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Synthesis and fungicidal activity of 3,5-dichloropyrazin-2(1*H*)-one derivatives

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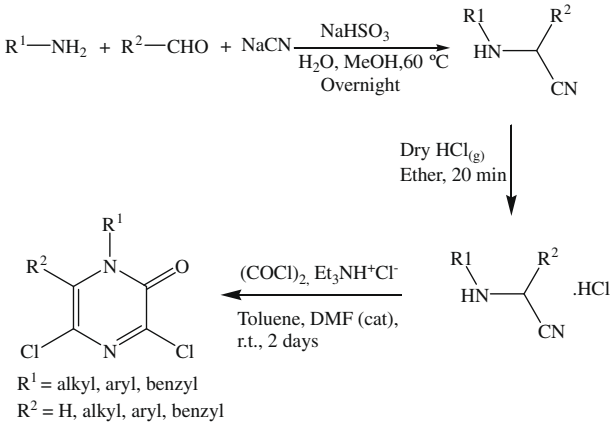
ABSTRACT

We synthesized a family of 3,5-dichloropyrazin-2(1*H*)-one derivatives and assessed their in vitro fungicidal activity against *Candida albicans*. Compounds **11** and **20** were most active against *C. albicans* and induced accumulation of reactive oxygen species in this pathogen. Using a genome-wide approach in the yeast *Saccharomyces cerevisiae*, we demonstrated that genes involved in vacuolar functionality and DNA-related functions play an important role in cellular mechanisms underlying the fungicidal activity of these compounds.

Since the early 1980s, fungi have emerged as major causes of human disease, especially among immunocompromised patients.[1] Several factors are associated with this increasing incidence of fungal infections, including the increasing use of invasive devices and implants that can be colonized by fungal biofilms and the larger population of immunocompromised patients. In the latter, fungi behave as opportunistic pathogens that can cause life-threatening infections, which are mainly caused by *Candida* spp. or *Aspergillus* spp. Some of the currently used antimycotics suffer from resistance occurrence and other pharmacological limitations, such as harmful drug–drug interactions, limited activity spectrum and/or high general cytotoxicity. Therefore, the search for new antifungal compounds with a novel mode of action is imperative.

In this context, we screened an in-house library, comprising of approx. 50 heterocyclic compounds including pyrazin-2(1*H*)-ones, oxazinones, pyridines and imidazopyrimidines, for fungicidal activity against the human fungal pathogen *Candida albicans*. To this end, we determined the minimal fungicidal activity of each compound against *C. albicans* strain CAI4.[2] The minimal fungicidal concentration (MFC) for each compound was determined in the saline buffer PBS and was calculated as the minimal concentration of the compound resulting in less than 0.1% survival of a *C. albicans* culture relative to the DMSO control.[3] It should be noted that determination of MFC values is preferred over MIC values, since the former reflects fungicidal activity whereas the latter may account for both fungistatic as well as fungicidal activity. As such, we identified two compounds, **1** and **2**, both 3,5-dichloropyrazin-2(1*H*)-one derivatives, with an MFC of 50 and 10 μg/mL, respectively. Until now, only few reports describing the antifungal activity of pyrazinone derivatives are available.[4] Hence, since these compounds could represent a novel class of antifungal compounds, we synthesized[5] several substituted 3,5-dichloropyrazin-2(1*H*)-one derivatives (Scheme 1) and analyzed their fungicidal activity against *C. albicans* (Table 1).

Table 1 demonstrates that compounds **11** and **20**[6] are characterized by the lowest MFC (i.e., 5 μg/mL); other compounds with



Scheme 1. Synthesis of 3,5-dichloropyrazin-2(1*H*)-ones.

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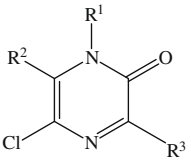
Table 1
Antifungal activity of pyrazin-2(1*H*)-one derivatives against *C. albicans*



Compds	R^1	R^2	R^3	MFC (μg/mL)	ROS
1	Benzyl	H	Cl	50	–
2	Phenyl	H	Cl	10	–
3	*p*-Methoxybenzyl	H	Cl	50	–
4	*p*-Methoxybenzyl	*p*-Methoxyphenyl	Cl	>100	–
5	*p*-Methoxybenzyl	Methyl	Cl	>100	–
6	Benzyl	Methyl	Cl	>100	–
7	Phenyl	*p*-Methoxyphenyl	Cl	>100	–
8	Phenyl	*o*-Methoxyphenyl	Cl	>100	–
9	Phenyl	*o*-Tolyl	Cl	>100	–
10	Phenyl	*p*-Carbomethoxyphenyl	Cl	>100	–
11	Phenyl	Chloromethyl	Cl	5	+
12	Phenyl	Methyl	Cl	>100	–
13	*p*-Methoxyphenyl	H	Cl	10	–
14	Phenethyl	H	Cl	50	–
15	Cyclohexyl	H	Cl	>100	–
16	Cyclohexylmethyl	H	Cl	100	–
17	*o*-Methoxybenzyl	Benzyl	Cl	>100	–
18	Methyl	*m*-Methoxyphenyl	Cl	>100	–
19	2-Methoxyethyl	Phenyl	Cl	>100	–
20	Phenyl	Bromomethyl	Cl	5	+
21	Phenyl	Chloromethyl	Methoxy	>100	–
22	Phenyl	Chloromethyl	Phenyl	>100	–

+ indicates compounds with a corrected fluorescence value (CFV)[6] >500 units when assayed at 100 μg/mL; – indicates compounds with a CFV<100 units when assayed at 100 μg/mL.

fungicidal activity were compounds **2** and **13** (MFC = 10 μg/mL); compounds **1**, **3** and **14** (MFC = 50 μg/mL); and compound **16** (MFC = 100 μg/mL). Replacing chlorine at 3-position of compound **11** by methoxy or phenyl resulted in a complete loss of fungicidal activity (compounds **21** and **22**,[6] respectively). This structure–activity relationship (SAR) study revealed that to detect high fungicidal activity, the pyrazin-2(1*H*)-one scaffold should bear both, a chlorine at the 3-position and a chloro- or bromomethyl at the 6-position. Note that MFC of fluconazole, a frequently used azole antimycotic, is greater than 200 μg/mL (data not shown).

We could recently demonstrate a link between fungicidal activity against *C. albicans* and induction of reactive oxygen species (ROS) in *C. albicans*.[7] As a first step to unravel the mode of antifungal action of the fungicidal 3,5-dichloropyrazin-2(1*H*)-ones, we determined their ROS induction capacity using 2′,7′-dichlorofluorescin diacetate staining (Table 1).[7] Compounds **11** and **20** induce ROS in *C. albicans*. We next focused on unraveling the cellular mechanisms underlying the fungicidal activity of compound **11**. In a first instance, we analyzed whether the induction of ROS by compound **11** is causally linked with its fungicidal activity. To this end, we determined the percentage survival of an overnight *C. albicans* culture after treatment with compound **11** in the presence and absence of the antioxidant ascorbic acid (AA). The low molecular weight antioxidant AA is one of the most studied and powerful antioxidants in plant cells.[8] AA can directly scavenge superoxide, ·OH and singlet oxygen and can reduce H_2O_2 to water via an ascorbate peroxidase reaction.[9] Percentage survival of *C. albicans* upon incubation with 5 μg/mL compound **11** was 0.009%, whereas the percentage survival of *C. albicans* upon coincubation with 5 μg/mL compound **11** and 80 mM AA was 64%. Control application of 80 mM AA alone did not influence the percentage (results not shown). These data point to the causal link between ROS production induced by compound **11** and its fungicidal activity.

In order to further unravel the mode of antifungal action of compound **11**, a genome-wide genetic approach in *Saccharomyces cerevisiae* was followed. This approach consisted of screening the complete haploid collection of 4853 *S. cerevisiae* deletion mutants (BY4741-Mat A collection, Invitrogen), individually deleted for non-essential genes, for mutants that were either resistant or hypersensitive towards compound **11** in agar. Theoretically, yeast mutants that are resistant to an antifungal compound are affected in a sensitivity gene, whereas yeast mutants that are hypersensitive to an antifungal compound are affected in a tolerance gene. The nature of sensitivity and tolerance genes provides complementary information regarding the mode of action of the antifungal compound. First, we determined the minimal inhibitory concentration (MIC) of compound **11** for the parental strain BY4741 in YPD agar plates as 2.5 μg/mL. Screening for compound **11** hypersensitive or resistant mutants was performed on YPD agar plates containing a compound **11** concentration which was fivefold lower (0.5 μg/mL) or higher (12.5 μg/mL), respectively, than the MIC.[10]

Using this genome-wide approach, we could only identify yeast mutants that were hypersensitive to compound **11**. The fact that no compound **11** resistant *S. cerevisiae* deletion mutants could be identified could imply that the target for compound **11** is encoded by an essential gene and as such, could point to a low frequency of resistance occurrence in vivo. However, note that resistance against an antifungal compound can also occur via other mechanisms than mutation/deletion of the target gene, namely via increased efflux of the compound, modulation of its target, or via a compensatory mutation in a related biochemical pathway, etc.

We identified 11 deletion mutants with at least fivefold increased sensitivity to compound **11** (listed in Table 2). Most genes fall into a limited set of functional classes, including (i) vacuolar functionality and assembly of vacuolar proton-translocating ATPase, (ii) DNA synthesis and repair, transcription and chromatin structure and (iii) meiotic cell division. We could further demonstrate that these compound **11** hypersensitive yeast deletion mutants are not hypersensitive to the structurally very similar non-ROS inducing compound **2** (results not shown), pointing to the specificity of the identified tolerance genes towards compound **11**. we will briefly discuss the two major functional classes of compound **11** tolerance genes in the next paragraphs.

(i) The largest proportion of compound **11** tolerance genes fall in the functional class of vacuolar functionality and more specifically in assembly and functionality of vacuolar H^+ ATPase (V-ATPase). V-ATPase has a role in the acidification of vacuoles,

Table 2
Compound **11** tolerance genes

Gene	Function
Vacuolar functionality	
VPS15	Serine/threonine protein kinase involved in vacuolar protein sorting
CUP5	Proteolipid subunit of the vacuolar H^+ proton-translocating ATPase (V-ATPase) V0 sector
VPH2	Membrane protein required for V-ATPase function
VMA7	Subunit of the V1 sector of V-ATPase
Transcription	
ADA2	Transcriptional co-activator, component of the ADA/SAGA complexes
HTL1	Component of the RSC chromatin remodeling complex
SPT7	Subunit of the SAGA transcriptional regulatory complex
Meiotic cell division	
GET2	Protein required for meiotic nuclear division
YKL118W	Hypothetical protein, involved in meiotic cell division
Varia	
REG1	Regulatory subunit of type 1 protein phosphatase Glc7p
AMD1	AMP deaminase, possibly involved in regulation of intracellular adenine nucleotide pools

controlling several antiport systems for ions and organic molecules and playing a role in maintaining cellular electrochemical equilibrium. A link between oxidative stress and V-ATPase was demonstrated in *Caenorhabditis elegans* in which a gene encoding a V-ATPase subunit was upregulated upon application of oxidative stress through incubation under high oxygen levels.[11] Additionally, V-ATPase was reported to play a role in resistance to cellular toxins,[12] the drug trifluoroperazine[13] and the antimicrobial peptide MiAMP1 isolated from *Macadamia integrifolia*.[14]

(ii) Yeast mutants affected in genes involved in DNA synthesis and repair, transcription and chromatin structure (including ADA/SAGA histone acetyltransferase complexes or SWI/SNF nucleosome remodeling complex) were previously identified as hypersensitive to a variety of stresses, including oxidative and chemical stress.[12,15] This finding highlights the requirement for de novo transcription in response to environmental stress.

In conclusion, we demonstrated high fungicidal activity against *C. albicans* of 3,5-dichloropyrazin-2(1*H*)-ones with chlorine at 3-position and chloromethyl (or bromomethyl) at 6-position (compounds **11** and **20**). Both compounds induced accumulation of ROS in *C. albicans*. Moreover, this ROS induction was causally linked with the antifungal activity of compound **11**. Additionally, in order to identify general cellular mechanisms underlying the fungicidal activity of compound **11**, we demonstrated that genes involved in vacuolar functionality (including V-ATPases) and DNA-related functions play an important role.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.06.024.

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3. An overnight *C. albicans* culture in YPD (1% yeast extract; 2% peptone; 2% glucose) was diluted in PBS ($\sim 2 \times 10^6$ cells/mL) and treated with various concentrations of the compounds of interest in 2% DMSO. After 5 h incubation at 37 °C, colony forming units (CFUs) were determined. Percentage survival was determined as the ratio of the number of CFUs after compound treatment to the number of CFUs after 2% DMSO (control) treatment.
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5. *General procedure for the synthesis of substituted 3, 5-dichloropyrazin-2(1H)-ones*. In a 500 mL round-bottomed flask $NaHSO_3$ (0.05 mol) was dissolved in water and aldehyde (0.05 mol) was added dropwise under an argon atmosphere. After stirring for 45 min, a solution of substituted amine (0.05 mol) in methanol was added dropwise to the reaction mixture and stirring was continued for 2 h at 60 °C. Subsequently, NaCN (0.05 mol) was added and the reaction mixture was left stirring overnight at 60 °C (under hood). The reaction mixture was cooled to room temperature and extracted with dichloromethane (3×50 mL). The organic phases were combined, washed with brine (50 mL), then dried for 2 h over Na_2SO_4 and concentrated. The residue was dissolved in dry ether (200 mL) and the resulting solution was cooled to 0 °C. Dry HCl gas was bubbled through the solution upon vigorous stirring for 20 min and the precipitate was filtered off and dried. Subsequently, the precipitate was suspended in 500 mL flask in dry toluene and oxalyl chloride (0.2 mol, 4.0 equiv) was added dropwise under an argon atmosphere. After stirring for 45 min, triethylamine hydrochloride (0.075 mol, 1.5 equiv) was added by small portions and DMF (0.1 equiv) as catalyst and the reaction mixture was kept stirring for 2 days. It was then concentrated under reduced pressure and the residue was purified by silica gel column chromatography (from 10% to 30% EtOAc in petroleum ether) to obtain substituted 3,5-dichloropyrazin-2(1*H*)-ones **1**–**10**, **12**–**19**.
6. See Supplementary data for the experimental procedure.
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10. The individual yeast deletion mutants were grown in 96 well microtiterplates containing 100 μL YPD. After 48 h incubation at 30 °C, the individual deletion mutants were spotted on YPD agar plates containing 0.5 μg/mL or 12.5 μg/mL compound **11** using a 96-pin replicator. The wild type strain BY4741 failed to grow on plates containing 2.5 μg/mL compound **11**. After 48–72 h incubation at 30 °C, plates were scored and hypersensitive mutants were identified. The identified compound **11** hypersensitive mutants were retested as follows. Five-microliter samples of fivefold serial dilutions of each yeast cell culture including the wild type strain BY4741 (grown to stationary phase in YPD in microtiterplates) were spotted on YPD plates containing 0 or 2.5 μg/mL compound **11**. Growth was assessed after 48 h of incubation at 30 °C. Only mutants displaying less growth than the wild type strain BY4741 in this assay were considered compound **11** hypersensitive mutants.
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