Article

Synthesis of New 4-Methyl-2-(4-pyridyl)-1,2,3,4-tetrahydroquinolines as Potent Antifungal Compounds

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A síntese, a caracterização espectroscópica e os resultados biológicos de novas séries de 2-(4-piridil)-1,2,3,4-tetrahidroquinolinas e seus precursores, -*N*-aril-*N*-[1-(4-piridil)but-3-enil] aminas, são descritos. Foi encontrado que ambos, precursores substituídos γ-piridil e os produtos finais, as tetrahidroquinolinas, demonstraram excelentes atividades antifúngicas contra *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Microsporum gypseun*, *Trichophyton rubrum* e *Trichophyton mentagrophytes*.

Synthesis, spectral characterization and biological results of new series of 2-(4-pyridyl)-1,2,3,4-tetrahydroquinolines and their closer precursors, -*N*-aryl-*N*-[1-(4-pyridyl)but-3-enyl] amines are reported. It was found that both γ-pyridyl substituted precursors and final products, tetrahydroquinolines, showed very good antifungal activities against *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger, Microsporum gypseun*, *Trichophyton rubrum* and *Trichophyton mentagrophytes*.

Keywords: homoallylamines, 2-(4-pyridyl)-1,2,3,4-tetrahydroquinolines, antifungal activity

Introduction

Both alicyclic and cyclic secondary amines are widely used throughout the chemical industry as basic intermediates to prepare fine chemicals, pharmaceuticals and agrochemicals.[1,2] Moreover, they are one of the most common structural features of naturally occurring biologically active compounds. Due to their unique biological properties, these compounds have played an important role in chemotherapeutic approaches to a variety of diseases, including fungal infectious diseases. These compounds serve as interesting pharmacological models in biomedicine studies. So, chemistry and biology of these compounds have received considerable attention from both the theoretical and practical points of view.

As a part of our ongoing drug discovery program we have been actively involved in determining the features that are important for antifungal activities of *N*-pyridylmethylanilines, and *N*-aryl-*N*-[1-(3-pyridyl)

but-3-enyl]amines ("homoallylamines"), easily accessible from aldimines. Both type of compounds displayed significant activity (MIC < 50 µg mL^{-1}) against some pathogenic dermatophytes.[3-6] We found also that some 4-methyl-1,2,3,4-tetrahydroquinolines are active against yeasts, hialohyphomycetes as well as dermatophytes. A point of connection between homoallylamines and tetrahydroquinolines, apart from their similar type of bioactivity, is that the first ones are suitable precursors for the corresponding 2,4-substituted 1,2,3,4-tetrahydroquinolines *via* acid-promoted intramolecular 6-exo-trig cyclization, that was described in 1994 by our team.[7,8] These facts encouraged us to develop other analogs of simple 4-methyltetrahydroquinoline derivatives with possible antifungal activity. Herein, we report the synthesis, spectral characterization and biological results of new series of 2-(4-pyridyl)-1,2,3,4-tetrahydroquinolines and their closer precursors, -*N*-aryl-*N*-[1-(4-pyridyl)but-3-enyl]amines that could be considered as interesting hits for antifungal drug development.

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Results and Discussion

The series of homoallylamines **6-10** were prepared using both standard Grignard and Barbier procedures[9] with prepared *in situ* allyl bromide magnesium in ether and with allyl bromide/indium/methanol system, respectively. Performing this later procedure, a mixture of allyl bromide (3 mmol), powder indio (1.5 mmol) and aldimines **1-5** (1 mmol) in dry MeOH was stirred at room temperature until the complete dissolution of metal, taking 1-4 h to obtain the desired products. Classical Grignard protocol consists in the addition of an ether solution of aldimines to preformed allyl magnesium bromide in ether that affords desired amine products. Thus, this nucleophilic addition was realized in ether (or THF) at 20 °C and, after stirring for 4 h, the reaction mixture was treated with saturated NH_4Cl solution. The organic products were extracted affording the homoallylamines **6-10** in 53-81% yields (Scheme 1, Table 1). It is worth noting that the use of ether is limited by the solubility of utilized aldimines.

Each of these procedures demonstrated some advantages and disadvantages: traditional Grignard protocol needs to use commercial, expensive organometallic reagent and dry solvents that are toxic and expensive, but magnesium is an easily available and inexpensive metal. Barbier protocol does not need to form organometallic reagent and dry solvents, but indium is still a very expensive metal to perform scale-up preparation of precursor compounds.

Homoallylamines **6-10** were easily characterized by IR (v_{NH} 3273 and 3293 cm^{-1}), ^{1}H-NMR, and Mass Spectra.

This later technique demonstrated that molecular ions were of low intensity (< 5%) and the principal fragmentation of these compounds were due to the lost of allyl radical [M-41]+, that generates ArNHCHPy+-cations.[10] ^{1}H-NMR spectra of homoallylamines **6-10** were very similar one each other, and were characterized by the presence of three groups of signals, which resonated in different zones (Table 2).

Preparation of desired 1,2,3,4-tetrahydroquinolines **11-14** consisted in the simple treatment of obtained homoallylamines mainly with sulfuric acid (85%). This mixture was heated at 80-90 °C during 1-5 h. Final products were isolated, and then purified by alumina column chromatography. In the case of homoallylamine **8**, PPA was used as mild cycling agent, because its treatment with sulfuric acid gave a complex mixture of inseparable products. It should be also noted that during the sulfuric acid catalysis of homoallylamine **10**, quinoline sulfonic acid **15** was formed as a sulfonation and oxidation product in considerable yields (Table 3). The structure of each final product was strongly confirmed by NMR (^{1}H, ^{13}C), IR and GC-MS data.

Analysing GC-MS and NMR data, it was noted that final products **11-14** presented a mixture of two diastereomers (*cis* and *trans*) from different spatial orientation of the substituents at C-2 and C-4 positions. GC-MS data indicated that the *cis*:*trans* ratio for comp. **11-14** were: 68:32; 79:21; 88:12 and 91:9, respectively. From NMR studies it was possible to assign *cis*-form for major isomer. This was corroborated by the protons 2-H, 3-H and 4-H coupling
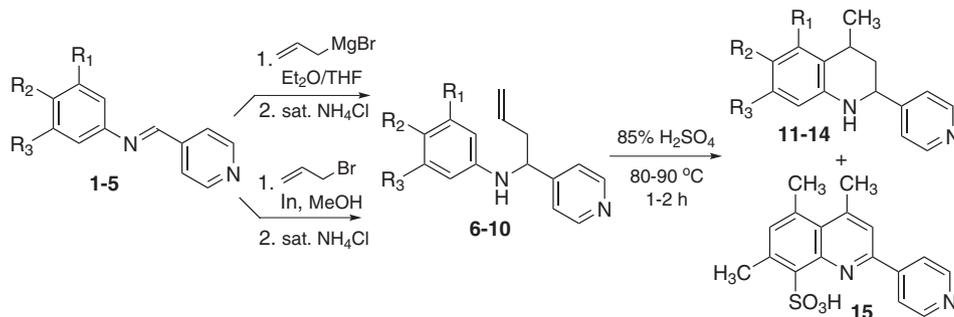
Scheme 1

Table 1. Physicochemical constants of homoallylamines **6-10**

| Comp.	R_1	R_2	R_3	Molecular formula	mp / °C	GC-MS		Yield / %[a]	
						t_R / min	M+, *m/z* (I, %)	Grignard protocol	Barbier protocol
6	H	H	H	$C_{15}H_{16}N_2$	oil	17.27	224 (3)	81	62
7	H	Me	H	$C_{16}H_{18}N_2$	95-96	19.27	238 (4)	73	nt[b]
8	H	OMe	H	$C_{16}H_{18}N_2O$	oil	21.32	254 (5)	60	nt[b]
9	H	Br	H	$C_{15}H_{15}BrN$	81-83	22.15	302 (5)	53	53
10	Me	H	Me	$C_{17}H_{20}N_2$	91-92	19.99	252 (3)	74	70

[a]isolated yield. [b]not tested.

Table 2. ¹H MNR data of 4-*N*-aryl-*N*-[1-(4-pyridyl)buten-3-yl]amines **6-10**

Comp.	Alkene part			Other		Py		Aryl		R
	CH₂=	=CH	-CH₂	-CH	N-H	2(6)-H$_{Py}$	3(5)-H$_{Py}$	2(6)-H$_{Ar}$	3(5)-H$_{Ar}$	
6	5.18-5.24 (m)	5.66-5.80 (m)	2.43-2.66 (m)	4.38 (dd) *J* 7.9, 5.0	4.19 (bs)	8.56 (dd) *J* 5.0, 1.6	7.31 (dd) *J* 4.4, 1.6	6.45 (d) *J* 8.5	7.10 (td) *J* 7.7, 1.0	4-H$_{Ar}$ 6.69 (t), *J* 7.3
7	5.18 (s) 5.21 (d) *J* 7.8	5.68-5.78 (m)	2.43-2.64 (m)	4.34 (dd) *J* 7.7, 5.0	4.09 (bs)	8.55 (dd) *J* 4.6, 1.4	7.31 (dd) *J* 4.7, 1.1	6.38 (d) *J* 8.4	6.91 (d) *J* 8.0	CH₃ 2.20 (s)
8	5.16 (s) 5.20 (d) *J* 6.0	5.67-5.77 (m)	2.41-2.61 (m)	4.29 (dd) *J* 8.0, 5.0	-	8.53 (dd) *J* 4.5, 1.5	7.29 (d) *J* 6.0	6.40 (d) *J* 8.9	6.68 (d) *J* 8.9	OCH₃ 3.68 (s)
9	5.17-5.23 (m)	5.63-5.78 (m)	2.42-2.67 (m)	4.34 (m)	4.19 (s)	8.55 (dd) *J* 4.4, 1.6	7.27 (dd) *J* 3.9, 1.7	6.31 (dd) *J* 8.8, 2.0	7.16 (dd) *J* 8.8, 2.0	-
10	5.20-5.15 (m)	5.65-5.76 (m)	2.41-2.62 (m)	4.35 (dd) *J* 7.7, 5.0	4.07 (s)	8.54 (dd) *J* 4.5, 1.5	7.29 (dd) *J* 4.7, 1.3	6.08 (s)	7.17 (td) *J* 7.4, 1.0	3(5)-CH₃ 2.15 (s) 4-H$_{Ar}$ 6.34 (s)

constants ($J_{2ax,3ax}$ 11.2-11.3 Hz, $J_{2ax,3eq}$ 2.3-2.9 Hz and $J_{4ax,3eq}$ 6.0-6.9 Hz, and $J_{4ax,3ax}$ 11.5-12.7 Hz), affirmation enough to indicate the equatorial-equatorial (*cis*) relationship to the case (Table 4). Figure 1 shows both diastereomeric forms. All our attempts in the separation of these diastereomers by gravity column chromatography failed in our hands, only the major diastereomer of comp. **13** was isolated and characterized as a pure *cis*-form.

The antifungal properties of compounds **6-14** were evaluated by the microbroth dilution method following the guidelines of the Clinical and Laboratory Standard Institute (CLSI, formerly National Committee for Clinical and Laboratory Standards (NCCLS) against a panel of ten fungal species: four human opportunistic pathogenic yeasts, three hialohyphomycetes and three dermatophytes. To carry out the antifungal evaluation, concentrations of compounds up to 250 µg mL⁻¹ were incorporated to
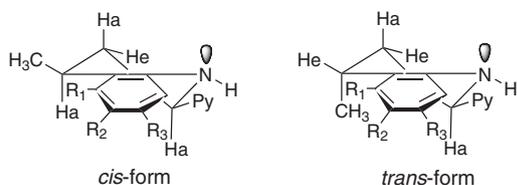
Figure 1. Spatial representation of *cis*-2eq,4eq and *trans*-2eq,4ax forms for the 2,4-disubstituted tetrahydroquinolines.

growth media according to published procedures.[11,12] The three most clinically used type of antifungal drugs were used as positive controls: the polyene Amphotericin B, the allylamine terbinafine and the azole ketoconazole. Biological results on fungal inhibition of the prepared molecules are summarized in Tables 5,6.

Regarding the activity displayed by homoallylamines **6-10** against the three most active fungal species, *M. gypseum*, *T. rubrum* and *T. mentagrophytes*, it appears that a substituent in the *p*-position produces an enhancement of the antifungal activity since 11 out the 12 MIC values of compounds **7-9** are lower than the corresponding MICs of compound **6**. In addition, the electronic properties of the *p*-substituents could play a role in the antifungal activity since compound **9** with a electron-withdrawing substituent (Br) was more active (MIC range = 16-31.2 µg mL⁻¹) than compounds **7** and **8**, which contain electron-donating groups (CH₃ and OCH₃) and their MICs range were 25-125 µg mL⁻¹. Another interesting observation is that methyl groups in both, the positions C-3 and C-5, do not significantly modify the activity (compare the activity of **6** with **10**).

Regarding tetrahydroquinolines, the high activity displayed by compound **11**, clearly showed that the closing of the ring dramatically enhanced the activity of the

Table 3. Physicochemical parameters of THQ **11-14** and quinoline **15**

Comp.	R₁	R₂	R₃	Molecular formula	mp / ºC	IR (v_{NH} / cm⁻¹)	GC-MS t$_R$ / min	M⁺, *m/z* (I / %)	Yield / %ᵃ
11	H	H	H	C₁₅H₁₆N₂	88-90	3266	20.06/20.29	224 (9)/244 (91)	84
12	H	Me	H	C₁₆H₁₈N₂	138-139	3299	20.78/21.84	238(10)/238(100)	72
13	H	OMe	H	C₁₆H₁₈N₂O	113-115	3267	22.74	254(100)	54
14	H	Br	H	C₁₅H₁₅BrN₂	104-106	3256	23.30/24.05	304(5)/304(97)	68
15	Me	H	Me	C₁₇H₁₆N₂O₃S	239-240	--	26.06	264(100)	56

ᵃisolated yield.

Table 4. [1]H MNR data of tetrahydroquinolines **11-14** and quinoline **15**

Comp.	Chemical shifts (δ/ppm, constants /Hz)												R
	Tetrahydroquinoline ring										Py		
	2-Hax	3-Hax	3-Heq	4-Hax	4-CH$_3$	N-H	5-H	6-H	7-H	8-H	2(6)-H$_{Py}$	3(5)-H$_{Py}$	
11	4.47 (dd) J 11.3, 2.8	1.70 (q) J 12.7	2.11 (ddd) J 12.9, 4.3, 3.0	3.13 (sp) J 6.0	1.34 (d) J 6.8	4.00 (s)	7.18 (d) J 7.7	6.74 (td) J 7.4, 0.9	7.03 (t) J 7.6	6.56 (dd) J 7.9, 0.9	7.35 (dd) J 4.7, 1.3	8.58 (dd) J 4.5, 1.5	-
12	4.43 (dd) J 11.3, 2.6	1.69 (q) J 11.6	2.10 (ddd) J 12.9, 5.3, 2.1	3.12 (sp) J 6.6	1.34 (d) J 6.8	3.86 (s)	7.00 (s)	-	6.86 (d) J 8.0	6.50 (d) J 8.0	7.35 (dd) J 4.5, 1.4	8.58 (dd) J 4.4, 1.6	6-CH$_3$ 2.26 (s)
13	4.40 (dd) J 11.2, 2.3	1.69 (q) J 11.6	2.11 (ddd) J 13.0, 5.5, 2.7	3.13 (sp) J 6.3	1.33 (d) J 6.8	-	6.79 (d) J 2.7	-	6.65 (dd) J 8.6, 2.8	6.56 (d) J 8.6	7.36 (dd) J 4.6, 1.6	8.58 (dd) J 4.6, 1.6	OCH$_3$ 3.76 (s)
14	4.46 (dd) J 11.2, 2.9	1.67 (q) J 11.5	2.09 (bt) J 13.1	3.10 (sp) J 6.4	1.33 (d) J 6.8	4.05 (bs)	7.27 (d) J 2.2	-	7.11 (dd) J 8.5, 2.3	6.45 (d) J 8.5	7.34 (dd) J 4.4, 1.6	8.59 (dd) J 4.5, 1.6	-
15	-	7.59 (s)		-	2.75 (s)		7.17 (s)	-	-	-	8.69 (dd) J 6.0, 1.0	7.93 (dd) J 5.2, 1.2	5-CH$_3$ 2.39 (s); 7-CH$_3$ 2.83 (s), O-H 9.17 (bs)

Table 5. Antifungal activity (MIC / µg mL^{-1}) of prepared molecules **6-14**

Comp.	Ca[1]	Ct[2]	Sc[3]	Cn[4]	Afu[5]	Afl[6]	An[7]	Mg[8]	Tr[9]	Tm[10]
6	250	250	125	250	250	250	250	125	250	62
7	>250	100	62	125	250	250	>250	25	50	50
8	>250	>250	>250	250	>250	>250	>250	62	125	125
9	>250	>250	250	>250	>250	>250	>250	16	16	32
10	250	250	250	>250	>250	>250	>250	125	250	125
11	125	125	125	125	32	32	32	8	8	8
12	>250	100	100	25	>250	>250	>250	25	50	25
13	125	125	125	125	32	125	250	62	62	62
14	>250	>250	>250	>250	>250	>250	>250	125	125	125
Amp.	1	0.5	0.5	0.25	0.5	0.5	0.5	-	-	-
Ket.	0.5	0.12	0.5	0.25	0.12	0.5	0.25	0.05	0.025	0.025
Terb.	-	-	-	-	-	-	-	0.04	0.01	0.04

[1]*Candida albicans* ATCC 10231; [2]*Candida tropicalis* C 131; [3]*Saccharomyces cerevisiae* ATCC 9763; [4]*Cryptococcus neoformans* ATCC 32264; [5]*Aspergillus fumigatus* ATCC 26934; [6]*Aspergillus flavus* ATCC 9170; [7]*Aspergillus niger* ATCC 9029; [8]*Microsporum gypseum* C 115; [9]*Trichophyton rubrum* C 110; [10]*Trichophyton mentagrophytes* ATCC 9972. Amp: Amphotericin B; Ket.: Ketoconazole; Terb.: Terbinafine.

unsubstituted homoallylamine **6**, although the same was not observed for substituted homoallylamines **7-9**. Compound **11** showed a broad spectrum of action inhibiting all fungi tested with MICs between 8 and 125 µg mL^{-1}. It was highly active against *Aspergillus* spp. (MIC = 31.2 µg mL^{-1}) and mainly against dermatophytes (MIC = 8 µg mL^{-1}).

The fact that tetrahydroquinoline molecule **11** inhibited *Aspergillus* spp. is highly interesting since it is worthwhile to take in account that previous homoallylamine-derived tetrahydroquinolines did not show activity against *Aspergillus* species up to 50 µg mL^{-1}.[3,4]

It is well known[13] that invasive aspergillosis has emerged as a leading cause of infection-related mortality among immunocompromised individuals, in particular among recipients of hematopoietic stem cell transplants or solid organ transplants. Historically, the drug of choice for the treatment of invasive aspergillosis has been amphotericin B, followed by the newer medications voriconazole and the recently approved caspofungin, which appeared more effective and with fewer side effects than the polyene. However, although the last drugs have advanced the management of these fungal infections, failure rates remain high, and emergence of intrinsically resistant fungi is a growing problem.[14] So, there is a general consensus that the discovery of new structures acting against *Aspergillus* spp. is highly necessary.

Regarding the activity of compounds **9** and **11** against dermatophytes, the most important findings are that these compounds display low MICs (16-32 and 8 µg mL^{-1}, respectively) against both, *T. rubrum* and *T. mentagrophytes*. It is worthwhile to take in account that approximately 80-93% of chronic and recurrent dermatophyte infections (tineas) are estimated to be caused by *Trichophyton spp.*[15] Although the treatment outcomes of these infections have now been greatly improved with the azole itraconazole or the allylamine terbinafine, there is unfortunately a considerable overlap of the strategies for treating the different tineas. This lack of specificity leads to chronic dermatophytoses or early re-infections after antifungal therapy and therefore, the superficial infections are very difficult to eradicate.[15]

The very low MIC displayed by compounds **9** and **11** against both *Trichophyton* spp. opens the possibility of using them as starting points for the development of new antifungal agents that selectively inhibit the most prevalent fungi in dermatomycoses. This hope is higher considering that both compounds not only inhibit but kill *Trichophyton* spp. (Minimum Fungicidal Concentration = 62 µg mL^{-1} for compound **9** and 32-62 µg mL^{-1} for compound **11**) (Table 6) adding one of the needed requirements for being good candidates for future development.

Table 6. MIC-100 and MFC (µg mL^{-1}) of compounds **9** and **11** against *M. gypseum*, *T. rubrum* y *T. mentagrophytes*

| Comp. | Activity / (µg mL^{-1}) | | |
| | *Mg*[1] | *Tr*[2] | *Tm*[3] |
	MIC$_{100}$/MFC	MIC$_{100}$/MFC	MIC$_{100}$/MFC
9	16/32	16/62	32/62
11	8/16	8/32	8/62

[1]*Microsporum gypseum* C 115; [2]*Trichophyton rubrum* C 110; [3]*Trichophyton mentagrophytes* ATCC 9972.

In summary, we found that both γ-pyridyl substituted precursors, homoallylamines, and final products, tetrahydroquinolines, showed very good antifungal activities against *A. fumigatus*, *A. flavus*, *A. niger* and the dermatophytes *M. gypseum*, *T. rubrum* and *T. mentagrophytes*. Among the compounds tested, the homoallylamine **9** and the tetrahydroquinoline **11** displayed the best activities against dermatophytes. Compound **11** showed also a very good activity against *Aspergillus spp*. In addition, **9** and **11** showed to be fungicide rather than fungistatic against dermatophytes, results that permit to consider them as good candidates for the further development of new antifungal agents.

Experimental

The melting points (uncorrected) were determined on a Fisher-Johns melting point apparatus. The IR spectra were recorded on a Lumex Infralum FT-02 spectrophotometer in KBr. ^{1}H NMR spectra were recorded on Bruker AM-400 spectrometer. Chemical shifts are reported in ppm (δ) relative to the solvent peak (CHCl$_3$ in CDCl$_3$ at 7.24 ppm for protons). Signals are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublets of doublets; t, triplet; td, triplet of doublets; q, quartet; sp, septet; m, multiplet; b, broad. On DEPT-135 spectra, the signals of CH$_3$, CH$_2$ and CH carbons are shown as positive (+) and negative (-), respectively. Quaternary carbons are not shown. A Hewlett Packard 5890a series II Gas Chromatograph interfaced to an HP 5972 Mass Selective Detector (MSD) with an HP MS Chemstation Data system was used for ms identification at 70 EV using a 60 m capillary column coated with HP-5 [5%-phenyl-poly(dimethyl-siloxane)]. Elemental analyses were performed on a Perkin Elmer 2400 Series II analyzer and were within ±0.4 of theoretical values. The reaction progress was monitored using thin layer chromatography on a silufol UV254 TLC aluminum sheet.

General procedure for the preparation of 4-N-aryl-N-[1-(4-pyridyl)buten-3-yl]amines

Grignard protocol

To an Et$_2$O solution (200 mL) of allyl magnesium bromide, prepared from allyl bromide (36.3 g, 0.30 mol) and magnesium (12.15 g, 0.50 mol), were added slowly aldimines **1-5** (0.10 mol), dissolved in absolute THF (50 mL). The mixture was kept during 4 h at room temperature, and then cooled to 0 °C to treat rapidly with water, and finally with saturated ammonium chloride solution. The organic layer was separated and the aqueous layer was extracted with ether (4 × 50 mL). The combined organic extracts were dried (Na$_2$SO$_4$) and concentrated. The residue was purified by Al$_2$O$_3$ column chromatography (eluent - hexanes/ethyl acetate) to give new homoallylamines **6-10**, those physicochemical characteristics are shown in Tables 1,2.

Barbier protocol

To a mixture of aldimines **1**, **4** and **5** (1 mmol) and powder indium (170 mg, 1.5 mmol) in methanol (10 mL) was added allyl bromide (365 mg, 3 mmol). The reaction mixture was stirred vigorously at room temperature until all the indium had dissolved (1 h to 4 h), at which time TLC indicated complete reaction. The reaction mixture was diluted with sat. NH$_4$Cl solution and extracted with

ethyl acetate. The extract was washed with brine and dried (Na_2SO_4). The product was purified by flash column chromatography on alumina, using hexanes/ethyl acetate as eluent. Physicochemical characteristics of the obtained homoallylamines **6**, **9** and **10** were in agreement with those obtained by Grignard method.

General procedure for the preparation of 4-methyl-2-(4-pyridyl)-1,2,3,4-tetrahydroquinolines

85% (v/v) sulfuric acid (4.0 mL) was added dropwise at 0 ℃ to the homoallylamines **6-10** (2.0 g) in minimal CH_2Cl_2 amount, and the resulting mixture was heated at 80-90 ℃ for 3 h while stirring vigorously. The reaction progress was monitored via TLC. At the end of the reaction the mixture was cooled down to room temperature and concentrated ammonium hydroxide solution was added to pH 10. Four 25 mL extractions with dichloromethane were performed. The organic layers were combined, dried (Na_2SO_4) and concentrated. The oily residue was purified by column chromatography over alumina to give new THQ **11-14** and quinoline sulfonic acid **15**. The synthesis of THQ **13** was realized using PPA. Physicochemical characteristics of the obtained compounds are given in Table 3.

Antifungal evaluation

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 en Micología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina were used: *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, A*spergillus flavus* ATCC 9170, *A. fumigatus* ATTC 26934, *A. niger* ATCC 9029, *Trichophyton rubrum* C 110, *T. mentagrophytes* ATCC 9972, and *M. gypseum* C 115.

Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 ℃, 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 ×10^3 cells/spores with colony forming units (CFU) mL^{-1}.[11,12]

Antifungal susceptibility testing

Minimal Inhibitory Concentration (MIC) of each compound was determined by using broth microdilution techniques according to the guideliness of the CLSI (formerly NCCLS) M27-A2[11] and for filamentous fungi M 38 A.[12] 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 ℃ for yeasts and hialohyphomycetes and at 28-30 ℃ 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.

Endpoints were defined as the lowest concentration of compound resulting in total inhibition (MIC_{100}) of visual growth compared to the growth control wells which contain culture medium and fungal inoculum but no antifungal drug.

For the assay, stock solutions of pure compounds were two-fold diluted with RPMI from 250-0.95 µg mL^{-1} (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 (Sigma Chem. Co.), Terbinafine (Novartis, Bs. As.) and Amphotericin B (Sigma Chem. Co.) were used as positive controls. All tests were made by duplicate.

The minimum fungicidal concentration (MFC) of each compound against each strain was determined as follows: After determining the MIC, an aliquot of 5 µL 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 MFC was recorded after 48 h. The MFC was defined as the lowest concentration of each compound that resulted in total inhibition of visible growth in the agarized plate.

Acknowledgments

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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