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Antifungal, cytotoxic and SAR studies of a series of *N*-alkyl, *N*-aryl and *N*-alkylphenyl-1,4-pyrrolediones and related compounds

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ABSTRACT

The synthesis, in vitro evaluation and SAR studies of 67 maleimides and derivatives acting as antifungal agents are reported. A detailed SAR study supported by theoretical calculations led us to determine that: an intact maleimido ring appears to be necessary for a strong antifungal activity, dissimilarly affected by the substituents in positions 2 and 3. The best activities were shown by 2,3-nonsubstituted followed by 2,3 dichloro- and 2-methyl-substituted maleimides. They all were fungicide rather than fungistatic enhancing the importance of their antifungal activity. 2,3-Dimethyl and 2,3-diphenyl-maleimides possessed marginal or null activity. The presence of a flexible connecting chain in *N*-phenylalkyl maleimides appears not to be essential for antifungal activity, although its length shows a correlation with the antifungal behavior, displaying maleimides with alkyl chains of $n = 3$ and $n = 4$ the best antifungal activities in most fungi. Different substituents on the benzene ring did not have a clear influence on the activity. Values of chemical potential properties as well as of energy do not sufficiently discriminate between active and inactive compounds. Nevertheless, it was found that, although log *P* alone is not strong enough to properly predict the antifungal activity, the comparison of its values for compounds within the same sub-type, showed an enhancement of antifungal activity along with an increment of lipophilicity. In addition, the LUMO's electronic clouds of the highly active compounds showed to be concentrated on the imido ring, indicating that their carbon atoms are potential sites for nucleophilic attack. Same results were obtained from MEPs. Most of the active compounds did not show cytotoxic activity against human cancer cell lines and no one possessed hemolytic activity, indicating that their activity is selective to pathogenic fungi and that they are not toxic at MIC concentrations.

1. Introduction

Fungal infections have emerged as a major cause of morbidity and often of mortality in immunocompromised and debilitated patients over the past two decades.[1] A matter of concern in the treatment of fungal infections is the limited number of efficacious antifungal drugs. Many of the currently available drugs are toxic, produce recurrence because they are fungistatic and not fungicide or lead to the development of resistance due in part to the prolonged periods of administration of the available antifungal drugs.[2] There is a clear need for the discovery of new structures which could be hits for the development of new antifungal drugs.

As part of our ongoing project on the detection of antifungal compounds, we recently reported[3] that non-substituted *N*-phenyl and *N*-phenylalkyl-1,4-pyrrolediones (common name: maleimides) **1**–**5** and 2,3-dichloro-derivatives **6**–**10**, with an alkyl chain length from $n = 0$ to 4 (Fig. 1 A and B), possess strong antifungal activities against a panel of clinically important fungi including yeasts, *Aspergillus* species and dermatophytes with minimum inhibitory concentrations (MICs) similar than some of the standard antifungal drugs.

Results obtained in that previous work showed that the antifungal activities of **1–5** were not dependent on the length of the connecting alkyl chain. In contrast, the antifungal behavior of **6–10** appeared to depend on the alkyl chain's length. From a conformational and electronic study of the last compounds, it was calculated that the optimum distance for the alkyl connecting chain was in the range 3.2–4.9 Å that corresponds to an alkyl chain of 3 carbon atoms.[3]

Unfortunately, this study[3] was performed with the agar dilution method which, as it is known, is a quantitative non-standardized test which could have generated not reproducible MICs.[4] In a recent paper, Cos et al.[5] stated that the use of a primary standardized

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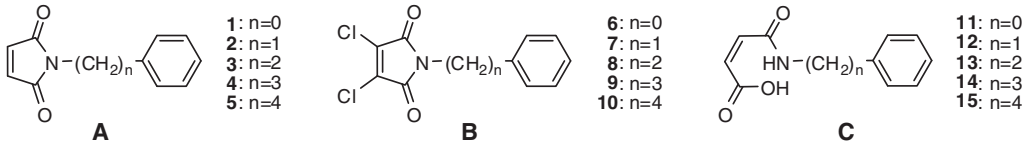


Figure 1. Structure of (A) *N*-phenyl and *N*-phenylalkyl-maleimides, (B) 2,3-dichloro-*N*-phenyl and *N*-phenylalkyl-maleimides and (C) *N*-phenyl and *N*-phenylalkyl-maleamic acids.

validated primary screening assay that generates confident results is essential to guarantee the reproducibility of the results. In this regard, the Clinical and Laboratory Standards Institute [CLSI, formerly National Committee for Clinical and Laboratory Standards (NCCLS)] established consensus' procedures to facilitate the agreement among laboratories in the measuring the antifungal susceptibility of yeasts[6] and filamentous fungi.[7] Parameters such as growth media, preparation and size of the inoculum, reference compounds, preparation of antifungal stock solutions, dilutions for testing, temperature and duration of incubation, end-point definitions, reference MIC ranges and quality controls were standardized in the CLSI documents.

So, in a further paper,[8] we re-evaluated maleimides **1–5** with the CLSI method only against *Candida* species and we corroborated that these compounds possess strong anticandidal activity, which was not dependent on the length of the alkyl chain. An important concern was clarified in that paper related to the stability of maleimides in the culture medium. Considering that in aqueous culture media, maleimide rings could undergo hydrolysis to the inactive maleamic acids **11–15** (Fig. 1C), we could demonstrate that **1–5** display antifungal activities with their intact maleimido ring and that they are not only fungistatic but fungicide with very low MICs and MFCs, killing the fungi in 90 min.

Taking into account the previous works,[3,8] maleimides appeared as excellent candidates for the development of antifungal drugs since they possess strong antifungal activities against important human opportunistic pathogenic fungi. Nevertheless, according to Barrett,[9] besides a strong activity, some other requirements are necessary to develop an antifungal agent. Among them, the possibility of performing chemical modifications on the structure is an important advantage to take into account when selecting an antifungal hit for developing a lead.

In that regard, we propose herein the syntheses and antifungal evaluation with a more ample fungal panel, with the CLSI methodologies, of an ample series of 67 maleimides and related compounds grouped in two main classes (Fig. 2): (I) compounds with an intact maleimido ring, in which the following variation of the substituents on positions 2,3 or modification of the N-substituents were performed: variation of the alkyl chain length in phenylalkyl derivatives, introduction of substituents on benzene ring of phenylalkylmaleimides and change of a phenylalkyl to an alkyl group; (II) compounds with a modified maleimido ring, in which breaking of the integrity of the maleimido ring, elimination of the double bond or reduction of one carbonyl group, were performed.

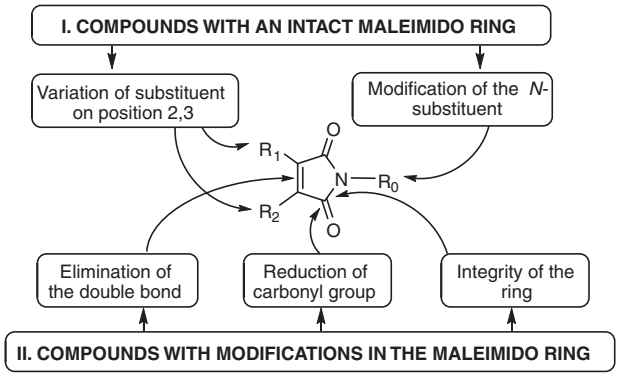


Figure 2. Types of synthesized compounds.

The 67 synthesized compounds include maleimides **1–10**, maleamic acids **11–15**, (of which **1–5** and **11–15** were tested previously with the CLSI methodology only against *Candida* spp. and **6–10** were tested only in agar dilution assays)[3,8] (Fig. 1) and other 52 derivatives **16–67** not tested previously as antifungal compounds, of which 17 are new ones. They were evaluated against a panel of 10 clinically important human pathogenic fungi including yeasts, *Aspergillus* spp. as well as dermatophytes. In addition we report a structure–activity relationship (SAR) study, including computational analyses which aid to determine the minimal structural requirements to produce the antifungal effect. The evaluation of the cytotoxic properties of the active compounds against a panel of three cellular lines was included in this study, too.

The study of the antifungal behavior of this ample series of maleimides and structural analogues against yeast and filamentous fungi seems appropriate for a more precise comprehension of the importance of maleimides and related compounds as antifungal hits.

2. Results and discussion

2.1. Chemistry

For the sake of clarity, the synthesized compounds were grouped according to their structural features (Fig. 3). Among compounds with an intact maleimido ring (I) the following structural types were synthesized, Type IA: 2,3-non-substituted *N*-phenyl and *N*-phenylalkyl-maleimides with a variable alkyl chain (n = 0–4) **1–5**; Type IB: 2,3-substituted *N*-phenyl and *N*-phenylalkyl-maleimides (**6–10**, **16–30**) (n = 0–4); Type IC: *N*-aryl-maleimides **31–43**; Type ID: *N*-alkyl-maleimides **44–47** (alkyl chain of 1 to 4 carbons).

Among compounds with modifications in the maleimido ring (II), Type IIA: *N*-phenyl and *N*-phenylalkyl-maleamic acids **11–15**; Type IIB: *N*-phenyl and *N*-phenylalkyl-succinimides **48–52** and Type IIC: *N*-phenyl and *N*-phenylalkyl-4-hydroxy-2,3-dehydro-γ-lactames **53–67** were prepared. Scheme 1 shows reactions and conditions for the obtainment of all synthesized compounds (**1–67**).[8,10]

2.1.1. Synthesis of type I-compounds

Reaction of maleic anhydrides **68–72** with aniline **73** or the appropriate phenylalkylamines **74–77** in $CHCl_3$ at room temperature generated the corresponding maleimides **1–10** (type IA) and **16–30** (type IB) via the corresponding maleamic acids (Scheme 1, plain arrows). Maleic anhydride **68** and substituted anilines **78–90** (Scheme 1, wavy arrows) or alkyamines **91–94** (Scheme 1, dashed arrows), produced *N*-aryl-maleimides **31–43** (type IC) and *N*-alkyl-maleimides **44–47** (type ID), respectively.

2.1.2. Synthesis of type II-compounds

N-Phenyl- and *N*-phenylalkyl-maleamic acids **11–15** (type IIA, Fig. 1) were obtained as intermediates of the synthesis of **1–5**. *N*-

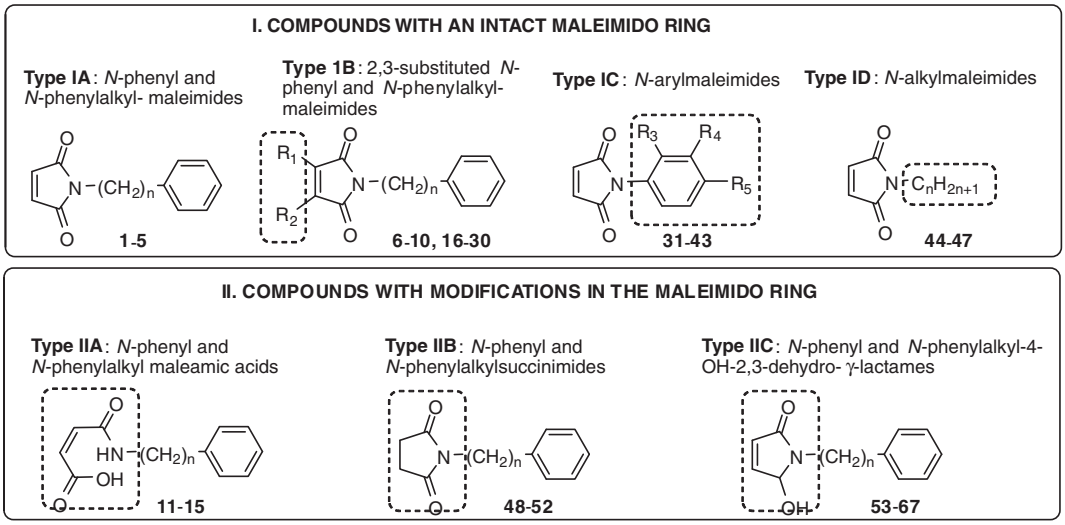


Figure 3. Structures of synthesized compounds.

Phenyl and *N*-phenylalkyl-succinimides **48–52** (type IIB) were synthesized utilizing the same procedure used for maleimides but with succinic anhydride **95** and aniline **73** (or phenylalkylamines **74–77**) as the starting material.

4-Hydroxy-2,3-dehydro-γ-lactames **53–57** (type IIC) were prepared from **1–5** by chemo-selective reduction with $NaBH_4$ in the presence of $CeCl_3{\cdot}7H_2O$, according to reported procedures (Scheme 1, bold arrows).[11] In turn, 4-hydroxy-3-methyl- and 4-hydroxy-2-methyl-2,3-dehydro-γ-lactames (**58–62** and **63–67**, respectively) were prepared by chemo- and regio-selective reduction of **16–20** with both, $NaBH_4$ and $NaBH_4$ plus $CeCl_3{\cdot}7H_2O$, respectively, according to reported procedures (Scheme 1, bold arrows).[11]

2.2. Biological activity

MICs were determined with the microbroth dilution methods M27-A3 and M38-A2 of CLSI,[6,7] against a panel of 10 fungal species comprising four yeasts (*Candida albicans*, *Candida tropicalis*, *Cryptococcus neoformans* and *Saccharomyces cerevisiae*), three *Aspergillus* spp. (*Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillus flavus*) and three dermatophytes (*Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum gypseum*. Compounds with MICs >250 μg/mL were considered inactive; MICs = 250 μg/mL, low active; between 62.5 and 125 μg/mL, moderately active and with MICs ⩽ 31.25 μg/mL, highly active. Particularly, structures displaying MICs ⩽ 10 μg/mL were considered of great interest for further development. Minimum fungicidal concentrations (MFCs) were accomplished by sub-culturing an aliquot from MIC wells showing no growth onto drug-free agar plates. Results are shown in Table 1.

2.3. Structure–activity relationships

(a) Influence of variation of the substituents on positions 2,3 (compounds **1–10**, **16–30**).

Results showed (Table 1) that compounds **1–5** (IA) displayed high antifungal activities against all yeasts (MICs = 0.97–15.63 ! μg/mL) and dermatophytes (MICs = 0.48–7.81 μg/mL) and different strengths of activities against all *Aspergillus* spp. (MICs = 7.81–250 μg/mL).[8] The introduction of substituents on positions 2,3 (IB, compounds **6–10**, **16–30**) produced a dissimilar influence on the antifungal activities, which appears to depend on the type of substituents introduced. In fact, the 2,3-dichloro maleimides **7–10** display strong antifungal effects against the whole panel, similar than those displayed by **1–5**. Regarding methylated derivatives, monomethylated maleimides **16–20** show a significant loss of activity respective the non-substituted analogues **1–5**. They possess moderate antifungal activity against yeasts and *Aspergillus* spp. (MICs = 15.63–125 μg/mL) although they maintain strong capacity for inhibiting dermatophytes (MICs = 1.95–31.25 μg/mL). Dimethyl derivatives **21–25** were almost inactive against yeasts and *Aspergillus* spp. and showed moderate to low activity against dermatophytes. In turn, diphenyl derivatives **26–30** were all inactive up to 250 μg/mL.

(b) Influence of the modification of the N-substituents.

(b1) Influence of the variation of the length of the alkyl chain.

The presence of a flexible alkyl chain in compounds **1–10**, **16–47** would not be essential for the antifungal effect, as it can be clearly seen in the activities displayed by compounds **1, 16** and **31–43**, which do not posses it.

In spite of this, it is observed that within the most active series, when the N-substituent did possess an alkyl chain (compounds **2–5**, **7–10**, **17–20**, **44–47**), its length appears to play a role in the activity, being three or four the optimum number of carbons, in most cases. So, among compounds **2–5**, the best activities were shown by compounds **4** ($n = 3$) and **5** ($n = 4$), although this difference was not observed against *C. albicans*.

Among the 2,3-dichloro derivatives **7–10**, maleimide **9** ($n = 3$) was the most active compound followed by **10**, coincident with the previously reported results.[3]

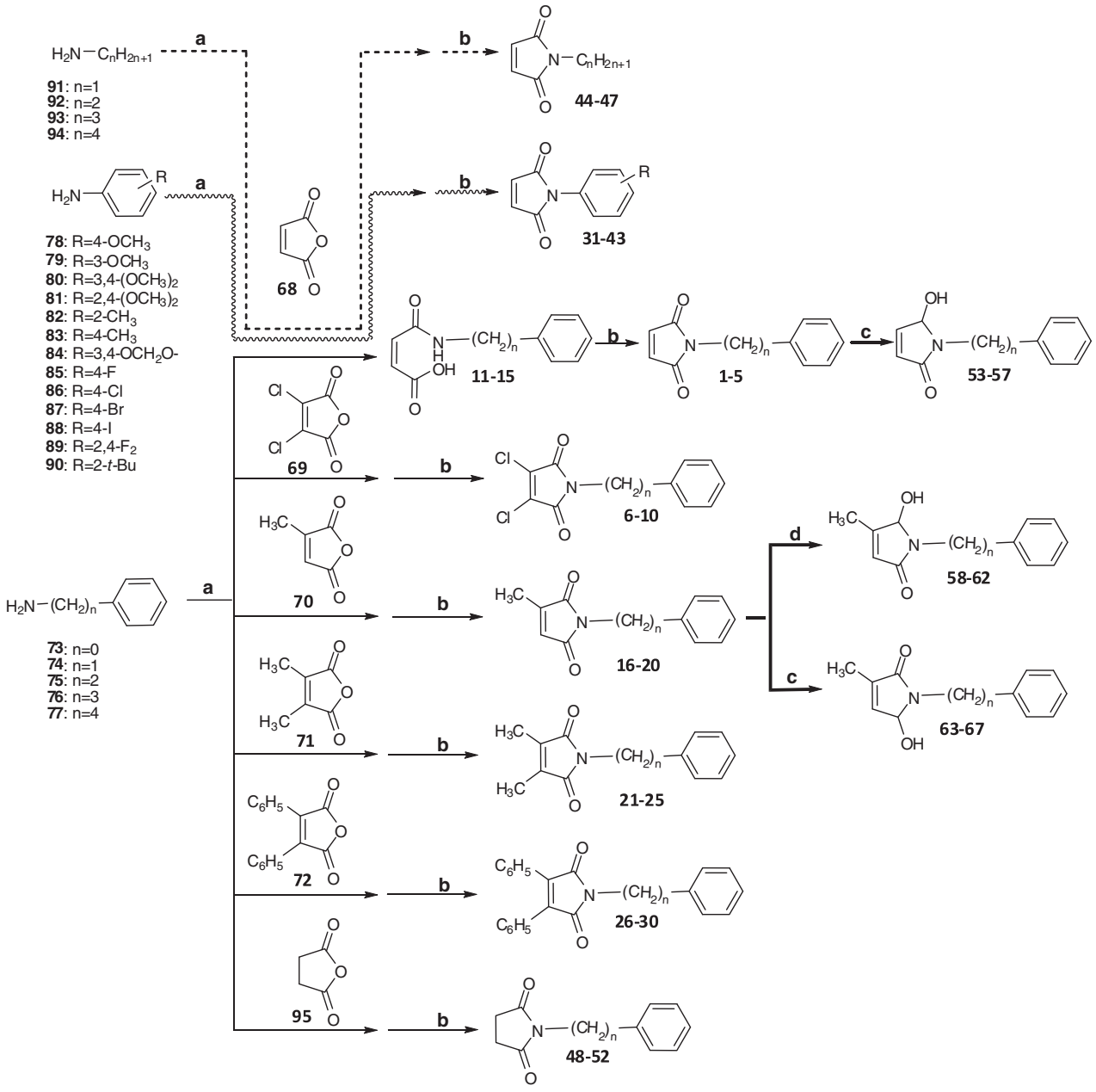
A similar relationship between length of the alkyl chain and antifungal activity was observed for *N*-butylmaleimide **47** ($n = 4$) that showed the lowest values of MICs among *N*-alkylmaleimides **44–47**.

(b2) Influence of the introduction of substituents on the benzene ring.

The comparison of the activities displayed by **1** with those of **31–43** showed that the presence of electron-withdrawing or electron-donor substituents, at any position of the phenyl ring does not exert a clear influence on the activity. The most striking result was the higher activity of **43** against *C. albicans* but this did not constitute a clear SAR neither.

(b3) Influence of the change of a phenylalkyl to an alkyl group (compounds 1D).

The activities displayed by these compounds demonstrated that the presence of a phenyl or an aryl moiety in the N-substituent was not essential to produce the biological effect.



Scheme 1. Reagents and conditions: (a) $CHCl_3$, 1 h, rt; (b) $(CH_3CO)_2O$, $NaOCOCH_3$, 1 h, reflux; (c) $NaBH_4$, $CeCl_3{\cdot}7H_2O$, CH_3OH, rt, 1 h; (d) $NaBH_4$, CH_3OH, rt, 1 h.

(II) Influence of the modification of the imido ring.

The antifungal activities of maleimides **1–5** (type IA) were compared to their open analogues (maleamic acids **11–15**, type IIA) and to their reduced analogues [succinimides **48–52** (type IIB) and 4-hydroxy-2,3-dehydro-γ-lactames **53–57** (type IIC)].

In contrast to type IA compounds **1–5**, type IIA maleamic acids **11–15** were inactive against *Candida* and *Aspergillus* spp. and *C. neoformans* and some of them displayed moderate to low activity against *S. cerevisiae* and dermatophytes. These results suggest that the intact imido ring would be necessary for the antifungal activity against the whole panel, in concordance with our previous findings for the same compounds against *Candida* spp.[8] In addition, the complete lack of activity of type IIB succinimides **48–52** (MIC >250 μg/mL) adds the data that a 2,3-unsaturated structure appears to be necessary for the activity.

The presence of both carbonyl groups appears to be important too, since the replacement of a carbonyl by a hydroxyl group in **1–5** gives 4-hydroxy-2,3-dehydro-γ-lactames **53–57** (type IIC) with MIC values from 31.25 to 250 μg/mL.

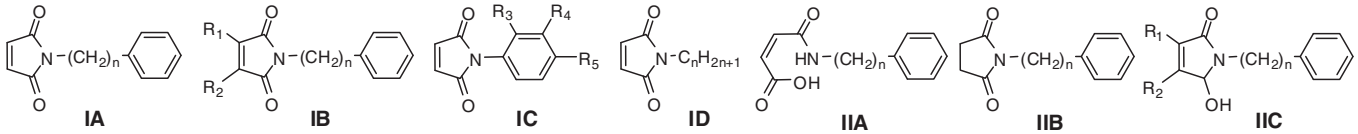
The methylation of **53–57** (type IIC) rendered two groups of regioisomers, the 3-methyl- and the 2-methyl-4-hydroxy-2,3-dehydro-γ-lactames, **58–62** and **63–68**, respectively which displayed marginal or null antifungal activity without differences between regioisomers, but with a clear decrease of activity respective the non-methylated analogues **53–57**.

At this stage of our SAR study, some general trends can be established:

(i) An intact maleimido ring appears to be necessary for a strong antifungal activity, dissimilarly affected by the substituents in positions 2 and 3.

(ii) Although the presence of a flexible connecting chain in *N*-phenylalkyl maleimides **1–10**, **16–20**, **44–47** appears not to be essential for antifungal activity, its length shows a

Table 1
Minimum inhibitory concentrations (MIC, in μg/mL)/minimum fungicidal concentrations (MFC, in μg/mL) of synthesized compounds



	Type	R_1	R_2	R_3	R_4	R_5	*n*	*Ca*	*Ct*	*Sc*	*Cn*	*Afu*	*Afl*	*An*	*Mg*	*Tr*	*Tm*
1	IA	—	—	—	—	—	0	3.90/ 7.81[a]	7.81/ 15.63[a]	3.90/ 7.81	15.63/ 62.5	250/ >250	62.5/ 125	31.25/ 62.5	7.81/ 15.63	3.90/ 7.81	7.81/ 7.81
2	IA	—	—	—	—	—	1	3.90/ 7.81[a]	15.63/ 32.25[a]	7.81/ 15.63	7.81/ 15.63	125/ 250	62.5/ 125	31.25/ 62.5	3.90/ 7.81	3.90/ 7.81	3.90/ 7.81
3	IA	—	—	—	—	—	2	3.90/ 7.81[a]	15.63/ 32.25[a]	3.90/ 7.81	7.81/ 15.63	31.25/ 31.25	31.25/ 31.25	15.63/ 15.63	3.90/ 7.81	1.95/ 3.90	7.81/ 15.63
4	IA	—	—	—	—	—	3	3.90/ 7.81[a]	3.90/ 15.63[a]	0.97/ 1.95	15.63/ 31.25	15.63/ 31.25	31.25/ 62.5	15.63/ 15.63	0.97/ 1.95	1.95/ 1.95	3.90/ 7.81
5	IA	—	—	—	—	—	4	3.90/ 7.81[a]	7.81/ 15.63[a]	0.97/ 1.95	15.63/ 31.25	15.63/ 31.25	7.81/ 15.63	7.81/ 7.81	0.97/ 1.95	0.48/ 0.97	3.90/ 7.81
6	IB	Cl	Cl	—	—	—	0	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
7	IB	Cl	Cl	—	—	—	1	15.63/ 31.25	31.25/ 31.25	7.81/ 15.63	7.81/ 15.63	15.63/ 31.25	31.25/ 31.25	31.25/ 62.5	15.63/ 31.25	7.81/ 15.63	7.81/ 15.63
8	IB	Cl	Cl	—	—	—	2	7.81/ 15.63	15.63/ 31.25	3.90/ 7.81	7.81/ 7.81	15.63/ 15.63	15.63/ 15.63	15.63/ 31.25	15.63/ 31.25	3.90/ 7.81	7.81/ 15.63
9	IB	Cl	Cl	—	—	—	3	7.81/ 15.63	7.81/ 15.63	3.90/ 3.90	3.90/ 3.90	15.63/ 31.25	15.63/ 31.25	15.63/ 15.63	15.63/ 15.63	3.90/ 7.81	3.90/ 7.81
10	IB	Cl	Cl	—	—	—	4	7.81/ 15.63	7.81/ 15.63	3.90/ 7.81	3.90/ 3.90	31.25/ 62.5	15.63/ 31.25	15.63/ 15.63	15.63/ 31.25	3.90/ 7.81	3.90/ 7.81
16	IB	CH_3	H	—	—	—	0	62.5/ 125	62.5/ 125	31.25/ 125	31.25/ 62.5	31.25/ 62.5	62.5/ 125	125/ 250	7.81/ 15.63	7.81/ 15.63	31.25/ 62.5
17	IB	CH_3	H	—	—	—	1	62.5/ 125	125/250	31.25/ 62.5	15.63/ 31.25	31.25/ 62.5	62.5/ 125	62.5/ 125	3.90/ 7.81	3.90/ 7.81	31.25/ 31.25
18	IB	CH_3	H	—	—	—	2	62.5/ 125	125/250	31.25/ 62.5	31.25/ 62.5	31.25/ 31.25	62.5/ 250	62.5/ 250	3.90/ 7.81	7.81/ 15.63	31.25/ 62.5
19	IB	CH_3	H	—	—	—	3	62.5/ 125	125/125	15.63/ 62.5	15.63/ 31.25	15.63/ 31.25	31.25/ 31.25	31.25/ 31.25	3.90/ 7.81	1.95/ 3.90	15.63/ 31.25
20	IB	CH_3	H	—	—	—	4	62.5/ 125	62.5/ 125	31.25/ 62.5	31.25/ 62.5	31.25/ 62.5	62.5/ 125	62.5/ 125	3.90/ 15.63	3.90/ 15.63	31.25/ 31.25
21	IB	CH_3	CH_3	—	—	—	0	*i*	*i*	*i*	125/ 250	*i*	*i*	*i*	250/ >250	62.5/ 125	125/ 250
22	IB	CH_3	CH_3	—	—	—	1	125/ >250	125/ >250	250/ >250	125/ 250	*i*	*i*	*i*	125/ 250	62.5/ 125	125/ 250
23	IB	CH_3	CH_3	—	—	—	2	*i*	*i*	*i*	250/ >250	*i*	*i*	*i*	250/ >250	62.5/ 250	250/ >250
24	IB	CH_3	CH_3	—	—	—	3	*i*	*i*	*i*	125/ 250	*i*	*i*	*i*	125/ 250	62.5/ 250	250/ >250
25	IB	CH_3	CH_3	—	—	—	4	*i*	*i*	*i*	*i*	*i*	*i*	*i*	250/ >250	125/ 250	125/ >250
26	IB	C_6H_5	C_6H_5	—	—	—	0	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
27	IB	C_6H_5	C_6H_5	—	—	—	1	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
28	IB	C_6H_5	C_6H_5	—	—	—	2	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
29	IB	C_6H_5	C_6H_5	—	—	—	3	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
30	IB	C_6H_5	C_6H_5	—	—	—	4	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
31	IC	—	—	H	H	OCH_3	0	62.5/ 125	62.5/ 125	31.25/ 62.5	15.63/ 15.63	62.5/ 125	62.5/ 125	31.25/ 62.5	3.90/ 7.81	3.90/ 3.90	3.90/ 7.81
32	IC	—	—	H	OCH_3	H	0	15.63/ 62.5	15.63/ 62.5	3.90/ 7.81	3.90/ 7.81	31.25/ 62.5	31.25/ 62.5	31.25/ 62.5	3.90/ 7.81	1.95/ 3.90	1.95/ 3.90
33	IC	—	—	H	OCH_3	OCH_3	0	31.25/ 62.5	15.63/ 15.63	7.81/ 15.63	7.81/ 15.63	62.5/ 125	62.5/ 125	62.5/ 125	3.90/ 7.81	3.90/ 7.81	3.90/ 7.81
34	IC	—	—	OCH_3	H	OCH_3	0	15.63/ 15.63	15.63/ 15.63	7.81/ 7.81	7.81/ 7.81	31.25/ 62.5	62.5/ 125	15.63/ 62.5	3.90/ 7.81	3.90/ 7.81	3.90/ 3.90
35	IC	—	—	CH_3	H	H	0	15.63/ 15.63	15.63/ 31.25	7.81/ 7.81	7.81/ 7.81	62.5/ 125	62.5/ 62.5	15.63/ 62.5	3.90/ 7.81	1.95/ 3.90	1.95/ 3.90
36	IC	—	—	H	H	CH_3	0	15.63/ 31.25	15.63/ 31.25	3.90/ 7.81	3.90/ 7.81	31.25/ 62.5	15.63/ 15.63	15.63/ 31.25	1.95/ 3.90	1.95/ 3.90	1.95/ 7.81
37	IC	—	—	H	OCH_2O		0	31.25/ 62.5	31.25/ 62.5	7.81/ 15.63	7.81/ 15.63	62.5/ 125	62.5/ 125	62.5/ 125	1.95/ 1.95	1.95/ 3.90	1.95/ 3.90
38	IC	—	—	H	H	F	0	15.63/ 15.63	15.63/ 15.63	3.90/ 7.81	7.81/ 15.63	15.63/ 31.25	15.63/ 15.63	7.81/ 7.81	3.90/ 7.81	3.90/ 7.81	3.90/ 7.81
39	IC	—	—	H	H	Cl	0	62.5/ 62.5	7.811/ 15.63	3.90/ 7.81	3.90/ 7.81	62.5/ 125	62.5/ 125	31.25/ 62.5	3.90/ 7.81	3.90/ 7.81	3.90/ 7.81
40	IC	—	—	H	H	Br	0	15.63/ 31.25	15.63/ 62.5	7.81/ 15.63	7.81/ 15.63	31.25/ 62.5	31.25/ 62.5	31.25/ 62.5	3.90/ 3.90	1.95/ 3.90	1.95/ 3.90

(*continued on next page*)

Table 1 (*continued*)

	Type	R_1	R_2	R_3	R_4	R_5	*n*	*Ca*	*Ct*	*Sc*	*Cn*	*Afu*	*Afl*	*An*	*Mg*	*Tr*	*Tm*
41	IC	—	—	H	H	I	0	31.25/62.5	31.25/62.5	7.81/7.81	7.81/15.63	62.5/125	62.5/125	62.5/125	3.90/7.81	3.90/7.81	3.90/7.81
42	IC	—	—	F	H	F	0	15.63/31.25	15.63/31.25	7.81/15.63	7.81/7.81	31.25/31.25	62.5/62.5	15.63/31.25	3.90/7.81	3.90/7.81	3.90/7.81
43	IC	—	—	*t*-Bu	H	H	0	3.90/3.90	3.90/3.90	3.90/7.81	3.90/7.81	15.63/31.25	62.5/62.5	62.5/125	1.95/3.90	1.95/3.90	1.95/3.90
44	ID	—	—	—	—	—	1	62.5/125	31.25/62.5	7.81/15.63	15.63/62.5	15.63/62.5	15.63/31.25	7.81/15.63	7.81/7.81	3.90/15.63	7.81/15.63
45	ID	—	—	—	—	—	2	62.5/250	62.5/125	7.81/15.63	15.63/31.25	15.63/31.25	15.63/15.63	7.81/15.63	7.81/7.81	3.90/3.90	7.81/15.63
46	ID	—	—	—	—	—	3	62.5/125	62.5/125	15.63/31.25	7.81/15.63	15.63/31.25	15.63/15.63	15.63/15.63	3.90/7.81	3.90/7.81	7.81/7.81
47	ID	—	—	—	—	—	4	3.90/15.63	3.90/3.90	1.95/1.95	3.90/7.81	1.95/1.95	1.95/7.81	3.90/3.90	3.90/3.90	1.95/1.95	1.95/3.90
11	IIA	—	—	—	—	—	0	*i*[a]	*i*[a]	125/>250	*i*	*i*	*i*	*i*	125/>250	62.5/250	250/>250
12	IIA	—	—	—	—	—	1	*i*[a]	*i*[a]	250/>250	*i*	*i*	*i*	*i*	*i*	*i*	*i*
13	IIA	—	—	—	—	—	2	*i*[a]	*i*[a]	*i*	*i*	*i*	*i*	*i*	250/>250	250/>250	*i*
14	IIA	—	—	—	—	—	3	*i*[a]	*i*[a]	*i*	*i*	*i*	*i*	*i*	250/>250	250/>250	*i*
15	IIA	—	—	—	—	—	4	*i*[a]	*i*[a]	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
48	IIB	—	—	—	—	—	0	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
49	IIB	—	—	—	—	—	1	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
50	IIB	—	—	—	—	—	2	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
51	IIB	—	—	—	—	—	3	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
52	IIB	—	—	—	—	—	4	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
53	IIC	H	H	—	—	—	0	*i*	*i*	*i*	250/>250	*i*	*i*	*i*	*i*	*i*	*i*
54	IIC	H	H	—	—	—	1	*i*	*i*	*i*	250/>250	*i*	*i*	*i*	*i*	*i*	*i*
55	IIC	H	H	—	—	—	2	62.5/125	250/>250	250/>250	62.5/>250	*i*	*i*	*i*	62.5/125	62.5/125	31.25/62.5
56	IIC	H	H	—	—	—	3	62.5/>250	125/250	62.5/125	62.5/>250	125/250	250/>250	*i*	62.5/125	62.5/250	31.52/62.5
57	IIC	H	H	—	—	—	4	*i*	*i*	*i*	250/>250	*i*	*i*	*i*	125/250	62.5/250	62.5/125
58	IIC	H	CH_3	—	—	—	0	*i*	*i*	*i*	250/>250	*i*	*i*	*i*	*i*	*i*	*i*
59	IIC	H	CH_3	—	—	—	1	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
60	IIC	H	CH_3	—	—	—	2	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
61	IIC	H	CH_3	—	—	—	3	*i*	*i*	*i*	250/>250	*i*	*i*	*i*	250/>250	250/>250	250/>250
62	IIC	H	CH_3	—	—	—	4	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
63	IIC	CH_3	H	—	—	—	0	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
64	IIC	CH_3	H	—	—	—	1	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
65	IIC	CH_3	H	—	—	—	2	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
66	IIC	CH_3	H	—	—	—	3	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
67	IIC	CH_3	H	—	—	—	4	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*	*i*
AMP								0.97/1.95	0.48/0.97	0.48/1.95	0.24/0.97	0.48/0.97	0.48/1.95	0.48/3.90	0.12/0.48	0.06/0.24	0.06/0.24
KTZ								0.48/1.95	0.12/0.97	0.48/0.97	0.24/1.95	0.12/3.90	0.48/3.90	0.24/1.95	0.06/0.24	0.03/0.12	0.03/0.12
TBF								—	—	—	—	—	—	—	0.03/0.06	0.01/0.03	0.03/0.06

Ca: *Candida albicans* ATCC 10213, *Ct*: *C. tropicalis* C 131 2000, *Sc*: *Saccharomyces cerevisiae* ATCC 9763, *Cn: Cryptococcus neoformans* ATCC 32264, *Afu: Aspergillus fumigatus* ATCC 26934, *Afl: A. flavus* ATCC 9170, *An: A. niger* ATCC 9029, *Mg: Microsporum gypseum* C 115 2000, *Tr: Trichophyton rubrum* C 113 2000, *Tm: T. mentagrophytes* ATCC 9972. C = CEREMIC (Centro de Referencia en Micología). ATCC: American Type Culture Collection AMP: Amphothericine B, KTZ: Ketoconazole, TBF: Terbinafine. *i*: inactive (>250 μg/mL).

[a] Results published in Ref.[8].

correlation with the antifungal behavior, showing alkyl chains of $n = 3$ and $n = 4$ the best antifungal activities in most fungi.

(iii) Results obtained for compounds possessing different substituents on the benzene ring showed that they would not have a clear influence in the activity. Nevertheless, **43** showed the highest antifungal activity of *N*-aryl-maleimides.

(iv) Considering that *C. albicans* produces the fourth most prevalent blood stream infection in immunocompromised hosts, representing more than 60% of isolates from clinical infections,[13] it is important to highlight that out of the 67 compounds tested, 2,3-non-substituted maleimides **1–5**, **43** and **47** showed the best antifungal activities against this clinically important fungus.

As an additional information to the SAR studies, it is important to take into account that MFC values of all active compounds were one or two dilutions higher than their respective MICs. MFC has

been pointed out as being more relevant than MIC to therapeutic outcome.[12]

2.4. Computational studies

To better understand the above experimental results, we performed both a conformational and an electronic study on one representative compound of each type reported here, which could allow discriminating between active and inactive structures. The purpose was to obtain more precise information on how closely these compounds resemble each other in terms of the spatial orientations of the essential moieties to produce the antifungal activity.

Regarding conformational studies, it was necessary at first to define the three-dimensional geometry of the relevant conformation of each molecule, which could be either that of the lowest-energy or the topographically congruent with any other active compound.

Once obtained the relevant conformations for the different compounds, we evaluated the electronic and hydrophobic aspects of the molecules, by using different electronic parameters as well as molecular electrostatic potentials (MEPs).

Theoretical calculations obtained for the representative compounds **1**, **2**, **5**, **7**, **12**, **17**, **22**, **27**, **31**, **36**, **38**, **42–47**, **49**, **54**, **59** and **64** are considered descriptive of the whole series and are shown in Table 2.

Thus, the first objective of the calculations was to study the chemical potential properties (CPP) of each selected compound that are defined by different variables tightly related among them: electron affinity (EA), ionization potential (IP), hardness (η), electronegativity (χ) and electrophilicity (ω). Such variables have different meanings but nevertheless, as a whole, they measure the tendency of a group to release or capture electrons; in fact, they constitute an index of potential chemical reactivity.[14] In addition, the energy of neutral and charged molecules (+1 and −1) are recorded in Table 2.

Results shown in Table 2 indicate that values of CPP as well as energy values do not sufficiently discriminate between active and inactive compounds.

So, other parameters such as dipolar moment and log *P* were calculated for all representative compounds. Results are showed in Table 3.

In general, the low-active or inactive compounds **12**, **22**, **27**, **49**, **54**, **59** and **64** showed relatively higher values of μ (range 1.90–4.75 D) than active compounds (**1, 2, 5, 7, 17, 31, 36, 38, 42–47**) (0.90–1.72 D) (table 4). The active compound **42** with $\mu = 2.11$ D is an exception.

Table 3
Dipolar moments and log *P* for representative compounds

Compound	μ (dipolar moment) (Debye)	log *P*
1	1.26	1.61
2	1.15	2.04
5	1.13	3.22
7	1.25	3.21
12	2.88	1.39
17	1.72	2.53
22	1.90	3.04
27	2.24	6.01
31	1.14	1.63
36	0.90	0.89
38	1.03	1.30
42	2.11	1.46
43	0.90	3.25
44	1.12	−0.52
45	1.03	−0.18
46	1.02	0.30
47[a]	0.91	1.31
47[b]	1.13	1.31
49	1.96	2.04
54	4.16	2.04
59	4.06	1.61
64	4.75	1.79

[a] Conformation extended.
[b] Conformation folded.

Table 2
Chemical potential properties and energy properties for representative compounds

Compd	Electro affinity EA = $E(0) - E(-1)$ (eV)	Ionization potential IP = $E(+1) - E(0)$ (eV)	Hardness $\eta = (I - A)/2$ (eV)	Electro negativity $\chi = (I + A)/2$ (eV)	Electro-philicity $\omega = \mu 2/2\eta$ (eV)	Energy of neutral molecule (0) (Hartree)	Energy of charged molecule (+1) (Hartree)	Energy of charged molecule (−1) (Hartree)
1	0.61	8.37	3.88	4.49	0.20	−590.48	−590.17	−590.50
2	0.46	8.77	4.15	4.61	0.16	−629.80	−629.47	−629.81
5	0.40	8.23	3.92	4.32	0.16	−747.74	−747.44	−747.75
7	1.02	8.66	3.82	4.84	0.20	−1548.98	−1548.66	−1549.02
12	0.03	8.43	4.20	4.23	0.99	−706.22	−705.91	−706.22
17	0.32	8.51	4.10	4.41	0.36	−669.12	−668.81	−669.13
22	0.19	8.49	4.15	4.34	0.44	−708.44	−708.13	−708.45
27	0.22	7.68	3.73	3.95	0.11	−819.52	−819.24	−819.53
31	0.54	8.46	3.96	4.49	0.16	−629.80	−629.49	−629.82
36	1.03	7.52	3.24	4.28	0.76	−1091.92	−1091.64	−1091.95
38	0.56	8.61	4.03	4.58	0.13	−689.71	−689.39	−689.73
42	0.65	8.64	4.00	4.64	0.56	−788.94	−788.62	−788.96
43	0.55	8.50	3.97	4.52	0.10	−747.72	−747.41	−747.74
44	0.32	9.80	4.74	5.06	0.13	−398.74	−398.38	−398.76
45	0.34	9.62	4.64	4.98	0.11	−438.06	−437.71	−438.07
46	0.36	9.50	4.57	4.93	0.11	−477.37	−477.03	−477.39
47[a]	0.35	9.39	4.52	4.93	0.14	−516.69	−516.34	−516.70
47[b]	0.41	9.45	4.52	4.87	0.09	−516.69	−516.34	−516.70
49	−1.31	8.61	4.96	3.65	0.25	−631.04	−630.72	−630.99
54	−0.79	8.28	4.54	3.74	1.90	−630.98	−630.67	−630.95
59	0.49	6.18	2.85	3.33	2.90	−669.66	−669.43	−669.67
64	0.27	5.90	2.82	3.08	4.00	−669.65	−669.43	−669.66

[a] Conformation extended.
[b] Conformation folded.

Table 4
Cytotoxic and hemolytic activity of active antifungal compounds

Compd	Cytotoxic activity GI_{50}			Hemolytic activity
	H-460	MCF-7	SF268	HC_{50}
1	>10.0	>10.0	>10.0	>250
2	>10.0	>10.0	>10.0	>250
3	4.7	3.6	4.1	>250
4	>10.0	5.2	5.8	>250
5	>10.0	>10.0	>10.0	>250
7	>10.0	>10.0	>10.0	>250
8	>10.0	>10.0	>10.0	>250
9	>10.0	>10.0	>10.0	>250
10	>10.0	>10.0	>10.0	>250
16	>10.0	>10.0	>10.0	>250
17	>10.0	>10.0	>10.0	>250
18	>10.0	>10.0	>10.0	>250
19	>10.0	>10.0	>10.0	>250
20	>10.0	>10.0	>10.0	>250
31	>10.0	>10.0	>10.0	>250
32	>10.0	>10.0	>10.0	>250
33	>10.0	>10.0	>10.0	>250
34	>10.0	>10.0	>10.0	>250
35	>10.0	>10.0	>10.0	>250
36	>10.0	>10.0	>10.0	>250
37	>10.0	>10.0	>10.0	>250
38	>10.0	>10.0	>10.0	>250
39	>10.0	>10.0	>10.0	>250
40	>10.0	>10.0	>10.0	>250
41	>10.0	>10.0	>10.0	>250
42	>10.0	>10.0	>10.0	>250
43	>10.0	>10.0	>10.0	>250
44	>10.0	>10.0	>10.0	>250
45	6.7	9.9	6.5	>250
46	>10.0	>10.0	>10.0	>250
47	>10.0	>10.0	>10.0	>250

GI_{50} (growth inhibition 50, in μg/mL) and HC_{50} (hemolytic concentration 50, in μg/mL).

Regarding log *P*, it was found that this descriptor alone is not strongly enough to properly predict the antifungal activity. The active compounds **5**, **43** and **47** displayed values of 3.22, 3.23 and 1.31, respectively, whereas the low-active or inactive compounds **22**, **49**, **54**, **59** and **64** showed values of 3.04, 2.04, 2.04, 1.61 and 1.79, respectively (Table 3). However, within compounds of the same type, the comparison of the values of log *P* shows that an enhancement of antifungal activity is directly related to the increment of lipophilicity. For instance, within compounds of each IA (**1–5**), IC (**31–43**) or ID (**44–47**) types, maleimides **5**, **43** and **47**, possess the highest log *P* values and, concomitantly, displayed the best antifungal activities.

In addition, it is known that the relative ordering of HOMO and/or LUMO contour plots provides a reasonable indication of the excitation properties of a compound.[15–17] Figure 4 gives a detailed description of LUMO orbitals, including spatial characteristics, nodal patterns, and individual atom contributions of **5**, **43** and **47**. It is interesting to note that the LUMO contour plot obtained for **5** (Fig. 1a) is closely related to those obtained from compounds **43** and **47** (Fig. 1b and c). We can observe that the LUMO's electronic clouds of the three compounds are concentrated on the imido ring, which is mostly composed by $\pi*$ orbitals (inter-ring antibonding character). This indicates that the carbon atoms in the imido ring are potential sites for nucleophilic attack, thus indicating that its contribution is crucial.

The same results can also be appreciated by observing the MEPs obtained from DFT calculations. MEPs have shown to provide reliable information, both on the interaction sites of molecules with point charges and on the comparative reactivities of these

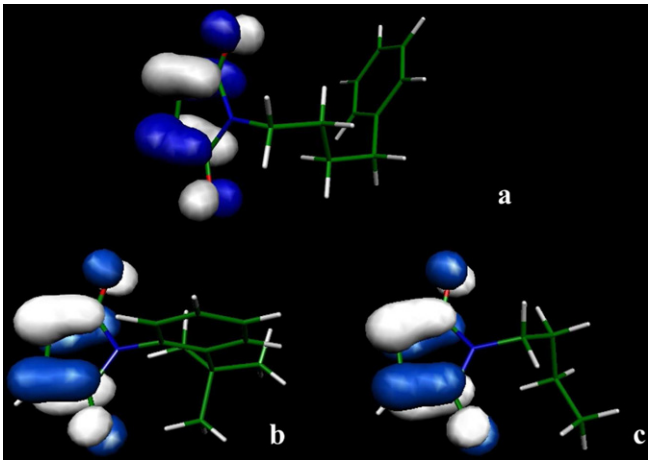


Figure 4. Spatial representation of the frontier molecular orbital LUMO of the compounds **5** (a), **43** (b) and **47** (c) at the ground state using B3LYP/6-31G(d) calculations.

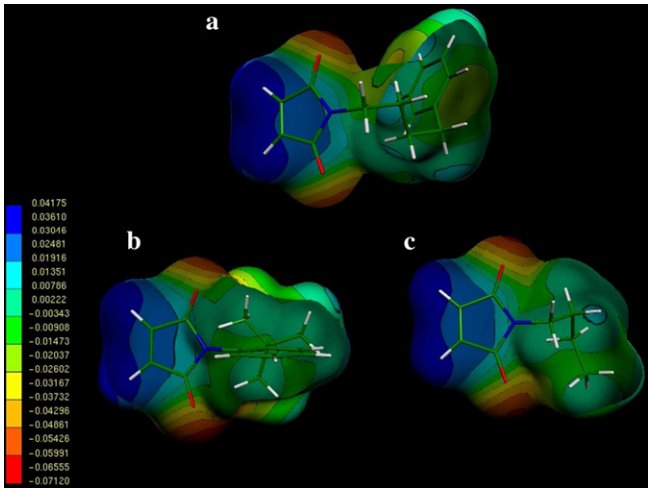


Figure 5. Electrostatic potential-encoded electron density surfaces of compounds **5** (a), **43** (b) and **47** (c). The surfaces were generated with GAUSSIAN 03 after B3LYP minimization with a 6-31G(d) basis set. The coloring represents electrostatic potential with red indicating the strongest attraction to a positive point charge and blue indicating the strongest repulsion. The electrostatic potential is the energy of interaction of the positive point charge with the nuclei and electrons of a molecule.

sites.[18–20] Figure 5 gives the MEPs obtained for compounds **5**, **43** and **47** which exhibit two clear minimum values (deep red zones) in the maleimido ring. This region is symmetrical with respect to the carbonyl groups and displays a deep and extensive negative potential zone with values of about −0.070 kcal/mol. The other relevant characteristic of these MEPs is the presence of a relatively extended hydrophobic zone (light blue and green zones).

2.5. Cytotoxic and hemolytic activity

In addition to the antifungal evaluation, cytotoxic activities against breast (MCF-7), lung (H-460) and central nervous system (SF-268) human cancer cell lines of the most active compounds (those whit MICs $\leqslant$ 10 μg/mL in at least one fungus) (**1–5**, **7–10**, **16–20**, **31–47**) were evaluated in order to determine the selectivity of maleimides towards the most sensitive fungi. Results showed (Table 4) that the cytotoxic activities (GI_{50}) were >10 μg/mL for all selected compounds, except for **3**, **4** and **45**, whose GI_{50} values were similar or higher than the antifungal ones (Table 4).

Then, considering the possibility that active compounds might have a simple lytic mode of action and be broadly cytotoxic against human cells, we determined the hemolytic activity of these compounds against human erythrocytes. Results indicate that no compounds induced hemoglobin release from red blood cells at concentrations up to 250 μg/mL, indicating that these compounds are not hemolytic and do not disturb membrane functions in erythrocytes at antifungal MICs (Table 4).

3. Conclusions

The synthesis, in vitro evaluation and SAR studies of 67 maleimides and derivatives acting as antifungal agents are reported. A detailed SAR study supported by theoretical calculations led us to determine the minimal structural requirements of this series to produce the antifungal effect: (i) an intact maleimido ring appears to be necessary for a strong antifungal activity; (ii) the substituents in positions 2 and 3 dissimilarly affected the antifungal activity. The best activities were shown by 2,3-nonsubstituted **1–5**, **31–47**, followed by 2,3 dichloro- **6–10** and by 2-methyl-substituted maleimides **16–20**. 2,3-Dimethyl- and 2,3-diphenyl-maleimides **21–30** possessed marginal or null activity; (iii) the presence of a flexible connecting chain in *N*-phenylalkyl maleimides appears not to be essential for antifungal activity, although its length shows a correlation with the antifungal behavior, displaying maleimides with alkyl chains of $n = 3$ and $n = 4$ the best antifungal activities in most fungi; (iv) different substituents on the benzene ring did not have a clear influence on the activity.

Values of CPP as well as of energy do not sufficiently discriminate between active and inactive compounds. Nevertheless, it was found that log *P*, although alone is not strong enough to properly predict the antifungal activity, the comparison of its values for compounds within the same type, showed an enhancement of antifungal activity along with an increment of lipophilicity.

In addition, the LUMO's electronic clouds of the highly active compounds **5**, **43** and **47** showed to be concentrated on the imido ring, indicating that their carbon atoms are potential sites for nucleophilic attack. Same results were obtained from MEPs.

Most active compounds did not show cytotoxic activity against human cancer cell lines and no one possessed hemolytic activity, indicating that their activity is selective to pathogen fungi and that they are not toxic at MIC concentrations.

Considering the high antifungal activities of types IA and IB compounds, a study of their mode of action constitutes a necessary next step for future research. In this regard, it is important to take into account that compound **9** showed, in a previous work,[3] to be inhibitory of the fungal cell-wall. Since fungal but not mammalian cells possess a wall, its inhibition is highly appreciated for the possible development of safe antifungal drugs.

In addition, a rational design that lead to further modifications of the unsaturated, and diketo ring system will allow to find new structure–activity relationships and will help to have a more profound and comprehensive knowledge of the potentiality of maleimide-related compounds as antifungal compounds.

4. Experimental

4.1. Chemistry

Solvents and reagents were purchased from Sigma (St. Louis, MO, USA) and Aldrich (Steinheim, Germany) and were purified in the usual manner. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 300 MHz (Bruker, Karlsruhe, Germany). Compounds were dissolved in deuterated solvents from commercial sources (Sigma) with tetramethylsilane (TMS) as the internal standard. Chemical shifts are reported in ppm (δ) relative to the solvent peak ($CHCl_3$ in $CDCl_3$ al 7.26 ppm for protons and at 77.0 for carbons). Signals are designated as follows: s, singlet; d, doublet; dd, doublets of doublets; t, triplet; dt; doublets of triplets; m, multiplet; quint, quintuplet. Mass spectra were obtained in a Turbo Mass Perkin Elmer, ionization energy 70 eV. Elemental analyses were performed on a Carlo Erba EA1108 analyzer. Percentages of C, H and N were in agreement with the product formula (within ±0.4% of theoretical values). Melting points were obtained in a Electrothermal apparatus (UK) and were uncorrected.

4.1.1. Synthesis of maleimides

The synthesis of maleimides **1–47** was performed by mixing an equimolar amount of the appropriate maleic anhydrides **68–72** in 5 mL of $CHCl_3$ and anilines **73**, **78–90**, amines **91–94** or phenylalkylamines **74–77** (5 mmol) dissolved in 1 mL of $CHCl_3$ and stirred during 1 h. The solid (maleamic acid) which precipitated out of the reaction mixture was filtered off. The whole amount of maleamic acid was dissolved in 5 mL of acetic anhydride and 100 mg of sodium acetate was added. The mixture was heated for 2 h under reflux. The reaction was cooled and quenched with water; then, the aqueous solution was extracted with Et_2O, dried with Na_2SO_4, filtered, and the solvent was evaporated. The product was purified by silica gel column chromatography using a mixture of hexane and ethyl acetate (9:1) as eluent. Compounds **1–10**, **16–26**, **31–34**, **36**, **38–42** and **43–47** were previously reported.[3,8,21–28]

4.1.1.1. 2,3-Diphenyl-*N*-benzylmaleimide 27. Yellow crystals. Mp 99–100 °C. Yield: 42%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.98 (2H, s, CH_2); 7.22–7.58 (15H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 46.5 (CH_2); 126.8; 127.1; 128.6; 128.7; 128.8; 129.8; 136.2; 136.5; 143.2; 170.5 ppm. EM: $m/z = 339$ (M^+). Anal. Calcd for $C_{23}H_{17}NO_2$: C, 81.3; H, 5.0; N, 4.1. Found: C, 81.2; H, 5.0; N, 4.1.

4.1.1.2. 2,3-Diphenyl-*N*-phenethylmaleimide 28. Yellow crystals. Mp 79–81 °C. Yield: 36%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.77 (2H, t, $J = 7.2$, ArCH_2); 3.92 (2H, t, $J = 7.2$, NCH_2); 7.18–7.52 (15H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 34.6 (ArCH_2); 40.0; 126.2; 128.8; 128.9; 129.8; 136.2; 138.1; 129.8; 170.6 ppm. EM: $m/z = 353$ (M^+). Anal. Calcd for $C_{24}H_{19}NO_2$: C, 81.5; H, 5.4; N, 4.0. Found: C, 81.6; H, 5.4; N, 4.0.

4.1.1.3. 2,3-Diphenyl-*N*-propylphenylmaleimide 29. Yellow crystals. Mp 82–84 °C. Yield: 35%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.79 (2H, quint, $J = 7.5$, $CH_2CH_2CH_2$); 2.66 (2H, t, $J = 7.5$, ArCH_2); 2.74 (2H, t, $J = 7.5$, NCH_2); 7.15–7.52 (15H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 33.2; 35.0; 41.6; 125.8; 125.9; 128.2; 128.4; 129.5; 135.4; 142.0; 142.0; 155.9; 170.3 ppm. EM: $m/z = 367$ (M^+). Anal. Calcd for $C_{25}H_{21}NO_2$: C, 81.6; H, 5.7; N, 3.8. Found: C, 81.4; H, 5.7; N, 3.8.

4.1.1.4. 2,3-Diphenyl-*N*-butylphenylmaleimide 30. Yellow crystals. Mp 150–152 °C. Yield: 41%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.28 (4H, m, $CH_2(CH_2)_2CH_2$); 3.01 (2H, t, $J = 7.5$, ArCH_2); 3.86 (2H, t, $J = 7.5$, NCH_2); 7.12–7.45 (15H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 22.4; 26.4; 34.6; 39.6; 125.3; 126.7; 128.8; 128.9; 129.4; 129.8; 136.2; 136.4; 138.1; 170.6 ppm. EM: $m/z = 381$ (M^+). Anal. Calcd for $C_{26}H_{23}NO_2$: C, 81.8; H, 6.0; N, 3.7. Found: C, 82.1; H, 6.0; N, 3.7.

4.1.1.5. *N*-(3′,4′-Dimethoxylphenyl) maleimide 33. Yellow crystals. Mp 92–94 °C Yield 85%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.90 (3H, s, 3′-OCH_3); 3.92 (3H, s, 4′-OCH_3); 6.85 (2H, s, H-2,3); 6.82–7.98 (3H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 55.0; 56.1; 110.0; 111.2; 119.0; 124.0; 134.2; 148.9; 149.3; 169.8 ppm.

Anal. Calcd for $C_{12}H_{11}NO_4$: C, 61.7; H, 4.7; N, 6.0. Found: C, 62.0; H, 4.7; N, 6.0.

4.1.1.6. *N*-(3′,4′-Methylendioxyphenyl)-maleimide 37. White crystals. Mp 147–148 °C Yield 74%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.03 (2H, s, OCH_2O); 6.79 (1H, dd, J = 9.0; 2.0, H-6′); 6.84 (s, 2H, H-2,3); 6.80–6.77 (2H, m, H-2′,5′) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 101.8; 107.7; 108.4; 120.2; 124.6; 134.2; 147.4; 148.1; 169.6 ppm. Anal. Calcd for $C_{11}H_7NO_4$: C, 60.8; H, 3.2; N, 6.4. Found: C, 61.0; H, 3.2; N, 6.5.

4.1.2. Synthesis of succinimides

4.1.2.1. *N*-Phenyl- and *N*-phenylalkylsuccinimides 48–52. They were prepared following the same procedure used for maleimides **1–47** utilizing succinic anhydride **95** as the reactive instead of maleic anhydrides. Compounds **48–50** were previously reported.[28]

4.1.2.2. *N*-Propylphenylsuccinimide 51. White crystals. Mp 110–113 °C Yield 71%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.96 (2H, quint, J = 7.8, $CH_2CH_2CH_2$); 2.57 (4H, s, H-2,3); 2.68 (2H, t, J = 7.8, $ArCH_2$); 3.78 (2H, t, J = 7.8, NCH_2); 7.13–7.38 (5H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 28.1; 28.4; 33.2; 38.7; 126.0; 128.2; 128.4; 141.0; 177.3 ppm. Anal. Calcd for $C_{13}H_{15}NO_2$: C, 71.8; H, 6.9; N, 6.4. Found: C, 72.0; H, 6.9; N, 6.5.

4.1.2.3. *N*-Butylphenylsuccinimide 52. White crystals. Mp 103–104 °C Yield 69%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.63 (4H, m, $CH_2(CH_2)_2CH_2$); 2.24 (2H, t, J = 7.2, $ArCH_2$); 2.70 (4H, s, H-2,3); 3.55 (2H, t, J = 6.9, NCH_2); 7.12–7.33 (5H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 27.3; 28.1; 28.6; 35.3; 38.6; 125.8; 128.3; 128.4; 141.9; 177.2 ppm. Anal. Calcd for $C_{14}H_{17}NO_2$: C, 72.6; H, 7.4; N, 6.1. Found: C, 72.5; H, 7.4; N, 6.0.

4.1.3. Synthesis of 4-hydroxy-2,3-dehydro-γ-lactames

4-Hydroxy-2,3-dehydro-γ-lactames **53–57** were prepared by dissolving maleimides **1–5** (1 mmol) in CH_3OH (2 mL) added with 2 mmol of $CeCl_3{\cdot}7H_2O$ and stirring (5 min); then $NaBH_4$ (2 mmol) was added portionwise and stirred for 1 h. The same procedure was used for obtaining **63–67** from **16–20**. 3-Methyl-4-hydroxy-2,3-dehydro-γ-lactames **58–62** were prepared similarly but without the addition of $CeCl_3$. The reaction mixture was quenched with ice water, CH_3OH was removed under reduced pressure and the solution was extracted with EtOAc, dried with Na_2SO_4, filtered, and the solvent was evaporated. The mixtures were subjected to silica gel column chromatography using a mixture of hexane and ethyl acetate (9:1) as eluent. Compounds **54, 58–59, 63–64** were previously reported.[11]

4.1.3.1. *N*-Phenyl-4-hydroxy-2,3-dehydro-γ-lactame 53. White crystals. Mp 117–120 °C. Yield: 88%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.00 (1H, d, H-4); 6.24 (1H, d, J = 6.0, H-2); 7.06 (1H, dd, J = 6,0; 1,7, H-3); 7.21 (1H, t, J = 7.9, H-4′); 7.42 (2H, t, J = 7.9, 3′,5′-H); 7.60–7.74 (2H, d, J = 7.9, H-2′,6′) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 84.1; 121.2; 125.1; 129.1; 129.2; 136.7; 144.9; 168.4 ppm. Anal. Calcd for $C_{10}H_9NO_2$: C, 68.5; H, 5.1; N, 8.0. Found: C, 68.6; H, 5.1; N, 8.0.

4.1.3.2. *N*-Phenethyl-4-hydroxy-2,3-dehydro-γ-lactame 55. White crystals. Mp 124–126 °C. Yield: 59%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2,71 (2H, t, J = 7,2, $ArCH_2$); 3,60 (1H, dt, J = 14,1; 7,2, C*H*aHb); 3.85 (1H, dt, J = 14.1; 7.2, CHa*H*b); 5.48 (1H, d, J = 10.7, H-4); 6.28 (1H, d, J = 5.1, H-2); 6.41 (1H, dd, J = 5.1; 1.6, H-3); 7.21–7.39 (5H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 33.7; 41.0; 85.4; 126.0; 128.4; 128.3; 128.4; 142.4; 146.7; 170.6 ppm. Anal. Calcd for $C_{12}H_{13}NO_2$: C, 70.9; H, 6.4; N, 6.9. Found: C, 70.7; H, 6.4; N, 6.9.

4.1.3.3. *N*-Propylphenyl-4-hydroxy-2,3-dehydro-γ-lactame 56. White crystals. Mp 125–127 °C. Yield: 76%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.92 (2H, m, $CH_2CH_2CH_2$); 2.39 (2H, m, $ArCH_2$); 3.22 (1H, dt, J = 14.1; 7.2, C*H*aHb); 3.60 (1H, dt, J = 14.1; 7.2, CHa*H*b); 5.38 (1H, d, J = 10.8, H-4); 6.08 (1H, d, J = 6.0, H-2); 6.91 (1H, dd, J = 6.0; 1.6, H-3); 7.11–7.37 (5H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 30.1; 33.3; 39.0; 83.4; 125.9; 128.3; 128.4; 128.4; 141.4; 145.7; 169.6 ppm. Anal. Calcd for $C_{13}H_{15}NO_2$: C, 71.8; H, 6.9; N, 6.4. Found: C, 71.7; H, 6.9; N, 6.5.

4.1.3.4. *N*-Butylphenyl-4-hydroxy-2,3-dehydro-γ-lactame 57. White crystals. Mp 132–134 °C. Yield: 74%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.58–1.77 (4H, m, $CH_2(CH_2)_2CH_2$); 2.64 (2H, t, J = 7.0, $ArCH_2$); 3.01 (1H, d, J = 11.4, O*H*); 3.34 (1H, dt, NC*H*aHb); 3.60 (1H, dt, NCHa*H*b); 5.19 (1H, d, J = 11.4, H-4); 7.09–7.35 (7H, m, H-2; H-3 and H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 27.7; 28.4; 35.3; 40.3; 82.1; 125.9; 126.9; 128.4; 128.4; 141.8; 142.8; 162.6 ppm. Anal. Calcd for $C_{14}H_{17}NO_2$: C, 71.8; H, 6.9; N, 6.4. Found: C, 72.0; H, 6.9; N, 6.5.

4.1.3.5. 3-Methyl-*N*-phenethyl-4-hydroxy-2,3-dehydro-γ-lactame 60. White crystals. Mp 143–145 °C. Yield: 69%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2,00 (3H, s, CH_3); 2,89 (2H, t, J = 7,2, $ArCH_2$); 3,47 (1H, dt, J = 14,2; 7,2, NC*H*aHb); 3,74 (1H, dt, J = 14,2; 7,2, NCHa*H*b); 4,99 (1H, d, J = 10,9, H-4); 5,68 (1H, s, H-2); 7,12–7,43 (5H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.5; 34.7; 40.8; 85.3; 122.3; 126.5; 128.6; 128.7; 138.9; 158.1; 170.4 ppm. Anal. Calcd for $C_{13}H_{15}NO_2$: C, 71.8; H, 6.9; N, 6.4. Found: C, 71.6; H, 6.9; N, 6.5.

4.1.3.6. 3-Methyl-*N*-propylphenyl-4-hydroxy-2,3-dehydro-γ-lactame 61. White crystals. Mp 112–114 °C. Yield: 71%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.89 (2H, m, $CH_2CH_2CH_2$); 2.04 (3H, s, CH_3); 3.63 (1H, d, J = 11.4, O*H*); 3.63 (2H, t, J = 7.8, $ArCH_2$); 3.26 (1H, dt, J = 14.2; 7,2, NC*H*aHb); 3.54 (1H, dt, J = 14.2; 7.2, NCHa*H*b); 5.08 (1H, d, J = 11.4, H-4); 5.68 (1H, s, H-2); 7.15–7.32 (5H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.5; 30.1; 33.1; 38.9; 84.9; 122.4; 125.9; 128.3; 128.4; 157.8; 141.4; 170.4 ppm. Anal. Calcd for $C_{14}H_{17}NO_2$: C, 72.6; H, 7.4; N, 6.1. Found: C, 72.4; H, 7.3; N, 6.0.

4.1.3.7. 3-Methyl-*N*-butylphenyl-4-hydroxy-2,3-dehydro-γ-lactame 62. White crystals. Mp 123–125 °C. Yield: 66%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1,62–164 (4H, m, $CH_2(CH_2)_2CH_2$); 1,88 (3H, s, CH_3); 2,65 (2H, t, J = 7,0 $ArCH_2$); 3,28 (1H, dt, NC*H*aHb); 3,56 (1H, dt, NCHa*H*b); 5,24 (1H, s, H-4); 6,53 (1H, s, H-2); 7,19–7,34 (5H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.5; 28.0; 28.6; 35.4; 38.8; 84.8; 122.4; 125.8; 128.3; 128.4; 142.1; 157.7; 170.3 ppm. Anal. Calcd for $C_{15}H_{19}NO_2$: C, 73.4; H, 7.7; N, 5.7. Found: C, 73.2; H, 7.8; N, 5.7.

4.1.3.8. 2-Methyl-*N*-phenethyl-4-hydroxy-2,3-dehydro-γ-lactame 65. White crystals. Mp 128–130 °C. Yield: 64%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.89 (3H, s, CH_3); 2.28 (1H, d, J = 11.1, O*H*); 2.93 (2H, t, J = 7.2, $ArCH_2$); 3.55 (1H, dt, J = 14.2; 7.2, NC*H*aHb); 3.79 (1H, dt, J = 14.2; 7.2, NCHa*H*b); 5.04 (1H, d, J = 11.1, H-4); 6.49 (1H, d, J = 1.5, H-3); 7.17–7.35 (5H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 10.9; 34.7; 41.1; 82.1; 126.5; 128.6; 128.8; 137.2; 138.2; 139.0; 170.3 ppm. Anal. Calcd for $C_{13}H_{15}NO_2$: C, 71.8; H, 6.9; N, 6.4. Found: C, 71.6; H, 6.9; N, 6.4.

4.1.3.9. 2-Methyl-*N*-propylphenyl-4-hydroxy-2,3-dehydro-γ-lactame 66. White crystals. Mp 115–117 °C. Yield: 70%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.87 (3H, s, CH_3); 1.91 (2H, m, $CH_2CH_2CH_2$);

2.85 (1H, d, J = 10.9, O*H*); 3.65 (2H, t, J = 7.8, ArCH_2); 3.33 (1H, dt, J = 14.2; 7.2, NC*H*aHb); 3.58 (1H, dt, J = 14,2; 7,2, NCHa*H*b); 5.25 (1H, d, J = 10.9, H-4); 6.53 (1H, d, J = 1.5, H-3); 7.21–7.32 (5H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 10.9; 30.1; 33.3; 39.3; 81.7; 125.9; 128.3; 128.4; 137.2; 138.2; 141.4; 170.4 ppm. Anal. Calcd for $C_{14}H_{17}NO_2$: C, 72.6; H, 7.4; N, 6.1. Found: C, 72.7; H, 7.3; N, 6.0.

4.1.3.10. 2-Methyl-*N*-butylphenyl-4-hydroxy-2,3-dehydro-γ-lactame 67. White crystals. Mp 122–124 °C. Yield: 71%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.52–1.72 (4H, m, $CH_2(CH_2)_2CH_2$); 2.04 (3H, s, CH_3); 2.45 (2H, t; J = 6.6, ArCH_2); 3.48 (1H, dt, NC*H*aHb); 3.53 (1H, dt, NCHa*H*b); 5.06 (1H, d, J = 1.5, H-4); 5,69 (1H, d, J = 1.5, H-3); 7.15–7.35 (5H, m, H_{Ar}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 10.9; 28.0; 28.7; 35.5; 39.2; 81.6; 125.8; 128.3; 128.4; 138.0; 138.0; 142.1; 170.3 ppm. Anal. Calcd for $C_{15}H_{19}NO_2$: C, 73.4; H, 7.7; N, 5.7. Found: C, 73.6; H, 7.8; N, 5.7.

4.2. Biological evaluation

4.2.1. Microorganisms and media

For the antifungal evaluation, reference strains from the American Type Culture Collection (ATCC), Rockville, MD, USA, and Culture Collection of Centro de Referencia en Micología-CEREMIC (CCC), Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina, were used: *C. albicans* ATCC 10231, *C. tropicalis* CCC 191, *S. cerevisiae* ATCC 9763, *C. neoformans* ATCC 32264, *A. flavus* ATCC 9170, *A. fumigatus* ATTC 26934, *A. niger* ATCC 9029, *M. gypseum* CCC 115, *T. rubrum* CCC 110, *T. mentagrophytes* ATCC 9972. Strains were grown on Sabouraud-chloramphenicol agar slants at 30 °C, 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 and adjusted to 1–5 × 10^3 colony forming units (CFU)/mL.[6,7]

4.2.2. Antifungal susceptibility testing

MIC of each compound was determined by using broth microdilution techniques following the guidelines of the CLSI for yeasts[6] and for filamentous fungi.[7] MIC values were determined in RPMI-1640 (Sigma) buffered to pH 7.0 with MOPS (Sigma). Microtiter trays were incubated at 35 °C for yeasts and hyalohyphomycetes and at 28 °C for dermatophyte strains in a moist, dark chamber; MICs were recorded at 48 h for yeasts, and at a time according to the control fungus growth, for the rest of fungi. The susceptibilities of the standard drugs Ketoconazole, Terbinafine, and Amphotericin B were defined as the lowest concentration of drug which resulted in total inhibition of fungal growth. For the assay, compound stock solutions were twofold diluted with RPMI-1640 from 250 to 0.24 μg/ml (final volume = 100 μL) and a final DMSO (Sigma) 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. MIC was defined as the minimum inhibitory concentration of the compound, which resulted in total inhibition of the fungal growth. MFC was determined by plating in duplicate 5 μL from each clear well of MIC determinations, onto a 150-mm SDA plate and incubated at the same conditions used previously. MFCs were determined as the lowest concentration of each compound showing no growth on the plates.

4.2.3. Cytotoxic assays

The cytotoxic activity was determined according to the method of Monks et al.[29] Briefly, the three human cells lines [breast (MCF-7), non-small cell lung (H-460), and central nervous system (SF-268)] obtained from US National Cancer Institute (NCI) were counted, diluted with fresh medium, and added to 96-well microtiter plates (100 μL/well) containing test materials (1 mg in 100 μL in DMSO). Test plates were incubated for 2 days at 37 °C in a 5% CO_2 incubator. All treatments were performed in duplicate. After incubation periods, cells were fixed by addition of 50 μL of cold 50% aqueous TCA solution (4 °C for 60 min), washed 4–5 times with tap water, and air-dried. The fixed cells were stained with 100 μL sulforhodamine B (SRB) (0.4% wt/vol in 1% acetic acid) for 15 min. Free SRB solution was then removed by rinsing with 1% acetic acid (5×). The plates were then air-dried, the bound dye was solubilized with 100 μL of 10 mM tris-base, and the absorbance was determined at 515 nm using an ELISA plate reader (Bio-Tek Instruments, Inc. Model ELX-800). Finally, the absorbance values obtained with each of the treatment procedures were averaged, and the average value obtained with the zero day control was subtracted measuring in this way the relative cell growth or inviability in treated and untreated cells. From the curves, growth inhibition and 50% inhibition of growth (GI_{50}) were calculated.[30]

4.2.4. Hemolysis assay

Stock solutions of compounds solubilized in DMSO were diluted with sterile 5% glucose to yield final test concentrations ranging from 250 to 0.24 μg/ml and added to 96-well microtiter plates (100 μL/well). Freshly obtained heparinized human red blood cells (RBC) were washed three times by centrifugation (2500 rpm for 10 min) in isotonic PBS, pH 7.0 at rt. Two milliliters of RBC were added to 50 mL of sterile 5% glucose to obtain a 4% suspension. One-hundred microliters of RBC suspension was added to each well, mixed, and plates incubated at 37 °C. The absorbance of the liberated hemoglobin was measured spectrophotometrically at 540 nm in a Versa Max spectrophotometer. HC_{50} was defined as the concentration at which there was 50% lysis in comparison with drug-free value.[31] The same amount of DMSO alone causes no lysis.

4.3. Computational methods

All the computational studies were carried out using density functional theory (DFT) methods implemented in the GAUSSIAN 03 suite of programs.[32] Correlation effects were included in the present work using DFT with the Becke3-Lee-Yang-Parr (B3LYP)[33–35] functional and the 6-31G(d) basis set. Thus, molecular geometry optimizations were performed at the DFT (B3LYP/6-31G(d) level of theory, using the GAUSSIAN 03 program employing standard basis set with no modifications. Convergence criteria were according to the limits imposed internally by GAUSSIAN 03. Low energy conformations were confirmed from a vibrational analysis using B3LYP/6-31G(d) calculations.

Molecular electrostatic potentials (MEPs) were calculated by using RB3LYP/6-31G(d) wave functions. MEP graphical presentations were created using the MOLEKEL program.[36] The calculated electronic properties for maleimides were: chemical potential properties (electron affinity, ionization potential, hardness, electronegativity, electrophilicity and dipolar moments. These properties were calculated from B3LYP/6-31G(d) calculations. All the calculations were performed in gas phase with the purpose of obtaining the intrinsic properties of the maleimides studied, free of any interaction. Calculations of the partition coefficient (log P) were performed from CHEM OFFICE 10.0 program.[37]

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