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Short communication

Naphthalimido derivatives as antifolate thymidylate synthase inhibitors

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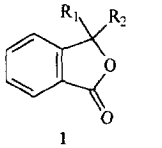
Summary — A new series of *N*-(substituted)benzyl-1,8-naphthalimides **4**, structurally related to the previously reported thymidylate synthase (TS) inhibitor naphthaleins **3**, were synthesized and compounds tested for their inhibition of several species of TS. Moreover, their in vitro cytotoxicity together with antimycotic and antibacterial properties were assayed. While no activity was detected in the antibacterial tests, the *m*-nitro (**4ae**) and the *p*-nitro (**4af**) derivatives were found able to partially inhibit TS at low micromolar concentrations. Introduction of nitro or (substituted)-amino groups in position 4 of the naphthalic ring always led to less active compounds.

thymidylate synthase / inhibition / naphthalimido derivatives

Introduction

Thymidylate synthase (TS) is an interesting enzyme target in cancer chemotherapy since its inhibition blocks the de novo synthesis of 2'-deoxythymidine-5'-monophosphate (dTMP): DNA is no longer synthesized and the cell replication stops [1]. A double substrate enzyme can be inhibited by compounds competing with either 2'-deoxyuridine-5'-monophosphate (dUMP) or a cofactor function. The antifolates are a very interesting tool because of their potential high structural specificity. In fact, selected modifications in the basic pteridinic ring give compounds with either antimicrobial or antitumor action [2]. TS-inhibiting agents are usually structurally related to the cofactor and follow a similar metabolic pathway. However, though favouring their accumulation in the cells and their affinity towards TS binding sites, this similarity is also the source of resistance for the best known folate analogues such as CB3717 [3]. Recently, some compounds non-structurally related to folate but with good affinity towards folate binding site of TS have been synthesized with the aim of overcoming the above-mentioned problems [4, 5].

We have previously described several classes of phthalido derivatives (**1–3**) provided with general competitive inhibition pattern and apparent inhibition constant in the low micromolar range against *Lactobacillus casei* TS (LcTS). While their activity was reported to slightly increase passing from **1** to **3**, the specific interactions with the enzyme are common



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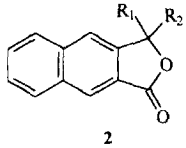

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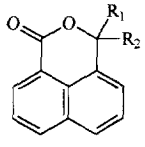

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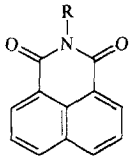

4

For **1, 2, 3**

$R_1 = R_2$ = (substituted)phenol
R_1 = OH R_2 = (substituted)phenol

For **4**

R = (substituted)benzyl

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to these ligands and involve the two phenolic hydroxy groups and the lactonic carbonyl [6, 7]. However, it should be noted that some derivatives with only one phenolic ring still retain LcTS inhibitory activity (Costi, unpublished results). On this basis, to further assess the real importance of these requirements, we have now modified the lactonic moiety of structure **3** to the imidic derivatives **4**. Besides giving more information on the essential requirements for these TS inhibitors, this substitution should increase their stability with respect to the models **1–3** [8, 9].

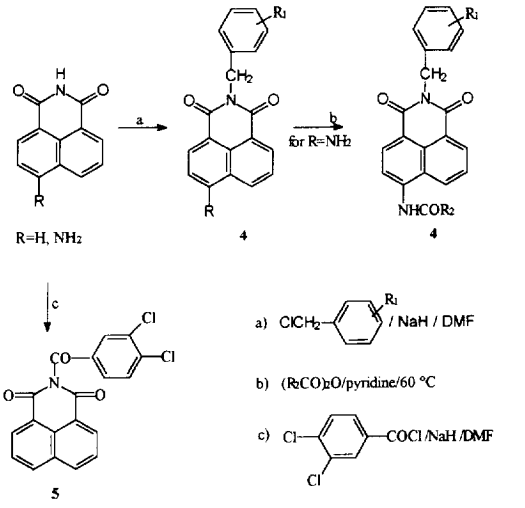
This paper describes the synthesis of compounds **4** and their ability to inhibit different species' TS. Moreover, several in vitro assays have been performed; cytotoxicity has been evaluated on MT-4 and C8166 cells together with their capability to stop the cell multiplication of various fungi and bacteria.

Chemistry

Compounds **4** were prepared following two alternatives pathways depicted in schemes 1 and 2. According to *Method a* (scheme 1), the appropriate 1,8-naphthalimide was condensed with the required benzyl chloride in the presence of sodium hydride, using DMF as the solvent. The desired **4** were isolated by suction after pouring the mixture onto ice/water. The acyl-substituted derivatives were easily obtained by condensing the corresponding amino substituted with acetic or butyric anhydride in pyridine at 60 °C. According to *Method b* (scheme 2), a solution of 4-nitro-1,8-naphthalic anhydride was refluxed overnight with the required benzylamino derivative in ethanol. All compounds were finally purified by trituration with hot ethanol or by silica-gel chromatography, eluting with dichloromethane/methanol (see table I for data).

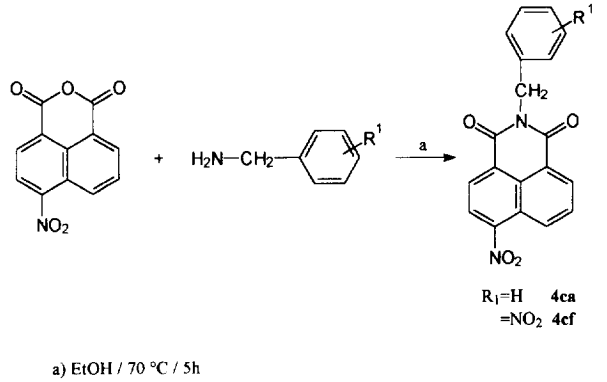
Biological assays

The compounds were tested for their inhibitory activity against LcTS. The most interesting derivatives were also screened against *Cryptococcus neoformans* TS (CnTS) and human TS. The inhibition assays were performed spectrophotometrically by standard methods (see table II). Moreover, they were evaluated in vitro for cytotoxicity against MT-4 and C8166 cell lines. Finally, the capability of compounds to inhibit the multiplication of various human pathogenic fungi (such as *Candida albicans, C parapsilosis, C paratropicalis, Trycophyton mentagrophytes, Microsporum canis*, and *Aspergillus fumigatus*) and bacteria (*Staphylococcus aureus*, group D *Streptococcus*, *Pseudomonas aeruginosa* and *Escherichia coli*) was tested. Econazole and streptomycin were used as reference compounds for antimycotic and antibacterial assays, respectively.



4	R	R_1	R_2	4	R	R_1	R_2
4aa	H	H	-	**4bb**	NH_2	3-OCH_3	-
4ab	H	3-OCH_3	-	**4bc**	NH_2	4-OCH_3	-
4ac	H	4-OCH_3	-	**4bd**	NH_2	4-F	-
4ad	H	4-F	-	**4be**	NH_2	3-NO_2	-
4ae	H	3-NO_2	-	**4bf**	NH_2	4-NO_2	-
4af	H	4-NO_2	-	**4de**	-	3-NO_2	CH_3
4ag	H	3-NO_2,4-Cl	-	**4df**	-	4-NO_2	CH_3
4ba	NH_2	H	-	**4ef**	-	4-NO_2	$(CH_2)_2CH_3$

Scheme 1. *Method a.*



Scheme 2. *Method b.*

Table I. Physicochemical properties of compounds **4**.

Compound	*R*	R_1	*Yield (%)*	*Mp (° C)*	*Formula*	*^{1}H-NMR δ (ppm)*
4aa	H	H	72	195–197	$C_{19}H_{13}NO_2$	5.4 (s, 2H), 7.3–7.4 (m, 5H), 7.9 (t, 2H, *J* = 7.7 Hz), 8.5 (dd, 4H, *J* = 7.3 and 8.2 Hz)
4ab	H	3-OCH_3	70	146–147	$C_{20}H_{15}NO_3$	3.8 (s, 3H), 5.3 (s, 2H), 6.8–6.9 (m, 1H), 7.1–7.2 (m, 3H), 7.7 (t, 2H, *J* = 7.8 Hz), 8.2 (d, 2H, *J* = 8.2 Hz), 8.6 (d, 2H, *J* = 7.2 Hz)
4ac	H	4-OCH_3	50	185–186	$C_{20}H_{15}NO_3$	3.7 (s, 3H), 5.3 (s, 2H), 6.7 (d, 2H, *J* = 8.4 Hz), 7.3 (d, 2H, *J* = 8.4 Hz), 7.7 (t, 2H, *J* = 7.7 Hz), 8.5 (dd, 4H, *J* = 7.7 and 9.0 Hz)
4ad	H	4-F	55	181–183	$C_{19}H_{12}FNO_2$	5.4 (s, 2H), 7.1 (t, 2H, *J* = 8.7 Hz), 7.3–7.4 (m, 2H), 7.9 (t, 2H, *J* = 7.7 Hz), 8.5 (dd, 4H, *J* = 6.9 and 7.7 Hz)
4ae	H	3-NO_2	53	207–209	$C_{19}H_{12}N_2O_4$	5.4 (s, 2H), 7.7 (dd, 1H, *J* = 7.9 and 8.0 Hz), 7.9 (dd, 3H, *J* = 7.7 and 8.0 Hz), 8.0–8.1 (m, 1H), 8.3 (s, 1H), 8.6 (dd, 4H, *J* = 7.0 and 7.9 Hz)
4af	H	4-NO_2	83	268–269	$C_{19}H_{12}N_2O_4$	5.4 (s, 2H), 7.7 (d, 2H, *J* = 8.4 Hz), 7.9 (t, 2H, *J* = 7.7 Hz), 8.2 (d, 2H, *J* = 8.4 Hz), 8.6–8.7 (m, 4H)
4ag	H	3-NO_2, 4-Cl	81	186–188	$C_{19}H_{11}ClN_2O_4$	5.4 (s, 2H), 7.5 (d, 1H, *J* = 8.5 Hz), 7.7 (d, 1H, *J* = 7.4 Hz), 7.9 (t, 2H, *J* = 7.8 Hz), 8.3 (d, 1H, *J* = 0.5 Hz), 8.5 (d, 4H, *J* = 7.7 Hz)
4ba	NH_2	H	60	240–241	$C_{19}H_{14}N_2O_2$	5.4 (s, 2H), 6.9 (d, 1H, *J* = 8.0 Hz), 7.2–7.3 (m, 5H), 7.4 (br s, 2H, exch with D_2O), 7.7 (t, 1H, *J* = 7.2 Hz), 8.3 (d, 1H, *J* = 8.0 Hz), 8.4 (d, 1H, *J* = 7.2 Hz), 8.7 (d, 1H, *J* = 8.0 Hz)
4bb	NH_2	3-OCH_3	54	214–216	$C_{20}H_{16}N_2O_3$	3.7 (s, 3H), 5.3 (s, 2H), 6.8–7.0 (m, 4H), 7.3 (dd, 1H, *J* = 7.6 and 8.0 Hz), 7.5 (s, 2H, exch with D_2O), 7.7 (dd, 1H, *J* = 7.6 and 8.0 Hz), 8.3 (d, 1H, *J* = 8.0 Hz), 8.5 (d, 1H, *J* = 7.0 Hz), 8.7 (d, 1H, *J* = 8.6 Hz)
4bc	NH_2	4-OCH_3	73	186–189	$C_{20}H_{16}N_2O_3$	3.7 (s, 3H), 5.3 (s, 2H), 6.7 (d, 3H, *J* = 8.0 Hz), 7.3 (d, 2H, *J* = 7.8 Hz), 7.5 (s, 2H, exch with D_2O), 7.7 (t, 1H, *J* = 6.9 Hz), 8.3 (d, 1H, *J* = 8.6 Hz), 8.5 (d, 1H, *J* = 6.9 Hz), 8.7 (d, 1H, *J* = 8.6 Hz)
4bd	NH_2	4-F	90	>260	$C_{19}H_{13}FN_2O_2$	5.3 (s, 2H), 6.9 (d, 1H, *J* = 8.6 Hz), 7.2 (t, 3H, *J*= 8.1 Hz), 7.3–7.4 (m, 2H), 7.5 (s, 2H, exch with D_2O), 7.7 (t, 1H, *J* = 8.1 Hz), 8.3 (d, 1H, *J* = 8.7 Hz), 8.5 (d, 1H, *J* = 7.1 Hz), 8.7 (d, 1H, *J* = 8.6 Hz)

Table I. *Continued.*

Compound	*R*	*R_1*	*Yield (%)*	*Mp (° C)*	*Formula*	*^{1}H-NMR δ (ppm)*
4be	NH_2	3-NO_2	95	268–269	$C_{19}H_{13}N_3O_4$	5.3 (s, 2H), 6.9 (d, 1H, *J* = 8.2 Hz), 7.6–7.8 (m, 5H), 8.2–8.3 (m, 3H), 8.5 (d, 1H, *J* = 7.2 Hz), 8.7 (d, 1H, *J* = 8.2 Hz)
4bf	NH_2	4-NO_2	91	280–282	$C_{19}H_{13}N_3O_4$	5.3 (s, 2H), 6.9 (d, 1H, *J* = 9.3 Hz), 7.6–7.7 (m, 5H), 8.2–8.3 (m, 3H), 8.5 (d, 1H, *J* = 7.3 Hz), 8.7 (d, 1H, *J* = 8.3 Hz)
4ca	NO_2	H	89		$C_{19}H_{12}N_2O_4$	5.3 (s, 2H), 7.2–7.4 (m, 5H), 8.1 (t, 1H, *J* = 8.1 Hz), 8.6–8.8 (m, 4H)
4cf	NO_2	4-NO_2	77	260–262	$C_{19}H_{11}N_3O_6$	5.4 (s, 2H), 7.7 (d, 2H, *J* = 8.1 Hz), 8.0–8.1 (m, 3H), 8.6–8.8 (m, 4H)
4de	$NHCOCH_3$	3-NO_2	46	186–188	$C_{21}H_{15}N_3O_5$	2.3 (s, 3H), 5.4 (s, 2H), 7.7 (t, 1H, *J* = 7.7 Hz), 7.9–8.0 (m, 2H), 8.2 (d, 1H, *J* = 7.7 Hz), 8.4–8.5 (m, 2H), 8.6 (t, 2H, *J* = 7.7 Hz), 8.8 (d, 1H, *J* = 7.7 Hz), 10.5 (s, 1H, exch with D_2O)
4df	$NHCOCH_3$	4-NO_2	45	>280	$C_{21}H_{15}N_3O_5$	2.3 (s, 3H), 5.4 (s, 2H), 7.7 (d, 2H, *J* = 8.6 Hz), 8.0 (t, 1H, *J* = 8.1 Hz), 8.2 (d, 2H, *J* = 8.6 Hz), 8.4 (d, 1H, *J* = 8.1 Hz), 8.7 (dd, 2H, *J* = 7.7 and 8.6 Hz), 8.8 (d, 1H, *J* = 8.6 Hz), 10.5 (s, 1H, exch with D_2O)
4ef	$NHCO(CH_2)_2CH_3$	4-NO_2	86	>280	$C_{23}H_{19}N_3O_5$	1.1 (t, 3H, *J* = 7 Hz), 1.6–1.8 (m, 2H), 2.6 (s, 2H), 5.4 (s, 2H), 7.7 (d, 2H, *J* = 8 Hz), 7.9 (t, 1H, *J* = 7.7 Hz), 8.2 (d, 2H, *J* = 8.0 Hz), 8.4 (d, 1H, *J* = 7.7 Hz), 8.6 (t, 2H, *J* = 7.7 Hz), 8.8 (d, 1H, *J* = 7.7 Hz), 10.3 (s, 1H, exch with D_2O)

Results and discussion

TS inhibition

The compounds were evaluated for their direct inhibitory properties against LcTS. Since their low water solubility prevented us from measuring detailed steady-state kinetic parameters, we determined the percentage of inhibition at the highest concentration compatible with their solubility (table II). Though the majority of the test compounds was found inactive in this assay, the *meta* (**4ae**) and *para* (**4af**) nitro derivatives were able to inhibit the enzyme by a value of about 35% at 5 μM. The presence of a chlorine atom in the *para* position of **4ae** (compound **4ag**) brought about a tenfold loss of activity. Moreover, it should be noted that the introduction of a nitro (**4cf**), amino (**4be**, **4bf**) or amido (**4de**, **4df**, **4ef**) group in position 4 of the naphthalic ring always lowered the inhibitory activity, though by different degrees. To verify the presence of a possible specificity, the most interesting compounds were also tested for their inhibitory properties against other species of TS, eg, CnTS and human TS. Their percentage inhibitions (table II) indicate that no significant specificity could be observed. However, compound **4af**, which shows almost the same inhibitory activity against LcTS and CnTS, did not inhibit human TS.

Table II. TS inhibition.

Compound	*Inhibition*[a]		
	LcTS	*Human TS*	*CnTS*
4ae	35 (5 μM)	NI (5 μM)	4 (5 μM)
4af	33 (5 μM)	NI (5 μM)	35 (5 μM)
4ag	30 (50 μM)	Nd	35 (50 μM)
4be	21 (50 μM)	Nd	NI (50 μM)
4bf	26 (50 μM)	Nd	NI (50 μM)
4cf	17 (5 μM)	Nd	NI (5 μM)
4de	16 (50 μM)	Nd	NI (50 μM)
4df	18 (50 μM)	Nd	NI (50 μM)
4ef	31 (50 μM)	Nd	Nd

[a]Percentage inhibition at a given concentration (in parentheses); NI: no inhibition; Nd: not determined.

Cell replication inhibition

None of the test compounds showed any significant activity in these assays up to a 300 μM concentration. The apparent inconsistency between these results and TS inhibition data for the most active compounds could possibly be explained assuming a bad cell membrane penetration, due to their low water solubility. Therefore, further efforts will be directed to improve this parameter.

Conclusion

In conclusion, the above results for this class indicate much lower activity with respect to the model in the enzymatic assays and absence of activity in the other in vitro assays. The better profile shown by the nitro-substituted **4ae** and **4af** seems to suggest that the presence of a strong electron-attracting group on the phenyl ring which could induce a significant polarization of the aromatic ring, is an essential requirement for TS-inhibiting properties of this class, thus confirming what has already been seen in the model [5–7]. However, contrary to the model, substitution of the naphthalene ring led to less active (**4cf**) or very weakly active (**4de**, **4df** and **4ef**) compounds. In this regard, it should be noted that the methylene bridge confers much more conformational freedom to the naphthalic ring, with respect to the quaternary carbon of the model **3**. Thus, assuming that the benzylic ring of compounds **4** will be anchored at the top of the folate active site as seen for the phenol ring of the reference compounds [7], the naphthalene ring of **4** could arrange in different ways with respect to the model in the large space of the binding pocket on the enzyme.

Experimental protocols

Chemistry

Melting points were determined on a Büchi 510 capillary melting points apparatus and are uncorrected. Analyses indicated by the symbols were within ±0.4% of the theoretical values. ^{1}H-NMR spectra were recorded on a Brüker AC200 spectrometer; chemical shifts are reported as δ (ppm) relative to tetramethylsilane as internal standard and coupling constants (*J*) are in Hz. Splitting patterns are designated as follows: s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. DMSO-d_6 was used as the solvent, unless otherwise noted. TLC on silica gel plates was used to check product purity. Silica gel 60 (Merck; 70-230 mesh) was used for column chromatography. The structures of all compounds were consistent with their analytical and spectroscopic data.

N-(Substituted)benzyl-1,8-naphthalimides ***4****. Method a*
To a solution of the required 1,8-naphthalimide (0.01 mol) in DMF (40 mL), NaH (0.02 mol) was added and the mixture stirred for 30 min at rt. The appropriate benzyl chloride was then added and the mixture stirred for further 3 h at rt. Water was added under cooling, the so-formed precipitate filtered by suction and thoroughly washed with ethanol (see table I for data).

N-(Substituted)benzyl-4-nitro-1,8-naphthalimides ***4****. Method b*
To a solution of 4-nitro-1,8-naphthalic anhydride (0.02 mol) in toluene (30 mL) a solution of the properly substituted benzylamine (0.02 mol) in ethanol (30 mL) was added dropwise and the mixture refluxed for 5 h. After evaporation of the solvent, the desired **4** were purified by column chromatography, eluting with CH_2Cl_2/MeOH 9:1 (see table I for data).

N-(Nitrobenzyl)-4-acyl-1,8-naphthalimides ***4****. General method*
A solution of the required **4** (0.01 mol) and the appropriate anhydride (0.05 mol) in pyridine (30 mL) was stirred overnight at 60 °C. After cooling, the mixture was poured onto water (30 mL) and extracted with CH_2Cl_2 (3 x 20 mL). The organic layer was washed in succession with 6 N HCl and water. After evaporation of the solvent, the desired **4** were purified by column chromatography, eluting with CH_2Cl_2/MeOH 9:1 (see table I for data).

N-(3,4-Dichlorobenzoyl)-1,8-naphthalimide ***5***
To a solution of 1,8-naphthalimide (0.2 g, 0.001 mol) in DMF (6 mL), NaH (0.033 g, 0.001 mol) was added in small portions and the mixture stirred for 30 min at rt. Equimolar 3,4-dichlorobenzoylchloride was then added and the mixture stirred for further 3 h at rt. Water (3 mL) was cautiously added and the so-formed precipitate filtered by suction to give 0.27 g (72%) of **5**. ^{1}H-NMR δ: 7.8–8.0 (m, 4H), 8.5 (d, 5H, J = 7.7 Hz). Anal $C_{19}H_9Cl_2NO_3$ (C, H, N).

Biology

Enzyme assays
Plasmids that express LcTS in *E coli* strain X2913 have been described previously [10]. The enzyme was purified by column chromatography method using phosphocellulose (P11, Biorad) and hydroxyapatite (HAP, Biorad) resin, with phosphate buffer as eluent [11]. CnTS and human TS have been purified as reported [12, 13]. The enzyme preparations were >95% homogeneous as shown by SDS-polyacrylamide gel electrophoresis. The purified enzyme have been stored at –80 °C in 10 mM phosphate buffer, pH 7.0, 0.1 mM EDTA until use. TS activity was monitored spectrophotometrically at 340 nm using a Perkin-Elmer λ15 spectrophotometer as previously described [14]. Assays were performed at 20 °C in the standard assay buffer, which contained 50 mM *N*-tris-(hydroxymethyl-2-aminoethane)sulfonic acid (TES), pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA, 75 mM β-mercaptoethanol; dUMP concentration was 120 μM and N_5,N_{10}-methylenetetrahydrofolate 82 μM. The inhibitory properties of the compounds were determined as percent inhibition at a given concentration.

In vitro assays

Compounds. The compounds were dissolved in DMSO at an initial concentration of 300 μM and they were serially diluted in culture medium.

Cells. MT-4 and C8166 cells were grown in RPMI-1640 containing 10% fetal calf serum (FCS), 100 IU/mL penicillin G and 100 μg/mL streptomycin. Cell culture were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco). Bacterial and fungal strains were obtained either from American Type Culture Collection (ATCC) or from Clinica Dermosifilopatica, Università di Cagliari.

Cytotoxicity. This was evaluated against the above cells and was based on the viability of Mock-infected cells, as monitored by MTT method [15].

Antimycotic assays. Yeast blastospores were obtained from a 30-h-old shaken culture incubated at 30 °C in Sabouraud dextrose broth, whereas the dermatophyte inoculum was scraped aseptically with a spatula from a 7-day-old agar culture. The macerate was then finely suspended in Sabouraud dextrose broth using a glass homogenizer. Glycerol, final concentration 10%, was added as a cryoprotective agent to both the yeast and the dermatophyte suspension, aliquots of which were then stored in liquid nitrogen. Test tubes were inoculated with 10^3 blastospores or colony forming units (cfu)/mL. The minimal inhibitory concentration (MIC) was determined by serial dilutions using Sabouraud dextrose broth (pH 5.7) and incubating at 37 °C. Microorganism growth was determined after 1 day (yeast) or 3 days (dermatophytes). The MIC was defined as the compound concentration at which no microscopic signs of fungal growth were detectable. Fungicidal activity was tested by subactivations of negative test tubes in Sabouraud dextrose agar.

Antibacterial assays. *S aureus*, group D *Streptococcus, E coli* and *P aeruginosa* were recent clinical isolates. Test were carried out in nutrient broth, pH 7.2. The inoculum consisted in 10^3 cells. MICs were determined after 18 h of incubation at 37 °C in the presence of different compounds concentrations.

Acknowledgments

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