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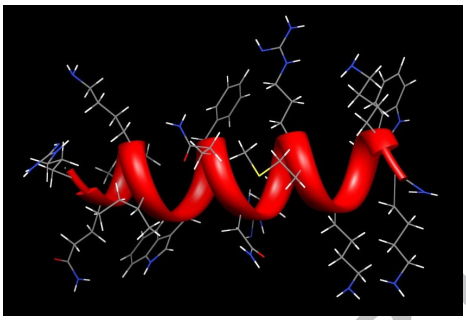

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We report the synthesis, the conformational study by using CD spectroscopy and the antifungal assays of penetratin analogues. These peptides displayed a significant antifungal activity against *C. albicans* and *C. neoformans*.

New antifungal peptides. Synthesis, bioassays and initial structure prediction by CD spectroscopy

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Abstract

The synthesis, in vitro evaluation and conformational study of KKWKMRRNQFWIKIQR-NH_2, HFRWRQIKIWFQNRRMKWKK-NH_2 and RQPKIWFPNRRKPWKK-NH_2 acting as antifungal agents are reported. These peptides displayed a moderate but significant antifungal effect against both pathogenic fungi *Candida albicans* and *Cryptococcus neoformans*. The conformational analysis of these peptides was carried out using both theoretical and experimental methods.

Keywords: peptides; antifungal; conformation; circular dichroism

Fungal infections pose a continuous and serious threat to human health and life especially to immunocompromised patients.[1-3] As part of our ongoing program aimed at identifying novel antifungal agents, we have reported several natural and synthetic compounds exhibiting antifungal activities against different human pathogenic fungi.[4-12] Among them, some peptides structurally related with α-melanocyte stimulating hormone (α-MSH) displayed significant antifungal effects. We have reported that His-Phe-Arg-Trp-NH_2, the 6–13 sequential unit of α-MSH, and additional structurally related tetrapeptides showed antifungal properties especially against *Cryptococcus neoformans* (*C. neoformans*).[9] A complete molecular modelling study has suggested that a half-extended molecular conformer family held important spatial orderings to fulfil the relevant bioactivity response for these peptides.[9,11] These results are in good agreement with the available experimental data reported by Carotenuto et al.[13]

We have recently reported that penetratin (**1**) (RQIKIWFQNRRMKWKK-NH_2), a well-known cell penetrating peptide, displays a significant antifungal effect against both *Candida albicans* (*C. albicans*) and *C. neoformans*, two important life-threatening infections of immunocompromised hosts.[12] Guided by the premise that a peptide-based antifungal agent should be as short as possible in order to reduce side-effects and cost, we decided to synthesize shorter derivatives of penetratin. We have synthesized shorter peptides structurally related to penetratin; however, they turned out to be inactive or presenting only a marginal effect. The tetrapeptide RQKK displayed a moderate antifungal activity against *C. neoformans*, but resulted almost inactive against *C. albicans*, limiting its potential use.[12] More recently, we have designed and synthesized small-size peptides of nine to eleven amino acids with antifungal activity.[14,15] Our related molecular modelling studies have suggested that a particular combination of cationic and hydrophobic residues, when adopting a definite spatial ordering, can translocate through the membrane from hydrophilic to hydrophobic phase, a necessary requirement of the antifungal activity. Thus, in contrast to the peptides structurally related to α-MSH, penetratin and its derivatives displayed a clear tendency to form helix-like secondary structures, at least in a membrane-mimetic environment (**Figure 1**).[12,14] These results are in agreement with those previously reported for penetratin and for its two derivatives detected from circular dichroism (CD) data.[16,17]

At this stage of our study we were particularly interested to determine if there was some direct relationship between the antifungal activity displayed by these peptides and their availability to adopt a fully helical secondary structural element. In order to obtain such information, three new analogues of penetratin were synthesized and tested, namely KKWKMRRNQFWIKIQR-NH_2 (peptide **2**), HFRWRQIKIWFQNRRMKWKK-NH_2 (peptide **3**) and RQPKIWFPNRRKPWKK-NH_2 (peptide **4**), (**Table 1**). As polypeptide **2** is the *retro-inverso* of penetratin (**1**), the position of the carbonyl and amino groups in each of the amide bonds of the polypeptide backbone was

reversed, conferring a strong resistance to the peptide toward various proteases.[18,19] Furthermore, peptide **3** was synthesized in order to investigate whether His-Phe-Arg-Trp-NH_2 (the 6–13 sequence of α-MSH) attached to penetratin might increase its antifungal activity. Finally, in peptide **4** (**Table 1**)**,** glutamine (Q) and methionine (M) were replaced by proline (P) residues. These replacements were performed in order to diminish the tendency of forming a helix-like secondary structure since proline is a well-known "helix breaker" residue. However, as only two amino acids were replaced by P in peptide **4** compared to penetratin, only a partial conformational shift was expected to occur.

In the present study we report the synthesis, antifungal activity and conformational behaviour of peptides **2**-**4**.

The solid phase synthesis of the peptides **2**-**4** was carried out manually on a *p*-methyl benzhydrylamine resin (1 g MBHA, 0.14 mmol/g) with standard methodology using Boc-strategy.[9,11,12] HPLC data of the peptides and additional experimental information are provided in the Supplementary data.

In the bioassays performed with both pathogenic fungi (*C. albicans* and *C. neoformans*) all three peptides (**2**-**4**) gave a significant antifungal response, despite quantitative differences (**Table 1**). The percentage of inhibition was performed in 96-well microplates; assays were carried out as previously described.[9,11,12] Clearly, the antifungal effects of all three peptides are very similar to those of penetratin previously reported (**Table 1**).[12]

To examine the correlation between chemical structure and antifungal activity, the conformational study of peptides **2**-**4** was performed. In a first step the conformational analysis of these peptides was carried out using Electrostatically Driven Monte Carlo (EDMC) calculations.[20] These calculations were conducted in the same way as previously reported.[12,14] EDMC results predicted that peptides **2** and **3** possessed a clear tendency to adopt helix-like secondary structures, being an α-helix structure the most representative form for these peptides. These conformations were characterized by hydrogen bonds between the carbonylic oxygen (residue *i*) and the NH group (residue $i + 4$). In contrast, the preferred conformation for peptide **4** corresponded to a structure that possessed residues 3 and 4 adopting a bend structure; residues 9, 10, 11, 13, 14 and 15 in a turn structure and residues 1, 2 and 16 without a stable structure. These results are summarized in **Tables 1S-4S** in the Supplementary data.

Second, an exploratory analysis was conducted by using two web-based services: AGADIR-helix content predictor[21] (**Table 5S**) and Advanced Protein Secondary Structure Prediction Server (APSSP2)[22] (**Table 6S**). The first server, AGADIR, suggested that peptide **2** and **3** have very little helix content (about 1.62, and 1.35 %, respectively) whereas peptide **4** has none (0.04%) (**Table 5S**). The second server, APSSP2, a protein sequence secondary structure predictor, showed that peptide **4** had mainly a random coil structure. For peptide **2**, two β-sheet regions were

predicted, connected by a short random coil sequence (the 7th and 8th residues, Arg-Asn), which suggested an overall hairpin structure. In the case of peptide **3**, the 4-12 residues were predicted to adopt a sheet structure, while the rest of the sequence was predicted to be random coil-like, very flexible residues. The above bioinformatics results did not predict the prevalence of any secondary structural element, and the dominance of α-helix was not observed. In addition, the same servers did not provide any significant correlation between the above sequences.

It is thus evident that the two procedures, EDMC and web-based services led to substantially different results. The first method predicted α-helix structure only for peptides **2** and **3** and a turn structure for **4**, while the second ones predicted that peptides **2** and **3** have very little helix content whereas peptide **4** has none. In the presence of such a situation a reasonable step to take is to look for an arbitrage by an experimental procedure. The experimental structure determination obtained by CD was interesting since this method is also sensitive to local conformational changes. In addition, CD may be used to monitor changes induced by the variation of the polarity of the molecular environment. Thus, for a better understanding of the conformational behaviour of peptides **2**-**4**, their structural ensemble was studied both in an extracellular matrix-mimetic and in a membrane-mimetic environment. The aqueous solutions simulated the extracellular matrix, while solutions containing trifluoroethanol mimicked a membrane-like molecular environment with respect to polypeptide folding. CD spectroscopic measurements were completed both in water and in a mixture of TFE and water (3/7). Peptides **2**-**4** were measured at room temperature by using the following conditions (pH adjusted by HCl/NaOH solutions): Peptide **2**: Concentration: 0.023 mM; pH=6.60**;** Peptide **3**: Concentration: 0.175 mM; pH=6.70; Peptide **4**: Concentration: 0.022 mM; pH=6.60.

The comprehensive spectral analysis revealed that peptides **2**-**4** showed little or no structural preference in water (**Figures 2**-**4** black lines). The "U"-type CD spectra reflected the presence of a very large number of different local conformations in a time average manner. Therefore, in water, from the shape of these U-type CD curves little characteristic secondary structure content could be extracted for any of these peptides (black lines). When the same peptides were recorded in the solvent mixture of 30% TFE and 70% H_2O, significant changes in the shape of the curves of peptides **2** and **3** were observed. As often detected for linear peptides, the CD curve of polypeptides **2** and **3** (see red lines in **Figures 2** and **3**, respectively) had spectral features similar to those of a C-type CD curve. This observation is in line with the predominance of type I/III β-turns like structures and/or with the appearance of helical structures. The "red curves" most probably reflected a conformational ensemble composed of α- or 3_{10}-helix combined with type I/III β-turns plus some percentage of still unstructured (or highly mobile) backbone foldamers. Nevertheless, as for most linear peptides of about 20 amino acid residues or more, the backbone structure may be

characterized as the ensemble of a multitude of opened folds in a rapid conformational equilibrium. Our results are in agreement with a previous study using CD spectroscopy[23] which has shown that, in aqueous solution, pseudin-2 existed predominantly as a random coil, but in 50% trifluoroethanol/water, the peptide adopted an α-helical conformation.

These results demonstrated that in the presence of a considerable amount of TFE, both peptides **2** and **3** adopted an increased amount of helical and/or type I/III β-turn secondary structure. Furthermore, as the TFE content increased, the atypical or random part of the backbone conformation decreased (**Figures 2** and **3**). In contrast to **2** and **3**, the inclusion of TFE did not initiate significant structural changes for peptide **4**; its conformational behaviour seemed to remain the same as in pure water (see red curve in **Figure 4**).

We have reported that at high TFE concentrations (an environment of low polarity), penetratin gave a class C-type spectrum[24,25] referring to β-turns (type I or III) and/or α- (or 3_{10}) helices.[16,17] In contrast to this, at high water concentration (a polar medium), U-type CD spectra[26] were recorded for this peptide. The results obtained for peptides **2** and **3** were closely related to those previously reported for penetratin. For these peptides, the inclusion of TFE as a co-solvent, clearly increased the helical and/or β–turn content and decreased the atypical or random coil part of the overall backbone conformation (**Figures 2** and **3**). In contrast, for peptide **4** the inclusion of TFE did not display a significant change in its conformational behaviour. However, it should be noted that peptide **4** as well as **3** and **2** displayed a strong antifungal activity against both *C. albicans* and *C. neoformans*. In fact, peptide **4** showed the strongest antifungal effect against *C. neoformans* (**Table 1**).

In conclusion, our present experimental data indicated that the three polypeptides studied here exhibit antifungal activities against both *C. albicans* and *C. neoformans.* Our results suggest that to adopt an α-helix structure is not a structural requirement to produce the antifungal activity. This is an interesting result from a medicinal chemistry point of view. Unlike peptide **2** and **3,** polypeptide **4** does not present any helical structural properties neither in water nor in the presence of 30% of TFE. Thus, the helical conformation and the antifungal effect show little or no correlation. In fact, substitution of Arg and Gln residues by Pro should decrease the stability of any helical conformation. Nevertheless, the antifungal activity remains at the same level. Therefore, as many antibacterial peptides have predominantly a random coil structure, the herein obtained results do not contradict any of the biological activity tests.

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Supplementary data

Supplementary data associated with this article can be found in the online version.

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Legends

Figure 1. Spatial view of the preferred form (α-helix structure) for peptide **1**

Figure 2. The CD spectrum of peptide **2** (KKWKMRRNQFWIKIQR-NH_2) in water (black line) and in TFE/H_2O (3/7) (red curve). The CD spectrum of peptide **1** is also shown on the top right side.

Figure 3. The CD spectrum of peptide **3** (HFRWRQIKIWFQNRRMKWKK-NH_2) in water (black line) and in TFE/H_2O (3/7) (red curve)

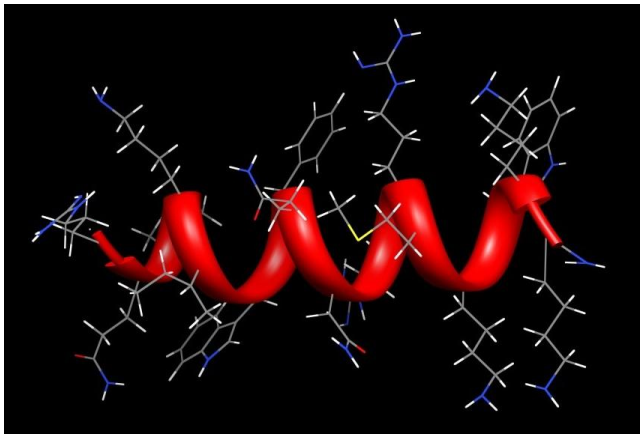
Figure 4. The CD spectrum of peptide **4** (KKWKMRRNQFWIKIQR-NH_2) in water (black line) and in TFE/H_2O (3/7) (red curve)

Table 1. Antifungal activity (% inhibition) of peptides **1**-**4** against *Candida albicans* ATCC 10231 and *Cryptococcus neoformans* ATCC 32264. (**1**) RQIKIWFQNRRMKWKK-NH_2; (**2**) KKWKMRRNQFWIKIQR-NH_2; (**3**) HFRWRQIKIWFQNRRMKWKK-NH_2 and (**4**) RQPKIWFPNRRKPWKK-NH_2. The percentage of inhibition higher than 60, are denoted in bold.

Peptide	*Candida albicans*						*Cryptococcus neoformans*					
	200 µM	100 µM	50 µM	25 µM	12.5 µM	6.25 µM	200µM	100 µM	50 µM	25 µM	12.5 µM	6.25 µM
1[a]	**100**	**100**	**95**±1.2	**91**±1.6	4.00 ±0.1	0	**100**	**100**	**100**	**100**	**90**±2.3	**60**±2.40
2	**100**	**100**	**100**	25.76±1.3	11.99±3.0	0	**100**	**100**	**98.99**±0.5	**94.06**±3.4	**89.50**±5.3	55.86±1.3
3	**100**	**100**	**93.36**±0.6	4.86± 1.4	3.85± 1.5	0.90±0.04	**100**	**100**	**87.27**±03.4	**85.88** ±05.8	**74.32**±7.0	51.02±2.6
4	**100**	**100**	41.37±3.4	15.37±6.3	6.10±7.18	0	**100**	**100**	**100**	**100**	**100**	**79.92**±5.9
Amph. B[b]	100	100	100	100	100	100	100	100	100	100	100	100
Ket[c]	100	100	100	100	100	100	100	100	100	100	100	100

[a] previously reported in reference 12
[b] Amphotericin B
[c] Ketoconazole

Figure 1



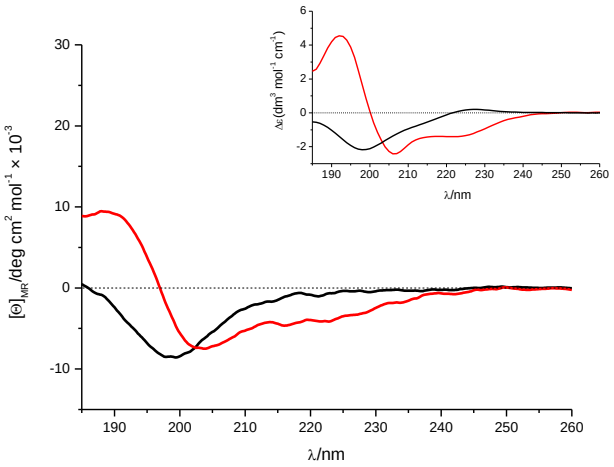


Figure 2

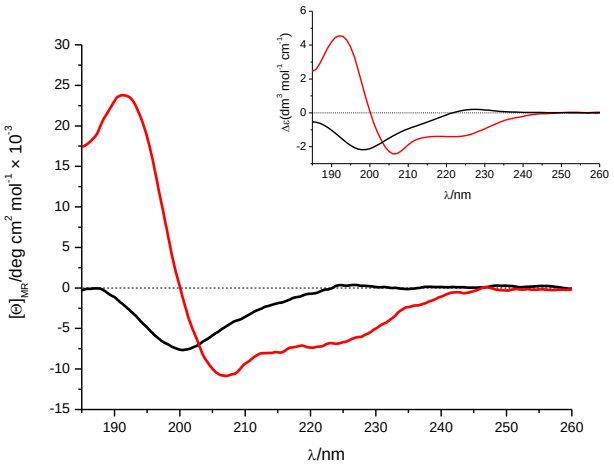


Figure 3

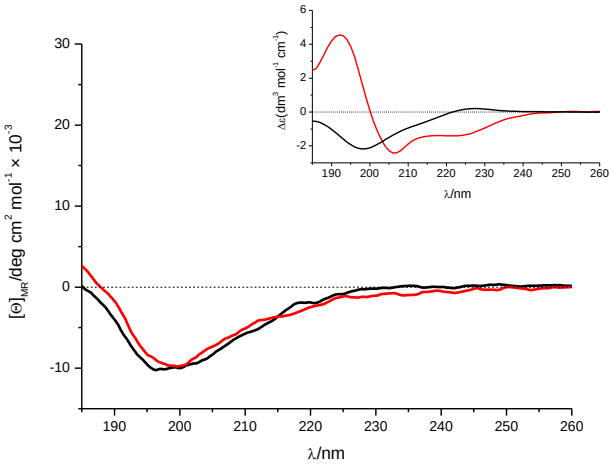


Figure 4