Structural study of melanocortin peptides by fluorescence spectroscopy: identification of β-(2-naphthyl)-d-alanine as a fluorescent probe

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Abstract

Several cyclic disulfide α-melanocyte stimulating hormone (α-MSH) analogues containing the aromatic fluorescent amino acid β-(2-naphthyl)-d-alanine (D-Nal) have high affinity and selectivity for the melanocortin (MC)-4 receptor. Considering the possible relevant role played by the lipid phase in the peptide-receptor interaction, the structures of two cyclic α-MSH analogues, containing both Trp and D-Nal fluorophores, were investigated by steady-state and time-resolved fluorescence spectroscopy, in aqueous solution and in the presence of dimyristoyl phosphatidylglycerol (DMPG) vesicles, and compared with that of the natural peptide. The amino acid D-Nal gives a unique de-excitation fluorescence profile, with an excited state lifetime much longer than those of Trp, allowing good distinction between the two fluorophores. The cyclic analogues’ aqueous structures seem to be adequate for membrane penetration, as Trp fluorescence indicates that, in both aqueous and lipid media, the Trp environment in the cyclic peptides is similar to that of α-MSH when incorporated in lipid bilayers. Trp, in the cyclic analogues, seems to penetrate deeper in the bilayer than in the native peptide. The amino acid D-Nal was also found to penetrate deep into the lipid bilayer, having its excited-state lifetime drastically changed from aqueous solution to lipid medium. The present work shows that D-Nal may serve as a fluorescent probe for studies of MC peptides and suggests that the high affinity and selectivity of the cyclic peptides to the MC4 membrane receptor could be related to their deeper penetration into the bilayer core.

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1. Introduction

Five different subtypes of melanocortin (MC) receptors have been identified by molecular cloning techniques (MC1 to MC5), belonging to the superfamily of receptors coupled to the G-protein, which stimulate the adenosine cyclic 3’,5’-phosphate (cAMP) signal transduction pathway [1]. The endogenous agonist ligands for these MC receptors are derived by cleavage of the precursor proopiomelanocortin (POMC): α-, β-, and γ-melanocyte stimulating hormones (MSH) and adrenocorticotropin (ACTH). All these MC peptide agonists contain a core His-Phe-Arg-Trp tetrapeptide that has been attributed to the ligand selectivity and stimulation of the MC receptors [2–5].

Among the natural MC peptides, ACTH is selective for the MC2 receptor, and the other peptides (α-, β- and γ-MSH) have no specific selectivity for the different MC subtypes [6–9]. MC1 receptor is known to be related to pigmentation processes in a variety of vertebrates, whereas MC2 receptor plays an important role in the regulation of steroid production in the adrenal gland [1]. The MC3 and MC4 are very important for central regulation of weight homeostasis [10,11], while the MC5 receptor is involved in exocrine gland function [12].

In order to get more information about the physiological roles of the MC receptors, α-MSH (Ac-Ser¹-Tyr²-Ser³-Met⁴-Glu⁵-His⁶-Phe⁷-Arg⁸-Trp⁹-Gly¹⁰-Lys¹¹-Pro¹²-Val¹³-NH₂) analogues have been synthesized and tested for the different receptors. Considering the known relevance of the sequence His⁶-Phe⁷-Arg⁸-Trp⁹, several analogues have been produced with modifications at this sequence [13–17]. Cyclic peptides, presenting a forced turn-structure at the 6–9 residues position, were shown to be potent ligands [14].

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The bulky aromatic β-(2-naphthyl)-d-alanine (d-Nal) substitution of the Phe residue in a cyclic peptide led to the discovery of a potent MC3 and MC4 receptor antagonist, SHU9119 (Ac-Ne^4-[Asp^5-His^6-d-Nal(2)^7-Arg^8-Trp^9-Lys^10]-NH₂ [15]. Recently [18–21], experimental studies in mouse MC receptors based on the template Ac-His-DPhe-Arg-Trp-NH₂ showed that the His^6 position can differentiate MC4 versus MC3 agonist selectivity, the inversion of chirality of the Phe to ω-Phe results in a dramatic increase in receptor potency and the removal of the Arg^8 guanidinyl side chain moiety decreases the receptor potency. Furthermore, modification of Trp^9 resulted in a change in MC1 potency up to a 220-fold, while at the MC4 and MC5 receptors the potency decreased up to a 9700-fold [21]. Moreover, the presence of a disulfide bridge between Cys residues in positions 3 (or 4) and 11 led to clearly selective antagonists for MC4 receptor [7,22].

Considering the possible relevant role played by the lipid phase in the peptide–receptor interaction, α-MSH and MC synthetic peptides were tested for lipid affinity and bilayer depth penetration. Using spin labels at different bilayer positions, we have shown that α-MSH interacts with low ionic strength dimeristoyl phosphatidylglycerol (DMPG) bilayers, changing the membrane packing and altering the amount of water molecules in the bilayer core [23,24]. Tryptophan fluorescence and its suppression by nitroxide moieties positioned at different bilayer depths have shown that α-MSH, and some potent analogues, partially penetrate DMPG bilayers [25,26]. The average depth of Trp penetration into DMPG bilayers was estimated to be around the carbons 6–8 of the lipid acyl chain [26].

In the present work, we report measurements of steady-state and time-resolved fluorescence spectroscopy of two cyclic analogues, HS032 (Ac-[Cys^3-Ne^4-Arg^5-His^6-DNal^-Arg^8-Trp^-Gly^10-Cys^11]-Pro^12-Pro^13-Lys^14-Asp^15-NH₂) and HS053 (Ac-[Cys^3-Ne^4-Lys^-His^6-o-Nal^7-Arg^8-Trp^-Gly^10-Cys^11]-NH₂), which were shown to present high affinity for the MC4 receptor [22]. The analogues were studied in aqueous solution and in the presence of anionic vesicles of DMPG, and compared with the natural peptide α-MSH. In the pH value used here (7.4), α-MSH is positive (approximately monovalent), whereas HS032 and HS053 possess net charge +2. α-MSH was monitored by the fluorescence of the Trp residue, whereas in the cyclic analogues the two fluorophores, Trp^9 and o-Nal^7, were analyzed. Though the Trp^9 and o-Nal^7 fluorescence properties only report the peptide structure in the vicinity of the residues, the information is rather relevant as the “core sequence” comprising residues 6–9 was shown to be essential for the peptides biological activity, as mentioned above. Considering the different packing regions of a biological membrane, the peptide/lipid interaction was studied with lipids in the highly packed and organized gel phase (DMPG at 10 °C), and with fluid lipids (DMPG at 40 °C) [27,28].

2. Materials and methods

2.1. Materials

α-MSH and the cyclic analogues, HS032 and HS053, were synthesized as previously described [22] and generously provided by Melacure Therapeutics AB, Uppsala, Sweden. The sodium salt of the phospholipid DMPG (1,2-dimyristoyl-sn-glycero-3-phospho-rac-glycerol) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). 4-(2-Hydroxyethyl)-1-piperizine ethanesulfonic acid (HEPES) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of MC peptides and lipid dispersion

Stock solutions (5 × 10^-3 M) of peptides and Boc-d-Nal were prepared at room temperature (23 °C) and diluted to the final concentration of 5 × 10^-5 M. The buffer was used to 10 mM HEPES + 2 mM NaCl, adjusted with NaOH to pH 7.4.

A film was formed from a chloroform solution of DMPG, dried under a stream of N₂ and left under reduced pressure for a minimum of 2 h, to remove all traces of the organic solvent. Liposomes were prepared by the addition of the desired peptide solution followed by vortexing at ca. 35 °C, above the lipid phase transition temperature. The DMPG final concentration was 1 × 10^-3 M.

2.3. Optical and fluorescence spectroscopy

Optical absorption measurements were performed with an HP 8452 A diode array spectrophotometer. For steady-state fluorescence experiments, a Fluorolog 3 Jobin Yvon-Spex spectrometer was employed. Excitation and emission slits of 1 and 2 nm bandpass, respectively, were used. Excitation wavelength was set to 296 nm.

Time-resolved experiments were performed using an apparatus based on the time-correlated single photon counting method. The excitation source was a Tsunami 3950 Spectra Physics titanium–sapphire laser, pumped by a 2060 Spectra Physics argon laser. The repetition rate of the 5-ps pulses was set to 800 kHz using the pulse picker 3980 Spectra Physics. The laser was tuned to give output at 888 nm, and a third harmonic generator BBO crystal (GWN-23PL Spectra Physics) gave 296-nm excitation pulses that were directed to an Edinburgh FL900 spectrometer. The I-format configuration of the spectrometer allowed detection of the emission at a right angle from the excitation. The emitted photons were detected by a refrigerated Hamamatsu R3809U microchannel plate photomultiplier. Measurements were made using a time resolution of 12 ps per channel for time scale of 50 ns and of 48 ps per channel for time scale of 200 ns. Software provided by Edinburgh Instruments was used to analyze the decay curves, and the adequacy of the multi-exponential decay fitting was judged by inspection of the plots of
weighted residuals and by statistical parameters, such as reduced $\chi^2$.

The decay curves were fitted according to $I(t) = \sum \alpha_i e^{-t/\tau_i}$, and the mean lifetime for the Trp de-excitation was calculated through the usual definition of mean value,

$$\langle \tau \rangle = \frac{\int_0^\infty t I(t) dt}{\int_0^\infty I(t) dt} \quad \text{or} \quad \langle \tau \rangle_{\text{Trp}} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i},$$

where $i = 1, 2$ and 3.

3. Results and discussion

3.1. Optical absorption and steady-state fluorescence spectroscopy

The peptides’ optical absorption spectra are shown in Fig. 1. In $\alpha$-MSH, Trp$^9$ (mainly), Tyr$^2$ and, in smaller extent, Phe$^7$ residues, are the chromophores responsible for the absorption band around 280 nm. The two cyclic peptides, HS032 and HS053, display identical optical absorption spectra (see Fig. 1), with both Trp$^9$ and $\tau$-Nal$^7$ contributing to the absorbance at 280 nm. The Boc-$\tau$-Nal optical spectrum is also shown in Fig. 1.

The hormone $\alpha$-MSH in aqueous solution, under excitation at 296 nm, presents a fluorescence emission band centered at 357 nm (Fig. 2a), typical of the Trp residue in a polar environment, as expected for a small peptide with no defined structure [25]. In the presence of DMPG vesicles, the Trp emission band is shifted to lower wavelengths, 342 nm, (Fig. 2b), probably indicating a less polar environment for the fluorophore [29], therefore its penetration into the lipid bilayer. The emission spectra of the two cyclic analogues, HS032 and HS053, are very similar, either in water medium or in lipid membranes (Fig. 2a and b). These spectra can be well reproduced by the addition of the $\alpha$-MSH (or Trp) and Boc-$\tau$-Nal emission fluorescence spectra (the latter also shown in Fig. 2a), the fraction of each spectrum ($\alpha$-MSH and $\tau$-Nal) being strongly dependent on the excitation wavelength.

In the $\alpha$-MSH/lipid ratio used here, the peptide molecules are nearly all bound to the lipid bilayer [25]. Considering that the cyclic peptides have higher positive charge than $\alpha$-MSH, they should also be mostly at the DMPG negative bilayer surface. Interestingly, the Boc-$\tau$-Nal emission spectrum clearly presents a vibrational structure, which is also observed in the emission spectra of the cyclic analogs when bound to DMPG vesicles (Fig. 2b). It is noteworthy that the cyclic peptides’ emission spectra in lipid medium are mostly due to the D-Nal fluorescence. This could be related to a significant increase in the D-Nal fluorescence intensity in lipid medium and/or to a considerable Trp fluorescence quenching. Time-resolved fluorescence supports the first hypothesis, as discussed below.
3.2. Time-resolved fluorescence

The peptides were also studied via time-resolved fluorescence spectroscopy, which has the advantage of avoiding artifacts due to light scattering of lipid dispersions. In both aqueous solution and DMPG dispersion, α-MSH fluorescence decay is well described by a three exponential function, as usually found for Trp-containing peptides [30,31]. This multi-exponential decay has been attributed to the different rotational conformers of the indole ring around the Cα–Cβ bond of the alanyl side chain [31,32]. For the cyclic peptides, in addition to the Trp excited state three lifetimes (τ₁, τ₂, τ₃), there is a long one (τ₄) due to D-Nal deexcitation. The peptides’ fluorescence time decay, at 25 °C, as well as the curve fitting (see Materials and methods) are shown in Fig. 3.

Considering that for a given peptide the decay times were nearly constant across the fluorescence spectrum, a global analysis of the data sets measured at different wavelengths (325 to 380 nm) was performed. Tables 1 and 2 present the parameters yielded by the global analysis of the data, at two temperatures, for the three peptides in aqueous solution and in DMPG dispersion: the excited state lifetimes (τᵢ), the mean lifetimes (τᵢ) Trp and the mean normalized pre-exponential factors (aᵢ). The last parameter is the arithmetic mean value calculated from the pre-exponential factors obtained for each wavelength in the interval 325–380 nm (the mean values and the mean values deviations are shown in Fig. 4).

For α-MSH, the lifetimes and pre-exponential factors are slightly different from those previously obtained with other solvents [25,33,34].

![Fluorescence time decay of α-MSH (A, —), HS032 (B, ●), and HS053 (C, ○) in (a) aqueous solution and (b) DMPG dispersion. HEPES buffer at 25 °C. Excitation wavelength 296 nm; emission wavelength 355 nm. The decay curves were fitted by multiexponential functions (data in Table 1). The quality of the fittings is indicated by the plots of the weighted residues shown below the fluorescence decays.](image)

Table 1
| Global analysis results: excited state lifetimes (τᵢ), mean normalized pre-exponential factors (aᵢ), and mean lifetimes (τᵢ) Trp for melanocortin peptides (5 × 10⁻⁵ M) in aqueous solution (HEPES buffer) |
|---|---|---|---|---|---|---|
| T (°C) | τ₁ (ns) | τ₂ (ns) | τ₃ (ns) | (τᵢ)Trp (ns) | a₁ | a₂ |
| α-MSH | 10 | 0.37 | 4.04 | 0.32 | 1.99 | 0.31 | 0.27 | 3.31 | 1.15 |
| | 40 | 0.29 | 2.19 | 0.44 | 1.36 | 0.27 | 1.70 | 1.07 |
| HS032 | 10 | 0.17 | 4.07 | 0.48 | 1.89 | 0.35 | 0.31 | 2.68 | 0.02 | 22.11 | 1.14 |
| | 40 | 0.04 | 3.70 | 0.53 | 1.33 | 0.41 | 0.29 | 1.59 | 0.02 | 12.68 | 1.02 |
| HS053 | 10 | 0.16 | 4.17 | 0.48 | 1.85 | 0.34 | 0.32 | 2.66 | 0.02 | 22.21 | 0.98 |
| | 40 | 0.03 | 4.33 | 0.48 | 1.31 | 0.48 | 0.33 | 1.58 | 0.01 | 13.32 | 0.97 |
| vₑ is the reduced χ² from the global analysis. |

Table 2
| Global analysis results: excited state lifetimes (τᵢ), mean normalized pre-exponential factors (aᵢ), and mean lifetimes (τᵢ) Trp for melanocortin peptides (5 × 10⁻⁵ M) in DMPG dispersion (HEPES buffer) |
|---|---|---|---|---|---|---|
| T (°C) | τ₁ (ns) | τ₂ (ns) | τ₃ (ns) | (τᵢ)Trp (ns) | a₁ | a₂ |
| α-MSH | 10 | 0.18 | 5.05 | 0.48 | 2.65 | 0.34 | 0.54 | 3.91 | 1.16 |
| | 40 | 0.08 | 4.20 | 0.46 | 1.71 | 0.46 | 0.27 | 1.70 | 1.14 |
| HS032 | 10 | 0.04 | 9.07 | 0.46 | 1.89 | 0.35 | 0.31 | 2.70 | 0.02 | 12.68 | 0.98 |
| | 40 | 0.04 | 6.09 | 0.48 | 1.31 | 0.48 | 0.33 | 1.58 | 0.01 | 13.32 | 0.97 |
| HS053 | 10 | 0.04 | 15.03 | 0.48 | 1.89 | 0.35 | 0.31 | 2.70 | 0.02 | 12.68 | 0.98 |
| | 40 | 0.03 | 9.98 | 0.48 | 1.31 | 0.48 | 0.33 | 1.58 | 0.01 | 13.32 | 0.97 |
| vₑ is the reduced χ² from the global analysis. |

*a Excitation wavelength 296 nm; emission interval 325 to 380 nm. |

b (τᵢ)Trp is the average of the Trp excited state lifetimes, τ₁, τ₂ and τ₃.
Tables 1 and 2).

and 40°C, j values) rather than to an actual decrease in the Trp factors Fig. 4. A graphical display of the Trp mean normalized pre-exponential percentages of each rotamer (\(\bar{\tau}_i\) values) are somewhat different, resulting in an \(\alpha\)-MSH (Trp) excited-state mean lifetime higher than those of the cyclic analogues (Table 1). For \(\alpha\)-MSH, at higher temperature (40°C), there is a significant decrease in the \(\tau_1\) and \(\tau_2\) values (usually attributed to an increase in the solvent collisional nonradiative deactivation processes upon temperature rise [29]) resulting in a rather short \(\langle \tau \rangle_{\text{Trp}}\) value. Though the \(\langle \tau \rangle_{\text{Trp}}\) values for the cyclic peptides at 40°C are lower than those obtained at 10°C, and lower than that of \(\alpha\)-MSH, those results are mainly due to the different percentages of each rotamer (\(\bar{\tau}_i\) values) rather than to an actual decrease in the Trp \(\tau_i\) values. Therefore, in aqueous solution, both at low and high temperatures, \(\beta\)-Nal in the cyclic peptides does not suppress the Trp fluorescence, as the Trp excited state lifetimes are not shorter for HS032 and HS053. On the contrary, \(\tau_1\) is significantly longer for the cyclic analogs at 40°C (see Table 1). An indication of a different structure at the Trp region for the cyclic peptides, as compared to the hormone, is the distinct equilibrium among the Trp rotamers, expressed in the mean normalized \(\bar{\tau}_i\) values (\(i = 1, 2, 3\)), with a considerable decrease in the percentage of the long decay time component for the analogues.

\(\beta\)-Nal excited state lifetime \(\tau_4\) is rather similar for the two cyclic peptides, presenting a considerable decrease upon increasing the temperature from 10 to 40°C. The values are remarkably lower than that of Boc-\(\beta\)-Nal in aqueous solution (\(\tau = 38.08\) ns at 10°C and 33.61 ns at 40°C) indicating a \(\beta\)-Nal fluorescence suppression. This could be related to a \(\beta\)-Nal energy transfer to Trp, a result of an overlap between \(\beta\)-Nal emission and Trp absorption bands. Thus, the energy transfer rate seems to be higher to the donor/acceptor pair \(\beta\)-Nal/Trp than to Trp/\(\beta\)-Nal.

3.2.2. In lipid medium

In the presence of DMPG dispersion, for the three peptides, Trp penetrates in the lipid bilayer, both in the gel and fluid phases of the membrane (10 and 40°C, respectively). This conclusion is based on the significant increase in the fluorophore lifetimes in DMPG dispersion, attributed to a reduction in collisional nonradiative deactivation processes with water molecules in the lipid phase [29] (see Tables 1 and 2). However, changes in the Trp excited state lifetimes can be partially ascribed to modifications in the microenvironment of the indole ring, owing to structural changes of the peptides on binding to the vesicles.

The percentages of increase of Trp and \(\beta\)-Nal lifetimes, from water to lipid environment, are listed in Table 3. Just considering the first hypothesis mentioned above (the most feasible), in the lipid gel phase, at 10°C, Trp in the cyclic analogues seems to penetrate deeper in the bilayer than the fluorophore in \(\alpha\)-MSH, as its lifetimes increase considerably more, with HS053 showing the greatest increase, probably related to a Trp deeper penetration. In the lipid fluid phase, at 40°C, HS053 still presents the largest increase of \(\tau_1\), \(\tau_2\) and \(\tau_3\), with similar changes observed for HS032 and \(\alpha\)-MSH (see Table 3). The lower variations found for the Trp lifetimes at 40°C (Table 3) could be related to the greater bilayer hydration in the lipid fluid state.

The possible deepest bilayer penetration of Trp in HS053 could be due to the smaller size of this peptide, as compared with HS032, and the presence of fewer charged residues, hence higher hydrophobicity. Hydrophobicity profiles, for the three peptides, are shown in Fig. 5, based on S. White's

\[
\begin{array}{cccc}
\text{Table 3} & \text{Variations} (%) \text{ of the Trp and } \beta\text{-Nal excited states lifetimes from aqueous to lipid environment}^a \\
\hline
10°C & \tau_1 & \tau_2 & \tau_3 & \tau_4 \\
\alpha\text{-MSH} & 50 & 33 & 100 & \\
HS032 & 123 & 51 & 126 & 128 \\
HS053 & 260 & 89 & 219 & 142 \\
40°C & \hline
\alpha\text{-MSH} & 92 & 26 & 44 & \\
HS032 & 64 & 34 & 41 & 159 \\
HS053 & 130 & 72 & 142 & 164 \\
\hline
\end{array}
\]

\(a\) \((\tau_{\text{lipid}} - \tau_{\text{aqueous}})/\tau_{\text{aqueous}} \times 100\).
work (see, for instance, Ref. [35] or http://blanco.biomol.uci.edu). In this diagram, a tentative value was attributed to D-Nal, considering that it should be more hydrophobic than Trp. The presence of D-Nal should facilitate the Trp penetration into the bilayer.

For both HS032 and HS053, there is a significant increase in the D-Nal lifetime in the presence of lipids (see Table 3), either in the gel or fluid phases, indicating that this amino acid also penetrates deeply into the bilayer, and/or is highly sensitive to changes in polarity environment. The increase in the $\tau_4$ value could also be partially related to a decrease in the D-Nal fluorescence suppression by Trp in lipid medium.

For a better evaluation and comparison of the distribution of the three Trp lifetime components in the three peptides, in aqueous and lipid environments, the mean values of $a_1$, $a_2$ and $a_3$, listed in Tables 1 and 2, are graphically displayed in Fig. 4. The three peptides in lipid medium acquire a conformation such that the percentage of the Trp long time decay component is rather low. Interestingly, this characteristic is also present in the cyclic analogues in aqueous medium, but not in the hormone, suggesting that the analogues’ structure in aqueous phase could favor their lipid interaction. The turn-structure forced by the Cys–Cys bridge is probably responsible for the similar structures assumed by HS032 and HS053 in aqueous and lipid environments. A turn-structure at (or close to) the region comprising the residues 6–9 has been associated with $\alpha$-MSH analogues’ superpotent biological activity [13,14]. It is noteworthy that the highly active cyclic lactam $\alpha$-MSH analogue, Ac-[Nle$^4$, Asp$^5$, D-Phe$^6$, Lys$^{10}$]-$\alpha$-MSH(4-10)-NH$_2$, also presents a rather low $\tau_4$ component in both aqueous and lipid environments [25]. Considering that $\alpha$-MSH is very flexible in aqueous medium, but seems to acquire a turn-structure in lipid environment [23,36], it has been suggested that this more restricted conformation could be important for the peptide biological activity [13,14].

![Fig. 5. Free energies of water/lipid transfer (kcal/mol) for $\alpha$-MSH (---), HS032 (--△--) and HS053 (---□---) residues (see text).](image-url)

![Fig. 6. DAS for $\alpha$-MSH (a), HS032 (b) and HS053 (c), in aqueous and DMPG dispersions, at 10 °C (left) and 40 °C (right), for $\tau_1$ (○), $\tau_2$ (△), $\tau_3$ (□) and $\tau_4$ (■) components. HEPES buffer.](image-url)
Maximum wavelength associated with each \( \tau \), component fluorescence emission band in the DAS analysis

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3.3. Decay-associated spectrum (DAS)

The emission spectrum associated with each lifetime, DAS [37], was generated for each peptide in both aqueous and lipid media, at 10 and 40 \(^\circ\)C, and are shown in Fig. 6. The fractional intensity of each rotamer at wavelength \( \lambda \) is given by

\[
f_i(\lambda) = \frac{a_i(\lambda)\tau_i}{\sum_j a_j(\lambda)\tau_j}
\]

and the emission spectrum of each component is \( I(\lambda) = f(\lambda)I(\lambda) \).

DAS evidences the contribution of D-Nal and each Trp rotamer to the fluorescence emission spectra, showing that the contributions are dependent on the temperature and the environment. The fluorescence spectra of the two cyclic analogues are similarly composed, and DAS makes clear the strong contribution of D-Nal to the peptides spectra in lipid medium (Fig. 6b and c).

In water, the positions \( \lambda_{\text{max}} \) of the Trp fluorescence bands associated with \( \tau_1 \), \( \tau_2 \) and \( \tau_3 \) are very similar (Fig. 6 and Table 4), indicating that the three Trp rotamers monitor similar hydrophilic environments in the three peptides. Considering that a blue shift in the fluorescence band is probably associated with a less polar environment for the receptor could be related to their deeper penetration into the bilayer core.

It is shown here that the amino acid D-Nal is an extremely interesting fluorescent probe for studies on peptides and proteins. It is present in very active MC peptides, presenting a high quantum yield, mainly in a bilayer medium. Moreover, its excited state lifetime (\( \tau_3 \)) is much longer than those of Trp, allowing a good distinction between the two fluorophores.

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