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Synthesis and biological evaluation of antifungal derivatives of enfumafungin as orally bioavailable inhibitors of β-1,3-glucan synthase

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In dedication to Professor Timothy Macdonald for his many contributions to the field of Organic and Medicinal Chemistry

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ABSTRACT

Orally bioavailable inhibitors of β-(1,3)-D-glucan synthase have been pursued as new, broad-spectrum fungicidal therapies suitable for treatment in immunocompromised patients. Toward this end, a collaborative medicinal chemistry program was established based on semisynthetic derivatization of the triterpenoid glycoside natural product enfumafungin in order to optimize in vivo antifungal activity and oral absorption properties. In the course of these studies, it was hypothesized that the pharmacokinetic properties of the semisynthetic enfumafungin analog **3** could be improved by tethering the alkyl groups proximal to the basic nitrogen of the C3-aminoether side chain into an azacyclic system, so as to preclude oxidative N-demethylation. The results of this research effort are disclosed herein.

The widespread medical use of immunosuppressive drug therapies and broad-spectrum antibacterial agents, in conjunction with the rise of viral infections that debilitate the human immune system such as AIDS, have contributed to an increasing annual death rate due to fungal infections (mycoses) over the last 30 years. During this same time period, frequent and indiscriminate use of antibacterial[1] and antifungal agents has led to increased antimicrobial resistance.[2] This alarming scenario has prompted a search for new, safe and effective human antifungal drugs.[3]

The semisynthetic antifungal drug CANCIDAS (caspofungin acetate)[4] was first approved by the US FDA in 2001 for invasive aspergillosis in patients who are intolerant of standard therapy. The drug is generally effective against systemic fungal infections in immunocompromised patients and is the primary treatment for a variety of *Candida* infections. CANCIDAS is derived from the echinocandin macrocyclic lipopeptide natural product, pneumocandin B_0.[5] The potent and broad-spectrum antifungal activity of the echinocandin family is attributed to inhibition of fungal cell wall synthesis via blockade of the biosynthesis of β-(1,3)-D-glucan, an essential cell wall component of pathogenic fungi.[2a] Given that the molecular target of CANCIDAS, β-1,3-D-glucan synthase, is unique to lower eukaryotes, antifungal agents that share this fungal-specific mode of action tend to exhibit low toxicity. Unfortunately, echinocandins and derivatives thereof are poorly absorbed when administered orally[6] and, therefore, are used only for parenteral administration. For this reason, *orally bioavailable* inhibitors of β-1,3-D-glucan synthase have, in recent years, been pursued[3b,c] as new, broad-spectrum fungicidal therapies suitable for treatment in immunocompromised patients.

Natural product screening efforts by Onishi and co-workers[7] identified four acidic terpenoid β-1,3-D-glucan synthase inhibitors that offer improved pharmacokinetic properties compared to those of the echinocandin-type lipopeptides. The in vitro antifungal activity of one of the screening hits, the triterpenoid glycoside enfumafungin (**1**, Fig. 1), was comparable to that of caspofungin acetate, particularly with regard to potency against the *Candida* and *Aspergillus* pathogenic fungal strains.[4,7] Moreover, in whole cells of *Candida albicans*, **1** selectively inhibits the incorporation of [^{14}C]-glucose into insoluble cell wall glucan without affecting

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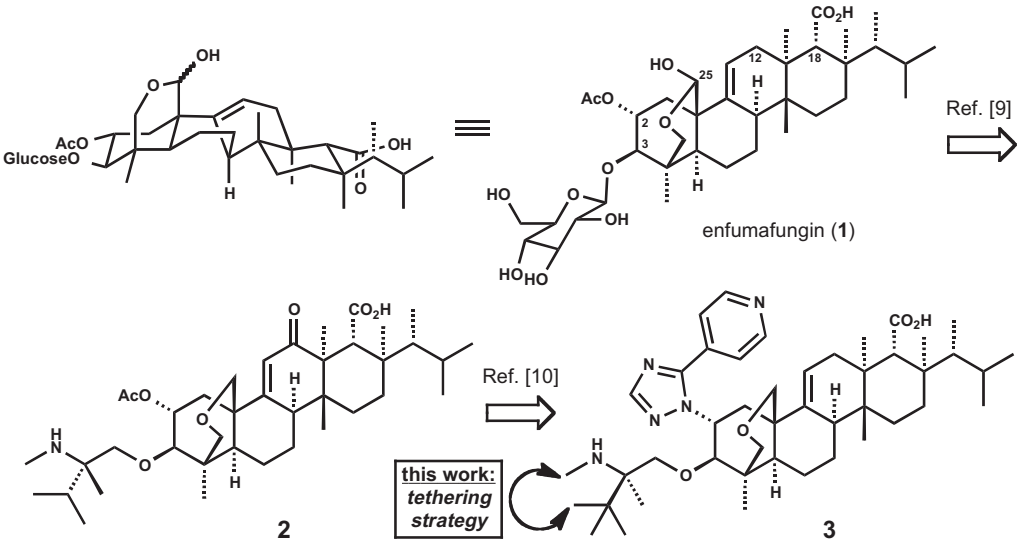


Figure 1. The triterpenoid glycoside, enfumafungin (**1**), and semisynthetic, orally bioavailable antifungal derivatives (**2**–**3**).

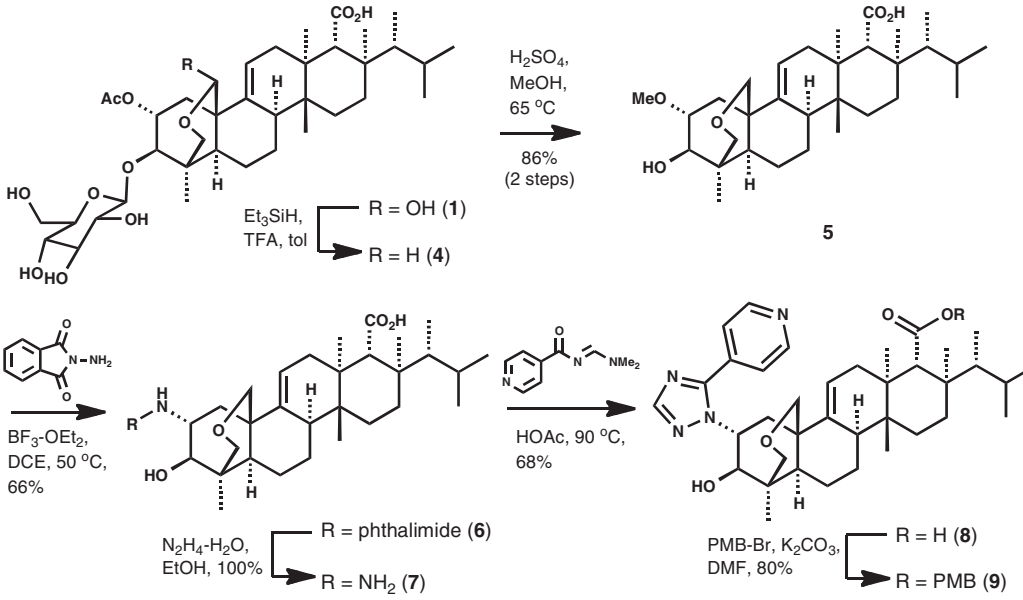
other components of the fungal cell wall, indicating that **1** is a specific inhibitor of glucan synthesis with an IC_{50} value that is similar in magnitude to the MIC.[7] The primary structure and relative stereochemistry of enfumafungin were determined mainly by detailed spectroscopic (two-dimensional NMR) studies and derivatization techniques.[8] The defining structural feature of the aglycone portion of **1** is the hemiacetal cyclization of C23–25 across the A ring (on the β-face of the molecule), which gives rise to interconverting diastereomers at the anomeric carbon position, C25. It was found that this unique bridging hemiacetal system enables a cationic S_N1-type substitution reaction to occur at C2 (vida infra), providing convenient and stereoselective access to A-ring-functionalized derivatives.

We, in collaboration with Merck Research Laboratories, established a medicinal chemistry program based on semisynthetic derivatization of the terpenoid natural product enfumafungin in order to modulate and optimize in vivo antifungal activity and oral absorption properties.[9] In the course of our work, chemical modification of C2, C3, C12, C18 and C25 was achieved and the resulting analogs were evaluated for in vitro antifungal activity and for oral efficacy in a mouse model of disseminated candidiasis. It was found that 12-oxo-25-deoxy derivatives bearing an ethanolamine-based side chain appended via an ether linkage to C3 (e.g., **2**) were efficacious in a candidiasis model when delivered orally.[9] Preliminary structure–activity relationship (SAR) studies indicated that oral activity is strongly dependent upon the nature of the steric environment proximal to the basic amino group of the C3 side chain. Eventually, by incorporating various heteroaryl systems at C2 on the α-face of the terpenoid framework, a series emerged with broad-spectrum fungicidal activity and markedly improved in vitro potency,[10] even in the absence of the 12-oxo moiety. In light of the previously observed sensitivity of oral efficacy and bioavailability to the steric nature of the C3-aminoether side chain, we hypothesized that the pharmacokinetic properties of the lead compound **3** could be improved by tethering the alkyl groups (designated in Fig. 1 with arrows) proximal to the basic nitrogen into an azacyclic system, so as to preclude oxidative N-demethylation. The results of this research effort are dislosed herein.

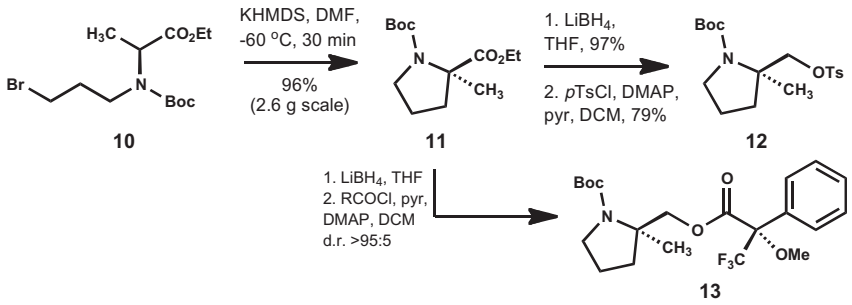
As depicted in Scheme 1, the epimeric C25 lactol of enfumafungin was removed by ionic reduction.[9,10] Although it is known that the cardenolide glycoside digoxin is therapeutically effective through an oral route of administration, enfumafungin does not exhibit an acceptable pharmacokinetic profile as a C3-glucoside. We therefore elected to replace the β-D-glucose moiety with a chemically and metabolically stable system that was more likely to confer oral bioavailability, a primary goal of the program. Acidic methanolysis of the glycoside linkage resulted in conversion to the 2α-methoxy-3β-hydroxy aglycone **5**. As alluded to above, the cationic substitution reaction at C2 is thought to be facilitated by the proximal bridging ether that simultaneously stabilizes a transient cationic species and sterically blocks the β-face, resulting in nucleophilic attack from the concave α-face. This unique reactivity pattern is exploited again in the subsequent operation wherein exposure of **5** to Lewis acidic conditions in the presence of N-aminophthalimide followed by treatment of the adduct with hydrazine secures the 2α-hydrazino intermediate **7**.[10] Heating of **7** with the isonicotinamide–formamidine then fashions the C2 triazole heteroaryl system and the resultant carboxylic acid intermediate (**8**) was protected as the corresponding C18 *para*-methoxybenzyl ester (**9**). It is notable in this sequence that the stereoselective hydrazine installation and heterocycle formation are conducted efficiently in the presence of unprotected hydroxyl and carboxylic acid functionality.[10]

The tethering approach encapsulated in Figure 1 suggested to us that a cyclic pyrrolidine system would be a beneficial motif for incorporation into the enfumafungin C3 side chain. Simple pyrrolidine systems were accessed by conversion of the enantiomeric forms of *N*-Boc-prolinol to their corresponding tosylates in preparation for a subsequent C3 O-alkylation condensation step with intermediate **9**. The requisite prolinol intermediates bearing a quaternary stereocenter adjacent to nitrogen were obtained as single stereoisomers by implementation of the 'memory of chirality' method of Kawabata.[11] Readily available *(S)*-alanine ethyl ester was converted into the *N*-Boc-*N*-(3-bromopropyl)-alanine derivative **10** (Scheme 2) in three straight-forward operations.[11a] The enantioselective cyclization of **10** proceeded in 96% yield on multi-gram scale to afford *N*-Boc-α-methyl proline ethyl ester (**11**) with exquisite enantioselectivity as determined by a Mosher ester analysis (cf. **11**→**13**). The cyclization is thought to proceed through the intermediacy of an axially chiral nonracemic enolate that undergoes intramolecular alkylation with a total retention of configuration.[11] Two additional steps generated the neopentyl tosylate electrophilic building block **12** in enantiomerically pure form.

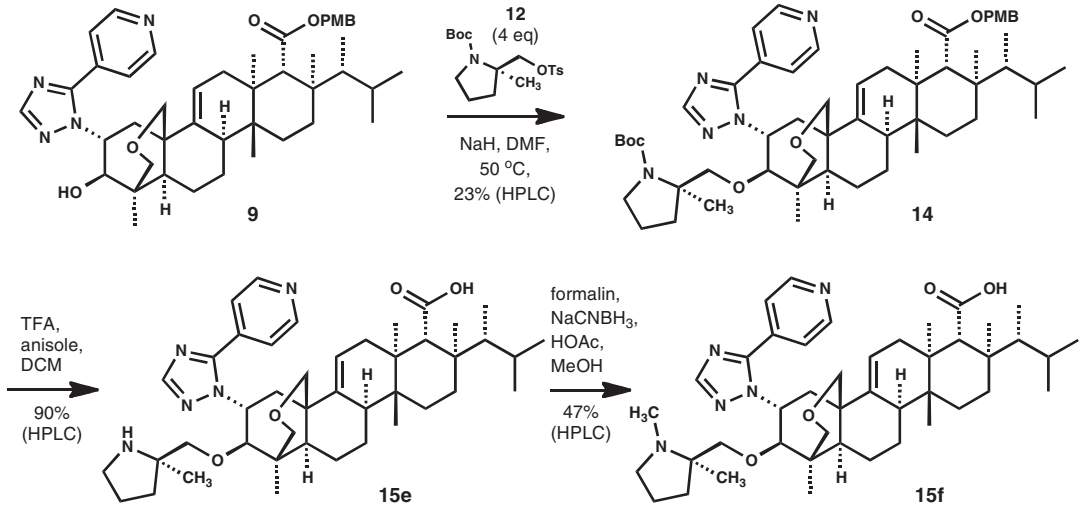
Completion of the partial synthesis of final compounds **15e** and **15f** (from **1**) is outlined in Scheme 3. Antifungal derivatives **15a–d**



Scheme 1. Semisynthetic preparation of the 2α-heteroaryl terpenoid derivative **9** from enfumafungin (**1**).



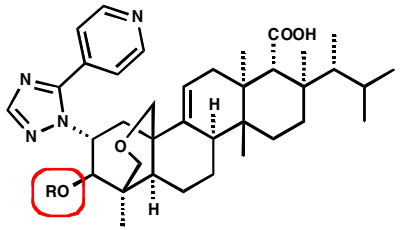
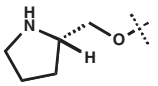
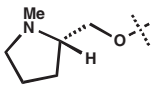
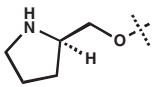
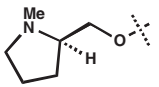
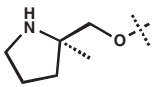
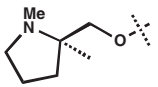
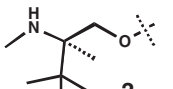
Scheme 2. Enantiospecific construction of an α-methyl prolinol building block (**12**) for eventual incorporation into the C3-aminoether side chain of semisynthetic enfumafungin derivatives.



Scheme 3. Completion of antifungal synthetic targets **15e–f**.

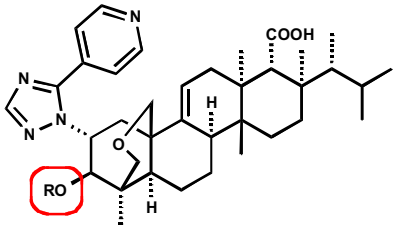
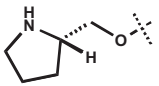
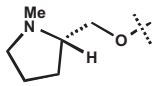
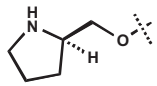
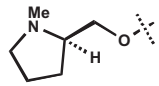
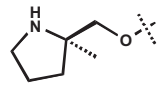
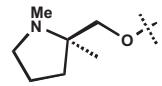
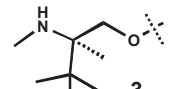
were obtained in a similar fashion.[10] The intermolecular C3 O-alkylation reaction between terpenoid derivative **9** and tosylate **12** was conducted using sodium hydride as a base under thermally forcing conditions. The modest isolated yield[12] is attributed to the relatively unreactive nature of the neopentyl tosylate electrophile due to steric hindrance. Utilization of *N*-Boc-α-methyl prolinol iodide or triflate species in the O-alkylation reaction returned only unreacted **9**, presumably due to decomposition of the electrophile

Table 1
In vitro inhibition of (1,3)-glucan synthase and antifungal activity against *C. albicans*

		 15a	 15b	 15c	 15d	 15e	 15f	 3
GS IC_{50}[a] (ng/mL)		18	61	22	19	4.0	6.4	2.0
MIC (ng/mL) *C. albicans*		1000	1000	500	500	125	125	30

[a] See Ref. 13 for assay conditions.

Table 2
Oral efficacy (TOKA) data and pharmacokinetic profiling (murine).

		 15a	 15b	 15c	 15d	 15e	 15f	 3
TOKA[a]								
Δ log CFU:								
at 12.5 mpk/IP		−2.18	N/D	−1.69	−1.84	−2.83	−1.80	−4.37
at 25 mpk/PO		−1.32		−2.09	−1.14	−2.16	−2.00	−4.25
at 12.5 mpk/PO		−0.26		−1.81	−0.90		−1.40	−3.88
at 6.25 mpk/po		−0.40		−0.27	−1.22		−0.90	−2.72
Mouse PK								
Clearance		N/D	N/D	16.3	13.1	19.2	N/D	16.0
po nAUC (μM × hr × kg/mg)				0.03	0.06	0.06		0.62
F (%)				1.7	23	4.7		42

N/D = not determined.
[a] See Ref. 14 for assay conditions.

under the vigorous reaction conditions. Alkylations of **9** with the enantiomeric forms of *N*-Boc-α-*H*-prolinol tosylate provided ca. 50% yields[10,12] of C3 O-alkylated products and these intermediates were further elaborated to final compounds **15a–d**.[10] In the penultimate step of the synthesis of **15f**, global deprotection of the acid-labile side chain *N*-Boc group and C18 *para*-methoxybenzyl ester was accomplished in a single operation (**14**→**15e**). Finally, the somewhat hindered pyrrolidine **15e** could be *N*-monomethylated in acceptable yield[12] under standard reductive alkylation conditions to complete the partial synthesis of derivative **15f**. The semisynthetic preparation of **15f** from enfumafungin depicted in Schemes 1–3 is representative of the general methodologies used to access other final compounds described in this work.[10]

Compounds **15a–f** were evaluated as direct inhibitors of β-(1,3)-glucan synthase, the essential fungal enzyme that forms β-(1,3)-glucan fibrils from UDP-glucose. In brief, the in vitro assay for inhibition of β-(1,3)-glucan synthase from *Candida albicans* quantifies the incorporation of radioactivity from UDP-[^{3}H]-glucose into precipitated glucan.[10,13] The IC_{50} of enfumafungin (**1**) in the glucan synthase inhibition assay is approximately 50 ng/mL, a value comparable to the MIC (200 ng/mL) against *Candida albicans*.[7,8] Semisynthetic enfumafungin derivatives **15a–f** are potent inhibitors of fungal cell wall glucan assembly (Table 1). The most potent in vitro inhibitors of glucan synthase from this series are compounds **15e** and **15f**, each bearing a quaternary stereocenter adjacent to the basic amine of the pyrrolidine in the C3-aminoether side chain. The IC_{50} potencies of **15e** and **15f** (4.0 and 6.4 ng/mL, respectively) in this assay are within an order of magnitude of the IC_{50} of the lead compound (**3**). Compounds **15a-d**, lacking the side chain quaternary carbon substitution, lose about one log order of potency against glucan synthase relative to **3**.

Antifungal susceptibility testing was conducted in order to determine minimum inhibitory concentrations (MIC values) for test compounds as a measurement of antifungal activity. Derivatives **15a–f** exhibited good to moderate MIC values against *C. albicans* (Table 1) that were generally comparable in potency to their corresponding IC_{50} values in the in vitro glucan synthase inhibition assay. As a point of reference, the MIC of enfumafungin (**1**) against *C. albicans* is 200 ng/mL.[7,8] Two of the compounds tested, **15e** and **15f**, displayed antifungal activity that was similar to that of **1**. However, the MIC values of these two derivatives (125 ng/mL for each) against *C. albicans* were less potent than that of the lead compound (**3**) by about one order of magnitude. In addition, the remaining analogs from this set (**15a–d**) were significantly less potent than **3**. Given the general loss of in vitro antifungal activity in the 'tethered' pyrrolidine series relative to **3**, it is evident that subtle structural modifications of the C3-aminoether side chain of enfumafungin derivatives can significantly modulate antifungal potency. In that respect, the C3 side chain of compound **3** is considered a highly desirable structural feature that contributes to potent in vitro fungicidal properties in the enfumafungin series.

In vivo anti-*Candida* activity and oral efficacy of the experimental enfumafungin derivatives were evaluated using the target organ kidney assay (TOKA) mouse model of disseminated candidiasis.[10,14] In brief, a disseminated *Candida* infection is induced in mice by intravenous inoculation of *C. albicans* into the lateral tail vein. Test compounds are then administered (ip or po) bid for 2 days and the reduction in kidney fungal burden compared to sham-treated control groups is determined. TOKA-active compounds generally lower the fungal colony forming units (CFU) per gram of kidney by 1–2 logarithmic units compared to sham-treated controls.[10] Enfumafungin derivatives **15a** and **15c–f** were shown to be efficacious in the mouse model of disseminated candidiasis when delivered orally (Table 2). In the pyrrolidine series, the configuration of the stereogenic carbon position in the C3-aminoether side chain influenced the magnitude of observed oral efficacy. Derivatives bearing the (*R*)-prolinol stereochemical configuration (**15c–f**) were more orally efficacious than the representative (*S*)-prolinol analog **15a**, reducing kidney fungal burden by ca. two logarithmic units when dosed orally at 25 mg/kg (mpk). Compound **15e**, the α-methyl *(R)*-prolinol derivative, exhibited notable oral efficacy in the TOKA mouse model (−2.16 log unit reduction at 25 mpk/po) but was considerably less active in this assay than lead compound **3**. The demonstrated oral efficacy of compounds **3**, **15e** and **15f** is consistent with the previously asserted[9c] notion that incorporation of a quaternary stereocenter adjacent to the basic amine of the C3 enfumafungin side chain confers improved oral activity in the TOKA mouse model of disseminated candidiasis.[9,10]

Based upon the promising oral efficacy observed in the pyrrolidine series, three compounds were advanced to murine pharmacokinetic profiling studies (Table 2).[10] Unfortunately, following oral administration of compounds **15c–e** in mice, poor plasma exposure was indicated by relatively low dose-normalized area under curve (nAUC) values in conjunction with high rates of clearance (13–19 L/h). Area under the curve values for **15c–e** were at least 10-fold less than the nAUC determined for lead compound **3** after identical po dosing in mice. Compound **3** was also significantly more bioavailable than derivatives from the pyrrolidine series (F = 42% for **3** vs 23% for **15d**). In spite of potent in vitro inhibitory properties against fungal β-(1,3)-glucan synthase, the moderate TOKA activity and low plasma exposure (for **15c–e**) short-circuited further development of C3-aminoether pyrrolidine enfumafungin derivatives as orally bioavailable antifungal agents. Pyrrolidine systems are known to undergo bioactivation involving hydroxylation at C5 (pyrrolidine numbering), leading to ring-opening of the resultant *N,O*-hemiaminal.[15] This metabolic process may be more detrimental to plasma exposure than oxidative *N*-demethylation in the case of **3**.

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12. The reported yield reflects an isolated quantity of HPLC-purified material from a single experiment and can, therefore, be considered unoptimized.
13. *Glucan synthase inhibition:* The in vitro evaluation of glucan synthase inhibitory activity of compounds was measured in a polymerization assay in 96-well format. Each well contained 100 μL of 3H-UDPG at 0.5 mM (6000–8000 dpm/nmol), 50 mM HEPES pH 7.5 (Sigma), 10% w/v glycerol (Sigma), 1.5 mg/mL bovine serum albumin (Sigma A 9647. Lot 44H0190), 25 mM KF (Fisher), 1 mM EDTA (Gibco ULTRAPURE), 25 μM GTP-γ-S, enzyme sufficient to give 3–6 nmol incorporation during the 60 min incubation at 22 °C, and test compound added from wells in threefold serial dilutions in 100% DMSO (1 μL/well). The reaction was stopped by the addition of 100 μL of 20% trichloroacetic acid. Plates were chilled for a minimum of 10 min, and precipitated glucan collected by filtration on GF/C plates (Packard UNIFILTER®-96), washed with 5 cycles of water (about 1 mL/well each cycle) using a Packard FILTERMATE HARVESTER. 40 μL/well scintillation fluid (Packard ULTIMA GOLD TM-XR) was added and the sealed plates counted in a WALLAC BETA counter in top-counting mode at an efficiency of approximately 40%.
14. *In vivo anti-candida activity:* A disseminated Candida infection is induced in DBA/2 mice by the iv inoculation of 0.2 mL of a yeast cell suspension containing 3.0×104 CFU of *C. albicans* MY1055 into the lateral tail vein. Therapy is initiated within 15–30 min after challenge. Mice are treated with test compound, either (1) ip, bid for a total of 2 days, or (2) po, bid for a total of 2 days. For each route of administration and diluent, an appropriate sham-treated control group is included. Kidneys from euthanized mice (4–5/group) are removed 4 days after challenge using aseptic techniques, weighed and placed in sterile WHIRL PAK bags containing 5 mL sterile saline. Kidneys are homogenized in the bags, serially diluted in saline and aliquots are plated on SD agar plates. Plates are incubated at 35 °C and enumerated after 30–48 h for *C. albicans* colony forming units (CFUs). Means from CFU/g of paired kidneys of treated groups are compared to the means from sham-treated controls.
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