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CHAPTER 2



SYNTHESIS & CONFORMATIONAL ANALYSIS OF HIS-PHE-ARG-TRP-NH₂ & ANALOGUES WITH ANTIFUNGAL PROPERTIES

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ABSTRACT

The synthesis, *in vitro* evaluation and conformational study of His-Phe-Arg-Trp-NH₂ and related derivatives acting as antifungal agents are reported. Among them, His-Phe-Arg-Trp-NH₂ and His-Tyr-Arg-Trp-NH₂ exhibited antifungal activity against *Cryptococcus neoformans*. Antifungal activity of these compounds appears to be closely related to the α-MSH effect. A conformational and electronic study allows us to propose a biologically relevant conformation for these tetrapeptides acting as antifungal agents. In addition, these theoretical calculations permit us to determine the minimal structural requirements to produce the antifungal response and may provide a guide for the design of compounds with this biological activity.

2.1 INTRODUCTION

In the early 1980s, it was believed that virtually any fungal infection could be successfully treated with the wide range of available antifungal agents. Nevertheless, this belief was soon proved to be false (Garber 2001). The continuing increase in the incidence of fungal infections together with the gradual rise in resistance mainly to azoles in the last two decades highlighted the need to search novel compounds not tested previously in antifungal assays (Fromling 1999; Kontoyiannis et al. 2003; Polak 1999; Zacchino et al. 2003). This event resulted in the identification of novel molecules of diverse structures, which could prove useful for a future development. Among them, some natural peptides have been recently reported as antifungal compounds. They showed to inhibit a broad spectrum of pathogens and microorganisms (Bulet and Stöcklin 2005; Hancock et al. 1995; Hancock and Lehrer 1998; Lee et al. 2003) and possess a very important characteristic, since they do not usually induce bacterial resistance (Hancock 1997). Regarding their structural features, they are cationic, but differing considerably in other characteristics such as size and presence of disulfide bonds and structural motifs (Epand et al. 1995; Epand and Vogel 1999).

Most of these peptides are believed to exert their antimicrobial activities either forming multimeric pores in the lipid bilayer of the cell membranes (Hancock 1997), or interacting with DNA or RNA after penetration into the cell (Boman et al. 1993; Cabiaux et al. 1994; Park et al. 1998). Nevertheless, α -melanocyte stimulating hormone (α -MSH) and its C-terminal tripeptide Lys-Pro-Val, which showed antimicrobial activity against two representative pathogens: *Staphylococcus aureus* and *Candida albicans* (Cutuli et al. 2000), appear to act through a mechanism substantially different from that of other natural antimicrobial peptides. Previous research suggests that the candidacidal effect of α -MSH is linked to the cAMP production in *C. albicans*

and the adenylyl cyclase inhibitor ddAdo partly reversed the candidacidal effect of the peptide (Cutuli et al. 2000; Grieco et al. 2003; Grieco et al. 2005). It was suggested by Eberle and Schwyzer that α -MSH might have two message sequences, the first active sequence centered around the central tetrapeptide His-Phe-Arg-Trp and the second active site centered around the C-terminal tripeptide Ac-Lys-Pro-Val-NH₂ (Eberle et al. 1975; Eberle and Schwyzer 1975, 1976). Thus, we focussed our attention on both sequences in order to evaluate their potential antifungal effects.

As part of our ongoing program aimed at identifying novel antifungal agents, we have reported several natural and synthetic compounds (Freile et al. 2003; Giannini et al. 2004; Karolyhazy et al. 2003; Lopez et al. 2005; Lopez et al. 2001; Suvire et al. 2006; Urbina et al. 2000; Vargas M et al. 2003; Zacchino et al. 1999; Zacchino et al. 1997) exhibiting antifungal activities against different human pathogenic fungi. However, peptide structures had not been tested during this program until now. In the present study we report the design, synthesis and antifungal activity of novel small-size peptides: fifteen tripeptides structurally related to Lys-Pro-Val (the 11-13 sequence of α -MSH) and thirteen tetrapeptides structurally related to His-Phe-Arg-Trp (the 6-9 sequence of α -MSH). We tested these peptides against various human pathogenic strains including yeasts, filamentous as well as dermatophyte fungi. In addition, a conformational and electronic study on the most representative peptides reported here was carried out in order to determine a possible biologically relevant conformation for these compounds acting as antifungal agents. Thus, one of the goals of this study was to identify a topographical and/or substructural template, which may be the starting structure for the design of new antifungal compounds.

2.2 RESULTS AND DISCUSSION

2.2.1 CHEMISTRY

Peptides **1-28** were prepared by manually solid-phase synthesis on a *p*-methylbenzhydrylamine resin (1 g MBHA, 0.39 mmol/g) and Merrifield resin (1 g, 1%, 200-400 mesh) with standard methodology using Boc-strategy. The peptides were cleaved from the resin with simultaneous side chain deprotection by acidolysis with anhydrous hydrogen fluoride containing 2% anisole, 8% dimethyl sulfide and indole. Details of the synthesis are given in Section 2.4.1 (SYNTHETIC METHODS)

2.2.2 ANTIFUNGAL ACTIVITY

To carry out the antifungal evaluation, concentrations of peptides up to 250 µg/ml were incorporated to growth media following the guidelines of the National Committee of Clinical and Laboratory Standards for yeasts (Pfaller 2002a) and for filamentous fungi (Pfaller 2002b). Compounds producing no inhibition of fungal growth at 250 µg/ml level were considered inactive.

In a first step we focussed our attention on the activity of the sequence Ac-Lys-Pro-Val-NH₂ (peptide **1**, SCHEME 2.1), which has been reported as relevant for both receptor activation (Hruby et al. 1987) and antimicrobial activity (Cutuli et al. 2000). However, this tripeptide did

not show any antifungal activity to the concentrations reported here against any of the strains of the fungal panel (yeasts: *Saccharomyces cerevisiae* and *Cryptococcus neoformans*; hialohyphomycetes: *Aspergillus flavus*, *A. niger* and *A. fumigatus*; dermatophytes: *Microsporum gypseum*, *Trichophyton rubrum* and *Trichophyton mentagrophytes*). It should be noted that compound **1** has been previously reported as antifungal against a strain of *C. albicans* (Cutuli et al. 2000). However, compound **1** was inactive against all the fungi tested here. Next, fourteen tripeptides with different residues (peptides **2-15**) were tested against the panel of opportunistic pathogenic fungi. It can be observed that none of these tripeptides showed antifungal activity against hialohyphomycetes or dermatophytes yeasts. It should be noted that peptide **6** was inactive against all the fungi tested here. This result is in agreement with those previously reported for peptide **6**, by Grieco et al. who reported only 2.7 % of inhibition of *C. albicans* for this tripeptide (Grieco et al. 2005).

In a second step of our study we focused on the tetrapeptide His-Phe-Arg-Trp-NH₂ (**16**), which is common to all melanocortin peptides and is important for binding to the known melanocortin receptors (Hruby et al. 1987). A very interesting result was that this tetrapeptide displayed a moderate but significant antifungal activity against *C. neoformans* (TABLE 2.1) as well as against dermatophyte fungi (MICs values of 125, 250 and 250 µg/ml against *T. mentagrophytes*, *M. gypseum* and *T. rubrum*, respectively). Peptide **16** was the only peptide reported here showing antifungal activity against dermatophytes.

The next step consisted in testing twelve tetrapeptides structurally related to peptide **16** (peptides **17-28**), in which different replacements in the His-Phe-Arg-Trp-NH₂ sequence were performed to determine the contribution of each amino acid to the antifungal activity. None of these tetrapeptides displayed significant effect against *C. albicans*, *Saccharomyces cerevisiae*, *Aspergillus*

SCHEME 2.1: Sequence of the peptides studied in CHAPTER 2.

TABLE 2.1: Antifungal activity (MIC) of tetrapeptide His-Phe-Arg-Trp-NH₂ and derivatives against *Cryptococcus neoformans* ATCC 32264

Peptide	Structure	MIC (μg/ml)
16	His-Phe-Arg-Trp-NH ₂	125
17	His-Tyr-Arg-Trp-NH ₂	125
18	His-Phe-Lys-Trp-NH ₂	250
19	His-Tyr-Lys-Trp-NH ₂	250
20	His-Phe-D-Arg-Trp-NH ₂	250
21	His-D-Phe-Arg-Trp-NH ₂	250
22	His-D-Phe-Lys-Trp-NH ₂	250
23	Ac-His-Phe-Arg-Trp-NH ₂	>250
24	Ac-His-Tyr-Arg-Trp-NH ₂	>250
25	Ac-His-Phe-Lys-Trp-NH ₂	>250
26	Ac-His-Tyr-Lys-Trp-NH ₂	>250
27	Ac-Trp-Arg-Phe-His-NH ₂	>250
28	For-His-Phe-Arg-Trp-NH ₂	>250
Standard drugs		
Amphotericin B		0.25
Ketoconazole		0.25

spp. and dermatophytes. However, six of these tetrapeptides (compounds 17–22) displayed antifungal activity only against *Cryptococcus neoformans* (TABLE 2.1). This is an important finding because cryptococcosis remains an important life-threatening complication for immunocompromised hosts and new compounds acting against this fungus are actually welcome (Polak 1999).

From the structure-activity analysis of the active peptides, it appears that the position of His and Trp-NH₂ in the amino acid sequence is essential for such activity, whereas the position of Phe and Arg are not. Replacement of the Phe

residue in position 2 by Tyr (16 → 17) was possible without any loss of activity. Replacing Arg in position 3 by Lys was tolerated but with loss of activity (compare 16 with 18). Peptide 19 showed that the replacement of the two central residues was tolerated but with loss of activity. An identical result was obtained replacing Arg with D-Arg (compare 16 with 20). Our results indicate that replacement of Phe by Tyr gives a peptide as potent as the starting structure, whereas replacing Phe by its isomer D-Phe gives an active tetrapeptide but with loss of activity (compare 16 with 21 and 22). In addition, all the acetylated (peptides 23–27) and formylated (28) derivatives were inactive compounds.

In order to better understand the above experimental results and to establish some basis to obtain more active compounds, we performed a conformational and electronic study on the most representative peptides reported here, using theoretical calculations.

2.2.3 CONFORMATIONAL AND ELECTRONIC STUDY OF HIS-PHE-ARG-TRP-NH₂ AND ANALOGUES

Linear peptides are highly flexible and therefore the determination of the biologically relevant conformations is not an easy task. It is necessary to perform an exhaustive conformational analysis for these structures. Therefore, eight tetrapeptides (16–19 and 23–26) were selected for energy calculations.

TABLE 2.2: Selected conformational search and clustering results for peptides 16–19 and 23–26 optimized at the EDMC/SRFOPT/ECCEP/3 level of theory

Pep	Elec.	Generated ^a				Accepted ^b				# F ^c	# F _{0.20%} ^d	% P ^e
		Ran.	Ther.	Total	Elec.	Ran.	Ther.	Total				
16	3808	57095	80	60983	576	4359	65	5000	442	55	86.76	
17	4592	65724	125	70441	651	4240	109	5000	436	51	87.84	
18	4602	73490	235	78327	653	4168	179	5000	512	66	86.12	
19	5403	78159	225	83787	747	4074	179	5000	449	37	87.72	
23	6158	94316	340	100814	536	4199	265	5000	715	45	79.64	
24	6517	91519	247	98283	743	4078	179	5000	623	59	82.22	
25	7514	102050	499	110063	448	4190	362	5000	786	49	78.82	
26	7104	95865	382	103351	518	4206	276	5000	659	51	81.94	

^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 as result of the clustering run.

^d # F_{0.20%}: Number of conformational families with populations above 0.20%

^e % P: Sum of the per cent relative population of # F_{0.20%}

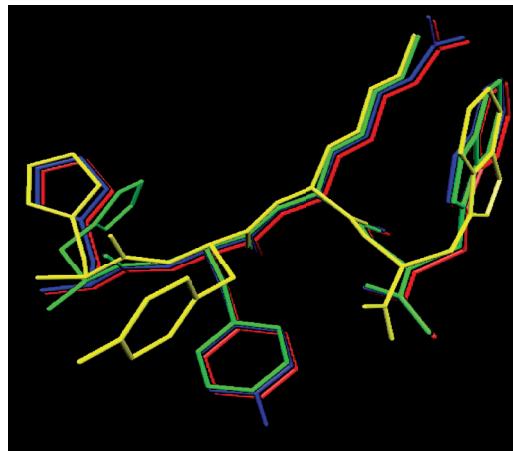


FIGURE 2.1: Stereoview of overlapping of conformer 1 for peptide **16** (red), conformer 1 for analogue **17** (blue), conformer 19 for analogue **18** (green) and conformer 19 for analogue **19** (yellow). All hydrogens are omitted for clarity.

Theoretical calculations were carried out as described in section 2.4.4 COMPUTATIONAL METHODS. These results are summarized in TABLE 2.2 and TABLES 2.1S-2.8S (available in APPENDIX A 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 60983 and 110063, and the number of those accepted was 5000 for all the cases. In the clustering procedure, an R.M.S.D (Root Mean Square Deviation) of 0.75 Å and a ΔE of 20 kcal mol⁻¹ were used. The number of families after clustering varied between 435 and 789. The total number of families accepted with a relative population higher than 0.20 % varied between 37 and 66. Their populations sum up to *ca* 80% of all conformations for each case (see TABLE 2.2).

All low-energy conformers of tetrapeptides studied here were then compared to each other. The comparison involved the spatial arrangements, relative energy and populations. It was found that all the active tetrapeptides **16-19** possess similar low-energy conformers. FIGURE 2.1 shows a spatial view of most populated and energetically preferred conformations of compounds **16** and **17**. These conformations have been

overlapped with conformation number 19 obtained for compounds **18** and **19** (TABLES 2.1S-2.8S in APPENDIX A). The conformers shown in FIGURE 2.1 are very close to one another considering the values of the dihedral angles for their backbones as well as comparing the dihedral angles defining the mutual spatial arrangement of side chains.

It is worth mentioning that the families 19 are not the preferred forms for peptides **18** and **19**, which possess 1.62 and 2.13 kcal mol⁻¹ above their respective global minimum, and also they are not the most populated forms. In other words, compounds **18** and **19** can adopt a very similar spatial ordering to those obtained for peptides **16** and **17** but this is not possible without some energetic cost. FIGURE 2.2 gives a spatial view of the preferred conformations obtained for compounds **16**, **18** and **19**. It is clear that there is not a complete conformational overlapping between these conformers. In this case, a good fit among the two first amino acids (His-Phe in peptides **16** and **18** and His-Tyr in peptide **19**) was obtained, but there is not a good overlapping between the two last amino acids (Arg-Trp for **16** and Lys-Trp in peptides **18** and **19**). Interesting enough, com-

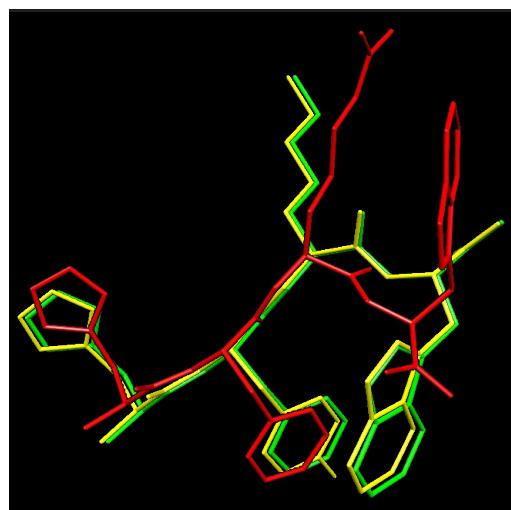


FIGURE 2.2: Stereoview of overlapping of conformer 1 for peptide **16** (red), conformer 1 for analogue **18** (green) and conformer 2 for analogue **19** (yellow). All hydrogens are omitted for clarity.

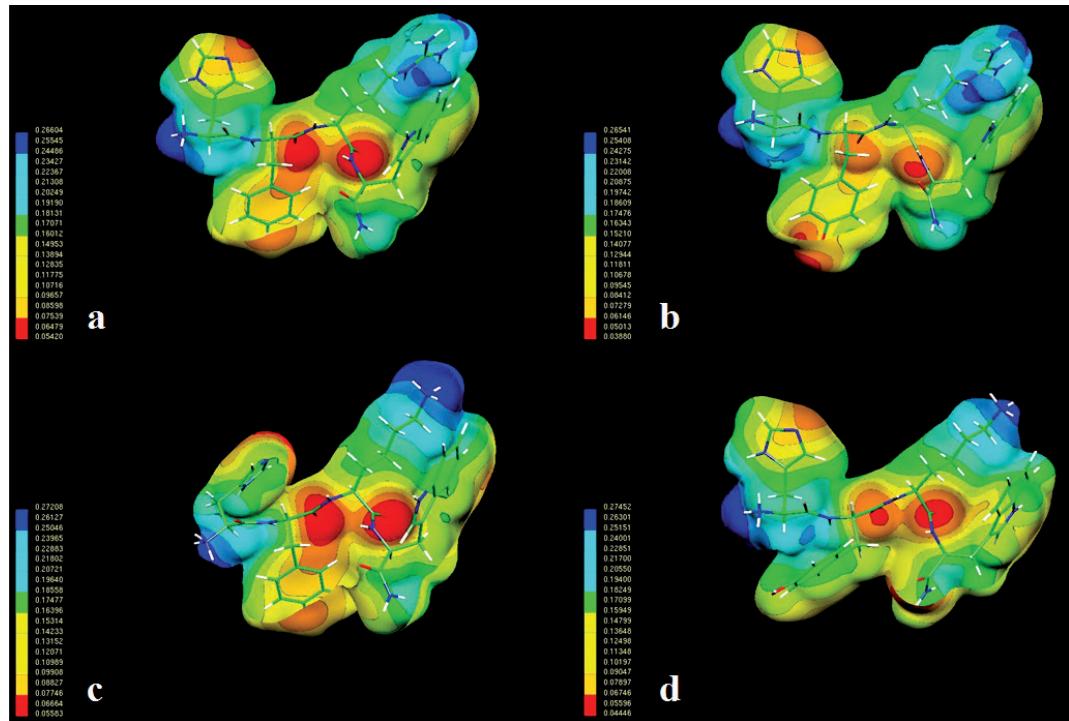


FIGURE 2.3: Electrostatic potential-encoded electron density surfaces of peptides **16** (a), **17** (b), **18** (c) and **19** (d). The surfaces were generated with Gaussian 03 after RHF/6-31G(d) single-point calculations. The coloring represents electrostatic potential with red indicating the strongest attraction to a positive point charge and blue indicating the strongest repulsion.

ounds **18** and **19** displayed half of the antifungal activity in comparison with compounds **16** and **17**. Thus, our results let us speculate that the different antifungal effect could be explained by this conformational difference.

Molecular recognition and the converse concept of specificity (Sarai 1989) are explained in mechanistic and reductionistic terms by a stereo-electronic “complementarity” between the ligand and the receptor (North 1989). In this context it is obvious that the knowledge of the stereo-electronic attributes and properties of His-Phe-Arg-Trp-NH₂ and its analogues will contribute significantly to the elucidation of the mechanism of action at the molecular level or at least, to determine a possible pharmacophoric pattern for these compounds acting as antifungal compounds. The intermolecular forces that contribute to both affinity and specificity can be schematically classified as hydrophobic and elec-

trostatic ones. Therefore, Molecular Electrostatic Potentials (MEPs) are of particular value because they permit the visualization and assessment of the capacity of a molecule to interact electrostatically with a putative binding site (Carrupt et al. 1991; Greling et al. 1996; Politzer and Truhlar 1981). MEPs can be interpreted in terms of a stereoelectronic pharmacophore condensing all available information on the electrostatic forces underlying affinity and specificity.

Once the low-energy conformations for the most active tetrapeptides 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 using MEPs. FIGURE 2.3 shows the MEPs obtained for compounds **16–19**. These results account for the general characteristics of the electronic behavior of active tetrapeptides reported here. The general pattern is similar for all of the active systems. The

MEPs exhibit clear minimum values (deep red zones) in the vicinity of the lone pair of nitrogen atom of His and near the carbonyl groups of the backbone. There are positive regions near the Arg residue (peptides **16** and **17**) or Lys residue (peptides **18** and **19**) and at the amino terminal group. Also three clear hydrophobic zones might be appreciated near the aromatic rings of His, Phe (or Tyr) and Trp.

All the acetylated derivatives tested here were inactive compounds. FIGURE 2.4b shows the spatial view obtained for the preferred conformation of peptide **23**. This conformer displays a very different spatial ordering with respect to the preferred conformation obtained for compound **16** (FIGURE 2.4a). While for active compounds, a partially extended (β -strand) conformation for the backbone is the preferred form, for peptide **23** the preferred form for the backbone is a folded conformation which is stabilized through hydrogen bonds. The interaction shown in FIGURE 2.4b is a bifurcated hydrogen bond between the carbonyl system of the acetyl group and the guanidine group of Arg residue.

This stabilizing interaction might be better appreciated in FIGURE 2.5. The different electronic distributions obtained for the acetylated peptides with respect to those attained for compounds **16-19** might be well appreciated com-

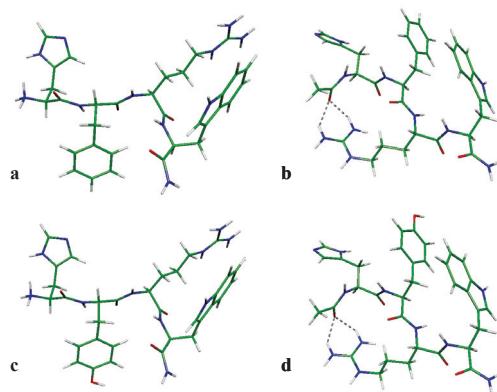


FIGURE 2.4: Spatial view of the preferred conformations obtained for peptides **16** (a), **23** (b), **17** (c) and **24** (d) using EDMC calculations. The hydrogen bonds are denoted by dotted lines.

paring FIGURES 2.3 with 2.5. All the acetylated and formylated derivatives (peptides **24-28**) displayed a conformational behavior closely related to that obtained for compound **23**. FIGURES 2.4c and d give a comparative spatial view between the preferred conformations of peptide **17** and its acetylated derivative (compound **24**). On the basis of our results, we can conclude that the lack of anti-fungal activity of these acetylated peptides could be explained at least in part, by their different conformational and electronic behaviors.

It is worthwhile to compare the biologically active conformation for the backbone of the His-Phe-Arg-Trp-NH₂ proposed in this study to that previously reported for the same sequence from proton NMR studies in aqueous solution (Sugg et al. 1986). Sugg et al. reported that the observed topology was consistent with non hydrogen-bonded β -like structure ($\phi=139^\circ$ and $\psi=135^\circ$ for *L*-amino acids) as the predominant solution conformation. This conformation is closely related to those obtained for the different conformers of the active peptides reported here. In FIGURE 2.6 we overlapped the conformation reported by Sugg et al. (in white) with three representative conformations obtained for peptide **16**. In the case of conformer **11** (FIGURE 2.6a) a complete overlap, not only with the backbone but also with the vici-

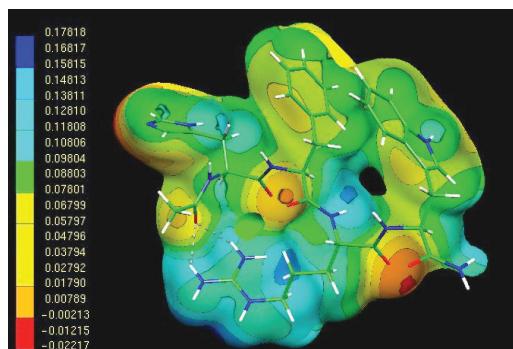


FIGURE 2.5: Electrostatic potential-encoded electron density surface of peptide **23**. The surface was generated with Gaussian 03 after RHF/6-31G(d) single-point calculation. The color-coded is shown at the left.

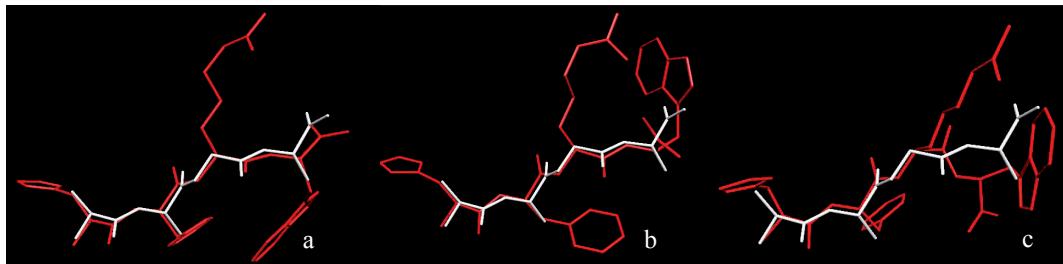


FIGURE 2.6: Overlapped stereoviews of the backbone conformation from Sugg et al in white and various conformations for compound 16 in red: (a) conformer 11, (b) conformer 5 and (c) conformer 1. All hydrogens are omitted for clarity.

nal side-chains was obtained. For conformers 5 and 1 (FIGURE 2.6b and c) although there is not a complete fit, the resemblance is still apparent.

At this stage of our study, some general trends might be established: (i) the smallest fragments with measurable antifungal activity were His-Phe-Arg-Trp-NH₂ and its derivatives 17-22. It has been shown that the minimal sequence possessing α -MSH-like activity is the central tetrapeptide His⁶-Phe⁷-Arg⁸-Trp⁹ (Hruby et al. 1987). This “core” sequence was suggested to be the “message” fragment for α -MSH, the rest of the molecule being regarded as the “address” sequence (Otsyka and Inouye 1964).

(ii) For the α -MSH activity, the importance of His⁶ was demonstrated by the fact that Ac- α -MSH₇₋₁₀-NH₂ lacked measurable activity. Trp⁹ was also critical for biological activity as demonstrated by the lack of biological activity of Ac- α -MSH₆₋₈. Our results suggest that the presence of His and Trp-NH₂ sequence appears to be a structural requirement for the antifungal activity

as well. In contrast, our results indicate that Phe and Arg could be replaced.

(iii) A. Catania’s group suggested that the antimicrobial effects of α -MSH are exerted through a unique mechanism different from that of other natural antimicrobial peptides (Cutuli et al. 2000; Grieco et al. 2003; Grieco et al. 2005). They suggested that the candidacidal effect of α -MSH is linked to the cAMP-inducing activity. Our results may be regarded as an additional support for that hypothesis.

(iv) It is interesting that the tetrapeptides 16-22, being the smallest fragment with antifungal activity, contains three aromatic residues. In this regard, aromatic amino acids are common in the active site of many hormones and are often considered critical for biological activity (Sabesan and Harper 1983). Thus, our conformational and electronic study indicates that a predominant β -conformation without internal stabilizing hydrogen bond could be the “biologically relevant conformation” for these compounds.

23 CONCLUSIONS

WE synthesized small-size peptides (tri and tetrapeptides) with different residues and tested their antifungal activity. Among the compounds tested, His-Phe-Arg-Trp-NH₂ and analogues displayed antifungal activities mainly against *Cryptococcus neoformans*. The antifungal activity of these compounds appears to be closely related to the α -MSH effect. These results are in a complete agreement with those previous-

ly reported by Grieco et al. against *Candida albicans* (Cutuli et al. 2000; Grieco et al. 2003; Grieco et al. 2005).

A detailed conformational and electronic study supported by theoretical calculations helped us to identify a possible “biologically relevant conformation” and understand the minimal structural requirements for the antifungal actions

of tetrapeptides reported here. Our results are very encouraging in that they show a great potential of His-Phe-Arg-Trp-NH₂ and analogues as a truly novel class of antifungal compounds particularly against the yeast *Cryptococcus neoformans*.

Although it is desirable to develop compounds having a broad spectrum of activity, it is also important to bear in mind that long treatments of fungal infections with the same broad spectrum antifungal agent may lead to a high resistance to the available antifungal agents (Ghannoum and Rice 1999). Thus, one of

the strategies for overcoming this problem is the treatment of fungal infections with the appropriate narrow spectrum agent when the ethological agent is known (Didomenico 1999). The selective antifungal activity reported in this paper for peptides **16–22** (and among them for compounds **16** and **17** in particular) opens an interesting field of research, which deals with the discovery and further development of new antifungal peptides useful for treating cryptococcosis, an important life-threatening infection for immuno-compromised hosts.

2.4 EXPERIMENTAL SECTION

2.4.1 SYNTHETIC METHODS

Solid phase synthesis of the peptides (His-Phe-Arg-Trp-NH₂, His-D-Phe-Arg-Trp-NH₂, His-Tyr-Arg-Trp-NH₂, His-Phe-Lys-Trp-NH₂, His-D-Phe-Lys-Trp-NH₂, His-Tyr-Lys-Trp-NH₂, His-Phe-D-Arg-Trp-NH₂, His-D-Phe-D-Arg-Trp-NH₂, His-Phe-D-Lys-Trp-NH₂, Arg-Pro-Val-NH₂, Lys-Phe-Val-NH₂, Lys-Pro-Val-NH₂, Ac-Arg-Pro-Val-NH₂, Ac-Lys-Phe-Val-NH₂, Ac-Val-Pro-Lys-NH₂, Ac-His-Pro-Val-NH₂, Ac-Lys-Pro-Val-NH₂, Ac-Lys-Gly-Gly-NH₂, Ac-Lys-Gly-Ala-NH₂, Ac-Lys-Ala-Gly-NH₂, Ac-His-Phe-Arg-Trp-NH₂, For-His-Phe-Arg-Trp-NH₂, Ac-Trp-Arg-Phe-His-NH₂, Ac-His-Tyr-Arg-Trp-NH₂, Ac-His-Phe-Lys-Trp-NH₂, Ac-His-Tyr-Lys-Trp-NH₂, Lys-Pro-Val-OH, Arg-Pro-Val-OH, Lys-Phe-Val-OH, Ac-Lys-Pro-Val-OH) were carried out manually on a p-methylbenzhydrylamine resin (1 g MBHA, 0.39 mmol/g) and Merrifield resin (1 g, 1%, 200–400 mesh), respectively 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 CH₂Cl₂ (5 ml) using DCC (2.5 equiv.) and HOBr (2.5 equiv.) until completion (3 h) judged by Kaiser ninhydrin test. After coupling of the appropriate amino acid, Boc deprotection was effected by use of TFA/CH₂Cl₂ (1:1, 5 ml) for 5 min first then repeated for 25 min. Following neutralization with 10% TEA/CH₂Cl₂ 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, 250x16 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, 250x4.6 mm; mobile phase: 80% acetonitrile, 0.1% TFA; flow rate: 1.2 ml/min, 220 nm). ESI-MS: Finnigan TSQ 7000 (TABLE 2.3).

2.4.2 MICROORGANISMS AND MEDIA

For the antifungal evaluation, strains from the American Type Culture Collection (ATCC), Rockville, MD, USA and CEREMIC (C), Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina were used: *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, *Aspergillus flavus* ATCC 9170,

TABLE 2.3: HPLC data of the synthesized peptides

Pep.	Sequence	Retention factor (min)	Gradient elution (%)
1.	His-Phe-Arg-Trp-NH ₂	12.912	5-60 (20 min)
2.	His-D-Phe-Arg-Trp-NH ₂	6.510	20-50 (15 min)
3.	Lys-Pro-Val-NH ₂	9.962	0-25 (15 min)
4.	His-Phe-Lys-Trp-NH ₂	8.048	20-50 (15 min)
5.	His-Tyr-Arg-Trp-NH ₂	6.523	20-50 (20 min)
6.	Arg-Pro-Val-NH ₂	5.552	5-50 (20 min)
7.	Lys-Phe-Val-NH ₂	10.570	5-50 (20 min)
8.	His-Phe-D-Lys-Trp-NH ₂	8.343	10-70 (20 min)
9.	His-D-Phe-D-Arg-Trp-NH ₂	9.598	10-70 (20 min)
10.	His-Phe-D-Arg-Trp-NH ₂	9.079	10-70 (20 min)
11.	His-Tyr-Lys-Trp-NH ₂	9.004	10-70 (20 min)
12.	His-D-Phe-Lys-Trp-NH ₂	6.068	20-50 (15 min)
13.	Ac-Lys-Gly-Gly-NH ₂	7.434	5-80 (20 min)
14.	For-His-Phe-Arg-Trp-NH ₂	12.750	10-45 (15 min)
15.	Ac-Lys-Phe-Val-NH ₂	11.878	5-80 (25 min)
16.	Ac-Lys-Gly-Ala-NH ₂	5.674	0-30 (10 min)
17.	Ac-Lys-Pro-Val-OH	8.311	10-45 (15 min)
18.	Ac-Lys-Pro-Val-NH ₂	8.153	5-25 (10 min)
19.	Ac-His-Tyr-Lys-Trp-NH ₂	8.919	15-45 (15 min)
20.	Ac-His-Phe-Lys-Trp-NH ₂	12.703	5-80 (25 min)
21.	Ac-His-Pro-Val-NH ₂	6.533	10-35 (10 min)
22.	Ac-Val-Pro-Lys-NH ₂	8.364	5-35 (10 min)
23.	Ac-Trp-Arg-Phe-His-NH ₂	11.770	5-80 (20 min)
24.	Ac-His-Tyr-Arg-Trp-NH ₂	7.297	20-50 (10 min)
25.	Ac-His-Phe-Arg-Trp-NH ₂	8.785	20-50 (10 min)
26.	Ac-Arg-Pro-Val-NH ₂	8.781	5-80 (25 min)
27.	Ac-Lys-Ala-Ala-NH ₂	6.235	0-40 (20 min)
28.	Lys-Pro-Val-OH	8.945	0-30 (15 min)
29.	Arg-Pro-Val-OH	9.254	5-20 (15 min)
30.	Lys-Phe-Val-OH	11.109	5-50 (20 min)

Aspergillus fumigatus ATCC 26934, *Aspergillus niger* ATCC 9029 and *Trichophyton mentagrophytes* ATCC 9972, *Trichophyton rubrum* C 113, *Microsporum gypseum* C 115.

Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Inocula of cell or spore suspensions were obtained according to reported procedures and adjusted to 10⁵ cells/spores with colony forming units (CFU) /mL (Wright et al. 1983).

2.4.3 ANTIFUNGAL SUSCEPTIBILITY TESTING

Minimal Inhibitory Concentration (MIC) of each compound was determined by using broth microdilution techniques according to the guidelines of the National Committee for Clinical Laboratory Standards for yeasts

(M27-A2) (Pfaller 2002a) and for filamentous fungi (M38-A). (Pfaller 2002b) MIC values were determined in RPMI-1640 (Sigma, St Louis, Mo, USA) buffered to pH 7.0 with MOPS. The starting inocula were 1x10³ to 5x10³ CFU/ml. Microtiter trays were incubated at 35 °C for yeasts and halo-hyphomycetes and at 28-30 °C for dermatophyte strains in a moist, dark chamber, and MICs were visually recorded at 48 h for yeasts, and at a time according to the control fungus growth, for the rest of fungi.

For the assay, stock solutions of peptides were two-fold diluted with RPMI 250-1 µg/ml (final volume = 100 µl) and a final DMSO concentration ≤ 1%. A volume of 100 µl of inoculum suspension was added to each well with the exception of the sterility control where sterile water was added to the well instead. MIC was defined as the minimum inhibitory concentration of pure compound which resulted in total inhibition of the fungal growth. Ketoconazole, Terbinafine and Amphotericin B were used as positive controls.

2.4.4 COMPUTATIONAL METHODS

2.4.4.1 EDMC CALCULATIONS

The conformational space of each peptide was explored using the method previously employed by Liwo et al. that included the Electrostatically Driven Monte Carlo (EDMC) method (Liwo et al. 1996b; Ripoll and Scheraga 1988, 1990). Conformational energy was evaluated using the ECEPP/3 force field (Némethy et al. 1992). This force field employs rigid valence geometry. Hydration energy was evaluated using a hydration-shell model with a solvent sphere radius of 1.4 Å and atomic hydration parameters that have been optimized using nonpeptide data (SRFOPT) (Vila et al. 1991; Williams et al. 1992). 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 (Li and Scheraga 1987). Piela's algorithm (Piela and Scheraga 1987), 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 may 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 to the energy of the parent conformation. If the new energy is lower, the new conformation is accepted unconditionally, otherwise the Metropolis criterion (Metropolis et al. 1953) 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 has been accepted. Sidor et al. has described this procedure in a very detailed fashion (Sidor et al. 1999).

In order to explore the conformational space extensively, we 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 conformations 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 was used for the simulations. A temperature jump of 50000 K

was 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 ($\omega \approx 180^\circ$) were considered.

The ensemble of obtained conformations was then clustered into families using the program ANALYZE (Meadows et al. 1994b; Post et al. 1990; Scheraga et al. 1983), which applies the minimal-tree clustering algorithm for separation, using all heavy atoms, energy threshold of 20 kcal mol⁻¹ and R.M.S.D. of 0.75 Å as separation criteria. This procedure allows for substantial reduction of the number of conformations and eliminates repetitions.

2.4.4.2 MOLECULAR ELECTROSTATIC POTENTIALS

The electronic study of the peptides was carried out using Molecular Electrostatic Potentials (MEPs). MEPs have shown to provide reliable information, both on the interaction sites of molecules with point charges and on the comparative reactivities of these sites (Carrupt et al. 1991; Greeling et al. 1996; Politzer and Truhlar 1981). These MEPs were calculated using RHF/6-31G(d) wave function from the Gaussian 03 program (Frisch et al. 2003). EDMC coordinates were imported to generate the wave functions; thus, RHF/6-31G(d) single-point calculations were performed from Gaussian 03 program. All MEPs graphical presentations were created using Molekel (Flükiger et al. 2000). ■

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*"Il pleut, c'est malheureux. Il pleut depuis ce matin. Il
veut s'emparer de mon être sans paraître malbonnête. Il
pleut dans ces gouttes de pluie mes doutes s'enfuient je
ne m'ennuie plus. Il pleut, mais ce n'est pas la pluie qui
occupe mes nuits"*
(E. Simon)



"The opportunist"

oil on paper (320x450 mm)

M.F. Masman 2010