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Design and Synthesis of a Series of Piperazine-1-carboxamidine Derivatives with Antifungal Activity Resulting from Accumulation of Endogenous Reactive Oxygen Species

Isabelle E. François,[a] Karin Thevissen,*[a] Klaartje Pellens,[a] Els M. Meert,[a] Jan Heeres,[b] Eddy Freyne,[c] Erwin Coesemans,[c] Marcel Viellevoye,[c] Frederik Deroose,[c] Sonia Martinez Gonzalez,[d] Joaquin Pastor,[d] David Corens,[c] Lieven Meerpoel,[c] Marcel Borgers,[e, f] Jannie Ausma,[e] Gerrit D. Dispersyn,[e] and Bruno P. Cammue[a]

In this study, we screened a library of 500 compounds for fungicidal activity via induction of endogenous reactive oxygen species (ROS) accumulation. Structure–activity relationship studies showed that piperazine-1-carboxamidine analogues with large atoms or large side chains substituted on the phenyl group at the R^3 and R^5 positions are characterized by a high ROS accumulation capacity in *Candida albicans* and a high fungicidal activity. Moreover, we could link the fungicidal mode of action of the piperazine-1-carboxamidine derivatives to the accumulation of endogenous ROS.

Introduction

The increasing global incidence of systemic fungal infections is caused by advances in medical technology and practice in organ transplantation, increasing prevalence of cytotoxic chemotherapeutic interventions, the widespread use of broad spectrum antimicrobials and indwelling catheters together with the increasing number of immunocompromised patients. The most common causes of these infections are *Candida* spp., of which *C. albicans* accounts for ~50%, and filamentous fungi such as *Aspergillus* spp.[1–3] The mortality rate associated with invasive *Candida* is approximately 40%,[4] whereas the mortality rate associated with invasive *Aspergillus* approaches 100% in solid organ transplant recipients.[5] Since there are no fungal vaccines currently licensed, the only clinical resource to combat fungal infections is the use of antifungal therapeutics (antimycotics). Among the currently used antimycotics, some offer only a limited activity spectrum, are available only in intravenous formulations, show harmful drug–drug interactions, or are associated with serious side effects such as nephrotoxicity.[6] Therefore, the search for new antifungal compounds with novel modes of action is imperative.

The imidazole miconazole was recently demonstrated to induce increased endogenous reactive oxygen species (ROS) levels in *C. albicans* prior to stabilization of the actin cytoskeleton.[7–9] Moreover, this ROS accumulation capacity was linked to fungicidal activity.[8] In this study, a target-based screening was designed in which compounds with antifungal activity and ROS accumulation capacity in *C. albicans* were identified. As such, we screened a compound library containing ~500 compounds from different classes, such as derivatives of azoles, pyridines and piperazines. Two piperazine-1-carboxamidine derivatives that cause accumulation of ROS in *C. albicans* were selected and used for further SAR studies in order to identify more potent antifungal compounds.

Results and Discussion

Screening

A compound library, consisting of ~500 compounds (provided by Barrier Therapeutics), was screened for antifungal activity and the capacity to induce accumulation of endogenous ROS in the human pathogen *C. albicans*. This compound library consisted of azoles, piperazines, pyrimidines and pyridines, as well as other compound classes. Antifungal activity and ROS accumulation capacity of these compounds was initially determined at a single concentration (100 $\mu g\,mL^{-1}$, as described in Experimental section). Compounds with antifungal activity at a higher minimal concentration (MIC) were considered to be in-

[a] *Dr. I. E. François, Dr. K. Thevissen, Dr. K. Pellens, E. M. Meert, Prof. Dr. B. P. Cammue*
Centre of Microbial and Plant Genetics, Katholieke Universiteit Leuven
Kasteelpark Arenberg 20, 3001 Heverlee (Belgium)
Fax: (+32) 16-32-19-66
E-mail: karin.thevissen@biw.kuleuven.be

[b] *Dr. J. Heeres*
Centre for Molecular Design, Janssen Pharmaceutica N.V.
Antwerpsesteenweg 37, 2350 Vosselaar (Belgium)

[c] *Dr. E. Freyne, Dr. E. Coesemans, Dr. M. Viellevoye, Dr. F. Deroose, Dr. D. Corens, Dr. L. Meerpoel*
Johnson & Johnson Pharmaceutical Research & Development, Division of Janssen Pharmaceutica N.V., Turnhoutseweg 30, 2340 Beerse (Belgium)

[d] *Dr. S. Martinez Gonzalez, Dr. J. Pastor*
Johnson & Johnson Pharmaceutical Research & Development, Division of Janssen-Cilag, C/ Jarama 75, Poligono Industrial, 45007 Toledo (Spain)

[e] *Dr. M. Borgers, Dr. J. Ausma, Dr. G. D. Dispersyn*
Barrier Therapeutics N.V., Cipalstraat 3, 2240 Geel (Belgium)

[f] *Dr. M. Borgers*
Department Molecular Cell Biology, CARIM, Maastricht University
Maastricht (The Netherlands)

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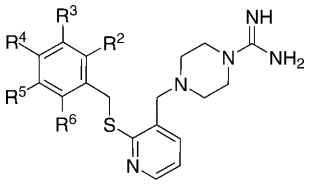
sufficiently potent or specific enough for potential use as antimycotics. We identified 60 compounds with antifungal activity. Of these 60 compounds, we could demonstrate that compounds **10**, with a Cl atom in the *para*-position of the phenyl group, and **4**, with a phenyl in the *meta*-position of the phenyl group (Table 1), induce increased endogenous ROS levels in *C. albicans*, not only at the 100 μg mL^{-1} concentration, but also in a dose-dependent manner (see below). Moreover, both compounds displayed antifungal activity with a fungicidal character, although to a different extent; the MFC (minimal fungicidal concentration) of compounds **4** and **10** is 50 and 100 μg mL^{-1}, respectively. This result points to the importance of the nature of the substituents of the phenyl group (namely Cl or phenyl) for the antifungal properties of the piperazine-1-carboxamidine derivatives.

Table 1. Antifungal activity of the piperazine-1-carboxamidine derivatives on *C. albicans*.



Compd	R^2	R^3	R^4	R^5	R^6	MIC_{50} [μg mL^{-1}]	MFC [μg mL^{-1}]	CFV[a]
1[b]	CN	H	H	H	H	>100	>100	0
2[b]	CH_3	H	H	H	H	>100	>100	47
3[c]	CH_2SO_2Ph	H	H	H	H	12.5	>100	155
4[d]	H	Ph	H	H	H	20	50	761
5[d]	H	H	Br	H	H	75	100	467
6[d]	H	OCF_3	H	H	H	80	100	338
7[b]	H	CN	H	H	H	>100	>100	0
8[c]	H	Cl	H	H	H	75	>100	311
9[b]	H	NO_2	H	H	H	>100	>100	0
10[d]	H	H	Cl	H	H	75	100	780
11[d]	H	H	CF_3	H	H	75	83	552
12[b]	H	H	CN	H	H	>100	>100	0
13[d]	H	H	Et	H	H	37.5	60	645
14[d]	H	H	Ph	H	H	20	50	780
15[b]	H	H	$MeSO_2$	H	H	>100	>100	0
16[b]	F	F	H	H	H	>100	>100	62
17[b]	F	H	F	H	H	>100	>100	114
18[b]	F	H	H	F	H	>100	>100	87
19[b]	F	H	H	H	F	>100	>100	1
20[b]	F	H	H	H	Cl	>100	>100	0
21[b]	F	H	F	H	F	>100	>100	0
22[b]	OCH_3	H	H	NO_2	H	>100	>100	59
23[d]	H	Br	H	Br	H	9.4	50	780
24[c]	H	F	H	F	H	50	>100	171
Miconazole						1.5	60	725

[a] Corrected fluorescence value at 100 μg mL^{-1} compound concentration. [b] Compounds without significant antifungal activity and CFV <100 (class III). [c] Compounds with fungistatic activity and intermediate CFV values (100 < CFV < 350; class II). [d] Compounds with fungicidal activity and CFV >350 (class I).

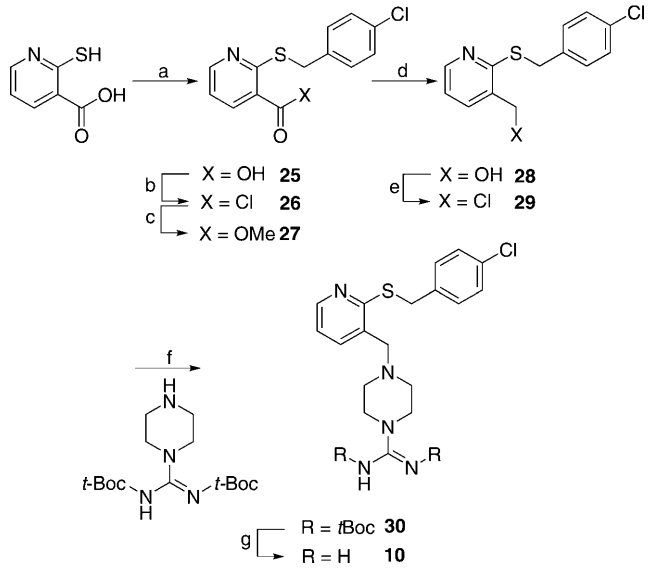
Synthesis of piperazine-1-carboxamidine derivatives

Compounds **1**–**24** were prepared following the route depicted in Schemes 1 and 2. Initially, the synthesis of the target compounds was based on a seven-step synthesis (for example, **10**; Scheme 1), but despite the straightforward nature of the route, it had major drawbacks such as moderate yields, lengthy reaction times, and was not amenable towards rapid exploration of the substituted mercaptobenzyl moiety. Based on the described chemoselective alkylation of thiols in the presence of amines and alcohols,[10a,b] we optimized a method that allowed chemoselective benzylation of the unprotected guanidinesintermediate **37**. The library, consisting of compounds **1**–**24** (Table 1), was achieved in parallel format starting from the fully deprotected intermediate **37** (Scheme 2). Coupling of compound **37** with the corresponding substituted benzyl halides, in the presence of an excess of polymer-bound DIPEA, gave *S*-Benzyl derivatives **1**–**24**. The final products were obtained with high levels of purity as determined by LC-MS. To our knowledge, chemoselective alkylation of thiols in the presence of unprotected guanidines has not been previously described in the literature.

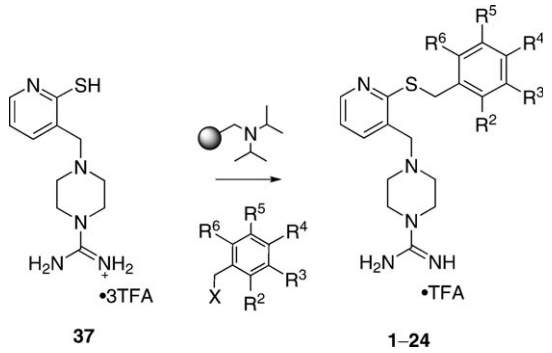
Accordingly, compound **37** was prepared following the synthetic route depicted in Scheme 3. Commercially available 2-mercaptonicotinic acid was allowed to react with *p*-bromobenzhydrol in the presence of methanesulfonic acid to give derivative **31** in excellent yield (95%). Reduction of the carboxylic acid group with borane–dimethylsulfide complex gave alcohol **32** in 90% yield. Activation of the hydroxy group in compound **32** and alkylation with *N*-Boc-piperazine under standard conditions afforded compound **34** in 52% overall yield over two steps. Trifluoroacetic acid (TFA)-mediated deprotection gave free amine **34**, which was subsequently guanylated according to standard methods using *N,N*-diBoc thiourea in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and diisopropylethylamine (DIPEA) to give compound **36** (82% yield).[11a,b] Finally, acidic treatment with TFA in dichloromethane (50% *v/v*) afforded the deprotected compound **37** in 65% yield.

Biological evaluation

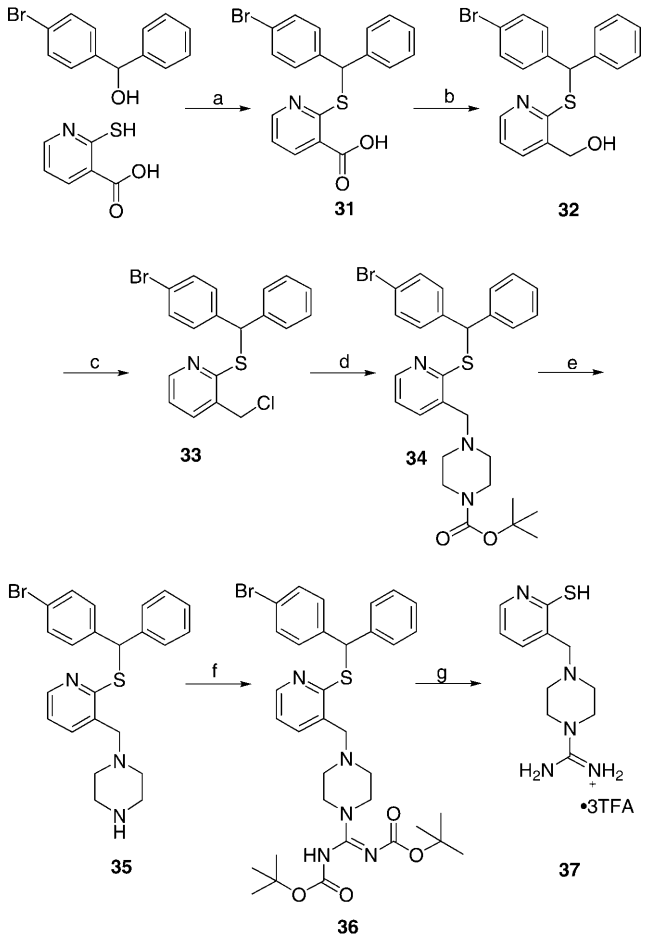
The derivatized compounds were screened for antifungal activity against, and ROS accumulation in, *C. albicans*. The identified



Scheme 1. Synthesis of target compounds. *Reagents and conditions*: a) 4-$ClPhCH_2Cl$, Na_2CO_3, Acetone, RT, 30 min, 39%; b) $SOCl_2$, reflux, 2 h, 99%; c) dry CH_3OH, RT, 16 h, 92%; d) 2 M $LiBH_4$/THF, THF, reflux, 16 h, 52%; e) MsCl, CH_2Cl_2, 0 °C→RT, 16 h, 99%; f) CH_3CN, TEA, 60 °C, 16 h, 13%; g) TFA/CH_2Cl_2 (1:1), RT, 2 h, 60%.



Scheme 2. General parallel synthesis procedure for compounds **1**–**24**, achieved using an Argonaut Quest 210. *Reagents and conditions*: substituted benzylhalide (1.2 equiv), polymer-bound DIPEA (3 equiv), DMF, 65 °C, 24 h.



Scheme 3. Synthesis procedure for compounds **31**–**37**. *Reagents and conditions*: a) $MeSO_3H$, RT, 18 h, 93%; b) BH_3·SMe_2, THF, 50 °C, 2.5 h, 90%; c) MsCl, TEA, CH_2Cl_2, 0 °C, 2 h; d) *N*-BOC-piperazine, AcCN, K_2CO_3, 80 °C, 16 h, 52% over two steps; e) TFA/CH_2Cl_2 (1:3), RT, 5 h, 65%; f) *N*,*N*′-bis-*tert*-butoxycarbonylthiourea, EDC, DIPEA, DMF, RT, 16 h, 82%; g) TFA/CH_2Cl_2 (1:1), RT, 3 h, 65%.

antifungal compounds were further tested for fungicidal activity (Table 1).

Antifungal activity

Among the 24 piperazine-1-carboxamidine derivatives tested (including the initially identified compounds **4** and **10**), 13 compounds were not active ($MIC_{50} > 100\ \mu g\,mL^{-1}$; class III) and 11 compounds were shown to have antifungal activity with MIC_{50} values ranging between 9.4 and 75 $\mu g\,mL^{-1}$ (Table 1). The antifungal compounds were further categorized as class I or class II agents depending on their fungicidal or fungistatic activity (see below).

SAR studies revealed that strong electron-withdrawing groups with hydrogen-bond acceptor capabilities, such as methyl sulfonyl (**15**), nitro (**9**) and cyano (**12**) groups, in the *ortho*-, *meta*- and *para*-position (R^4) of the phenyl ring generated inactive compounds ($MIC_{50} > 100\ \mu g\,mL^{-1}$). However, compounds containing a bulky trifluoromethyl group (**11**) or a halogen atom like chlorine (**10**) or bromine (**5**) in the *para*-position, exhibit only weak antifungal activity at ~75 $\mu g\,mL^{-1}$. Improvement in antifungal activity was observed upon introduction of an ethyl moiety (**13**, $MIC_{50} = 37.5\ \mu g\,mL^{-1}$) in this position. A voluminous phenyl moiety (**14**) enhanced antifungal activity even more ($MIC_{50} = 20\ \mu g\,mL^{-1}$). Shifting the phenyl or chlorine substituent from the *para*- to the *meta*-position (**4** and **8**) had no effect on antifungal activity ($MIC_{50} = 20$ and 75 $\mu g\,mL^{-1}$, respectively). Overall, introduction of small substituents in the *ortho*-position such as nitro (**1**), methyl (**2**) and fluorine (**16**–**21**) abolished the antifungal activity. Surprisingly, inclusion of the much more bulky phenylsulfonylmethyl substituent (**3**) led to significantly better antifungal activity ($MIC_{50} = 12.5\ \mu g\,mL^{-1}$). Yet another breakthrough was obtained with disubstituted derivatives. In comparison with the *para*-bromo analogue (**5**, $MIC_{50} = 75\ \mu g\,mL^{-1}$) or the *meta*-chloro derivative (**8**, $MIC_{50} = 75\ \mu g\,mL^{-1}$) an eightfold increase in potency

was obtained with the dibromo compound (**23**, MIC_{50} = 9.4 μg mL^{-1}). Moreover, the importance of the 3,5-dihalosubstitution pattern was further confirmed by compound **24** (MIC_{50} = 50 μg mL^{-1}), which showed a significantly improved potency compared with the difluoro analogues (**16**–**19** and **24**) and the *meta*-chloro derivative (**8**, MIC_{50} = 75 μg mL^{-1}). Overall, these results indicate that substitution with large groups, such as 3- or 4-phenyl, 3,5-dibromo, and 2-phenylsulfonylmethyl, on the benzyl group has a favorable effect on antifungal activity.

Additionally, all piperazine-1-carboxamidine derivatives were tested for their cytotoxic activity in vitro on HeLaM cells. None of these compounds displayed high cytotoxic activity; their pIC_{50} (i.e. −log of the half maximal (50%) inhibitory concentration) was typically lower than 4 (i.e. below 60 μg mL^{-1}). Two compounds, **14** and **21**, had a pIC_{50} value between 4 and 6, indicating moderate cytotoxicity.

Fungicidal character

We determined the fungicidal or fungistatic character of the compounds. Derivatives were considered fungicidal only when they induced cell death in 99.9% of a *C. albicans* culture. Based on these data, we could classify the compounds in three classes (Table 1). Compounds belonging to class I (i.e. compounds **4–6, 10, 11, 13, 14** and **23**, and miconazole) are fungicidal, with MFC values ranging between 50 and 100 μg mL^{-1}. Compounds belonging to class II (i.e. compounds **3, 8** and **24**) are fungistatic (MFC > 100 μg mL^{-1}) and those belonging to class III (i.e. compounds **1, 2, 7, 9, 12, 15**–**22**) do not have significant antifungal activity (MIC_{50} > 100 μg mL^{-1}).

We can conclude that substitution of the phenyl group at positions R^3 and R^5 in the piperazine-1-carboxamidine derivatives with large atoms (such as Br) or large side chains (such as phenyl) is beneficial for the fungicidal activity and ROS accumulation capability of the piperazine-1-carboxamidine derivatives. Furthermore, these data demonstrate that substitution of the phenyl group 1) at the R^3 and R^4 position with small atoms (e.g. F atoms), 2) at the R^2 and R^3 positions (independent of the size of the substituent) and 3) at more than one position simultaneously, is not desirable since this results in loss of fungicidal and even antifungal activity.

Accumulation of endogenous ROS

We measured the endogenous ROS levels after induction by the piperazine-1-carboxamidine derivatives on *C. albicans* as described previously.[8] These results are shown in Table 1. Interestingly, the extent of increased endogenous ROS production can be linked to antifungal activity as follows: compounds with a high capacity to accumulate ROS at a specific concentration (corrected fluorescence value (CFV) > 350) are fungicidal at this concentration (class I), the only exception being compound **10**, which is not fungicidal at 50 μg mL^{-1} although its CFV is higher than 350 at this concentration. Compounds with low or no capacity to induce ROS accumulation (CFV < 100) are not antifungal (class III). Class II compounds **3**, **8** and **24** have an intermediate ROS-accumulation capacity (100 < CFV < 350) and are fungistatic. Hence, these results demonstrate that compounds with high ROS accumulation capacity (CFV > 350) in *C. albicans* are fungicidal, linking fungicidal activity with endogenous ROS accumulation.

Involvement of ROS in fungicidal activity

In order to demonstrate the direct involvement of ROS accumulation in the fungicidal activity of these compounds, we determined their fungicidal activity in the presence of an antioxidant, namely ascorbic acid (AA). This low-molecular-weight antioxidant is one of the most studied and powerful antioxidants in plant cells.[12–16] AA can directly scavenge superoxide, HO$^{\circ}$ and singlet oxygen and can reduce H_2O_2 to H_2O via an ascorbate peroxidase reaction.[13] We observed antagonism between AA and the ROS-accumulating fungicidal compounds (**4**–**6**, **10**, **11**, **13**, **14** and **23**). Co-incubation of these compounds at their MFC value with AA (8 mM) alleviated their fungicidal action. While administration of the compounds alone at their MFC resulted in less than 0.1% survival of the *C. albicans* culture, the percentage survival of *C. albicans* cultures co-incubated with the compounds and AA (8 mM) was more than 50%. This result points towards the direct involvement of ROS in the fungicidal activity of the compounds.

Conclusions

To treat fungal infections, fungicidal compounds are favored over fungistatic compounds since the latter probably contribute to the development of drug resistance.[17] We previously demonstrated that the accumulation of endogenous ROS and fungicidal activity in *C. albicans* are linked, thus, we screened a compound library for agents able to induce ROS accumulation in *C. albicans* in an attempt to identify new fungicidal compounds. We identified two piperazine-1-carboxamidine derivatives able to induce ROS accumulation and confirmed that these compounds are fungicidal agents. In order to identify more potent piperazine-1-carboxamidine derivatives, we derivatized the phenyl group of these compounds resulting in the synthesis of 22 piperazine-1-carboxamidine derivatives. Among these derivatives, compounds **23**, **14** and **4** are the most potent since their MFC values were the lowest of all compounds tested. Moreover, they induce accumulation of endogenous ROS to the highest extent, linking fungicidal activity with high ROS accumulation capability. ROS involvement in fungicidal activity of the compounds was further demonstrated by the abolishment of the fungicidal activity of these compounds when co-incubated with an antioxidant. Note that compound **14** was characterized by moderate cytotoxicity, whereas cytotoxic activity of compounds **23** and **4** was low.

SAR studies of these piperazine-1-carboxamidine derivatives revealed that substitution of the phenyl group with large substituents, such as Br atoms or phenyl side chains, enhance antifungal activity and ROS inducing capability, as in compound **23**. Future research will be directed at assessing the fungicidal activity of dimethylated, dichlorinated or dimethoxy derivatives of compound **23**. Whether the increase of endogenous ROS

levels is caused by an enhanced ROS production, for example, by stimulation of NADPH oxidase activity, and/or by a decreased ROS breakdown, for example, by inhibiting the activity of antioxidant enzymes such as catalases and peroxidases remains to be determined, as does the extent to which the nature of the substitution of the phenyl group interferes with these systems.

An elevated endogenous ROS level is a phenotypical marker of apoptosis in yeast, for example, in *Saccharomyces cerevisiae* and in *C. albicans*.[18] Whether the piperazine-1-carboxamidine derivatives induce apoptosis in *C. albicans* needs to be investigated further. Other compounds or stimuli that have been shown to trigger apoptosis in *C. albicans* are AcOH, H_2O_2, the polyene antifungal drug amphotericin B, 1,10-phenantroline metal complexes, silver–coumarin complexes, diallyl disulphide, or disruption of γ-glutamylcysteine synthase.[19–23] Additionally, lactoferrin, a protein present in mammalian mucosal secretions with antifungal and antibacterial activity, was also demonstrated to induce apoptotic cell death in *C. albicans*.[24] Moreover, it should be noted that ROS inducing compounds are currently being used as antitumor therapeutics (e.g. procarabazine).[25] Therefore, future research will include the evaluation of the most potent ROS inducing antifungal compounds for potential antitumor activity.

Experimental Section

Chemistry

General methods: Melting points were measured in open capillaries on a Buchi B545 instrument and are uncorrected. ^{1}H NMR spectra were recorded with Bruker Avance DPX 400, 360 and 300 spectrometers and chemical shifts (δ) are expressed in parts per million (ppm) with TMS as internal standard. Elemental analyses were performed with a Carlo–Erba EA1110 analyzer. Silica gel thin-layer chromatography was performed on precoated plates Kieselgel 60F 254 (Merck, Germany). Silica gel column chromatography was performed with Kiesel gel 60 (0.063 to 0.200 mm; Merck, Germany).

Mass spectra were obtained on a Waters-Micromass ZQ mass spectrometer with an electrospray ionization (ESI) source operated in positive and negative ionization modes. Mass spectra were acquired by scanning from 100 to 1000 mass units in 1 s using a dwell time of 0.1 s. The capillary needle voltage was 3 kV and the source temperature was maintained at 140 °C. Nitrogen was used as the nebulizer gas. Cone voltage was 10 V for positive ionization mode and 20 V for negative ionization mode.

HPLC was performed on a Waters Alliance HT 2790 system with a column heater set at 40 °C. Flow from the column was split to a Waters 996 photodiode array (PDA) detector and a Waters Micromass ZQ mass spectrometer with an ESI source. Reversed phase HPLC was carried out on a Xterra MS C18 column (3.5 mm, 4.6× 100 mm) with a flow rate of 1.6 mL min^{-1}. Three mobile phases (A: ammonium acetate (25 mM)/CH_3CN, 95:5; B: CH_3CN; C: CH_3OH) were employed to run a gradient condition from A (100%) → B/C (1:1) over 6.5 min, → B (100%) over 1 min, → C (100%) for 1 min and re-equilibrate with A (100%) for 1.5 min. An injection volume of 10 μL was used. MsCl (methanesulfonyl chloride), EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride), DIPEA (*N,N*-Diisopropylethylamine), TFA (trifluoroacetic acid), and DIPE (diisopropyl ether).

General parallel synthesis procedure for compounds 1–24: All reactions were conducted using an Argonaut QUEST 210 under a N_2 atmosphere. The reaction tubes were loaded with a suspension of polystyrene-bound DIPEA (1.3 mmol g^{-1}, 0.6 mmol) in anhyd DMF (8 mL). Upon agitation, **37** (118.7 mg, 0.2 mmol) and the appropriate substituted benzylbromide, or benzylchloride in the case of **9, 13, 14**, and **20**, (0.22 mmol) were added. The reaction mixture was heated at 65 °C for 24 h. After cooling, the mixture was filtered and the resin was washed with DMF. The combined filtrates were concentrated to give the target compounds **1**–**24** as TFA salts. LC-MS analysis confirmed high levels of purity (81–100%; see Supporting Information).

4-[[2-[[(4-Chlorophenyl)methyl]thio]-3-pyridinyl]methyl]-1-piperazinecarboximidamide trifluoroacetate (1:2) (10): A solution of TFA in CH_2Cl_2 (10 mL, 50%) was added to **30** (111 mg, 0.143 mmol). The mixture was stirred at RT for 2 h. The solvent was removed and the residue was stirred in 2-propanol/DIPE (1:3). The precipitate was filtered off and recrystallized (CH_3OH/Et_2O; 1:10) yielding compound **10** (0.042 g, 60%) as a solid; mp: 181.6–183.8 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 2.40$–2.47 (m, 4H), 3.36–3.43 (m, 4H), 3.46 (s, 2H), 4.43 (s, 2H), 7.17 (dd, $J = 7.5$, 4.8 Hz, 1H), 7.35 (d, $J = 8.6$ Hz, 2H), 7.37 (br s, 3H), 7.42 (d, $J = 8.6$ Hz, 2H), 7.66 (dd, $J = 7.5$, 1.8 Hz, 1H), 8.42 ppm (dd, $J = 4.8$, 1.7 Hz, 1H); Anal. calcd for $C_{18}H_{22}ClN_5S{\cdot}2C_2HF_3O_2$: C 43.75, H 4.01, N 14.61, found: C 37.68, H 3.61, N 14.80.

2-[[(4-Chlorophenyl)methyl]thio]-3-pyridinecarboxylic acid (25): A mixture of 2-mercapto nicotinic acid (5 g, 0.032 mol) and Na_2CO_3 (3.8 g, 0.035 mol) in acetone (50 mL) was stirred at RT. 4-Chlorobenzylchloride (5.1 g, 0.032 mol) was added dropwise and the reaction mixture was stirred at RT for 30 min. The mixture was poured into water (250 mL) and the solution was acidified to pH 6 with aq HCl (10%), and extracted with CH_2Cl_2 (×2). The combined organic layer were washed with H_2O, the combined organic layers were dried ($MgSO_4$), filtered and concentrated. The residue was crystallized from 2-propanol to give **25** (3.46 g, 38.9%); ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 4.36$ (s, 2H), 7.26 (dd, $J = 7.8$, 4.8 Hz, 1H), 7.35 (d, $J = 8.8$ Hz, 2H), 7.44 (d, $J = 8.8$ Hz, 2H), 8.22 (dd, $J = 7.8$, 1.8 Hz, 1H), 8.65 (dd, $J = 4.8$, 1.9 Hz, 1H), 13.48 ppm (br s, 1H).

Methyl 2-[[(4-chlorophenyl)methyl]thio]-3-pyridinecarboxylate (27): A mixture of **25** (3.36 g, 0.012 mol) in thionylchloride (40 mL) was refluxed while stirring for 2 h. The reaction mixture was concentrated in vacuo. Dry toluene (50 mL) was added then removed in vacuo to give **26** (3.55 g, 99%), which was used in the next step without purification. A mixture of **26** (3.1 g, 10 mmol) in dry CH_3OH (150 mL) was stirred at RT for 16 h. The solvent was evaporated and the residue was purified by column chromatography (CH_2Cl_2/hexane; 50:50). The resultant product was recrystallized from DIPE/hexane yielding **27** (2.7 g, 92%); mp: 77 °C; ^{1}H NMR (360 MHz, $CDCl_3$): $\delta = 3.91$ (s, 3H), 4.40 (s, 2H), 7.07 (dd, $J = 7.78$, 4.74 Hz, 1H), 7.24 (d, $J = 8.41$ Hz, 2H), 7.37 (d, $J = 8.40$ Hz, 2H), 8.21 (dd, $J = 7.82$, 1.87 Hz, 1H), 8.57 ppm (dd, $J = 4.74$, 1.88 Hz, 1H); Anal. calcd for $C_{14}H_{12}ClNO_2S$: C 57.24, H 4.12, N 4.77, found: C 57.21, H 4.18, N 4.67.

2-[[(4-Chlorophenyl)methyl]thio]-3-pyridine methanol (28): A solution of **27** (3.4 g, 0.012 mol) in THF (200 mL) was treated with $LiBH_4$ (29 mL, 0.058 mol, 2 M in THF) and the solution was refluxed for 16 h. The reaction was poured into water (300 mL), acidified with aq HCl (1 N) and extracted with CH_2Cl_2. The organic layer was separated, dried ($MgSO_4$), filtered and concentrated. The residue

was recrystallized from petroleum ether (PE) and CH_2Cl_2 (1:1) yielding **28** (1.68 g, 52%); mp: 89.6 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.06 (s, 1H), 4.46 (s, 2H), 4.64 (s, 2H), 7.06 (dd, J = 7.5, 4.9 Hz, 1H), 7.24 (d, J = 8.4 Hz, 2H), 7.33 (d, J = 8.4 Hz, 2H), 7.63 (dd, J = 7.5, 1.8 Hz, 1H), 8.39 ppm (dd, J = 4.9, 1.8 Hz, 1H). Anal. calcd for $C_{13}H_{12}ClNOS$: C 58.75, H 4.55, N 5.27, found: C 58.74, H 4.54, N 5.15.

2-[[(4-Chlorophenyl)methyl]thio]-3-pyridine methylchloride (29): Et_3N (1 mL) was added at 0 °C under a flow of N_2 to a solution of **28** (1.6 g, 0.012 mol) in dry CH_2Cl_2 (30 mL). After stirring for 15 min, MsCl (0.93 mL, 0.012 mol) was added dropwise. The mixture was warmed to RT and stirred for an additional 16 h. The reaction mixture was poured into water (100 mL) and extracted with CH_2Cl_2 (× 2). The combined organic layer was dried ($MgSO_4$), filtered, and concentrated. The residue was recrystallized from PE and CH_2Cl_2 (15:1) yielding **29** (1.7 g, 99%); mp: 67 °C; ^{1}H NMR (360 MHz, $CDCl_3$): δ = 4.48 (s, 2H), 4.58 (s, 2H), 7.06 (dd, J = 7.6, 4.8 Hz, 1H), 7.25 (d, J = 8.4 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 7.61 (1H, dd, J = 7.6, 1.7 Hz, 1H), 8.42 ppm (dd, J = 4.8, 1.7 Hz, 1H); Anal. calcd for $C_{13}H_{11}Cl_2NS$: C 54.94, H 3.90, N 4.93, found: C 55.10, H 3.99, N 4.97.

bis(1,1-Dimethylethyl)[(*Z*)-[4-[[2-[[(4-chlorophenyl)methyl]thio]-3-pyridinyl]methyl]-1-piperazinyl]methylidyne]biscarbamate (30): A solution of **29** (0.43 g, 0.00152 mol) in CH_3CN (25 mL) was added dropwise to a solution of 1-[*N,N'*-bis(tert-butoxycarbonyl)amidino]-piperazine (0.5 g, 0.00152 mol) in CH_3CN (25 mL) and Et_3N (0.25 mL).[26a,b] KI (25 mg) was added and the mixture was stirred at 60 °C for 16 h. The reaction mixture was poured into water (200 mL) and extracted with CH_2Cl_2. The organic layer was separated, dried ($MgSO_4$), filtered and concentrated. The residue (0.75 g) was purified by HPLC using a gradient elution (0.5% NH_4OAc in H_2O/CH_3CN (9:1)/CH_3OH/CH_3CN; 23:42:35→0:30:70→0:100:0) to give **30** (0.111 g, 13%); mp: 70.4–73.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.49 (br s., 18H), 2.51 (t, J = 4.89 Hz, 4H), 3.46 (s, 2H), 3.59 (br s., 4H), 4.42 (s, 2H), 7.01 (dd, J = 7.40, 4.89 Hz, 1H), 7.25 (d, J = 8.30 Hz, 2H), 7.34 (d, J = 8.28 Hz, 2H), 7.54 (dd, J = 7.40, 1.38 Hz, 1H), 8.38 (dd, J = 4.77, 1.51 Hz, 1H), 10.17 ppm (br s., 1H); Anal. calcd for $C_{28}H_{38}ClN_5O_4S$: C 58.37, H 6.65, N 12.16, found: C 58.84, H 6.64, N 12.24.

2-[[(4-bromophenyl)phenylmethyl]thio]-3-pyridinecarboxylic acid (31): A mixture of 2-mercapto nicotinic acid (17.8 g, 0.115 mol) and 4-bromo benzhydrol (0.115 mol) in MsOH (150 mL) was stirred at RT for 18 h and then poured into water (800 mL). The mixture was stirred for 30 min. The precipitate was filtered off, washed with H_2O and hexane, and recrystallized from EtOAc yielding compound **31** as a white solid (42.6 g, 93%); mp: 208.1–208.6 °C; ^{1}H NMR (360 MHz, $CDCl_3$): δ = 6.47 (s, 1H), 7.06 (dd, J = 7.8, 4.7 Hz, 1H), 7.22 (t, J = 7.3 Hz, 1H), 7.30 (t, J = 7.4 Hz, 2H), 7.37 (d, J = 8.8 Hz, 2H), 7.38–7.44 (m, 4H), 8.28 (dd, J = 7.8, 1.9 Hz, 1H), 8.48 ppm (dd, J = 4.7, 1.9 Hz, 1H); Anal. calcd for $C_{19}H_{14}BrNO_2S$: C 57.01, H 3.53, N 3.50, found: C 55.71, H 3.22, N 3.87.

2-[[(4-bromophenyl)phenylmethyl]thio]-3-pyridine methanol (32): $BH_3{\cdot}SMe_2$ (12.7 mL, 0.127 mol, 10 M in THF) was added carefully to a solution of **31** (42.6 g, 0.106 mol) in dry THF (100 mL). The mixture was stirred at 50 °C for 2.5 h and then brought to RT. Saturated aq $NaHCO_3$ (100 mL) was added carefully and the mixture was diluted with CH_2Cl_2 (200 mL). The organic layer was separated, dried (Na_2SO_4), filtered and concentrated to yield compound **32** as a brown foam (37 g, 90%); ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 4.45 (d, J = 5.4 Hz, 2H), 5.50 (t, J = 5.3 Hz, 1H), 6.42 (s, 1H), 7.12 (dd, J = 7.6, 4.7 Hz, 1H), 7.22 (t, J = 7.2 Hz, 1H), 7.30 (t, J = 7.2 Hz, 2H), 7.37–7.51 (m, 6H), 7.68 (d, J = 7.5 Hz, 1H), 8.25 ppm (d, J = 4.9 Hz, 1H); Anal. calcd for $C_{19}H_{16}BrNOS.2H_2O$: C 54.03, H 4.77, N 3.32, found: C 59.31, H 4.72, N 3.17.

2-[[(4-Bromophenyl)phenylmethyl]thio]-3-chloromethyl-pyridine (33): A solution of **32** (37 g, 0.096 mol) in CH_2Cl_2 (400 mL) was cooled to 0 °C. MsCl (11 mL, 0.144 mol) and Et_3N (15.8 mL) were added and the mixture was stirred at RT for 2 h. The reaction was quenched with saturated aq NH_4Cl (50 mL). The organic layer was separated, dried (Na_2SO_4), filtered and concentrated. The crude residue was used without further purification in the next reaction; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.60 (s, 2H), 6.51 (s, 1H), 7.00 (dd, J = 7.3, 4.8 Hz, 1H), 7.22 (t, J = 7.3 Hz, 1H), 7.29 (t, J = 7.5 Hz, 2H), 7.33–7.43 (m, 6H), 7.58 (dd, J = 7.5, 1.6 Hz, 1H), 8.32 ppm (dd, J = 4.9, 1.6 Hz, 1H).

4-{2-[(4-Bromo-phenyl)-phenyl-methyl]thio]-pyridin-3-ylmethyl}-piperazine-1-carboxylic acid *tert*-butyl ester (34): A mixture of **33** (~0.096 mol), *tert*-butyl 1-piperazinecarboxylate (17.9 g, 0.096 mol) and K_2CO_3 (13.2 g, 0.096 mol) in CH_3CN (400 mL) was stirred at 80 °C overnight. The solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2 (200 mL) and then washed with H_2O (2×100 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/hexane; 1:3) to yield **34** as a light yellow foam (28 g, 52%).

1-{2-[(4-Bromo-phenyl)-phenyl-methyl]thio]-pyridin-3-ylmethyl}-piperazine (35): A solution of **34** (17.2 g, 0.031 mol) in TFA (25% in CH_2Cl_2 *v/v*; 150 mL) was stirred at RT for 5 h. The mixture was basified to pH 10–12 with aq NaOH (2 N), then CH_2Cl_2 (200 mL) was added. The organic layer was separated, dried (Na_2SO_4), filtered and concentrated. The residue was purified by column chromatography (CH_2Cl_2/(CH_3OH/NH_3); 7:1) and then by preparative HPLC to yield **35** (9.2 g, 65%).

bis(1,1-Dimethylethyl)[(*Z*)-[4-[[2-[[(4-bromophenyl)phenylmethyl]thio]-3-pyridinyl]methyl]-1-piperazinyl]methylidyne]biscarbamate (36): A mixture of **35** (7.5 g, 0.0165 mol), *N,N'*-bis-*tert*-butoxycarbonylthiourea (4.5 g, 0.0165 mol),[11a,b] EDC (3.8 g, 0.0198 mol) and DIPEA (3.5 mL, 0.0198) in DMF (60 mL) was stirred for 16 h at RT. The mixture was partitioned between aq NH_4Cl (50 mL, 1 N) and Et_2O (200 mL). The organic layer was separated, dried (Na_2SO_4), filtered and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/hexane; 1:3→2:3) to yield **36** as a white foam (9.5 g, 82%); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.35 (s, 9H), 1.40 (s, 9H), 2.33–2.43 (m, 4H), 3.33–3.41 (m, 4H), 3.49 (s, 2H), 6.41 (s, 1H), 7.10 (dd, J = 7.5, 4.9 Hz, 1H), 7.23 (t, 1H), 7.31 (t, J = 7.5 Hz, 2H), 7.40–7.49 (m, 6H), 7.64 (dd, J = 7.5, 1.6 Hz, 1H), 8.29 (dd, J = 4.8, 1.8 Hz, 1H), 9.54 ppm (s, 1H); Anal. calcd for $C_{34}H_{42}BrN_5O_4S.H_2O$: C 57.14, H 6.21, N 9.80, found: C 57.83, H 6.22, N 9.49.

4-[(2-mercapto-3-pyridinyl)methyl]-1-piperazinecarboximidamide trifluoroacetate (1:2) 37: A solution of **36** (0.0136 mol), triethylsilane (10 mL) in TFA (50% in CH_2Cl_2 *v/v*; 100 mL) was stirred for 3 h at RT. Hexane and Et_2O were added and the deprotected product was precipitated as a yellow solid, which was filtered off, washed with Et_2O and dried to yield compound **37** (6.3 g, 65%); ^{1}H NMR (400 MHz, [D$_6$]DMSO, 125 °C): δ = 2.82 (t, J = 5.14 Hz, 4H), 3.57 (t, J = 5.17 Hz, 4H), 3.92 (s, 2H), 6.78 (t, J = 6.69 Hz, 1H), 7.36 (br s., 4H), 7.63 (d, J = 6.23 Hz, 1H), 7.66 ppm (d, J = 7.18 Hz, 1H); Anal. calcd for $C_{11}H_{17}N_5S.2C_2HF_3O_2$: C 37.58, H 3.99, N 14.61, found: C 37.46, H 3.61, N 14.80.

Biological evaluation

Strains and media: The yeast strain used in this study was *C. albicans* strain SC5314 CAI.[27] The medium used was YPD (1% yeast extract, 2% peptone, 2% glucose) unless stated otherwise.

Antifungal activity: The antifungal activity of the compounds was determined at 100 μg mL^{-1}. Compounds were dispensed in microtiterplates at a final concentration of 500 μg mL^{-1} in 10% DMSO. *C. albicans* cell culture grown overnight in YPD (diluted in fresh YPD to ~10^6 cells mL^{-1}) was added to 20 μL of the compound solution in 2% DMSO. After 24 h incubation at 37 °C, antifungal activity of the compounds on *C. albicans* was assessed by microspectrophotometry of liquid cultures grown in microtiterplates as described previously.[28,29]

Compounds displaying antifungal activity were analyzed further to determine their MIC_{50} (concentration of compound required to inhibit 50% yeast growth). A twofold dilution series (20 μL) of the test compounds were prepared in DMSO. Subsequently 180 μL of MilliQ was added giving a dilution series of compound in 10% DMSO. *C. albicans* cell culture (80 μL) grown overnight in YPD (diluted in fresh YPD to ~10^6 cells mL^{-1}) was added to 20 μL of these dilution series (leading to a dilution series of compound in 2% DMSO) and incubated for 24 h at 37 °C. Subsequently, antifungal activity of the compounds against *C. albicans* was assessed by microspectrophotometry of liquid cultures grown in microtiterplates as described previously.[28,29]

Fungicidal action of antifungal compounds: An overnight *C. albicans* culture in YPD was diluted in 200 μL PBS to a cell density of ~10^6 cells mL^{-1} and incubated in the presence of a compound or DMSO. To examine whether ROS is involved in the fungicidal process (at the MFC value of the compounds, Table 1), incubations were conducted in parallel in the absence and presence of 8 mM ascorbic acid (AA). Administration of AA resulted in a PBS pH decrease from 7.2 to 6.0. After 0 h and 5 h of incubation at 37 °C, 100 μL aliquots were plated on YPD plates and colony forming units (CFUs) were counted after 2 d of incubation at 37 °C. Percentage survival was calculated as the ratio of the number of CFUs after treatment with the compound as compared to the number of CFUs of the initial inoculum. The minimal fungicidal concentration (MFC) of a compound is defined as the concentration leading to reduction of 99.9% of the viability of the initial inoculum.[30]

Cytotoxic activity in vitro: A volume of 180 μL of Eagle's minimum essential medium (supplemented with 5% fetal calf serum and 20 mM Hepes buffer) was dispensed in flat-bottomed 96-well plates. Test compound (45 μL) was added and serial fivefold compound dilutions were made. Additionally, 50 μL of medium and 50 μL of HeLaM cell suspension (2×10^5 cells mL^{-1}) were added. The microtiterplates were incubated at 37 °C over 7 d in a 5% CO_2 atmosphere. The viability of the cells was quantified spectrophotometrically by a tetrazolium colorimetric method (MTT assay). Briefly, to each well of the microtiterplate, 25 μL of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added, followed by incubation for 2 h at 37 °C and removal of the medium. The formazan crystals were solubilized by adding 200 μL 2-propanol and shaking. Finally, the absorbance was measured at 690 nm and 540 nm. To eliminate the effects of nonspecific absorption, the absorbance at 690 nm was subtracted from the values at 540 nm. The pIC_{50} was defined as −log of the half maximal (50%) inhibitory concentration for cytotoxicity of a compound.

Measurement of ROS production: Endogenous ROS levels were measured by a fluorometric assay with 2′,7′-dichlorofluorescin diacetate (DCFHDA; Molecular Probes Inc. USA) as described previously.[8] Briefly, 5 mL of an early log-phase yeast culture in YPD (grown at 37 °C) was centrifuged. The cell pellet was washed with PBS and resuspended in 5 mL PBS. Aliquots of the yeast cell suspension (40 μL) were mixed with 20 μL of compound at a final concentration of 100 μg mL^{-1} in 2% DMSO (initial screening to identify compounds that induce ROS at 100 μg mL^{-1}) or with 20 μL of a twofold dilution series of compounds (prepared as described above) or DMSO and incubated in white 96-well microtiterplates (PE white; Perkin–Elmer, USA). After incubation for 1 h at 37 °C, 40 μL aliquots of DCFHDA stock solution (25 μM in PBS) were added to the cell suspensions. Fluorescence emitted by the cells in the microtiterplates was measured with a Perkin–Elmer LS 50 B fluorescence spectrometer at an excitation wavelength of 485 nm (2.5 nm slit) and an emission wavelength of 540 nm (2.5 nm slit). Fluorescence was measured after incubation for 1, 3 and 5 h at 37 °C in the dark. Fluorescence values of the samples were corrected by subtracting the fluorescence value of the antifungal compound in the corresponding concentration without cells but with DCFHDA. These corrected fluorescence values (CFVs) can hence be considered as a measure to determine the extent of increased endogenous ROS levels.

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Keywords: antifungal agents · *Candida albicans* · piperazine-1-carboxamidines · reactive oxygen species · structure–activity relationships

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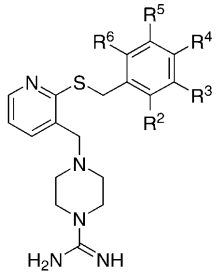
Antifungal agents: A series of piperazine-1-carboxamidine derivatives was synthesized and evaluated for antifungal activity against *Candida albicans*. Seven of the compounds synthesized induce accumulation of reactive oxygen species (ROS) in *C. albicans*, which correlated well with their antifungal activity, indicating this as the fungicidal mode of action. Compounds **4**, **14** and **23** were the most potent derivatives, with the lowest MFC values and greatest induction of endogenous ROS accumulation.



4 R^2=H; R^3=Ph; R^4=H; R^5=H; R^6=H
14 R^2=H; R^3=H; R^4=Ph; R^5=H; R^6=H
23 R^2=H; R^3=Br; R^4=H; R^5=Br; R^6=H

I. E. François, K. Thevissen, K. Pellens, E. M. Meert, J. Heeres, E. Freyne, E. Coesemans, M. Viellevoye, F. Deroose, S. Martinez Gonzalez, J. Pastor, D. Corens, L. Meerpoel, M. Borgers, J. Ausma, G. D. Dispersyn, B. P. Cammue*

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Design and Synthesis of a Series of Piperazine-1-carboxamidine Derivatives with Antifungal Activity Resulting from Accumulation of Endogenous Reactive Oxygen Species

