2-(2-oxo-morpholin-3-yl)-acetamide derivatives as broad-spectrum antifungal agents

2-(2-Oxo-morpholin-3-yl)-acetamide Derivatives as Broad-Spectrum Antifungal Agents

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KEYWORDS

Antifungal, fungicidal, *Candida albicans*, *Aspergillus fumigatus*, dermatophyte, morpholin-2-one

ABSTRACT

From a fungicidal screen, we identified 2-(2-oxo-morpholin-3-yl)-acetamide derivatives as fungicidal agents against *Candida* species, additionally characterized by antifungal activity against *Aspergillus* species. However, development of this series was hampered by low plasmatic stability. Introduction of a *gem*-dimethyl on the 6-position of the morpholin-2-one core led to considerable improvement in plasmatic stability while maintaining *in vitro* antifungal activity. Further optimization of the series resulted in the discovery of *N*-(biphenyl-3-ylmethyl)-2-(4-ethyl-6,6-dimethyl-2-oxomorpholin-3-yl)acetamide (**87**), which in addition to fungicidal activity against *Candida* species, shown promising and broad antifungal *in vitro* activity against various fungi species, such as molds and dermatophytes. *in vivo* Efficacy was also demonstrated in a murine model of systemic *C. albicans* infection with a significant fungal load reduction in kidneys.

INTRODUCTION

Invasive fungal infections are caused by yeast pathogenic species (e.g. *Candida albicans*, *C. glabrata*) or filamentous pathogens (e.g. *Aspergillus fumigatus*). These infections are an increasing cause of morbidity and mortality in hospitalized patients. Candidaemia has been reported to occur in general as approximately 1 case per 1000 hospital admissions.[1] The overall mortality associated with candidaemia is 30 to 40%.[2-3] While *C. albicans* remains the most common pathogen, non-albicans *Candida* species, like *C. glabrata* and *C. krusei*, with greater resistance to triazoles are being increasingly isolated.[4] Invasive aspergillosis is an important cause of mortality in patients with hematologic malignancies and occurs in about 25% of these patients; the associated mortality is 40 to 50%. Moreover, invasive aspergillosis appears to be

gaining a foothold in the intensive care unit in patients without classical risk factors. Approximately 80% of invasive *Aspergillus* infections are caused by *A. fumigatus*.[5] In general, the mortality associated with fungal infections depends on the severity of the underlying disease, the infecting species, and on the timing and choice of antifungal treatment.

Currently, several structurally distinct antifungal drug classes are available.[6-8] These compound classes exert their antifungal effects by targeting different cell components. Polyenes, such as amphotericin B, bind to ergosterol, resulting in disruption of the cell membrane. In contrast, the azoles and allylamines block ergosterol biosynthesis by inhibiting the enzymes cytochrome P450 and squalene oxidase, respectively. Nucleoside analogs, such as flucytosine, inhibit DNA and RNA synthesis whereas griseofulvin inhibits mitotic spindle formation. In addition, echinocandins, such as caspofungin, inhibit glucan synthase, thereby blocking cell wall synthesis.[6] The limited efficacy of standard treatments, the associated toxicity and the increase of fungal resistance[9] have stimulated the search for new antifungal drugs.

Fluconazole, a standard fungistatic antimycotic, exerts a strong activity by inhibiting the growth of fungal species while other drugs such as amphotericin B and caspofungin are considered as fungicidal agents that can kill pathogens. When designing or screening for novel antifungal drugs, fungicidal activity is generally preferred over fungistatic activity since it pinpoints to inhibition of targets that are essential for fungal growth or induction of an active cell death pathway, such as apoptosis.[10] Moreover, Wong *et al.* recently reported[11] that an ideal antifungal agent should be fungicidal in order to avoid or minimize the emergence of resistance. The minimum inhibitory concentration (MIC), i.e. the lowest concentration that inhibits the fungal growth is used to characterize *in vitro* fungistatic activity. Fungicidal activity can be evaluated *in vitro* by determination of the minimum fungicidal activity (MFC) resulting in at least 99% killing

of the inoculum. In order to know whether compounds showing a 2-log reduction in inoculum size *in vitro* could translate in a clear *in vivo* efficacy, such fungicidal compounds should be tested extensively *in vivo* to make sure that the infection could effectively be cleared.

In an effort to identify new small molecules with broad antifungal activity (more specifically fungicidal activity), a screening was performed with a compound library of about 34,000 compounds, resulting in the identification of 2-(4-ethyl-2-oxomorpholin-3-yl)-*N*-(4-isopropylphenyl)acetamide (Compound (**1**), Figure 1) as a promising starting point. This compound was characterized by a good fungicidal activity against *C. albicans* (MFC = 12.5 µg/mL) and an interesting inhibitory activity against *A. fumigatus* (MIC = 12.5 µg/mL). Due to the presence of the lactone moiety, we investigated the plasmatic stability of compound **1**, which was very low upon incubation in mouse and human plasma ($t_{1/2}$ = 32 and 22 min, respectively). In this study, we describe the structural modifications of the morpholin-2-one core to improve the plasmatic stability. In addition we report on further optimization of the series resulting in compounds showing *in vitro* broad antifungal activity and *in vivo* efficacy in a mouse candidiasis model.

CHEMISTRY

The synthesis of compound **4** where the morpholin-2-one core was replaced by a morpholine was conducted from the commercially available building block ethyl 2-(morpholin-3-yl)acetate (**2**) (Scheme 1). Reductive amination with acetaldehyde afforded the *N*-ethyl morpholine **3**. Subsequent methyl ester hydrolysis followed by amide formation with HATU as coupling agent provided **4**.

We designed a 5-step synthetic route to prepare compounds substituted in 5- and/or 6-position of the morpholin-2-one core (Scheme 2). The amino-alcohols **12**-**18** were prepared either by reductive amination[12] or by epoxide opening[13] with ethylamine. Condensation with glyoxal led to morpholin-2-ones **19**-**25**. Alkylation in 3-position with α-bromoacetate proceeded smoothly after deprotonation with LHMDS leading to the desired compounds as racemic mixtures. The esters **26**-**32** were cleaved in acidic or basic conditions and the corresponding carboxylic acids were converted to the desired amide derivatives **33**-**39** by reaction with 4-isopropylaniline under standard HATU-coupling conditions.

Scheme 3 illustrates the synthesis of compounds where the morpholin-2-one core is substituted in 4-position with alkyl, cycloalkyl, phenyl, acyl and sulfonyl groups. A synthetic route was developed to allow introduction of *N*-substituents at the last step. The *N*-benzylmorpholin-2-one (**41**) was synthesized by alkylation of the amino-alcohol **40** with *tert*-butyl bromoacetate, followed by lactonization[14] with a catalytic amount of *p*TsOH. Alkylation in 3-position, ester cleavage, amide formation and benzyl removal by hydrogenation led to the key intermediate **44**. Reductive amination of **44** with aldehydes and ketones provided *N*-alkyl derivatives **45**-**47** and *N*-cycloalkyl derivatives **50**-**51**. Compounds **48** and **49** were synthesized by alkylation of **44** with 1-fluoro-2-iodoethane and 2,2,2-trifluoroethyltrifluoromethanesulphonate respectively. Acylation and sulfonylation of **44** provided compounds **52** and **53**. The *N*-phenyl analogue **57** was obtained in 4 steps starting from 2-methyl-1-(phenylamino)propan-2-ol[15] in a similar strategy as applied for compound **43**.

To determine the effect of the 4-isopropylaniline replacement by different amines, compounds **59**-**87** were synthesized in two steps from the *tert*-butyl ester **30**, as depicted in Scheme 4. The *tert*-butyl group was readily removed using trifluoroacetic acid to give the carboxylic acid **58**,

which was converted into the desired compounds *via* HATU-mediated coupling reactions with various amines.

The preparation of compound **89** with a *gem*-dimethyl in α-position of the amide was achieved following the synthetic sequence outlined in Scheme 5, where the key step was the alkylation of **23** using *tert*-butyl 2-bromoisobutyrate.

RESULTS AND DISCUSSION

Improvement of plasmatic stability. Despite its interesting *in vitro* fungicidal activity against *C. albicans*, the development of **1** was limited due to its poor plasmatic stability ($t_{1/2} \leq 32$ min in human and mouse plasma). Therefore, we first directed our chemistry towards the identification of new compounds with an improved plasmatic stability while keeping antifungal activity. Since we strongly suspected that the measured plasmatic instability was due to the presence of the lactone moiety (lactones are known to be hydrolyzed by esterases[16] contained in plasma), we decided to synthesize (i): compound **4** which was only lacking the keto function compared to compound **1** and (ii): a series of compounds (**33-39**) substituted on the 6-position of the morpholin-2-one core to see whether these structural modifications could lead to the identification of active and more stable compounds. It is indeed known that the introduction of steric hindrance close to an ester or a lactone moiety is a well-described strategy to block or significantly reduce hydrolysis of these functional groups.[17-18] From a *C. albicans* activity point of view (Table 1), it was clear that the lactone moiety was absolutely required since compound **4** was found to be inactive as opposed to **1**. On the other hand, the presence of a methyl (**33-34**), a phenyl (**35-36**) or a gem-dimethyl (**37**) on the 6-position of the morpholin-2-one was well tolerated whereas introduction of a gem-diethyl (**38**) led to complete loss of activity. Compound

39, in which an additional gem-dimethyl on the 5-position was introduced, was also found to be inactive. From a stereochemistry point of view, it is worth noticing that the absolute configuration of the substituents on the 6-position was not crucial for the antifungal activity since paired compounds (**33** and **34**) and (**35** and **36**) exhibited exactly the same MFC values (12.5µg/mL). However, we do not know whether the chiral center on the 3-position (where the acetamide moiety is connected) plays a crucial role in the intrinsic activity of the compounds since we could not separate the enantiomers by chiral preparative HPLC (no chiral synthesis was envisaged at that time). The human plasmatic stability of compounds which retained fungicidal activity was assessed (Table 1). Slight improvement in plasmatic stability was observed for compounds incorporating only one substituent on the 6-position ($t_{1/2}$ < 60 min). However, compound **37** with two methyl groups on the 6-position shown a significantly enhanced plasmatic stability ($t_{1/2}$ > 240 min). This plasma stability increase is probably due to the formation of a steric shield by the 2 methyl groups around the lactone and consequently prevent its hydrolysis. Based on this excellent result, compound **37** was selected as starting point for further development of the series.

Investigation of structure activity relationships. Having achieved a significant improvement in plasmatic stability compared to the initial hit **1**, we next focused on the optimization of the series based on compound **37**. First, we investigated the role of the *N*-ethyl group from the morpholin-2-one core. We therefore synthesized several derivatives bearing modifications on this position (free NH, *N*-alkyl, -cycloalkyl, -phenyl, -acyl and -sulfonyl groups). As indicated in Table 2, none of the synthesized compounds displayed fungicidal activity below 25 µg/mL, providing evidence that the *N*-ethyl group was essential for activity. Remarkably, even a slight

modification such as the substitution of an hydrogen by a fluorine on the ethyl group (**48**) yielded a compound devoid of any fungicidal activity at 25 μg/mL.

We also investigated alternatives to the 4-isopropylaniline moiety from compound **37**. It is indeed well known[19-20] that some aniline containing compounds can sometimes be associated to (geno)toxicity[21] upon metabolic oxidative cleavage (generation of reactive metabolites). For that particular reason and in view of improving the biological activity of the compound **37**, the 4-isopropylaniline moiety was replaced by various amines. All the newly synthesized compounds (Table 3) were tested for their fungicidal activity against *C. albicans*. Among all the derivatives with a heteroarylamine, only compound **65** (*N*-methylindole) retained potency. Replacement of the aniline by a benzylamine (**67**) was well tolerated, whereas a further elongation of the chain (phenethylamine derivative **68**) led to a two-fold decrease of fungicidal activity. Derivatives bearing alkylamines **70**-7**2** and cycloalkylamines **74**-**75** were also associated to a two-fold decrease in potency. Compound **76** with a morpholine proved to be inactive. *N*-Alkylation of the amide group (**69**, **73**) led to complete loss of activity, pointing towards a crucial role of the NH of the amide group indicating that it might be engaged in an hydrogen bond. Furthermore, introduction of a *gem*-dimethyl in α-position of the amide (**89**) was detrimental for fungicidal activity.

On the basis of the interesting activity of **67**, the benzylamine group was further explored. Phenyl replacements as well as substitutions were investigated (Table 4). Bioisosteric replacement[22-23] of the phenyl ring by a thienyl (**77**) or a furyl (**78**) led to compounds showing decreased fungicidal activity. Similarly, compound **79** with a cyclohexyl was also found to be less active. In order to evaluate the effect of substituents on the phenyl ring, we synthesized compounds **80**-**87**. Comparing the activities of the *ortho*-, *meta*- or *para*-chloro derivatives **80**-

82, the *meta*-position seemed to be preferred since compound **81** shown a two-fold increase in activity compared to compound **67**. Moreover, dichloro-substitution (**83**) did not result in further improvement in potency. Introduction in *meta*-position of other electron-withdrawing (**85**) or electron-donating groups (**86**) led to reduced activity whereas the presence of a methyl (**84**) or a phenyl (**87**) was well tolerated.

Broad spectrum activity. Representative compounds, selected based on their fungicidal activities against *C. albicans* and chemical differences (**37**, **65**, **82** and **87**) were screened against a panel of fungal species, including the fluconazole-resistant pathogenic yeast *C. glabrata,* the filamentous pathogens *A. fumigatus* and *A. flavus* and dermatophytes (Table 5). Onychomycosis is a common nail ailment associated with significant physical and psychological morbidity. Dermatophytes are the most commonly implicated etiologic agents, particularly *Trichophyton rubrum* and *Trichophyton mentagrophytes*, followed by *Candida* species.[24] Commonly used oral therapeutic agents include terbinafine, fluconazole and itraconazole. Hence, in this context, we tested the above selected compounds along with the reference antimycotic terbinafine and miconazole for activity against 4 dermatophytes (Table 5). The four selected compounds shown antifungal activity against all tested species. Overall, **87** turned to be the most promising compounds of the selection since it could inhibit the growth of all tested species, with a MIC value below 3 µg/mL against *A. fumigatus* and IC_{50} values between 1 and 3 µg/mL against the 4 tested dermatophytes. **87** was however modestly active against *A. flavus* (MIC=25 µg/mL), like the other compounds except **37** which shown a 2 fold increase in MIC. In addition to this broad spectrum antifungal evaluation, the cytotoxicity of compounds **37**, **65**, **82** and **87** was assessed in a MRC-5 cell line (see biological section). None of the compounds shown significant cytotoxicity at the highest tested concentration (CC_{50} > 20 µg/mL). However, to have an exact

indication of their therapeutic window, higher concentrations of the compounds should be tested with regard to cytotoxicity. Next, we assessed the fungicidal activity of **87** against the CA2 high-persister *C. albicans* clinical isolate.[25] This *C. albicans* isolate is characterized by a high abundance of miconazole-tolerant persister cells and miconazole is not fungicidal against this isolate (MFC miconazole > 100 µg/ml). Most importantly, persister cells can survive high doses of an antimicrobial agent which partly explains the recalcitrance of chronic infections against antimicrobial therapy. Hence, it is very important to eradicate such isolates. Interestingly, **87** was equally fungicidal against this high-persister clinical isolate (MFC=12.5µg/mL) as compared to the reference *C. albicans* strain (MFC=12.5µg/mL), indicating that **87** is also fungicidal against high-persister clinical isolates, which is of importance to combat antifungal drug resistance.

Pharmacological evaluation. 37 and **87** were selected as representative derivatives of the series for a detailed evaluation of *in vitro* ADMET properties (Table 6). The data illustrate that both compounds displayed a good aqueous kinetic solubility and a moderate to high permeability without severe efflux as determined in the Caco2 assay. Despite the presence of the lactone moiety (sometimes easily hydrolysable in mild-strong acid conditions), both **37** and **87** shown a good chemical stability in a simulated gastric fluid medium (contains 800000 Units/L of pepsin at pH=1.2). The compounds were also tested in a simulated intestinal fluid medium (contains 1000000 Units/L of pancreatin at pH=6.8) and appeared to be stable in these conditions although **87** was a bit more degraded than **37**. In addition to the chemical stability, the good plasmatic stability of **87** was confirmed ($t_{1/2}$ > 240 min) demonstrating the beneficial stabilizing effect of the *gem*-dimethyl on the morpholin-2-one core. Neither **37** nor **87** shown significant hERG inhibition at 10 µM (11 and 25.5 respectively). Moreover **37** did not inhibit major cytochrome P450 enzymes involved in drug metabolism (< 40% inhibition at 10 µM for CYP1A2, CYP2C9,

CYP2C19, CYP2D6 and CYP3A4). However, microsomal stability appeared as a major concern for the series. While **37** presented a reasonable human microsomal stability ($t_{1/2}$ = 29 min), **87** was rapidly metabolized as demonstrated by its short half-life ($t_{1/2}$ = 6 min). In view of performing a mouse in vivo efficacy study, the stability of **37** and **87** was also assessed in Mouse Liver Microsomes (MLM). Similarly to the human data, **37** appeared to be more stable than **87** but both compounds were rapidly metabolized in the MLM assay ($t_{1/2}$ = 6 min and 1 min, for compounds **37** and **87** respectively). Some additional compounds within the series were profiled in the MLM assay (data not shown) but unfortunately all of them were also metabolized very rapidly ($t_{1/2}$ < 10 min). In order to better understand this observation, we conducted a metabolite identification study in MLM with compound **37**. After incubation of **37** (10μM) in MLM for 90 min, the sample was analyzed by LC-MS/MS which led to the identification of 4 major putative metabolites. Two oxidative metabolites at 349 m/z (+16 m/z difference from parent compound), and 365 m/z (+32 m/z difference from parent compound) were observed. Although the data did not allow for definitive structures determination, the MS/MS analysis let us think that these hydroxylations were most likely occurring on the substituted aniline moiety. The metabolite observed at 305 m/z (-28 m/z difference from the parent) clearly seemed to be a result of N-deethylation. Finally, the metabolite at 331 m/z (-2 m/z difference from parent compound) was likely a result of a dehydrogenation but it was impossible to conclude from the product ion spectrum where this dehydrogenation occurred. Taking into account these results as well as the available Structure Activity Relationship (SAR), one of the best option to improve the MLM stability may be to block the oxidative hot spots from the aromatic amides (ortho, meta and para substitutions are tolerated as can be seen from table 3 and 4). Alternatively, some additional

compounds bearing modifications of the N-ethyl substituent might be synthesized although the generated data point towards a sharp SAR at this position (see table 2).

Despite the fact that **37** was rapidly metabolized in mouse microsomes, but taking into account its fungicidal activity, we were wondering whether it could demonstrate some *in vivo* efficacy in a murine model of systemic candidiasis. Therefore, BALB/c mice were infected intravenously with *C. albicans* SC5314 and were treated intraperitoneally during 5 days, starting 16 h after the infection, with (i) compound **37** at a dose of 10 mg/kg/day, (ii) with fluconazole at a dose of 10 mg/kg/day and with (iii) the vehicle. To determine the efficacy of therapy in murine models, a determination of renal fungal burden as CFU is commonly used. In a recent study, using bioluminescent *C. albicans* reporter strains aiming at real-time non-invasive imaging to monitor infection *in vivo*, the kidneys were confirmed as the main target organ.[26] Hence, at the end of the study, mice were sacrificed and tissue burdens of infection in the kidneys were assessed. As shown in Figure 2, both Fluconazole (10mg/kg) and **37** (10mg/kg) yielded a significantly lower fungal load in the kidneys ($p < 0.05$) compared to treatment with the vehicle. Encouraged by these promising results, we also decided to assess the *in vivo* activity of another compound from the series. Hence, compound **87** was tested in the same murine model of systemic *C. albicans* infection with a daily intraperitoneal injection of 10 mg/kg. A positive control group (Fluconazole 10mg/kg) and a vehicle control group were also added to the study. After 5 day treatment, Compound **87** exhibited a marked *in vivo* antifungal effect similar to the effect of fluconazole and seemed effective in controlling the fungal infection. The fungal load in kidneys was significantly reduced and as expected, no fungi was detected in liver and spleen (Figure 3). Despite their low mouse microsomal stability, both **37** and **87** shown very good *in vivo* efficacy in our *C. albicans* infection mouse model. Although these results clearly demonstrate the

potential for this new chemical series, more medicinal chemistry efforts are necessary to bring it to a further development stage. Indeed, the *in vitro* fungicidal activities remain modest compared to marketed fungicidal compounds such as Amphotericin B. Furthermore, the overall metabolic stability of the series is low both in mouse and human microsomes and should therefore be optimized. To this end, one option may be to reduce or block the N-deethylation cleavage as well as the oxidation sites on the aromatic amide moiety observed in the metabolite identification study. Additionally, it will be interesting to separate the pure enantiomers of compounds **37** and **87** and see whether the chiral center on the 3-position plays a role on the *in vitro* activities as well as on the overall ADMET properties.

Mode of action study. In order to start unravelling the exact mechanism of action of these compounds, we assessed potential antagonisms and synergies between compound **87** and a selection of 96 chemical compounds affecting diverse cellular processes. To this end, we used a phenotype microarray assay, and tested **87** at 2 concentrations of compound, namely 12.5 µg/mL (which equals the MFC on *C. albicans*) and 50 µg/mL. Both concentrations resulted in a significant growth reduction of the model yeast *Saccharomyces cerevisiae* (data not shown). We found that sodium cyanide (respiration inhibitor), sodium thiosulfate (calcium remover), chlortetracycline hydrochloride (calcium binder), potassium chromate (oxidative agent) or monothioglycerol (respiration inhibitor) antagonized the antifungal activity of compound **87** at 12.5 µg/mL and 50 µg/mL against *S. cerevisiae*. These data point towards the fact that **87** requires calcium and a functional respiratory chain to exert its antifungal activity against *S. cerevisiae*. However, the precise mechanism of action could not be determined yet and therefore additional studies will be required in the future.

CONCLUSION

In summary, we discovered and optimized a new class of morpholin-2-one derivatives as antifungal agents. Hit **1** identified from a screening campaign shown interesting fungicidal properties against *C. albicans* but its further development was limited by its plasma instability. By introducing a *gem*-dimethyl on the 6-position of the morpholin-2-one core, we succeeded in obtaining a compound that combined both fungicidal activity and excellent plasmatic stability. Synthetic routes were developed to allow introduction of structural modifications at the last step of the sequence. Extensive SAR revealed that the aniline moiety could be replaced by various amines from which *meta* substituted benzylamines turned to be the best. However, all the modifications brought to the ethyl residue led to inactive compounds showing that this *N*-ethyl substituent was crucial towards fungicidal activity. Compounds from this new chemical series also shown a broad-spectrum *in vitro* activity against resistant *Candida* isolates and against various fungal species including dermatophytes. The preliminary mechanism of action study demonstrated that compound **87** requires calcium and a functional respiratory chain to exert its antifungal activity against *S. cerevisiae*. Finally, compound **37** and **87** could demonstrate a clear *in vivo* efficacy when evaluated in a murine systemic candidiasis model.

EXPERIMENTAL PART

Chemistry. All reagents and solvents were purchased from commercial sources and used without further purification. Flash chromatography purifications were performed on Biotage prepacked silica gel columns using Biotage Isolera or SP4 instruments. TLC was carried out with Macherey-Nagel Alugram Sil G/UV_{254} plates. TLC plates were revealed with UV light, $KMnO_4$, *p*-anisaldehyde or ninhydrine solutions. ^{1}H NMR spectra were recorded on a 300 MHz Bruker

spectrometer. Proton chemical shifts are reported in parts per million (δ) using TMS as a standard. Electrospray mass (ESI) measurements were obtained on a Brucker Esquire 6000 mass spectrometer. The purity of all compounds screened in biological assays was > 95 %. Purity was determined by LC-MS recorded on a system consisting of a Dionex Ultimate 3000 HPLC equipped with a PDA detector and a Brucker Esquire 6000 mass spectrometer, using a C-18 column (SunFire C18, 3.5 µm, 3.0 x 100 mm or XBridge C18, 3.5 µm, 3.0 x 100 mm).

Ethyl 2-(4-ethylmorpholin-3-yl)acetate (3). A mixture of ethyl 2-(morpholin-3-yl)acetate (**2**) (0.300 g; 1.73 mmol), sodium acetate (0.226 g; 2.75 mmol) and acetaldehyde (0.291 mL; 5.19 mmol) in MeOH (2 mL) was stirred at room temperature for 1 h. The reaction mixture was concentrated under reduced pressure. The residue was taken up with EtOAc, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel using a gradient of MeOH (0 to 10%) in CH_2Cl_2 to yield 0.256 g (73%) of the title compound as a yellow oil. ESI/APCI(+): 202 (M+H).

2-(4-Ethylmorpholin-3-yl)-*N*-(4-isopropylphenyl)acetamide (4). To a solution of **3** (0.040 g; 0.199 mmol) in a mixture of dioxane/water (2 mL; 1/1) was added 1N LiOH (0.400 mL; 0.400 mmol). After 45 min at room temperature, the reaction mixture was acidified (pH =5) with 1N HCl, concentrated under reduced pressure and coevaporated with toluene to give the carboxylic acid which was used without further purification.

To a solution of the carboxylic acid (0.199 mmol) in DMF (1.5 mL) cooled at 0 °C were added HATU (0.140 g; 0.369 mmol) and DIPEA (0.103 mL; 0.590 mmol). After 1 h at 0 °C, 4-isopropylaniline (0.034 mL; 0.249 mmol) was added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was partitioned between CH_2Cl_2 and

saturated $NaHCO_3$. The phases were separated. The organic phase was washed with water and brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel using a gradient of MeOH (0 to 10%) in CH_2Cl_2 to yield 0.016 g (28%) of the title compound as a yellow oil. ESI/APCI(+): 291 (M+H); 313 (M+Na). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 10.55 (1H, br s), 7.44 (2H, d, *J* = 8.4 Hz), 7.18 (2H, d, *J* = 8.5 Hz), 3.85 (2H, m), 3.65 (2H, m), 2.8-3.1 (5H, m), 2.25-2.5 (3H, m), 1.23 (1H, d, *J* = 6.9 Hz), 1.17 (3H, t, *J* = 7.2 Hz).

General procedure for epoxide opening with an amine. Exemplified for 1-(ethylamino)-2-methylpropan-2-ol (16). To a solution of isobutylene oxide (3.5 mL, 39.7 mmol) in MeOH (60 mL) in a sealed tube was added a 70% ethylamine solution in water (7.2 mL; 104 mmol). The reaction mixture was heated overnight at 100 °C. The reaction mixture was concentrated under reduced pressure. Purification by distillation under reduced pressure furnished 4.42 g (95%) of the title compound as a colorless liquid. ESI/APCI(+): 118 (M+H). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.70 (2H, q, *J* = 7.5 Hz), 2.53 (2H, s), 1.17 (6H, s), 1.11 (3H, t, *J* = 7.5 Hz).

General procedure for morpholin-2-one formation. Exemplified for 4-ethyl-6,6-dimethylmorpholin-2-one (23). To a mixture of a 40% glyoxal solution in water (4.5 mL; 39.2 mmol) in toluene (20 mL) cooled at 10 °C was added a solution of **16** (4.4 g; 37.7 mmol) in toluene (11 mL). After 2 h at 10 °C, the reaction mixture was refluxed to azeotrope out solvents. Purification by distillation under reduced pressure furnished 4.72 g (80%) of the title compound as a colorless liquid. ESI/APCI(+): 158 (M+H); 180 (M+Na). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.21 (2H, s), 2.47 (2H, s), 2.44 (2H, q, *J* = 7.3 Hz), 1.43 (6H, s), 1.09 (3H, t, *J* = 7.3 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 168.5, 99.8, 81.5, 59.3, 54.6, 51.0, 26.9, 11.7.

General procedure for morpholin-2-one alkylation in 3-position. Exemplified for *tert*-butyl 2-(4-ethyl-6,6-dimethyl-2-oxomorpholin-3-yl)acetate (30). To a solution of **24** (2.0 g; 12.7 mmol) in THF (80 mL) cooled at -70 °C was added dropwise a 1M LHMDS solution in THF (12.7 mL; 12.7 mmol). After 1 h at -70 °C, *tert*-butyl bromoacetate (2.1 mL; 14.1 mmol) was added dropwise and the reaction mixture was stirred at -70 °C for 4 h. The reaction was quenched by addition of saturated NH_4Cl. After warming to room temperature, the solids were filtered and the filtrate was concentrated under reduced pressure. The residue was taken up with EtOAc, dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification by flash chromatography on silica gel using a gradient of EtOAc (10 to 40%) in heptane furnished 2.61 g (75%) of the title compound as a white solid. ESI/APCI(+): 272 (M+H); 294 (M+Na). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.32 (1H, m), 3.00 (1H, m), 2.75-2.85 (3H, m), 2.30-2.40 (2H, m), 1.50 (3H, s), 1.46 (9H, s), 1.38 (3H, s), 1.05 (3H, t, J = 7.1 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 170.4, 170.0, 81.0, 80.4, 60.9, 57.5, 47.2, 36.5, 28.1, 27.3, 26.4, 11.1.

General procedure for *tert*-butyl ester cleavage. Exemplified for 2-(4-ethyl-6,6-dimethyl-2-oxomorpholin-3-yl)acetic acid (58). To a solution of **30** (0.300 g; 1.11 mmol) in CH_2Cl_2 (5 mL) was added TFA (1.7 mL). After 5 h at room temperature, the reaction mixture was concentrated and coevaporated with toluene to give quantitatively the title compound under its trifluoroacetate salt form which was used in the next step without further purification.

General procedure for amide formation. Exemplified for 2-(4-ethyl-6,6-dimethyl-2-oxomorpholin-3-yl)-*N*-(4-isopropylphenyl)acetamide (37). To a solution of **58** (0.442 mmol) in DMF (2.9 mL) were added DIPEA (0.385 mL; 2.20 mmol) and HATU (0.203 g; 0.534 mmol). After 30 min at room temperature, 4-isopropylaniline (0.074 mL; 0.541 mmol) was added and the reaction mixture was stirred overnight at room temperature. The solvent was evaporated

under reduced pressure. The residue was partitioned between CH_2Cl_2 and saturated $NaHCO_3$. The phases were separated. The organic phase was washed with water and brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification by flash chromatography on silica gel using a gradient of EtOAc (30 to 80%) in heptane furnished 0.132 g (90%) of the title compound as a beige powder. ESI/APCI(+): 333 (M+H); 355 (M+Na). ESI/APCI(-): 331 (M-H). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.49 (1H, br s), 7.40 (2H, d, *J* = 8.4 Hz), 7.16 (2H, d, *J* = 8.4 Hz), 3.41 (1H, m), 2.8-3.1 (5H, m), 2.4-2.55 (2H, m), 1.47 (3H, s), 1.41 (3H, s), 1.22 (6H, d, *J* = 6.8 Hz), 1.11 (3H, t, *J* = 7.1 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 171.1, 167.9, 144.9, 135.6, 126.8, 120.1, 81.0, 61.3, 57.6, 47.4, 37.6, 33.6, 27.2, 26.7, 24.0, 10.9.

2-(6,6-Dimethyl-2-oxomorpholin-3-yl)-*N*-(4-isopropylphenyl)acetamide (44). To a mixture of 2-(4-benzyl-6,6-dimethyl-2-oxomorpholin-3-yl)-*N*-(4-isopropylphenyl) acetamide (**43**) (0.500 g; 1.27 mmol) in methyl acetate (20 mL) was added 10% Pd/C (0.500 g). The reaction mixture was stirred at room temperature for 5 h under a hydrogen atmosphere and then was filtered through celite. The filtrate was concentrated under reduced pressure. Purification by flash chromatography on silica gel using a gradient of MeOH (3 to 8%) in CH_2Cl_2 furnished 0.345 g (89%) of the title compound as a white foam. ESI/APCI(+): 305 (M+H); 327 (M+Na); 609 (2M+H); 631 (2M+Na). ESI/APCI(-): 303 (M-H). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.61 (1H, br s), 7.38 (2H, d, *J* = 8.4 Hz), 7.17 (2H, d, *J* = 8.4 Hz), 3.80 (1H, m), 2.8-3.1 (6H, m), 1.52 (3H, s), 1.40 (3H, s), 1.22 (6H, d, *J* = 6.7 Hz).

General procedure for reductive amination. Exemplified for *N*-(4-isopropylphenyl)-2-(4,6,6-trimethyl-2-oxomorpholin-3-yl)acetamide (45). To a solution of **44** (0.080 g; 0.263 mmol) in THF (1.4 mL) and CH_3CN (3.8 mL) was added a 37% formaldehyde solution in water (0.098 mL; 1.34 mmol). After 15 min at room temperature, $NaBH_3CN$ (0.033 g; 0.525 mmol)

was added portionwise. The reaction mixture was stirred for an additional 15 min and acetic acid (0.031 mL; 0.542 mmol) was added. The reaction mixture was stirred at room temperature for 1 h. The reaction was quenched by addition of saturated $NaHCO_3$ and the reaction mixture was diluted with EtOAc. The phases were separated. The organic phase was washed with water and brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification by flash chromatography on silica gel using a gradient of EtOAc (30 to 80%) in heptane furnished 0.045 g (54%) of the title compound as a white foam. ESI/APCI(+): 319 (M+H); 341 (M+Na); 659 (2M+Na). ESI/APCI(-): 317 (M-H). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.21 (1H, s), 7.40 (2H, d, *J* = 8.4 Hz), 7.15 (2H, d, *J* = 8.4 Hz), 3.05-3.15 (2H, m), 2.80-2.95 (3H, m), 2.49 (1H, m), 2.47 (3H, s), 1.49 (3H, s), 1.39 (3H, s), 1.21 (6H, d, *J* = 7.4 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 170.5, 167.7, 144.9, 135.5, 126.8, 120.1, 80.8, 63.8, 62.4, 43.4, 37.5, 33.6, 27.1, 26.9, 24.0.

General procedure for *N*-alkylation. Exemplified for 2-(4-(2-fluoroethyl)-6,6-dimethyl-2-oxomorpholin-3-yl)-*N*-(4-isopropylphenyl)acetamide (48). To a solution of **44** (0.059 g; 0.194 mmol) in THF (3 mL) heated at 80 °C were added progressively DIPEA (0.266 mL; 1.52 mmol) and 1-fluoro-2-iodoethane (0.266 g; 1.53 mmol) over 6 days. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in EtOAc and washed with saturated $NaHCO_3$. The organic phase was washed with water and brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification by flash chromatography on silica gel using a gradient of EtOAc (20 to 80%) in heptane furnished 0.022 g (32%) of the title compound as a white foam. ESI/APCI(+): 351 (M+H); 373 (M+Na); 723 (2M+Na). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.99 (1H, br s), 7.39 (2H, d, *J* = 8.4 Hz), 7.16 (2H, d, *J* = 8.4 Hz), 4.71 (1H, m), 4.55 (1H, m), 3.58 (1H, m), 2.7-3.2 (6H, m), 2.65 (1H, m), 1.46 (3H, s), 1.40 (3H, s), 1.22

(6H, d, J = 7.1 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 170.9, 167.8, 145.0, 135.4, 126.8, 120.2, 83.5, 81.2, 61.4, 59.1, 53.4, 38.4, 33.6, 26.9, 26.4, 24.0.

2-(4-Acetyl-6,6-dimethyl-2-oxomorpholin-3-yl)-*N*-(4-isopropylphenyl)acetamide (52). To a mixture of **44** (0.080 g; 0.263 mmol) and Na_2CO_3 (0.084 g; 0.793 mmol) in CH_2Cl_2 (5 mL) was added acetyl chloride (0.028 mL; 0.394 mmol). After 3 h at room temperature, Na_2CO_3 (0.042 g; 0.396 mmol) and acetyl chloride (0.028 mL; 0.394 mmol) were added again. The reaction mixture was stirred overnight at room temperature. Water was added and the phases were separated. The organic phase was washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification by flash chromatography on silica gel using a gradient of MeOH (0 to 8%) in CH_2Cl_2 followed by recrystallization from EtOAc furnished 0.053 g (58%) of the title compound as a white powder. ESI/APCI(+): 347 (M+H); 369 (M+Na); 715 (2M+Na). ESI/APCI(-): 345 (M+H). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.69 (1H, br s), 7.39 (2H, d, J = 8.4 Hz), 7.16 (2H, d, J = 8.4 Hz), 4.90 (1H, t, J = 3.8 Hz), 4.11 (1H, m), 3.55 (1H, m), 3.31 (2H, m), 2.86 (1H, m), 2.14 (3H, s), 1.50 (3H, s), 1.37 (3H, s), 1.21 (6H, d, J = 7.0 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 169.8, 168.6, 168.0, 145.2, 135.2, 126.9, 120.4, 81.0, 51.9, 50.6, 38.6, 33.6, 26.0, 24.8, 24.0, 21.7.

2-(6,6-Dimethyl-4-(methylsulfonyl)-2-oxomorpholin-3-yl)-*N*-(4-isopropylphenyl)acetamide (53). To a solution of **44** (0.080 g; 0.263 mmol) and DIPEA (0.092 mL; 0.527 mmol) in CH_2Cl_2 (5 mL) cooled at 0 °C was added mesyl chloride (0.031 mL; 0.401 mmol). After 4 h at 0 °C, DIPEA (0.092 mL; 0.527 mmol) and mesyl chloride (0.031 mL; 0.401 mmol) were added again. The reaction mixture was stirred at 0 °C for 4 h and overnight at room temperature. The reaction mixture was diluted with CH_2Cl_2 and washed with saturated $NaHCO_3$. The organic phase was washed with water and brine, dried over Na_2SO_4, filtered and concentrated under reduced

pressure. Purification by flash chromatography on silica gel using a gradient of EtOAc (20 to 60%) in heptane furnished 0.076 g (76%) of the title compound as a white foam. ESI/APCI(+): 383 (M+H); 405 (M+Na); 787 (2M+Na). ESI/APCI(-): 381 (M+H). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.39 (1H, br s), 7.35 (2H, d, *J* = 8.5 Hz), 7.18 (2H, d, *J* = 8.5 Hz), 4.78 (1H, t, *J* = 4.0 Hz), 3.60 (2H, m), 3.21 (2H, m), 3.06 (3H, s), 2.87 (1H, m), 1.49 (3H, s), 1.43 (3H, s), 1.22 (6H, d, *J* = 7.1 Hz). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 168.0, 167.3, 145.7, 134.8, 127.0, 120.5, 82.5, 52.5, 50.1, 41.7, 39.7, 33.6, 25.9, 25.5, 24.0.

Biological methods.

Microorganisms and materials. The yeast strains used in this study were *Saccharomyces cerevisiae* BY4741 (Euroscarf, Germany), *Candida albicans* strain SC5314[27] and *Candida glabrata* strain BG2[28]. The fungal strains used in this study were *Aspergillus fumigatus* (CBS 117202), *Aspergillus flavus* (CBS111.45), *Trichophyton rubrum* (B68183), *T. mentagrophytes* (B70554), *Microsporus canis* (B68128) and *Sporothrix schenkii* (B62482). Spore suspensions were prepared as previously described.[29] The fungal isolates were obtained from the Scientific Institute of Public Health (IHEM, Brussels, Belgium) and cultivated on Sabouraud dextrose agar (SDA) (Oxoid). PBS consists of 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4 (pH 7.4). Methylcellulose 0.5% was from Sigma (St. Louis, US). YPD consists of YPD 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose).

Antifungal and fungicidal activity tests. The fungicidal activity of the compounds against *C. albicans* and *C. glabrata* was determined in PBS and the MFC for each compound was calculated according to the definition of Thevissen and coworkers[30]. To this end, overnight

cultures of *C. albicans* or *C. glabrata* in YPD (1% yeast extract, 2% peptone and 2% glucose) were 1/200 and 1/400 diluted in PBS, respectively, and treated with the compounds or DMSO (2.5% as solvent control) for 24 h at 37 °C. After 24 h, the MFC was calculated by counting CFUs.[31] Antifungal activity of protein samples against the filamentous fungi was assayed by microspectrophotometry of liquid cultures grown in microtiter plates as described previously[29]. To determine the MIC of a compound against *A. fumigatus* or *A. flavus*, a 2-fold dilution series of the compound was incubated with the corresponding spore suspension in PDB (potato dextrose broth, Difco) (2 x 10^4 spores/mL). After 24 h incubation at 25 °C, MIC was determined as the minimal concentration that inhibits growth of the fungus.

The *in vitro* susceptibility screens with dermatophytes were performed as previously described.[32] Briefly, 10 µL of prediluted compound solution was spotted onto 96-well plates (U-bottom; Greiner Bio-One) with 64 µM as the highest concentration; 10^3 CFU in 200 µL RPMI-MOPS was added to each well. After incubation, growth inhibition was measured after adding 10 µL/well 0.005% (wt/vol) resazurin (Sigma), allowing fluorimetric reading (λex, 550 nm; λem, 590 nm). Activity is expressed as IC_{50}, i.e., the concentration that inhibits growth for 50% compared to non-treated controls. Miconazole and terbinafine were included as reference antifungals and they were purchased from Sigma. Three independent replicates were performed for each observation.

Cytotoxicity assay.[32] MRC-5 SV2 cells, human fetal lung fibroblast, were cultivated in MEM, supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate, and 5% FCS at 37 °C and 5% CO_2. For the assay, 10^4 MRC-5 cells/well were seeded onto the test plates containing the prediluted compounds and incubated at 37 °C and 5% CO_2 for 72 h. After 72 h of incubation, parasite growth was assessed fluorimetrically by adding resazurin for 24 h at 37 °C.

Fluorescence was measured using a GENios Tecan fluorimeter (excitation 530 nm, emission 590 nm). CC_{50} values are calculated from duplicate determinations with relative difference below 25%.

ADMET assays. The chemical stability (SIF and SGF medium), plasma stability, microsomal stability and plasma protein binding assays were performed by Anthem Biosciences (http://www.anthembio.com/). The CYP inhibition, hERG inhibition, Caco2 permeability assays and the metabolite identification study were performed by CEREP (www.cerep.fr).

Mouse systemic candidiasis model. BALB/c, 6- to 8-week-old male mice, were bred at the University of São Paulo animal facility under specific-pathogen-free conditions. All animals were handled in accordance with good animal practice as defined by the relevant national animal welfare bodies and all *in vivo* testing was approved by the Institutional Animal Care and Use Committee of the University of São Paulo. BALB/c mice were treated during the experiment with 100 mg/kg cyclophosphamide (Sigma), at 4 days and 24 hours before onset of infection, and an additional dose at 3 days post infection. To establish the *C. albicans* infection, 2 x 10^3 *C. albicans* SC5314 cells from an overnight culture in brain heart infusion (Difco) at 37 °C, were suspended in 100 µL of sterile saline and injected intravenously in BALB/c mice. Compound **37** or **87** formulated in DMSO/0.5% methylcellulose in water (5/95) was administered by intraperitoneal injection at 10 mg/kg/day. As control, mice were treated by IP injection with fluconazole (10mg/kg/day) formulated in water and the vehicle alone [DMSO/0.5% methylcellulose in water (5/95)]. Administration of the compounds/vehicle started 16 h after the challenge with *C. albicans* and was continued daily for 5 days.

To evaluate the fungal burden, kidney, spleen and liver of the mice were dissected aseptically on day 7 after infection, weighed and homogenized in 1 mL of PBS. Aliquots of the homogenate

(100 μL) were seeded in infusion of brain and heart (BHI, Difco) containing 2% agar. After incubation for 18 h at 37 °C, the number of CFU was determined. The effectiveness of different treatments was determined by considering the number of CFU per gram of tissue of treated animals compared with the number of CFU per gram of tissue of animals treated with vehicle alone. ANOVA with the post-Tukey test was used to evaluate the statistical significance of results obtained in all experiments. The differences between the results obtained by treatment with the compounds compared to the control were considered statistically significant when the p value was less than 0.05.

Phenotype microarray (PM) assay. The PM22-PM25 chemical sensitivity test microplates for fungi were used to compare the cellular phenotypes of *S. cerevisiae* BY4741 treated with or without compound 87 under 96 different conditions. The layout and contents of these plate set can be viewed at http://www.biolog.com/pdf/pm_lit/PM21-PM25.pdf. Preparation of the different IF (Inoculating Fluids; proprietary formulation supplied by BIOLOG, Hayward, CA, US) solutions and inoculation of the PM plates was performed according to the BIOLOG PM protocol for yeast/fungi, using an overnight *S. cerevisiae* culture grown in YPD (final culture dilution was 1/500 in the plates). Plates were incubated at 37°C for 24 h in the Omnilog plate reader (BIOLOG).

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ASSOCIATED CONTENT

Supporting Information Available: Experimental and analytical data of all intermediates and final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

MFC, minimal fungicidal concentration; MIC, minimal inhibitory concentration; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate; LHMDS, lithium hexamethyldisilazide; *p*TsOH, *p*-toluene sulfonic acid; DIPEA, *N*,*N*-diisopropylethylamine; CFU, colony forming unit; QD, quaque die (once a day); BID, bis in die (twice a day); PM, phenotype microarray.

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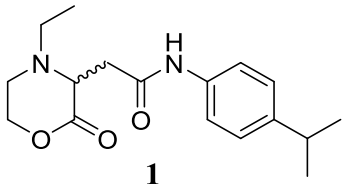
Legends of the figures

Figure 1. Structure and properties of hit compound **1**

Figure 2. *in vivo* Efficacy of compound **37** in a candidiasis mouse model (*C. albicans* SC 5314). Colony forming units (CFU) from kidneys (K) of BALB/c infected mice (n = 5) after 5-day intraperitoneal treatment with fluconazole (10mg/kg), **37** (10mg/kg), and vehicle alone (DMSO/0.5% methylcellulose (5/95)). * $p < 0.05$

Figure 3. *in vivo* efficacy of compound **87** in a candidiasis mouse model (*C. albicans* SC 5314). CFU from kidneys (K), spleen (S) and liver (L) of BALB/c infected mice (n = 10) after 5-day intraperitoneal

administration of 10 mg/kg/day of **87** or vehicle alone **(**DMSO/0.5% methylcellulose (5/95)) or 10 mg/kg/day intraperitoneal fluconazole. *** $p < 0.0001$



1

C. albicans MFC = 12.5 μg/mL
C. glabrata MFC = 12.5 μg/mL
A. fumigatus MIC = 12.5 μg/mL

Plasmatic stability (Human) $t_{1/2}$ = 22 min
Plasmatic stability (Mouse) $t_{1/2}$ = 32 min

Figure 1.

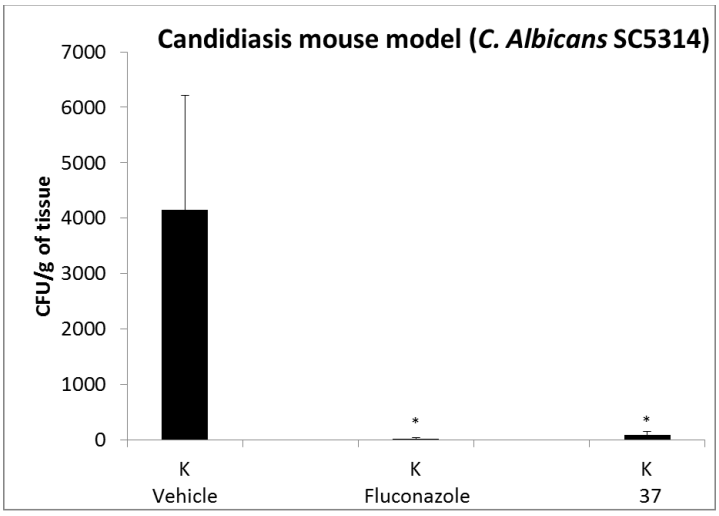


Figure 2.

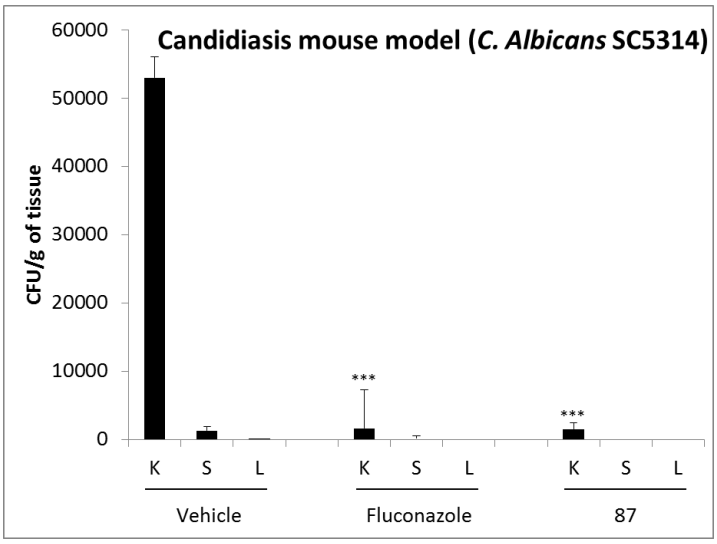
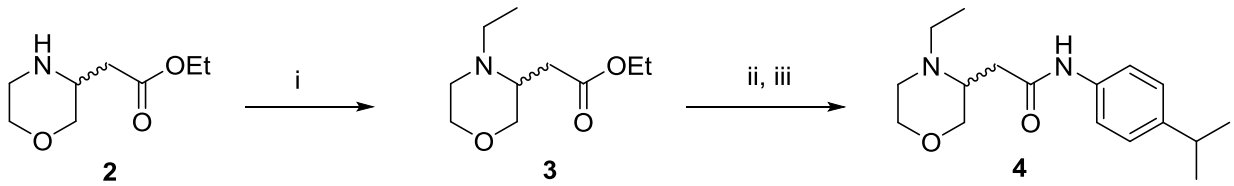


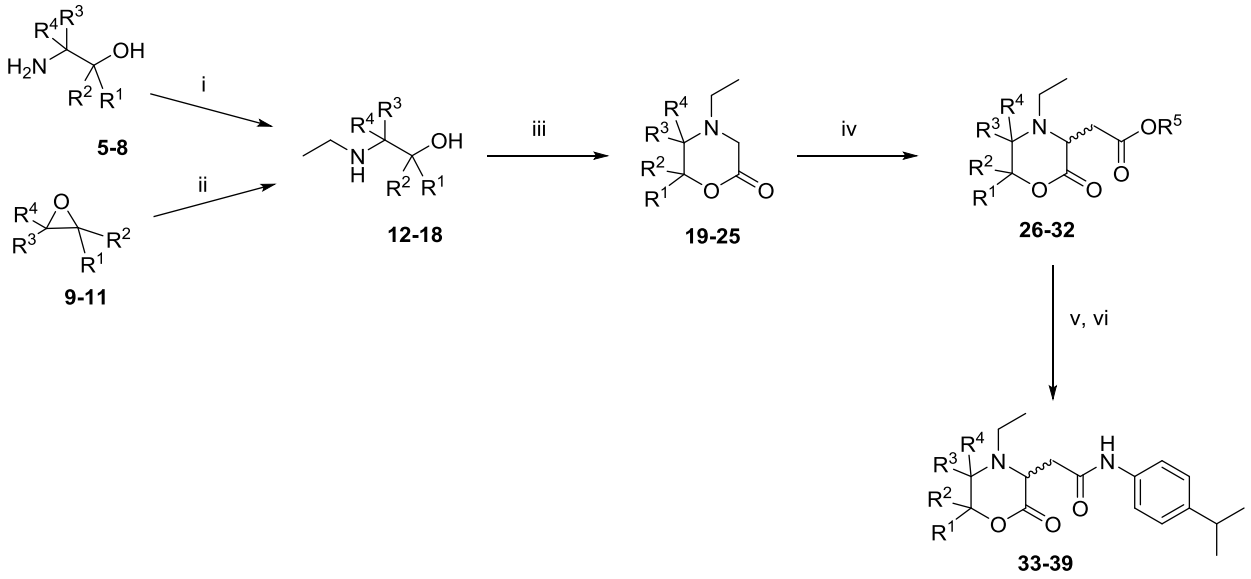
Figure 3.

Scheme 1. Synthesis of the morpholine analogue **4**[a]



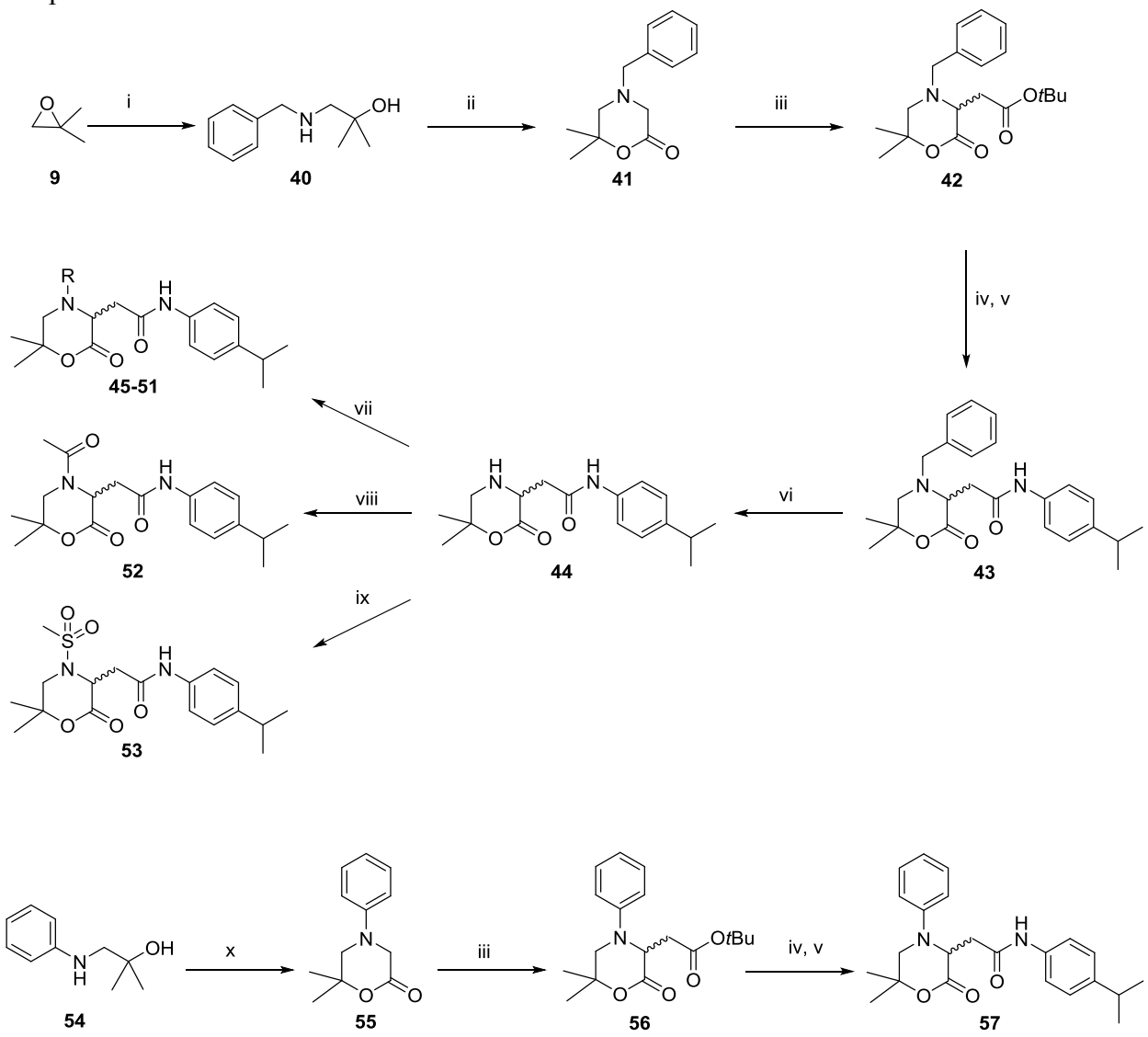
[a]Reagents and conditions: i) acetaldehyde, sodium acetate, $NaBH_3CN$, MeOH, rt, 1 h; ii) 1M LiOH, dioxane, water, rt, 45 min; iii) HATU, DIPEA, 4-isopropylaniline, DMF, rt, overnight.

Scheme 2. Synthesis of derivatives **33-39**[a] with substituents in 5- and/or 6-position on the morpholin-2-one core



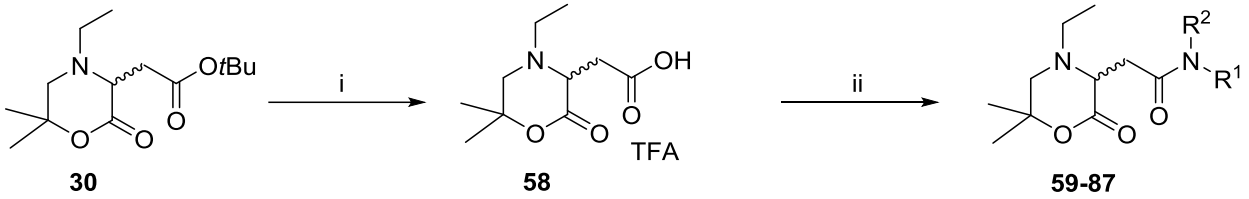
[a]Reagents and conditions: i) acetaldehyde, $NaBH_4$, EtOH, 0 °C, 4 h; ii) 70% $EtNH_2$, MeOH, sealed tube, 100 °C, overnight; iii) 40% glyoxal, toluene, 10 °C, 2-3 h; iv) a) 1M LHMDS, THF, -70 °C, 1 h; b) $BrCH_2COOEt$ or $BrCH_2COOtBu$, -70 °C, 3-5 h; v) 1N LiOH, dioxane, water, rt, 0.5-1 h (R^5 = Et) or TFA, CH_2Cl_2, rt, 5 h (R^5 = *t*Bu); vi) HATU, DIPEA, 4-isopropylaniline, DMF, rt, overnight.

Scheme 3. Synthesis of compounds **43-53** and **57**[a] with different *N*-substituents on the morpholin-2-one core



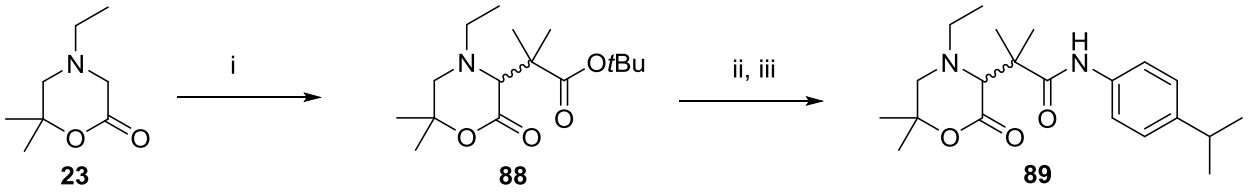
[a]Reagents and conditions: i) benzylamine, MeOH, sealed tube, 100 °C, overnight; ii) a) $BrCH_2COOEt$, DIPEA, CH_3CN, rt to 55 °C, 7.5 h; b) *p*TsOH, toluene, reflux; iii) a) 1M LHMDS, THF, -70 °C, 1 h; b) $BrCH_2COOtBu$, -70 °C, 4 h; iv) TFA, CH_2Cl_2, rt, 6 h; v) HATU, DIPEA, 4-isopropylaniline, DMF, rt, overnight; vi) 10% Pd/C, H_2, methyl acetate, rt, 5 h; vii) aldehyde or ketone, $NaBH_3CN$, acetic acid, THF, CH_3CN, rt, 1-18 h (for R = Me, Pr, *i*Pr, cyclopentyl) or (1-ethoxycyclopropoxy)trimethylsilane, 3Å molecular sieves, $NaBH_3CN$, THF, rt to reflux, 26 h (R = cyclopropyl) or 1-fluoro-2-iodoethane, DIPEA, THF, 88 h (R = CH_2CH_2F) or 2,2,2-trifluoroethyltrifluoromethanesulphonate, DIPEA, THF, reflux, 66 h (R = CH_2CF_3); viii) AcCl, Na_2CO_3, CH_2Cl_2, rt, 24 h; ix) MsCl, DIPEA, CH_2Cl_2, 0 °C to rt, 24 h; x) $BrCH_2COOEt$, K_2CO_3, DMF, 110 °C, 7 h.

Scheme 4. Synthesis of compounds **59-87**[a] with modification on the amide part



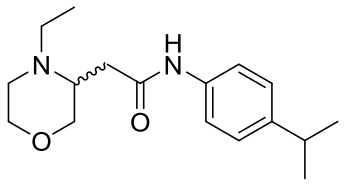
[a]Reagents and conditions: i) TFA, CH_2Cl_2, rt, 5 h; ii) HATU, DIPEA, R^1R^2NH, DMF, rt, overnight.

Scheme 5. Synthesis of compound **89**[a]



[a]Reagents and conditions: i) a) 1M LHMDS, THF, -70 °C, 1 h; b) *tert*-butyl 2-bromoisobutyrate, -70 °C to -15 °C, 3 h ii) TFA, CH_2Cl_2, rt, 5 h; iii) HATU, DIPEA, 4-isopropylaniline, DMF, rt, overnight.

Table 1. Fungicidal activity and human plasmatic stability for compounds **1**, **4** and **33-39**



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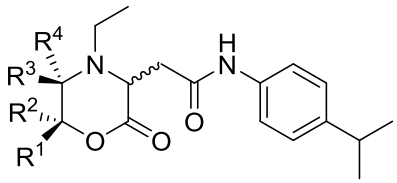
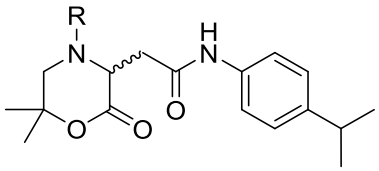


1; 33-39

					C. albicans[a]	Human plasma stability
Compd	R^1	R^2	R^3	R^4	MFC[b] (μg/mL)	$t_{1/2}$ (min)
1	H	H	H	H	12.5	22
4	-	-	-	-	> 50	ND[c]
33	Me	H	H	H	12.5	57
34	H	Me	H	H	12.5	55
35	Ph	H	H	H	12.5	49
36	H	Ph	H	H	12.5	31
37	Me	Me	H	H	12.5	>240
38	Et	Et	H	H	> 25	ND
39	Me	Me	Me	Me	> 25	ND
AmB[d]					1.6	ND
Fluc[e]					> 100	ND

[a] *Candida albicans* SC5314 strain
[b] MFC: Minimal Fungicidal Concentration that results in 99% killing of the inoculum.
[c] ND: not determined
[d] AmB: Amphotericin B
[e] Fluc: Fluconazole

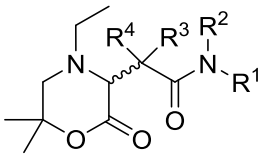
Table 2. Fungicidal activity of compounds **37**, **43-53** and **57**

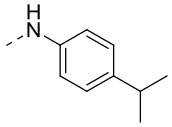
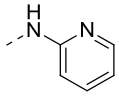
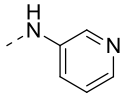
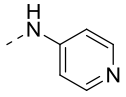
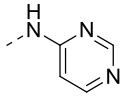
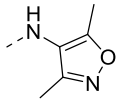
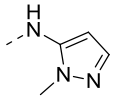
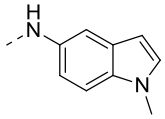
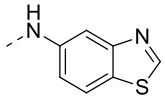
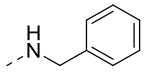


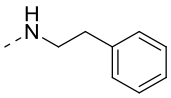
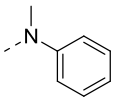
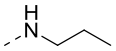
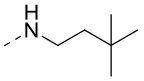
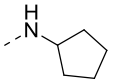
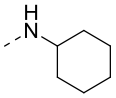
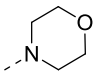
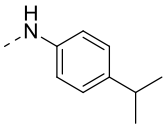
Compd	R	*C. albicans*[a] MFC[b] (μg/mL)
37	Ethyl	12.5
43	Benzyl	> 50
44	H	> 25
45	Methyl	> 25
46	Propyl	> 25
47	*i*-Propyl	> 25
48	2-Fluoroethyl	> 25
49	Trifluoroethyl	> 25
50	Cyclopropyl	> 25
51	Cyclopentyl	> 25
52	Acetyl	> 25
53	Mesyl	> 25
57	Phenyl	> 50

[a]*Candida albicans* SC5314 strain
[b]MFC: Minimal Fungicidal Concentration that results in 99% killing of the inoculum.

Table 3. Fungicidal activity of compounds **37**, **59-76** and **89**

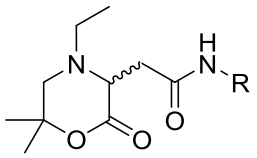


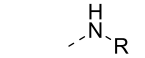
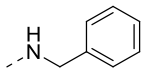
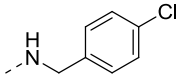
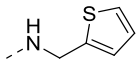
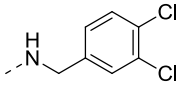
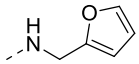
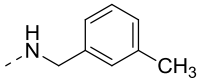
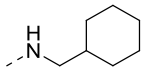
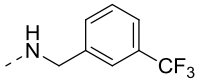
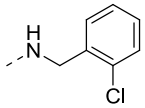
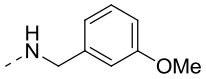
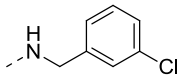
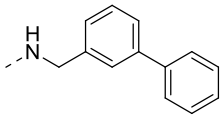
Compd		R^3	R^4	*C. albicans*[a] MFC[b] (μg/mL)
37		H	H	12.5
59		H	H	> 50
60		H	H	> 50
61		H	H	> 50
62		H	H	> 50
63		H	H	> 50
64		H	H	> 50
65		H	H	12.5
66		H	H	> 50
67		H	H	12.5

Compd		R^3	R^4	*C. albicans*[a] MFC[b] (μg/mL)
68		H	H	25
69		H	H	> 50
70		H	H	50
71		H	H	25
72		H	H	25
73		H	H	> 50
74		H	H	50
75		H	H	50
76		H	H	> 50
89		Me	Me	> 50

[a]*Candida albicans* SC5314 strain
[b]MFC: Minimal Fungicidal Concentration that results in 99% killing of the inoculum.

Table 4. Fungicidal activity of compounds **67** and **77-87**



Compd		*C. albicans*[a] MFC[b] (μg/mL)	Compd		*C. albicans*[a] MFC[b] (μg/mL)
67		12.5	**82**		12.5
77		25	**83**		12.5
78		50	**84**		12.5
79		25	**85**		25
80		25	**86**		25
81		6.25	**87**		12.5

[a]*Candida albicans* SC5314 strain
[b]MFC: Minimal Fungicidal Concentration that results in 99% killing of the inoculum.

Table 5. Antifungal and fungicidal activity of a selection of compounds against yeast, filamentous molds and dermatophytes.

Strain	*C. albicans*	*C. glabrata*	*A. fumigatus*	*A. flavus*	*T. mentagrophytes*	*T. rubrum*	*M. canis*	*S. schenckii*
Compound	MFC[a] (µg/mL)	MFC[a] (µg/mL)	MIC[b] (µg/mL)	MIC[b] (µg/mL)	IC_{50}[c] (µg/mL)	IC_{50}[c] (µg/mL)	IC_{50}[c] (µg/mL)	IC_{50}[c] (µg/mL)
37	12.5	25	1.6	12.5	10.6	6.3	6.6	11.8
65	12.5	50	3.1	25	11	11	6.2	10
82	12.5	12.5	6.2	25	2.7	2.0	2.0	2.2
87	12.5	12.5	≤ 3.1	25	3.0	0.70	2.4	2.7
terbinafine					0.02	0.02	ND	ND
miconazole					ND	ND	0.02	0.12

[a]MFC: Minimal Fungicidal Concentration that results in 99% killing of inoculum.
[b]MIC: Minimal Inhibitory Concentration that inhibits growth of the fungus
[c]IC_{50}: Minimal Concentration that inhibits growth for 50% compared to non-treated controls
[d]ND: not determined

Table 6. ADMET properties of compounds **37** and **87**

	37	**87**
Kinetic solubility at pH = 7.4 (µM)	50	50
Permeability [P_{app} (10^{-6} cm.s^{-1})]	65 (A-B) 35 (B-A)	22 (A-B) 13 (A-B)
Human protein plasma binding (% bound)	93.9	99.7
Chemical stability		
SGF[a] [half-life (min)]	> 240	> 240
SIF[b] [half-life (min)]	> 240	223
Human plasma stability [half-life (min)]	> 240	> 240
Human liver microsomal stability [half-life (min)]	29	6
Mouse liver microsomal stability [half-life (min)]	6	1
hERG (% inhibition at 10µM)	11	25.5

[a]SGF: Simulated Gastric Fluid
[b]SIF: Simulated Intestinal Fluid

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