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Structure–antifungal activity relationship of His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 and analogues

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Abstract—The synthesis, in vitro evaluation and conformational study of His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 and analogues acting as antifungal agents are reported. Among them, His-Phe-Lys-Trp-Gly-Arg-Phe-Val-NH_2 exhibited a moderate but significant antifungal activity against *Cryptococcus neoformans*, *Candida albicans* and *Candida tropicalis*. A theoretical study allows us to propose a biologically relevant conformation for these octapeptides acting as antifungal agents. In addition, these theoretical calculations allow us to determine the minimal structural requirements to produce the antifungal response and can provide a guide for the design of compounds with this biological activity.

1. Introduction

The prevalence of systemic and dermal fungal infections has significantly increased during the past two decades and remains an important cause of great morbi-mortality of immunocompromised patients.[1] In a recent study, McNeil et al.[2] found a dramatic increase in mortality from 1980 to 1997 due to mycoses originated in multiple causes which were mainly associated with *Candida*, *Aspergillus* and *Cryptococcus* spp. infections. Added to these fungal spp., new emerging fungal pathogens appear every year as the cause of morbidity and life-threatening infections in the immunocompromised hosts.[3,4]

Although different antifungal agents are available for the treatment of fungal infections, some of them have undesirable side effects, are ineffective against new or re-emerging fungi or develop resistance mainly due to the broad use of antifungal agents.[5] As a consequence, there is an urgent need of a next generation of new antifungal agents, which may overcome the above disadvantages.

Natural and synthetic peptides have gained attention as potential new antifungal agents.[6–9] They have shown to inhibit a broad spectrum of pathogens and microorganisms[10–12] and possess the important characteristic that they do not usually induce bacterial or fungal resistance.[13] Most of these peptides are believed to exert their antimicrobial activities either forming multimeric pores in the lipid bilayer of the cell membranes,[13] or interacting with DNA or RNA after penetration into the cell.[14–16] In contrast, α-melanocyte stimulating hormone (α-MSH) and its C-terminal tripeptide Lys-Pro-Val, which showed antimicrobial activity against the two representative pathogens *Staphylococcus aureus* and *Candida albicans*,[17] appear to act through a mechanism substantially different from that of other natural antimicrobial peptides.

Catania et al. have reported the antifungal activity of several peptides.[17–20] They have indicated that the antimicrobial effects of α-MSH are exerted through a

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unique mechanism different from that of other natural peptides, suggesting that the candidacidal effect of these compounds is linked to the cAMP-inducing activity. As part of our ongoing program aimed at identifying novel antifungal compounds,[21–23] we have recently reported the synthesis and antifungal properties of His-Phe-Arg-Trp-NH_2 and structurally related tetrapeptides.[24] In this paper, we report the synthesis and conformational analysis of His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2 (**1**), which is the 6–13 sequence of α-MSH and structurally related octapeptides with antifungal properties. The aim of our work is to develop more potent antifungal peptides and to understand the conformational features that enhance potency and selectivity and their relation to the amino acid sequence. For this purpose, ten new analogues with single or double replacements within the α-MSH(6–13) sequence were designed, prepared and tested (peptides **2**–**11** in Table 1).

For the α-MSH activity, the importance of the presence of His-6 has been previously demonstrated by the fact that Ac-α-$MSH_{7–10}$-NH_2 lacked measurable activity.[25] Trp-9 was also critical for activity as demonstrated by the lack of biological activity of Ac-α-$MSH_{6–8}$-NH_2.[25] More recent structure–activity relationship (SAR) studies including physiological effects of α-MSH might be obtained from references.[26–29] Recently, we reported that the presence of His-6 and Trp-9 residues appears to be a structural requirement for the antifungal activity as well. In contrast, our results indicated that Tyr and Lys could replace Phe-7 and Arg-8, respectively.[24] It is well known that the receptor binding and transducing properties of melanocortins depend on separate structural and conformational characteristics.[30] For example, Phe-7 plays a key role in receptor binding whereas Lys-11 and Pro-12 are more important for receptor stimulation.[30] Thus, the positions 7, 8, 11 and 12 were systematically mutated in the series presented here. The resulting peptides were tested against three human pathogenic strains (*C. albicans*, *Cryptococcus neoformans* and *Candida tropicalis*). In addition, an exhaustive conformational analysis and an electronic properties study on all the peptides reported here was carried out in order to determine a possible biologically relevant conformation for these compounds acting as antifungal agents.

2. Results and discussion

The principal goal was to find α-MSH analogues with greater antifungal activity and to reach a better understanding of the peptide structure–antifungal relationship through design, synthesis and testing of novel octapeptide analogues in which several modifications were made. As starting structure, we chose the sequence α-MSH(6–13), His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, which contains the invariant 'core' sequence His-Phe-Arg-Trp (6–9), common to all melanocortin receptors,[25] and the sequence Lys-Pro-Val (11–13), which has been reported to be relevant to antimicrobial activity.[17] Previously, we tested the antifungal activity of tetrapeptide His-Phe-Arg-Trp-NH_2 and Lys-Pro-Val-NH_2 against a panel of pathogenic fungi.[24] Lys-Pro-Val-NH_2 did not show antifungal activity; in contrast, the tetrapeptide His-Phe-Arg-Trp displayed a moderated but significant antifungal effect, in particular against *C. neoformans*.[24] To better characterize the structure–antifungal activity relationship of octapeptides **1**–**11** reported here, the present research explored influences of amino acid substitutions on its antifungal activity.

2.1. Antifungal activity

According to Section 4, concentrations of peptides at 100 and 200 μM were incorporated to growth media in order to carry out the antifungal evaluation. Compounds producing no inhibition of fungal growth at 200 μM level were considered inactive.

In a first step of our study, we focused our attention on the C-terminal moiety (α-MSH(11–13) sequence), synthesizing and evaluating compounds **1**–**4**. Our results

Table 1. Antifungal activity (% inhibition = mean ± SD) of peptides against *Candida albicans* ATCC 10231, *Candida tropicalis* C 131 2000 and *Cryptococcus neoformans* ATCC 32264

Peptide	Sequence	*Cryptococcus neoformans*		*Candida albicans*		*Candida tropicalis*	
		% inhib. 200 μM	% inhib. 100 μM	% inhib. 200 μM	% inhib. 100 μM	% inhib. 200 μM	% inhib. 100 μM
1	His-Phe-Arg-Trp-Gly-Lys-**Pro**-Val-NH_2	24 ± 5	9 ± 2	0	0	0	0
2	His-Phe-Arg-Trp-Gly-Lys-**Phe**-Val-NH_2	**77 ± 30**	15 ± 3	**61 ± 23**	5 ± 2	19 ± 10	9 ± 3
3	His-Phe-Arg-Trp-Gly-Lys-**Tyr**-Val-NH_2	**99 ± 1**	**58 ± 1**	43 ± 10	0	21 ± 10	0
4	His-Phe-Arg-Trp-Gly-**Arg-Phe**-Val-NH_2	**94 ± 6**	**72 ± 7**	**80 ± 0.6**	1 ± 0.2	**64 ± 12**	1 ± 3
5	His-Phe-**Lys**-Trp-Gly-Lys-**Phe**-Val-NH_2	**97 ± 3**	38 ± 6	**72 ± 8**	0	22 ± 5	0
6	His-Phe-**Lys**-Trp-Gly-**Arg-Phe**-Val-NH_2	**97 ± 4**	**98 ± 3**	**100 ± 0.4**	**80 ± 1**	**92 ± 4**	**58 ± 6**
7	His-Phe-**Lys**-Trp-Gly-Lys-**Tyr**-Val-NH_2	**83 ± 20**	**50 ± 6**	15 ± 12	0	0	0
8	His-**Tyr-Lys**-Trp-Gly-**Arg-Phe**-Val-NH_2	**89 ± 5**	47 ± 4	11 ± 3	0	23 ± 8	0
9	His-**Tyr-Lys**-Trp-Gly-Lys-**Tyr**-Val-NH_2	**63 ± 15**	42 ± 1	0	0	3 ± 3	0
10	His-**Tyr-Lys**-Trp-Gly-Lys-**Phe**-Val-NH_2	41 ± 10	25 ± 1	0	0	1 ± 2	0
11	His-**Tyr**-Arg-Trp-Gly-Lys-**Phe**-Val-NH_2	35 ± 10	21 ± 7	10 ± 6	0	1 ± 3	0
Amph B		100	100	100	100	100	100

The main mutation performed in the peptide sequences and the percentage of inhibition higher than 50, are denoted in bold.
Amph B, amphotericin B.

showed that octapeptide **1** did not inhibit *C. albicans* up to a concentration = 200 μM (Table 1). This result is in contrast to that of Grieco et al.[19] who reported that peptide **1** possessed a moderate inhibitory activity against *C. albicans* (59.4%) at 100 μM. Peptide **1** did not inhibit *C. tropicalis* either, but showed a low inhibitory effect against *C. neoformans* (24%) at 200 μM. The replacement of Pro-12 by Phe in peptide **2** produced an enhancement of the antifungal activity against all fungi tested mainly at 200 μM. These results were in agreement with those of Grieco et al.[19] who reported a higher inhibitory effect for peptide **2** in comparison to peptide **1** against *C. albicans*. The replacement of Pro-12 of peptide **1** by Tyr in peptide **3** gave a higher enhancement of activity (compare **3** with **2** and **1**) only against *C. neoformans*. We then replaced Lys-11 in **2** by Arg obtaining peptide **4**, which displayed an enhancement of the antifungal activity at 200 μM in the three fungi tested.

In a second step of our study, we focused our efforts on the introduction of modifications in the sequence α-MSH(6–9). Replacement of Arg-8 by Lys in **2** and **4** gave peptides **5** and **6**, respectively. This change produced a significant enhancement ($p < 0.01$) of antifungal activity, from 15% (peptide **2**) to 38% (peptide **5**) and from 72% (peptide **4**) to 98% (peptide**6**) of inhibition at 100 μM against *C. neoformans*. The same trend was observed for compounds **5** and **6** when tested against *C. albicans* and *C. tropicalis*. It is interesting to emphasize that peptide **6** was the most active peptide in this series, showing a significant antifungal activity against all the fungi tested, including *C. albicans*. Next, we replace Phe by Tyr-12 in peptide **5** obtaining peptide **7**. Although peptide **7** displayed a similar activity against *C. neoformans* to that of **5** and **6** at 200 μM, its inhibitory effect against this yeast at 100 μM, as well as its activity against *C. albicans* and *C. tropicalis* was markedly lower. Peptides **8**–**11** were obtained replacing Phe-7 by Tyr in peptides **6**, **7**, **5** and **2**, respectively. In general, these peptides displayed a lower inhibitory effect with respect to their congeners, in particular peptides **10** and **11**, which displayed only a marginal effect.

In order to have an overview of the potential of these peptides as antifungal agents, we show the percentage of inhibition of all compounds at concentrations below 100 μM, in comparison to the behaviour of amphotericin B. Peptides **1**–**11** were almost devoid of activity against species of *Candida* genus between 50 and 0.4 μM (results not shown), but they possess moderate percentage of inhibition against *C. neoformans* (Table 2).

In order to better understand the above experimental results, we performed an exhaustive conformational and electronic properties study of all the peptides reported here using theoretical calculations. These results are presented in the next section and might be useful to perform a structure–activity relationship on this series.

2.2. Theoretical calculations

2.2.1. Conformational study. Linear peptides are highly flexible and, therefore, to determine the biologically relevant conformations is not an easy task. It is necessary to perform an exhaustive conformational analysis for these structures to obtain a good perspective about their conformational intricacies. Thus, a conformational study on all peptides reported here was carried out using EDMC calculations.[31,32] Theoretical calculations were carried out as described in calculation methods section. These results are summarized in Table 3 and Tables S1–S11 (as Supplementary material).

Calculations yielded a large set of conformational families for each peptide studied. The total number of conformations generated for each peptide varied between 94,082 and 110,569, and the number of those accepted was 5000 for all cases. In the clustering procedure, a route mean square deviation (RMSD) of 0.75 Å and a ΔE of 30 kcal mol^{-1} were used. The number of families after clustering varied between 221 and 366. The total number of families accepted with a relative population higher of 0.20% varied between 8 and 20. Their populations summed up to ca. 90% of all conformations in each case (see Table 3).

Table 2. % of inhibition of *Cryptococcus neoformans* by peptides **1**–**11** at concentrations between 50 and 0.4 μM

Peptide	% inhibition							
	50 μM	25 μM	12.5 μM	6.25 μM	3.25 μM	1.62 μM	0.81 μM	0.40 μM
1	0	0	0	0	0	0	0	0
2	15 ± 2	2 ± 1	0	0	0	0	0	0
3	56 ± 18	41 ± 0.6	28 ± 5	24 ± 9	10 ± 5	6 ± 4	0	0
4	67 ± 19	26 ± 5	26 ± 3	18 ± 10	12 ± 4	11 ± 3	10 ± 2	7 ± 1
5	23 ± 2	0	0	0	0	0	0	0
6	96 ± 2	95 ± 0.7	84 ± 1	84 ± 3	57 ± 8	45 ± 4	32 ± 8	36 ± 7
7	50 ± 1	49 ± 0.4	40 ± 0.1	31 ± 3	36 ± 10	33 ± 1	31 ± 5	26 ± 0,2
8	n.t.[a]	n.t.	n.t.	19 ± 8	17 ± 4	21 ± 3	2 ± 2	0
9	39 ± 17	22 ± 3	19 ± 2	10 ± 2	n.t.	n.t.	n.t.	n.t.
10	43 ± 2	38 ± 2	34 ± 1	0	0	0	0	0
11	37 ± 6	24 ± 1	21 ± 6	16 ± 7	16 ± 7	5 ± 3	0	0
Amph B[b]	100	100	100	100	100	100	100	100

[a] n.t., not tested.
[b] Amphotericin B.

Table 3. Selected conformational search and clustering results for peptides **1**–**11** optimized at the EDMC/SRFOPT/ECCEP/3 level of theory

Peptide	Generated[a]				Accepted[b]				# F[c]	# $F_{0.20\%}$[d]	% P[e]
	Electrost.	Random	Thermal	Total	Electrost.	Random	Thermal	Total			
1	7051	95,871	237	103,159	650	4158	192	5000	271	15	92.16
2	6450	88,021	181	94,652	501	4356	143	5000	254	20	92.86
3	7165	96,921	258	104,344	552	4246	202	5000	221	8	92.48
4	7277	96,253	275	103,805	622	4169	209	5000	298	16	91.38
5	6372	87,496	214	94,082	565	4266	169	5000	252	13	93.06
6	6525	87,475	208	94,208	468	4378	154	5000	272	14	92.08
7	7437	10,1014	267	108,718	628	4164	208	5000	274	16	92.00
8	7419	100,395	317	108,131	500	4261	239	5000	305	13	91.52
9	7032	96,579	258	103,869	553	4284	163	5000	227	12	93.84
10	7547	102,709	313	110,569	530	4218	252	5000	366	20	88.74
11	6752	92,559	227	99,538	571	4247	182	5000	283	16	92.60

[a] Number of conformations generated electrostatically, randomly and thermally during the conformational search.
[b] Number of conformations accepted from those generated electrostatically, randomly and thermally during the conformational search.
[c] # F, total number of conformational families a as result of the clustering run.
[d] # $F_{0.20\%}$, number of conformational families with populations above 0.20%.
[e] % P, sum of the percent relative population of # $F_{0.20\%}$.

Our results indicate that these peptides are highly flexible, showing equilibrium of statistical-coil[33] structures. All low-energy conformers of octapeptides studied here were then compared to each other. The comparison involved the spatial arrangements, relative energy and populations.

The preferred fully-folded form with 42% of population (global minimum) obtained for peptide **1** is comparable to the conformation reported by Caratenuto et al.[20] from RMN spectroscopic data. The torsional angles obtained for the fragment Arg^{8}-Trp^{9}-Gly^{10}-Lys^{11}-Pro^{12} are the following: $\phi_8 = -65$ (-67 ± 5), $\psi_8 = -41$ (-41 ± 3); $\phi_9 = -89$ (-76 ± 3), $\psi_9 = -29$ (-26 ± 2); $\phi_{10} = 94$ (-84 ± 5), $\psi_{10} = 39$ (-1 ± 15); $\phi_{11} = -63$ (-67 ± 13), $\psi_{11} = -58$ (-46 ± 6); $\phi_{12} = -68$, $\psi_{12} = -19$ (-3 ± 45). The experimental data are given in brackets. As can be observed, there is a very good correlation between the theoretical and experimental dihedral angles for Arg-8, Trp-9, Lys-11 and Pro-12. Nevertheless, the values obtained for Gly-10 are quite shifted. Whereas from the theoretical calculations it appears that this residue adopts a bend structure, the experimental results suggest a turn form. However, it must be pointed out that Gly is a very flexible residue as was previously highlighted by Carotenuto et al.[20] and therefore this difference is not unexpected.

It was found that the active octapeptides **2**–**9** possess low-energy conformers which were similar to each other. They showed mainly two preferred conformations considering the relative energy and population. These were a fully-folded conformation and a partially extended form (see Fig. 1). The fully-folded conformation displayed a turn along 7–10 residues, a bend at residue 11 and the rest of the residues (6, 12 and 13) in a non-stable structure (Fig. 1a). The partially extended conformation showed an extended form encompassing the first three residues, a turn along 9–12 residues and the last residue in a non-stable structure (Fig. 1b). In addition, peptides **7**–**9** displayed an α-helix conformation with a relatively relevant population. In general, the relative populations obtained for the fully-folded and partially extended forms were in equilibrium, giving at least 50% of the whole population. It is interesting to note that peptide **5**, which was one of the most active peptides in this series, displayed the partially extended conformation as the highly preferred form with 65% of the whole population. In contrast, peptides **1**, **10** and **11**, which were inactive or displayed only marginal activity, did not adopt the partially extended conformation among the preferred forms. These peptides displayed the fully-folded form as the highly preferred conformation, and a mixture between bend and coil conformations, as the second preferred form (see % of population in Tables S1, S10 and S11 in Supplementary material). Thus, our results permit us speculate that the low antifungal activity obtained for peptides **1**, **10** and **11** could be explained at least in part by this conformational difference. Interestingly enough, in the fully-folded conformations, a stabilizing π-stacking interaction between the aromatic residue at 7 position (Phe-7 or Tyr-7) and the other aromatic residue located at 12 position (Phe-12 or Tyr-12) was observed.

Molecular recognition and the converse concept of specificity[34] are explained in mechanistic and reductionistic terms by a stereoelectronic 'complementarity' between the ligand and the receptor.[35] In this context, it is obvious that the knowledge of the stereoelectronic attributes and properties of octapeptides and its analogues will contribute significantly to the elucidation of a possible pharmacophoric patron for these compounds acting as antifungal compounds. Once the low-energy conformations for the octapeptides reported here were obtained and, in an attempt to find the potentially reactive sites for the ligands, we evaluated the electronic aspects of the molecules under study using molecular electrostatic potentials (MEP).[36]

2.2.2. Molecular electrostatic potentials (MEP). MEP are of particular value because they allow the visualization and assessment of the capacity of a molecule to interact electrostatically with a putative-binding site.[36–38] MEP

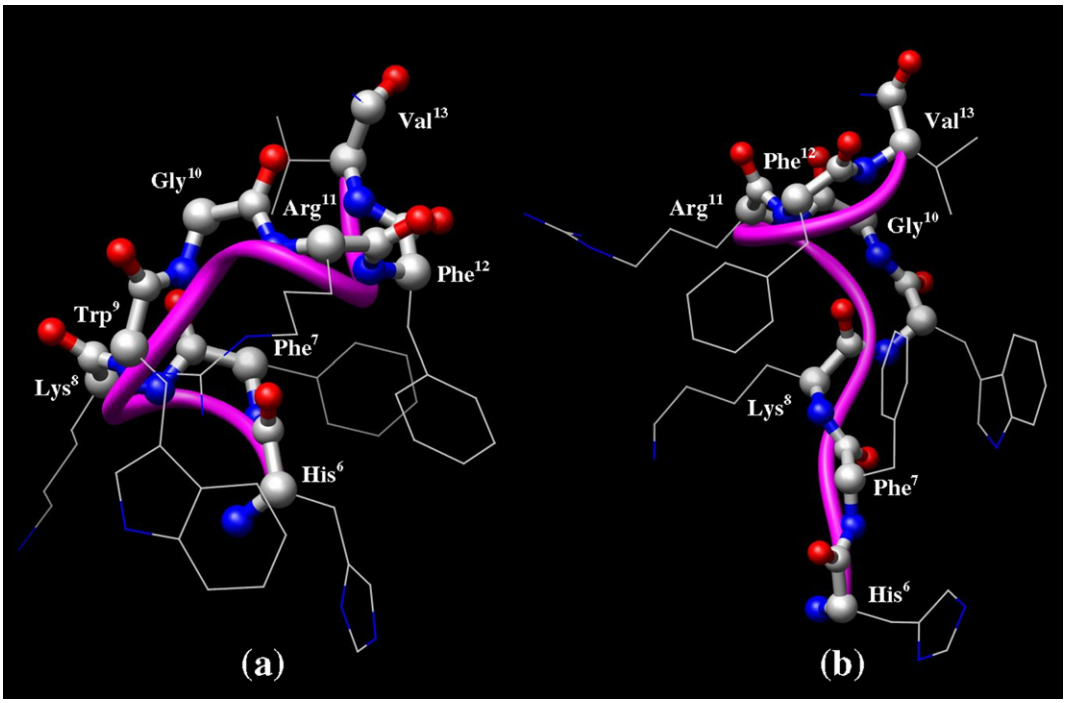

Figure 1. Spatial view of the preferred forms obtained for peptide **6**: (a) fully-folded conformation and (b) partially extended conformation. The backbones are shown as balls and sticks whereas the side chains are shown as wire representations. All hydrogens are omitted for clarity.

can be interpreted in terms of a stereoelectronic pharmacophore condensing all available information on the electrostatic forces underlying affinity and specificity.

Figure 2 gives the MEP obtained for compounds **1** and **3**, showing the C-terminal portion of these peptides. The different electronic behavior obtained for this moiety of peptide **1** with respect to **3** and the rest of the octapeptides reported here can be appreciated in this figure. Note that the electrostatic potentials near to the C-terminal portion of **1** are markedly more negative than those obtained for peptide **3.** Thus, there is not only a different conformational behavior between peptide **1** and the rest of peptides but also a clear different electronic distribution due to the replacement of an aromatic residue (Phe or Tyr) by Pro-12. The different conformational and electronic behavior observed for **1** could explain the lack of antifungal effect observed for this peptide.

Figure 3 gives the MEP obtained for peptides **5**, **9** and **10** showing, in this case, a spatial view of the stabilizing π-stacking interaction between the aromatic residues located at positions 7 and 12. The π-stacking interaction between Phe-7 and Phe-12 in peptide **5** (Fig. 3a) displays both aromatic residues in a parallel orientation and it is located far away in comparison to the similar interaction observed in peptides **9** and **10** (Fig. 3b and c, respectively). It should be noted that the π-stacking interactions involving Tyr residues (either at positions 7 or 12) adopted a perpendicular orientation between the aromatic residues and also displayed a nearby spatial position. These results suggest that π-stacking interactions involving Tyr residues appear to be stronger than those in which only Phe residues participate. The preference for fully-folded structures observed for peptides **3**, **7**–**11** may be, in principle, related to the stronger stabilizing π-stacking interaction present in these structures. It appears that the replacement of Phe-7 or Phe-12 by Tyr could be the responsible for such difference. It is interesting to note that the most active peptides (**4**–**6**) possessing preferred partially extended forms, do not have Tyr in their respective sequence.

2.2.3. Structure–antifungal activity relationships. In a previous paper we demonstrated that the smallest fragment with measurable antifungal activity was His^6-Phe^7-Arg^8-Trp^9-NH_2 and some of its derivatives.[24] It has been shown that the minimal sequence possessing α-MSH-like activity is the central tetrapeptide His-Phe-Arg-Trp.[25] This 'core' sequence was suggested to be the 'message' fragment for α-MSH, the rest of the molecule being regarded as the 'address' sequence.[39]

Keeping in mind that peptides **2**–**9** can be considered active, whereas peptides **1**, **10** and **11** have reduced inhibitory effect, it is noteworthy that the partially extended conformations as preferred forms were only present in active analogues, whereas fully-folded conformations were present both in active and inactive peptides. This suggests that the partially extended conformations could be operative in the recognition process between these peptides and their receptor. Figure 4 shows a spatial view of the most populated and energetically preferred conformation of peptide **5**. This conformation has been overlapped with the partially extended conformations of the rest of the active peptides **2**–**4** and **6**–**9**. The conformers shown in Figure 4 are very close to each other considering the values of the dihedral angles obtained for their backbones of the N-terminal portion (residues 6–9). From the analysis of Figure 4, it is clear that there

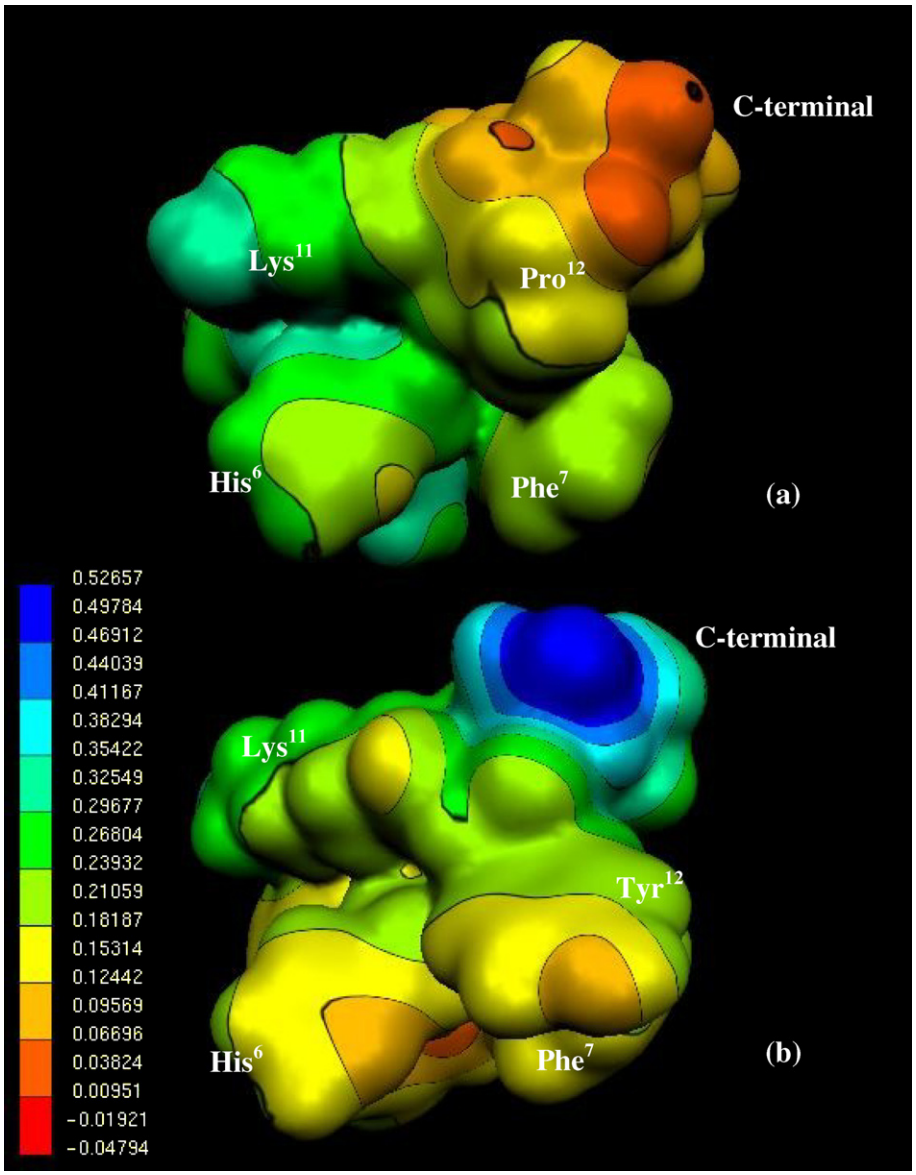

Figure 2. Electrostatic potential-encoded electron density surfaces of peptides **1** (a) and **3** (b). The surfaces were generated with Gaussian 03 using RB3LYP/6-31G(d,p) single point calculations. The coloring represents electrostatic potential with red indicating the strongest attraction to a positive 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. It provides a representative measure of overall molecular charge distribution. The color-coded is shown on the left.

is not a complete conformational overlapping between these conformers. However, all the active peptide adopted a very similar spatial ordering for the 'core' 6–9 sequence. A good fit among the four first amino acids was obtained, but there was not a good overlapping between the last four amino acids (residues 10–13). Thus, the backbone conformations of C-terminal tripeptide for the partially extended forms do not appear to be essential for the general spatial organization of these octapeptides. This situation might be well appreciated in Figure 4. In contrast, our results indicate that in the fully-folded forms of these peptides, the spatial ordering adopted by the C-terminal tripeptide (11–13 sequence), in particular, the aromatic amino acid Phe-12 or Tyr-12 could play a determinant role for the whole conformation of the octapeptide making a stabilizing π-stacking interaction.

These results are in good agreement with our previous ones postulate a partially extended conformations as the biologically relevant form for His-Phe-Arg-Trp-NH_2 and analogues.[24] Interestingly, the acetylation of the same peptides led to inactive compounds possessing a highly preferred fully-folded conformation.[24]

Besides, these results agree fairly well with those previously reported by Sugg et al.[40] postulating a partially extended conformation for the His-Phe-Arg-Trp 'core' sequence as the biologically active α-MSH conformation. Thus, it is worthwhile to compare the 'biologically active conformation' proposed in this study for the backbone of the His-Phe-Arg-Trp-NH_2 portion, with that previously reported for the same sequence from proton NMR studies in aqueous solution.[40] Sugg et al. reported that the observed topology was consistent with

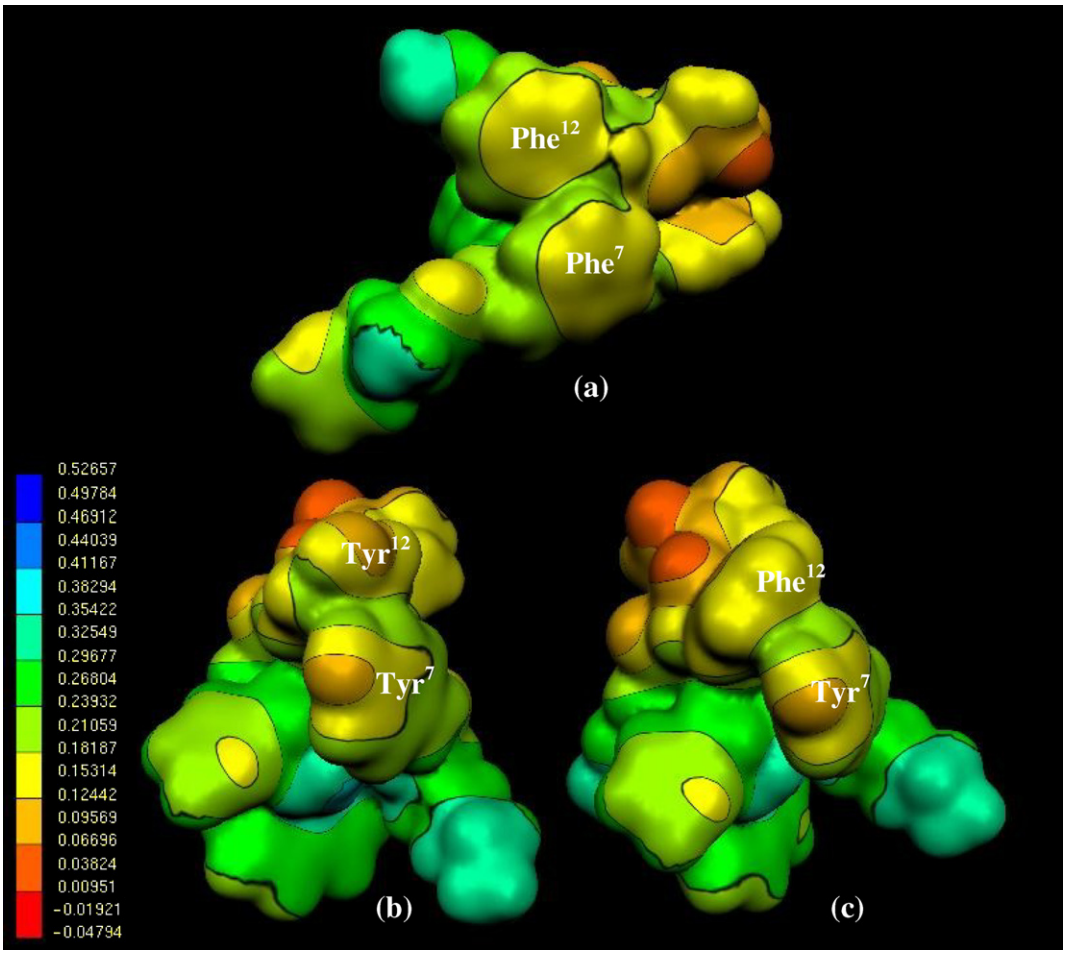

Figure 3. Electrostatic potential-encoded electron density surfaces of peptides **5** (a), **9** (b) and **10** (c). The color-coded is shown on the left.

a non-hydrogen-bonded β-like structure ($\phi = -139°$ and $\psi = 135°$ for L-amino acids) as the predominant conformation in solution. This conformation is closely related to those obtained for the 'core portion' (6–9 sequence) of the different conformers of the active peptides. In Figure 5, we overlapped the conformation reported by Sugg et al. (in orange) with the partially extended conformations obtained for all the active peptides.

Grieco et al.[17–20] suggested that the antimicrobial effects of α-MSH could be exerted through a unique mechanism different from that of other natural antimicrobial peptides. They suggested that the candidacidal effect of α-MSH is linked to the cAMP-inducing activity using toluene permeabilized cells. They proposed that there is some parallelism between the α-MSH and antifungal effects. Our results, using entire cells, showed some parallelism with those previously reported by Grieco et al.; however, it should be noted that there are interesting differences as well. Previous structure–activity studies on the α-MSH(11–13) sequence disclosed the significance of Pro-12 to the anti-inflammatory influence of the tripeptide.[41] Indeed, substitution of Pro-12 with its D-isomer abolished the anti-inflammatory effect.[41] Subsequently, researches on C-terminally modified analogues of α-MSH confirmed the important role of Pro-12 for binding and activity at the MC1 receptor.[42] In contrast, our results indicate that the presence of Pro-12 is not relevant for the antifungal activity. Replacement of Pro-12 by Phe or Tyr gives a clear enhancement of inhibitory effect. A striking difference can be observed between the roles of Pro-12 of these peptides when acting as anti-inflammatory or antifungal compounds. On the other hand, the 'core' 6–9 sequence (His-Phe-Arg-Trp) of melanocortins has long been considered essential for melanocortin receptor recognition and activation.[43] Alanine replacement studies in B16 melanoma cells showed that when each amino acid in the 6–9 sequence was replaced by alanine there was a substantial decrease in receptor recognition and activation by α-MSH.[44] Our results indicate that the 'core' 6–9 sequence, or a very similar structure, is also a structural requirement to produce the antifungal activity.

Carotenuto et al.[20] hypothesized that the N-terminal portion (7–10) is responsible for receptor activation while the C-terminal moiety (10–13) works only enforcing the binding to the receptor through positive interactions with an auxiliary-binding site, for the candidacidal activity.[20] Our results are in a relatively good agreement with such hypothesis. It is clear that the fully-folded conformations, stabilized by π-stacking interactions, do not show an adequate spatial ordering for the hypothetical receptor possessing two-binding sites. On one

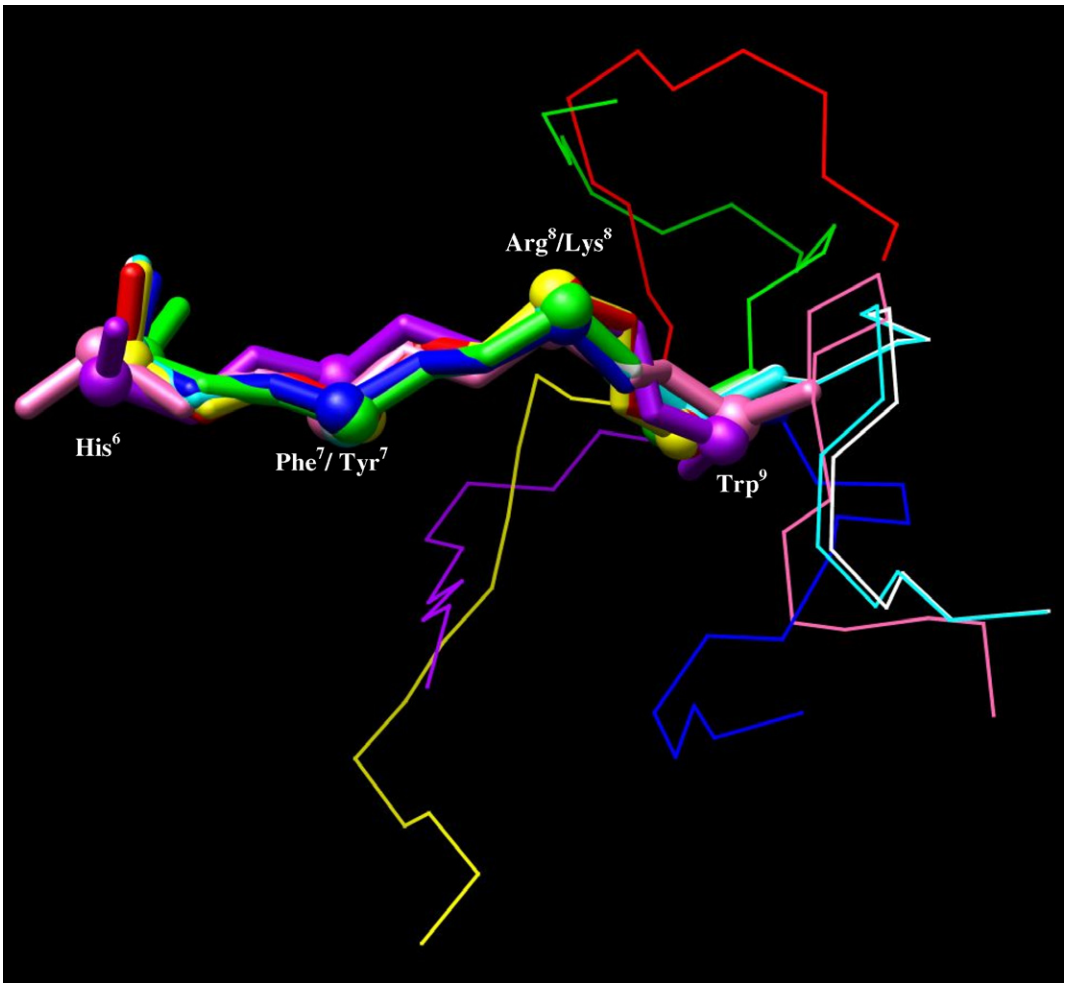

Figure 4. Stereoview of the overlapping of peptides **2**–**9** partially extended conformers. The backbone 'core' 6–9 sequence is shown in sticks indicating each alpha carbon with spheres, the rest of the backbone structures are shown in wire representations. Peptide **2** is shown in red, **3** (blue), **4** (white), **5** (pink), **6** (cyan), **7** (yellow), **8** (green) and **9** (purple). All hydrogens and side chains are omitted for clarity.

hand, they cannot reach the adequate distances for both interactions and, on the other hand, the intra-molecular interactions could affect the binding of the N-terminal portion proposed as responsible for the receptor activation. A similar explanation could be applied for the inactive acetylated derivatives previously reported.[24] The fact that His-Phe-Arg-Trp-NH_2 is the smallest fragment with significant antifungal activity could be an additional support to consider the N-terminal portion as responsible for receptor activation. However, caution is required for such speculations, in particular, because we are hypothesizing on a still unknown receptor. Although the actual existence of melanocortin receptors in yeasts has not been established, our results showing the effects of amino acid substitutions on critical portions, suggest that the presence of such receptors or more probably a structurally related receptor is highly plausible.

3. Conclusions

We designed and synthesized 11 α-MSH octapeptide analogues with different residues in the core and in the C-terminal sequences and tested their antifungal activity. Among compounds tested, His-Phe-Lys-Trp-Gly-Arg-Phe-Val-NH_2 (**6**) displayed the best antifungal activity against *C. neoformans*, *C. albicans* and *C. tropicalis*. Other octapeptide analogues reported here displayed moderate but still significant antifungal effects against *C. neoformans*.

A detailed conformational and electronic properties study supported by theoretical calculations helped us to identify a possible 'biologically relevant conformation' and also understand the minimal structural requirements for the antifungal actions of octapeptides reported here. Our results are very encouraging since they show a great potential of His-Phe-Lys-Trp-Gly-Lys-Phe-Val-NH_2 and its derivatives as a truly novel class of antifungal compounds particularly against the yeast *C. neoformans*.

The general picture emerging from our results is that there is some parallelism between the recognition process for the α-MSH receptor and the hypothetical receptor involved in the antifungal response. However, in this regard it is interesting to note that there are also struc-

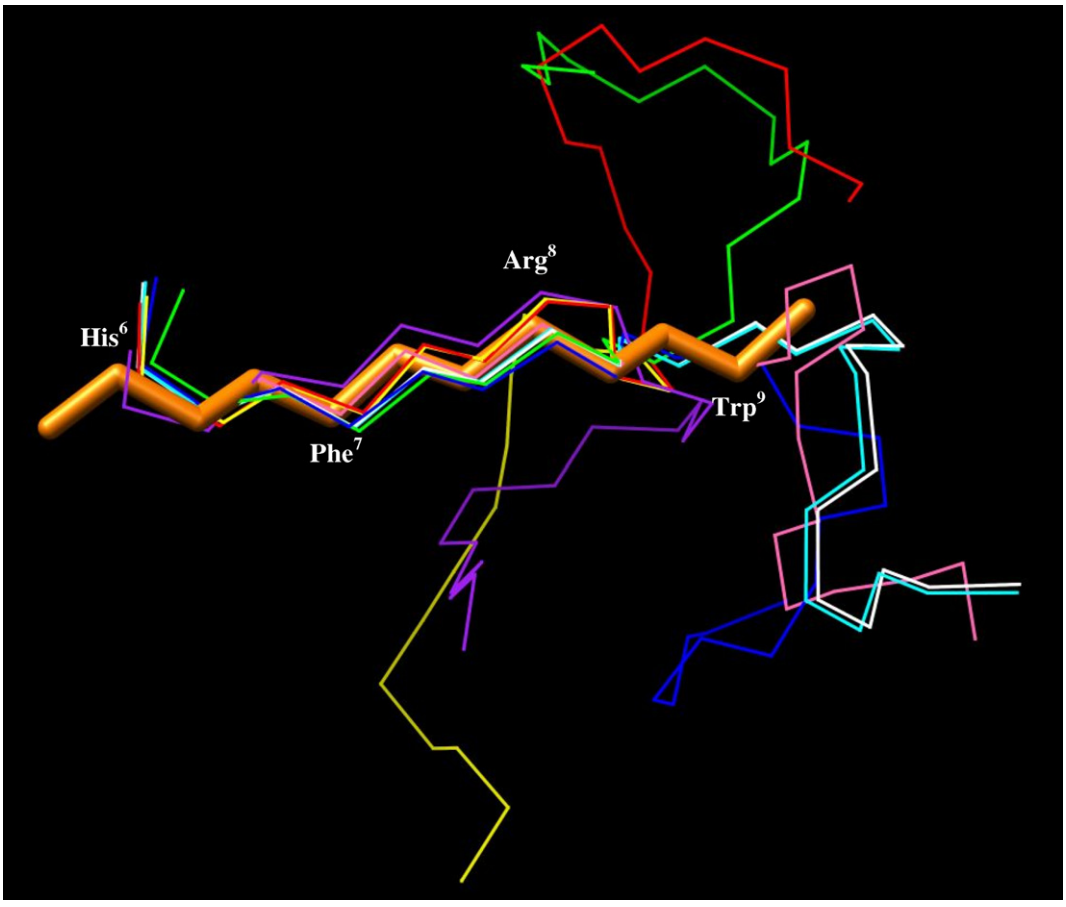

Figure 5. Stereoview of the overlapping of the backbone 'core' 6–9 sequence conformation from Ref. 40 in orange sticks, and the partially extended backbone conformers of peptides **2**–**9** in wire representations. Peptide **2** is shown in red, **3** (blue), **4** (white), **5** (pink), **6** (cyan), **7** (yellow), **8** (green) and **9** (purple). All hydrogens and side chains are omitted for clarity.

tural differences between the respective ligands, suggesting some structural difference between the α-MSH receptor and the putative yeast's receptor.

4. Experimental

4.1. Synthetic methods

Solid phase synthesis of the peptides was carried out manually on a *p*-methylbenzhydrylamine resin (1 g MBHA, 0.39 mmol/g) with standard methodology using Boc-strategy. Side chain protecting groups were as follows: Arg(Tos), His(Tos), Lys(2Cl-Z), Tyr(2Br-Z). All protected amino acids were coupled in CH_2Cl_2 (5 ml) using DCC (2.5 equiv) and HOBt (2.5 equiv) until completion (3 h) judged by Kaiser[45] ninhydrin test. After coupling of the appropriate amino acid, Boc-deprotection was effected by the use of TFA/CH_2Cl_2 (1:1, 5 ml) for 5 min first then repeated for 25 min. Following neutralization with 10% TEA/CH_2Cl_2 three times (5–5 ml of each), the synthetic cycle was repeated to assemble the resin-bond protected peptide. The peptides were cleaved from the resin with simultaneous side chain deprotection by acidolysis with anhydrous hydrogen fluoride (5 ml) containing 2% anisole, 8% dimethyl sulfide and indole at 5 °C for 45 min. The crude peptides were dissolved in aqueous acetic acid and lyophilized. Preparative and analytical HPLC of the crude and the purified peptides were performed on an LKB Bromma apparatus (for preparative HPLC, column: Lichrosorb RP C18, 7 μm, 250 × 16 mm; gradient elution: 30–100%, 70 min; mobile phase: 80% acetonitrile, 0.1% TFA; flow rate: 4 ml/min, 220 nm, for analytical HPLC, column: Phenomenex Luna 5C18(2), 250 × 4.6 mm; mobile phase: 80% acetonitrile, 0.1% TFA; flow rate: 1.2 ml/min, 220 nm). ESI-MS: Finnigan TSQ 7000.

HPLC data of the synthesized peptides:

	Retention factor (min)	Gradient elution (%)
His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (**1**)	5.757	30–55 (15 min)
His-Phe-Arg-Trp-Gly-Lys-Phe-Val-NH_2 (**2**)	8.786	25–50 (15 min)
His-Phe-Arg-Trp-Gly-Lys-Tyr-Val-NH_2 (**3**)	7.019	25–50 (15 min)
His-Phe-Arg-Trp-Gly-Arg-Phe-Val-NH_2 (**4**)	8.831	25–50 (15 min)
His-Phe-Lys-Trp-Gly-Lys-Phe-Val-NH_2 (**5**)	8.524	25–50 (15 min)
His-Phe-Lys-Trp-Gly-Arg-Phe-Val-NH_2 (**6**)	8.633	25–50 (15 min)
His-Phe-Lys-Trp-Gly-Lys-Tyr-Val-NH_2 (**7**)	6.992	25–50 (15 min)
His-Tyr-Lys-Trp-Gly-Arg-Phe-Val-NH_2 (**8**)	7.087	25–50 (15 min)
His-Tyr-Lys-Trp-Gly-Lys-Tyr-Val-NH_2 (**9**)	5.173	25–50 (15 min)
His-Tyr-Lys-Trp-Gly-Lys-Phe-Val-NH_2 (**10**)	6.820	25–50 (15 min)
His-Tyr-Arg-Trp-Gly-Lys-Phe-Val-NH_2 (**11**)	6.947	25–50 (15 min)

4.2. Microorganisms and media

Candida spp. and *Cryptococcus* sp. from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used. The panel included *Candida albicans* 10231, *Candida tropicalis* C 131 and *C. neoformans* ATCC 32264. Strains were grown on Sabouraud-chloramphenicol agar slants for 24 h at 35 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid). Inocula of cell suspensions were obtained according to reported procedures and adjusted to $1–5 \times 10^3$ cells with colony forming units (CFU)/mL.[46]

4.3. Antifungal evaluation

The test was performed in 96 wells-microplates. Peptide test wells (PTW) were prepared with stock solutions of each peptide in DMSO (maximum concentration $\leqslant$2%), diluted with RPMI-1640 to final concentrations 200 and 100 μM. Inoculum suspension (100 μl) was added to each well (final volume in the well = 200 μL). A growth control well (GCW) (containing medium, inoculum, the same amount of DMSO used in PTW, but compound-free) and a sterility control well (SCW) (sample, medium and sterile water instead of inoculum) were included for each strain tested. Microtiter trays were incubated in a moist, dark chamber at 35 °C, 24 or 48 h for *Candida* spp. or *Cryptococcus* sp., respectively. Microplates were read in a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). Amphotericin B (Sigma Chemical Co., St. Louis, MO, USA) was used as positive control (100% inhibition). Tests were performed by duplicate. Reduction of growth for each peptide concentration was calculated as follows: % of inhibition: $100 - (OD_{405}$ PTW $- OD_{405}$SCW)/OD_{405} GCW $- OD_{405}$ SCW.

4.4. Statistical analysis

Data were statistically analyzed by both, one-way analysis of variance and Student's test. A $p < 0.05$ was considered significant.

4.5. Computational methods

4.5.1. EDMC calculations. The conformational space of each peptide was explored using the method previously employed by Liwo et al.[47] that included the electrostatically driven Monte Carlo (EDMC) method.[31,32] The conformational energy was evaluated using the ECEPP/3 force field.[48] This force field employs rigid valence geometry. The hydration energy was evaluated using a hydration-shell model with a solvent sphere radius of 1.4 Å and atomic hydration parameters that have optimized using non-peptide data (SRFOPT).[49,50] In this model in addition to a sum of electrostatic, non-bonding, hydrogen-bond and torsional energy terms, the total conformational energy includes terms accounting for loop closing and peptide solvation. The conformation with minimized energy was subsequently perturbed by changing its torsional ϕ and ψ angles using the Monte Carlo method.[51] Piela's algorithm,[52] which was also applied at this stage, greatly improves the acceptance coefficient. In this algorithm ϕ and ψ angles are changed in a manner which allows the corresponding peptide group to find the most proper orientation in the electrostatic field of the rest of the peptide chain. The energy of the new conformation is minimized, compared to the previous one and can be accepted or discarded on the basis of energy and/or geometry. If the new energy-minimized conformation is similar in shape and in energy to the starting conformation, it is discarded. Otherwise, the energy of the new conformation is compared with the energy of the parent conformation. If the new energy is lower, the new conformation is accepted unconditionally, otherwise the Metropolis criterion[53] is applied in order to accept or reject the new conformation. If the new conformation is accepted, it replaces the starting one; otherwise another perturbation of the parent conformation is tried. A temperature jump may be included if the perturbation is not successful for an arbitrarily chosen number of iterations. The process is iterated, until a sufficient number of conformations have been accepted. The detailed procedure is described in Ref. 54.

In order to do an extensive exploration of the conformational space, we have been carried out 10 different runs, each of them with a different random number, for each peptide studied. Since the EDMC procedure uses random numbers, there is a need to initialize the random number generator by providing an integer. Therefore, we collected a total of 5000 accepted conformation for each peptide studied. Each EDMC run was terminated after 500 energy-minimized conformations had been accepted. The parameters controlling the runs were the following: a temperature of 298.15 K had been used for the simulations. A temperature jump of 50,000 K had been used; the maximum number of allowed repetitions of the same minimum was 50; the maximum number of electrostatically-predicted conformations per iteration was 400; the maximum number of random-generated conformations per iteration was 100; the fraction of random/electrostatically-predicted conformations was 0.30; the maximum number of steps at one increased temperature was 20; and the maximum number of rejected conformations until a temperature jump is executed was 100. Only trans peptide bonds ($\omega \cong 180°$) had been considered.

The ensemble of obtained conformations was then clustered into families using the program ANALYZE,[55–57] which applies the minimal-tree clustering algorithm for separation, using backbone atoms, energy threshold of 30 kcal mol^{-1} and RMSD of 0.75 Å as separation criteria. This procedure allows for substantial reduction of the number of conformations and eliminates repetitions.

4.5.2. Molecular electrostatic potentials and molecular interaction calculations. Quantum mechanics calculations were carried out using the Gaussian 03 program.[58] We use low-energy conformations of peptide **1**–**11** obtained from EDMC calculations. Subsequently, single point DFT (RB3LYP/6-31G(d,p)) calculations were carried out. The electronic study was carried out using

molecular electrostatic potentials (MEP).[36] These MEP were calculated using RB3LYP/6-31G(d,p) wave functions and MEP graphical presentations were created using the MOLEKEL program.[59]

Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).[60]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.02.072.

References and notes

1. Vicente, M. F.; Basilio, A.; Cabello, A.; Peláez, F. *Clin. Microbiol. Infect.* **2003**, *9*, 15.
2. McNeil, M. M.; Nash, S. L.; Hajjeh, R. A.; Phelan, M. A.; Conn, L. A.; Plikaytis, B. D.; Warnock, D. W. *Clin. Infect. Dis.* **2001**, *33*, 641.
3. Pfaller, M. A.; Diekema, D. J. *Clin. Microbiol. Rev.* **2007**, *20*, 133.
4. Walsh, T. J.; Groll, A.; Hiemenz, J.; Fleming, R.; Roilides, E.; Anaissie, E. *Clin. Microbiol. Infect.* **2004**, *10*, 48.
5. Pfaller, M. A.; Diekema, D. J. *J. Clin. Microbiol.* **2004**, *42*, 4419.
6. Weinberg, A.; Krisanaprakornkit, S.; Dale, B. A. *Crit. Rev. Oral Biol. Med.* **1998**, *9*, 399.
7. Maly, W. L.; Kari, U. P. *Biopolymers* **1995**, *37*, 105.
8. Epand, R. M.; Vogel, H. J. *Biochim. Biophys. Acta* **1999**, *1462*, 11.
9. Tossi, A.; Sandri, L.; Giangaspero, A. *Biopolymers* **2000**, *55*, 4.
10. Hancock, R. E.; Talla, W. T.; Brown, M. H. *Adv. Microb. Physiol.* **1995**, *37*, 135.
11. Hancock, R. E.; Leher, R. I. *Trends Biotechnol.* **1993**, *16*, 82.
12. Bulet, P.; Stöcklin, R. *Protein Peptide Lett.* **2005**, *12*, 3.
13. Hancock, R. E. *Lancet* **1997**, *349*, 418.
14. Park, C. B.; Kim, H. S.; Kim, S. C. *Biochem. Biophys. Res. Commun.* **1998**, *244*, 253.
15. Boman, H. G.; Agerberth, B.; Boman, A. *Infect. Immun.* **1993**, *61*, 2978.
16. Cabiaux, V.; Agerberth, B.; Johansson, J.; Homble, F.; Goormaghtigh, E.; Ruysschaert, J. M. *Eur. J. Biochem.* **1994**, *224*, 1019.
17. Cutuli, M.; Cristiani, S.; Lipton, J. M.; Catania, A. J. *Leukocyte Biol.* **2000**, *67*, 233.
18. Grieco, P.; Rossi, C.; Colombo, G.; Gatti, S.; Novellino, E.; Lipton, J. M.; Catania, A. *J. Med. Chem.* **2003**, *46*, 850.
19. Grieco, P.; Rossi, C.; Gatti, S.; Colombo, G.; Carlin, A.; Novellino, E.; Lama, T.; Lipton, J. M.; Catania, A. *J. Med. Chem.* **2005**, *48*, 1384.
20. Carotenuto, A.; Saviello, M. R.; Auriemma, L.; Campiglia, P.; Catania, A.; Novellino, E.; Grieco, P. *Chem. Biol. Drug Des.* **2007**, *69*, 68.
21. Kouznetsov, V. V.; Urbina, J.; Palma, A.; López, S.; Ribas, J.; Enriz, R. D.; Zacchino, S. *Bioorg. Med. Chem.* **2000**, *8*, 691.
22. Vargas, L. Y.; Castelli, M.; Kouznetsov, V.; Urbina, J. M.; Lopez, S.; Sortino, M.; Enriz, R. D.; Ribas, J. C.; Zacchino, S. *Bioorg. Med. Chem.* **2003**, *11*, 1531.
23. Suvire, F. D.; Sortino, M.; Kouznetsov, V. V.; Vargas, M. L. Y.; Zacchino, S. A.; Cruz, U. M.; Enriz, R. D. *Bioorg. Med. Chem.* **2006**, *14*, 1851.
24. Masman, M. F.; Rodríguez, A. M.; Svetaz, L.; Zacchino, S. A.; Somlai, C.; Csizmadia, I. G.; Penke, B.; Enriz, R. D. *Bioorg. Med. Chem.* **2006**, *14*, 7604.
25. Hruby, V. J.; Wilkes, B. C.; Hadley, M. E.; Al-Obeidi, F.; Sawyer, T. K.; Staples, D. J.; de Vaux, A. E.; Dym, O.; Castrucci, A. M.; Hintz, M. F.; Riehm, J. P.; Ranga Rao, K. *J. Med. Chem.* **1987**, *30*, 2126.
26. Cai, M.; Mayorov, A. V.; Ying, J.; Stankova, M.; Trivedi, D.; Cabello, C.; Hruby, V. J. *Peptides* **2005**, *26*, 1481.
27. Fung, S.; Hruby, V. J. *Curr. Opin. Chem. Biol.* **2005**, *9*, 352.
28. Grieco, P.; Cai, M.; Mayorov, A. V.; Trivedi, D.; Hruby, V. J. *Peptides* **2006**, *27*, 472.
29. Cain, J. P.; Mayorov, A. V.; Cai, M.; Wang, H.; Tan, B.; Chandler, K.; Lee, Y.-S.; Petrov, R. R.; Trivedi, D.; Hruby, V. J. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5462.
30. Eberle, A. N. In *The Melanotropins: Chemistry, Physiology and Mechanisms of Action*; Karger, Basel: Switzerland, 1988; pp 393–394.
31. Ripoll, D. R.; Scheraga, H. A. *Biopolymers* **1988**, *27*, 1283.
32. Ripoll, D. R.; Scheraga, H. A. *Biopolymers* **1990**, *30*, 165.
33. Vila, J. A.; Ripoll, D. R.; Baldoni, H. A.; Scheraga, H. A. *J. Biol. NMR* **2002**, *24*, 245.
34. Sarai, A. J. *Theor. Biol.* **1989**, *140*, 137.
35. North, A. C. T. *J. Mol. Graphics.* **1989**, *7*, 67.
36. Politzer, P.; Daiker, K. C. In *The Force Concept in Chemistry*; Deb, M. B., Ed.; Van Nostrand Reinhold Co.: New York, 1981.
37. Náray-Szabó, G.; Ferenczy, G. G. *Chem. Rev.* **1995**, *95*, 829.
38. Murray, S.; Politzer, P. In *Molecular Orbital Calculations for Biological Systems*; Sapse, A.-M., Ed.; Oxford University Press: New York, 1998; pp 49–84.
39. Otsuka, H.; Inouye, K. *Bull. Chem. Soc. Jpn.* **1964**, *37*, 289.
40. Sugg, E. E.; Cody, W. L.; Abdel-Malek, Z.; Hadley, M. E.; Hruby, V. J. *Biopolymers* **1986**, *25*, 2029.
41. Hiltz, M. E.; Catania, A.; Lipton, J. M. *Peptides* **1991**, *12*, 767.
42. Peng, P. J.; Sahm, U. G.; Doherty, R. V.; Kinsman, R. G.; Moss, S. H.; Pouton, C. W. *Peptides* **1997**, *18*, 1001.
43. Holder, J. R.; Haskell-Luevano, C. *Med. Res. Rev.* **2004**, *24*, 325.
44. Sahm, U. G.; Olivier, G. W.; Branch, S. K.; Moss, S. H.; Pouton, C. W. *Peptides* **1994**, *15*, 1297.

45. Kaiser, E.; Colescott, R. L.; Bossiuger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.
46. NCCLS, National Committee for Clinical Laboratory Standards, Method M27-A2, 2nd ed., Wayne Ed.; 2002, Vol. 22, pp 1–29.
47. Liwo, A.; Tempczyk, A.; Oldziej, S.; Shenderovich, M. D.; Hruby, V. J.; Talluri, S.; Ciarkowski, J.; Kasprzykowski, S.; Lankiewicz, L.; Grzonka, Z. *Biopolymers* **1996**, *38*, 157.
48. Némethy, G.; Gibson, K. D.; Palmer, K. A.; Yoon, C. N.; Paterlini, G.; Zagari, A.; Rumsey, S.; Scheraga, H. A. *J. Phys. Chem.* **1992**, *96*, 6472.
49. Vila, J.; Williams, R. L.; Vásquez, M.; Scheraga, H. A. *Proteins Struct. Funct. Genet.* **1991**, *10*, 199.
50. Williams, R. L.; Vila, J.; Perrot, G.; Scheraga, H. A. *Proteins Struct. Funct. Genet.* **1992**, *14*, 110.
51. Li, Z.; Scheraga, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6611.
52. Piela, L.; Scheraga, H. A. *Biopolymers* **1987**, *26*, S33.
53. Metropolis, N.; Rosenbluth, A. W.; Rosenbluth, M. N.; Teller, A. H.; Teller, E. J. *Chem. Phys.* **1953**, *21*, 1087.
54. Sidor, M.; Wójcik, J.; Pawlak, D.; Izdebski, J. *Acta Biochim. Pol.* **1999**, *46*, 641.
55. Meadows, R. P.; Post, C. B.; Luxon, A. B.; Gorenstein, D. G. MORASS 2.1; Purdue University: W. Lafayette, 1994.
56. Post, C. B.; Meadows, R. P.; Gorenstein, D. G. *J. Am. Chem. Soc.* **1990**, *112*, 6796.
57. Scheraga, H. A.; Ripoll, D. R.; Liwo, A.; Czaplewski, C. User Guide ECEPPAK and ANALYZE Programs.
58. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. Gaussian 03, revision B.01; Gaussian, Inc.: Wallingford, CT, 2004.
59. Flükiger, P.; Lüthi, H. P.; Portmann S.; Weber, J. MOLEKEL 4.0; Swiss Center for Scientific Computing: Manno, Switzerland, 2000.
60. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. *J. Comput. Chem.* **2004**, *25*, 1605.