new antifungal agents.

Haloprogin, which is used to treat athlete's foot and other fungal infections, was marketed over the counter primarily to treat tinea infections of the skin. Its mechanism of action is unknown [146]. Allicin, the main parent antifungal compound in garlic, appears to have a mode of action related to its ability to cross the cell membrane and combine with sulfur-containing groups in amino acids and proteins and interfering with cell metabolism. Thus, it appears that human cells are poisoned by allicin derivatives due to their interaction with glutathione. In addition to their biochemical mechanism, these derivatives appear to stimulate cellular immunity, an important ability lacking in conventional antifungal chemotherapy [147]. Tea tree oil, citronella oil, lemon grass, orange oil, palmarosa oil,

patchouli, lemon myrtle, neem seed oil and coconut oil all exhibit antifungal activity, although their mechanisms of action are currently unknown. However, it is hypothesized that they might act by altering membrane properties and compromising membrane-associated functions [148]. Olive leaf acts by interfering with the pathogen's amino acid properties and prevents the pathogen from reproducing and creating more microbes in the body and directly stimulating their phagocytosis [149]. Zinc pyrithione (ZnP), (Fig. **21**), also known as pyrithione zinc, is an antifungal agent best known for its use in treating dandruff and seborrheic dermatitis. It is believed to act through the disruption of membrane transport by blocking the proton pump that energizes the transport mechanism. Fungi are capable of inactivating pyrithione in low concentrations [150].

AGENTS EXHIBITING ANTIFUNGAL ACTIVITY, WITH UNKNOWN MECHANISM OF ACTION

Schizotrin A (Fig. **22**) is a cyclic undecapeptide produced by cyanobacterium [151] which has activity against *C. albicans* and *C. tropicalis* and has also been found to inhibit the radial growth of *F. oxysporum*. Cepacidines, comprising cepacidines A_1 and A_2 (Fig. **22**) have shown potent antifungal properties comparable to amphotericin B [152]. However, the mechanisms by which these activities occur have not been reported.

Leucinostatin-trichopolyn Group

Leucinostatin A (Fig. **22**) represents a group of products of submerged cultures of *Penicillium lilacinum* [153]. Several leucinostatins isolated from *Paecilomyces marquandii* (Massee) Hughes, have been reported to display biological activities including antifungal activity against *C. neoformans* and *Candida* species [154]. Other natural products, including trichopolyns A and B from *Trichoderma polysporum* [155] and helioferins A and B, products of *Mycogone rosea*, inhibit *C. albicans* [156]. Histatins, produced by human salivary glands, are naturally occurring peptides that have shown several activities including antifungal effects against pathogens such as *C. albicans* and *C. neoformans*. While their antifungal mechanism of action is not fully understood, some studies have suggested interaction of Histatin H5 with heat-shock-related proteins in *C. albicans* [157]. Thiarubrine A and B classified as dithiins, are natural products isolated from plants of the family *Compositae* that have shown antifungal activity [158] while aspirochlorine, lissoclinotoxin A and related analogs were reported to have potent antifungal activities [159].

Benzophenanthridines 1, 2, 3 and 4 (Fig. **23**) have also been reported to have moderate antifungal activity [160]. Cryptolepine, an indoloquinoline alkaloid with a variety of biological activities including antifungal activities [161], has served as a scaffold for the development of novel antifungal agents [162–172]. 3-Dimethylallylindole has displayed a moderate antifungal activity *in vitro* [173]. Hamacanthine A and B [174] and melinonine F [175] display good antifungal activity and compounds such as β-cyanoglutamic acid [176], benzothiazole derivatives [177], bguaine, a pyrrolidine alkaloid from *Arisarum vulgare*, pyrrololinosalicyclic acid derivatives and melithiazole A have all been shown to have antifungal activities [1]. Lipopeptides such as cyclopeptolide [178] and jasplankinolide [179] (Fig. **23**) are known to display broad antifungal activities. Disorazoles as represented by

disorazole A$_1$ [180], xanthone isoquinoline derivatives such as SCH54445 [181], LL-Z1271 alpha [182], sonomolide B [183], coniochaetone A, 1-dehydroxyarthrinone [184, 185], pseudolaric acid B [186], soraphen A1α [187], and clabistrin A [188], all display antifungal activities, several of which are comparable to amphotericin B. Fusarielin A displays antifungal activity against *A. fumigatus* [189]. The structures of several key compounds are shown in Fig. **23**.

FUTURE APPROACHES FOR IDENTIFICATION OF NOVEL DRUG TARGETS

Significant effort has been expended on optimizing the specificity of current drugs in an attempt to make more selective anti-fungal agents which are less toxic to the patient [190]. However, chemical modifications of drugs have inherent limitations which may compromise their effectiveness. In addition, the development of drug resistance as a result of mutations in the microorganism considerably limits the useable lifetime of drugs aimed at inhibiting a specific target. For these reasons, the need to identify novel, fungal-specific targets is currently a major undertaking. Ideal targets require two major features. First they need to be essential to cell viability, and second, they need to be unique to the fungal organism.

Annotated genome databases can serve as the main sources of information to identify genes with the potential to be therapeutic targets. These databases gather information on gene structure and function, which is compiled from published works and curated for accuracy. Currently, the most complete database is the *Saccharomyces* Genome Database (SGD) [191]. The SGD genome inventory, as of June 29, 2015, indicates that budding yeast contain 6,604 Open Reading Frames or ORFs (gene sequences which are predicted to encode for a protein) and 78% have been verified. Of these, 1,284 genes are essential indicating that their inactivation leads to cellular inviability [192]. Further analysis can indicate which of these essential genes are unique to the organism and absent in humans.

Similarly, databases for pathogenic fungi are available and are updated with genomic information as it becomes available. For example, the *Candida* Genome Database [193] collects information for *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. dubliniensis*. At this time, *C. albicans* has 6,218 ORFs but only 1,548 ORFs (24.9%) have been verified. As sequences become available and the ORF function including viability of the null alleles is determined, potential therapeutic targets can be identified. The *Aspergillus* Genome Database [194] collects genomic information for *A. nidulans*, *A. fumigatus*, *A. niger* and *A. oryzae*. A genome snapshot of *A. nidulans* indicates it has 10,678 ORFs, of which only 1,198 (11.2%) have been verified so far [194].

The growing number of organisms subjected to genomic sequencing is generating large amounts of data and this has required the consolidation of the findings in the Fungal and Oomycete Genomics Resources Database, known as FungiDB [195]. However, since the most investigated organism is the budding yeast *S. cerevisiae*, the SGD provides a tool that restricts BLAST searches to fungal genomes [196] which can serve as the starting point to identify potentially essential genes in other fungi.

An alternative approach to increasing the effectiveness of current anti-fungal drugs involves the identification of genes which when inactivated render the organism hypersensitive to the agent [197]. These genes are identified through genome-wide screens which are performed using libraries of deletion strains arranged in an array, usually in a 96-well format. The library is replicated onto both media containing the drug and media without the drug (as a control for the different growth rates of each strain). Those strains that do not grow in the plate containing the drug are classified as sensitive, and the identity of the deleted gene in the strain is determined by their position in the array or by PCR. These genes are potential targets of therapeutic value since their inactivation results in hypersensitivity to the anti-fungal agent tested.

Currently, the majority of genome-wide screens are performed in *S. cerevisiae* for which deletion strain libraries are available. The information derived from these screens can prove useful when other fungi contain the same genes identified in the screen, or at least provide additional information regarding the mechanism of action of the anti-fungal agent and the cellular response required to protect the cell from its toxicity [197].

CONCLUSION

Opportunistic fungal infections are frequently observed in immunocompromised patients and present serious life-threatening risks. Significant advances in our understanding of fungal biology have provided numerous potential targets that may yield more effective antifungal agents of potential use in the clinic. In parallel with laboratory evaluation of safety and efficacy, it is hoped that the availability of new knowledge of the mechanisms of action of the existing antifungal agents will spur the discovery of new and effective antifungal agents. The advent of genomics in the investigation of pathogenic fungi is expected to reveal new targets that are unique to fungi and essential for their survival, and hopefully lead to the development of novel antifungal agents.

Acknowledgments

Biography

S.Y. Ablordeppey

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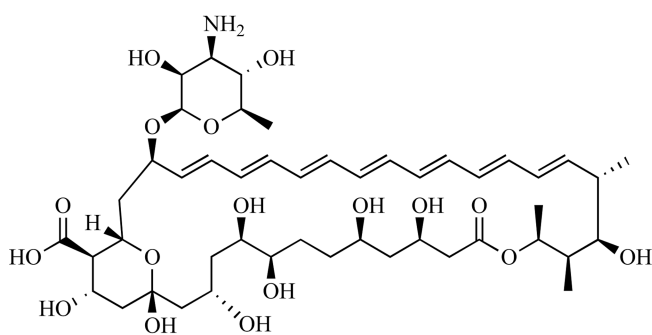
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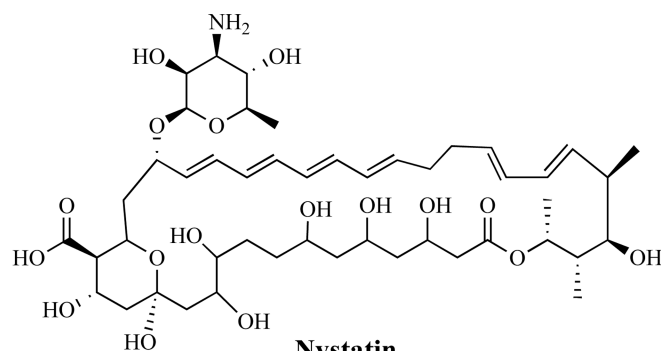
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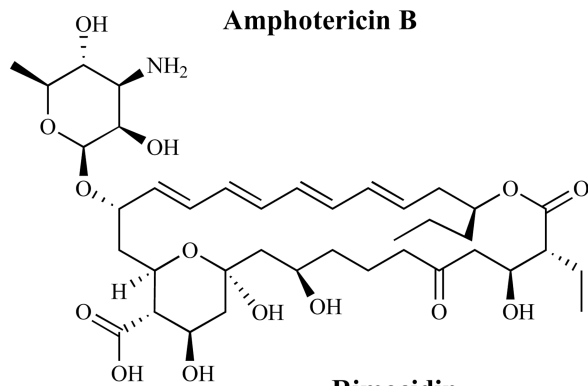
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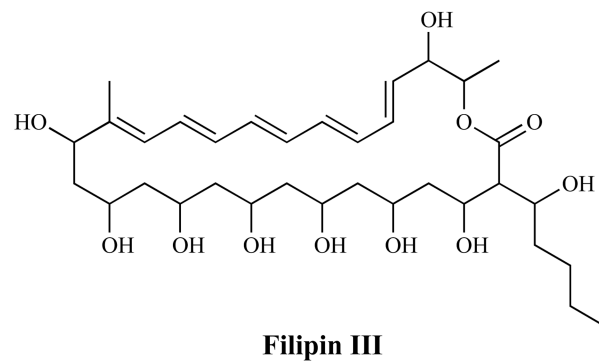
Filipin III

Fig. (1).
Polyene Antifungal Agents.

Isoconazole   **Oxiconazole**   **Sertaconazole**   **Sulconazole**

Tioconazole   **Fluconazole**   **Itraconazole**   **Isavuconazole**

Ravuconazole   **Posaconazole**

Voriconazole   **Terconazole**   **TAK-187**

Fig. (2).
Azole Antifungal Agents.

erg 10
erg 13
*hmg*1/ *hmg*2
erg 12
erg 8
erg 19
idi 1
erg 20

Acetyl CoA

Farnesyl Pyrophosphate

erg 9

Squalene

erg 1 ————————— Allylamines

Squalene Epoxide

erg 7

Lanosterol

erg 11 ————————— Azoles

4,4-dimethylcholesterol-8,14.24-trienol

erg 24

4,4-dimethylzymosterol

erg 25
erg 26
erg 27
erg 6

Fecosterol

——— Morpholines

erg 2

Episterol

erg 3

Ergosta-5,7,24(28)-trienol

erg 5
erg 4

Ergosterol ——————— Polyenes

Fig. (3).

Linear model of the ergosterol biosynthetic pathway adapted from *Saccharomyces cerevisiae*. Significant intermediates are in boldface, genes involved in the step of biosynthesis appear on the left and inhibitors are shown on the right. CoA = coenzyme A [34].
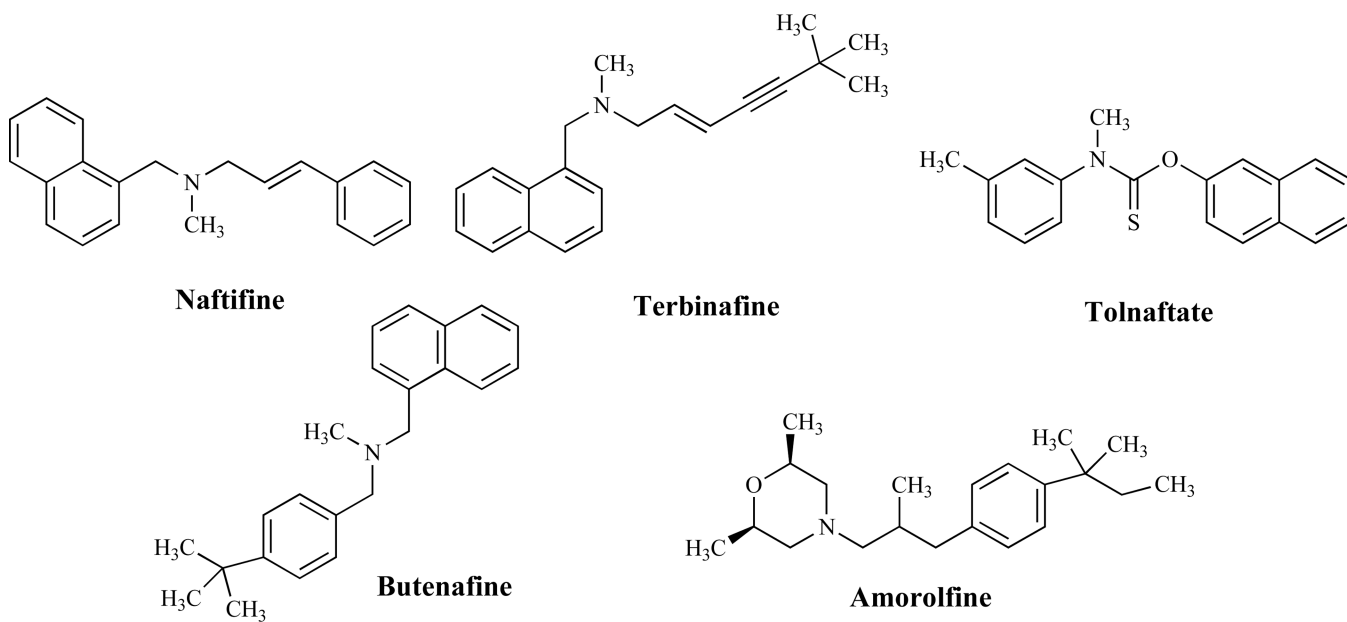
Fig. (4).
Allylamines, Thiocarbamates and Morpholines.
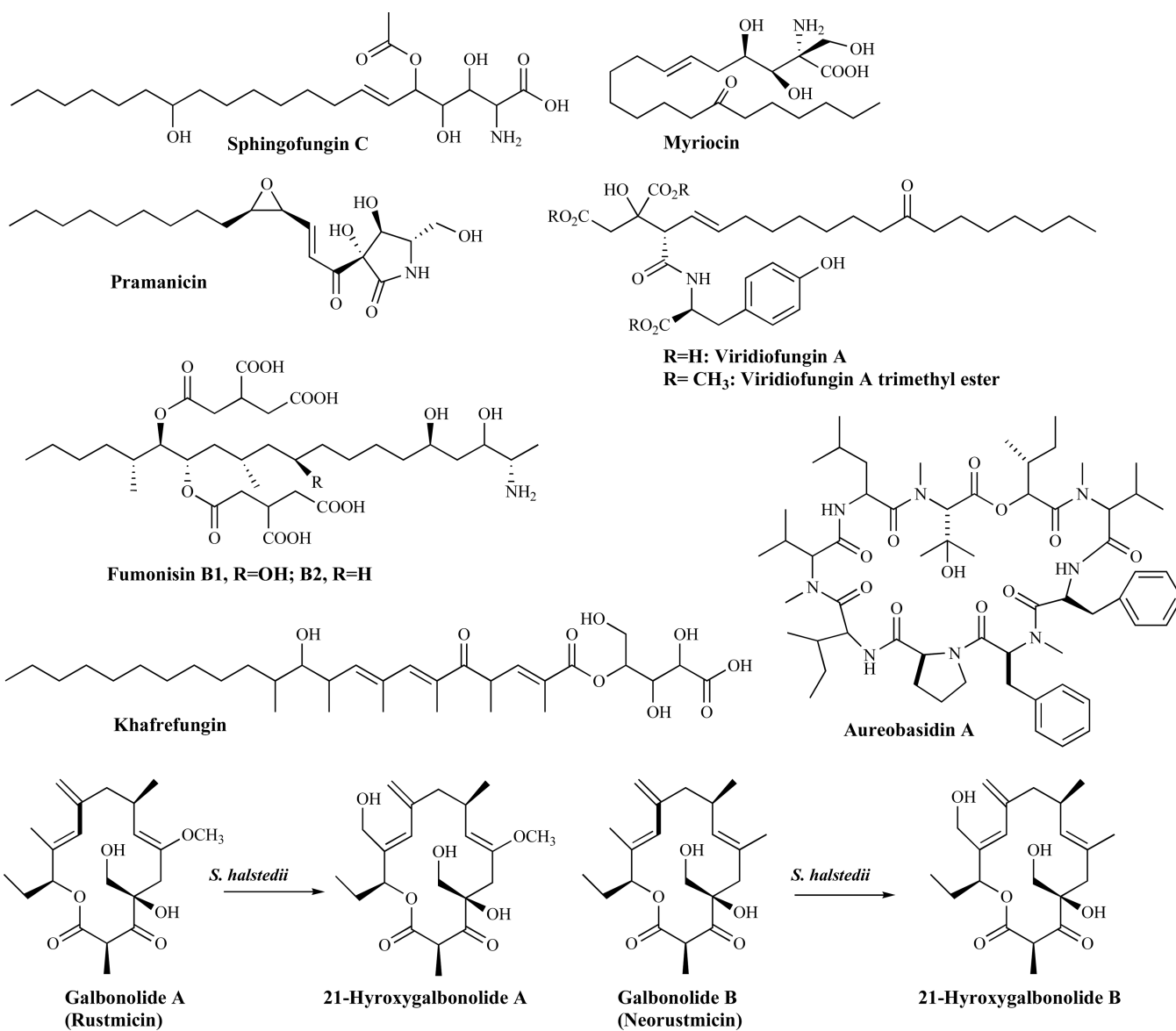
Fig. (5).
Inhibitors of Sphingolipid Synthesis.

Folimycin (Concanamycin A)

Bafilomycin A1

Ebselen

NC1175

Omeprazole
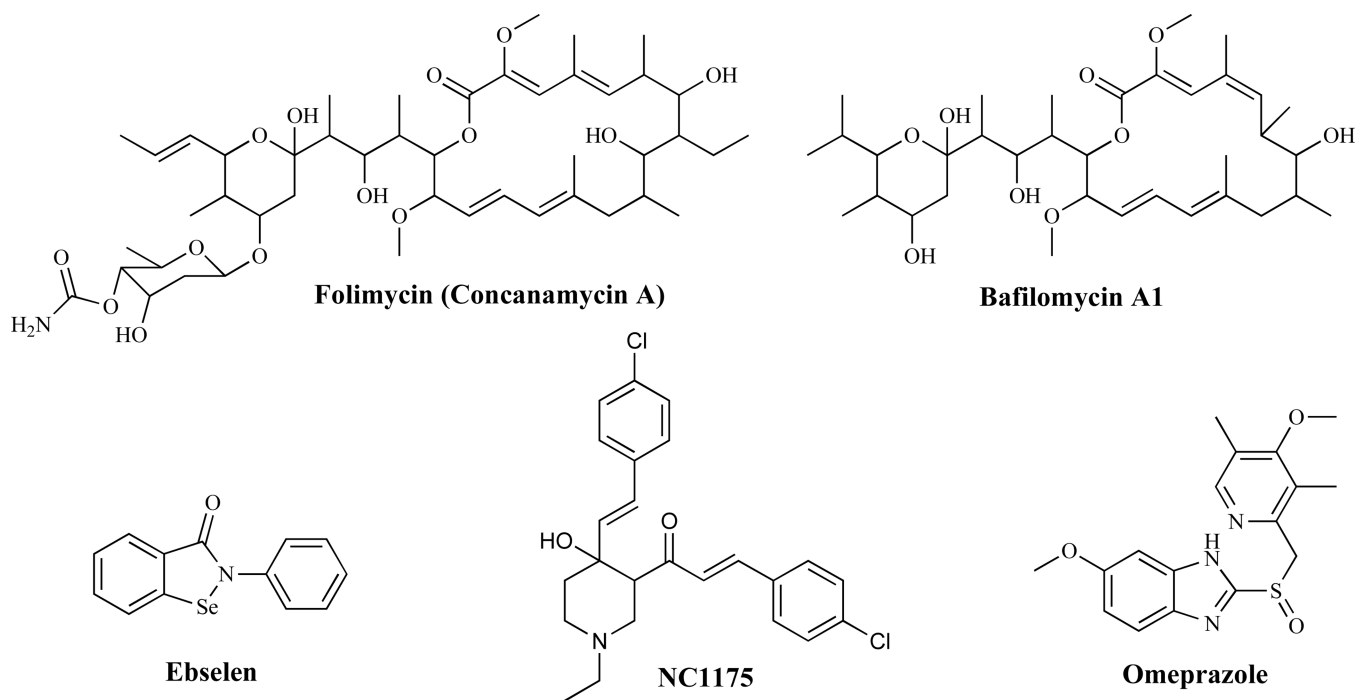
Fig. (6).
Proton ATPase Inhibitors.
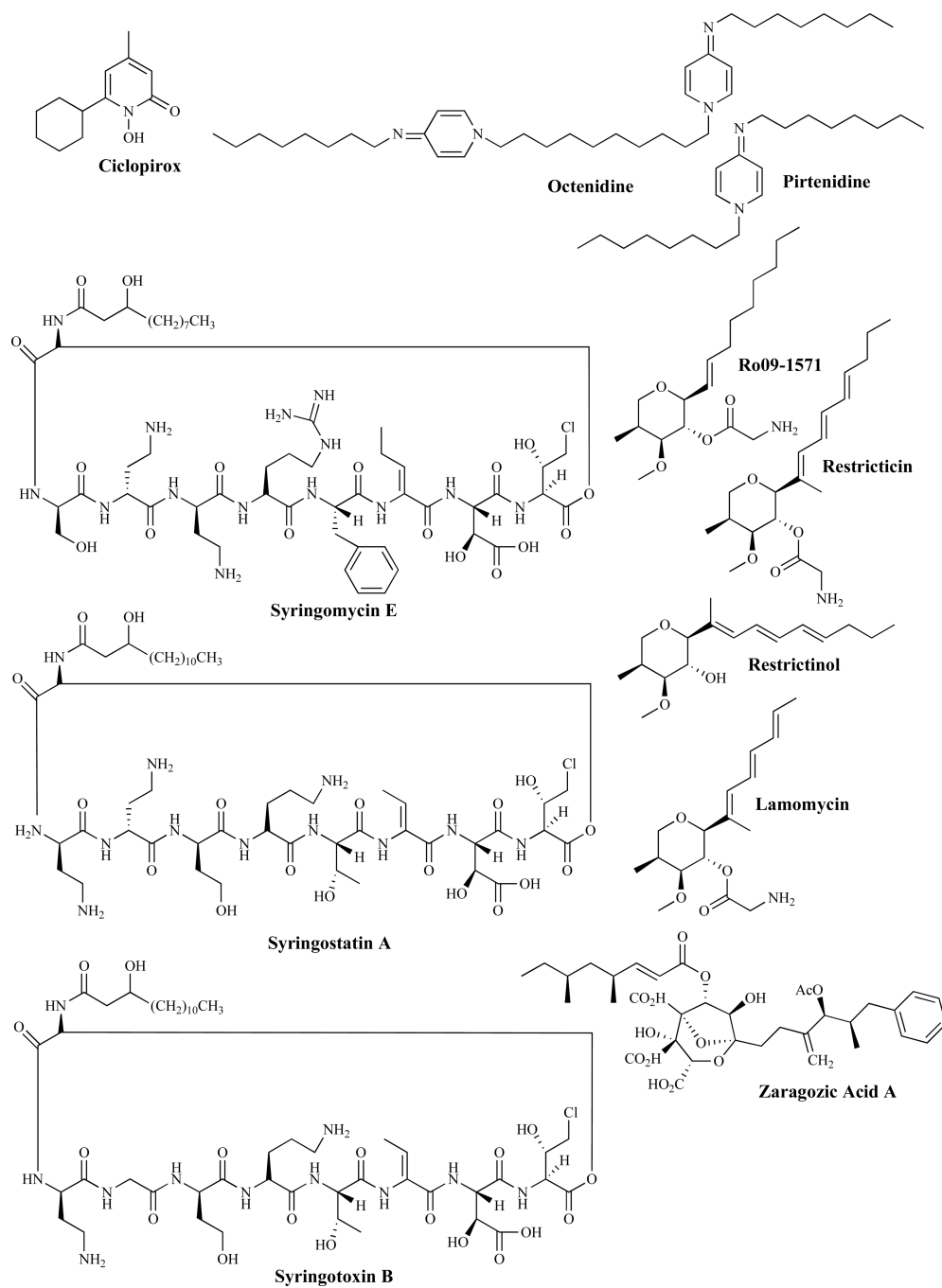
Fig. (7).
Other Antifungal Agents That Interfere With the Cell Membrane.

Caspofungin

Micafungin

Anidulafungin

Mulundocandin

L-693,989

L-733, 560

L-731, 373

Corynecandin

(a).

Mer-WF3010

Fusacandin A

Solanine, a saponin
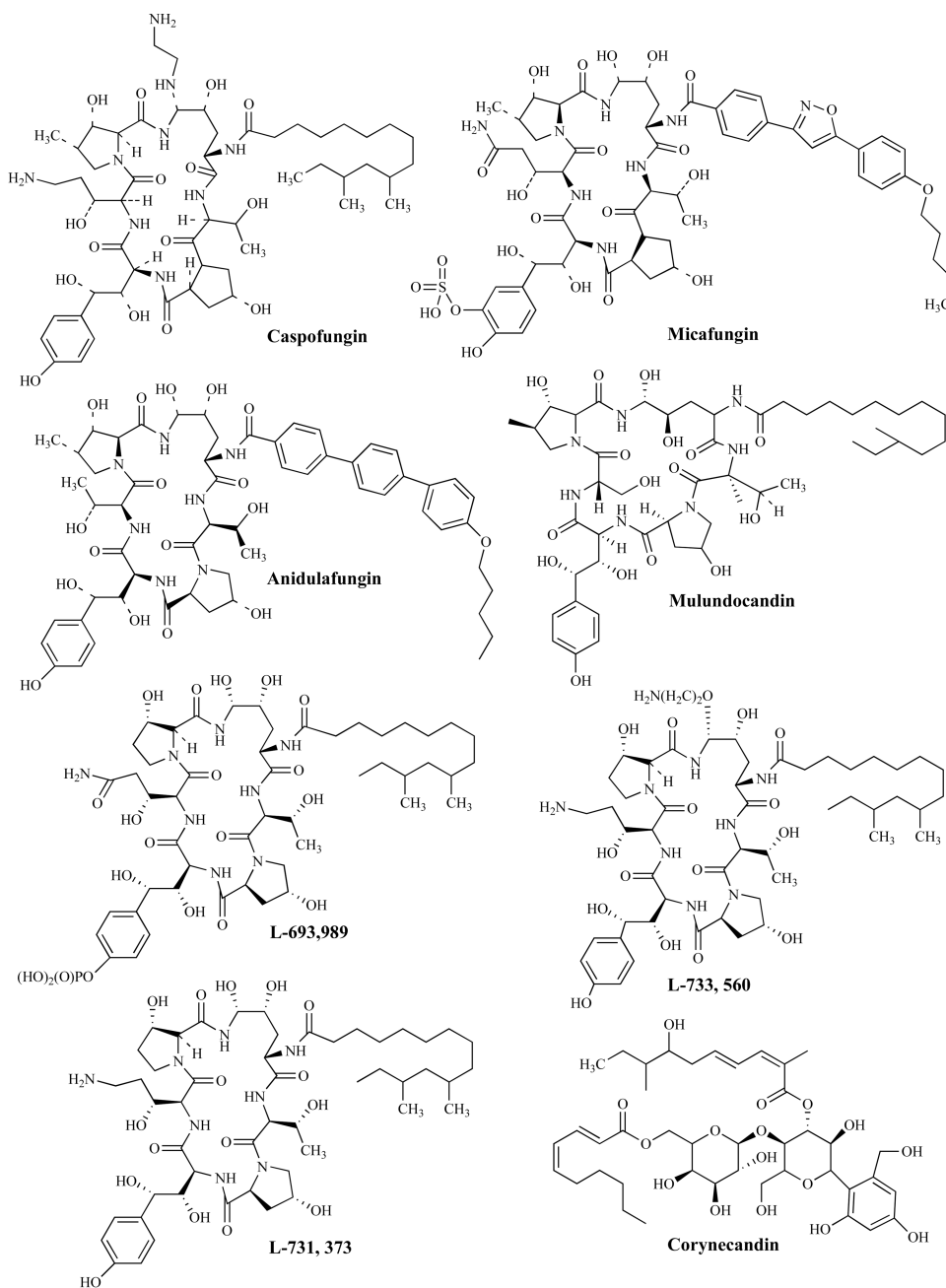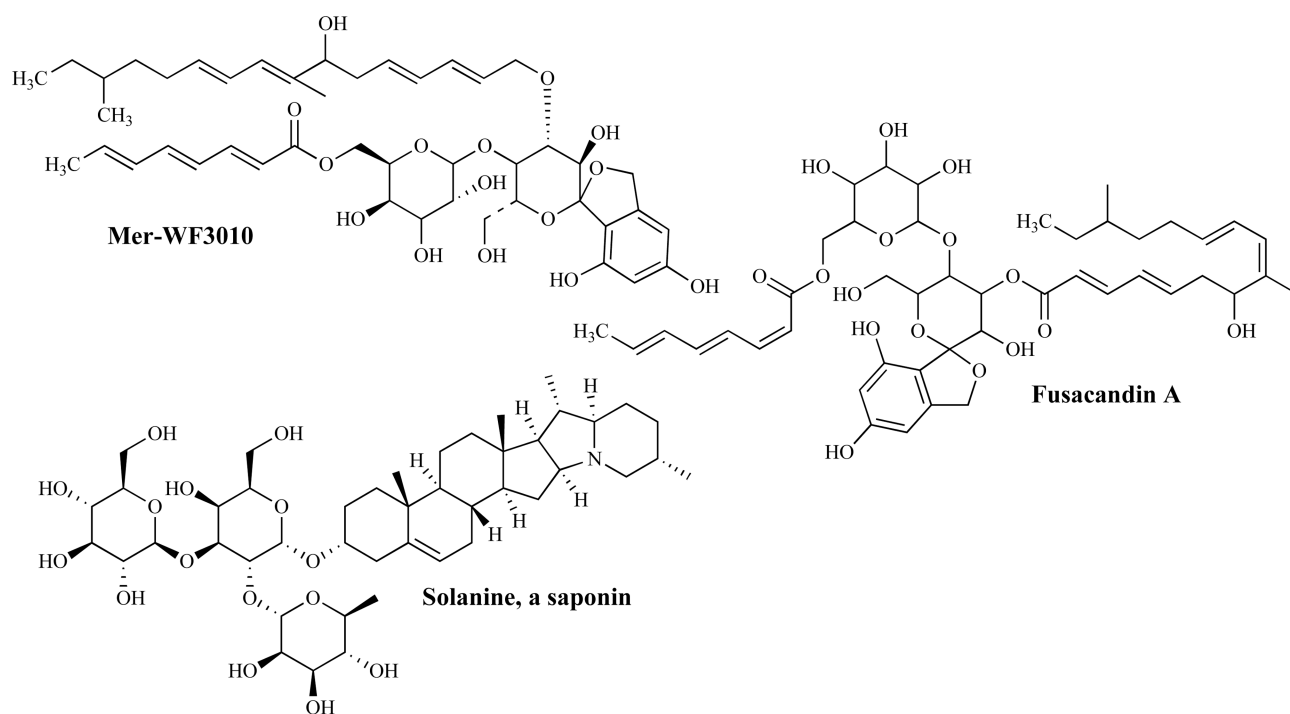
(b).

Fig. (8).
Fig. (a). Inhibitors of Glucan Biosynthesis.
Fig. (b). Inhibitors of Glucan Biosynthesis.
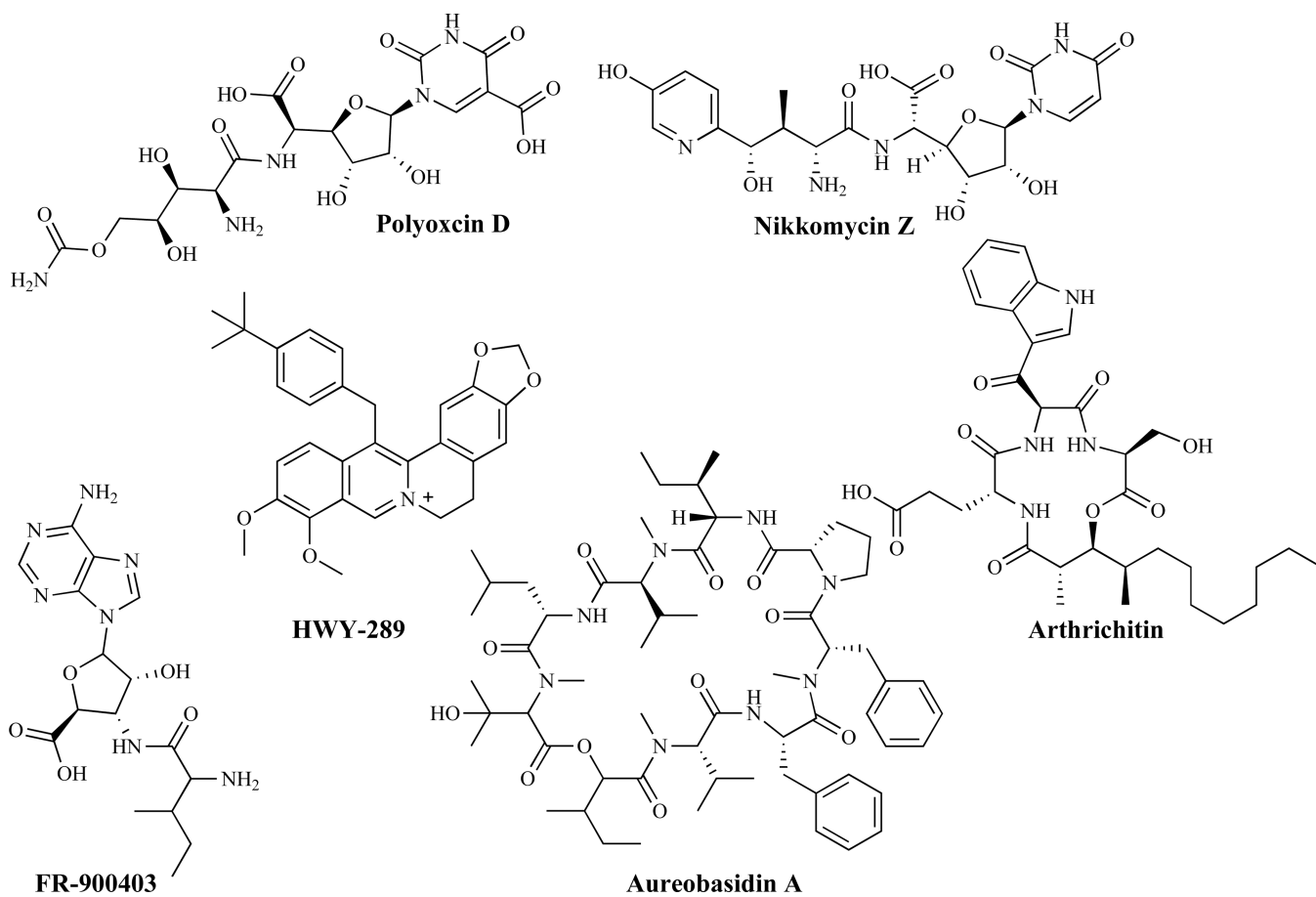
Fig. (9).
Inhibitors of Chitin Biosynthesis.

Benanomicin A Methyl ester

Pradimicin A; R₁ = CH₃, R₂ = NHCH₃

$\text{Pradimicin A; } R_1 = CH_3, R_2 = NHCH_3$

$\text{BMY-28864; } R_1 = CH_2OH, R_2 = N(CH_3)_2$
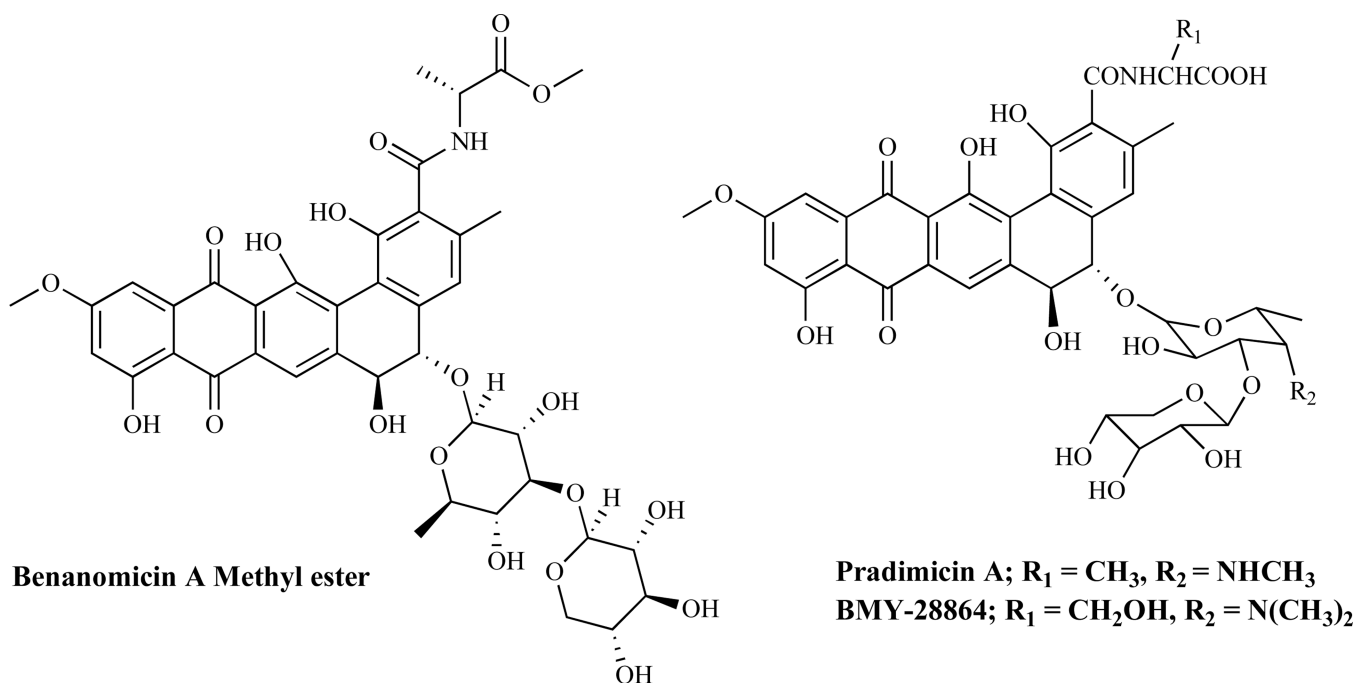
Fig. (10).
Mannoprotein Biosynthesis Inhibitors.
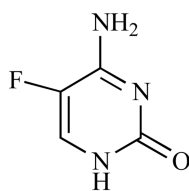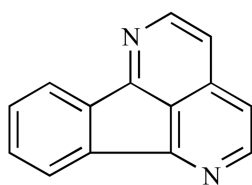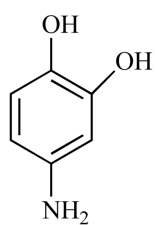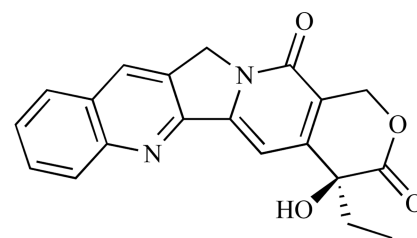
Fig. (11).
Antimetabolites and Topoisomerase Inhibitors.
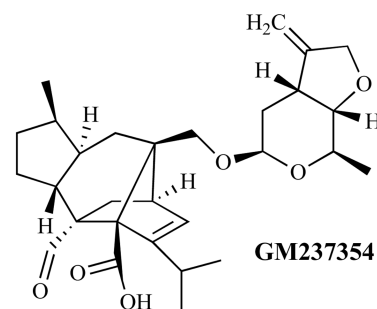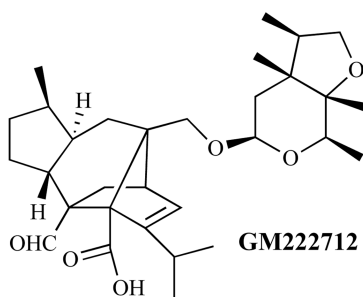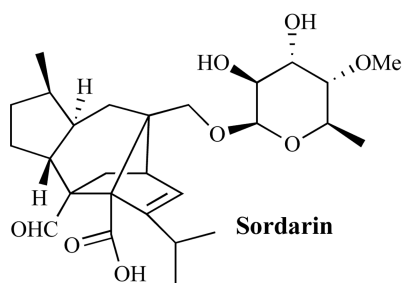
Fig. (12).
Elongation Factor Inhibitors.
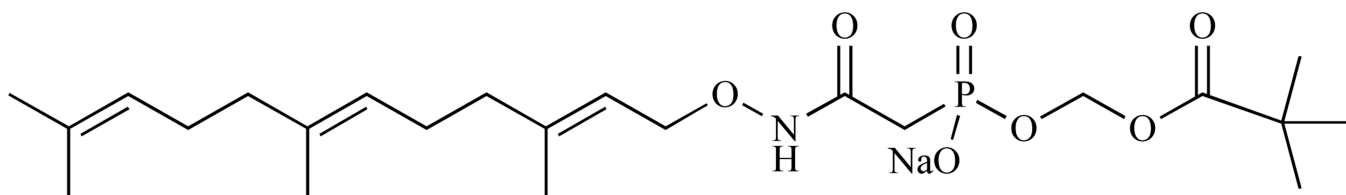
FPT Inhibitor III

Fig. (13).
A Protein-farnesyltransferase Inhibitor
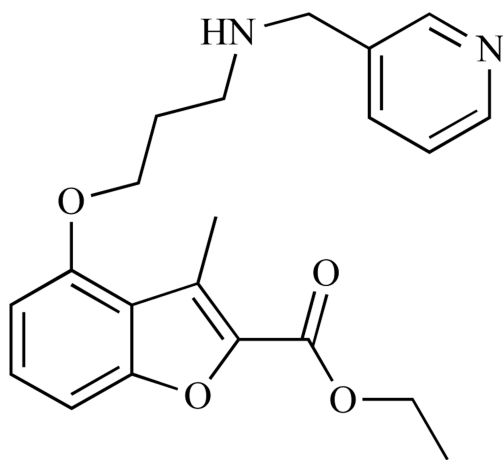
RO-09-4609
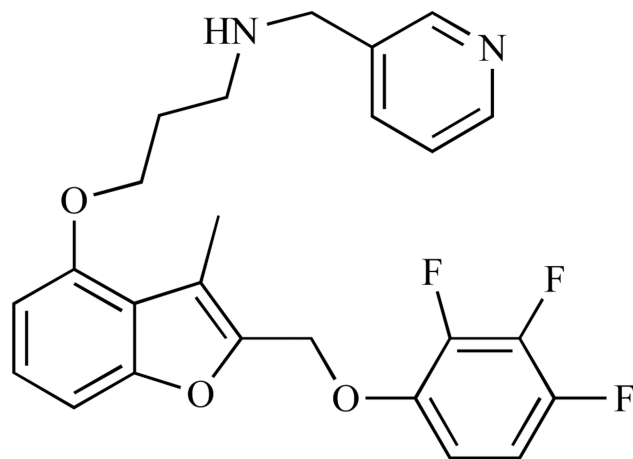
RO-09-4879

Fig. (14).
N-myristoyltransferase (NMT) Inhibitors.

Sampangine **Meridine** **Onychine** **Berberine**
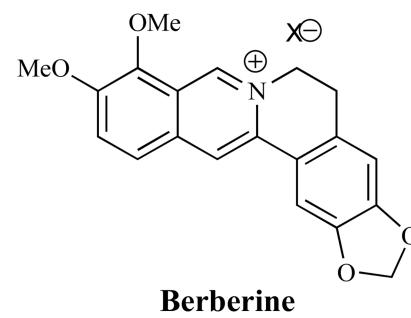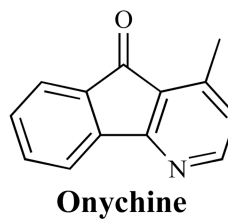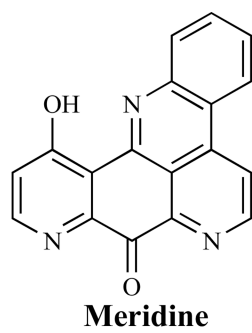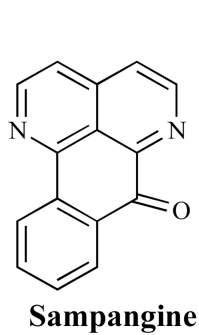
Fig. (15).
Inhibitors of Nucleic Acid Biosynthesis.
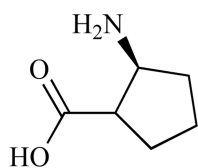
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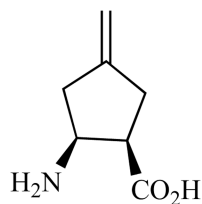
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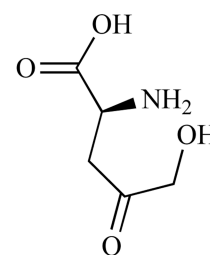
RI-331
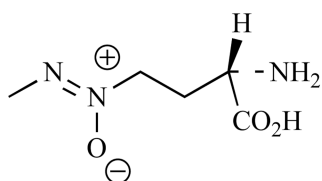
Azoxybacilin
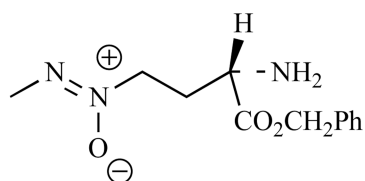
Ro09-1824
[Azoxybacilin Phenylmethyl Ester]
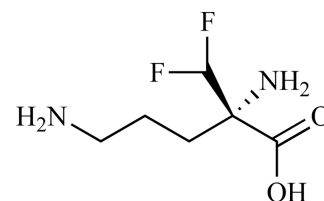
Eflornithine

Fig. (16).
Inhibitors of Amino Acid and Polyamine Biosyntheses.
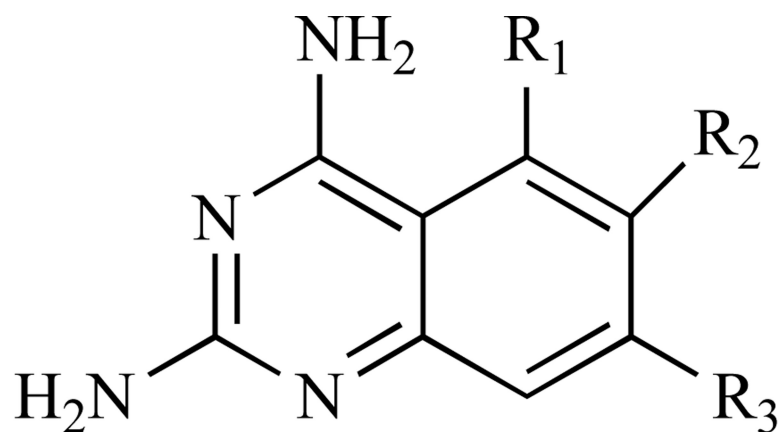
2,4-diaminoquinazolines

R_1 = H, CH_3, CF_3, F, Cl, Br, I, OC_3H_7, OCH_2CF_3, $OCH_2C_2F_5$, $OCH_2C_3F_7$, or SCH_3

R2 = H, CH_3, CF_3, F, Cl, Br, I, or OCH_3

R3 = H, CH_3, CF_3, F, Cl, or Br

Fig. (17).
Inhibitors of Folic Acid Biosynthesis.
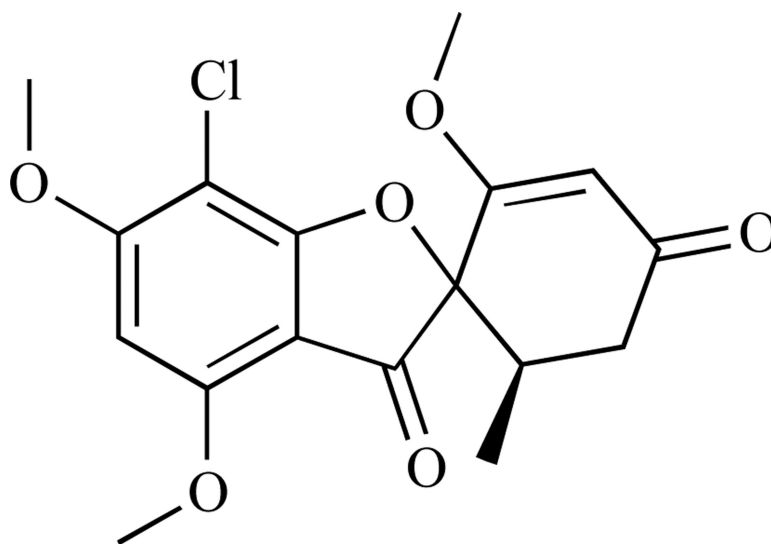
Griseofulvin
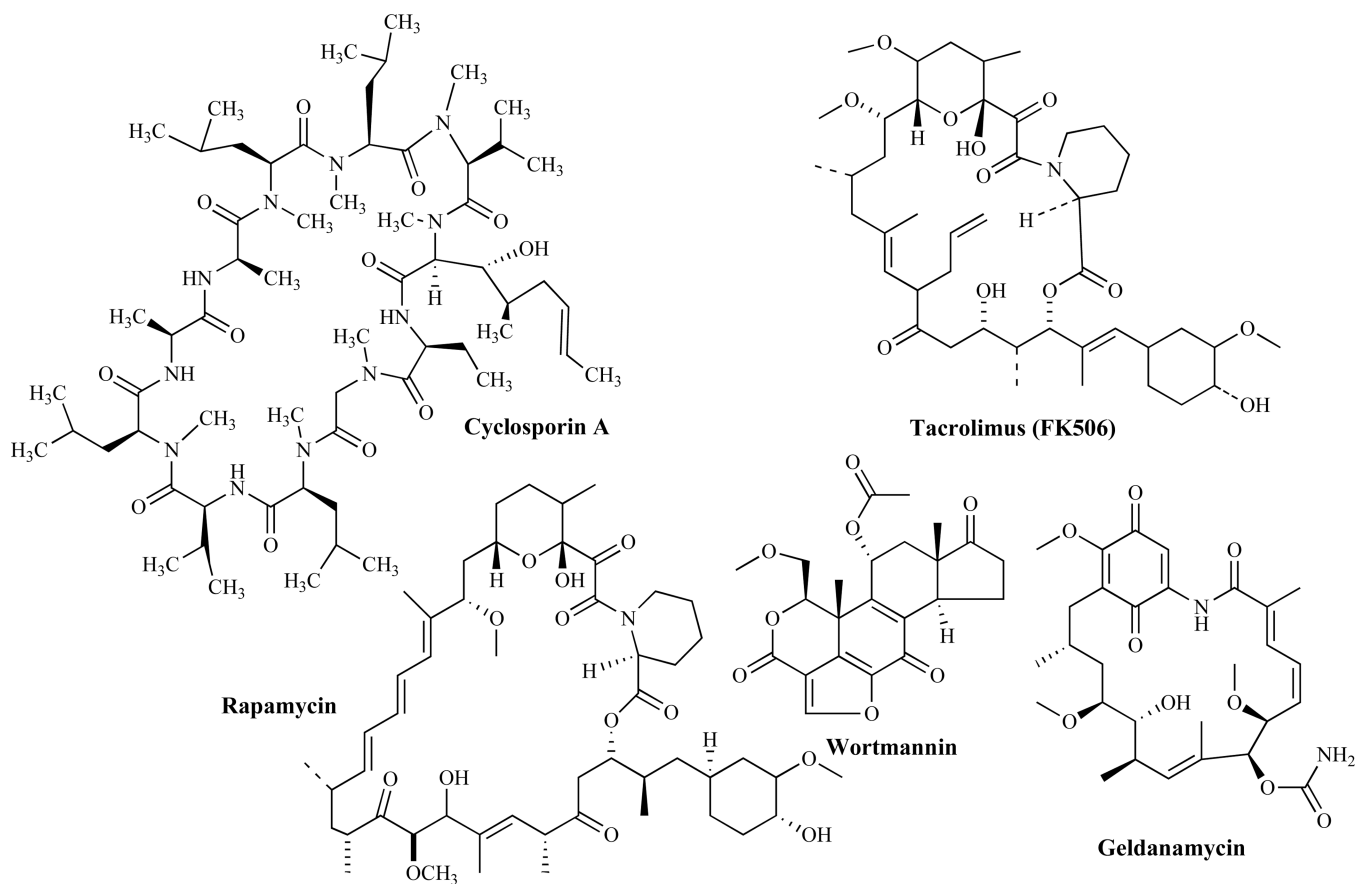
Fig. (18).
An Inhibitor of Microtubule Aggregation.
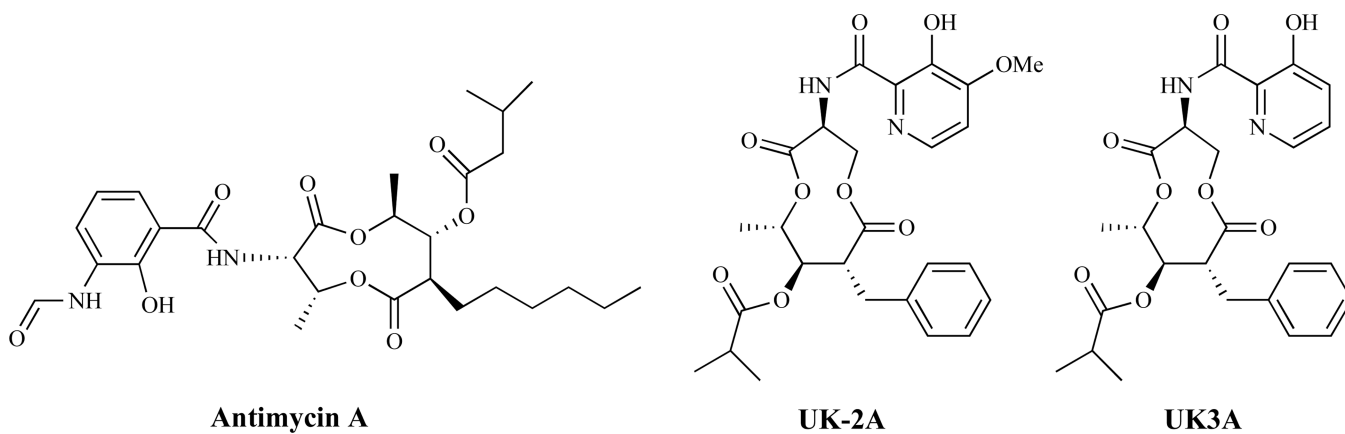
Cyclosporin A

Tacrolimus (FK506)

Rapamycin

Wortmannin

Geldanamycin

Fig. (19).
Inhibitors of Signal Transduction Pathways.

Antimycin A   **UK-2A**   **UK3A**

Fig. (20).
Inhibitors of Electron Transport.

Caprylic acid

Salicylic acid

Undecyclenic acid

Benzoic acid

Sorbic acid

Biotin

Haloprogin

Allicin

Zinc Pyrithione

Fig. (21).
Miscellaneous Antifungal Agents.

Schizotrin A

Cepacidine A1; R = OH
Cepacidine A2; R = H

Leucinostatin A
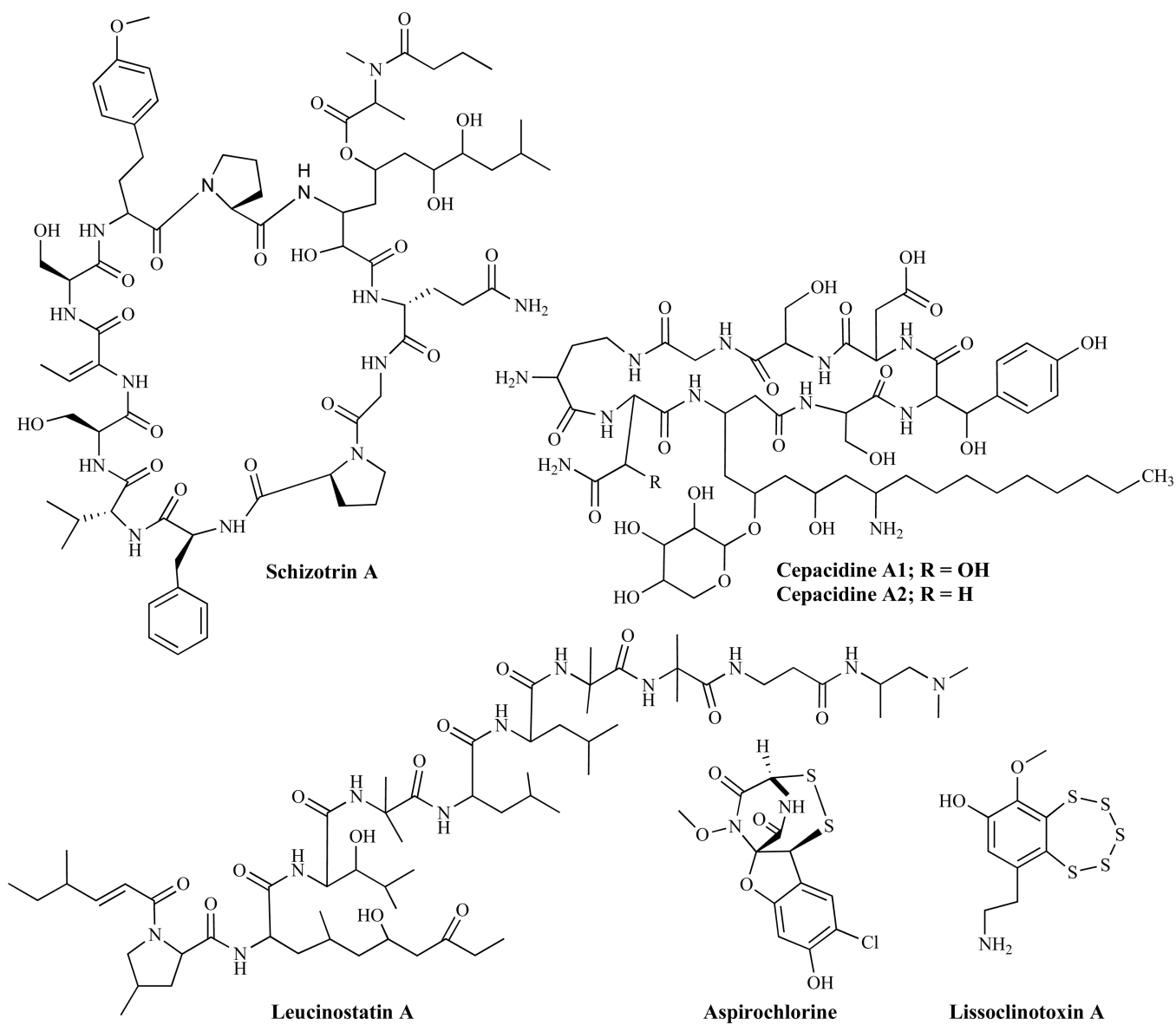
Aspirochlorine

Lissoclinotoxin A
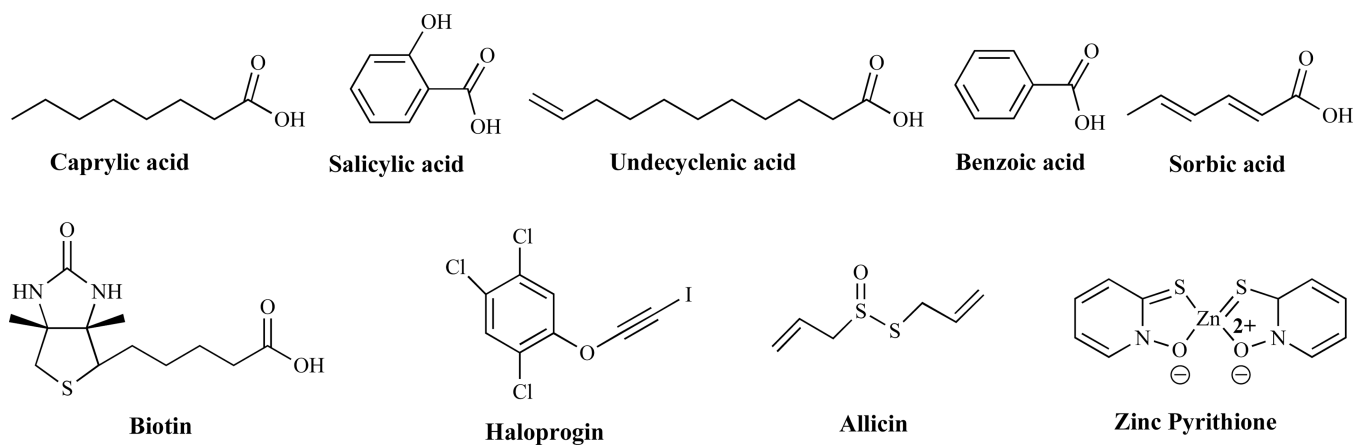
Fig. (22).
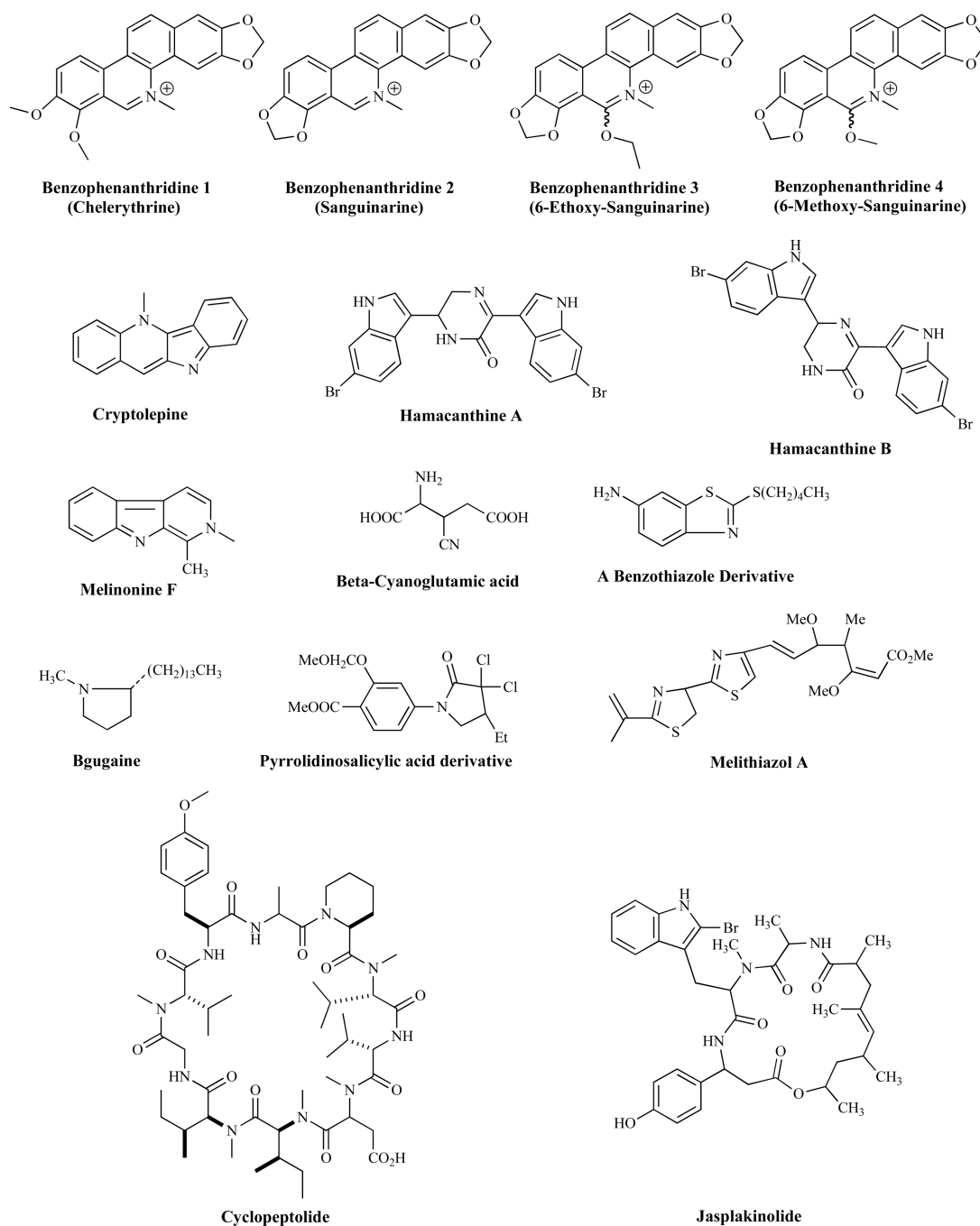Agents Exhibiting Antifungal Activity, with Unknown Mechanism of Action.

Fig. (23).
Agents Exhibiting Antifungal Activity, with Unknown Mechanism of Action.