United States Patent [19]

Bartizal et al.

[11] **Patent Number: 5,026,554**

[45] **Date of Patent: Jun. 25, 1991**

[54] **METHOD OF INHIBITING FUNGAL GROWTH USING SQUALENE SYNTHETASE INHIBITORS**

[75] Inventors: **Kenneth F. Bartizal**, Somerset; **Janet C. Onishi**, Mountainside, both of N.J.

[73] Assignee: **Merck & Co., Inc.**, Rahway, N.J.

[21] Appl. No.: **582,303**

[22] Filed: **Sep. 13, 1990**

[51] **Int. Cl.**[5] **A61K 31/335**

[52] **U.S. Cl.** **424/404**; 424/79; 424/405; 424/409; 514/450; 514/464; 514/824; 549/363; 549/397

[58] **Field of Search** 549/363, 397; 514/450, 514/824, 464; 424/79, 404, 405, 409

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,734,757	5/1973	Wiles et al.	424/404 X
4,790,989	12/1988	Hunter et al.	424/404
4,865,844	9/1989	Blank et al.	424/404 X
4,871,721	10/1989	Biller	514/102
4,915,940	4/1990	Saitoh et al.	424/404 X

OTHER PUBLICATIONS

S. A. Biller et al., *J. Med. Chem.*, 31, 1869, (1988).

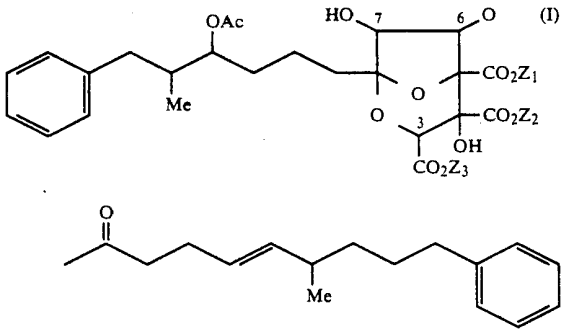
C. D. Poulter et al., *J. Am. Chem. Soc.*, 111, 3734, (1989).

E. J. Corey et al., *J. Am. Chem. Soc.*, 98, 1291, (1976).

Primary Examiner—Thurman Page
Assistant Examiner—Donald R. McPhail
Attorney, Agent, or Firm—Melvin Winokur; Joseph F. DiPrima

[57] **ABSTRACT**

This invention relates to a method of inhibiting fungal growth by employing an antifungal amount of a compound of formula (I):



6 Claims, No Drawings

METHOD OF INHIBITING FUNGAL GROWTH USING SQUALENE SYNTHETASE INHIBITORS

BACKGROUND OF THE INVENTION

Hypercholesterolemia is known to be one of the prime risk factors for ischemic cardiovascular disease, such as arteriosclerosis. Bile acid sequestrants have been used to treat this condition; they seem to be moderately effective but they must be consumed in large quantities, i.e. several grams at a time and they are not very palatable.

MEVACOR® (lovastatin), now commercially available, is one of a group of very active antihypercholesterolemic agents that function by limiting cholesterol biosynthesis by inhibiting the enzyme, HMG-CoA reductase.

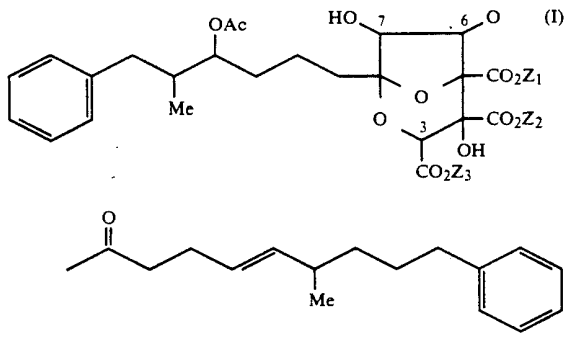
Squalene synthetase is the enzyme involved in the first committed step of the de novo cholesterol biosynthetic pathway. This enzyme catalyzes the reductive dimerization of two molecules of farnesyl pyrophosphate to form squalene. The inhibition of this committed step to cholesterol should leave unhindered biosynthetic pathways to ubiquinone, dolichol and isopentenyl t-RNA.

Previous efforts at inhibiting squalene synthetase have employed pyrophosphate or pyrophosphate analog containing compounds such as those described in P. Ortiz de Montellano et al, J. Med Chem. 20, 243 (1977) and E. J. Corey and R. Volante, J. Am. Chem. Soc., 98, 1291 (1976). S. Biller (U.S. Pat. No. 4,871,721) describes isoprenoid (phosphinylmethyl)phosphonates as inhibitors of squalene synthetase.

The present invention provides nonphosphorus containing inhibitors of squalene synthetase.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to novel compounds of structural formula (I) which are squalene synthetase inhibitors:



wherein Z_1, Z_2 and Z_3 are each independently selected from;

(a) H;

(b) C_{1-5}alkyl;

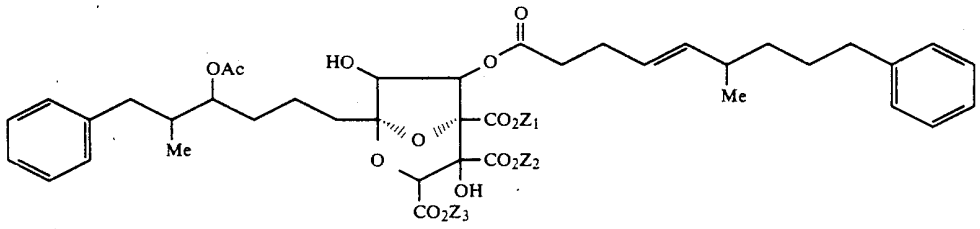
(c) C_{1-5}alkyl substituted with a member of the group consisting of:

(i) phenyl,

(ii) phenyl substituted with methyl, methoxy, halogen (Cl, Br, F, I) or hydroxy; or

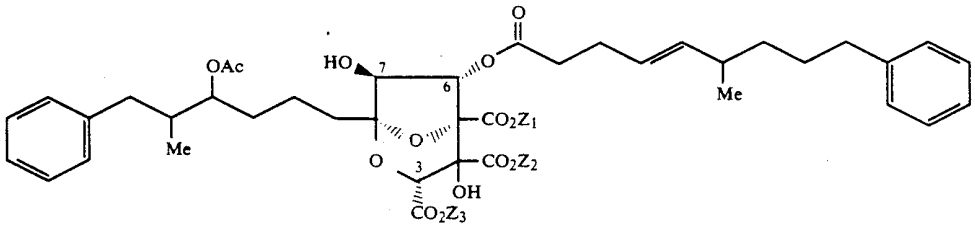
a pharmaceutically acceptable salt of a compound of of formula (I).

In one embodiment of the present invention are those compounds of formula (I) wherein the relative stereochemical configuration of the 2,8-dioxabicyclo[3.2.1]octane ring is as shown below:

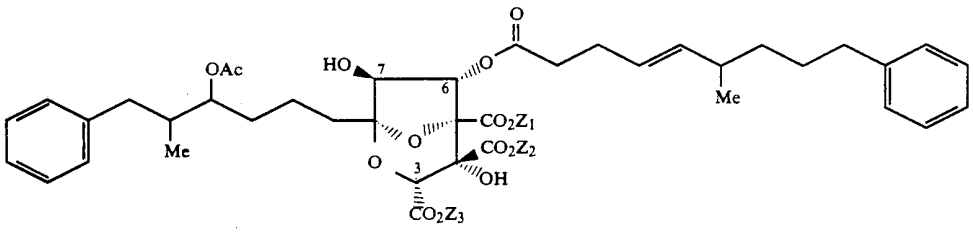


Throughout this specification and claims where stereochemistry is described for the dioxabicyclo[3.2.1]octane ring the configuration implied is relative. The actual configuration may be as shown or that of its enantiomer.

Further illustrating this embodiment are those compounds of structural formula (I) wherein the relative configuration at positions 3,6 and 7 is as shown below:



In one class of this embodiment are those compounds of structure (I) wherein the relative configuration at the 4-position is as shown below:



Exemplifying this class is the compound wherein Z_1, Z_2 and Z_3 are each hydrogen or a pharmaceutically acceptable salt thereof. The compound wherein Z_1, Z_2 and Z_3 are each hydrogen is hereafter referred to as Compound A.

Further illustrating this class are those compounds in which one or more of Z_1, Z_2 or Z_3 is C_{1-5}alkyl or C_{1-5}alkyl substituted with phenyl or substituted phenyl wherein the substituent is methyl, methoxy, halogen or hydroxy. In a specific illustration, Z_1, Z_2 and Z_3 are each methyl. This compound is hereafter referred to as Compound B.

The compounds of formula (I) are prepared in an aerobic fermentation procedure employing a novel fungal culture, MF5465, identified as *Leptodontium elatius*. Although the use of this organism is specifically described herein, other species of the genus Leptodontium including mutants of the above described organism are also capable of producing compounds of this invention.

The culture MF5465 is that of a fungus, a lignicolous Hyphomycete, *Leptodontium elatius*, isolated from wood in the Joyce Kilmer Memorial Forest in North Carolina. This culture has been deposited with the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Md. 20852 as ATCC 74011.

The culture MF5465, identified as *Leptodontium elatius* exhibits the following morphological features.

Colonies attaining 12–15 mm in 7 days on oatmeal agar (Difco), with both aerial and submerged mycelium. Colony surface flat to appressed in side view, minutely velvety with a metallic sheen towards the margins, dull towards the center, hyaline at the margin, but soon becoming pale to dark gray, finally black, often developing olivaceous colors in age, Pallid Neutral Gray, Light Gull Gray, Deep Gull Gray, Dark Gull Gray, Slate-Gray, Deep Olive-Gray, Olive-Gray, (capitalized color names from Ridgway, R. 1912. Color Standards and Nomenclature, Washington, D.C.), with similar reverse pigmentation, without exudates diffusible pigments or odors.

Conidiogenous cells holoblastic, arising as the terminal cells of relatively undifferentiated conidiophores, with tapered, subulate apices, with the conidiogenous loci confined to the extreme apex. Occasionally with undifferentiated conidiogenous loci directly on vegetative hyphae. Developing conidia adhere to conidiophore terminus in a thin, irregular to ladder-like rachis in groups of up to 4–15 conidia. Conidiophores originating as undifferentiated branches at right or subacute angles from vegetative hyphae, gradually elongating, remaining simple or forming 1–3-branch points, usually at right to subacute angles, usually clustered in small groups when viewed from above, 1–3 septate, cylindrical to conical with tapered apices hyaline when young but developing olivaceous to olivaceous gray pigments from the base upward in age, with walls slightly thicker than those of vegetative hyphae, 20–65×3–5 μm. Co- oatmeal, malt extract, or corn meal agar, 3.5–5 μm×1–2 μm, aseptate, smooth, thin-walled, allantoid, suballantoid, to short cylindrical, or narrowly elliptical, often with a small proximal scar or apiculus, without visible slime or gelatinous materials. Hyphae septate, branched, cylindrical or occasionally inflated, up to 5 μm in diameter.

Compounds of this invention can be obtained by culturing the above noted microorganism in an aqueous nutrient medium containing sources of assimilable carbon and nitrogen, preferably under aerobic conditions. Nutrient media may also contain mineral salts and defoaming agents.

The preferred sources of carbon in the nutrient medium are carbohydrates such as glucose, glycerin, starch, dextrin, and the like. Other sources which may be included are maltose, mannose, sucrose, and the like. In addition, complex nutrient sources such as oat flour, corn meal, millet, corn and the like may supply utilizable carbon. The exact quantity of the carbon source which is used in the medium will depend, in part, upon the other ingredients in the medium, but is usually found in an amount ranging between 0.5 and 5 percent by weight. These carbon sources can be used individually in a given medium or several sources in combination in the same medium.

The preferred sources of nitrogen are amino acids such as glycine, methionine, proline, threonine and the like, as well as complex sources such as yeast extracts (hydrolysates, autolysates), dried yeast, tomato paste, soybean meal, peptone, corn steep liquor, distillers solubles, malt extracts and the like. Inorganic nitrogen sources such as ammonium salts (eg. ammonium nitrate, ammonium sulfate, ammonium phosphate, etc.) can also be used. The various sources of nitrogen can be used alone or in combination in amounts ranging between 0.2 to 90 percent by weight of the medium.

The carbon and nitrogen sources are generally employed in combination, but need not be in pure form. Less pure materials which contain traces of growth factors, vitamins, and mineral nutrients may also be used. Mineral salts may also be added to the medium such as (but not limited to) calcium carbonate, sodium or potassium phosphate, sodium or potassium chloride, magnesium salts, copper salts, cobalt salts and the like. Also included are trace metals such as manganese, iron, molybdenum, zinc, and the like. In addition, if necessary, a defoaming agent such as polyethylene glycol or silicone may be added, especially if the culture medium foams seriously.

The preferred process for production of compounds of this invention consists of inoculating spores or mycelia of the producing organism into a suitable medium and then cultivating under aerobic condition.

The fermentation procedure generally is to first inoc-

medium and to obtain, sometimes through a two step process, growth of the organisms which serve as seeds in the production of the active compounds. After inoculation, the flasks are incubated with agitation at temperatures ranging from 20° to 30° C., preferably 25° to 28° C. Agitation rates may range up to 400 rpm, preferably 200 to 220 rpm. Seed flasks are incubated over a period of 2 to 10 days, preferably 2 to 4 days. When growth is plentiful, usually 2 to 4 days, the culture may be used to inoculate production medium flasks. A second stage seed growth may be employed, particularly when going into larger vessels. When this is done, a portion of the culture growth is used to inoculate a second seed flask incubated under similar conditions but employing shorter time.

After inoculation, the fermentation production medium is incubated for 3 to 30 days, preferably 4 to 14 days, with or without agitation (depending on whether liquid or solid fermentation media are employed). The fermentation is conducted aerobically at temperatures ranging from 20° to 40° C. If used, agitation may be at a rate of 200 to 400 rpm. To obtain optimum results, the temperatures are in the range of 22° to 28° C., most preferably 24° to 26° C. The pH of the nutrient medium suitable for producing the active compounds is in the range of 3.5 to 8.5, most preferably 5.0 to 7.5. After the appropriate period for production of the desired compound, fermentation flasks are harvested and the active compound isolated.

An alcoholic solvent is employed to extract a compound of this invention from the solid fermentation medium. The preferred solvent for extraction of the solid fermentation is methanol. The mixture of alcoholic solvent and fermentation broth is vigorously stirred and filtered, and water added to the filtrate. The aqueous methanol extract is then adsorbed on an anion exchange resin. The preferred resin is Dowex-1 (Cl^-). The active compound can be eluted from Dowex-1 using a high salt eluant; the preferred eluant is 3% ammonium chloride in 90% methanol/water. After elution from the ion exchange resin, the active compound may be recovered from the eluate by diluting the eluate with water, lowering the pH to 2.5, and extracting into an organic solvent; the preferred solvent for extraction is dichloromethane. The organic extract is then evaporated to afford partially purified active compound.

The active compound is further purified by chromatographic separation which may be carried out by employing reverse phase chromatagraphy. The preferred adsorbent for this chromatography is a C8 bonded phase silica gel. The preferred eluant for reverse phase chromatography is a mixture of acetonitrile and water buffered at a low pH, such as 0.1% phosphoric acid, or trifluoroacetic acid.

The present invention is also directed to a method of inhibiting cholesterol biosynthesis which comprises the administration to a subject in need of such treatment a nontoxic therapeutically effective amount of a compound represented by structural formula (I) and pharmaceutically acceptable salts thereof. Specifically, the compounds of this invention are useful as antihypercholesterolemic agents for the treatment of arteriosclerosis, hyperlipidemia, familial hypercholesterolemia and the like diseases in humans. They may be administered orally or parenterally in the form of a capsule, a tablet, an injectable preparation or the like. It is usually desirable to use the oral route. Doses may be varied, depending on the age, severity, body weight and other conditions of human patients, but daily dosage for adults is within a range of from about 20 mg to 2000 mg (preferably 20 to 100 mg) which may be given in two to four divided doses. Higher doses may be favorably employed as required.

The pharmaceutically acceptable salts of the compounds of this invention include those formed from cations such as sodium, potassium, aluminum, calcium, lithium, magnesium, zinc, and from bases such as ammonia, ethylenediamine, N-methyl-glucamine, lysine, arginine, ornithine, choline, N,N'-dibenzylethylenediamine, chloroprocaine, diethanolamine, procaine, N-benzylphenethylamine, diethylamine, piperazine, tris(hydroxymethyl)aminomethane, and tetramethylammonium hydroxide. The salts included herein encompass those wherein one, two or all three of the carboxyl groups are in the salt form.

The compounds of this invention may also be coadministered with pharmaceutically acceptable nontoxic cationic polymers capable of binding bile acids in a non-reabsorbable form in the gastrointestinal tract. Examples of such polymers include cholestyramine, colestipol and poly[methyl-(3-trimethylaminopropyl)iminotrimethylene dihalide]. The relative amounts of the compounds of this invention and these polymers is between 1:100 and 1:15,000.

The intrinsic squalene synthetase inhibitory activity of representative compounds of this invention was measured by the standard in vitro protocol described below:

Preparation of Microsomes

Male, Charles River CD rats (120 to 150 g) were fed a diet containing 0.1% lovastatin for 4 days. The livers from these rats were homogenized in 5 volumes (ml/g) of ice cold 50 mM HFPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), 5 mM EDTA(ethylenediaminetetraacetic acid) pH 7.5 with a Potter-Elvehjem type tissue grinder. The homogenate was centrifuged twice at 20,000×g for 15 minutes at 4° C., discarding the pellet each time. The supernatant was then centrifuged at 100,000×g for 1 hour at 4° C. The resulting microsomal pellet was resuspended in a volume of the above homogenizing buffer equal to one-fifth the volume of the original homogenate. This microsomal preparation has a protein concentration of about 7 mg/ml. The microsomal suspensions were stored in aliquots at −70° C. Squalene synthetase activity in these aliquots is stable for at least several months.

Partial Purification of Prenyl Transferase

Prenyl transferase was purified to use in the enzymatic synthesis of radiolabelled farnesyl pyrophosphate. Prenyl transferase was assayed by the method of Rilling (Methods in Enzymology 110, 125–129 (1985)) and a unit of activity is defined as the amount of enzyme that will produce 1 μ mole of farnesyl pyrophosphate per minute at 30° C. in the standard assay.

The livers of 23 forty-day old male rats that had been fed 5% cholestyramine plus 0.1% lovastatin were homogenized in a Waring blender in 1 liter of 10 mM mercaptoethanol, 2 mM EDTA, 25 μM leupeptin, 0.005% phenylmethyl sulfonyl fluoride pH 7.0 containing 0.1 trypsin inhibitor units of aprotinin/ml. The homogenate was centrifuged at 20,000×g for 20 minutes. The supernatant was adjusted to pH 5.5. with 6N HOAc and centrifuged at 100,000×g for 1 hour. This supernatant was adjusted to pH 7.0 with 3N KOH and a 35–60% ammonium sulfate fraction taken. The 60%

pellet was redissolved in 60 ml of 10 mM potassium phosphate, 10 mM mercaptoethanol, 1 mM EDTA pH 7.0 (Buffer A) and dialyzed against two 1 liter changes of Buffer A. This dialyzed fraction was applied to a 12.5×5 cm column of DEAE-sepharose 4B equilibrated with Buffer A. The column was washed with 700 ml of Buffer A and a 1 liter gradient from Buffer A to 100 mM potassium phosphate, 10 mM mercaptoethanol, 1 mM EDTA pH 7.0. Fractions having a specific activity greater than 0.20 units/mg were combined, solid ammonium sulfate was added to bring to 60% saturation and pelleted. The pellet was dissolved in 8 ml of 10 mM Tris, 10 mM β-mercaptoethanol pH 7.0 (Buffer B). The redissolved pellet was taken to 60% saturation with ammonium sulfate by adding 1.5 volumes of saturated ammonium sulfate in Buffer B. This ammonium sulfate suspension contained 3.5 units/ml with a specific activity of 0.23 units/mg and was free of isopentenyl pyrophosphate isomerase activity. This ammonium sulfate suspension was used for the synthesis of [4-^{14}C]farnesyl-pyrophosphate and its activity was stable stored at 4° C. for at least 6 months.

Enzymatic Synthesis of [4-^{14}C]farnesyl-pyrophosphate

The solvent (ethanol: 0.15N NH_4OH, 1:1) was removed from 55 μCi of [4-^{14}C]isopentenyl pyrophosphate(47.9 μCi/μmole) by rotary evaporation. Six hundred microliters of 100 mM Tris, 10 mM $MgCl_2$, 4 mM dithiothreitol pH 7.5 was added and the solution was transferred to a 1.5 ml Eppendorf centrifuge tube. Geranyl-pyrophosphate, 250 μl of a 20 mM solution, and 50 μl of the ammonium sulfate suspension of prenyl transferase were added to initiate the reaction. This incubation contained 5 μmoles of geranyl pyrophosphate, 1.15 μmoles of isopentenyl pyrophosphate, 6 μmoles of $MgCl_2$ of 0.18 units of prenyl transferase in a volume of 900 μl. The incubation was conducted at 37° C. During the incubation, the mix turned cloudy white as the newly formed magnesium complex of farnesyl pyrophosphate precipitated out of solution. The [4-^{14}C]farnesyl pyrophosphate was collected by centrifugation for 3 minutes at 14,000 rpm in an Eppendorf centrifuge tube, the supernatant removed, and the pellet was dissolved in 1.0 ml of 50 mM HEPES, 5 mM EDTA, pH 7.5 . The yield was 50.7 μCi (92%) of [4-^{14}C]farnesyl pyrophosphate. The [4-^{14}C]farnesyl pyrophosphate was stored in aliquots at −70° C.

Squalene Synthetase Assay

Reactions were performed in 16×125 mm screw cap test tubes. A batch assay mix was prepared from the following solution:

		μl per assay	volume for 50 assays
1.	250 mM Hepes pH 7.5	20	1000
2.	NaF 110 mM	10	500
3.	$MgCl_2$ 55 mM	10	500
4.	Dithiothreitol 30 mM	10	500
5.	NADPH 10 mM (made fresh)	10	500
6.	[4-^{14}C]farnesyl-pyrophosphate 47.9 μCi/μmole, and 0.025 μCi/3.0 μl	3.0	150
7.	H_2O	24	1200

This assay mix was degassed under a vacuum and flushed with N_2. Solutions of the squalene synthetase inhibitors were prepared either in DMSO or MeOH and a 1:120 dilution of the microsomal protein was made with the original homogenizing buffer. For each reaction, 87 μl of the assay mix was taken with 3 μl of an inhibitor solution (DMSO or MeOH in the controls), warmed to 30° C. in a water bath and then the reaction was initiated by the addition of 10 μl of the 1:120 dilution of microsomal protein (0.6 μg protein total in the assay). The reactions were stopped after 20 minutes by the addition of 100 μl of a 1:1 mix of 40% KOH with 95% EtOH. The stopped mix was heated at 65° C. for 30 minutes, cooled, 10 ml of heptane was added and the mix was vortexed. Two g of activated alumina was then added, the mix vortexed again, the alumina allowed to settle and 5 ml of the heptane layer was removed. Ten ml of scintillation fluid was added to the heptane solution and radioactivity was determined by liquid scintillation counting.

Percent inhibition is calculated by the formula:

$$\left[1 - \frac{[\text{Sample} - \text{Blank}]}{[\text{Control} - \text{Blank}]}\right] \times 100$$

IC_{50} values were determined by plotting the log of the concentration of the test compound versus the percentage inhibition. The IC_{50} is the concentration of inhibitor that gives 50% inhibition as determined from these plots.

Representative of the intrinsic squalene synthetase inhibitory activities of the compounds of this invention is the IC_{50} data tabulated below:

Compound	Squalene Synthetase IC_{50}
Compound A	9 nM

The present compounds also demonstrate broad spectrum antifungal activity as determined by broth and agar dilution methods. The compounds are particularly active towards filamentous fungi and yeasts including *Candida albicans* and *Cryptococcus neoformans*. The sensitivity of filamentous fungi and yeast was determined using inhibitor dilution assays in microtiter format. The compounds were dissolved in DMSO at 2 mg/ml and serially diluted in 0.1M phosphate buffer, pH 7.0 in the microtiter dish from 100 to 0.006 μg/ml. A standardized spore suspension for testing the filamentous fungi was prepared by inoculating Antibiotic Medium #3 containing 1.5% agar with spores such that 1.5×10^3 colony forming units were added per well. The microtiter wells were filled with 50 μl of buffer containing compound and 50 μl of inoculated medium.

The sensitivity of yeasts was determined by inoculating yeast nitrogen base containing 1% dextrose (YNB/G) with aliquots of an overnight yeast culture grown in Yeast Morphology (YM) media at 35° C. and diluting in YNB/G to yield a final concentration of $1.5\text{–}7.5\times10^3$ colony forming units/well. To test the sensitivity of yeast, compound was solubilized in 10 percent aqueous DMSO at 2.56 mg/ml. The compound was diluted serially in YNB/G from 128 to 0.06 μg/ml and further diluted 1:10 in YNB/G. The wells were filled with 150 μl of media containing drug. The minimum inhibitory concentration (MIC) is defined as the lowest concentration to prevent growth after an incubation for 42 hours, at 28° C. for the filamentous fungi and 24 to 48 hours, at 35° C. for the yeasts. Representative

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.