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Original article

Synthesis of novel thiazole-based 8,9-dihydro-7*H*-pyrimido[4,5-*b*][1,4]diazepines as potential antitumor and antifungal agents

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ABSTRACT

A new series of novel thiazole-based 8,9-dihydro-7*H*-pyrimido[4,5-*b*][1,4]diazepines **6a–g** and **7a–g** were obtained with high regioselectivity from the reaction of triamino- or tetraaminopyrimidines **4** and **5** with α,β-unsaturated carbonyl compounds **3a–g** based on 2,4-dichlorothiazol-5-carbaldehyde **1**. Twelve of the synthesized compounds were selected and tested by US National Cancer Institute (NCI) for their antitumor activity against 60 different human tumor cell lines. Compounds **7d** and **7g** showed important GI_{50} ranges of 1.28–2.98 μM and 0.35–2.78 μM respectively under *in vitro* assays. In addition, **6a–g** and **7a–g** were tested for antifungal properties against the clinical important fungi *Candida albicans* and *Cryptococcus neoformans*. Although these compounds showed moderate activities against *C. albicans*, the 2-amino derivatives **7a–g** and mainly **7a** and **7b**, showed high activity against standardized and clinical isolates of *C. neoformans* with MIC_{50} = 7.8–31.2 μg/mL, MIC_{80} = 15.6–31.2 μg/mL and MIC_{100} = 15.6–62.5 μg/mL. In addition, since both compounds were fungicide rather than fungistatic these thiazole-based 8,9-dihydro-7*H*-pyrimido[4,5-*b*][1,4]diazepines appear as good candidates for further development not only as antifungal but also as antitumor drugs.

1. Introduction

The seven-membered nitrogen-containing heterocyclic compounds [1,4]-diazepines are considered very important targets in drug discovery due to their broad spectrum of pharmacological activities [1] such as anti-schistosomal [2], antibacterial, antioxidant [3], anti HIV [4,5], anticonvulsant [6], and anticancer [7–9]. Recently, heterocyclic rings like pyrimidines which have showed several biological activities [10–14] were fused to a [1,4]-diazepine system through the regioselective reaction of α,β-unsaturated carbonyl compounds and several 5,6-diaminopyrimidines [15–17]. This promising modification led to the formation of novel pyrimido[4,5-*b*][1,4]diazepines with remarkable antitumor activity against different human cell lines [18–20].

On the other hand, previous studies have shown that the use of thiazole-based compounds as starting materials in organic synthesis led to the formation of several thiazole-derivatives with a wide spectrum of biological activities [21,22] such as antifungal [23,24], antimicrobial [25–27], antimalarial [28] and anticancer [29–35].

Continuing with our current research on the synthesis and biological activities of [1,4]-diazepines, we are reporting here the efficient synthesis of two series of novel thiazole-based 8,9-dihydro-7*H*-pyrimido[4,5-*b*][1,4]diazepines **6a–g** and **7a–g**, several of these compounds were evaluated for their antitumoral activity by the US National Cancer Institute (NCI) against 60 different human tumor cell lines (i. e. leukemia, melanoma, lung, colon, brain, ovary, breast, prostate, and kidney cancers) Also, all synthesized compounds were tested for antifungal properties against a panel of standardized as well as clinical isolates of clinically important fungi.

2. Results and discussion

2.1. Chemistry

The heating under reflux of equimolar amounts of thiazole-based α,β-unsaturated carbonyl compounds **3a–g** and 4,5,6-

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triaminopyrimidine **4** or 2,4,5,6-tetraaminopyrimidine **5** in methanol afforded the expected compounds **6a**–**g** and **7a**–**g** in good yields, Scheme 1.

The thiazole-based pyrimido[4,5-*b*][1,4]diazepines synthesis proceeded with high Regioselectivity under the reaction conditions. This fact may be explained by the higher nucleophilicity of the C-5 amino group than the others amino groups in aminopyrimidines **4** and **5** due to electronic effects of the pyrimidine ring [16,17,36]. In this sense, a nucleophilic condensation between C-5 amino group of compounds **4** and **5** with the carbonyl group of the α,β-unsaturated ketones **3a**–**g** occurred follow by intramolecular cyclization type Michael addition of the C-4 amino group to the beta-carbon of **3a**–**g**.

Structure elucidation of all synthesized compounds was performed by spectroscopic techniques, FT-IR, NMR, EI-MS and elemental analysis. All diazepines **6a**–**g** and **7a**–**g** showed similar spectroscopic data (see Experimental section).

All compounds of the series **6a**–**g** showed FT-IR absorption bands in two ranges: 3471–3218 cm^{-1} assigned to –NH and C-4–NH_2 groups and 1649–1557 cm^{-1} assigned to the C=N and C=C functionalities. Compounds **7a**–**g** showed absorption bands in the ranges of 3476–3309 cm^{-1} assigned to –NH, C-2-NH_2 and C-4–NH_2 groups and 1661–1548 cm^{-1} assigned to the C=N and C=C functionalities.

Regarding the NMR spectra, the seven **6a**–**g** compounds showed similar NMR shifts and therefore we discuss here the spectroscopic data only of **6c** as the representative of this series. Its ^{1}H NMR spectrum showed an AMX spin system for the protons on C-7 and on the stereogenic carbon atom C-8 of the diazepine moiety. So, the diastereotopic proton H_{7A} appeared at 3.00 ppm as a double-doublet with geminal and vicinal coupling constants of $^2J_{AM}$ = 14.5 Hz and $^3J_{MX}$ = 5.7 Hz while the signal of the diastereotopic proton H_{7M} appeared at 3.83 ppm as a double-doublet with geminal and vicinal coupling constants of $^2J_{AM}$ = 14.5 Hz and $^3J_{AX}$ = 2.1 Hz. The signal of the proton H_{8X} is observed at 5.76 ppm as a multiplet. The signal of the 4-NH_2 protons appeared at 6.66 ppm as a broad singlet while the signal of the –NH was observed as a doublet at 7.73 ppm with ^3J = 6.2 Hz, this coupling constant corroborating the vicinal position to C-8 proton. Within the aromatic region, a multiplet at 7.19 ppm due to H_m and a double-doublet at 7.88 ppm associated to proton H_o with coupling constants $^3J_{HF}$ = 5.5 Hz and $^3J_{HH}$ = 9.0 Hz, were observed. The signal corresponding to C-2 proton appeared as a singlet at 7.83 ppm.

In turn, compounds **7a**–**g** showed in NMR ^{1}H and ^{13}C experiments broad singlets in the range 4.91–5.89 ppm associated to the 2-NH_2 group on C-2 carbon atom.

Analysis of ^{13}C, DEPT-135 and two dimensional heteronuclear NMR spectra allowed the final structural elucidation of all synthesized compounds **6a**–**g** and **7a**–**g**. For compound **6c** the ^{13}C NMR spectrum with the help of DEPT-135 experiment showed the signal associated to the methylene carbon atom C-7 at 36.8 ppm and the signal associated to C-8 at 56.0 ppm. Signals of quaternary carbon atoms C-4a and C-9a were observed at 108.4 ppm at 152.5 ppm respectively. Signals of all carbon atoms on *p*-fluorophenyl substituent appeared as doublets at 115.5, 129.5, 136.2 and 161.7 ppm with coupling constants of $^2J_{CF}$ = 21.5, $^3J_{CF}$ = 8.6, $^4J_{CF}$ = 3.0 and $^1J_{CF}$ = 246.9 Hz assigned to C_m, C_o, C_i and C_p carbon atoms, respectively. Finally, the signal of carbon C-2 appeared at 156.0 ppm.

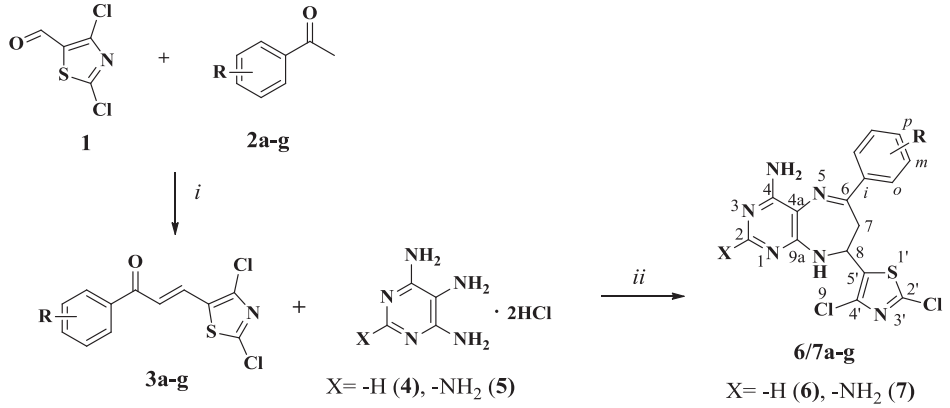
HMBC experiment for compound **6c** confirmed the high regioselectivity of the reaction in which three-bond correlations between quaternary bridge carbon atom C-9a and protons 2-H and 8-H were observed. This correlation supports the sequence of steps for the formation of compounds **6** and **7** proposed above.

In Fig. 1 the Ortep drawing of compound **6b** has confirmed the regioselectivity of the reaction. In the X-ray structure of compound **6b** the diazepine ring adopts a conformation close to the twist-boat form [37], Fig. 1.

Mass spectra of compounds **6a**–**g** and **7a**–**g** show well-defined molecular ions type **I**, with a characteristic fragmentation pattern involving the loss of a chlorine atom from the thiazole unit affording the cation **II** and the loss of the 5-vinylthiazol **III** to afford the stabilized purine species **IV** [38] (Scheme 2).

2.2. Antitumor activity

To determine the antitumor activity, compounds were first evaluated against 60 cell lines derived from nine cancer types: leukemia, lung, melanoma, colon, CNS, ovary, renal, breast and prostate cancers at a single dose of 10 μM. The output from the



Compound	6a/7a	6b/7b	6c/7c	6d/7d	6e/7e	6f/7f	6g/7g
Yield (%)	57/59	68/72	71/78	55/59	56/53	50/49	62/58
R	H	4-Cl	4-F	4-CH_3	4-OCH_3	3,4,5-*tri*OCH_3	3,4-methylenedioxy

Scheme 1. Synthesis of novel 8,9-dihydro-7*H*-pyrimido[4,5-*b*][1,4]diazepines **6a**–**g** and **7a**–**g**. *i* = AcOH, H_2SO_4, r.t. 36 h. *ii* = MeOH, reflux, 24–36 h.

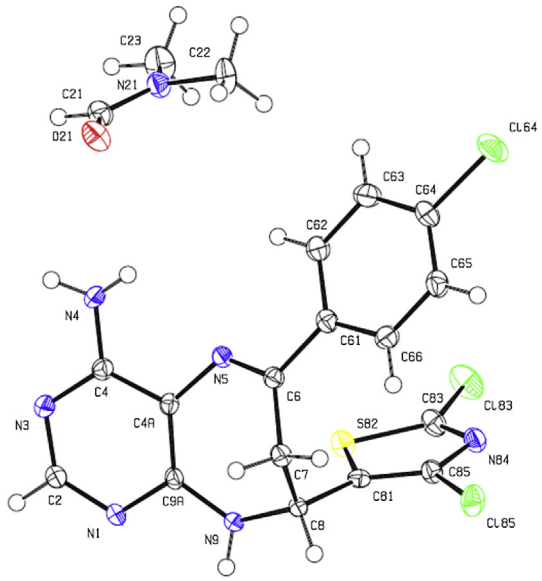


Fig. 1. Ortep drawing of compound **6b**.

single dose screen was reported as a mean graph available for analysis by the COMPARE program. These results showed that compounds **6d** and **6g** were active. With these results, a secondary assay was performed; in this case, compounds **6d** and **6g** were tested against the same 60 cell lines at concentrations of 100, 10, 1.0, 0.1 and 0.01 μM in order to determine their cytostatic activity. The test consisted of a 48 h continuous drug exposure protocol in which sulforhodamide B (SRB) protein assay was used to estimate cell growth according to published procedures [39,40]. As shown in Table 1, compounds **6d** and **6g** showed the most prominent values of GI_{50} against several lines. Structure **6d** showed activity against the following lines: K-562 of leukemia with GI_{50} = 1.68 μM and LC_{50} > 100 μM; SND-75 of CNS cancer with GI_{50} = 1.63 μM and LC_{50} = 38.1 μM; MDA-MB-435 of melanoma with GI_{50} = 1.47 μM and LC_{50} = 39.0 μM and A498 of renal cancer with GI_{50} = 1.28 μM and LC_{50} = 31.2 μM. In turn, **6g** was the most active compound against the leukemia cell line K-562 with GI_{50} value of 0.54 μM and LC_{50} > 100 μM, and the melanoma cell line MDA-MB-435 with GI_{50} value of 0.35 μM and LC_{50} > 48.4 μM. Also **6g** showed low GI_{50} values against other cell lines such as HCT-15 of colon cancer with GI_{50} = 1.07 μM and LC_{50} > 100 μM; SNB-75 of CNS cancer with GI_{50} = 1.39 μM and LC_{50} = 61.4 μM; RXF393 of renal cancer with GI_{50} = 1.40 μM and LC_{50} = 36.5 μM and MDA-MB-468 of breast cancer with GI_{50} = 1.65 μM and LC_{50} > 100 μM.

2.3. Antifungal activity

Considering that some nitrogen-containing heterocyclic compounds containing compounds like 1,4-diazepine moiety have demonstrated antifungal activity in previous reports [41–46], compounds **6a**–**g** and **7a**–**g** were tested for antifungal activities against two clinically important fungal species *Candida albicans* and *Cryptococcus neoformans*.

C. albicans is the cause of over 60% of all isolates from nosocomial infections [47], and *C. neoformans* is the fungal species that produces cryptococcal meningitis that has killed more than 650,000 immunocompromised patients worldwide and whose treatment is based on drugs discovered nearly 50 years ago [48].

Since the only difference between compounds **6** and **7** is the amino group in position 2, we performed at first a comparative antifungal evaluation of each compound of the series **6** (i.e **6a**, **6b**, and so on) with its analogs (**7a**, **7b** and so on) in order to determine the role of the NH_2 group in the antifungal behavior and then deepen the study of the most relevant ones. Results are showed in Figs. 2 and 3.

From the analysis of Fig. 2 (A–G), it is clear that compounds **7a**–**g**, that possess an NH_2 group on C-2, displayed better activities in both the *Candida albicans* (Fig. 2$\mathbf{A_{Ca}}$–$\mathbf{G_{Ca}}$) and the *Cryptococcus neoformans* (Fig. 2$\mathbf{A_{Cn}}$–$\mathbf{G_{Cn}}$) strains, than the analogs **6a**–**g** that do not possess the amino group.

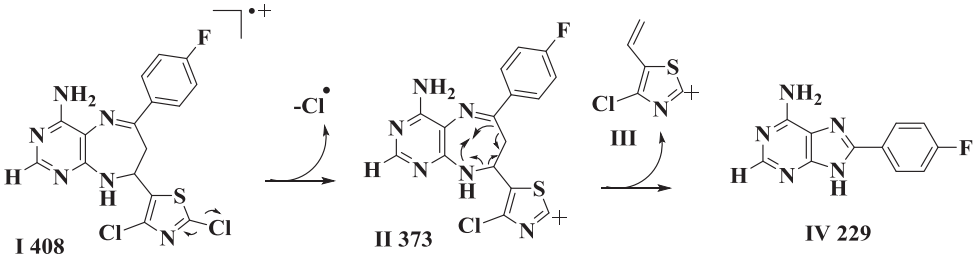
Based on the results of Fig. 2, we constructed dose–response curves for compounds **7a**–**g**, against each fungus *C. albicans* and *C. neoformans* (Fig. 4A and B) in order to compare their activities. The values of the inhibition percentages plotted in Fig. 4 are recorded in Table 2.

As it can be observed in Table 2, there is a clear difference in the activity of **7a**–**g** against both fungi. With regard to *Candida albicans*, not any compound displayed 80% inhibition at concentrations below 250 μg/mL, being **7b**–**g** only moderately active (25.48–78.73 % inhibition) at 250 μg/mL while compound **7a** showed the best activity displaying a 100% growth inhibition at 250 μg/mL. Instead, **7a**–**7g** showed a better inhibition capacity against *Cryptococcus neoformans* and, among them, **7a** and **7b** completely inhibited *C. neoformans* growth at 31.25 and 15.62 μg/mL respectively. The remaining compounds were moderately active reaching 14.96–62.64% inhibition at 250 μg/mL. These findings clearly highlight that when these thiazole-based diazepines possess a non-substituted or a 4-Cl substituted benzene ring, the compounds showed the best activities suggesting that **7a** and **7b** could be promising anticryptoccocal hits for further development.

2.4. Second-order studies with clinical isolates

In order to gain insight into the extent of inhibitory capacity of **7a** and **7b** against *C. neoformans*, both compounds were tested in a new panel of 10 clinical *C. neoformans* strains isolated from patients suffering from mycoses.

The Minimum Inhibitory Concentration (MIC) of **7a** and **7b** was determined against this new panel by using three endpoints: MIC_{100}, MIC_{80} and MIC_{50} (defined as the minimum



Scheme 2. Fragmentation pattern of compound **6** and **7**.

Table 1
In vitro cytotoxic effects of compounds **6d** and **6g** against NCI's *in vitro* disease-oriented human tumor cell line screen.

Panel/Cell line	6d		6g	
	GI_{50}[a] (μM)	LC_{50}[b] (μM)	GI_{50}[a] (μM)	LC_{50}[b] (μM)
Leukemia				
CCRF-CEM	3.21	>100	3.02	>100
HL-60(TB)	2.04	>100	36.1	>100
K-562	1.68	>100	0.54	>100
MOLT-4	4.06	>100	5.91	>100
RPMI-8226	4.99	>100	4.66	>100
SR	2.25	76.7	1.90	>100
Non-small cell lung				
A549/ATCC	5.02	52.5	6.47	>100
HOP-62	3.72	48.9	5.32	62.6
HOP-92	3.72	45.4	4.25	77.5
NCI–H226	13.7	90.3	17.7	>100
NCI–H23	8.23	58.7	8.87	>100
NCI–H460	4.06	49.4	3.99	>100
NCI–H522	4.05	42.1	2.51	55.3
Colon Cancer				
COLO205	4.18	42.1	3.48	45.6
HCC-2998	10.3	46.8	6.11	48.8
HCT-116	3.70	37.3	3.42	44.5
HCT-15	3.05	50.1	1.07	>100
KM12	3.80	40.4	3.93	73.6
SW-620	3.42	40.7	2.98	>100
CNS Cancer				
SF-268	5.68	67.3	9.84	92.2
SF-295	3.06	36.4	2.21	39.8
SF-539	2.69	33.1	2.22	33.6
SNB-19	5.51	47.2	4.83	>100
SNB-75	1.63	38.1	1.39	61.4
Melanoma				
LOXIMVI	5.39	43.2	7.02	75.6
MALME-3M	6.33	44.0	14.3	59.2
M14	2.64	35.6	2.01	44.6
MDA-MB-435	1.47	39.0	0.35	48.4
SK-MEL-2	2.98	35.6	5.19	48.6
SK-MEL-28	4.26	42.8	7.63	91.6
SK-MEL-5	3.69	37.3	3.43	40.8
UACC-257	6.76	48.6	14.6	>100
UACC-62	3.05	37.2	2.17	63.9
Ovarian Cancer				
IGROV1	7.08	63.2	10.4	>100
OVCAR-3	3.60	43.4	3.94	66.8
OVCAR-5	10.2	46.8	6.03	>100
OVCAR-8	4.80	80.7	4.86	>100
NCI/ADR-RES	3.13	58.5	3.55	>100
SK-OV-3	3.60	43.0	2.78	>100
Prostate Cancer				
PC-3	5.89	73.1	5.15	>100
DU-145	11.2	52.4	8.45	>100
Breast Cancer				
MCF7	3.56	97.6	2.81	>100
MDA-MB231/ATCC	3.86	42.1	5.81	55.7
HS578T	2.44	>100	2.35	>100
BT-549	3.93	40.6	3.79	53.9
T-47D	3.35	>100	5.36	>100
MDA-MB-468	2.12	41.6	1.65	>100

[a] GI_{50} was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation, determined at five concentration levels (100, 10, 1.0, 0.1 and 0.01 mM).
[b] LC_{50} is a parameter of cytotoxicity and reflects the molar concentration needed to kill 50% of the cells.

concentration of compounds that inhibit 100, 80 and 50% of growth respectively). The application of a less stringent endpoint such as MIC_{80} and MIC_{50} have shown to consistently represent the *in vitro* activity of the tested compounds and many times provides a better correlation with other measurements of antifungal activity [49].

In addition to MIC determinations, the evaluation of the Minimum Fungicidal Concentration (MFC) of **7a** and **7b** against this panel was accomplished by sub-culturing a sample from MIC tubes showing no growth, onto drug-free agar plates. These results are shown in Table 3.

Results of Table 3 corroborate that **7a** and **7b** display interesting activities against *C. neoformans* since the activity found in clinical isolates were similar to those displayed against the standardized strain ATCC 32264. In addition, this table adds the data that both compounds are fungicide rather than fungistatic, which signifies that they kill fungi in addition to inhibit them. This characteristic is highly appreciated in an antifungal drug; since recurrences and the appearance of resistance are mostly avoided due to long treatments are not longer necessary.

3. Conclusions

A new series of novel thiazole-based 8,9-dihydro-7*H*-pyrimido[4,5-*b*][1,4]diazepines **6a**–**g** and **7a**–**g** were efficiently synthetized by the reaction of tri or tetra-aminopyrimidines with thiazole-based α,β-unsaturated carbonyl compounds **3**. Compounds **6** and **7** were obtained in good yields and the reactions proceeded regioselectively being the correct structural orientation demonstrated by 1D- and 2D-NMR experiments and X-ray diffraction. Antitumor activity studies showed that two compounds **7d** and **7g** exhibited values of GI_{50} in a range of 1.28–1.68 μM and 0.35–1.65 μM, respectively. The studies on antifungal activity showed that compounds **7** are more active than compounds **6** and of **7a**–**g**, **7a** and **7b** showed the highest activity against standardized and clinical isolates of *C. neoformans* with MIC_{50} between 7.8 and 31.2 μg/mL, MIC_{80} = 15.6–31.2 μg/mL and MIC_{100} in the range 15.6–62.5 μg/mL.

4. Experimental

4.1. General

Reagents and solvents used were obtained from commercial sources. Melting points were measured using a Stuart SMP3 melting point device and are uncorrected. IR spectra were obtained with a Shimadzu IRAffinity-1. The ^{1}H and ^{13}C NMR spectra were run on a BRUKER DPX 400 spectrometer operating at 400 and 100 MHz respectively, using $CDCl_3$ as solvent and TMS as internal standard. The mass spectrum was obtained on a SHIMADZU-GCMS-QP2010 spectrometer operating at 70 eV. The elemental analyses were obtained using a Thermo Finnigan Flash EA1112 CHN (STIUJA) elemental analyzer. Thin layer chromatography (TLC) was performed on a 0.2-mm pre-coated plates of silica gel 60GF254 (Merck).

4.2. Chemistry

General procedure for synthesis of *4-amino-8-(2,4-dichlorothiazol-5-yl)-6-aryl-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepines* (**6a–g**) and *2,4-diamino-8-(2,4-dichlorothiazol-5-yl)-6-aryl-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepines* (**7a**–**g**):

A mixture of 0.5 mmol of compounds **3a**–**g**, synthesized by a methodology reported previously [50], and 0.6 mmol of the corresponding hydrochloride of triaminopyrimidine **4** or tetraaminopyrimidine **5** in methanol, was subjected to reflux during 24–30 h. The reaction progress was monitored by TLC. After complete reaction the resultant suspension was quenched with NH_4OH 6% until neutralization and the crude was extracted with CH_2Cl_2. Solvent was eliminated under reduced pressure and the product was purified by column chromatography employing 60:1 of CH_2Cl_2:CH_3OH as eluant. All compounds were obtained as beige solids.

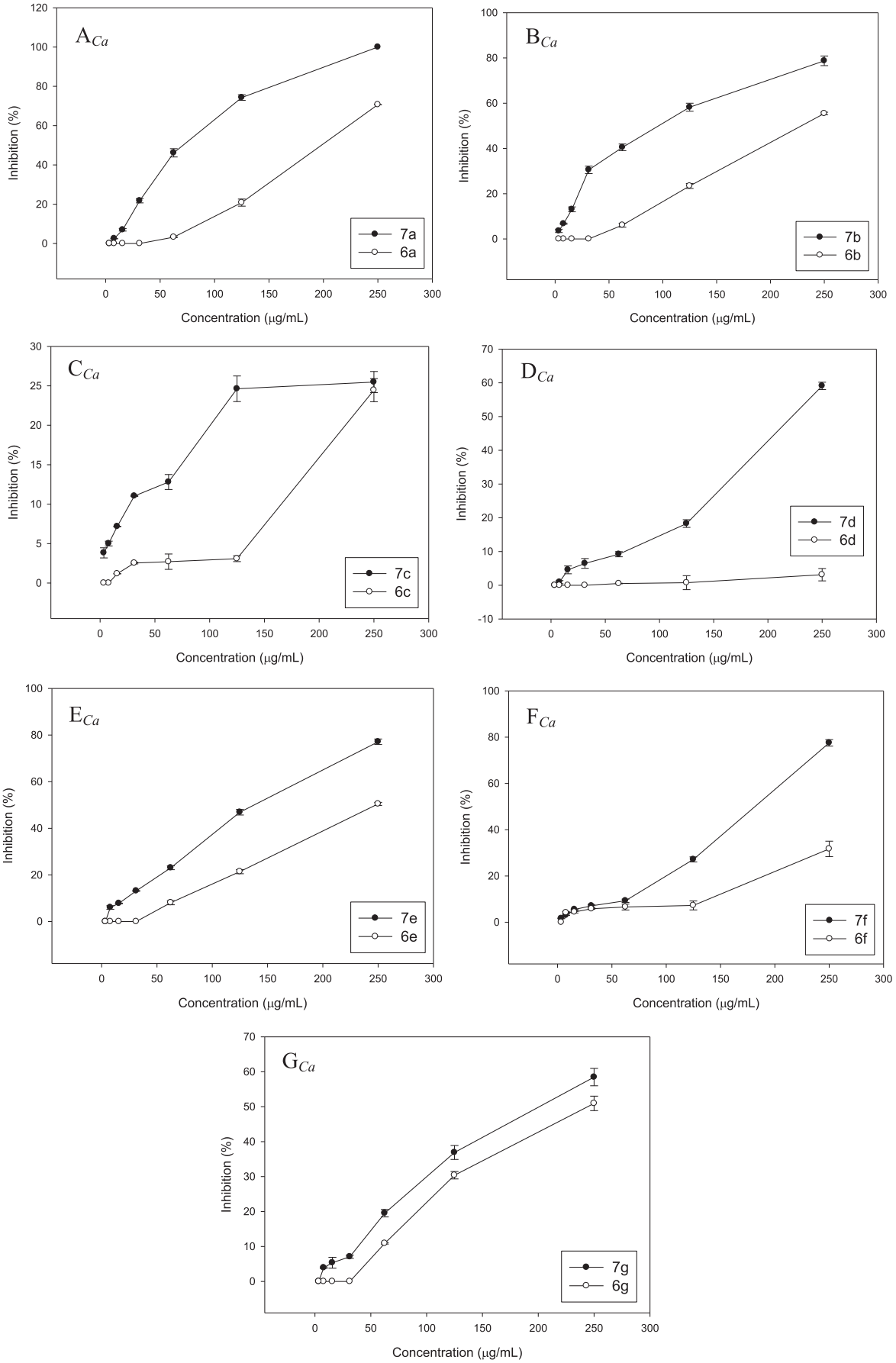


Fig. 2. Comparative dose response curves against *C. albicans* ATCC 10231 of **7a**–**6a** (A_{Ca}); **7b**–**6b** (B_{Ca}); **7c**–**6c** (C_{Ca}); **7d**–**6d** (D_{Ca}): **7e**–**6e** (E_{Ca}); **7f**–**6f** (F_{Ca}); **7g**–**6g** (G_{Ca}). Amphotericin B, used as the standard drug, displayed 100% of inhibition at all concentrations tested (curve not shown).

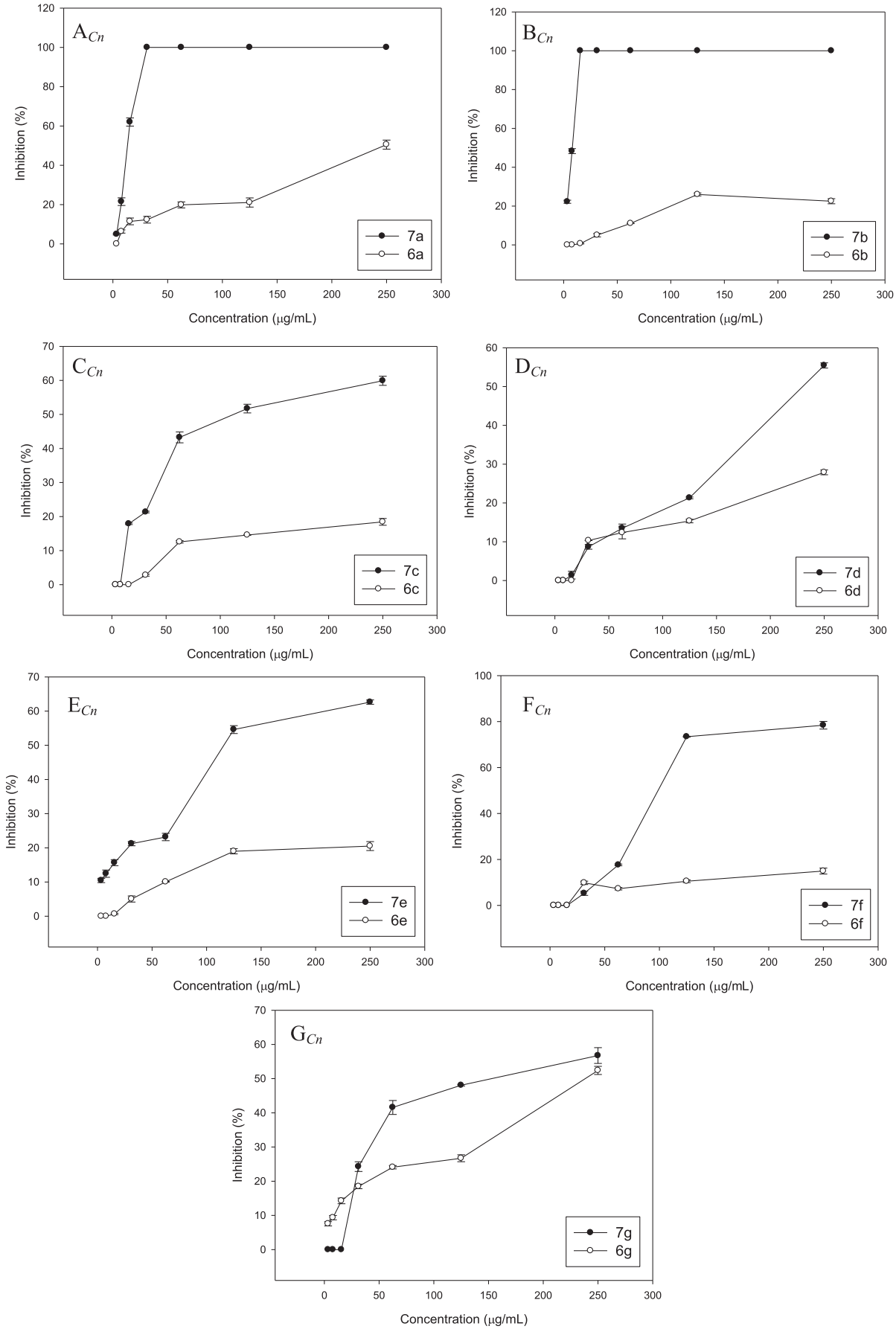


Fig. 3. Comparative dose response-curves against *C. neoformans* ATCC 32264 of **7a**–**6a** (A_{Cn}); **7b**–**6b** (B_{Cn}); **7c**–**6c** (C_{Cn}); **7d**–**6d** (D_{Cn}): **7e**–**6e** (E_{Cn}); **7f**–**6f** (F_{Cn}); **7g**–**6g** (G_{Cn}). Amphotericin B, used as the standard drug, displayed 100% of inhibition at all concentrations tested (curve not shown).

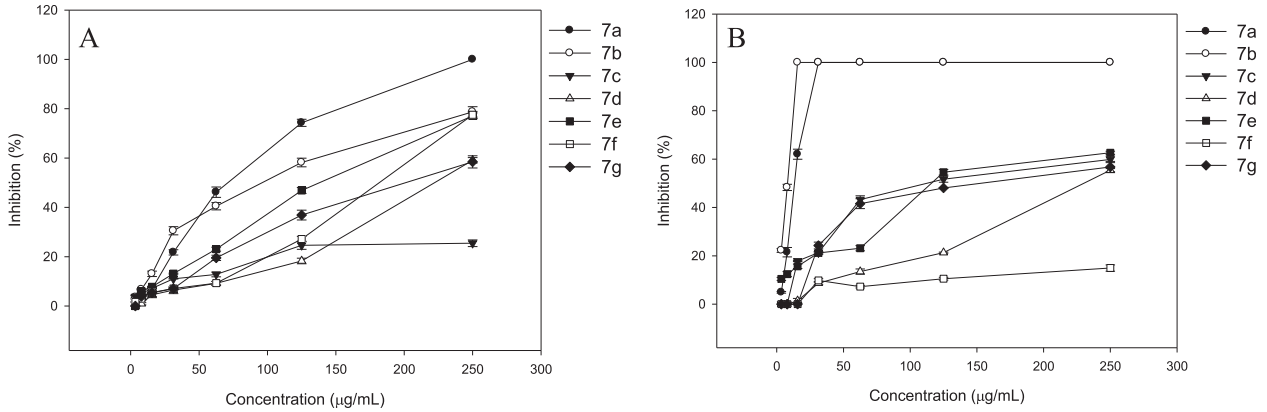


Fig. 4. Dose-response curves of compounds **7a**–**7g** against (A) *C. albicans* ATCC 10231 and (B) *C. neoformans* ATCC 32264. Amphotericin B inhibited 100% of both fungi at 1 μg/mL.

4.2.1. 4-Amino-8-(2,4-dichlorothiazol-5-yl)-6-phenyl-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (6a)

m.p 242–243 °C; FT-IR (KBr), υ(cm^{-1}): 3462, 3278 and 3231 (NH, 4-NH_2), 1631, 1591 and 1564 (C═N and C═C). NMR-^{1}H (400 MHz, DMSO-d_6): δ 3.00 (dd, J = 14.4, 3.0 Hz, 1H, H_{7A}), 3.84 (dd, J = 14.4, 5.6 Hz, 1H, H_{7M}), 5.49 (dd, J = 5.6, 3.0 Hz, 1H, H_{8X}), 6.65 (s, 2H, 4-NH_2), 7.31–7.45 (m, 3H, H_m y H_p), 7.74 (d, J = 6.0 Hz, 1H, −NH) 7.81 (dd, J = 7.9, 1.5 Hz, 2H, H_o) 7.84 (s, 1H, H_2) ppm. NMR-^{13}C (100 MHz, DMSO-d_6): δ 36.5 (CH_2), 56.1 (CH), 108.5 (C_c), 127.04 (CH), 128.7 (CH), 130.35 (CH), 130.4 (C_c), 139.6 (C_c), 139.8 (C_c), 149.3 (C_c), 152.5 (C_c), 156.0 (CH), 162.1 (C_c), 162.4 (C_c) ppm. MS (IE, 70 eV) m/z (%): 392/390 [M^+] (27.0/40.4), 357/355 (33.6/87.7), 211 (100). Anal. Calcd. $C_{16}H_{12}Cl_2N_6S$: C, 49.11; H, 3.09; N, 21.48; Found: C, 49.28; H, 2.50; N, 21.53.

4.2.2. 4-Amino-6-(4-chlorophenyl)-8-(2,4-dichlorothiazol-5-yl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (6b)

m.p 238–239 °C; FT-IR (KBr), υ(cm^{-1}): 3462, 3278 and 3231 (−NH, 4-NH_2), 1629, 1590 and 1563 (C═N and C═C). NMR-^{1}H (400 MHz, DMSO-d_6): δ2.99 (dd, J = 14.5, 3.0 Hz, 1H, H_{7A}), 3.84 (dd, J = 14.5, 5.7 Hz, 1H, H_{7M}), 5.46 (dd, J = 5.7, 3.0 Hz, 1H, H_{8X}), 6.69 (s, 2H, 4-NH_2), 7.42 (d, J = 8.7 Hz, 2H, H_m), 7.79 (d, J = 6.2 Hz, 1H, −NH), 7.84 (d, J = 8.7 Hz, 2H, H_o) 7.86 (s, 1H, H_2) ppm. NMR-^{13}C (100 MHz, DMSO-d_6): δ36.5 (CH_2), 55.7 (CH), 108.3 (C_c), 128.7 (CH), 128.9 (CH), 130.5 (C_c), 135.2 (C_c), 138.4 (C_c), 139.0 (C_c), 149.9 (C_c), 152.6 (C_c), 156.2 (CH), 160.6 (C_c), 162.5 (C_c) ppm. MS (IE, 70 eV) m/z (%):428/426/424 [M^+] (10/29/30), 391/389 (58/80), 247/245 (31/100). Anal. Calcd. $C_{16}H_{11}Cl_3N_6S$: C, 45.14; H, 2.60; N, 19.74; Found: C, 45.30; H, 2.77; N, 19.95.

4.2.3. 4-Amino-8-(2,4-dichlorothiazol-5-yl)-6-(4-fluorophenyl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (6c)

m.p 244–245 °C; FT-IR (KBr), υ(cm^{-1}): 3460, 3272 and 3218 (−NH, 4-NH_2), 1630, 1586 and 1563 (C═N and C═C). NMR-^{1}H(400 MHz, DMSO-d_6): δ 3.00 (dd, J = 14.5, 2.1 Hz, 1H, H_{7A}), 3.83 (dd, J = 14.4, 5.7 Hz, 1H, H_{7M}), 5.45–5.49 (m, 1H, H_{8X}), 6.66 (s, 2H, 4-NH_2), 7.17–7.21 (m, 2H, H_m), 7.73 (d, J = 6.2 Hz, 1H, −NH), 7.83 (s, 1H, H_2), 7.88 (dd, J = 9.0 y J_{HF} = 5.5 Hz, 2H, H_o) ppm. NMR-^{13}C (100 MHz, DMSO-d_6): δ 36.5 (C_7), 56.0 (C_8), 108.4 (C_{4a}), 115.5 (d, $^2J_{CF}$ = 21.5 Hz, C_m), 129.5 (d, $^3J_{CF}$ = 8.6 Hz, C_o), 130.4 ($C_{4'}$), 136.2 (d, $^4J_{CF}$ = 3.0 Hz, C_i), 139.7 ($C_{5'}$), 149.4 (C_4), 152.5 (C_{9a}), 156.0 (C_2), 161.7 (d, $^1J_{CF}$ = 246.9 Hz), 162.4 (C═N), 164.9 ($C_{2'}$) ppm. MS (IE, 70 eV) m/z (%): 410/408 [M^+] (20.4/30.8), 375/373 (28.7/77.9), 229 (100). Anal. Calcd. $C_{16}H_{11}Cl_2FN_6S$: C, 46.95; H, 2.71; N, 20.53; Found: C, 46.99; H, 2.89; N, 20.51.

4.2.4. 4-Amino-8-(2,4-dichlorothiazol-5-yl)-6-(p-tolyl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (6d)

m.p 250–251 °C; FT-IR (KBr), υ(cm^{-1}): 3408, 3301 and 3237 (−NH, 4-NH_2), 1630, 1589 and 1557 (C═N and C═C). NMR-^{1}H(400 MHz, DMSO-d_6): δ 2.31 (s, 3H, $p$$CH_3$), 2.97 (dd, J = 14.3, 2.0 Hz, 1H, H_{7A}), 3.81 (dd, J = 14.3, 5.6 Hz, 1H, H_{7M}), 5.46–5.50 (m, 1H, H_{8X}), 6.62 (s, 2H, 4-NH_2), 7.17 (d, J = 8.2 Hz, 2H, H_m), 7.67 (d, J = 6.2 Hz, 1H, −NH), 7.71 (d, J = 8.2 Hz, 2H, H_o), 7.83 (s, 1H, H_2) ppm. NMR-^{13}C (100 MHz, DMSO-d_6): δ21.3 (CH_3), 36.3 (CH_2), 55.3 (CH), 108.5 (C_c), 127.0 (CH), 129.3 (CH), 130.4 (C_c), 136.9 (C_c), 139.8 (C_c), 140.1 (C_c), 149.3 (C_c), 152.5 (CH), 155.8 (C_c), 162.8 (C_c), 162.4 (C_c) ppm.MS (IE, 70 eV) m/z (%): 406/404 [M^+] (26.2/35.1), 371/369 (24.4/63.7), 225 (100). Anal. Calcd. $C_{17}H_{14}Cl_2N_6S$: C, 50.38; H, 3.48; N, 20.74; Found: C, 50.32; H, 3.76; N, 20.75.

4.2.5. 4-Amino-8-(2,4-dichlorothiazol-5-yl)-6-(4-methoxyphenyl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (6e)

m.p 226–227 °C; FT-IR (KBr), υ(cm^{-1}): 3468, 3280 and 3229 (−NH, 4-NH_2), 1629, 1590 and 1563 (C═N and C═C). NMR-^{1}H (400 MHz, DMSO-d_6): δ 2.96 (dd, J = 14.3, 2.1 Hz, 1H, H_{7A}) 3.84–3.75 (m, 4H, H_{7M} y $p$$OCH_3$), 5.45–5.49 (m, 1H, H_{8X}), 6.58 (s, 2H, 4-NH_2), 7.63 (d, J = 6.0 Hz, 2H, H_m), 7.68 (d, J = 6.0 Hz, 1H, −NH) 7.79 (d, J = 8.9 Hz, 2H, H_o), 7.81 (s, 1H, H_2) ppm. NMR-^{13}C (100 MHz, DMSO-d_6): δ36.1 (CH_2), 55.7 (CH_3), 56.4 (CH), 108.7 (C_c), 114.0 (CH), 128.7 (CH), 130.3 (C_c), 132.2 (C_c), 139.9 (C_c), 149.2 (C_c), 152.4 (CH), 155.5 (C_c), 161.2 (C_c), 161.8 (C_c), 162.2 (C_c) ppm. MS (IE, 70 eV) m/z (%): 422/420 [M^+] (42.4/58.7), 387/385 (32.4/87.7), 371/369 (6.5/16.1), 241 (100). Anal. Calcd. $C_{17}H_{14}Cl_2N_6OS$: C, 48.46; H, 3.35; N, 19.95; Found: C, 48.73; H, 3.51; N, 19.93.

4.2.6. 4-Amino-8-(2,4-dichlorothiazol-5-yl)-6-(3,4,5-trimethoxyphenyl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (6f)

p.f. 249–250 °C; FT-IR (KBr), υ(cm^{-1}): 3395, 3288 and 3256 (−NH, 4-NH_2), 1669, 1583 and 1561 (C═N and C═C). NMR-^{1}H (400 MHz, DMSO-d_6): δ 3.04–2.92 (m, 1H, H_{7A}), 3.69 (s, 3H, $p$$OCH_3$), 3.92–3.72 (m, 7H, H_{7M} y $m$$OCH_3$), 5.53 (d, J = 3.9 Hz, 1H, H_{8X}), 6.62 (s, 2H, 4-NH_2), 7.01 (s, 2H, H_o), 7.59 (d, J = 6.1 Hz, 1H, −NH), 7.84 (s, 1H, H_2) ppm. NMR-^{13}C (100 MHz, DMSO-d_6): δ36.1 (CH_2), 56.3 (CH), 57.7 (CH_3), 60.5 (CH_3), 104.6 (CH), 108.9 (C_c), 130.2 (C_c), 135.0 (C_c), 139.8 (C_c), 140.4 (C_c), 149.2 (C_c), 152.5 (C_c), 152.9 (C_c), 155.7 (CH), 162.0 (C_c), 162.6 (C_c) ppm. MS (IE, 70 eV) m/z (%): 482/480 [M^+] (66.1/96.3), 447/445 (38.8/100), 431/429 (7.6/21.9), 301 (68.2), 286 (45.1). Anal. Calcd. $C_{19}H_{18}Cl_2N_6O_3S$: C, 47.41; H, 3.77; N, 17.46; Found: C, 47.48; H, 3.85; N, 17.53.

4.2.7. 4-Amino-8-(2,4-dichlorothiazol-5-yl)-6-(3,4-methylenedioxyphenyl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4] diazepine (6g)

p.f. 261–262 °C; FT-IR (KBr), υ(cm^{-1}): 3471, 3288 and 3256 (−NH, 4-NH_2), 1675, 1581 and 1573 (C═N and C═C). NMR-^{1}H

Table 2
Inhibition percentages of **7a**–**g** against C. albicans ATCC 10231 and C. neoformans ATCC 32264 at the range of compound concentrations 250–3.9 μg/mL.

Concentrations	250 μg/mL	125 μg/mL	62.5 μg/mL	31.25 μg/mL	15.62 μg/mL	7.81 μg/mL	3.9 μg/mL
Compound	Inhibition percentages (%) against *C. albicans* ATCC 10231						
7a	100	100	100	100	62.03 ± 2.08	21.51 ± 1.94	4.92 ± 0.36
7b	100	100	100	100	100	48.31 ± 1.27	22.24 ± 0.80
7c	59.90 ± 1.35	51.72 ± 1.27	43.26 ± 1.59	21.33 ± 0.25	17.89 ± 0.23	0	0
7d	55.45 ± 0.67	21.31 ± 0.23	13.49 ± 1.09	8.70 ± 0.62	1.41 ± 0.99	0	0
7e	62.64 ± 0.64	54.60 ± 1.15	23.17 ± 1.08	21.22 ± 0.62	15.66 ± 0.89	12.45 ± 1.06	10.45 ± 0.62
7f	14.96 ± 1.31	10.53 ± 0.59	7.26 ± 0.53	9.84 ± 0.73	0	0	0
7g	56.75 ± 2.31	48.07 ± 0.19	41.58 ± 2.04	24.27 ± 1.41	0	0	0
	Inhibition percentages against *C. neoformans* ATCC 32264						
7a	100	74.31 ± 1.44	21.77 ± 1.08	7.0 ± 0.62	2.53 ± 0.13	0	0
7b	78.73 ± 2.12	58.21 ± 1.76	40.48 ± 1.49	30.53 ± 1.65	13.08 ± 1.09	3.56 ± 0.73	0
7c	25.48 ± 1.34	24.63 ± 1.63	12.81 ± 0.95	11.04 ± 0.05	7.17 ± 0.04	5.01 ± 0.30	3.83 ± 0.64
7d	59.09 ± 1.10	18.29 ± 1.14	9.20 ± 0.77	6.47 ± 1.45	4.57 ± 1.16	0.93 ± 0.46	0
7e	77.11 ± 1.18	46.88 ± 1.20	23.06 ± 0.81	13.15 ± 0.21	7.79 ± 0.12	6.04 ± 0.77	0
7f	77.53 ± 1.39	27.16 ± 1.08	9.28 ± 0.72	7.09 ± 0.57	5.52 ± 0.19	2.99 ± 0.03	1.65 ± 0.11
7g	58.47 ± 2.48	36.91 ± 1.99	19.53 ± 1.07	7.07 ± 0.44	5.35 ± 1.57	3.92 ± 0.21	0

(400 MHz, DMSO-d_6): δ 2.95 (d, J = 14.1 Hz, 1H, H_{7A}), 3.75 (dd, J = 14.1, 5.4 Hz, 1H, H_{7M}), 5.46 (s, 1H, H_{8X}), 6.07 (s, 2H, O–CH_2–O), 6.63 (s, 2H, 4-NH_2), 6.88 (d, J = 8.1 Hz, 1H, H_m), 7.20 (d, J = 8.1 Hz, 1H, H_o), 7.58 (s, 1H, –NH), 7.63 (d, J = 5.9 Hz, 1H, $H_{o'}$), 7.81 (s, 1H, H_2) ppm. NMR-^{13}C (100 MHz, DMSO-d_6): δ36.3 (CH_2), 56.4 (CH), 101.9 (CH_2), 107.1 (CH), 108.0 (CH), 108.6 (C_c), 121.8 (CH), 130.4 (C_c), 134.2 (C_c), 139.8 (C_c), 148.1 (C_c), 149.3 (C_c), 149.4 (C_c), 152.5 (C_c), 155.6 (CH), 161.5 (C_c), 162.2 (C_c) ppm. MS (IE, 70 eV) *m/z* (%): 436/434 [M^+] (54.5/83.1), 401/399 (45.1/100), 385/383 (8.9/22.3), 255 (30.0). Anal. Calcd. $C_{17}H_{12}Cl_2N_6O_2S$: C, 46.91; H, 2.78; N, 19.31; Found: C, 47.09; H, 2.99; N, 19.35.

4.2.8. 2,4-Diamino-8-(2,4-dichlorothiazol-5-yl)-6-phenyl-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (7a)

p.f. 172–173 °C; FT-IR (KBr), υ(cm^{-1}): 3468, 3444 and 3310 (–NH, 2-NH_2, 4-NH_2), 1551, 1579 and 1548 (C=N and C=C). NMR-^{1}H (400 MHz, $CDCl_3$): δ 2.89 (dd, J = 14.6, 1.8 Hz, 1H, H_{7A}), 3.81 (dd, J = 14.6, 5.5 Hz, 1H, H_{7M}), 5.31–5.42 (m, 1H, H_{8X}), 5.89 (s, 2H, 2-NH_2), 6.36 (s, 2H, 4-NH_2), 7.28–7.40 (m, 4H, –NH, H_m y H_p), 7.65–7.75 (m, 2H, H_o) ppm. NMR-^{13}C (100 MHz, $CDCl_3$): δ 36.7 (CH_2), 53.5 (CH), 102.3 (s), 126.4 (CH), 128.6 (CH), 129.6 (CH), 130.4 (C_c), 132.3 (C_c), 140.6 (C_c), 153.9 (CH), 154.7 (C_c), 160.8 (C_c), 163.8 (C_c), 167.4 (C_c) ppm.MS (IE, 70 eV) *m/z* (%): 407/405 [M^+] (4.1/25.3), 372/370 (7.1/17.1), 226 (56.5), 91 (100). Anal. Calcd. $C_{16}H_{13}Cl_2N_7S$: C, 47.30; H, 3.23; N, 24.13; Found: C, 47.59; H, 3.50; N, 24.32.

4.2.9. 6-(4-Chlorophenyl)-2,4-diamino-8-(2,4-dichlorothiazol-5-yl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (7b)

m.p 215–216 °C; FT-IR (KBr), υ(cm^{-1}): 3476, 3446 and 3311 (–NH, 2-NH_2, 4-NH_2), 1648, 1585 and 1549 (C=N and C=C). NMR-^{1}H (400 MHz, $CDCl_3$): δ 3.06 (dd, J = 14.6, 2.0 Hz, 1H, H_{7A}), 3.67 (dd, J = 14.6, 6.1 Hz, 1H, H_{7M}), 5.16 (s, 2H, 2-NH_2), 5.34 (d, J = 4.5 Hz, 1H, H_{8X}), 5.88 (s, 2H, 4-NH_2), 6.48 (s, 1H, –NH), 7.31 (d, J = 8.7 Hz, 2H, H_m), 7.60 (d, J = 8.7 Hz, 2H, H_o) ppm. NMR-^{13}C (100 MHz, $CDCl_3$): δ37.6 (CH_2), 53.0 (CH), 103.0 (C_c), 127.4 (CH), 128.6 (CH), 131.9 (C_c), 135.6 (C_c), 136.3 (C_c), 138.4 (C_c), 151.0 (C_c), 153.4 (C_c), 155.2 (C_c), 160.4 (C_c), 163.9 (C_c) ppm. MS (IE, 70 eV) *m/z* (%): 443/441/439 [M^+] (29/57/58), 406/404 (35.8/52.4), 370/368 (11.2/24.6), 262/260 (48.3/100). Anal. Calcd. $C_{16}H_{12}Cl_3N_7S$: C, 43.60; H, 2.74; N, 22.25; Found: C, 43.71; H, 2.85; N, 22.45.

4.2.10. 2,4-Diamino-8-(2,4-dichlorothiazol-5-yl)-6-(4-fluorophenyl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (7c)

m.p 214–215 °C; FT-IR (KBr), υ(cm^{-1}): 3475, 3446 and 3309 (–NH, 2-NH_2, 4-NH_2), 1651, 1587 and 1552 (C=N and C=C). NMR-^{1}H (400 MHz, $CDCl_3$): δ 3.06 (dd, J = 14.5, 1.9 Hz, 1H, H_{7A}), 3.68 (dd, J = 14.4, 5.7 Hz, 1H, H_{7M}), 5.20 (s, 2H, 2-NH_2), 5.34 (d, J = 3.9 Hz, 1H, H_{8X}), 5.89 (s, 2H, 4-NH_2), 6.53 (s, 1H, –NH), 6.98–7.08 (m, 2H, H_m), 7.65 (dd, J = 8.9 y J_{HF} = 5.3 Hz, 2H, H_o) ppm. NMR-^{13}C (100 MHz, $CDCl_3$): δ 37.6 (C), 53.2 (C_8), 103.7 (C_{4a}), 115.4 (d, $^2J_{CF}$ = 21.7 Hz, C_m), 128.1 (d, $^3J_{CF}$ = 8.4 Hz, C_o), 131.8 ($C_{4'}$), 136.2 (d, $^4J_{CF}$ = 3.1 Hz, C_i), 136.4 ($C_{5'}$), 150.9 (C_4), 153.3 (C_{9a}), 155.6 (C_2), 160.3 (C=N), 163.1 (d, $^1J_{CF}$ = 246.9 Hz), 164.9 ($C_{2'}$) ppm. MS (IE, 70 eV) *m/z* (%): 425/423 [M^+] (7.1/12.5), 390/388 (16.0/40.6), 352 (19.5), 244 (100). Anal. Calcd. $C_{16}H_{12}Cl_2FN_7S$: C, 45.29; H, 2.85; N, 23.11; Found: C, 45.36; H, 2.89; N, 23.21.

4.2.11. 2,4-Diamino-8-(2,4-dichlorothiazol-5-yl)-6-(p-tolyl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (7d)

m.p 217–218 °C; FT-IR (KBr), υ(cm^{-1}): 3471, 3439 and 3313 (–NH, 2-NH_2, 4-NH_2), 1641, 1588 and 1550 (C=N and C=C). NMR-^{1}H (400 MHz, $CDCl_3$): δ 2.36 (s, 3H, pCH_3) 3.06 (dd, J = 14.6, 2.0 Hz, 1H, H_{7A}), 3.69 (dd, J = 14.6, 6.1 Hz, 1H, H_{7M}), 5.16 (s, 2H, 2-NH_2), 5.35 (d, J = 4.3 Hz, 1H, H_{8X}), 5.89 (s, 2H, 4-NH_2), 6.48 (s, 1H, –NH), 7.14 (d, J = 8.2 Hz, 2H, H_m), 7.57 (d, J = 8.2 Hz, 2H, H_o) ppm. NMR-^{13}C (100 MHz, $CDCl_3$): δ 21.2 (CH_3), 37.5 (CH_2), 53.3 (CH), 103.2 (C_c), 126.2 (CH), 129.2 (CH), 131.8 (C_c), 136.7 (C_c), 137.3 (C_c), 138.7 (C_c), 150.8 (C_c), 153.3 (C_c), 156.8 (C_c), 160.2 (C_c), 163.8 (C_c) ppm. MS (IE, 70 eV) *m/z* (%): 421/419 [M^+] (19.1/54.0), 386/384 (10.8/31.4), 370/368 (6.1/15.9), 240 (100). Anal. Calcd. $C_{17}H_{15}Cl_2N_7S$: C, 48.58; H, 3.60; N, 23.33; Found: C, 48.83; H, 3.89; N, 23.57.

4.2.12. 2,4-Diamino-8-(2,4-dichlorothiazol-5-yl)-6-(4-methoxyphenyl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (7e)

m.p 202–203 °C; FT-IR (KBr), υ(cm^{-1}): 3460, 3433 and 3309 (–NH, 2-NH_2, 4-NH_2), 1648, 1584 and 1551 (C=N and C=C). NMR-^{1}H (400 MHz, $CDCl_3$): δ 3.06 (d, J = 13.3, 1H, H_{7A}), 3.69 (dd, J = 14.4, 6.0 Hz, 1H, H_{7M}), 3.83 (s, 3H, $pOCH_3$), 5.06 (s, 2H, 2-NH_2), 5.36 (s, 1H, H_{8X}), 5.82 (s, 2H, 4-NH_2), 6.31 (s, 1H, –NH), 6.86 (d, J = 8.8 Hz, 2H, H_m), 7.64 (d, J = 8.8 Hz, 2H, H_o) ppm. NMR-^{13}C (100 MHz, $CDCl_3$): δ37.1 (CH_2), 53.8 (CH), 55.3 (CH_3), 103.3 (C_c), 113.8 (CH), 127.8 (CH), 131.6 (C_c), 132.66 (C_c), 136.8 (C_c), 150.84 (C_c), 153.2 (C_c), 156.8 (C_c), 160.7 (C_c), 160.9 (C_c), 163.6 (C_c) ppm.MS (IE, 70 eV) *m/z* (%): 437/435 [M^+] (15.4/21.8), 401.90/399.95 (14.7/41.9), 386/384 (2.3/6.3), 269 (31.7), 256 (100). Anal. Calcd. $C_{17}H_{15}Cl_2N_7OS$: C, 46.80; H, 3.47; N, 22.47; Found: C, 47.09; H, 3.61; N, 22.46.

4.2.13. 2,4-Diamino-8-(2,4-dichlorothiazol-5-yl)-6-(3,4,5-tri methoxyphenyl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4]diazepine (7f)

m.p 223–224 °C; FT-IR (KBr), υ(cm^{-1}): 3476, 3439 and 3311 (–NH, 2-NH_2, 4-NH_2), 1661,1581 and 1556 (C=N and C=C).

Table 3
Minimum Inhibitory Concentrations (MIC_{100}, MIC_{80} and MIC_{50}) and Minima Fungicidal Concentrations (MFC) of **7a** and **7b** against clinical isolates of *C. neoformans*.

C. neoformans voucher spp	7a MICs in μg/mL				7b MICs in μg/mL				Amph. B
	MIC_{100}	MIC_{80}	MIC_{50}	MFC	MIC_{100}	MIC_{80}	MIC_{50}	MFC	MIC_{100}
ATCC 32264	31.2	31.2	15.6	31.2	15.6	15.62	7.8	31.2	0.12
IM 983040	31.2	31.2	7.8	62.5	31.2	31.2	31.2	62.5	0.25
IM 972724	31.2	31.2	15.6	31.2	15.6	15.6	7.8	31.2	0.25
IM 042074	31.2	31.2	15.6	62.5	31.2	31.2	15.6	62.5	0.12
IM 983036	31.2	31.2	15.6	31.2	31.2	15.6	15.6	31.2	0.25
IM 00319	62.5	31.2	31.2	62.5	15.6	15.6	7.8	31.2	0.50
IM 972751	62.5	31.2	31.2	62.5	62.5	31.2	31.2	62.5	1.00
IM 031631	62.5	31.2	31.2	62.5	31.2	15.6	7.8	31.2	0.12
IM 031706	62.5	31.2	31.2	62.5	62.5	31.2	7.8	62.5	0.50
IM 961951	31.2	31.2	15.6	31.2	15.6	15.6	7.8	62.5	1.00
IM 052470	62.5	31.2	7.8	62.5	62.5	31.25	15.6	62.5	0.50

MIC_{100}, MIC_{80} and MIC_{50}: concentration of a compound that caused 100, 80 or 50% reduction of the growth control, respectively. Within voucher specimen: IM: Malbrán Institute (Buenos Aires, Argentina). Amph B: amphotericin B.

NMR-^{1}H (400 MHz, $CDCl_3$): δ 3.06 (d, J = 14.4, 1H, H_{7A}), 3.70 (dd, J = 14.3, 5.9 Hz, 1H, H_{7M}), 3.89 (b.s, 9H, $pOCH_3$, $mOCH_3$), 4.89 (s, 2H, 2-NH_2), 5.42 (s, 1H, H_{8X}), 5.69 (s, 2H, 4-NH_2), 6.15 (s, 1H, −NH), 6.87 (s, 2H, H_o) ppm. NMR-^{13}C (100 MHz, $CDCl_3$): δ37.3 (CH_2), 54.7 (CH), 56.2 (CH_3), 60.9 (CH_3), 103.4 (C_c), 103.7 (CH), 131.8 (C_c), 135.5 (C_c), 137.2 (C_c), 139.8 (C_c), 150.8 (C_c), 153.0 (C_c), 153.3 (C_c), 157.3 (C_c), 160.2 (C_c), 163.5 (C_c) ppm. MS (IE, 70 eV) m/z (%): 497/495 [M^+] (63.4/100), 462/460 (11.1/28.2), 446/444 (7.7/20.5), 316 (55.6). Anal. Calcd. $C_{19}H_{19}Cl_2N_7O_3S$: C, 45.97; H, 3.86; N, 19.75; Found: C, 45.99; H, 3.97; N, 19.81.

4.2.14. 2,4-Diamino-8-(2,4-dichlorothiazol-5-yl)-6-(3,4-methylenedioxyphenyl)-8,9-dihydro-7H-pyrimido[4,5-b][1,4] diazepine (7g)

m.p 189–190 °C; FT-IR (KBr), υ(cm^{-1}): 3467, 3440 and 3313 (−NH, 2-NH_2, 4-NH_2), 1657, 1578 and 1552 (C=N and C=C). NMR-^{1}H(400 MHz, $CDCl_3$): δ 3.04 (d, J = 14.5, 1H, H_{7A}), 3.68 (dd, J = 14.5, 6.0 Hz, 1H, H_{7M}), 4.91 (s, 2H, 2-NH_2), 5.34 (d, J = 4.0 Hz, 1H, H_{8X}), 5.73 (s, 2H, 4-NH_2), 6.01 (s, 2H, O−CH_2−O) 6.13 (s, 1H, −NH), 6.76 (d, J = 8.2 Hz, 1H, H_m), 7.09 (d, J = 8.2 Hz, 1H, H_o), 7.33 (s, 1H, $H_{o'}$) ppm. NMR-^{13}C (100 MHz, $CDCl_3$): δ37.3 (CH_2), 53.6 (CH), 101.4 (CH_2), 103.0 (C_c), 106.4 (CH), 107.8 (CH), 120.8 (CH), 131.8 (C_c), 134.3 (C_c), 136.5 (C_c), 148.1 (C_c), 149.1 (C_c), 150.9 (C_c), 153.2 (C_c), 156.2 (C_c), 160.1 (C_c), 163.6 (C_c) ppm. MS (IE, 70 eV) m/z (%): 451/449 [M^+] (56.0/78.3), 416/414 (13.3/34.1), 400/398 (7.6/19.3), 283 (19.9), 270 (100). Anal. Calcd. $C_{17}H_{13}Cl_2N_7O_2S$: C, 45.34; H, 2.91; N, 21.77; Found: C, 45.57; H, 3.11; N, 21.79.

4.3. Microorganisms and media

For the antifungal evaluation, standardized strains from the American Type Culture Collection (ATCC), Rockville, MD, USA, *C. albicans* ATCC 10231 and *C. neoformans* ATCC 32264 were used. Clinical isolates of *C. neoformans* were provided by Malbrán Institute [(IM), Av. Velez Sarsfield 563, Buenos Aires]. The isolates included ten strains *C. neoformans* whose voucher specimens are presented in Table 3. 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 subcultured every 15 days to prevent pleomorphic transformations. Inocula were obtained according to reported procedures [51] and adjusted to 1–5 × 10^3 cells with colony forming units (CFU)/mL.

4.4. Fungal growth inhibition percentage determination

Broth microdilution techniques were performed in 96-well microplates according to the Clinical and Laboratory Standards Institute Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard M27-A3 [51]. For the assay, compound test wells (CTWs) were prepared with stock solutions of each compound in DMSO (maximum concentration ≤ 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 (SIGMA-ALDRICH, St Louis, MO, USA) 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, with SigmaPlot 11.0 software.

4.5. MIC_{100}, MIC_{80} and MIC_{50} determinations

Three endpoints were defined from the assay explained above and the dose–response curves. Minimum Inhibitory concentration (MIC) resulting in total fungal growth inhibition was named MIC_{100} while MIC_{80} and MIC_{50} were defined as the minimum concentration that inhibits 80 or 50% of the fungal growth respectively.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.01.053.

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