Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 560–568
Bioorganic & Medicinal Chemistry

N-Phenyl and *N*-phenylalkyl-maleimides acting against *Candida* spp.: Time-to-kill, stability, interaction with maleamic acids

Maximiliano Sortino,[a] Valdir Cechinel Filho,[b] Rogério Corrêa[b] and Susana Zacchino[a,*]

[a]*Farmacognosia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina*
[b]*Núcleo de Investigações Químico-Farmacêuticas (NIQFAR), Universidade do Vale do Itajaí (UNIVALI), 88.302-202, Itajaí (SC), Brazil*

Received 4 June 2007; revised 14 August 2007; accepted 20 August 2007
Available online 24 August 2007

Abstract—*N*-Phenyl and *N*-phenylalkyl maleimides (alkyl chain = $(CH_2)n$; $n = 0–4$) and their respective open derivatives (maleamic acids) were evaluated against *Candida* spp. with the microbroth dilution method following the guidelines of CLSI (formely NCCLS). MIC values of maleimides without pre-incubation and submitted to different pre-incubation times into the growth media, time-to-kill studies as well as a time-dependent UV-spectroscopy study of the maleimides in water, led to determine that maleimides display antifungal activities with their intact maleimide ring, being in addition their activities not dependent on the length of the alkyl chain. They are not only fungistatic but fungicidal with very low MICs and MFCs, displaying strong fungicide activities not only against standardized but also clinical isolates of *Candida albicans* and non-*albicans Candida* spp.

1. Introduction

Candida spp. are usually present in skin, mucous membranes, gastrointestinal tract, and genitourinary organs of healthy human beings. Nevertheless, they produce serious infections in patients with immunodeficiencies such as individuals submitted to organ transplantations or antineoplastic chemotherapy, those suffering the acquired immunodeficiency syndrome (AIDS), extremely aged persons and patients in intensive care units, among others.[1]

Candidosis has been shown to be the fourth most common nosocomial blood stream infection, *Candida albicans* representing more than 60% of all isolates from clinical infections.[2,3] In the last years, there has been an increase in the percentage of *Candida* infections caused by non-*albicans Candida* spp. such as *C. glabrata*, *C. tropicalis* and *C. parapsilosis*, and in a lower percentage *C. guillermondii*, *C. lusitaneae*, *C. krusei*, *C. dubliniensis*, and *C. rugosa*, among others.[1,4,5]

Although amphotericin B, fluocytosine and azoles such as ketoconazole, fluconazole or itraconazole have been considered efficient for the treatment of candidosis, some of these drugs show toxicity, produce recurrence because they are fungistatic and not fungicide, or lead to development of resistance due in part to the intensive prophylactic use of antifungal drugs.[6]

There is, therefore, a clear need for the discovery of new structures with anti-*Candida* activity, which could constitute alternatives for the management of candidosis mainly in immunocompromised hosts.[7]

As part of our ongoing project on the detection of antifungal compounds,[8–12] we recently reported that *N*-phenyl and *N*-phenylalkylmaleimides (Fig. 1) possess strong antifungal activities when tested in agar dilution assays against a panel of 11 strains of clinically important fungi including *Candida* spp.[13]

In contrast to results obtained with *N*-phenyl and *N*-phenylalkyl-3,4-dichloromaleimides, the antifungal activity of *N*-phenyl and *N*-phenylalkylmaleimides appeared not to be dependent on the presence or on the length of the alkyl chain. Nevertheless, the fact that they

Keywords: Maleimides; Antifungal; Stability; *Candida*.
* Corresponding author. Fax: +54 341 4375315; e-mail: szaabgil@citynet.net.ar

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.08.030

showed Minimum Inhibitory Concentrations (MICs) lower than 12.5 μg/mL, 6.25 μg/mL or 3.25 μg/mL in 40, 30 or 18 tests, respectively, out of the 55 agar dilution tests performed[13] prompted us to continue with the study of the antifungal behavior of *N*-phenyl and *N*-phenylalkylmaleimides, results that are presented here. The MICs and Minimum Fungicidal Concentrations (MFCs) of maleimides along with time-to-kill values were determined at first against *C. albicans* ATCC 10231, by using the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical and Laboratory Standards, NCCLS) approved reference method for antifungal susceptibility of yeasts.[14]

Then, considering the possibility that in the aqueous culture media, the maleimide ring could undergo hydrolysis to maleamic acids as it was demonstrated for non *N*-substituted maleimide in alkaline medium and under certain conditions[15] (Fig. 2), we performed a study on the time-dependant stability of each compound in the growth media to determine if the maleimides under study (with the intact ring) are the true responsible for the antifungal activity or instead, their open analogues contribute to their antifungal behavior. In addition, the type of interaction (synergistic, antagonistic or additive)[16] that could exist between maleamic acids and the respective maleimide, and a time-dependent UV spectroscopy study of the five maleimides in water were performed too.

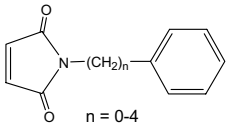


Figure 1. General structure of *N*-phenyl and *N*-phenylalkylmaleimides.

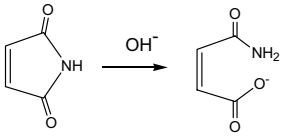


Figure 2. Opening of maleimide to maleamic acid under basic conditions.

At last, to gain insight into a future clinical application of these structures, MICs and MFCs of maleimides against an extended panel of clinical isolates of *C. albicans* and non-*albicans Candida* spp. were determined.

2. Results and discussion

2.1. Chemistry

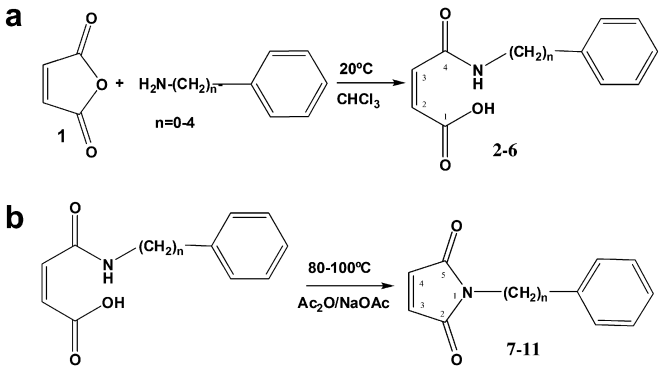
The syntheses of *N*-substituted maleamic acids and maleimides were achieved by a two steps procedure previously reported.[17] Reactions of maleic anhydride **1** with the appropriate amine (Ph-$(CH_2)_n$-NH_2; $n = 0$–4) in $CHCl_3$ at room temperature (rt) generated the corresponding *N*-substituted maleamic acids **2**–**6** in near quantitative yields. Compounds **5** and **6** have not been reported up to date in the literature. Maleamic acids were subsequently cyclized to the corresponding *N*-phenyl and *N*-phenylalkylmaleimides **7**–**11** by heating **2**–**6** in the presence of acetic anhydride containing catalytic amounts of sodium acetate (Scheme 1).

^1H and ^{13}C NMR spectra were coincident with the expected structures. A variation of the NMR signals was observed in the first step due to the lost of symmetry in the reaction. Cyclization in the second step was proven by NMR signals in agreement with the symmetrical structures of *N*-arylmaleimides obtained.

2.2. MIC and MFC determinations

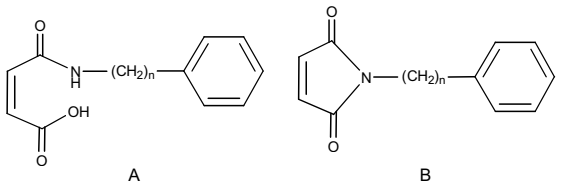
The MICs of maleamic acids **2**–**6** and maleimides **7**–**11** were determined against *C. albicans* ATCC 10231 by using the standardized microbroth dilution method M-27 A2 for yeasts according to the guidelines of the CLSI (formerly NCCLS)[14] which assures confident and reproducible results.[18]

Results showed that maleimides **7**–**11** possessed strong antifungal activities against *C. albicans* (all MICs = 3.9 μg/mL; Table 1, first row), also corroborating our previous findings that the activity did not depend on the length of the alkyl chain. Regarding maleamic acids **2**–**6**, they did not possess any antifungal activity at concentrations up to 250 μg/mL. MFCs of maleimides **7**–**11** were accomplished afterwards by



Scheme 1.

Table 1. Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) values (μg/mL) of *N*-phenyl- and *N*-phenylalkyl-maleamic acids and maleimides acting against *Candida* spp.



Candida spp.	Voucher number	Maleamic acids (type A)					Maleimides (type B)										St. drug	
		n = 0	*n* = 1	*n* = 2	*n* = 3	*n* = 4	*n* = 0		*n* = 1		*n* = 2		*n* = 3		*n* = 4			
		2	3	4	5	6	7		8		9		10		11		Amp B	Ket
		MIC	MIC	MIC	MIC	MIC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MIC
C. albicans 1	ATCC 10231	>250	>250	>250	>250	>250	3.9	7.8	3.9	7.8	3.9	7.8	3.9	7.8	3.9	7.8	1.0	0.5
C. albicans 2	C 125-2000	>250	>250	>250	>250	>250	0.48	1.95	0.97	1.95	1.95	1.95	0.97	1.95	0.97	1.95	0.78	6.25
C. albicans 3	C 126-2000	>250	>250	>250	>250	>250	0.97	3.9	0.97	3.9	3.9	7.81	0.97	1.95	0.97	1.95	1.56	1.56
C. albicans 4	C 127-2000	>250	>250	>250	>250	>250	0.97	1.95	1.95	3.9	1.95	3.9	1.95	3.9	1.95	3.9	0.78	6.25
C. albicans 5	C 128-2000	>250	>250	>250	>250	>250	0.97	3.9	1.95	3.9	3.9	7.81	3.9	7.81	0.97	1.95	1.56	6.25
C. albicans 6	C 129-2000	>250	>250	>250	>250	>250	0.48	1.95	1.95	3.9	1.95	1.95	1.95	3.9	0.97	1.95	0.78	12.5
C. albicans 7	C 130-2000	>250	>250	>250	>250	>250	0.48	1.95	0.97	1.95	1.95	1.95	0.97	1.95	1.95	3.9	0.39	6.25
C. glabrata	C 115-2000	>250	>250	>250	>250	>250	1.95	3.9	1.95	1.95	1.95	3.9	1.95	3.9	1.95	3.9	0.39	1.56
C. parapsilosis	C 124-2000	>250	>250	>250	>250	>250	1.95	3.9	1.95	3.9	3.9	3.9	3.9	3.9	1.95	7.8	0.78	0.78
C. lusitaniae	C 131-2000	>250	>250	>250	>250	>250	3.9	7.8	1.95	3.9	3.9	7.8	1.95	7.8	3.9	3.9	0.39	25
C. colliculosa	C 122-2000	>250	>250	>250	>250	>250	1.95	3.9	1.95	7.81	1.95	7.81	3.9	32.25	3.9	3.9	0.39	0.78
C. krusei	C 117-2000	>250	>250	>250	>250	>250	1.95	1.95	3.9	3.9	3.9	7.8	3.9	7.8	7.8	15.75	0.39	50
C. kefyr	C 123-2000	>250	>250	>250	>250	>250	1.95	3.9	3.9	3.9	3.9	3.9	15.75	7.81	1.95	7.81	0.78	0.78
C. tropicalis	C 137-1997	>250	>250	>250	>250	>250	7.81	15.75	15.75	32.25	15.75	32.25	15.75	32.25	15.75	32.25	0.12	0.12

ATCC, American Type Culture Collection (Rockville, MD, USA); C, Centro de Referencia en Micología (CEREMIC, Rosario, Argentina); Amp B, amphotericin B; Ket, ketoconazole.

sub-culturing an aliquot from MIC wells showing no growth, onto drug-free agar plates. MFCs for all tested maleimides were 7.8 μg/mL (Table 1, first row).

2.3. Time-to-kill studies

The times needed for compounds **7**–**11** to kill *C. albicans* (time-to-kill studies), were determined by enumerating viable cells of fungal suspensions exposed to 1× MFC of each compound at different exposition times (0–24 h) as explained in the Experimental section. Plots of UFC/mL versus time of incubation for maleimides **7**–**11** are shown in Figure 3 (times longer than 120 min are not shown). Results revealed that the fungus was killed by all compounds within 90 min of incubation.

2.4. MICs of maleimides submitted to different pre-incubation times

Each maleimide was pre-incubated at 37 °C into growth media during 0–7 days prior to inoculation. Figure 4 shows the 96-wells microplate that was used for determining the MICs of maleimide **7** at different pre-incubation times. Each line, which contains a series of twofold dilutions (1000–0.98 μg/mL) of the tested maleimide, was prepared in 7 consecutive days beginning for bottom to top. At the end of the seventh day, plates were inoculated with *C. albicans* ATCC 10231 and incubated 24 h at 37 °C. Results showed that the MIC of maleimide **7** was 3.9 μg/mL up to 2 days of contact with the growth media, suggesting that during this period the maleimide would remain intact. The MIC gently raised to a value of 500 μg/mL after 7 days of pre-incubation suggesting a gradual opening of the ring. Similar results were obtained for all maleimides tested (Table 2).

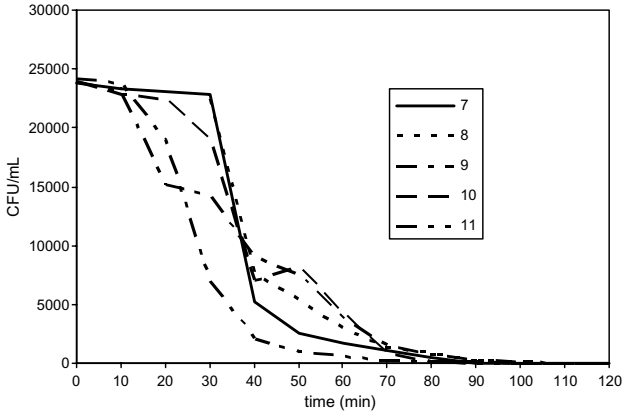


Figure 3. Time-to-kill curves of maleimides **7**–**11** at 1 × MFC against *C. albicans* ATCC 10231.

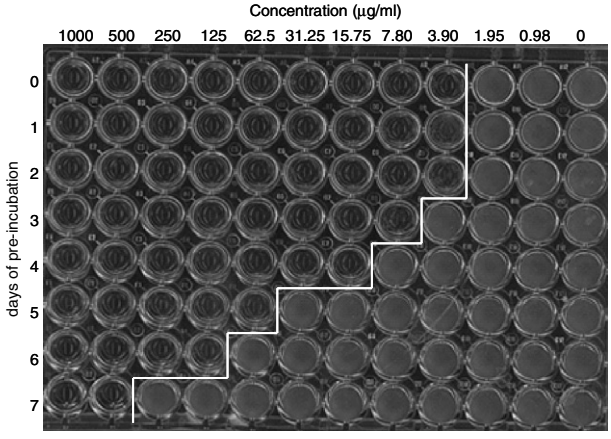


Figure 4. Microtiter plate showing MIC values in μg/mL (white line) of *N*-phenylmaleimide **7** without pre-incubation (0 days) and with different times (1–7 days) of pre-incubation into the growth media, against *Candida albicans* ATCC 10231 using the CLSI (formerly NCCLS) M27 A2 method.

2.5. Interaction of maleimides with their corresponding maleamic acids

For a correct interpretation of the above results, it seemed in order to determine if the interaction of maleimides and their respective maleamic acids produce synergism, antagonism, or no effect in the case of acting together as antifungal agents.

Chequerboard experiments were used to determine the Fractional Inhibitory Concentration (FIC, in μg/mL) of the combination of each maleimide with its correspondent maleamic acid for *C. albicans* ATCC 10231 and to calculate the Fractional Inhibitory Concentration Index (FICI). FICI value for each maleimide/maleamic acid was interpreted as follows: FICI $\leqslant$ 0.5, synergistic; 0.5 < FICI $\leqslant$ 4, indifferent; FICI > 4, antagonistic.[19]

Results showed that each maleimide and its maleamic acid do not interact with each other and therefore only an additive effect can be expected (Table 3).

Table 2. MIC values (μg/mL) against *Candida albicans* ATCC 10231 of maleimides **7**–**11**, without pre-incubation (0 days) and with different times (1–7 days) of pre-incubation at 37 °C in the growth media, using the CLSI (formerly NCCLS) M27 A2 method

T.P.	**7**	**8**	**9**	**10**	**11**
0	3.90	3.90	3.90	3.90	3.90
1	3.90	3.90	3.90	3.90	3.90
2	3.90	7.80	7.80	3.90	3.90
3	7.80	7.80	7.80	15.75	15.75
4	15.75	15.75	31.25	15.75	31.25
5	62.50	125	125	62.5	125
6	125	250	250	125	250
7	500	500	500	1000	1000

T.P., Time of pre-incubation of maleimides in the growth media.

Table 3. Fractional Inhibitory Concentration Indexes (FICI) of maleimides **7**–**11** in combination with maleamic acids **2**–**6** against *Candida albicans* ATCC 10231

Combination of Compounds	FICI	Result
7/2	0.80	Indifferent
8/3	1.125	Indifferent
9/4	0.75	Indifferent
10/5	1.00	Indifferent
11/6	0.75	Indifferent

Considering that each maleimide and its open derivative are indifferent when co-existing in a solution, mixtures of maleimides/maleamic acids at different concentrations were prepared and their MICs were determined and compared with those of Table 2, in order to estimate the % of hydrolysis that would have been produced at the different pre-incubation times. Results showed (Table 4) that for all pairs of compounds tested, maleimides with up to 48 h of pre-incubation (Table 2) could have suffered a maximum of 10–20% of hydrolysis (see Table 4). According to these results, the hydrolysis of maleimides **7**–**11** at the time required to kill the fungi (90 min) would have been negligible.

2.6. Stability of maleimides 7–11 in water

Considering that the culture media is an aqueous solution, we corroborated the above results by analyzing the stability of maleimides **7**–**11** in water by spectrophotometric measurements. The UV spectra of maleimides dissolved in water was plotted (250–350 nm) at intervals from 2 h to 3 days taking into account that a typical absorption wavelength for a cyclic imide group is 300 nm, while an amide group does not absorb here. So, a decrease in the absorption peak at 300 nm should demonstrate the cleavage of the imide bond.[15]

Spectra of pure maleimides **7**–**11** and pure maleamic acids **2**–**6** at time = 0 h are presented in Figure 5a–e. Figure 5a′–e′ shows the spectra of the aqueous solutions of maleimides after 0, 2, 24, 48, and 72 h. In these figures, it is observed that all maleimides spectra were almost identical at the beginning of the experiment (0 h) as well as after 2 h, suggesting that at the time needed for killing the fungus (90 min), the hydrolysis would be almost negligible for *N*-phenylmaleimides and *N*-phenylalkylmaleimides **7**–**11**, confirming that maleimides **7**–**11** act with their intact maleimide ring against *C. albicans*.

2.7. Antifungal activity of maleimides against clinical isolates of the *Candida* genus

The high activity shown by maleimides against the ATCC standardized *C. albicans* strain and the demonstration that they are truly responsible for the observed antifungal activity, prompted us to test compounds **7**–**11** in a second panel conformed by thirteen clinical isolates of the *Candida* genus. Six of them were *C. albicans* and seven were non-*albicans Candida* spp., including *C. tropicalis*, *C. glabrata*, *C. parapsilopsis*, *C. krusei*, *C. kefyr*, *C. colliculosa* and *C. lusitaneae*. The inclusion of non-*albicans Candida* spp. was due to the high incidence of infections by *non-albicans Candida* spp. observed in recent years in immunocompromised patients as it was stated in the Introduction.[1]

Results showed (Table 1) that maleimides **7**–**11** possessed strong antifungal activities against all the tested *Candida* strains with MICs between 0.48 and 3.90 μg/mL, values that are similar to those of amphotericin B (0.12—1.56 μg/mL) and in some cases better that those of ketoconazole (0.12–6.25 μg/mL). In addition, these maleimides killed *C. albicans* and most non-*albicans Candida* strains with MFCs between 0.97 and 7.8 μg/mL and 1.95–7.8 μg/mL, respectively. The maximum MFC value observed was 32.25 μg/mL.

Table 4. MIC values (μg/mL) of combinations of maleimides **7**–**11** (100% → 0%) with maleamic acids **2**–**6** (0% → 100%)

%		Maleimide/Maleamic acid				
Maleimide	Maleamic Acid	**7/2**	**8/3**	**9/4**	**10/5**	**11/6**
100	0	3.90	3.90	3.90	3.90	3.90
90	10	3.90	3.90	3.90	3.90	3.90
80	20	3.90	3.90	3.90	7.80	7.80
70	30	7.80	7.80	7.80	7.80	7.80
60	40	7.80	7.80	7.80	7.80	7.80
50	50	7.80	7.80	7.80	7.80	15.75
40	60	7.80	7.80	15.75	15.75	15.75
30	70	15.75	15.75	15.75	15.75	15.75
20	80	31.25	31.25	31.25	31.25	31.25
10	90	62.5	62.5	31.25	62.5	62.5
0	100	750	750	1000	1000	1000

3. Conclusion

These studies deepen the knowledge of the behavior of *N*-phenyl- and *N*-phenylalkylmaleimides **7**–**11** as anticandidal agents demonstrating that (a) they display antifungal activities with their intact maleimide ring; (b) the opening of the maleimide ring would lead to a loss of antifungal activity; (c) they are not only fungistatic but fungicidal with very low MICs and MFCs, killing the fungi in about 90 min; (d) they display strong fungicidal activities not only against standardized but also clinical isolates of *C. albicans* and non-*albicans Candida* spp., enhancing the importance of their antifungal behavior.

Taking into account that fungicidal compounds, mainly acting against *Candida* spp., are highly needed, these results open an important possibility for the development of new promising anticandidal drugs.

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 200 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 $CDCl_3$ at 7.26 ppm for protons and at 77.0 ppm for carbons). Signals are designated as follows: s, singlet; d, doublet; dd, doublets of doublets; t, triplet; dt: doublets of triplets; m, multiplet; q, quartet; quint, quintuplet; b, broad. All gas chromatograms were obtained in a CG-MS Turbo Mass Perkin Elmer, column PE1 30m × 0.25 mm of inner diameter, film 0.1 μm, ionization energy 70 eV.

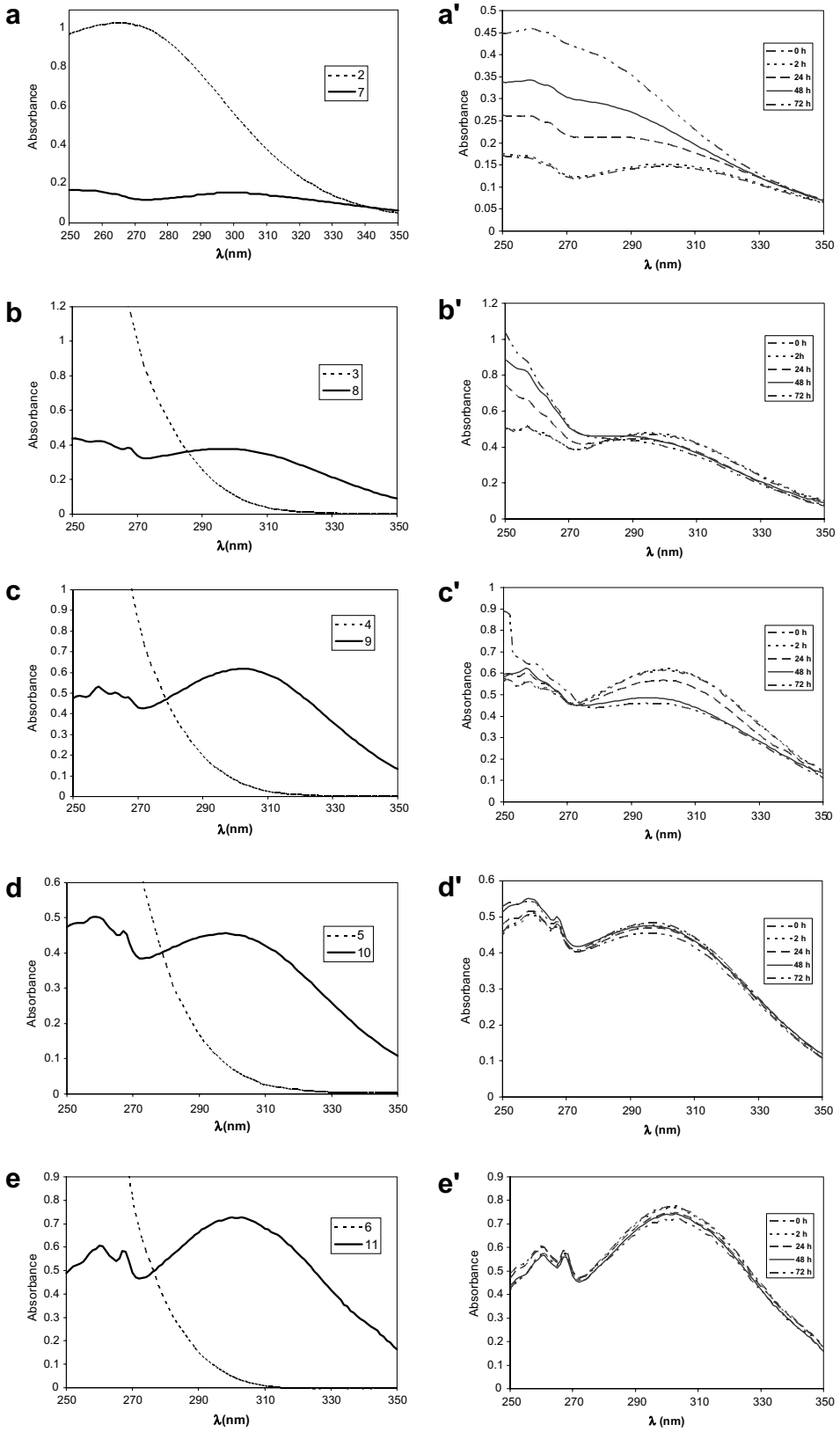


Figure 5. (a–e) UV spectra of each maleimide and its respective maleamic acid in recently prepared water solutions. In detail: (a) *N*-phenylamaleimide **7** and *N*-phenylmaleamic acid **2**. (b) *N*-bencylmaleimide **8** and *N*-bencylmaleamic acid **3**. (c) *N*-phenethylmaleimide **9** and *N*-phenethylmaleamic acid **4**. (d) *N*-phenylpropylmaleimide **10** and *N*-phenylpropylmaleamic acid **5**. (e) *N*-phenylbutylmaleimide **11** and *N*-phenylbutylmaleamic acid **6**. (a′–e′) UV spectra of each maleimide (250 μg/mL) **7**–**11** in aqueous solutions, after different times of incubation (0 to 72 h).

UV–vis spectra were recorded in a Beckman DU-640 spectrophotometer (USA). Elemental analyses were performed on a Perkin-Elmer 2400 series II analyzer (Norwalk, CT, USA). 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 (U.K.) and were uncorrected.

4.2. General procedure for the synthesis of maleamic acids 2–6

The compounds were obtained according to published methodologies[17] with yields ranging from 90 to 98%. Reaction mixtures containing maleic anhydride (**1**) (500 mg, 5.1 mmol in $CHCl_3$ (5 mL)) and equimolar amounts of the appropriate amine (Ph-$(CH_2)_n$-NH_2; n = 0-4) dissolved in $CHCl_3$ (1 mL) were stirred during 1–2 h. The solid which precipitated out of the reaction mixture was filtered and washed thoroughly with water. *N*-phenyl maleamic acid **2** and *N*-phenylalkylmaleamic acids **3** and **4** have been previously reported[20,21]. To our knowledge, their spectroscopic data were not described previously. For the numbering of maleamic acids, see Scheme 1.

4.2.1. *N*-Phenyl maleamic acid (2). White crystals; yield 98%; mp: 203.0–203.6 °C. UV (MeOH) λ_{max} (log ε): 265 (2.9).^{1}H RMN (200 MHz) δ 6.33 (1H, d, J = 12.3, H-2), 6.51 (1H, d, J = 12.3 Hz, H-3), 7.45 (5H, m, H_{Ar}); ^{13}C RMN (50 MHz) δ 119.4 (C-2″,6″), 123.8 (C-4″), 128.7 (C-3″,5″), 130.3 (C-1″), 131.4 (C-2), 138.5 (C-3), 163.2 (*C*ONHR), 163.2 (*C*OOH); GC: t_R = 9.18 min. EIMS *m*/*z* 173 (M^+-H_2O) (100), 129 (28), 93 (27), 54 (52). Anal for $C_{13}H_{15}NO_3$. Calcd (%): C, 62.82; H, 4.74; N, 7.33. Found (%): C, 62.93; H, 4.75; N, 7.34.

4.2.2. *N*-Benzyl maleamic acid (3). White crystals; yield 96%; mp: 142.2–142.9 °C. UV (MeOH): not maximum appears from 350 to 250 nm; at λ < 310 the absorbance increases rapidly up to 250 nm. ^{1}H RMN (200 MHz) δ 4.47 (2H, s, H-1′), 6.26 (1H, d, J = 12.6, H-2), 6.41 (1H, d, J = 12.6 Hz, H-3), 7.45 (5H, m, H_{Ar}); ^{13}C RMN (50 MHz) δ 44.7 (C-1′), 128.6 (C-4″), 129.0 (C-2″,6″), 129.7 (C-3″,5″), 130.9 (C-2), 133.4 (C-3), 134.2 (C-1″), 167.7 (*C*ONHR), 176.8 (*C*OOH); GC: t_R = 19.0 min. EIMS *m*/*z* 106 (100), 91 (65). Anal for $C_{13}H_{15}NO_3$. Calcd (%): C, 64.38; H, 5.40; N, 6.83. Found (%): C, 64.47; H, 5.41; N, 6.85.

4.2.3. *N*-Phenylethyl maleamic acid (4). White crystals; yield 96%; mp: 138.0–138.7 °C. UV (MeOH): not maximum appears from 350 to 250 nm; at λ < 310 the absorbance increases rapidly up to 250 nm. ^{1}H RMN (200 MHz) δ 2.92 (2H, t, J = 7.2 Hz, H-2′), 3.68 (2H, dt (appearing as a q), J = 7.0 Hz, H-1′), 6.10 (1H, d, J = 12.8, H-2), 6.36 (1H, d, J = 12.8 Hz, H-3), 7.20 (5H, m, H_{Ar}); ^{13}C RMN (50 MHz) δ 34.7 (C-2′), 41.7 (C-1′), 126.7 (C-4″), 128.6 (C-2″,6″), 128.7 (C-3″,5″), 132.5 (C-2), 135.4 (C-3), 138.0 (C-1″), 166.3 (*C*ONHR), 166.7 (*C*OOH); GC: t_R = 10.71 min. EIMS *m*/*z* 201 (M^+-H_2O) (25), 110 (34), 104 (100), 91 (40). Anal for $C_{13}H_{15}NO_3$. Calcd (%): C, 65.74; H, 5.98; N, 6.39. Found (%): C, 65.87; H, 6.99; N, 6.40.

4.2.4. *N*-Phenylpropyl maleamic acid (5). White crystals; yield 90%; mp: 101.3–101.6 °C. UV (MeOH): not maximum appears from 350 to 250 nm; at λ < 310 the absorbance increases rapidly up to 250 nm. ^{1}H RMN (200 MHz) δ 1.95 (2H, dt (appearing as a quint), J = 7.4 Hz, H-2′), 2.69 (2H, t, J = 7.2 Hz, H-3′), 3.41 (2H, dt (appearing as a q) J = 7.0 Hz, H-1′), 6.28 (1H, d, J = 12.8, H-2), 6.41 (1H, d, J = 12.8 Hz, H-3), 7.20 (5H, m, H_{Ar}), 7.81 (1H, bs, NH); ^{13}C RMN (50 MHz) δ 29.9 (C-3′), 33.0 (C-2′), 40.1 (C-1′), 126.0 (C-4″), 128.3 (C-2″,6″), 128.4 (C-3″,5″), 132.7 (C-2), 135.3 (C-3), 140.9 (C-1′), 166.3 (*C*ONHR), 166.7 (*C*OOH); GC: t_R = 12.36 min. EIMS *m*/*z* 215 (M^+-H_2O) (25), 117 (100), 111 (45), 91 (38), 82 (25). Anal for $C_{13}H_{15}NO_3$. Calcd (%): C, 66.94; H, 6.48; N, 6.02. Found (%): C, 66.74; H, 6.45; N, 6.04.

4.2.5. *N*-Phenylbutyl maleamic acid (6). White crystals; yield 92%; mp: 96.7–97.3 °C. UV (MeOH): not maximum appears from 350 to 250 nm; at λ < 310 the absorbance increases rapidly up to 250 nm. ^{1}H RMN (200 MHz) δ 1.67 (4H, m, H-2′,3′), 2.65 (2H, t, J = 7.2 Hz, H-4′), 3.39 (2H, dt (appearing as a q) J = 6.8 Hz, H-1′), 6.28 (1H, d, J = 12.8 Hz, H-2), 6.43 (1H, d, J = 12.8 Hz, H-3), 7.24 (5H, m, H_{Ar}), 7.76 (1H, bs, NH); ^{13}C RMN (50 MHz) δ 28.1 (C-3′), 28.5 (C-2′), 35.3 (C-4′), 40.3 (C-1′), 125.8 (C-4″), 128.2 (C-2″,6″), 128.3 (C-3″,5″), 135.2 (C-2), 135.2 (C-3), 141.8 (C-1″), 166.2 (C-4), 166.3 (C-1); GC: t_R = 13.55 min. EIMS *m*/*z* 229 (M^+-H_2O; 47%), 131 (35), 110 (30), 91 (100), 82 (24). Anal for $C_{14}H_{17}NO_3$. Calcd (%): C, 68.00; H, 6.93; N, 5.66. Found (%): C, 68.16; H, 6.94; N, 5.68.

4.3. General procedure for the synthesis of maleimides 7–11

A reaction mixture containing the appropriated maleamic acid in 5 mL of acetic anhydride and 100 mg of sodium acetate was heated for 2 h under reflux, according to previous reports.[17,22] The reaction was cooled and quenched with water. The aqueous solution was extracted with Et_2O, dried with Na_2SO_4, filtered, and the solvent was evaporated. *N*-phenylmaleimide **7** and *N*-phenylalkylmaleimides **8**–**11** were obtained with yields ranging from 45% to 98%. Their spectroscopic data were identical to reported data.[23]

4.4. Antifungal evaluation

4.4.1. Microorganisms and media. Fungal species from the American Type Culture Collection (ATCC, Rockville, MD, USA), and clinical isolates from the Centro de Referencia en Micología (C, CEREMIC, Rosario, Argentina) were used. The panel included 14 strains of *Candida* spp. (seven of them were *C. albicans* and seven were non-*albicans Candida* spp.); voucher specimens are detailed in Table 1. Strains were grown on Sabouraud-chloramphenicol agar slants for 24 h at 37 °C, and maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid). Inocula of cell suspensions were obtained according to reported procedures and adjusted to 1–5×10^4 colony forming units (CFU)/mL.[14]

4.4.2. MIC and MFC determinations of maleimides and maleamic acids. Stock solutions of compounds (100 μL) were diluted twofold with RPMI-1640 (Sigma, St. Louis, MO, USA) (final concentrations 250–0.98 μg/mL and a final DMSO concentration ⩽2%) according to the M27-A2 method recommended by CLSI (formerly NCCLS),[14] with the modification that the inoculum used was $1–5 \times 10^4$ instead of $1–5 \times 10^3$ CFU/mL. About 100 μL of the inoculum suspension was added to each well. Ketoconazole (Janssen Pharmaceutical, Titusville, NJ, USA) and amphotericin B (Sigma) were used as positive controls. MIC was defined as the lowest concentration of a compound that induced total inhibition of growth as read visually. MFC was determined by plating in duplicate 5 μL from each clear well of MIC determinations, onto a 150-mm SDA plate. After 48 h at 37 °C, MFCs were determined as the lowest concentration of each compound showing no growth.

4.4.3. MIC determinations of maleimides pre-incubated in the growth media. Five empty 96-microwell plates were used for this assay. Each row of the plates was filled-in during 7 consecutive days (see Fig. 4). (a) Day 0: twofold diluted series (1000–0.98 μg/mL) of each maleimide (with all ingredients as to determine MIC except the inoculum) were put into the first row of each plate, therefore having the five plates with only the first row filled-in. (b) Day 1: a second series of dilutions of each maleimide was prepared and put into the second row of the plates. (c) Days 2–7: The same procedure was performed during six more consecutive days at the same time of the day on the six following rows.

At the end of the seventh day, all wells of each plate were inoculated with *C. albicans* ATCC 10231 (inocula size $1–5 \times 10^4$ CFU/mL) and put at 37 °C (Dalvo Incubator, Buenos Aires) for 24 h. A control of growth was added to each row. The MIC of each maleimide tested with 0–7 days pre-incubation times was recorded. The experiment was performed in duplicate.

4.4.4. Time-to-kill assays. The time-course of fungicidal activity of each maleimide was determined by enumerating viable cells in 5 mL of RPMI-1640 medium containing an inoculum of *C. albicans* ATCC *10231* ($2–2.5 \times 10^4$ CFU/mL) and 1× MFC of the tested compound. The culture vials were incubated with agitation at 37 °C.[24,25] Samples of 10 μL were removed from each vial at fixed times (0, 30, 60, 90, and 120 min, 4, 8, 12, and 24 h), diluted appropriately if necessary, and plated onto drug-free SDA plates. Colony were counted after incubation of the plates at 37 °C for 24–48 h. All time-to kill experiments were conducted in duplicate.

4.4.5. Chequerboard method. Interactions of maleimides **7–11** with maleamic acids **2–6** when acting against *C. albicans* ATCC *10231* were determined by using the microbroth dilution chequerboard method with incubations at 37 °C for 24 h.[26,27] Seven twofold dilutions of each maleimide and seven twofold dilutions of maleamic acids at such a range of drug concentrations that encompassed the MIC of each compound used were tested in combination. Microbroth dilution wells were inoculated with *C. albicans* ($1–5 \times 10^4$ CFU/mL) in a 200 μL final volume.

The FICI was calculated by adding the FIC of each maleimide (M) to the FIC of maleamic acid (MA) for each combination using the following formula:[28]

$$\begin{aligned} \mathrm{FICI} &= \mathrm{FIC_M} + \mathrm{FIC_{MA}} \\ &= (\mathrm{MIC_{M\ combination}}/\mathrm{MIC_{M\ alone}}) \\ &\quad + (\mathrm{MIC_{MA\ combination}}/\mathrm{MIC_{MA\ alone}}). \end{aligned}$$

The experiments were performed by duplicate and results are the mean of both values.

4.4.6. MIC determinations of different mixtures of maleimide/maleamic acids. Stock solutions of mixtures at different ratios (0–100%/100–0%) of each pair of maleimide/maleamic acid, namely **7/2**, **8/3**, **9/4**, **10/5**, and **11/6** (100 μL), were twofold diluted with RPMI-1640 to obtain a series of concentrations (250–0.98 μg/mL and a final DMSO concentration ⩽2%). About 100 μL of inoculum suspension was added to each well and MIC was determined according to CLSI guidelines (formerly NCCLS) as explained above.

4.4.7. Spectrophotometric experiments. The absorption spectra were obtained scanning the samples between 250 and 350 nm with a Beckman DU-640 spectrophotometer. Each experiment was started with a solution of each maleimide in water at 250 μg/mL (with the addition of minimal quantities of DMSO to assure their solubility). Beer's law was confirmed for each maleimide in a range between 0.10 and 1000 μg/mL.

An aliquot was quickly transferred to a quartz cell kept pre-incubated (37 °C) in the thermostatic cell-holder of the spectrophotometer. The progress of hydrolysis was followed by monitoring the variability of the peak at 300 nm at regular intervals (0, 2, 24, 48, and 72 h). UV spectra of maleamic acids were obtained under the same conditions as maleimides without incubation.

Acknowledgments

S.Z. and M.S. are grateful to ANPCyT (PICTR 260) and CONICET, CYTED (RIBIOFAR network/RT 0284) and UNR (1BIO133). V.C.F. thanks CNPq/Brazil.

References and notes

1. Pfaller, M.; Diekema, D. *Clin. Microb. Rev.* **2007**, *20*, 133.
2. Beck-Sagué, C. M.; Jarvis, W. R. The National Nosocomial Infections Surveillance System. *J. Infect. Dis.* **1993**, *167*, 1247.
3. Edmond, M. B.; Wallace, S. E.; McClish, D. K.; Pfaller, M. A.; Jones, R. N.; Wenzel, R. P. *Clin. Infect. Dis.* **1999**, *29*, 239.
4. Pfaller, M.; Diekema, D. *J. Clin. Microb.* **2004**, *42*, 4419.
5. Nucci, M.; Marr, K. *Clin. Infect. Dis.* **2005**, *41*, 521.

6. Cuenca-Estrella, M.; Rodriguez, D.; Almirante, B.; Morgan, J.; Planes, A. M.; Almela, M.; Mensa, J.; Sanchez, F.; Ayats, J.; Giménez, M.; Salvado, M.; Warnock, D.; Pahissa, A.; Rodriguez-Tudela, J. L. On behalf of the Barcelona Candidemia Project Study Group. *J. Antimicrob. Chemother.* **2005**, *55*, 194.
7. Vicente, M. F.; Basilio, A.; Cabello, A.; Peláez, F. *Clin. Microbiol. Infect.* **2003**, *9*, 15.
8. López, S.; Ramallo, I.; González Sierra, M.; Zacchino, S.; Furlán, R. *PNAS* **2007**, *104*, 441.
9. Sortino, M.; Delgado, P.; Juárez, S.; Quiroga, J.; Abonía, R.; Insuasty, B.; Nogueras, M.; Rodero, L.; Garibotto, F.; Enriz, R.; Zacchino, S. *Bioorg. Med. Chem.* **2007**, *15*, 484.
10. Massman, M.; Somlai, C.; Rodríguez, A.; Sortino, M.; Zacchino, S.; Enriz, R. *Bioorg. Med. Chem.* **2006**, *14*, 7604.
11. Suvire, F.; Sortino, M.; Kouznetsov, V.; Vargas, L.; Zacchino, S.; Enriz, R. *Bioorg. Med. Chem.* **2006**, *14*, 1851.
12. Vargas, L.; Castelli, M.; Kouznetsov, V.; Urbina, J.; López, S.; Sortino, M.; Enriz, R.; Ribas, J. C.; Zacchino, S. *Bioorg. Med. Chem.* **2003**, *11*, 1531.
13. López, S.; Castelli, M. V.; de Campos, F.; Corrêa, R.; Cechinel Filho, V.; Yunes, R.; Zamora, M.; Enriz, R.; Ribas, J. C.; Furlán, R.; Zacchino, S. *Arzn Forsch (Drug Res)*. **2005**, *55*, 123.
14. NCCLS, National Committee for Clinical and Laboratory Standards, Method M27-A2, 2nd ed., Wayne Ed.; 2002, Vol. 22 (15), pp 1–29.
15. Khan, M. *J. Pharm. Sci.* **1984**, *73*, 1767.
16. Johnson, M.; McDougall, C.; Ostrosky-Zeichner, L.; Perfect, J.; Rex, J. *Antimicrob. Agents Chemother.* **2004**, *48*, 693.
17. Kalgutkar, A.; Crews, B. C.; Marnett, L. J. *J. Med. Chem.* **1996**, *39*, 1692.
18. Odds, F.; Motyl, M.; Andrade, R.; Bille, J.; Cantón, E.; Cuenca-Estrella, M.; Davidson, A.; Durussel, C.; Ellis, D.; Foraker, E.; Fothergill, A.; Ghannoum, M.; Giacobbe, R.; Gobernado, M.; Hadke, R.; Laverdière, M.; Yang, W.; Merz, W.; Ostrosky, L.; Peman, J.; Perea, S.; Perfect, J.; Pfaller, M.; Proia, L.; Rex, J.; Rinaldi, M.; Rodriguez, J.; Schell, W.; Shields, C.; Sutton, D.; Verjweij, P.; Warnock, D. *J. Clin. Microb.* **2004**, *42*, 3475.
19. Quan, H.; Cao, Y.; Xu, Z.; Zhao, J.; Gao, P.; Qin, X.; Jiang, Y. *Antimicrob. Agents Chemother.* **2006**, *50*, 1096.
20. Metha, N.; Phillips, A.; Fu, F.; Brooks, R. *J. Org. Chem.* **1960**, *25*, 1012.
21. Frankel, M.; Liwschitz, Y.; Amiel, Y. *J. Am. Chem. Soc.* **1953**, *75*, 330.
22. Cava, M.; Deana, A.; Muth, K.; Mitchell, M. *Org. Synth.* **1973**, *5*, 944.
23. Cechinel Filho, V.; Queiroz, E.; Lima, E.; Pinheiro, T.; Nunes, R.; Yunes, R. *Quim Nova.* **1996**, *19*, 590.
24. Klepser, M.; Wolfe, E.; Jones, R.; Hightingale, C.; Pfaller, M. *Antimicrob. Agents Chemother.* **1997**, *41*, 1392.
25. Klepser, M.; Ernst, E.; Lewis, R.; Ernst, M.; Pfaller, M. *Antimicrob. Agents Chemother.* **1998**, *42*, 1207.
26. Rand, K.; Houck, H.; Brown, P.; Bennett, D. *Antimicrob. Agents Chemother.* **1993**, *37*, 613.
27. Lewis, R.; Diekema, D.; Messer, S.; Pfaller, M.; Klebser, M. *J. Antimicrob. Chemother.* **2002**, *49*, 345.
28. Arikan, S.; Lozano-Chiu, M.; Paetznick, V.; Rex, J. *Antimicrob. Agents Chemother.* **2002**, *46*, 245.