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Synthesis of New Linear Guanidines and Macrocyclic Amidinourea Derivatives Endowed with High Antifungal Activity against *Candida* spp. and *Aspergillus* spp.†

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Abstract: New linear and cyclic guanidines were synthesized and tested in vitro for their antifungal activity toward clinically relevant strains of *Candida* species, in comparison to fluconazole. Macrocyclic compounds showed a minimum inhibitory concentration in the micromolar range and a biological activity profile in some cases better than that of fluconazole. One macrocyclic derivative was also tested against *Aspergillus* species and showed high antifungal activity comparable to that of amphotericin B and itraconazole.

The opportunistic human pathogen *Candida albicans* and other non-*albicans* species have acquired considerable clinical significance as infectious agents in immunocompromised patients, being important causes of morbidity and mortality.[1] The pathogenic species of *Candida* derive their importance from the severity of their infections and from their ability to develop resistance against a variety of antifungal agents.[2] In addition to candidosis, other invasive filamentous fungal infections, such as aspergillosis, are a major problem for certain groups of patients. Clinically, candidosis and aspergillosis account for 80−90% of systemic fungal infections in immunocompromised patients. However, whereas many drugs have proven to be effective against *Candida* infections, aspergillosis (including infections caused by the mostly encountered *A. fumigatus*, *A. flavus*, and *A. terreus*) remains very hard to overcome. The antifungal agents currently used in therapy are azoles (e.g., fluconazole and itraconazole), polyenes (e.g., amphotericin B), echinocandins, and allylamines.[3] Among them, azoles are fungistatic and orally active agents against most yeasts and filamentous fungi. Moreover, fluconazole shows good antifungal activity with relatively low toxicity and is preferred as firstline antifungal therapy. However, it has suffered severe drug resistance and is not effective against invasive aspergillosis,[3b] unlike amphotericin B and itraconazole, although wide use of itraconazole is hampered by variable oral absorption and low bioavailability. For these reasons, a drug active against *Candida* and *Aspergillus* pathogens and endowed with high bioavailability would be highly desirable. Accordingly, in recent years, new structural classes of antifungals have been reported,[4] among which guanidine derivatives proved to have a very interesting inhibitory activity.[5] As an example, guazatine (a mixture of guanidines and polyamines used in agriculture as fungicide) was classified as a moderately hazardous antifungal agent, while results from in vivo animal studies demonstrated a high potential for guazatine and related compounds as antifungal agents.[6] In this context, we have recently reported that single components of guazatine (compounds **1**−**6**, Figure 1) are able to act toward *albicans* and non-*albicans Candida* strains,[7] laying the foundation for designing new agents endowed with antifungal activity. On the basis of such results, we focused our efforts on the synthesis of guanidine and polyamine derivatives related to guazatine with the aim of identifying new potential antifungal agents. Herein, we describe the synthesis of novel linear guanidine and macrocyclic amidinourea derivatives and their biological evaluation against clinical isolates of *Candida* spp. Moreover, one of the amidinourea derivatives (**13d**), with the best antifungal activity toward the entire panel of *Candida* isolates, was also tested in vitro for its activity toward fungal isolates representative of clinically relevant *Aspergillus* spp.

The natural components **3** and **5** of guazatine,[7] showing good antifungal activity toward *Candida* strains, were chosen as models for the synthesis of the new derivatives. The 1,17-diamino-9-azaheptadecane **7** was selected as a building block for the synthesis of guanidine derivatives and was reacted with *N*,*N*′-di-Boc-*S*-methylisothiourea, leading to the monoguanylated intermediate **8** (Scheme 1).[8] The aminoguanidine **8** was then reacted with different *N*,*N*′-di-Boc-*N*-alkyl-*S*-methylisothioureas **9a**−**f** (previously synthesized via Mitsunobu reaction or via phase-transfer catalyzed N-alkylation reaction, Scheme 2),[9] leading to desired linear guanidines **10** and/or to the macrocyclic amidinoureas **11**, in agreement with our recent studies on the synthesis of macrocyclic amidinoureas[10] showing that linear aminoguanidines bearing a di-Boc-guanidine and a primary or secondary amine moiety, such as **8** or **10**, are generally converted into macrocyclic amidinoureas when heated in THF.

In some cases, because of the different rates of the guanylation/cyclization steps of **8**, the linear guanidines **10** were the only product obtained. On the other hand, since the cyclization of **10** into **11** started as soon as the guanylation step was completed, amidinoureas **11** were obtained in most cases as the only/major product, together with only a small amount of **10**.[11] The reaction of **7** with an excess of the isothiourea **9f** led to **14**. Compounds **10**, **11**, and **14** were finally reacted with 10% TFA to afford deprotected linear guanidines **12** and **15** and cyclic amidinourea **13** in quantitative yield. The reaction mixtures of **12** and **13** were then purified by semipreparative HPLC, and the structure of the macrocyclic amidinoureas was confirmed by exact mass analysis (MALDI-TOF).

A total of eight clinical isolates of five different *Candida* species (obtained from respiratory specimens, with each strain representing a single isolate from a patient) were tested. Regarding chemical components found in the guazatine mixture, molecular doubling of the guanidinooctyl moiety

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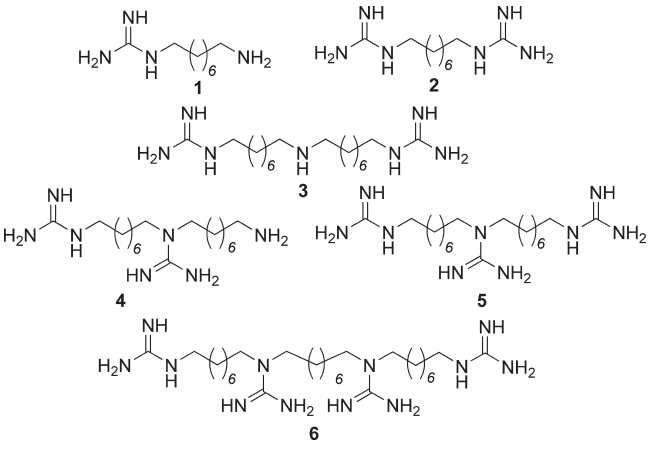
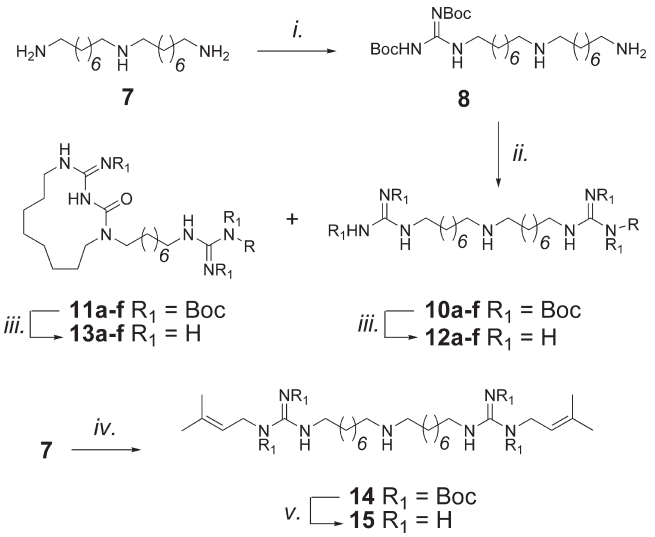


Figure 1. Structures of major components isolated from guazatine mixture.

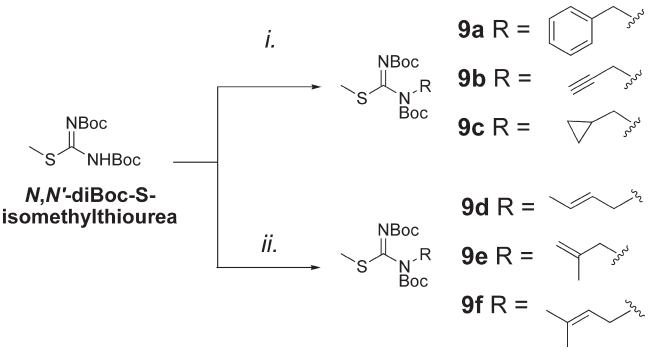
Scheme 1[a]



[a] Reagents and conditions: (i) *N*,*N*′-di-Boc-*S*-methylisothiourea, THF/MeOH (5:3), 50 °C; (ii) *N*,*N*′-di-Boc-*N*-alkyl-*S*-methylisothioureas **9a**−**f**, THF, 70 °C; (iii) 10% TFA, dry CH_2Cl_2, 24 h, room temp; (iv) THF/MeOH (5:3), **9f**, 50 °C; (v) 10% TFA, dry CH_2Cl_2, 24 h, room temp. Compounds: **9a**−**13a**, R = benzyl; **9b**−**13b**, R = propargyl; **9c**−**13c**, R = cyclopropylmethyl; **9**−**13d**, but-2-enyl; **9e**−**13e**, R = isobutenyl; **9f**−**13f**, R = prenyl.

of **1** by an amino linkage led to iminoctadine (**3**) that showed measurable antifungal activity toward many of the fungal strains (Table 1), with a very good inhibition of *C. tropicalis* (1.25 μM).[7] Moreover, introduction of an additional guanidino moiety instead of the central amino group led to **5**, found to be much more efficient than **3** against *C. albicans*, while comparable or lower activity was measured toward the remaining fungal strains. Prenylation of one of the terminal guanidino groups of **3** led to **12f**, characterized by a general decrease in activity, with the sole slight improvement of activity toward *C. albicans* 15T. The corresponding double prenylated **15** showed an additional decrease in antifungal activity with respect to **3** and **12f**, suggesting that prenylation of terminal guanidino groups of linear compounds was detrimental for antifungal activity toward clinical or reference strains tested. On the other hand, transforming the prenyl group of **12f** into a cyclopropylmethyl moiety (**12c**) allowed a significant enhancement of activity toward *C. albicans*, the

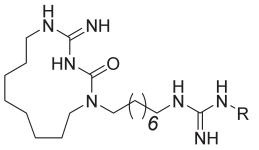
Scheme 2[a]



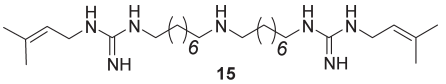
[a] Reagents and conditions: (i) ROH, DIAD, PPh_3, THF; (ii) RBr, KOH, Bu_4NBr, DCM, H_2O.

C. krusei reference strain, *C. parapsilosis* 64E, *C. glabrata*, and *C. tropicalis*. Activity of **12c** was comparable to that of **12f** against *C. albicans* 15T, *C. krusei* 193T, and both the reference and 81E strains of *C. parapsilosis*. Among cyclic derivatives, **13f**, bearing the bulkiest acyclic side chain (a prenyl group), showed interesting activity toward *C. albicans*, *C. krusei*, and *C. tropicalis* (10−40 μM) and lower activity against *C. parapsilosis* and *C. glabrata* (40−80 μM). By reduction of the size of the unsaturated moiety to the corresponding unbranched chain (the butenyl group of **13d**), the activity underwent a significant increase toward *C. albicans* (1.25−2.5 μM) and *C. tropicalis* (1.25 μM) and toward *C. krusei* and *C. parapsilosis* (5 μM). The lowest activity of **13d** was toward *C. glabrata* (20 μM). Shortening and branching the butenyl chain into an isobutenyl moiety (**13e**) caused a dramatic loss of activity against all fungal strains. The best activity for this compound was found toward the reference and 15T strains of *C. albicans* (20 μM). Transforming the isobutenyl side chain to a linear propargyl moiety as in **13b** restored good activity toward *C. albicans* (with the exception of *C. albicans* 4T that was resistant to such a compound) and *C. tropicalis* (5 μM). Finally, aromatization of the side chain to a benzyl group (**13a**) led to activity data comparable in some cases (*C. albicans* and *C. tropicalis*) to those found for the butenyl derivative **13d**. In summary, both the butenyl and benzyl derivatives showed the best antifungal activity, followed by the propargyl analogue that retained interesting activity toward a wide number of fungal strains. Moreover, it seems that linear side chains of cyclic derivatives are better for activity in comparison to branched moiety (compare the prenyl compound **13f** with the butenyl analogue **13d**, and the isobutenyl **13e** with the propargyl derivative **13b**). Fluconazole, used as the reference compound, showed a 0.8 μM activity toward the reference strain of *C. albicans*.[12] Interesting activity was also retained against *C. parapsilosis* and, in part, toward *C. tropicalis*, while activity against the remaining C. albicans strains and against *C. krusei* and *C. glabrata* was unimportant. Biological results showed that **13a**, **13d**, and the linear derivative **12c** were characterized by very good activity values toward all the strains tested (with the sole exception of the standard *C. parapsilosis*). They also showed a biological activity profile better than that of fluconazole against *C. albicans* (except the reference strain), *C. krusei*, *C. glabrata*, and *C. tropicalis*. In contrast, activity of **13a** and **12c** toward the reference and the 81E strains of *C. parapsilosis* was in general lower, while a significantly better activity was found toward the 64E strain, in comparison to fluconazole.

Table 1. Anticandidal Activity of Guazatine Components and Linear and Cyclic Guanidino Derivatives



13a R = Benzyl; **13b** R = Propargyl; **13d** R = But-2-enyl;
13e R = Isobutenyl; **13f** R = Prenyl



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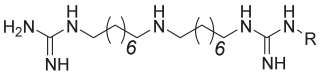

12c R = Methylcyclopropyl
12f R = Prenyl

	antifungal activity (MIC, μM)[a]											
Candida species	G[b]	**3**	**5**	**12c**[c]	**12f**[c]	**13a**	**13b**	**13d**	**13e**	**13f**	**15**	F[d]
C. albicans ATCC 60193	40	80	5	20	80	2.5	2.5	2.5	20	40	> 80	0.8
C. albicans 4T	20	80	5	10	80	2.5	80	1.25	40	20	80	209
C. albicans 53T	40	40	20	10	80	2.5	5	2.5	40	20	80	418
C. albicans 15T	80	80	10	20	40	5	2.5	1.25	20	20	> 80	209
C. krusei ATCC 14243	20	20	10	5	40	20	80	5	40	10	40	209
C. krusei 193T	20	20	40	10	20	10	40	5	80	20	80	418
C. parapsilosis ATCC 34136	10	> 80	> 80	80	> 80	80	40	5	> 80	> 80	> 80	6.5
C. parapsilosis 64E	20	40	> 80	5	> 80	20	40	5	> 80	> 80	> 80	32
C. parapsilosis 81E	20	20	> 80	20	40	20	80	5	> 80	40	> 80	13
C. glabrata 70E	40	40	> 80	20	80	40	80	20	80	80	> 80	209
C. tropicalis 86E	10	1.25	1.25	5	20	2.5	5	1.25	40	20	> 80	52

[a] MIC values were determined at 24 h both visually and spectrophotometrically. [b] G is the guazatine mixture. [c] Compounds **12d** and **12e** were isolated in amounts not enough for assays. [d] F is fluconazole.

Table 2. Activity of **13d** toward clinical isolates of *Aspergillus* spp.

	antifungal activity (μM)[a]							
compd	*A. niger*[d]	*A. niger*[e]	*A. fumigatus*[d]	*A. fumigatus*[g]	*A. versicolor*[d]	*A. versicolor*[f]	*A. terreus*[g]	*A. flavus*[d]
13d	18	18	73	36	18	4	73	36
AMB[b]	0.27	0.135	0.135	0.27	0.54	0.135	1	2.1
ITRA[c]	0.35	0.35	0.35	0.04	0.02	0.35	0.35	0.04

[a] MIC values were determined visually at 48 h. [b] AMB is amphotericin B. [c] ITRA is itraconazole. [d] Isolated from sputum. [e] Isolated from nasal swab. [f] Isolated from skin. [g] Isolated from bronchoalveolar lavage fluid.

Morevoer, **13d** proved to be active toward all the *C. parapsilosis* strains with MIC[a] values better than or comparable to that of fluconazole. Among the guanidine derivatives, the most active compound **13d** was selected and tested in vitro against clinical isolates belonging to five different *Aspergillus* species that were obtained from respiratory and nonrespiratory specimens. In all cases, **13d** showed a very good activity against *Aspergillus* species, even if lower than that of amphotericin B and itraconazole (Table 2). In particular, the best activity was found toward *A. niger* and *A. versicolor*, with MIC ranging from 4 to 18 μM. In vitro cytotoxicity of **13d** was checked in human lymphocytes at 25 and 37 °C with compound concentrations between 0 and 50 μM. Preliminary results suggest that **13d** does not induce cellular damage at doses up to 30 μM (100% viability), while it interacts with the cellular membrane at the highest concentrations (40 and 50 M), thus reducing the lymphocyte viability to 78% and 43%, respectively. As expected, temperature increases the toxic effects.[13] Under these experimental conditions, the therapeutic indices (TI_{50}) related to the various *Candida* species were evaluated on the basis of the 50% viability and MIC_{50}.

As a result, TI_{50} was between 38 and 2.4 μM at 25 °C and between 30 and 1.9 μM at 37 °C. Hence, the broad spectrum antifungal activity and high solubility in aqueous medium and low cytotoxicity make **13d** and its derivatives a very attractive class of compounds in the treatment of topical and systemic fungal infections.

In conclusion, this study demonstrates that analogues of guazatine components have a high potential as antifungal agents, being characterized by a low toxicity in preliminary cell-based assays and by a good inhibitory profile against common fungal pathogens, thus showing an activity profile in many cases better than fluconazole. Fungi often infect the skin surface and subsequently invade the stratum corneum to avoid being shed from the skin surface by desquamation. Pharmacologic agents applied to the surface of the skin in the form of creams, lotions, or sprays readily penetrate the stratum corneum to kill the fungi (fungicidal agents) or at least render them unable to grow or divide (fungistatic agents).[14] In this context, biological activity data reported here suggest a potential role of the new guanidines for the topical treatment of *Candida* infections and contribute to the active and challenging research on development of novel antifungal agents. However, the development of new antifungal compounds and the use of alternative approaches in immunocompromised patients should be pursued through well-designed laboratory and clinical studies. For this purpose, additional studies are required to clearly understand the mechanism of action of guazatine derivatives and to define precisely the role of

[a] Abbreviations: G, guazatine mixture; F, fluconazole, MIC, minimum inhibitory concentration; AMB, amphotericin B; ITRA, itraconazole, LD_{50}, lethal dose 50%; NOAEL, no observed adverse effect level.

these new agents alone or in combination with other antifungal agents. Further functional characterization of the hit compounds **12c**, **13a**, and **13d** is planned, and their results will be reported in due time.

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Supporting Information Available: General procedures and experimental data for compounds **9**−**15**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Chamilos, G.; Kontoyiannis, D. P. The rationale of combination antifungal therapy in severely immunocompromised patients: empiricism versus evidence-based medicine. *Curr. Opin. Infect. Dis.* **2006**, *19*, 380–385.

(2) Klepser, M. E. Candida resistance and its clinical relevance. *Pharmacotherapy* **2006**, *26*, 68S–75S.

(3) (a) Aperis, G.; Myriounis, N.; Spanakis, E. K.; Mylonakis, E. Develop in the treatment of candidiasis: more choices and new challenges. *Expert Opin. Invest. Drugs* **2006**, *15*, 1319–1336. (b) Hoffman, H. L.; Ernst, E. J.; Klepser, M. E. Novel triazole antifungal agents. *Expert Opin. Invest. Drugs* **2000**, *9*, 593–605.

(4) Pauli, A. Anticandidal low molecular compounds from higher plants with special reference to compounds from essential oils. *Med. Res. Rev.* **2006**, *26*, 223–268.

(5) (a) Buxbaum, A.; Kratzer, C.; Graninger, W.; Georgopoulos, A. Antimicrobial and toxicological profile of the new biocide Akacid plus. *J. Antimicrob. Chemother.* **2006**, *58*, 193–197. (b) Jana, G. H.; Jain, S.; Arora, S. K.; Sinha, N. Synthesis of some diguanidino 1-methyl-2,5-diaryl-1H-pyrroles as antifungal agents. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3592–3595. (c) Martin, D. W.; Schurr, M. J.; Mudd, M. H.; Govan, J. R.; Holloway, B. W.; Deretic, V. Mechanism of conversion to mucoidy in Pseudomonas aeruginosa infecting cystic fibrosis patients. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8377–8381.

(6) Guazatine produces severe local irritation, and single oral doses are of moderate toxicity, with an oral LD_{50} value in rats of 280 mg/kg body weight. In a number of short term studies in rats, the overall NOAEL of guazatine was 200 ppm, equivalent to 10 mg/kg bw per day. In a 1-year study in dogs, the NOAEL was 25 ppm, equal to 0.8 mg/kg bw per day. Several studies in rats concluded that guazatine is not genotoxic and carcinogenic, while in a study of developmental toxicity in rabbits there were no signs of fetotoxicity or teratogenicity. FAO Corporate Document Repository.

(7) Dreassi, E.; Zizzari, A. T.; D'Arezzo, S.; Visca, P.; Botta, M. Analysis of guazatine mixture by LC and LC−MS and antimycotic activity determination of principal components. *J. Pharm. Biomed. Anal.* **2007**, *46*, 1499–1506.

(8) Delle Monache, G.; Botta, B.; Delle Monache, F.; Espinal, R.; De Bonnevaux, S. C.; De Luca, C.; Botta, M.; Corelli, F.; Carmignani, M. Novel hypotensive agents from *Verbesina caracasana*. 2. Synthesis and pharmacology of caracasanamide. *J. Med. Chem.* **1993**, *36*, 2956–2963.

(9) (a) Dodd, D. S.; Kozikowski, A. P. Conversion of alcohols to protected guanidines using the Mitsunobu protocol. *Tetrahedron Lett.* **1994**, *35*, 977–980. (b) Corelli, F.; Dei, D.; Delle Monache, G.; Botta, B.; De Luca, C.; Botta, M.; Volpe, A. R.; Carmignani, M. Synthesis and preliminary pharmacological evaluation of analogues of caracasanamide, a hypotensive natural product. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 653–658. (c) Cona, A.; Manetti, F.; Leone, R.; Corelli, F.; Tavladoraki, P.; Polticelli, F.; Botta, M. Molecular basis for the binding of competitive inhibitors of maize polyamino oxidase. *Biochemistry* **2004**, *43*, 3426–3435.

(10) Castagnolo, D.; Raffi, F.; Giorgi, G.; Botta, M. Macrocyclization of di-Boc-guanidino-alkylamines related to guazatine components: discovery and synthesis of innovative macrocyclic amidinoureas. *Eur. J. Org. Chem.* **2009**, *3*, 334–337.

(11) For further details, see Supporting Information.

(12) The low MIC_{50} value (0.8 μM) of fluconazole toward *C. albicans* ATCC 60193 was in disagreement with literature reports showing such a strain as fluconazole-resistant (Carballeira, N. M.; Sanabria, D.; Cruz, C.; Parang, K.; Wan, B.; Franzblau, S. 2,6-Hexadecadiynoic acid and 2,6-nonadecadiynoic acid: novel synthesized acetylenic fatty acids as potent antifungal agents. *Lipids* **2006**, *41*, 507–511). However, determination of the MIC_{80} value (>418 μM) allowed us to classify the strain as fluconazole-resistant, according to the literature .

(13) Drug toxicity, particularly nephrotoxicity, is a significant problem, as amphotericin B also damages mammalian cell membranes.

(14) Kyle, A. A.; Dahl, M. V. Topical therapy for fungal infections. *Am. J. Clin. Dermatol.* **2004**, *5*, 443–451.