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Synthesis and antifungal activity of (*Z*)-5-arylidenerhodanines

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Abstract—An efficient microwave-assisted synthesis of new (*Z*)-5-arylidenerhodanines under solvent-free conditions is described and their in vitro antifungal activity was evaluated following the CLSI (formerly NCCLS) guidelines against a panel of both standardized and clinical opportunistic pathogenic fungi. An analysis of the structure–activity relationship (SAR) along with computational studies showed that the most active compounds (F- and CF_3-substituted rhodanines) possess high log *P* values and low polarizability. Mechanism-based assays suggest that active compounds neither would bind to ergosterol nor would produce a damage to the fungal membrane.

1. Introduction

Fungal infections have emerged as a major cause of morbidity and often of mortality in immunocompromised and debilitated patients over the past two decades.[1,2] A matter of concern in the treatment of fungal infections is the limited number of efficacious antifungal drugs. Many of the currently available drugs are toxic, produce recurrence because they are fungistatic and not fungicides or lead to the development of resistance due in part to the prolonged periods of administration of the available antifungal drugs. Although the use of a new generation of triazoles, the available polyenes in lipid formulations, the use of echinocandins or the combination therapy have been introduced as alternatives in the last ten years, fungal infections remain difficult to eradicate.[3] There is, therefore, a clear need for the discovery of new structures with antifungal properties, which could lead to the development of new drugs for the management of fungal infections.

Compounds containing the 2-thioxothiazolidin-4-one ring [rhodanine (**1**) and derivatives] have showed a wide range of pharmacological activities, which includes antimicrobial,[4–9] antiviral,[10] and anticonvulsant[11] effects. Additionally, rhodanine-based molecules have been popular as small molecule inhibitors of numerous targets such as HCV NS3 protease,[12] aldose reductase,[13,14] β-lactamase,[15] UDP-*N*-acetylmuramate/L-alanine ligase,[16] antidiabetic agents,[17] cathepsin D,[18] and histidine decarboxylase.[19]

As part of our ongoing project devoted to the synthesis of heterocycles including the use of bi-electrophiles in condensation reactions with heterocyclic amines,[20–22] we prepared a series of benzylidene derivatives of rhodanine (**1**) by using microwave-induced (MW) reactions. This methodology has demonstrated to be effective for synthesizing various and structurally diverse heterocyclic compounds[23–26] and a few examples of MW-assisted preparation of rhodanine derivatives have been previously described.[27,28]

In the present paper, we report the synthesis of fourteen (*Z*)-5-arylidenerhodanines, their antifungal activity including Minimum Inhibitory Concentrations and

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Minimum Fungicidal Concentrations against standardized as well as clinical isolated fungi along with an analysis of the structure–activity relationship (SAR). Some studies on the mode of action are included too.

2. Results and discussion

2.1. Chemistry

The reaction between rhodanine **1** and aldehydes **2a**–**2n** was carried out by microwave irradiation (600 watts) during 3 min giving (*Z*)-5-arylidene-2-thioxothiazolidin-4-ones **3a**–**3n** in 47–88% yield (Scheme 1).

The structures of compounds **3a**–**3n** were assigned by ^{1}H and ^{13}C NMR spectra and mass spectrometric data (see Section 4) which are consistent with the proposed 5-arylidenerhodanine structures. For example, the ^{1}H NMR and of compound **3a** shows two singlets at 13.80, 7.65 ppm, with integrals in the ratio 1:1, which were readily assigned to the N-H proton (deuterium exchangeable proton) of rhodanine ring and to the =C–H proton, respectively, and one multiplet integrating for 5 protons at 7.50–7.58 ppm, corresponding to aromatic protons of phenyl ring. Regarding ^{13}C NMR spectra, DEPT experiments allowed us the assignment of the signals belonging to quaternary, tertiary, secondary, and primary carbon atoms of compounds **3a**–**3n**.

The unequivocal assignment of signals in the ^{1}H and ^{13}C NMR spectra of compounds **3a**–**3n** was deduced from the concerted application of both, direct and long-range heteronuclear chemical shift correlation experiments. One-bond proton-carbon chemical shift correlations were established using the HSQC[29] and the long-range correlation responses over to two and three bonds (^{2}J or ^{3}J couplings) using the HMBC[30] technique.

Regarding MS, the fragmentation pattern is characteristic for all compounds with a base peak corresponding to $[M^{+} - C_2HNOS]$ $[M^{+} - 87]$ which is the dipole generated by a retrocycloaddition of the cyclic system and formation of the dipolarophile ArCH=C=S.[31]

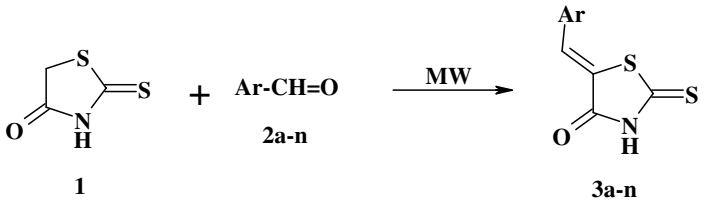
The isolation of single crystals for some compounds permitted us to corroborate the postulated structures including their Z-configuration by single crystal X-ray diffraction analysis.[32,33]

2.2. Antifungal activity and structure–activity relationship studies

To carry out the antifungal evaluation, concentrations of rhodanines**3a**–**3n** up to 250 μg/mL were incorporated to growth media according to CLSI (formerly NCCLS) guidelines.[34]

Table 1 summarizes the concentration of rhodanines **3a**–**3n** that completely inhibited the growth of 10 opportunistic pathogenic fungi including yeasts (*Candida albicans*, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae*), hialohyphomycetes (*Aspergillus* spp.) as well as dermatophytes (*Microsporum* and *Trichophyton* spp.) (MIC_{100}). As main observations, it can be stated that only compounds with structure A showed interesting antifungal activities (MICs < 20 μg/mL). Among them, compounds **3a**, **3b**, **3d**, **3e**, **3f**, and **3g** showed significant activities against *C. albicans*, *C. neoformans*, *S. cerevisiae*, and/or dermatophytes each one showing MICs as low as 3.9–15.6 μg/mL unless in one fungal species.

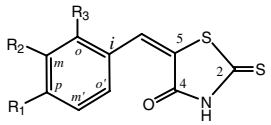
From the analysis of the structures and the activities displayed, some structure–activity relationships can be extracted: (a) the 2-thioxothiazolidin-4-one ring could be a structural requirement but not by itself sufficient for antifungal activity as it is clearly suggested by the lack of activity of some compounds of types A and all structures of type B. The same can be applied to the en-one linkage, considering that some structures type A and all of type B are inactive; (b) a substituted benzene ring but not a pyridine ring (α, β or γ) is a necessary moiety for these compounds to possess antifungal activity (compare structures type A possessing a substituted benzene ring and type B containing pyridine); (c) the type of substituents on the benzene ring appears to play an important role in activity, and this behavior depends on the fungal species to which they are faced to. The activity of this series against *C. albicans* and *S. cerevisiae* seems to be related to the presence of a F or a CF_3 (see activity of **3d** and **3e**). Nevertheless the replacement of F by Cl was well tolerated (compound **3b**). Compounds without substituents (**3a**) and possessing a Br atom (**3c**) suffered a clear loss of activity. In general, structures



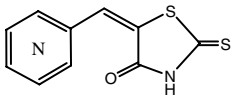
1 3a-n

Scheme 1. General methodology for the preparation of arylidenerhodanines **3a**–**3n**. Ar = **a**: C_6H_5; **b**: 4-ClC_6H_4; **c**: 4-BrC_6H_4; **d**: 4-FC_6H_4; **e**: 4-$CF_3C_6H_4$; **f**: 2-FC_6H_4; **g**: 2-$CF_3C_6H_4$; **h**: 4-$CH_3C_6H_4$; **i**: 4-$CH_3OC_6H_4$; **j**: 3,4-$OCH_2OC_6H_3$; **k**: 3,4,5-tri-$CH_3OC_6H_2$; **l**: 2-pyridyl; **m**: 3-pyridyl; **n**: 4-pyridyl.

Table 1. MIC values (μg/mL) of aryliden-rhodanines **3a**–**3n** acting against human opportunistic pathogenic fungi



A



B

Compound	Type	R_1	R_2	R_3	N	Ca	Sc	Cn	Afu	Afl	Ani	Mg	Tr	Tm
3a	**A**	**H**	**H**	**H**	—	**125**	**125**	**62.5**	**62.5**	**62.5**	**125**	**7.8**	**3.9**	**3.9**
3b	**A**	**Cl**	**H**	**H**	—	**250**	**250**	**15.6**	**125**	**125**	**250**	**62.5**	**62.5**	**62.5**
3c	**A**	**Br**	**H**	**H**	—	**>250**	**250**	**62.5**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**
3d	**A**	**F**	**H**	**H**	—	**7.8**	**7.8**	**7.8**	**250**	**250**	**250**	**15.6**	**32**	**7.8**
3e	**A**	**CF_3**	**H**	**H**	—	**15.6**	**15.6**	**3.9**	**125**	**62.5**	**62.5**	**15.6**	**15.6**	**3.9**
3f	**A**	**H**	**H**	**F**	—	**250**	**250**	**15.6**	**>250**	**>250**	**>250**	**3.9**	**3.9**	**3.9**
3g	**A**	**H**	**H**	**CF_3**	—	**250**	**250**	**15.6**	**>250**	**>250**	**>250**	**3.9**	**3.9**	**3.9**
3h	**A**	**CH_3**	**H**	**H**	—	**>250**	**>250**	**125**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**
3i	**A**	**OCH_3**	**H**	**H**	—	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**
3j	**A**	**O–CH_2-O**		**H**	—	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**
3k	**A**	**OCH_3**	**OCH_3**	**OCH_3**	—	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**
3l	**B**	—	—	—	α	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**
3m	**B**	—	—	—	β	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**
3n	**B**	—	—	—	γ	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**	**>250**
Amph B						0.78	0.50	0.25	0.50	0.50	0.50	0.125	0.075	0.075
Terbinafine						1.56	3.12	0.39	0.78	0.78	1.56	0.04	0.01	0.025
Ketoconazole						**0.5**	**0.5**	0.25	0.125	0.50	0.25	0.05	0.025	0.025

Ca: *Candida albicans* ATCC 10231, Sc: *Saccharomyces cerevisiae* ATCC 9763, Cn: *Cryptococcus neoformans* ATCC 32264; An: *Aspergillus niger* ATCC 9029, Afu: *Aspergillus fumigatus* ATCC 26934; Afl: *Aspergillus flavus* ATCC 9170, Mg: *Microsporum gypseum* C 115; Tr: *Trichophyton rubrum* C113, Tm: *Trichophyton mentagrophytes* ATCC 9972; Amph B: Amphotericin B.

with donor substituents (**3h**, **3i**, **3j**, **3k**) showed marginal or null activity; (d) the position of substituents appears to play an important role in activity too, since the change of F from *para* to *ortho* positions (**3d** → **3f** and **3e** → **3g**) led to a decrease of activity.

2.3. Second order studies and studies with clinical isolates

In order to gain insight into the spectrum of activity, the rhodanines that showed the best fungistatic activity (**3d** and **3e**) were tested against an extended panel of clinical isolates of some clinically important fungal species.

The MIC values of the most active rhodanines were determined against this new panel by using three end-points: MIC_{100}, MIC_{80}, and MIC_{50} (the minimum concentration of compounds that inhibit 100%, 80%, and 50% of growth, respectively). The application of a less stringent end point such as MIC_{80} and MIC_{50} has been showed to consistently represent the in vitro activity of compounds[34] and many times provide a better correlation with other measurements of antifungal activity such as the minimum fungicide concentration (MFC).[35,36]

In addition to MIC determinations, the evaluation of MFC of each active compound against this extended panel was accomplished by subculturing a sample of media from MIC tubes showing no growth, onto drug-free agar plates.

So, compounds **3d** and **3e** were tested against fourteen other *Candida* strains, including six clinical isolates of *C. albicans* and the rest, non-*albicans Candida* spp, all of them provided by CEREMIC (see Section 4) and 10 strains of *C. neoformans* provided by Malbrán Institute (Buenos Aires).

The selection of *Candida* strains was due to the importance that this fungal genus possesses in the epidemiology of fungal infections. It is known that *Candida* spp. are among the leading causes of nosocomial bloodstream infections worldwide and although *C. albicans* was in the past the usual spp. associated with invasive infection, at present non-*albicans Candida* spp such as *C. tropicalis*, *Candida glabrata*, *Candida parapsilopsis*, *Candida krusei*, and others comprise more than half of the isolates of candidosis.[2,3] In turn, *C. neoformans* remains an important life-threatening complication for immunocompromised hosts, particularly for patients who have undergone transplantation of solid organs and therefore, new compounds acting against this fungus are highly welcome.[1,37]

Results are shown in Table 2. The MIC values of these two compounds against all *Candida* isolates and *C. neoformans* were very low especially against this last species (medians between 2.98 and 6.47 μg/mL). Interesting enough, the two compounds were fungicide against all yeasts tested, with median MFC values between 11.7 and 40.17 μg/mL.

In addition, compounds displaying MIC values <20 μg/mL (**3a**, **3d**–**3g**) against the dermatophytes of the first panel (see Table 1) were tested against clinical isolates of *Trichophyton mentagrophytes* and *Trichophyton rubrum* (Table 3). Interesting enough, the five rhodanines displayed very strong activities with MIC values between

Table 2. Minimal inhibitory concentrations (MIC_{100}, MIC_{80} and MIC_{50}) and minimal fungicidal concentration (MFC) of rhodanines **3d** and **3e** against clinical isolates of *Candida* genus and *C. neoformans*

Strain	Voucher specimen	**3d**				**3e**				Amph. B	Ket.	Itz	5FC	Vcz
		MIC_{100}	MIC_{80}	MIC_{50}	CFM	MIC_{100}	MIC_{80}	MIC_{50}	CFM					
C. albicans 1	ATCC 10231	7.8	7.8	3.9	15.6	15.6	15.6	7.8	31.2	1.0	0,5	—	—	—
C. albicans 2	C 125-2000	15.6	7.8	7.8	31.2	31.2	31.2	15.6	62.5	0.78	6.25	—	—	—
C. albicans 3	C 126-2000	15.6	15.6	3.9	31.2	15.6	15.6	15.6	31.2	1.56	1.56	—	—	—
C. albicans 4	C 127-2000	15.6	15.6	7.8	31.2	15.6	15.6	7.8	31.2	0.78	6.25	—	—	—
C. albicans 5	C 128-2000	31.2	15.6	3.9	62.5	15.6	15.6	15.6	31.2	1.56	6.25	—	—	—
C. albicans 6	C 129-2000	15.6	7.8	3.9	31.2	31.2	31.2	15.6	31.2	0.78	12.5	—	—	—
C. albicans 7	C 130-2000	15.6	7.8	3.9	31.2	15.6	15.6	7.8	62.5	0.39	6.25	—	—	—
Median		16.71	8.91	5.01	33.44	20.05	20.05	12.25	40.17					
C. glabrata	C 115-2000	7.8	3.9	3.9	15.6	3.9	3.9	3.9	15.6	0.39	1.56	—	—	—
C. parapsilopsis	C 124-2000	7.8	3.9	7.8	15.6	31.2	31.2	3.9	62.5	0.78	0.78	—		—
C. lusitaniae	C 131-2000	7.8	3.9	3.9	15.6	31.2	31.2	15.6	62.5	0.39	25	—	—	—
C. colliculosa	C 122-2000	7.8	3.9	3.9	15.6	31.2	31.2	15.6	62.5	0.36	0.78	—	—	—
C. krusei	C 117-2000	31.2	7.8	3.9	62.5	15.6	15.6	7.8	31.2	0.39	50	—	—	—
C. kefyr	C 123-2000	31.2	7.8	3.9	31.2	7.8	7.8	3.9	15.6	0.78	0.78	—	—	—
C. tropicalis	C 131-1997	7.8	7.8	3.9	15.6	15.6	15.6	7.8	31.2	0.5	0.125	—	—	—
Median		14.48	5.57	4.45	24.52	20.06	20.06	8.35	40.17					
C. neoformans	ATCC 32264	7.8	7.8	3.9	31.2	3.9	3.9	3.9	7.8	0.25	0.25	0.15		
C. neoformans	IM 983040	15.6	1.9	1.9	31.2	7.8	7.8	1.9	15.6	0.13	—	<0.015	7.8	<0.015
C. neoformans	IM 972724	15.6	7.8	3.9	31.2	7.8	7.8	1.9	7.8	0.06	—	0.25	3.9	<0.015
C. neoformans	IM 042074	7.8	3.9	3.9	31.2	7.8	7.8	3.9	7.8	0.25	—	<0.015	3.9	<0.015
C. neoformans	IM 983036	15.6	7.8	7.8	62.5	7.8	7.8	1.9	7.8	0.13	—	<0.015	7.8	<0.015
C. neoformans	IM 00319	7.8	1.9	1.9	15.6	7.8	3.9	3.9	7.8	0.25	—	<0.015	n.t.	<0.015
C. neoformans	IM 972751	7.8	1.9	1.9	62.5	7.8	3.9	1.9	7.8	0.25	—	<0.015	15.6	0.030
C. neoformans	IM 031631	7.8	3.9	1.9	31.2	7.8	7.8	3.9	15.6	0.13	—	0.25	7.8	0.25
C. neoformans	IM 031706	7.8	3.9	1.9	31.2	7.8	7.8	7.8	31.2	0.25	—	0.50	7.8	0.50
C. neoformans	IM 961951	7.8	7.8	1.9	15.6	7.8	3.9	1.9	7.8	0.06	—	<0.015	3.9	0.015
C. neoformans	IM 052470	7.8	1.9	1.9	31.2	7.8	7.8	1.9	12.5	0.50	—	<0.015	7.8	0.030
Median		9.92	4.59	2.98	32.63	7.44	6.47	3.16	11.77					

For the sake of comparison the MIC and MFC of both compounds against an ATCC standardized strain of *C. albicans* and *C. neoformans* are included.

MIC_{100}, MIC_{80} and MIC_{50}: concentration of a compound that provoked (induced) 100%, 80% or 50% reduction of the growth control, respectively.

Within Voucher specimen: ATCC = American Type Culture Collection (Illinois, USA); C = Center of Mycological Reference (Rosario, Argentina); IM = Malbran Institute (Buenos Aires, Argentina). *C. albicans* = *Candida albicans*; *C. glabrata* = *Candida glabrata*; *C. parapsilopsis* = *Candida parapsilopsis*; *C. lusitanae* = *Candida lusitanae*; *C. colliculosa* = *Candida colliculosa*; *C. krusei* = *Candida krusei*; *C. kefyr* = *Candida kefyr*; *C. tropicalis* = *Candida tropicalis*; *C. neoformans* = *Cryptococcus neoformas*. Amph B = Amphotericin B; Ket = Ketoconazole; Itz = Itraconazole; 5FC = 5-Fluocitosine; Vcz = Voriconazole.

Table 3. Minimum inhibitory concentration (MIC_{100}, μg/mL) of rhodanines **3a**, **3d**–**3g** against clinical isolates of *Trichophyton* genus

Strain	Voucher specimen	3a	3d	3e	3f	3g	Terb
T. rubrum	C 110	3.9	n.t.	n.t.	1.95	3.9	0.006
T. rubrum	C 135	3.9	n.t.	n.t.	1.95	3.9	0.006
T. rubrum	C 136	3.9	n.t.	n.t.	1.95	3.9	0.006
T. rubrum	C 137	3.9	n.t.	n.t.	0.97	1.95	0.006
T. rubrum	C 139	3.9	n.t.	n.t.	1.95	3.9	0.012
T. rubrum	C 140	3.9	n.t.	n.t.	0.97	3.9	0.003
Median		3.9			1.62	3.57	
T. mentagrophytes	C 108	3.9	1.95	1.95	1.95	1.95	0.006
T. mentagrophytes	C 364	3.9	0.97	0.97	1.95	0.97	0.006
T. mentagrophytes	C 539	3.9	1.95	1.95	0.97	1.95	0.006
T. mentagrophytes	C 738	1.95	1.95	1.95	1.95	1.95	0.006
T. mentagrophytes	C 943	3.9	0.97	0.97	0.97	0.97	0.006
Median		3.51	1.17	1.17	1.17	1.17	

C = Center of Mycological Reference (Rosario, Argentina), Terb. = Terbinafine; n.t. = not tested.

1.17 and 3.57 μg/mL. This result is very interesting since these fungi are responsible for approximately 80–93% of chronic and recurrent dermatophyte infections in human beings. They are the etiological agent of tinea unguium (producer of invasive nail infections), tinea manuum (palmar and interdigital areas of the hand infections), and tinea pedis (Athlete's foot), the last one being the most prevalent fungal infection in developed countries, and the first one accounting for 50% and 90% of all fingernail and toenail infections, respectively.[38]

2.4. Computational studies

In order to correlate the qualitative structure–activity relationships described above, with quantitative parameters, we calculated the log *P* for all rhodanines (**3a**–**3n**) and attempted to find a relationship between these values and their MICs (in μg/mL) against *C. neoformans* that is the fungi that was inhibited by a higher number of compounds (8/14). Results are shown in Figure 1.

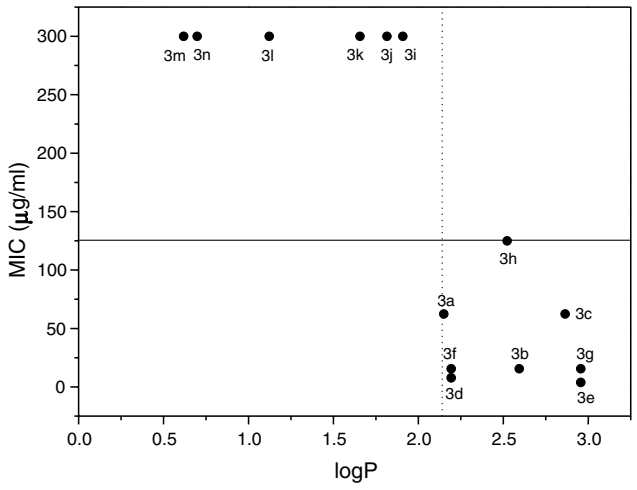


Figure 1. Log *P* vs. MIC values of aryliden-rhodanines **3a**–**3n** against *C. neoformans* ATCC 32264.

It is known that log *P* describes the macroscopic hydrophobicity of a molecule which is a factor that determines its ability to penetrate the membranes of fungal cells and to reach the interacting sites, thus influencing the antifungal activity of compounds.[39]

From Figure 1 it is clear that active compounds against *C. neoformans* (**3a**–**3h**, MIC values ⩽250 μg/mL) possessed higher log *P* values (between 2.15 and 2.95) than the rest of rhodanines. In contrast, compounds possessing log *P* values lower than 1.89 (compounds **3i**–**3n**) were inactive.

In addition, we studied the polarity of arylidenerhodanines through the calculation of the dipole moment, which is a useful parameter to quantify the polarity.[40,41] The most active compounds **3e** and **3d** possess dipole moment values (μ) of 1.72 and 3.09 Debyes, respectively, whereas inactive compounds **3j**, **3k** and **3i** possess a higher polarity with dipole moment values of 5.40, 5.43, and 6.49 D each, suggesting that the antifungal activity is related to a low polarity. Compounds **3a**, **3b**, **3c**, **3f**, **3g**, and **3h** displayed 4.17, 2.49, 2.61, 4.12, 3.93, and 4.19 D, respectively, which are intermediate values in accordance with experimental results. However, it is necessary to keep in mind that the total dipole moment reflects only the global polarity of a molecule but not local dipole moments which, at a first approximation, can be obtained by considering the atomic charges in localized regions of the molecule. The electron distribution can also be used to quantitatively map the electrostatic potential generated by a molecule in the different regions surrounding it.[42] In the case of rhodanines, since the main differences between active and inactive compounds are the nature and position of substituents on the benzene ring, it was worthwhile to investigate the distribution of charge within this ring when it supports different R_1 substituents and to depict the molecular electrostatic potentials (MEPs)[43–45] of these compounds. Details of the calculations (including a brief explanation on the conformational study previously performed) are given in Computational Methods section. The MEP energy isosurfaces of compounds **3d**, **3j**, and **3k** are shown in Figures 2a–c. Electron den-

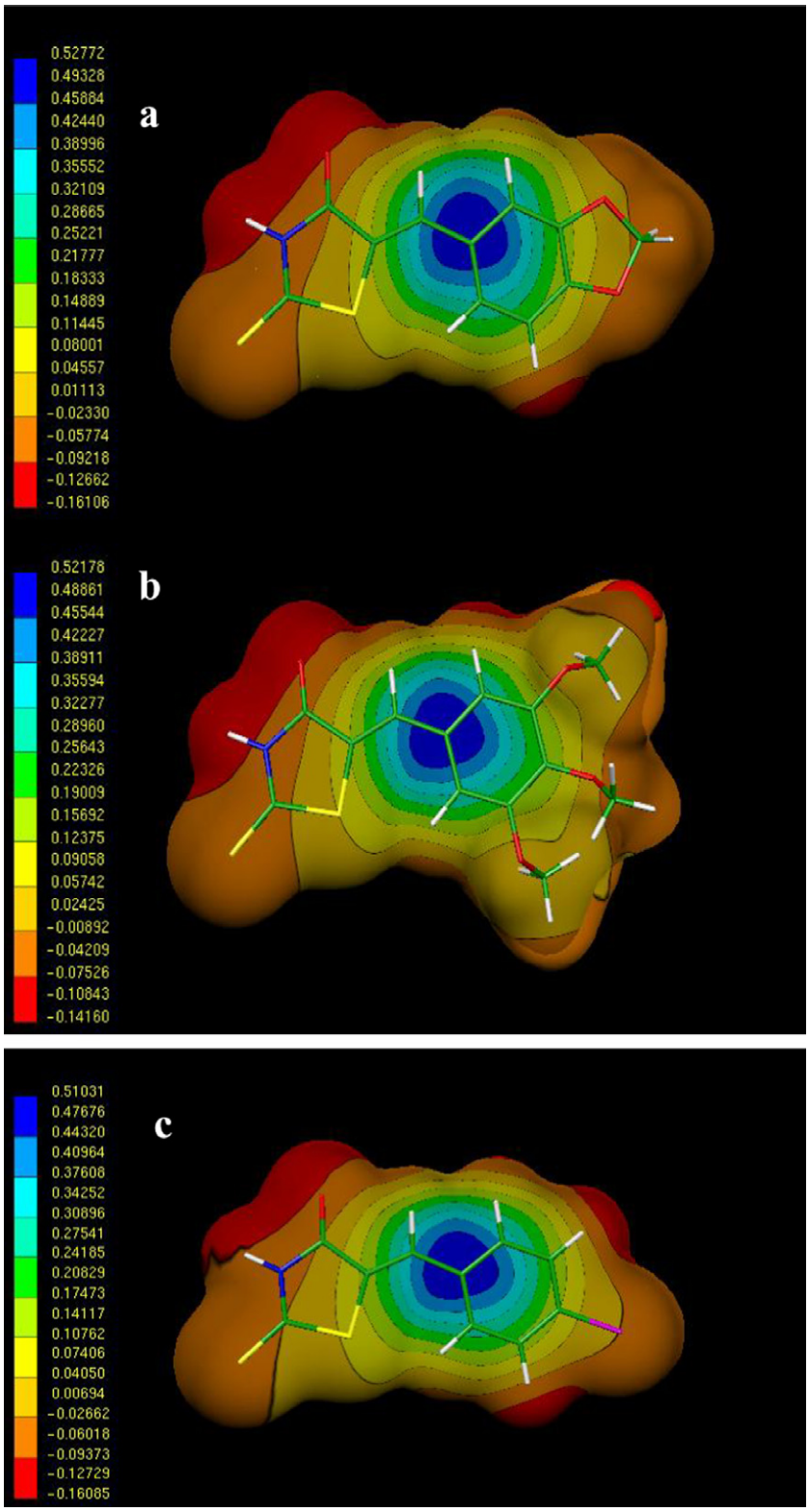


Figure 2. Electrostatic potential-encoded electron density surfaces of the core structures of compounds **3j** (a), **3k** (b), and **3d** (c). The surfaces were generated with Gaussian 03 using B3LYP/aug-cc-pVDZ//B3LYP/6-31G(d) single-point calculations after B3LYP/6-31G(d) minimization. The coloring represents electrostatic potential with red indicating the strongest attraction to a positive point charge and blue indicating the strongest repulsion. The electrostatic potential is the energy of interaction of the positive point charge with the nuclei and electrons of a molecule. It provides a representative measure of overall molecular charge distribution.

sity surfaces encoded with the electrostatic potential obtained by B3LYP/aug-cc-pVDZ//B3LYP/6-31G(d) calculations demonstrate that the inactive compounds **3j** and **3k** have a highly polarized electron distribution at ring A (Figs. 2a and b), while the analogous graph for the most active compound **3d** shows a different and

much less polarized electrostatic potential surface in this ring (Fig. 2c). These results account for the general characteristics of this correlation since the decrease or enhancement of antifungal activity of the rest of rhodanines, when they possess donating or attracting groups, respectively, is in agreement with this electronic study. Calculations have been carried out for the rest of the compounds reported here, with results of Figures 2a–d being considered representative of the overall phenomenon.

2.5. Studies on mode of action

The fact that the most active compounds possess a high lipophilicity and a low polarity suggests that they would be able to penetrate the fungal membrane and to bind to ergosterol or to other lipophilic components such as the lipid tail of membrane phospholipids producing damage (or eventually a pore). This possibility prompted us to study if the most active rhodanines, **3d** and **3e** bind to ergosterol, by using the 'Ergosterol Effect Assay'. In this assay, we determined the MIC of rhodanine **3d** and **3e** in the presence of different concentrations of exogen ergosterol. As already known, the binding of a compound to exogen ergosterol should produce an enhancement of MIC values of compounds which acts by binding to membrane ergosterol as its mechanism of antifungal action.[46] Results showed (Fig. 3) that MIC values of compounds **3d** and **3e** against *C. albicans* did not change in the presence of up to 400 μg/mL of ergosterol, therefore suggesting that these compounds would not act by binding to ergosterol.

As a complement of the above assay, the action of rhodanines **3d** and **3e** on the permeability of yeast cells was tested by measuring the intracellular-components' release material that absorb at 260 nm (nucleic acids adenine, timine, guanine, cytosine, uracil, and aromatic aminoacids). Concentrations of 4× and 10× CFM were added to cell suspensions as reported previously, and samples were examined at several time intervals (2, 4, and 6 h).[46] The results are showed in Figure 4. The OD_{260} of filtrates containing testing compounds were not significantly different of control suspensions after an incubation period of 6 h, therefore suggesting that rhodanines **3d** and **3e** were neither completely lysed nor partially damaged. Perchloric acid-treated cells (see Section 4) were considered 100% cellular leakage.

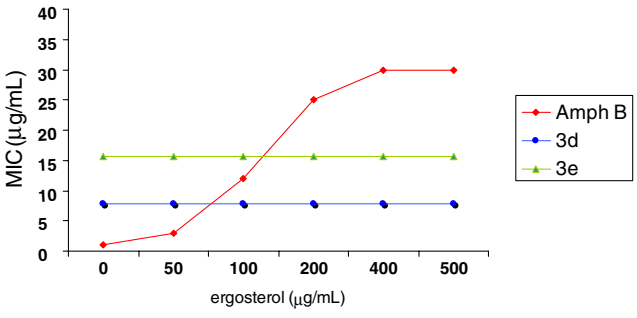


Figure 3. Effect of exogen ergosterol (50–500 μg/mL) on the MIC of rhodanines **3d** and **3e** against *C. albicans* ATCC 10231 (Amph B = Amphotericin B).

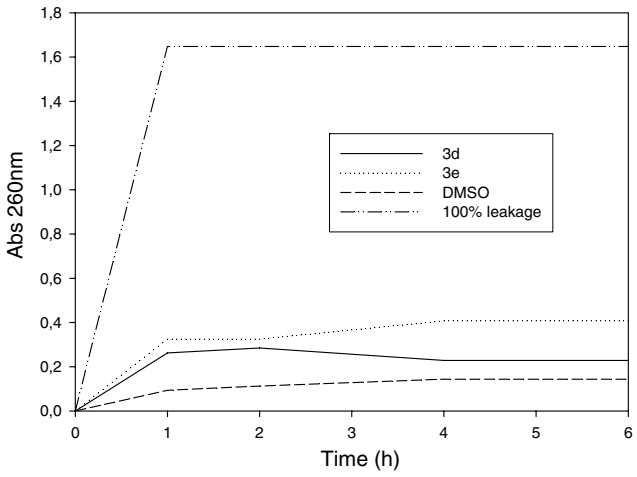


Figure 4. Release of 260-UV absorbing materials from cells of *S. cerevisiae* incubated with 10 × CFM of compounds **3d** and **3e** for 2, 4, and 6 h.

3. Conclusion

We report here a group of benzyliden-rhodanines acting as antifungal agents. Among them, compounds **3d** and **3e** showed to be fungicides and were the most active against *Candida* genus and *C. neoformans* including clinical isolates. Compounds **3a**, **3d**, **3e**, **3f**, and **3g** showed also a very good activity against dermatophytes.

SAR studies on the rhodanine compounds including the use of log *P*, dipole moments, and MEPs allowed to understand the minimal structural requirements for rhodanines to display antifungal activity. It appears that a 2-thioxothiazolidin-4-one ring and an enone linkage are indispensable for activity. In addition, our results indicate that compounds possessing both a relatively high log *P* value and a weakly polarized ring A were the most active compounds in this series.

Regarding their mode of action, our results indicate that the most active compounds would not either bind to the ergosterol of the fungal membrane or would produce a damage in it because there was not observed a cellular leakage of 260 nm-absorbing materials at concentrations as high as 10-fold MFC of **3d** and **3e**.

Since there exist many known biochemical and molecular targets for antifungal compounds and many efforts are being directed toward the identification and development of new ones, it is not possible to suggest a possible mechanism of action for benzyliden-rhodanines. Nevertheless, their lipophilic character added to the fact that they do not produce a damage in the membrane lead to the conclusion that they may traverse the membranes and interact with intracellular targets such as pathways of the intermediary metabolism (nucleic acid, amino acid or polyamine synthesis), microtubule formation

or any other process that takes place inside the fungal cells.[47,48]

4. Experimental

4.1. Chemistry

Melting points were determined in a Büchi Melting Point Apparatus and are uncorrected. The ^{1}H and ^{13}C NMR spectra were run on a Bruker DPX 300 spectrometer operating at 300 and 75 MHz, respectively, using dimethylsulfoxide-d_6 as solvent and tetramethylsilane as internal standard. The mass spectra were scanned on a Hewlett Packard HP Engine-5989 spectrometer (equipped with a direct inlet probe) operating at 70 eV. High Resolution Mass Spectra (HRMS) were recorded in a Waters Micromass AutoSpec NT spectrometer (STIUJA).

4.1.1. Synthesis of (*Z*)-aryl-2-thioxothiazolidin-4-ones (3a–3n). Equimolar amounts of 2-thioxothiazolidin-4-one (1 mmol) and the appropriate benzaldehyde (1 mmol) were placed in open Pyrex-glass vessels in the absence of any solvent and irradiated (3 min) in a domestic microwave oven (at 600 W); the reactions were monitored by thin-layer chromatography. Considering that this very interesting technique for chemists is suffering from the non-controlled conditions of domestic microwaves, the reactions were repeated in three different domestic microwave ovens to prove reproducibility, and no significant deviation was found. The products **3a**–**3n** were recrystallized from ethanol.

4.1.2. (*Z*)-5-Phenyl-2-thioxothiazolidin-4-one (3a). This compound was obtained according to general procedure as a block orange crystal, yield 58%, mp 205 °C (204–206 °C[49]). δ_H (400 MHz, DMSO) 7.65 (s, 1H, =CH), 7.50 (m, 2H, H*m*), 7.54 (d, 1H, H*p*, J = 8.1 Hz), 7.58 (t, 2H, H*o*), 13.80 (s, 1H, N-H); δ_C (100 MHz, DMSO) 133.0 (C*i*), 129.4 (C*o*), 130.4 (C*m*), 130.7 (C*p*), 131.4 (C=C–H), 125.5 (C-5), 169.4 (C-4), 195.7 (C-2). MS: (70 eV) *m*/*z* (%) = 134 (100, M^+-C_2HNOS), 108 (4), 89 (8), 51 (4), 77 (2). Anal. calcd for $C_{10}H_7NOS_2$: C, 54.28; H, 3.19; N, 6.33. Found C, 54.37; H, 3.23; N, 6.21.

4.1.3. (*Z*)-5-(4-Chlorobenzyliden)-2-thioxothiazolidin-4-one (3b). This compound was obtained according to general procedure as a yellow powder, yield 67%, mp 228 °C (231–232 °C[49]). δ_H (400 MHz, DMSO) 7.55 (s, 1H, =CH), 7.58 (m, 4H, H*o*,*m*); δ_C (100 MHz, DMSO) 132.2 (C*i*), 129.4 (C*o*), 129.0 (C*m*), 127.8 (C*p*), 131.9 (C=C–H), 135.0 (C-5), 171.35 (C-4), 196.4 (C-2). MS: (70 eV) *m*/*z* (%) = 255 (28, M^+), 170 (40), 168 (100, M^+-C_2HNOS), 133 (25), 89(38).

4.1.4. (*Z*)-5-(4-Bromobenzyliden)-2-thioxothiazolidin-4-one (3c). This compound was obtained according to general procedure as an orange powder, yield 62%, mp 231 °C. δ_H (400 MHz, DMSO) 7.61 (s, 1H, =CH), 7.73 (d, 2H, H*o*, J = 8.6 Hz), 7.53 (d, 2H, H*m*, J = 8.6 Hz), 13.82 (s, 1H, N-H); δ_C (100 MHz, DMSO) 124.7 (C*i*), 132.4 (C*o*), 132.2 (C*m*), 124.3 (C*p*), 130.2 (C=C–H), 126.4 (C-5), 169.3 (C-4), 195.4 (C-2). MS: (70 eV) *m*/*z* (%) = 301/299 (31/29, M^+), 212 (27, M^+-C_2HNOS), 133 (24), 89 (100). HRMS (EI): $C_{10}H_6BrNOS_2$ requires: 298.9067. Found: 298.9074.

4.1.5. (*Z*)-5-(4-Fluorobenzyliden)-2-thioxothiazolidin-4-one (3d). This compound was obtained according to general procedure as lath orange crystals, yield 88%, mp 219 °C (226–227 °C[50]). δ_H (400 MHz, DMSO) 7.65 (s, 1H, =CH), 7.67 (d, 2H, H*o*, J = 9.3 Hz, $J_{H\text{-}F}$ = 4.3 Hz), 7.30 (d, 2H, H*m*, J = 9.3 Hz, $J_{H\text{-}F}$ = 6.1 Hz), 13.81 (s, 1H, N-H); δ_C (100 MHz, DMSO) 161.8 (C*i*), 130.5 (C*o*), 132.9 (C*m*), 125.3 (C*p*), 116.6 (C=C–H), 164.3 (C-5), 169.4 (C-4), 195.7 (C-2). MS: (70 eV) *m*/*z* (%) = 239 (24, M^+), 152 (100, M^+-C_2HNOS), 107 (20).

4.1.6. (*Z*)-5-(4-α,α,α-Trifluoromethylbenzyliden)-2-thioxothiazolidin-4-one (3e). This compound was obtained according to general procedure as lath orange crystals, yield 62%, mp 215 °C. δ_H (400 MHz, DMSO) 7.70 (s, 1H, =CH), 7.88 (d, 2H, H*o*, J = 8.4 Hz), 7.78 (d, 2H, H*m*, J = 8.4 Hz), 13.91 (s, 1H, N-H); δ_C (100 MHz, DMSO) 128.6 (C*i*), 129.6 (C*o*), 126.1 (C*m*), 136.9 (C*p*), 130.6 (C=C–H), 122.5 (C-5), 169.2 (C-4), 195.4 (C-2). MS: (70 eV) *m*/*z* (%) = 289 (100, M^+), 152 (35), 138 (11). HRMS (EI): $C_{11}H_6F_3NOS_2$ requires: 288.9840. Found: 288.9843.

4.1.7. (*Z*)-5-(2-Fluorobenzyliden)-2-thioxothiazolidin-4-one (3f). This compound was obtained according to general procedure as lath orange crystals, yield 53%, mp 165 °C (201–203 °C[50]). δ_H (400 MHz, DMSO), 7.59 (s, 1H, =CH), 7.53–7.32 (m, 4H, aromatics), 13.88 (s, 1H, N-H); δ_C (100 MHz, DMSO) 133.1, 125.6, 122.5, 116.3, 120.1 and 161.9 (C-aromatics), 129.5 (C=C–H), 128.1 (C-5), 169.2 (C-4), 195.5 (C-2). MS: (70 ev) *m*/*z* (%) = 239 (4, M^+), 152 (100), 108 (2). Anal. calcd for $C_{10}H_6FNOS2$: C, 50.19; H, 2.53; N, 5.85. Found C, 50.25; H, 2.49; N, 5.81.

4.1.8. (*Z*)-5-(2-α,α,α-trifluoromethylbenzyliden)-2- thioxothiazolidin-4-on (3g). This compound was obtained according to general procedure as a purple power, yield 55%, mp 187 °C. δ_H (400 MHz, DMSO) 7.59 (s, 1H, =CH), 7.56 (m, 1H, H*m*′, J = 7.2 and 8.2 Hz), 7.48 (d, 1H, H*m*, J = 7.7 Hz), 7.38 (d, 1H, H*o*′, J = 7.2 Hz), 7.34 (m, 1H, H*p*, J = 8.2 and 7.7 Hz), 13.90 (s, 1H, N-H); δ_C (100 MHz, DMSO) 159.4 (C*o*), 161.9 (C*i*), 133.1 (C*m*′), 129.3 (C*m*), 128.1 (CF_3), 125.5 (C*p*), 122.3 (C=C–H), 120.8 (C-5), 116.3 (C*o*′), 169.1 (C-4), 195.4 (C-2). MS: (70 eV) *m*/*z* (%) = 289 (40, M^+), 202 (100), 182 (16), 152 (20), 138 (7). HRMS (EI): $C_{11}H_6F_3NOS_2$ requires: 288.9840. Found: 288.9843.

4.1.9. (*Z*)-5-(4-Methylbenzyliden)-2-thioxothiazolidin-4-one (3h). This compound was obtained according to general procedure as lath orange crystals, yield 55%, mp 215 °C (219–220 °C[49]); δ_H (400 MHz, DMSO) 2.34 (s,3H, CH_3), 7.57 (s, 1H, =CH), 7.46 (d, 2H, H*o*,

J = 8.2 Hz), 7.33 (m, 2H, H*m*, J = 8.2 Hz), 13.77 (s, 1H, N-H); δ_C (100 MHz, DMSO) 133.2 (C*i*), 130.5 (C*o*), 130.0 (C*m*), 141.2 (C*p*), 131.8 (C=C–H), 124.2 (C-5), 169.3 (C-4), 195.6 (C-2). MS: (70 eV) *m*/*z* (%) = 235 (39, M^+), 148 (100, M^+-C_2HNOS), 115 (10), 91 (9), 77 (4), 59 (9). HRMS (EI): $C_{11}H_9NOS_2$ requires: 235.0131. Found: 235.0126.

4.1.10. (*Z*)-5-(4-Methoxlbenzyliden)-2-thioxothiazolidin-4-one (3i). This compound was obtained according to general procedure as plate orange crystals, yield 87%, mp 243 °C (509–502 °C[49]). δ_H (400 MHz, DMSO) 3.94 (s, 3H, OCH_3), 7.08 (m, 2H, H*m*, J = 8.4 Hz), 7.52 (s, 1H, =CH), 7.48 (d, 2H, H*o*, J = 8.4 Hz), 13.77 (s, 1H, N-H); δ_C (100 MHz, DMSO) 124.6 (C*i*), 131.2 (C*o*), 132.1 (C*m*), 125.4 (C*p*), 129.8 (C–C=H), 125.3 (C-5), 168.9 (C-4), 195.5 (C-2). MS: (70 eV) *m*/*z* (%) = 164 (100, M^+-C_2HNOS), 149 (61), 122 (11), 89 (17), 77 (34), 63 (12).

4.1.11. (*Z*)-5-Benzo[1,3]dioxol-4-ilmethylen-2-thioxothiazolidin-4-one (3j). This compound was obtained according to general procedure as a yellow powder, yield 57%, mp 300 °C. δ_H (400 MHz, DMSO) 7.54 (s, 1H, =CH), 7.05 (d, 1H, Hb, J = 8.9 and 1.4 Hz), 7.09 (dd, 1H, Hc, J = 8.9 Hz), 7.14 (d, 1H, Hd, J = 8.7 and 1.4 Hz), 6.11 (s, 2 H, CH_2), 13.72 (s, 1H, N-H); δ_C (100 MHz, DMSO) 127.1 (C*o*), 102.1 (C*i*), 126.7 (C*o*′), 109.4 (C*m*′), 149.3 (C*p*), 148.3 (C*m*), 102.1 (OCH_2O), 131.9 (C=C–H), 122.8 (C-5), 169.3 (C-4), 195.3 (C-2). MS: (70 eV) *m*/*z* (%) = 265 (6, M^+), 22 (15), 121 (37), 120 (100), 94 (27). HRMS (EI): $C_{11}H_7NO_3S_2$ requires: 264.9899. Found: 264.9867.

4.1.12. (*Z*)-5-(2,3,4-Trimethoxybenzyliden)-2-thioxothiazolidin-4-one (3k). This compound was obtained according to general procedure as plate orange crystals, yield 85%, mp 201 °C. δ_H (400 MHz, DMSO), 3.82 (s, 9H, OCH_3), 7.57 (s, 1H, =CH), 6.88 (s, 2H, H*o*) 13.80 (s, 1H, N-H); δ_C (100 MHz, DMSO) 139.8 (C-*i*), 108.0 (C*o*), 153.2 (C*m*), 128.4 (C*p*), 132.0 (C=C–H), 124.3 (C-5), 169.2 (C-4), 195.4 (C-2). MS: (70 eV) *m*/*z* (%) = 313 (M^++2), 312 (M^++1), 311 (88, M^+), 224 (100, M^+-C_2HNOS), 209 (94), 181 (16), 166 (9). HRMS (EI): $C_{13}H_{13}NO_4S_2$ requires: 311.0289. Found: 311.0286.

4.1.13. (*Z*)-5-(Pyridin-2-ylmethylen)-2-thioxothiazolidin-4-one (3l). This compound was obtained according to general procedure as a green powder, yield 47%, mp 268 °C (262 °C[51]). δ_H (400 MHz, DMSO) 7.65 (s, 1H, =CH), 7.41 (dd, 1H, Hγ, J = 6.2 and 7.4 Hz), 7.88 (d, 1H, Hβ, J = 6.2 Hz), 7.92 (dd, 1H, Hβ′, J = 7.4 and 4.7 Hz), 8.78 (d, 1H, Hα′, J = 4.7 Hz), 13.62 (s, 1H, N-H); δ_C (100 MHz, DMSO) 151.0 (C-α), 123.4 (C-β), 128.1 (C-γ), 127.4 (C-β′), 149.5 (C-α′),137.5 (C=C–H), 129.7 (C-5), 169.3 (C-4), 201.9 (C-2). MS: (70 eV) *m*/*z* (%) = 163 (15), 135 (100, M^+-C_2HNOS), 108 (11), 91 (12), 78 (18), 51 (6).

4.1.14. (*Z*)-5-(Pyridin-3-ylmethylene)-2-thioxothiazolidin-4-one (3m). This compound was obtained according to general procedure as a yellow powder, yield 52%, mp 295 °C. δ_H (400 MHz, DMSO) 7.60 (s, 1H, =CH), 8.77 (s, 1H, Hα), 7.88 (d, 1H, Hγ, J = 8.0 Hz), 7.52 (m, 1H, Hβ′, J = 8.0 and 7.7 Hz), 8.60 (d, 1H, Hα′, J = 7.7 Hz); δ_C (100 MHz, DMSO) 149.8 (C-α), 128.3 (C-β), 126.9 (C-γ), 123.5 (C-β′), 150.6 (C-α′), 136.3 (C=C–H), 168.6 (C-4), 193.5 (C-2). MS: (70 eV) *m*/*z* (%) = 135 (100, M^+-C_2HNOS), 108 (11), 91 (12), 82 (15), 69 (18). Anal. calcd for $C_9H_6N_2OS2$: C, 48.63; H, 2.72; N, 12.60. Found C, 48.53; H, 2.78; N, 12.53.

4.1.15. (*Z*)-5-(Pyridin-4-ylmethylene)-2-thioxothiazolidin-4-one (3n). This compound was obtained according to general procedure as a yellow powder, yield 55%, mp 296 °C[51] δ_H (400 MHz, DMSO) 7.58 (s, 1H, =CH), 7.52 (d, 1H, Hβ, J = 5.5 Hz), 8.77 (d, 1H, Hα, J = 5.5 Hz), 13.68 (s, 1H, N-H); δ_C (100 MHz, DMSO) 133.1 (C-β), 149.8 (C-γ), 150.6 (C-α), 135.6 (C=C–H), 127.6 (C-5), 168.3 (C-4), 193.7 (C-2). MS: (70 eV) *m*/*z* (%) = 163 (14), 135 (100, M^+-C_2HNOS), 108 (14), 91 (17), 78 (21), 69 (15), 51 (7).

4.2. Antifungal evaluation

4.2.1. Microorganisms and media. For the antifungal evaluation, standardized strains from the American Type Culture Collection (ATCC), Rockville, MD, USA, and CEREMIC (C), Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina, were used in a first instance of screening: *C. albicans* ATCC 10231, *S. cerevisiae* ATCC 9763, *C. neoformans* ATCC 32264, *A. flavus* ATCC 9170, *A. fumigatus* ATTC 26934, *A. niger* ATCC 9029, *T. rubrum* C 110, *T. mentagrophytes* ATCC 9972, and *M. gypseum* C 115.

Active compounds were tested against clinical isolates from CEREMIC [(C) Reference Center in Micology, National University of Rosario, Suipacha 531, Rosario] and Malbrán Institute [(M), Av. Velez Sarsfield 563. Buenos Aires]. The isolates included 14 strains of *Candida* spp. (10 of them *C. albicans* and 10 strains were *Candida* non-*albicans*) and 10 strains of *C. neoformans*. The number of voucher specimens is presented in Table 2. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid), and subcultured every 15 days to prevent pleomorphic transformations. Inocula of cell or spore suspensions were obtained according to reported procedures and adjusted to $1–5 \times 10^3$ cells/spores with colony forming units (CFU)/mL.[34]

4.2.2. Antifungal susceptibility testing. Minimal inhibitory concentration (MIC) of each extract or compound was determined by using broth microdilution techniques according to the guidelines of the National Committee for Clinical Laboratory Standards for yeasts (M27-A2) and for filamentous fungi (M 38 A).[34] MIC values were determined in RPMI-1640 (Sigma, St Louis, Mo, USA) buffered to pH 7.0 with MOPS. Microtiter trays were incubated at 35 °C for yeasts and hialohyphomycetes and at 28–30 °C for dermatophyte strains in a moist, dark chamber, and MICs were visually recorded at

48 h for yeasts, and at a time according to the control fungus growth, for the rest of fungi.

For the assay, stock solutions of pure compounds were twofold diluted with RPMI from 256 to 0.98 μg/mL (final volume = 100 μL) and a final DMSO concentration ⩽1%. A volume of 100 μL of inoculum suspension was added to each well with the exception of the sterility control where sterile water was added to the well instead. Ketoconazole, Terbinafine, Amphotericin B, Fluconazole, Voriconazole, Itraconazole, and 5-Flucytosine were used as positive controls.

Endpoints were defined as the lowest concentration of drug resulting in total inhibition (MIC_{100}) of visual growth compared to the growth in the control wells containing no antifungal. MIC_{80} and MIC_{50} were defined as the lowest concentration of a compound that provoked 80% or 50% reduction of the growth control, respectively (culture media with the microorganism but without the addition of any compound), and were determined spectrophotometrically with the aid of a VERSA Max microplate reader (Molecular Devices, USA).

The minimum fungicidal concentration (MFC) of each compound against each isolate was also determined as follows: After determining the MIC, an aliquot of 5 μL sample was withdrawn from each clear well of the microtiter tray and plated onto a 150-mm RPMI-1640 agar plate buffered with MOPS (Remel, Lenexa, Kans.). Inoculated plates were incubated at 30 °C, and MFCs were recorded after 48 h. The MFC was defined as the lowest concentration of each compound that resulted in total inhibition of visible growth.

4.3. Ergosterol effect

MIC of compounds **3d** and **3e** against *C. albicans* was determined following the guidelines of CLSI as explained above, in the absence and in the presence of different (50, 100, and 200 μg/mL each) concentrations of ergosterol (SIGMA Chemical Co) added to the assay medium, in different lines of the same microplate.[46] Amphotericin B was used as control drug. MIC was read at 24 h according to the control fungus growth.

4.4. Cellular leakage effect

Cells of *S. cerevisiae* ATCC 9763 were grown 18 h, harvested, washed with MOPS, and then resuspended in cold clean MOPS and adjusted to a concentration of 5×10^7 cells/mL at pH 6. Eppendorfs containing inocula and either 4× MFC and 10× MFC of any of the compounds **3d** or **3e** were left for 2, 4, or 6 h. After each period of time, the eppendorfs were centrifuged (5 min at 3000 rpm) and the supernatant was dropped into the wells of a 96-well microplates, thoroughly mixed. The absorbance of each well was read at 260 in a Beckman Multimode detector DTX 880. A negative control (fungal cells with DMSO) was prepared in the same conditions. The maximal extractable UV absorbing materials was determined after treatment of cells with 1.2 N perchloric acid at 100 °C, for 30 min. The absorbance of this sample was taken as 100% release.

4.5. Computational methods

Molecular geometry optimizations were performed at the DFT (B3LYP/6-31G(d) level of theory, using the Gaussian 03[52] program employing standard basis set with no modifications. Correlation effects were included in the present work using DFT with the Becke3-Lee-Yang-Parr[53–55] (B3LYP) functional and the 6-31G8d) basis set. Convergence criteria were according to the limits imposed internally by Gaussian 03.

First an exploratory conformational analysis was carried out using DFT calculations. Compounds reported here look like a simple conformational problem with only one torsion angle (the single C–C bond at the connecting chain). Thus, only two different conformations were obtained for these compounds: a *cis* conformation possessing the single C–C bond near to 0° and a *trans* conformation with this torsion angle near to 180°. In the case of compound **3a**, B3LYP/6-31G(d) calculations predict that the *trans* conformation is energetically preferred by 3.95 kcal/mol (similar results were obtained for the rest of compounds in this series). Thus, the electronic and hydrophobic studies for these compounds were carried out on their respective *trans* conformations.

The electronic study of the rhodanine derivatives was carried out using Molecular Electrostatic Potentials (MEPs). After obtaining the optimized structures, the most reliable, flexible basis set aug-cc-pvDZ single-point calculations on these geometries were used to evaluate the MEPs. B3LYP/6-31G(d) coordinates were imported to generate the wave functions; thus, B3LYP/aug-cc-pVDZ//B3LYP/6-31G(d) single-point calculations were performed from Gaussian 03 program. All MEP graphical presentations were created using Molekel.[56] Calculations of the partition coefficient ($\log P$) were performed from Chem Office 4.5 program.[57]

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