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ORIGINAL ARTICLE

Solvent-free microwave-assisted synthesis of novel pyrazolo[4′,3′:5,6]pyrido[2,3-*d*]pyrimidines with potential antifungal activity

Paola Acosta [a], Braulio Insuasty [a], Alejandro Ortiz [a], Rodrigo Abonia [a], Maximiliano Sortino [b], Susana A. Zacchino [b], Jairo Quiroga [a,*]

[a] *Heterocyclic Compounds Research Group, Department of Chemistry, Universidad del Valle, A.A. 25360 Cali, Colombia*
[b] *Área Farmacognosia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina*

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o-Aminonitriles;
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Microwave irradiation;
Antifungal activity

Abstract Novel fused pyrazolo[4′,3′:5,6]pyrido[2,3-*d*]pyrimidines **5** were prepared by a solvent-free microwave assisted reaction of heterocyclic *o*-aminonitriles **3** and cyanopyridines **4** in the presence of *t*BuOK as catalyst. This protocol provides a versatile procedure for the synthesis of the title compounds with the advantages of easy work-up, mild reaction conditions and good yields. All compounds were also tested for antifungal properties against two clinically important fungi; *Candida albicans* and *Cryptococcus neoformans*. Several compounds showed moderate activity against both fungi, being **5a** the most active compound. Analysis of the antifungal behavior of properly grouped compounds allowed to determine that the position of the N in the pyrimidyl moiety *per se* does not play a role in the activity. In turn, the type of 4-R substituent appears to influence the activity. In addition to the above considerations, the lipophilicity of compounds measured as log *P* showed to be not related to the activity and regarding the dipole moment (*D*), no net correlation was observed, although it is the most active compounds (% inhibition > 50%) that have a $D \geqslant 7.5$, mainly against *C. albicans*.

1. Introduction

The pyrimidine core has been widely studied due to its presence in numerous natural products and structurally diverse synthetic derivatives (Lawen, 2003; Choudhury et al., 2008). Among pyrimidine-containing compounds, fused pyrimidines, particularly pyrido[2,3-*d*]pyrimidine derivatives (i.e. deazapteridines) have attracted much attention because they showed interesting bioactivities (Lunt et al., 1984; Bagley

* Corresponding author. Fax: +57 2 3339240.
E-mail address: jairo.quiroga@correounivalle.edu.co (J. Quiroga).
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et al., 2001; Devi et al., 2003; Devi et al., 2004; Kanth et al., 2006; Bulicz et al., 2006; Tu et al., 2006; Tu et al., 2008) such as antipyretic, antibacterial, antitumor, antihistaminic (Piper et al., 1986; Kuyper et al., 1996; Quintela et al., 1997; Cordeu et al., 2007), diuretic, antifolate, calcium-channel-antagonist, anti-inflammatory (Parish et al., 1982; Pastor et al., 1994; Rosowsky et al., 1995).

The pyrimido[2,3-*d*]pyrimidine-7-ones **I,** piritrexim **(II)** and [1-(2-amino-6-aryl-pyrido[2,3-*d*]pyrimidin-7-yl)ureas] **(III)** showed to be inhibitors of cyclin-dependent kinases (Toogood, 2001), dihydrofolate reductase (Gangjee et al., 2003; Chan and Rosowsky, 2005; Chan et al., 2005) and receptor and non-receptor tyrosine kinases (Hamby et al., 1997; Dorsey et al., 2000; Wissing et al., 2004), respectively (Fig. 1).

The incidence of fungal infection has increased dramatically in recent years. The widespread use of antifungal drugs and their resistance against fungal infections has led to serious health hazards (Tandon et al., 2009). Although there are diverse available drugs for the treatment of systemic and superficial mycoses, they are not completely effective for their eradication (Brown and Wright, 2005). In addition, they all possess a certain degree of toxicity and quickly develop resistance due to the large-scale use. There is, therefore, an urgent need for new antifungal chemical structures alternatives to the existing ones (Mukherjee et al., 2003). In this sense, the pyrido[2,3-*d*]pyrimidine ring system is present in biologically active compounds which possess high antifungal properties. More specifically some of them show activity against dermatophytes, fungi causing the most important superficial mycoses in human beings (Quiroga et al., 2006).

Microwave irradiation (MWI) has emerged as a powerful tool for high-throughput organic synthesis. This source of energy can improve the yield and purity of the desired compounds in short reaction times through the precise control of parameters such as power irradiation, pressure and temperature (Martins et al., 2009; Kappe, 2004; Quiroga et al., 2010; Quiroga et al., 2012).

Due to our interest in the synthesis of potentially bioactive nitrogen-containing six-membered heterocyclic compounds (Insuasty et al., 2008; Insuasty et al., 2010; Quiroga et al., 1998), herein we report a versatile and efficient method for the preparation of pyrazolo[4′,3′:5,6]pyrido[2,3-*d*]pyrimidine derivatives, *via* cyclocondensation reaction between heterocyclic *o*-aminonitriles **3** and cyanopyridines **4**. The starting *o*-aminonitriles **3** (6-aminopyrazolo[3,4-*b*]pyridine-5-carbonitriles) were obtained by a modified method described in the literature (Quiroga et al., 1999), through the interaction of 5-amino-3-methyl-1-phenylpyrazole **1** with different benzylidenemalononitriles **2**, using ethanol as solvent and acetic acid as catalyst (Scheme 1).

2. Results and discussion

2.1. Chemistry

In our study, several conditions were tested at first including diverse solvents, temperatures and power of the microwave source in order to find the best reaction conditions for the synthesis of **5a**. In all cases, reactions were carried out from *o*-aminonitrile **3a** (R = Cl) and 4-cyanopyridine **4** as a model reaction (Scheme 2). When ethanol was used as the solvent and the mixture was subjected to reflux, the desired product **5a** was obtained in low yield (30%, **entry 1**) after 9 h. When DMF was used as the refluxing solvent, almost the same yield was obtained (31%, **entry 2**) after 8 h. Significative improvements were obtained when the reaction was performed under MWI using DMF as the solvent (11 min, yield = 36%, **entry 3**) or under solvent-free MWI (10 min, yield = 41%, **entry 4**). The presence of *t*BuOK in the reaction media, which is a typical catalyst for such reactions (Olivieria et al., 2008), increased the efficiency of the MW as well as the reflux reactions. In MW under solvent-free conditions or with solvents or in reflux reactions, the improvement was evidenced for

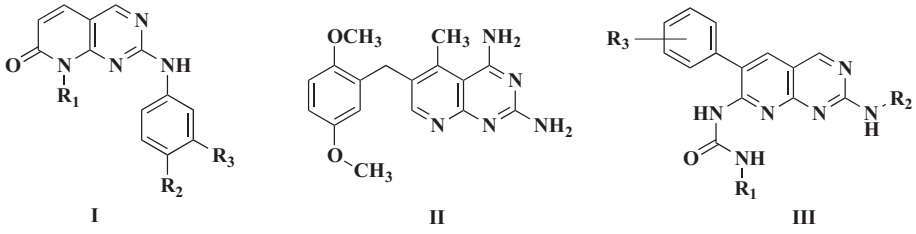
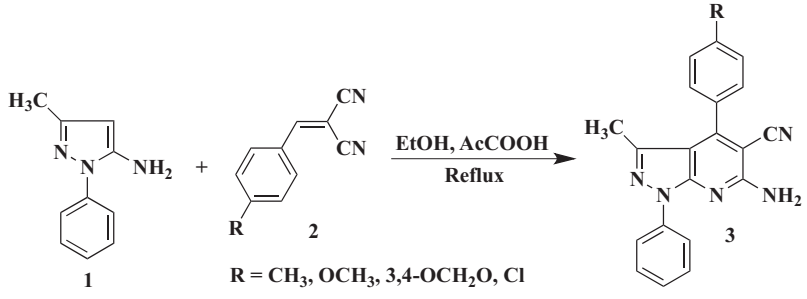
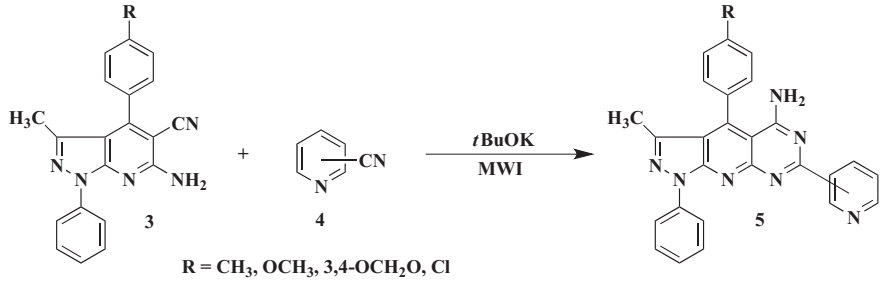


Figure 1 Structures of some pyrido[2,3-*d*]pyrimidine derivatives with biological activity.



Scheme 1 Synthesis of heterocyclic *ortho*-aminonitrile derivatives **3**.



Scheme 2 Synthesis of pyrazolo[4′,3′:5,6]pyrido[2,3-*d*]pyrimidine derivatives **5**.

shorter times of reaction and higher yields (compares **entries 4/5; 3/10; 1/8; 2/9**). Another important finding was that when the MW potency is raised in the presence of *t*BuOK (**entries 5–7**), shorter times and higher yields were obtained (Table 1).

It is worth mentioning that the lower yields were obtained when using 2-cyanopyridine in all cases (Table 2).

Formation of the pyrazolo[4′,3′:5,6]pyridine[2,3-*d*]pyrimidine system was unequivocally established by NMR data of the products. The chemical shifts and multiplicities of the protons were in accordance with the expected values. For example signals for the protons of the phenyl and pyridine rings of compounds **5** were found between 7.05 and 9.60 ppm. The signal for NH_2 appears as a broad singlet between 5.12 and 5.40 ppm, and the signals of the protons of CH_3 appear as singlets between 1.70 and 1.90 ppm.

A possible mechanism of this cyclo-condensation reaction is outlined in Scheme 3. Presumably, the initial step is the addition of the amino group of the *o*-aminonitrile **3** to the nitrile group of the cyanopyridine **4** to amidine intermediate **6**; the final step should be the amine–nitrile intramolecular condensation in **6** to afford **5a–k** (Scheme 3).

2.2. Antifungal activity

In order to have a look into the potential usefulness of these compounds as hits and heads of series for the development of antifungal drugs, we investigated the antifungal properties of compounds **5a**–**5k** against two clinical important fungal species, *Cryptococcus neoformans* and *Candida albicans*. At first we used standardized strains of the American Type Culture Collection (ATCC) as the targets for testing antifungal activity and then, the most active compounds were tested against an expanded panel of clinical isolates in order to know the actual activity of the selected compounds against not only fungi from culture collections but from patients with fungal infections.

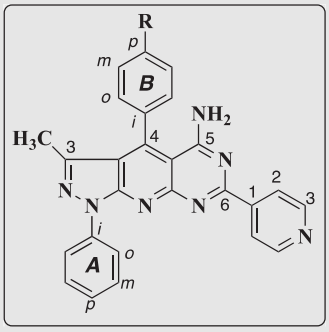
Results were expressed as the percentages of inhibition of each fungus in the range 250–0.98 μg/mL by using the standardized microbroth dilution method M-27A3 of Clinical and Laboratory Standards Institute (CLSI document, 2008), which assures confident and reproducible results.

The selection of *C. neoformans* was due to the fact that this opportunistic fungus is the main cause of cryptococcal meningoencephalitis, which has a high mortality rate among patients with profoundly impaired infections (Trpković et al., 2012).

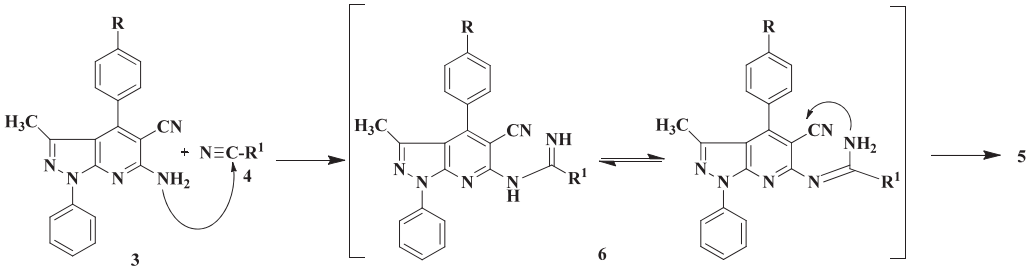
Table 1 Optimization of the reaction of the 6-amino-4-(4-chloro-phenyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carbonitrile **3a** with 4-cyanopyridine **4**.

Entry	Solvent	Conditions	Time (min)	Yield (%)
1	Ethanol	Reflux	540	30
2	DMF	Reflux	480	31
3	DMF	MW (80 °C, 100 W)	11	36
4	Solvent-free	MW (100 °C, 150 W)	10	41
5	Solvent-free	*t*BuOK, MW (100 °C, 150 W)	8	52
6	Solvent-free	*t*BuOK, MW (100 °C, 200 W)	6	59
7	Solvent-free	*t*BuOK, MW (100 °C, 250 W)	5	61
8	Ethanol	*t*BuOK, Reflux	240	36
9	DMF	*t*BuOK, Reflux	210	40
10	DMF	*t*BuOK, MW (80 °C, 100 W)	9	48

Table 2 Synthesis of pyrazolo[4′,3′:5,6]pyrido[2,3-*d*]pyrimidines **5**.



Compound	R	Product	Time (min)	Yield (%)
5a	Cl	Pyridin-4-yl	5	61
5b	Cl	Pyridin-3-yl	1	57
5c	Cl	Pyridin-2-yl	1	42
5d	OCH_3	Pyridin-4-yl	2	60
5e	OCH_3	Pyridin-3-yl	3	61
5f	OCH_3	Pyridin-2-yl	2	42
5g	CH_3	Pyridin-4-yl	5	62
5h	CH_3	Pyridin-3-yl	6	56
5i	CH_3	Pyridin-2-yl	3	48
5j	3,4-OCH_2O	Pyridin-4-yl	5	59
5k	3,4-OCH_2O	Pyridin-3-yl	5	50



Scheme 3 Possible mechanistic route for the synthesis of compounds **5**.

Even though new antifungal drugs have been developed in recent years, the availability of antifungal agents with anticryptococcal activity is still limited and sometimes the strains develop quickly resistance (Perkins et al., 2005). This scenario has motivated the search of new compounds that present antifungal properties against this fungus (Aguiar et al., 2012).

In turn, *C. albicans* is the fourth leading cause of nosocomial bloodstream infection (BSI) in intensive care units, causing fatal invasive candidiasis in a high percentage of patients (Pfaller and Diekema, 2007). As a consequence, new chemical structures with anticandidal activities are highly welcome.

For a more comprehensive analysis of the results, we grouped the compounds in two series: series (i) includes compounds with different pyridinyl moieties (pyridin-4-yl, 3-yl and 2-yl) and same R (Cl, OCH_3 CH_3 or 3,4-OCH_2O) which allowed to have a look on the influence of the position of the N of the pyridinyl moiety on the antifungal activity; and series (ii) that includes compounds with same pyridinyl moiety (pyridin-4-yl, 3-yl or 2-yl) but different R (Cl, OCH_3 CH_3 and 3,4-OCH_2O) which allowed to analyze the role played by the different R substituents in the antifungal activity. Compounds of series (i) were sub-divided in four sub-groups: (i.1) with R = Cl (**5a, 5b, 5c**); (i.2) with R = OCH_3 (**5d, 5e, 5f**); (i.3) with R = CH_3 (**5g, 5h, 5i**) and (i.4) with R = 3,4-OCH_2O (**5j, 5k**). Comparative growth inhibition percentages of the compounds of each sub-group can be observed in Fig. 2.

In Fig. 2, sub-group i.1 (R = Cl) the highest activity was displayed by **5a** which possesses a pyridin-4-yl moiety. Instead, in sub-groups i.2 and i.4 (R = OCH_3 and —OCH_2O— respectively) compounds with 4-pyridinyl moiety showed the lowest activity and compounds with 3-pyridinyl (**5e**, **5k**) and 2-pyridinyl (**5f**) moieties were the most active ones. Compounds of group i.3 show dissimilar behavior against *C. albicans* and *C. neoformans*. From these results it is clear that the position of the N in pyridinyl moiety *per se*, does not play a role in the activity since i.e. compounds with pyridin-4-yl moieties are not the most active structures within each sub-series against *C. albicans* or *C. neoformans*. The same can be observed for compounds with pyridin-3-yl or 2-yl moieties (see Table 3 for values of the inhibition percentages).

Then, we tried to investigate the role (if any) played by the different R substituents in the activity. So, we compared compounds with same pyridinyl moiety (pyridin-4-yl, 3-yl or 2-yl) but different R (Cl, OCH_3 CH_3 and 3,4-OCH_2O) [series (ii)] as follows: in Fig. 3, ii.1 we compared the compounds with pyridin-4-yl moiety but different R: **5a** (Cl), **5d** (OCH_3), **5g** (CH_3) and **5j** (OCH_2O) against each fungi; in ii.2, compounds with pyridin-3-yl moiety **5b, 5e, 5h** and **5k** and in ii.3, compounds with pyridin-2-yl moiety **5c, 5f** and **5i.**

From Fig. 3 (whose percentages of inhibition can be queried in Table 4), we see that within compounds of group ii.1 **5a**, **5d**, **5g** and **5j** those with R = Cl (**5a**) and R = CH_3 (**5g**) (Fig. 3, ii.1) showed the best activity (91.7% and 57.3% inhibition growth against *C. neoformans* and 78.3% and 76.9% against *C. albicans*). Since chloro and methyl groups have nearly the same volume, the size of the substituent and not its electronic properties would seem to play a role in the antifungal activity in this ii.1 group. In contrast, within compounds of group ii.2 with pyridin-3-yl moiety, those with R = OCH_3 (**5e**) and OCH_2O (**5k**) showed the best activity against both fungi (72.5% and 64.5% against *C. neoformans* and 62.2% and 50.2% against *C. albicans*). The methylated derivative **5h** also showed good activity but only against *C. albicans*. Within group ii.3, the substituents appear not to influence the antifungal activity since **5c**, **5f** and **5i** show almost the same activity against *C. neoformans* and the behavior is dissimilar with *C. albicans*.

To deepen this analysis, the quantitative parameters *log P* and dipole moment (*D*) of each compound (**5a**–**5k**) were calculated and correlated with the activity. Both parameters were calculated using quantum mechanical at semiempirical level using Mopac, with the parametric method 3 (PM3). The molecular modeling was prepared using CS Chem-Office Software version 9.0 (Cambridge software) (C.S. Chem office, 2005). It is known that log *P* (logarithm of the partition coefficient in a biphasic system, *e.g. n*-octanol/water) describes the macroscopic hydrophobicity of a molecule which is a factor that many times determines its ability to penetrate the membranes of fungal cells and to reach the interacting sites, thus influencing the antifungal activity of compounds (Voda et al., 2004). *D*, that is the measure of net molecular polarity, tell us about the charge separation in a molecule. The larger the difference in electronegativities of bonded atoms, the larger the dipole moment.

Table 4 shows log *P* and *D* for all compounds tested along with the percentages of inhibition against both fungi at 250 μg/mL.

To determine if the log *P* has some influence in the activity, this parameter was plotted *vs* antifungal activity (against *C. neoformans* and *C. albicans*) in Fig. 4.

From Fig. 4, it is clear that log *P*, that is to say the lipophilicity of compounds, has no relationship with the activity, since compounds with the same log *P* such as **5a** and **5b** display completely different activities against both *C. neoformans* and *C. albicans*. Another clear example is the

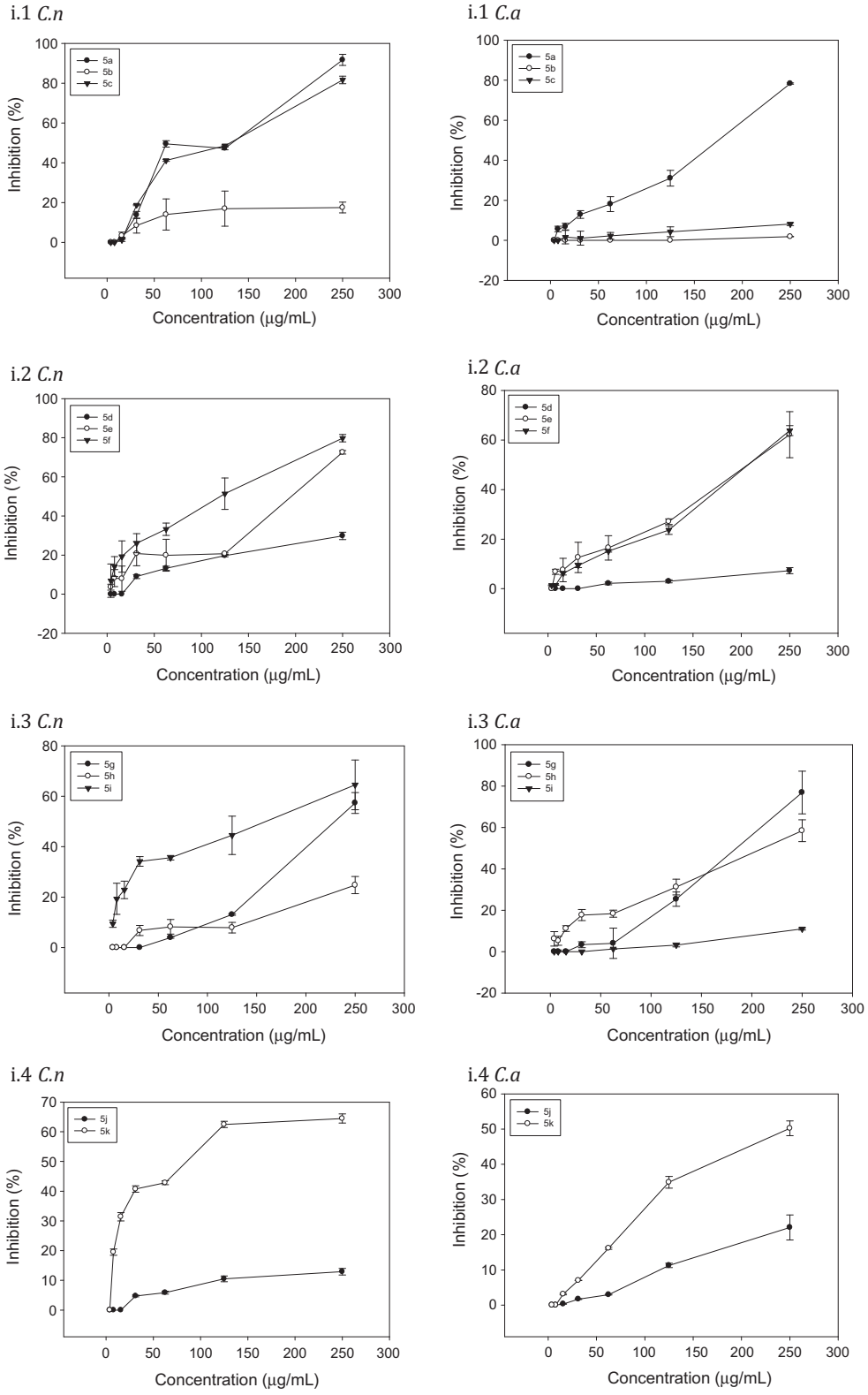


Figure 2 Comparative antifungal activities of compounds of series (i) with different pyridin-yl moieties and similar R in position 4: (i.1) 5a, 5b and 5c; (i.2) 5d, 5e and 5f; (i.3) 5g, 5h and 5i; or in 3,4 (i.4): 5j and 5k against *Cryptococcus neoformans* [*C.n.*] or *Candida albicans* [*C.a*]. Amphotericin B (Amp) inhibits 100% growth at 1.0 μg/mL against *C. albicans* and 0.5 μg/mL against *C. neoformans* (curves of Amp are not included).

Table 3 Percentages of inhibition of **5a–5k** against *C. albicans* ATCC 10231 and *C. neoformans* ATCC 32264 at the range 250–3.9 μg/mL.

Sub-series	Moiety	R	Cp	Concentrations in μg/mL						
				250	125	62.5	31.25	15.62	7.81	3.9
C. neoformans ATCC 32264										
i.1	4-pyridinyl	Cl	**5a**	91.7 ± 2.8	57.3 ± 0.7	49.5 ± 1.6	13.75 ± 1.8	2.17 ± 0.4	0	0
	3-pyridinyl	Cl	**5b**	17.5 ± 2.7	16.9 ± 1.8	14.0 ± 1.8	8.4 ± 1.3	3.4 ± 1.7	0	0
	2-pyridinyl	Cl	**5c**	81.6 ± 1.9	48.6 ± 0.38	41.1 ± 0.5	18.8 ± 0.31	1.21 ± 0.79	0	0
i.2	4-pyridinyl	OCH_3	**5d**	29.9 ± 1.9	19.7 ± 0.6	13.2 ± 1.08	9.04 ± 1.03	0	0	0
	3-pyridinyl	OCH_3	**5e**	72.5 ± 0.9	20.7 ± 0.4	19.9 ± 1.21	19.7 ± 1.2	7.9 ± 1.4	6.2 ± 1.3	3.6 ± 1.5
	2-pyridinyl	OCH_3	**5f**	79.7 ± 1.8	51.3 ± 1.5	33.2 ± 1.2	26.1 ± 1.9	19.3 ± 1.9	14.1 ± 1.1	6.9 ± 0.5
i.3	4-pyridinyl	CH_3	**5g**	57.3 ± 1.1	33.09 ± 0.3	23.9 ± 0.3	0	0	0	0
	3-pyridinyl	CH_3	**5h**	24.7 ± 1.4	7.9 ± 2.1	7.2 ± 1.9	6.8 ± 1.9	0	0	0
	2-pyridinyl	CH_3	**5i**	64.5 ± 2.9	44.6 ± 1.6	35.6 ± 0.9	34.1 ± 1.9	22.9 ± 1.5	19.3 ± 1.1	9.4 ± 1.4
i.4	4-pyridinyl	$-OCH_2O-$	**5j**	12.9 ± 1.1	10.5 ± 0.9	5.9 ± 0.5	4.7 ± 0.3	0	0	0
	3-pyridinyl	$-OCH_2O-$	**5k**	64.5 ± 1.5	62.5 ± 1.0	42.8 ± 0.6	40.7 ± 1.1	31.4 ± 1.4	19.5 ± 1.1	0
	Amphotericin B at 2 μg/mL			100	100	100	100	100	100	100
C. albicans ATCC 10231										
i.1	4-pyridinyl	Cl	**5a**	78.3 ± 0.3	31.0 ± 1.9	18.1 ± 1.7	12.9 ± 1.2	6.9 ± 1.6	5.7 ± 1.4	0
	3-pyridinyl	Cl	**5b**	1.85 ± 0.1	0	0	0	0	0	0
	2-pyridinyl	Cl	**5c**	8.12 ± 0.7	4.3 ± 0.4	2.3 ± 0.7	1.6 ± 0.4	1.1 ± 0.1	0	0
i.2	4-pyridinyl	OCH_3	**5d**	7.3 ± 1.2	3.0 ± 0.6	2.1 ± 0.5	0	0	0	0
	3-pyridinyl	OCH_3	**5e**	62.2 ± 2.3	27.1 ± 1.1	16.5 ± 1.9	12.6 ± 0.1	0	0	0
	2-pyridinyl	OCH_3	**5f**	63.8 ± 2.0	23.7 ± 1.8	15.1 ± 0.4	9.4 ± 0.9	6.2 ± 0.7	1.4 ± 0.1	1.4 ± 0.2
i.3	4-pyridinyl	CH_3	**5g**	76.9 ± 1.3	25.4 ± 1.5	24.1 ± 1.3	3.4 ± 0.5	0	0	0
	3-pyridinyl	CH_3	**5h**	58.5 ± 1.3	31.2 ± 1.8	18.4 ± 1.7	17.7 ± 2.8	11.1 ± 1.4	6.3 ± 1.1	5.3 ± 0.4
	2-pyridinyl	CH_3	**5i**	11.0 ± 0.6	3.2 ± 0.9	1.3 ± 0.1	0	0	0	0
i.4	4-pyridinyl	$-OCH_2O-$	**5j**	22.1 ± 1.5	11.3 ± 0.6	2.9 ± 0.1	1.7 ± 0.1	0.3 ± 0.1	0	0
	3-pyridinyl	$-OCH_2O-$	**5k**	50.2 ± 2.1	34.9 ± 1.7	16.2 ± 0.3	7.0 ± 0.1	3.2 ± 0.2	0	0
	Amphotericin B			100	100	100	100	100	100	100

comparison of activities of **5e** and **5d,** which have almost the same log *P* and different activities.

In turn, to determine if *D* has some influence in the activity, *D* was plotted *vs* antifungal activity (against *C. neoformans* and *C. albicans*) in Fig. 5.

In Fig. 5, we can observe that there is not a net correlation between *D* and antifungal activity, but it is observed that most active compounds (% inhibition $>50\%$) have a $D \geqslant 7.5$, mainly against *C. albicans*.

From Table 3 and both Figs. 4 and 5, it is clear that compound **5a** showed the best activity against both fungi inhibiting more than 90% of the growth of *C. neoformans* and 78% of *C. albicans* and therefore, this compound deserves further attention.

2.2.1. Second order studies with clinical isolates

In order to gain insight into the potential of **5a** not only against standardized strains but on clinical isolates of medical important fungi, **5a** was tested at 250, 125, 62.5, 31.2 and 16.2 μg/mL against an extended panel of *C. albicans* and *C. neoformans* strains isolated from immunocompromised patients suffering fungal infections. Results are recorded in Table 5.

As it can be observed in Table 5, compound **5a** exerts more than 80% of inhibition on 3 out the 5 *C. albicans* strains at 250 μg/mL, and more than 50% inhibition in 4 of the 5 strains at 125 μg/mL. Moreover, compound **5a** produces more than 80% inhibition on 8/10 isolates of *C. neoformans* at 250 μg/mL, more than 50% inhibition in 9/10 isolates at 125 μg/mL and in 4/10 strains at 62.5 μg/mL, clearly indicating that **5a** is a better inhibitor of *C. neoformans* than of *C. albicans*.

3. Conclusion

In this article we described the microwave-assisted synthesis of novel pyrazolo[4′,3′:5,6]pyrimido[2,3-*d*]pyrimidines **5** under solvent-free conditions. The described synthesis represents a versatile, practical and environmental friendly method for the preparation of compounds **5** with the advantages of easy work-up, mild reaction conditions and good yields. Regarding the antifungal activity, several compounds showed moderate activity against *C. albicans* and *C. neoformans,* being **5a** the most active compound. Analysis of the antifungal activity of properly grouped compounds allowed to determine that the position of the N in the pyrimidyl moiety *per se* does not play a role in the activity. In turn, the type of 4-R substituent appears to play a role in the activity. Within compounds with pyridin-4-yl moiety (**5a**, **5d**, **5g** and **5j**) those with R = Cl (**5a**) and R = CH_3 (**5g**) showed the best activity. In contrast, within compounds with pyridin-3-yl moiety, those with R = OCH_3 (**5e**) and OCH_2O (**5k**) showed the highest inhibition percentage against both fungi. Within compounds with pyridin-2-yl, the

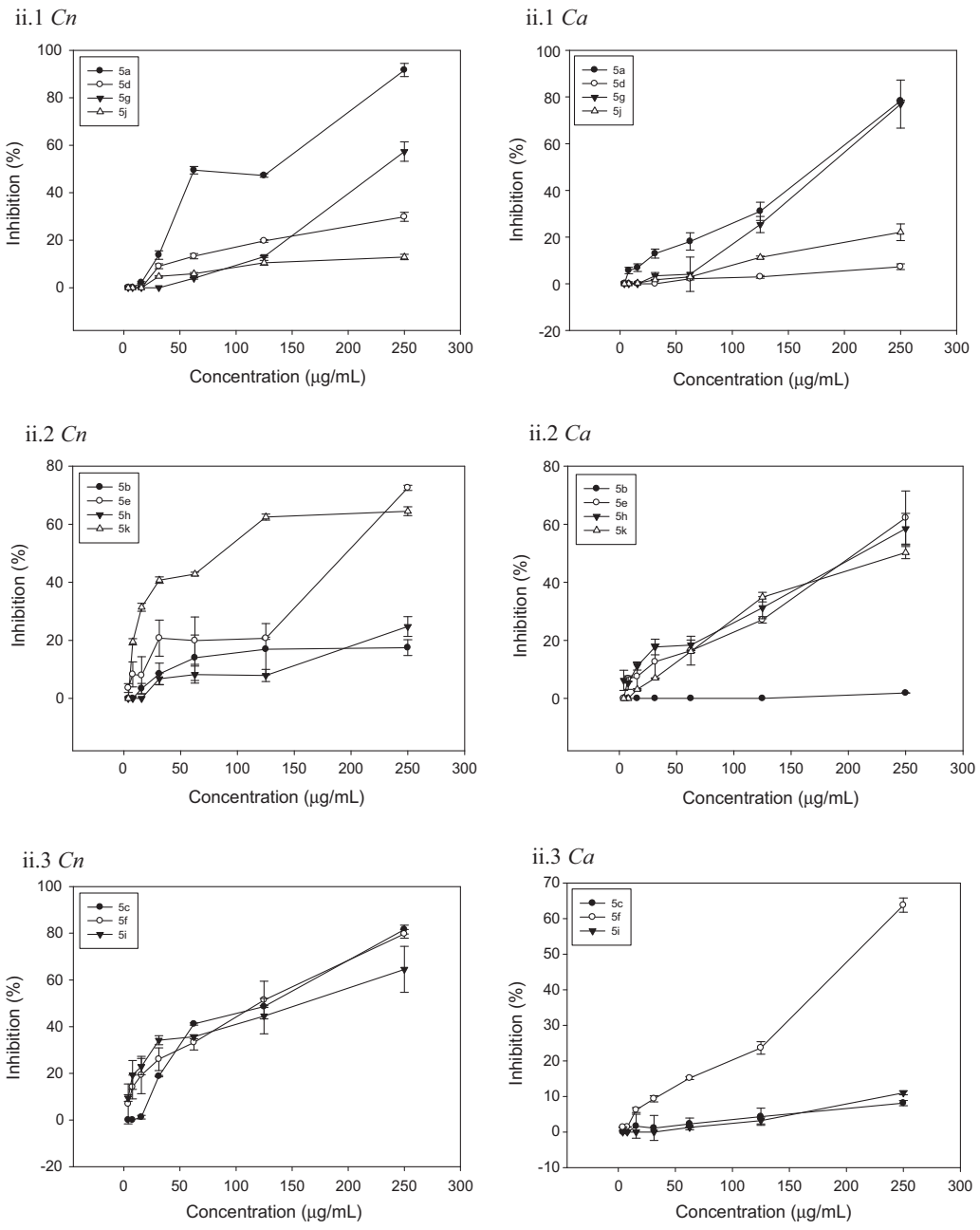


Figure 3 Comparative antifungal activities of compounds of series (ii) with different pyridin-yl moiety and similar 4-R: (ii.1) compounds with pyridine-4-yl and Cl (5a), OCH_3 (5d), CH_3 (5g) and 3,4-OCH_2O (5j); (ii.2) compounds with pyridine-3-yl moiety 5b (Cl), 5e (OCH_3), 5h (CH_3) and 5k (3,4-OCH_2O); (ii.3) compounds with pyridine-2-yl moiety 5c (Cl); 5f (OCH_3); 5i (CH_3) against *Cryptococcus neoformans* [*C.n.*] or *Candida albicans* [*C.a.*]. Amphotericin B inhibits 100% at 1.0 μg/mL against *C. albicans* and 0.5 μg/mL against *C. neoformans*.

substituents appear not to exert any influence in the antifungal activity. In addition to the above considerations, the lipophilicity of compounds measured as log *P* showed to be not related to the activity and there is not observed a net correlation between D and antifungal activity, although it is observed that the most active compounds (% inhibition $>50\%$) have a $D \geqslant 7.5$, mainly against *C. albicans*.

4. Experimental

Commercially available starting materials, reagents and solvents were used as supplied. The TLC analysis was performed on Merck TLC-plates aluminum silica gel 60 F254. Melting point was measured using a Büchi melting point apparatus and was uncorrected. Microwave reactions were performed in glass vessels (10 mL) using a CEM Focused Microwave Synthesis System™ apparatus, Model Discover, with power output from 0 to 300 W. The IR analysis was performed on a Shimadzu FTIR 8400 spectrophotometer in KBr disks. ^{1}H and ^{13}C NMR spectra were run on a Bruker DPX 400 spectrometer operating at 400 MHz and 100 MHz respectively, using dimethyl sulfoxide-d_6 as solvent and tetramethylsilane as internal standard. Mass spectra were obtained from Shimadzu GCMS-QP 2010 spectrometer (equipped with a

Table 4 The *in vitro* activity (% inhibition in *Cryptococcus neoformans* (*C.n.*) and *Candida albicans* (*C.a.*) at 250 μg/mL of compounds **5a–k**.

Compound	Log *P*	Dipole (*D*)	*C.n.*[1] (% Inh)	*C.a.*[1] (% Inh)
5a	7.27	7.6032	91.7	78.3
5b	7.27	6.9140	17.5	1.8
5c	7.69	7.1909	81.6	8.1
5d	6.58	8.4704	29.9	7.2
5e	6.58	9.2600	72.5	62.1
5f	7.01	9.2013	79.7	63.7
5g	7.20	8.5322	57.3	76.9
5h	7.20	7.9098	24.7	58.4
5i	7.62	8.6168	64.5	11.0
5j	6.49	7.4864	12.9	22.0
5k	6.46	8.9442	64.4	50.3

direct inlet probe) operating at 70 eV. Elemental analysis was carried out using a Thermo Finnigan Flash EA1112 CHN (STIUJA) elemental analyzer.

4.1. General procedure for the preparation of pyrazolo[4′,3′:5,6]pyrido[2,3-d]pyrimidines 5

All experiments were carried out using a focused microwave reactor (CEM Discover TM). A mixture of *ortho*-aminonitrile **3** (0.3 mmol), cyanopyridine **4** (0.4 mmol) and *t*BuOK (10 mol%), was exposed to microwave irradiation from 1 to 6 min at 100 °C, a power of 250 W and 30 PSI of pressure. Then, the reaction mixture was treated with ethanol and the excess of solvent was removed under reduced pressure. Purification of products was performed using column chromatography in a mixture $CHCl_3$/EtOH (20:1) as eluent.

4.1.1. 4-(4-Chlorophenyl)-3-methyl-1-phenyl-7-(pyridin-4-yl)-1H-pyrazolo[4′,3′:5,6]pyrido[2,3-d]pyrimidin-5-amine ***5a***

Yellow solid, yield 61%, mp > 350. FTIR (KBr) υ(cm^{-1}): 3496 (NH), 3038 (═C─H), 1600, 1569 (C═N and C═C). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.78 (s, 3H, CH_3), 5.13 (brs, 2H, NH_2), 7.36 (t, 1H, J = 7.3 Hz, HA*p*), 7.61 (t, 2H, J = 7.7 Hz, HA*o*,), 7.76–7.86 (m, 4H, HA*m*, HB*m*), 8.29 (d, 2H, J = 4.6 Hz, H-2Py), 8.33(d, 2H, J = 8.1 Hz, HB*o*), 8.75 (d, 2H, J = 4.7 Hz, H-3Py). ^{13}C NMR (100 MHz DMSO-d_6) δ: 14.5 (CH_3), 103.6 (C), 116.8 (C), 121.1 (CH), 122.6 (CH), 126.1 (CH), 129.6 (CH), 130.0 (CH), 131.0 (CH), 134.3 (C), 135.7 (C), 139.4 (C), 145.1 (C), 145.2 (C), 145.6 (C), 150.6 (CH), 152.6 (C), 159.6 (C), 162.4 (C), 164.9 (C). HR-MS calcd for $C_{26}H_{18}ClN_7$ 463.1312, found [M^+ + K] 501.7842. [M^+ + H] 463.8283. Anal. Calcd for $C_{26}H_{18}ClN_7{\cdot}H_2O$: C, 64.90; H, 4.08; N, 20.34; found: C, 65.13; H, 3.82; N, 20.38.

4.1.2. 4-(4-Chlorophenyl)-3-methyl-1-phenyl-7-(pyridin-3-yl)-1H-pyrazolo[4′,3′:5,6]pyrido[2,3-d]pyrimidin-5-amine ***5b***

Yellow solid, yield 57%, mp: 317–318. FTIR (KBr) υ(cm^{-1}): 3470 (NH), 3058 (═C─H), 1550, 1510 (C═N and C═C). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.74 (s, 3H, CH_3), 5.10 (brs, 2H, NH_2), 7.35 (t, 1H, J = 7.4 Hz HA*p*), 7.51–7.56 (m, 1H, H-2Py), 7.60 (t, 2H, J = 7.9 Hz, HA*o*), 7.79 (s, 4H, HA*m*, HB*m*), 8.33 (d, 2H, J = 7.9 Hz, HB*o*), 8.68–8.73 (m, 2H, H-3Py, H-4Py). 9.55 (s, 1H, H-5Py) ^{13}C NMR (100 MHz DMSO-d_6) δ: 14.5 (CH_3), 103.4 (C), 116.6 (C), 121.2 (CH), 123.8 (CH), 126.2 (CH), 129.5 (CH), 130.0 (CH), 130.1 (CH), 133.8 (C), 134.8 (C), 135.7 (C), 136.0 (CH), 139.5(C), 145.0 (C), 145.2 (C), 150.2 (CH), 151.8 (CH), 152.3 (C), 159.7 (C), 162.7 (C), 164.7 (C). HR-MS calcd for $C_{26}H_{18}ClN_7$ 463.1312, found [M^+ + K] 501.8709. [M^+ + H] 463.9121.

4.1.3. 4-(4-Chlorophenyl)-3-methyl-1-phenyl-7-(pyridin-2-yl)-1H-pyrazolo[4′,3′:5,6]pyrido[2,3-d]pyrimidin-5-amine ***5c***

Yellow solid, yield 42%, mp > 350. FTIR (KBr) υ(cm^{-1}): 3485 (NH), 3040 (═C─H), 1574, 1547 (C═N and C═C). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.70 (m, 3H, CH_3), 7.36 (t, 1H, J = 7.3 Hz, HA*p*), 7.52–7.56 (m, 2H, HA*o*), 7.61–7.63 (m, 4H, HA*m*, HB*m*), 8.37 (d, 2H, J = 7.8 Hz, HB*o*), 8.58–8.63 (m, 2H, H-2Py, H-3Py), 8.83–8.70 (m, 2H, H-4Py, H-5Py). Not observed (brs, 2H, NH_2). Compound **5c** is barely soluble in dimethyl sulfoxide or any other solvent normally used for NMR spectroscopy; thus, made the registration of a high resolution ^{13}C NMR spectrum impossible. HR-MS calcd for $C_{26}H_{18}ClN_7$ 463.1312, found [M^+ + Na] 486.1490. [M^+ + H] 463.3628.

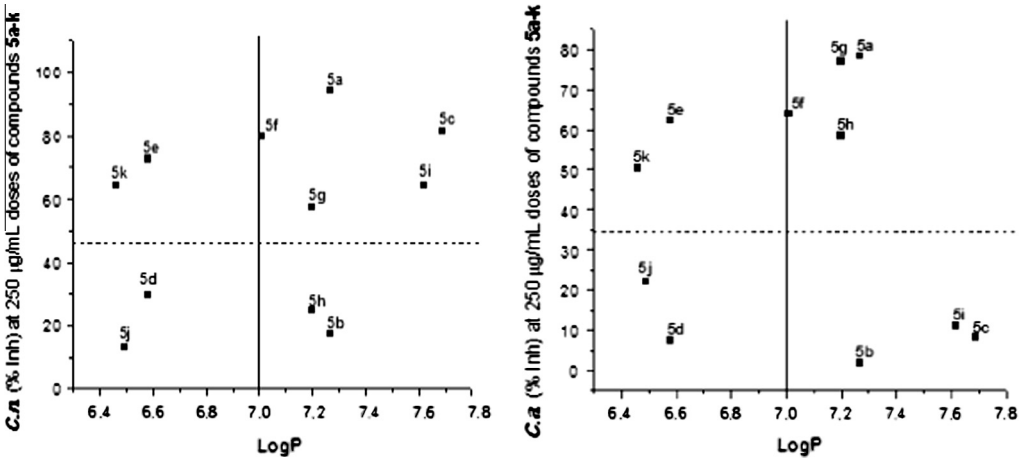


Figure 4 Log *P vs* inhibition percentage of *Cryptococcus neoformans* (left) and *C. albicans* (right) growth, by **5a–k** at 250 μg/mL.

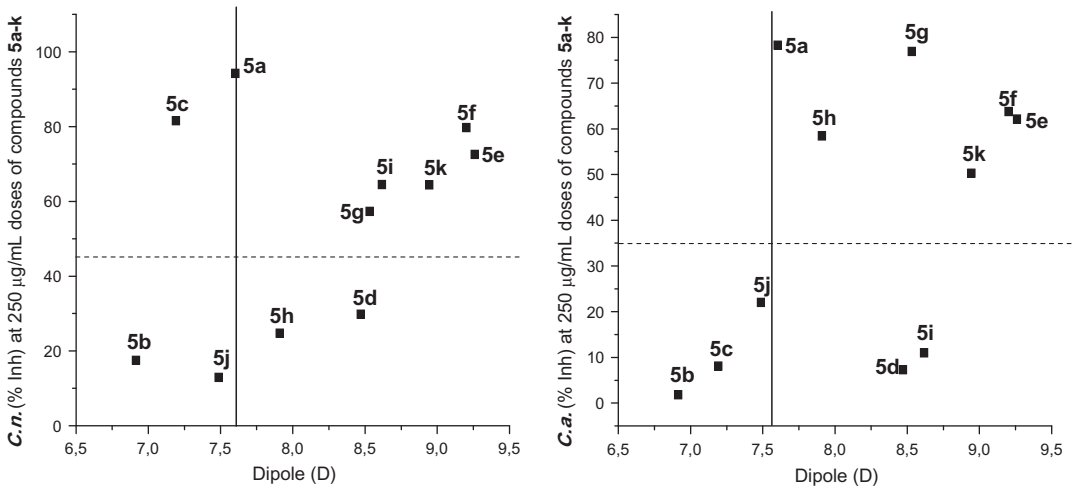


Figure 5 Dipole (*D*) *vs* inhibition percentage of the *C. neoformans* (left) and *C. albicans* (right) growth by **5a–k** each at 250 µg/mL.

Table 5 Antifungal activity (inhibition percentage) of **5a** against clinical isolates of *Candida albicans* and *Cryptococcus neoformans*.

		Inhibition % of compound **5a**					Amp
Strain		250 µg/mL	125 µg/mL	62.5 µg/mL	331.2 µg/mL	15.6 µg/mL	1
Ca	ATCC10231	78.3 ± 0.3	31.0 ± 1.9	18.1 ± 1.7	12.9 ± 1.2	6.9 ± 1.6	100
Ca	CCC 126	80.1 ± 0.4	58.2 ± 2.1	34.2 ± 1.5	14.3 ± 0.2	5.3 ± 0.6	100
Ca	CCC 127	70.8 ± 2.3	45.4 ± 1.0	32.7 ± 1.2	10.7 ± 0.8	4.2 ± 1.0	100
Ca	CCC 128	73.3 ± 0.8	55.0 ± 1.6	46.3 ± 1.4	17.4 ± 0.7	12.2 ± 1.3	100
Ca	CCC 129	84.3 ± 1.2	62.5 ± 1.1	50.7 ± 1.4	33.9 ± 0.2	10.9 ± 2.0	100
Ca	CCC 130	80.2 ± 1.2	53.2 ± 0.4	44.7 ± 1.3	22.5 ± 0.7	12.6 ± 1.3	100
Cn	ATCC 32264	91.7 ± 2.8	57.3 ± 0.7	49.5 ± 1.6	13.7 ± 1.8	2.1 ± 0.4	100
Cn	IM 983040	94.3 ± 1.4	68.3 ± 3.4	54.9 ± 2.3	23.4 ± 1.2	5.2 ± 0.7	100
Cn	IM 972724	97.8 ± 2.4	77.2 ± 2.3	35.5 ± 3.3	13.1 ± 1.3	0.3 ± 0.1	100
Cn	IM 042074	84.4 ± 1.7	76.8 ± 1.5	55.9 ± 2.6	33.6 ± 1.8	7.8 ± 1.2	100
Cn	IM 983036	92.3 ± 1.3	80.4 ± 1.0	52.4 ± 1.3	32.5 ± 2.4	12.3 ± 1.8	100
Cn	IM 00319	88.3 ± 1.2	68.5 ± 1.6	53.7 ± 1.4	39.3 ± 0.2	16.9 ± 2.5	100
Cn	IM 972751	83.4 ± 1.9	54.5 ± 1.5	22.7 ± 0.4	13.7 ± 1.3	6.7 ± 0.4	100
Cn	IM 031631	74.3 ± 1.5	55.8 ± 3.1	35.1 ± 1.9	15.2 ± 0.2	5.7 ± 0.2	100
Cn	IM 031706	87.5 ± 2.1	46.5 ± 1.2	37.1 ± 2.2	28.8 ± 1.2	12.3 ± 1.2	100
Cn	IM 961951	79.7 ± 2.8	69.5 ± 2.9	45.6 ± 1.2	20.9 ± 1.2	9.9 ± 0.3	100
Cn	IM 052470	88.2 ± 1.7	76.3 ± 1.1	45.3 ± 1.5	31.2 ± 0.3	14.7 ± 1.5	100

For the sake of comparison, standardized strains of *C. albicans* ATCC 10231 and *C. neoformans* ATCC 32264 are included in the table. *Ca*: *Candida albicans*; *Cn*: *Cryptococcus neoformans*; IM: Instituto Malbrán, Buenos Aires; ATCC: American Type Culture Collection, Manassas, USA; CCC: Reference Center in Mycology, Rosario, Argentina. Amp = Amphotericin B.

4.1.4. 4-(4-Methoxyphenyl)-3-methyl-1-phenyl-7-(pyridin-4-yl)-1H-pyrazolo[4′,3′:5,6]pyrido [2,3-d]pyrimidin-5-amine ***5d***

Yellow solid, yield 60%, mp: 332–333. υ(cm^{-1}): 3494 (NH), 3044 (=C–H), 1620, 1574 (C=N and C=C). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.90 (s, 3H, CH_3), 3.94 (s, 3H, OCH_3), 5.13 (brs, 2H, NH_2), 7.28 (d, 2H, J = 8.6 Hz, HB*o*), 7.35 (t, 1H, J = 7.4 Hz, HA*p*), 7.55–7.63 (m, 4H, HA*m*, HB*m*), 8.32 (d, 2H, J = 5.9 Hz, H-2Py), 8.36 (d, 2H, J = 7.8 Hz, HA*o*), 8.75 (d, 2H, J = 5.9 Hz, H-3Py). ^{13}C NMR (100 MHz DMSO-d_6) δ: 14.4 (CH_3), 56.1 (OCH_3), 104.0 (C), 115.6 (CH), 117.6 (C), 121.1 (CH), 122.6 (CH), 126.1 (CH), 127.4 (C), 129.5 (CH), 130.0 (CH), 139.6 (C), 145.4 (C), 145.8 (C), 146.8 (C), 150.6 (CH), 152.4 (C), 159.7 (C), 161.3 (C), 162.4 (C), 165.1 (C). HR-MS calcd for $C_{27}H_{21}N_7O$ 459.1808, found [M^+ + K] 498.9195. [M^+ + H] 459.9754.

4.1.5. 4-(4-Methoxyphenyl)-3-methyl-1-phenyl-7-(pyridin-3-yl)-1H-pyrazolo[4′,3′:5,6]pyrido [2,3-d]pyrimidin-5-amine ***5e***

Yellow solid, yield 61%, mp: 306–307. FTIR (KBr) υ(cm^{-1}): 3468 (NH), 3056 (=C–H), 1575, 1508 (C=N and C=C). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.81 (s, 3H, CH_3), 3.92 (s, 3H, OCH_3), 5.26 (brs, 2H, NH_2), 7.28 (d, 2H, J = 8.4 Hz, HB*o*), 7.36 (t, 1H, J = 7.3 Hz, HA*p*), 7.51–7.58 (m, 1H, H-3Py) 7.58–7.67 (m, 4H, HA*m*, HB*m*), 8.35 (d, 2H, J = 8.0 Hz, HA*o*), 8.73 (d, 2H, J = 7.3 Hz, H-2Py, H-4Py), 9.57 (s, 1H, H-6Py). ^{13}C NMR (100 MHz DMSO-d_6) δ: 14.4 (CH_3), 56.1 (OCH_3), 103.8 (C), 115.6 (CH), 117.0 (C), 121.1 (CH), 123.7 (CH), 126.1 (CH), 127.5 (C), 129.5 (CH), 130.3 (CH), 133.9 (C), 136.0 (CH), 139.6 (C), 145.4 (C), 146.7 (C) 150.2 (CH), 151.7 (CH), 152.4 (C), 159.7 (C), 161.3 (C), 162.7 (C), 164.9 (C). HR-MS calcd for $C_{27}H_{21}N_7O$ 459.1808, found [M^+ + K] 498.8764. [M^+ + H] 459.9340.

4.1.6. 4-(4-Methoxyphenyl)-3-methyl-1-phenyl-7-(pyridin-2-yl)-1H-pyrazolo[4′,3′:5,6]pyrido [2,3-d]pyrimidin-5-amine ***5f***

Yellow solid, yield 42%, mp > 350. FTIR (KBr) υ(cm^{-1}): 3484 (NH), 3055 (═C─H), 1579, 1547 (C═N and C═C). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.86 (s, 3H, CH_3), 3.94 (s, 3H, OCH_3), 7.27 (d, 2H, *J* = 8.1 Hz, HB*o*), 7.35 (t, 1H, *J* = 7.6 Hz, HA*p*), 7.53 (t, 1H, *J* = 6.2 Hz, H-4Py) 7.61 (d, 4H, *J* = 7.8 Hz, HA*m,* HB*m*), 7.97 (t, 1H, *J* = 7.8 Hz, H-3Py), 8.39 (d, 2H, *J* = 8.1 Hz, HA*o*), 8.48 (d, 1H, *J* = 7.9 Hz, H-2Py), 8.73 (d, 1H, 4.6 Hz, H-5Py). Not observed (brs, 2H, NH_2). Compound **5f** is barely soluble in dimethyl sulfoxide or any other solvent normally used for NMR spectroscopy; thus, made the registration of a high resolution ^{13}C NMR spectrum impossible. HR-MS calcd for $C_{27}H_{21}N_7O$ 459.1808, found [M^+ + K] 498.9088. [M^+ + H] 459.9754.

4.1.7. 3-Methyl-1-phenyl-7-(pyridin-4-yl)-4-(p-tolyl)-1H-pyrazolo[4′,3′:5,6]pyrido[2,3-d]pyrimidin-5-amine ***5g***

Yellow solid, yield 62%, mp: 339–340. FTIR (KBr) υ(cm^{-1}): 3498 (NH), 3042 (═C─H), 1589, 1559 (C═N and C═C). ^{1}H NMR (400 MHz, DMSO-d_6) δ:1.82 (s, 3H, CH_3), 5.22 (brs, 2H, NH_2), 7.36 (t, 1H, *J* = 7.3 Hz, HA*p*), 7.49–7.72 (m, 6H, HB*o*, HA*m*, HB*m*), 8.31 (d, 2H, *J* = 5.2 Hz, H-3Py), 8.35 (d, 2H, *J* = 8.0 HA*o*), 8.76 (d, 2H, *J* = 5.1 Hz, H-2Py). ^{13}C NMR (100 MHz DMSO-d_6) δ: 14.3 (CH_3), 21.4 (CH_3), 103.8 (C), 116.9 (C), 121.1 (CH), 122.6 (CH), 126.1 (CH), 128.7 (CH), 129.5 (CH), 130.5 (CH), 132.7 (C), 139.6 (C), 140.4 (C), 145.4 (C), 145.8 (C), 146.8 (C), 150.6 (CH), 152.4 (C), 159.7 (C), 162.5 (C), 165.1 (C). HR-MS calcd for $C_{27}H_{22}N_7$ 443.1858, found [M^+ + K] 482.8695. [M^+ + H] 443.9281.

4.1.8. 3-Methyl-1-phenyl-7-(pyridin-3-yl)-4-(p-tolyl)-1H-pyrazolo[4′,3′:5,6]pyrido[2,3-d]pyrimidin-5-amine ***5h***

Yellow solid, yield 56%, mp: 305–306. FTIR (KBr) υ(cm^{-1}): 3468 (NH), 3054 (═C─H), 1564, 1547 (C═N and C═C). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.81 (s, 3H, CH_3), 5.21 (brs, 2H, NH_2), 7.37 (t, 1H, *J* = 7.3 Hz, HA*p*), 7.58–7.66 (m, 7H, HB*o*, HA*m*, HB*m*, H-3Py), 8.35 (d, 2H, *J* = 8.0 Hz, HA*o*), 8.69–8.78 (m, 2H, H-2Py, H-4Py), 9.58 (s, 1H, H-6Py) ^{13}C NMR (100 MHz DMSO-d_6) δ: 14.5 (CH_3), 21.5 (CH_3), 103.4 (C), 116.6 (C), 120.9 (CH), 124.1 (CH), 126.1 (CH), 128.6 (CH), 129.7 (CH), 130.5 (CH), 132.6 (C), 133.5 (C), 136.1 (CH), 139.3 (C), 140 (C), 145.4 (C), 146.9 (C), 150.0 (CH), 151.9 (CH), 152.0 (C), 159.5 (C), 162.5 (C), 164.7 (C). EI MS (70 eV): *m*/*z*: 443(M^+, 18), 354(16), 236 (17). Anal. Calcd for $C_{27}H_{21}N_7$: C, 73.12; H, 4.77; N, 22.11; found: C, 73.07; H, 4.72; N, 22.06.

4.1.9. 3-Methyl-1-phenyl-7-(pyridin-2-yl)-4-(p-tolyl)-1H-pyrazolo[4′,3′:5,6]pyrido[2,3-d]pyrimidin-5-amine ***5i***

Yellow solid, yield 48%, mp > 350. FTIR (KBr) υ(cm^{-1}): 3481 (NH), 3057 (═C─H), 1569, 1544 (C═N and C═C). ^{1}H NMR (400 MHz, DMSO-d_6): 1.86 (s, 3H), 7.36 (t, 1H, *J* = 7.4 Hz, HA*p*), 7.52–7.57 (m, 4H, HB*o*, HB*m*), 7.95–8.01 (m, 2H, HA*m*), 8.03 (d, 2H, *J* = 7.7 Hz, H-3Py, H-4Py), 8.39 (d, 2H, *J* = 7.7 Hz, HA*o*), 8.63 (d, 2H, *J* = 4.6 Hz, H-2Py, H-5Py). Not observed (brs, 2H, NH_2). Compound **5i** is barely soluble in dimethyl sulfoxide or any other solvent normally used for NMR spectroscopy; thus, made the registration of a high resolution ^{13}C NMR spectrum impossible. EI MS (70 eV): *m*/*z*: 443(M^+, 83), 354(1), 236 (1). Anal. Calcd for $C_{27}H_{21}N_7$: C, 73.12; H, 4.77; N, 22.11; found: C, 73.09; H, 4.74; N, 22.08.

4.1.10. 4-(Benzo[d][1,3]dioxol-5-yl)-3-methyl-1-phenyl-7-(pyridin-4-yl)-1H-pyrazolo[4′,3′:5,6] pyrido[2,3-d]pyrimidin-5-amine ***5j***

Yellow solid, yield 59%, mp: 316–317. FTIR (KBr) υ(cm^{-1}): 3488 (NH), 3045 (═C─H), 1570, 1559 (C═N and C═C). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.82 (s, 3H, CH_3), 5.36 (brs, 2H, NH_2), 6.15 (s, 2H, OCH_2O), 7.06 (d, 1H, *J* = 7.8 Hz, HA*p*), 7.16 (d, 1H, *J* = 7.9 Hz, HB*o*), 7.19–7.30 (m, 2H, HB*o*′, HB*m*), 7.51 (t, 2H, *J* = 7.7 Hz, HA*m*) 8.20–8.27 (m, 4H, H-2Py, HA*o*), 8.67 (d, 2H, *J* = 5.3 Hz, H-3Py). ^{13}C NMR (100 MHz DMSO-d_6) δ: 14.1 (CH_3), 102.0 (CH_2), 103.3 (C), 108.9 (CH), 109.1(CH), 116.6 (C), 120.2 (CH), 122.0 (CH), 125.6 (CH), 127.8 (C), 129.2 (CH), 138.8 (C), 144.8 (C), 145.0 (C), 145.9 (C), 148.1 (C), 148.6 (C), 150.1 (CH), 151.4 (C), 158.9 (C), 161.6 (C), 164.3 (C). EI MS (70 eV): *m*/*z*: 473(M^+, 46), 369 (61), 313 (23), 236 (30). Anal. Calcd for $C_{27}H_{19}N_7O_2$: C, 68.49; H, 4.04; N, 20.71; found: C, 68.39; H, 4.01; N, 20.61.

4.1.11. 4-(Benzo[d][1,3]dioxol-5-yl)-3-methyl-1-phenyl-7-(pyridin-3-yl)-1H-pyrazolo[4′,3′:5,6] pyrido[2,3-d]pyrimidin-5-amine ***5k***

Yellow solid, yield 50%, mp: 325–326. FTIR (KBr) υ(cm^{-1}): 3478 (NH), 3058 (═C─H), 1571, 1518 (C═N and C═C). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.88(s, 3H, CH_3), 5.13 (brs, 2H, NH_2), 6.24 (s, 2H, OCH_2O), 7.16 (d, 1H, *J* = 9.0 Hz, HA*p*), 7.25 (d, 1H, *J* = 7.9 Hz, HB*o*), 7.32–7.38 (m, 2H, HB*o*′, HB*m*), 7.46–7.69 (m, 4H, H-2Py, H-3Py, HA*m*), 8.34 (d, 2H, *J* = 8.0 Hz, HA*o*), 8.72 (t, 2H, *J* = 6.1 Hz, H-4PY, H-6Py). ^{13}C NMR (100 MHz DMSO-d_6) δ: 14.0 (CH_3), 101.9 (CH_2), 103.1 (C), 108.9 (CH), 109.1 (CH), 116.3 (C), 120.2 (CH), 122.0 (CH), 123.5 (CH), 125.5 (CH), 127.9 (C), 129.1 (CH), 133.0 (C), 135.5 (CH), 138.8 (C), 144.8 (C), 145.9 (C), 148.1 (C), 148.6 (C), 149.5 (C), 151.4 (CH), 158.9 (C), 161.8 (C), 164.7 (C). EI MS (70 eV): EI MS: *m*/*z*: 473(M^+, 100), 368 (11), 313 (12), 236 (19). Anal. Calcd for $C_{27}H_{19}N_7O_2$: C, 68.49; H, 4.04; N, 20.71; found: C, 68.41; H, 4.02; N, 20.62.

4.2. Biological evaluation

4.2.1. Antifungal activity

4.2.1.1. Microorganisms and media. For the antifungal evaluation, standardized strains from the American Type Culture Collection (ATCC), Rockville, MD, USA, Reference Center in Mycology (CEREMIC, CCC, Rosario, Argentina) and Instituto Malbrán (IM, Av. Velez Sarsfield 563, Buenos Aires) were used. Standardized strains: *C. albicans* ATCC 10231 and *C. neoformans* ATCC 32264; clinical isolates of *C. albicans* were provided by CCC and of *C. neoformans* were provided by IM. Voucher specimens of the isolated are presented in Table 6. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C, were maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and sub-cultured every 15 days to prevent pleomorphic transformations. Inocula were obtained according to reported

procedures [29] and adjusted to $1–5 \times 10^3$ cells with colony forming units (CFU)/mL.

4.2.1.2. Fungal growth inhibition percentage determination. Broth microdilution techniques were performed in 96-well microplates according to the guidelines of the Clinical and Laboratory Standards Institute for yeasts (M27-A3) (Clinical and Laboratory Standards Institute). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard [29]. For the assay, compound test wells (CTWs) were prepared with stock solutions of each compound in DMSO (maximum concentration $\leqslant 1\%$), diluted with RPMI-1640, to final concentrations of 250–0.98 μg/mL. An inoculum suspension (100 μL) was added to each well (final volume in the well = 200 μL). A growth control well (GCW) (containing medium, inoculum, and the same amount of DMSO used in a CTW, but compound-free) and a sterility control well (SCW) (sample, medium, and sterile water instead of inoculum) were included for each fungus tested. Microtiter trays were incubated in a moist, dark chamber at 30 °C for 48 h for both yeasts. Microplates were read in a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). Amphotericin B was used as positive control. Tests were performed in triplicate. Reduction of growth for each compound concentration was calculated as follows: % of inhibition = 100 − (OD 405 CTW − OD 405 SCW)/(OD 405 GCW − OD 405 SCW). The means ± SEM were used for constructing the dose–response curves representing% inhibition *vs* concentration of each compound. Dose–response curves were constructed with SigmaPlot 11.0 software.

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