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Photophysics of phenylalanine analogues Part 1. Constrained analogues of phenylalanine modified at phenyl ring

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Abstract

Photophysics of constrained analogues of phenylalanine (Phe) modified at phenyl ring: tetrahydroisoquinoline-3-carboxylic acid (Tic), (5-methyl) tetrahydroisoquinoline-3-carboxylic acid ((5-Me)Tic), (2′-methyl)phenylalanine ((2′-Me)Phe) and (2′,6′-dimethyl)phenylalanine ((2′,6′-Me_2) Phe) and their simple derivatives obtained by a modification of amino group (acetylation) or carboxylic group (amidation), or by both modifications at the same time was the subject of our investigations. The synthesized compounds were used to study the influence of (i) sterical constraints (cyclization or incorporation of methyl substituent(s) into phenyl ring), (ii) the N-acetylation of the amino group and (iii) the transformation of the carboxylic group into the amide on the photophysical properties of the phenylalanine analogue.

Keywords: Phenylalanine; Photophysics; Constrained amino acids; Fluorescence

1. Introduction

The fluorescence and ultraviolet absorption of peptides and proteins is mostly due to the presence of three aromatic amino acids: tryptophan, tyrosine and phenylalanine [1,2]. Therefore, the spectroscopy and photophysics of these amino acids have been extensively studied to prove their utility as optical probes in conformational studies of peptides and proteins [3–5]. However, most of the efforts have been focused on tryptophan and tyrosine. Phenylalanine has got less attention because of its 'worse' photophysical behaviour (low absorption and quantum yield) and because of the fact that many native proteins and peptides contain large numbers of phenylalanine residues which makes usage of phenylalanine fluorescence in conformational studies rather difficult. But on the other hand, many native biopolymers contain one or two phenylalanines as the only aromatic amino acid [6]. Hence, knowledge of photophysical parameters of phenylalanine is essential for conformational studies of proteins and peptides, especially for the estimation of interchromophoric distances [7].

The photophysical properties of the aromatic amino acids are different and they are also not identical to those of the corresponding aromatic chromophores, e.g. benzene, phenol and indole and even to the methyl-substituted corresponding chromophores. It means that the presence of the carboxylic and amino groups in the molecule affects the photophysical properties of the aromatic chromophore although the interacting fragments are separated by two methylene groups. Additionally, the status of the carboxylic and amino groups (neutral, protonated, ionized, protected) also affects fluorescence of the aromatic amino acid.

In the case of Tyr and Trp containing compounds multiexponential kinetics of the fluorescence decay were observed [8,9]. For amidated tyrosine derivatives biexponential fluorescence decays were found, whereas for amidated tryptophan derivatives monoexponential ones were observed [9]. On the other hand for Tyr or Trp incorporated into peptide and protein chains even more complex kinetics of the decays were observed; like the triexponential one for tyrosine fluorescence in arginine-vasopressin and oxytocin [10,11]. Our previous studies of the analogues of Tyr have revealed quite reverse and still complicated behaviour of fluorescence decays [12–14]. For example, for acetylated β-homo-tyrosine, in contrary to respective tyrosine derivative, biexponential fluorescence decay was observed [12,15]. Several explana-

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tions for the complex process of the fluorescence decay of aromatic amino acid derivatives were delivered like the rotamer model [16,17], the involvement of ^1L_b and ^1L_a states [18] and excited state reactions [19]. The strong support for the rotamer model of fluorescence decays of aromatic amino acids have been published lately [20–23] including also, similar to our work, studies of constrained tryptophan [24–26], tryptophan in proteins crystals [27,28] and in peptides with defined secondary structure [29].

Lately, there is a great interest in syntheses of sterically constrained amino acids and peptides [30] in order to stabilize a conformation of peptides of interest, to improve receptor selectivity, and decrease enzymatic degradation [31,32]. One of the most convenient amino acid to achieve conformation restrictions by various modifications is phenylalanine. And one of the most interesting conformational restriction of aromatic amino acids is based on the Pictet–Spengler reaction [33]. In order to use phenylalanine analogues in conformational studies of peptides by means of fluorescence it is necessary to know their photophysical behaviour.

In this communication we present photophysical properties of phenylalanine and its conformationally constrained analogues: tetrahydroisoquinoline-3-carboxylic acid (Tic), (5-methyl) tetrahydroisoquinoline-3-carboxylic acid ((5-Me)Tic), (2′-methyl) phenylalanine ((2′-Me)Phe) and (2′,6′-dimethyl)phenylalanine ((2′,6′-Me_2) Phe) in order to explain influence of status of quenching groups and level of constrain on fluorescence parameters of an analogue.

2. Experimental details

2.1. Synthetic methods

All studied analogues of phenylalanine (Tic, (5-Me)Tic, (2′-Me)Phe and (2′,6′-Me_2)Phe) were prepared according to the procedures described in literature [34]. The synthetic scheme is presented in Fig. 1.

N-methylamides of the amino acids were prepared from N-Boc (Boc, *tert*-butyloxycarbonyl) protected precursors using PyBOP ((benzotriazolyl)-*N*-oxy-pyrrolidino-phosphonium hexafluorophosphate) as a coupling reagent and CH_3NH_2·HCl as amine source in the presence of TEA (triethylamine) [35]. Acetylation of the amino group was realized by reaction with Ac_2O (acetic anhydride) in tetrahydrofuran-H_2O. The chemical homogeneity of the synthesized compounds was assessed by thin layer chromatography (TLC) (precoated Silicagel 60 plates, Merck; *n*-BuOH-AcOH-H_2O=4:1:1, $CHCl_3$-MeOH-AcOH=85:10:5, $CHCl_3$-MeOH=9:1, AcOEt-*n*-hexane=1:1, visualization: UV-lamp (254 nm) or ninhydrin), ^{1}H NMR spectra, analytical reversed-phase high performance liquid chromatography (RP-HPLC) (a linear gradient of 0–80% CH_3CN in 0.1%TFA (trifluoroacetic acid) in H_2O over 60 min at flow rate of 1 ml/min, column, Vydac C-18, 4.6 mm×250 mm, 5 mm) and mass spectrometry (FDMS, field desorption or FABMS, fast atom bombardment).

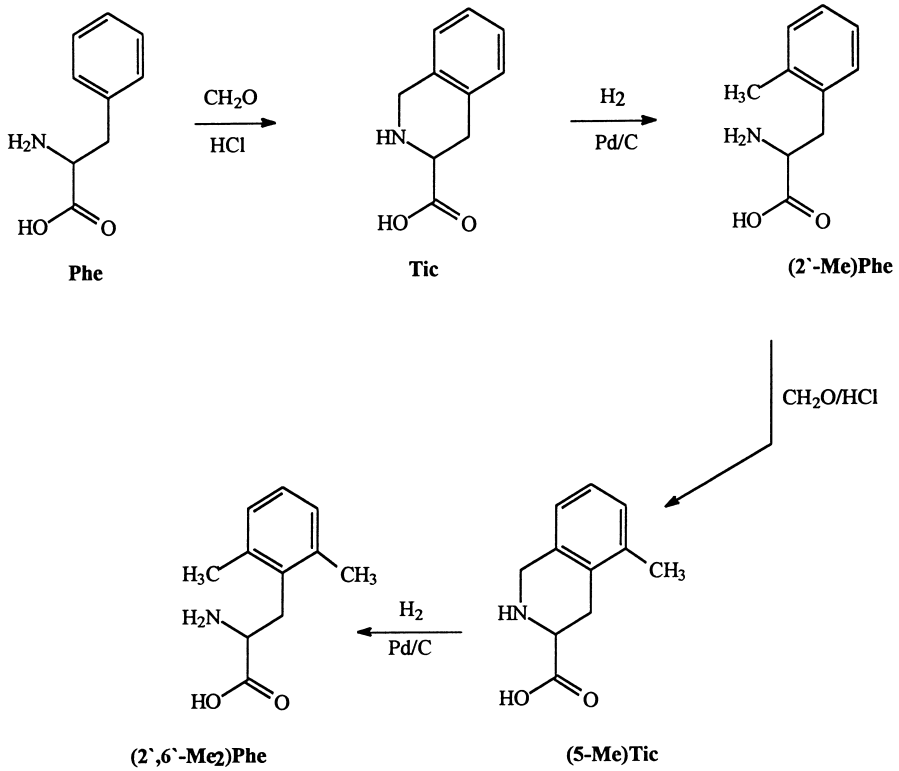

Fig. 1. Synthetic scheme.

2.2. Spectroscopic measurements

Photophysical parameters were obtained as it is described in our previous similar studies of 7-hydroxy-tetrahydroisoquinoline-3-carboxylic acid and its derivatives [12]. The absorption spectra were obtained using Beckman DU-600 spectrophotometer. Fluorescence decays were collected by the time-correlated single-photon counting techniques on an Edinburgh Analytical Instrument type CD-900 fluorometer interfaced with an IBM PC AT. The excited source was a flash lamp filed with 0.5 atm hydrogen, operated at 40 kHz with about 6.5 kV across a 1 mm gap. The half width of the instrument response was 1.0 ns. The excitation (258 mm) and emission (290 nm) wavelengths were selected by means of monochromators (about 10 nm bandwidth). The steady-state spectra were obtained on a Perkin–Elmer LS-50B spectrofluorimeter with 2.5 nm bandwidth for excitation and emission. The excitation wavelength was 258 nm. The quantum yields were measured relative to a value of 0.14 for tyrosine in water at room temperature [36]. In steady state measurements, the sample concentrations was about 2×10^{-4} and 5×10^{-4} M in time-resolved experiments. All measurements were performed in double deionized water (pH=7 or 1) at room temperature.

3. Results and discussion

Absorption spectra of the studied amino acids are presented in Fig. 2 (the spectrum of Phe is added for comparison).

The new phenylalanine analogues have longwave shift of absorption spectra (batochromic shift) and higher molar coefficients than phenylalanine which is advantageous for fluorescence studies and consistent with literature data [37,38]. Fluorescence spectra of Phe and its studied analogues are shown in Fig. 3.

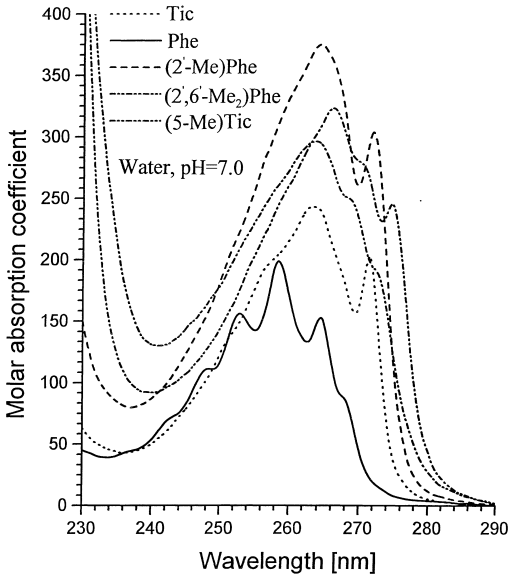

Fig. 2. Absorption spectra of Tic, Phe, (2′-Me)Phe, (2′,6′-Me_2)Phe, (5-Me)Tic in water at room temperature, at pH=7.

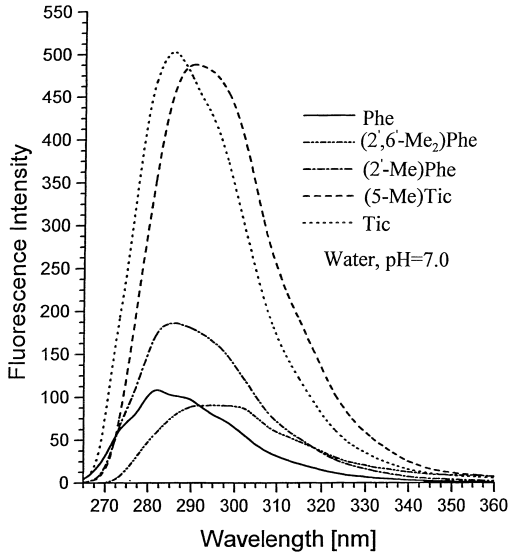

Fig. 3. Emission spectra of Phe, (2′,6′-Me_2)Phe, (2′-Me)Phe, (5-Me)Tic and Tic in water at room temperature, at pH=7.

Fluorescence intensity observed for Tic is around six times higher than Phe but the maxima of emission for both molecules are at the same wavelength. For other analogues fluorescence intensities were lower than Tic and additionally small longwave shift of emission maxima were observed (Fig. 3). The fluorescence decay curves of selected amino acids are shown in Figs. 4 and 5. As can be seen, the decays of fluorescence for all compounds are monoexponential.

It is worth to state at that point that the photophysical parameters obtained in our measurements for unmodified phenylalanine are the same, as described in literature [7,36,39]. Incorporation of one substituent into aromatic ring of phenylalanine analogue (e.g. (2′-Me)Phe and Tic) increases quantum yield (Φ) and decay time (τ) (Table 1 and Figs. 4 and 5).

On the other hand the presence of two substituents in the phenyl ring [(5-Me)Tic and (2′,6′-Me_2)Phe] decreases the quantum yield and fluorescence decay time. Photophysical properties of Tic (long decay time τ=20 ns, quantum yield Φ=0.12 and batochromic shift of the fluorescence spectrum) are making this particular amino acid a suitable donor for energy transfer measurements. For comparison, a very popular donor in such studies, tyrosine, has shorter fluorescence decay time τ=3.38 ns [16,40] and comparable with Tic the quantum yield, Φ=0.14. Moreover, fluorescence decay time and quantum yield for Tyr incorporated into a peptide chain strongly depends on micro surrounding and/or rotamers population [9,10] — the decay becomes multiexponential and quantum yield decreases, which is not observed in the case of Tic. As it was observed for derivatives of tyrosine

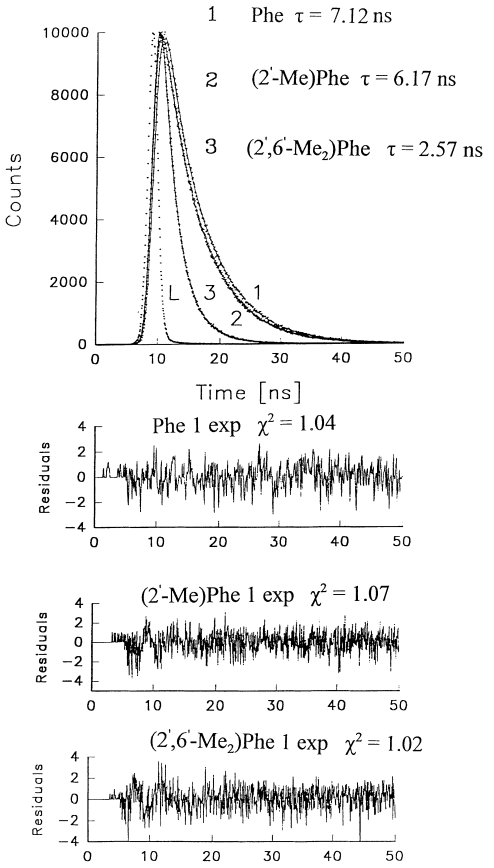

Fig. 4. Measured (doted line) and fitted decay curves (solid line) vs time (left set of points L-lamp profile) for 1-Phe, 2-(2′-Me)Phe, 3-(2′,6′-Me_2)Phe. The weighted residuals are plotted at the lower part of the figure.

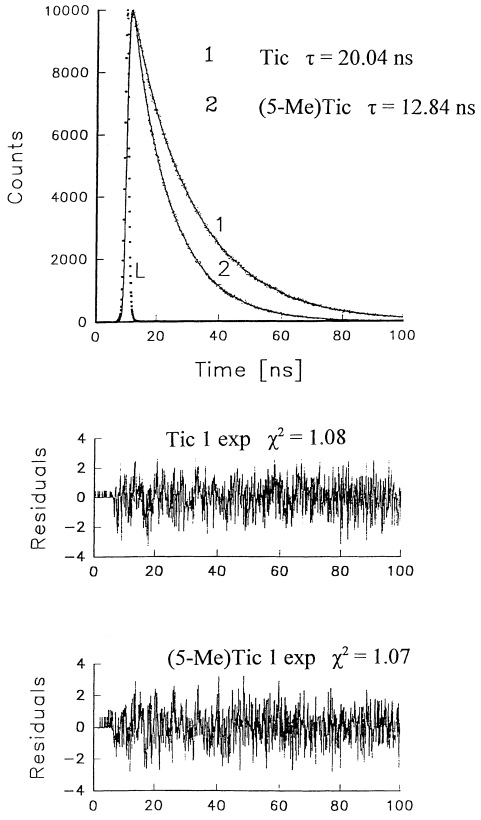

Fig. 5. Measured (doted line) and fitted decay curves (solid line) vs time (left set of points L-lamp profile) for 1-Tic, 2-(5-Me)Tic. The weighted residuals are plotted at the lower part of the figure.

and their analogues conversion of carboxylate to amide group [9,12–16,40] or protonation of the carboxylic group [9,40,41] caused changes of their photophysical properties. Protonation of carboxylic group of analogues of phenylalanine also results in decrease of the fluorescence quantum yield and lifetime, (Table 1) but the shape of absorption and emission spectra are not changed. Decrease of quantum yield (Φ) and fluorescence lifetime (τ) at acidic solution (pH=1) testifies that the protonated carboxylic group works as an efficient quencher of fluorescence of the aromatic residue of amino acid. The fluorescence quenching proceeds according to the electron transfer mechanism with the protonated carboxylic group as an electron (charge) acceptor in a relation to the excited phenyl chromophore [42].

The comparison of the fluorescence parameters of cyclic analogues of Tyr and Phe, e.g. Tic(OH) ((7-hydroxy)tetrahydroisoquinolino-3-carboxylic acid) [12] and Tic, respectively, seems to be interesting. For both Tic(OH) and Tic

Table 1
Photophysical properties of phenylalanine and its analogues and derivatives

Compound	Quantum yield, Φ		Lifetime, τ (ns)		$k_f \times 10^{-6}$ (s^{-1}) ($k_f = \Phi/T$) (pH=7)	$k_{nr} \times 10^{-8}$ (s^{-1}) ($k_{nr} = (1-\Phi)/T$) (pH=7)
	pH=1	pH=7	pH=1	pH=7		
Phe[a]	0.014	0.020	4.54	7.12	2.81	1.38
Phe-NHMe	–	0.015	–	5.14	2.92	1.92
Ac-Phe-NHMe	–	0.018	–	5.90	2.78	1.66
(2′-Me)Phe	–	0.046	–	6.17	7.45	1.55
(2′,6′-Me_2)Phe	–	0.012	–	2.57	4.67	3.84
Tic[a]	0.115	0.120	16.75	20.04	5.99	0.44
Tic-NHMe[a]	–	0.119	–	19.81	6.01	0.44
(5-Me)Tic	–	0.048	–	12.94	3.71	0.74
(p-F)Phe	0.085	0.144	4.87	7.24	19.89	1.18

[a] Acetylation of amino group does not change the photophysical properties (at pH=7).

steric constrains e.g. space filling of all fragments are very similar but in the case of Tic(OH) derivatives quantum yields and fluorescence decay times are lower than for 'mother' Tyr. In contrary, for some Tic derivatives, substantial increases of the fluorescence parameters were found (Table 1). Moreover, for Tic(OH) derivatives, simple monoexponential fluorescence decays were observed, which is different from that of tyrosine derivatives. In the case of Phe and Tic, cyclization did not influence the nature of kinetics of fluorescence decay so dramatically — for both compounds the kinetics is monoexponential and only kinetics parameters were changed (Table 1). Fluorescence decay times for Phe-NHMe (τ=5.14 ns) and Ac-Phe-NHMe (τ=5.90 ns) are shorter than for Phe (τ=7.21 ns) and the decays are still monoexponential. On the other hand, for Tic derivatives (amidated ones), the fluorescence decay was still monoexponential and the decay time was almost the same as observed for nonamidated precursor (τ=19.8 ns).

Acetylation of amino group of phenylalanine (free carboxylic group) or Tic and *N*-methylamide derivative of Tic did not cause changes, within the range of experimental errors, their photophysical properties. Moreover, N-acetyl group is a weak quencher of aromatic amino acid fluorescence. After N-acetylation, decrease of the fluorescence lifetime was observed for about 1.2 times for (*p*-hydroxy)phenyl glycine [43], 1.05 times for tyrosine [16,39] and 1.01 times for Tic(OH) [12]. Additionally, acetylation of amino group of phenylalanine *N*-methylamide, like in the case of tyrosine amide [44,45] decreases the quenching ability of amide group (Table 1).

Conformational studies of rotational isomerism of phenylalanine and its analogues by circular dichroism (CD) and nuclear magnetic resonance (NMR) techniques have discovered possibilities of the presence of stable rotamers in ground state [37,38,46,47], which should also be revealed by fluorescence (according to the rotamer theory [16,17,44]). But because of either similar decay times of different rotamers or low energy of rotamers interconversion, the fluorescence decays for phenylalanine and its analogues are monoexponential. In the case of more rotationally restricted analogues (Tic and analogues) one could explain the monoexponential decays due to the presence of one stable rotamer but our previous studies of (7-hydroxy)-tetrahydroisoquinoline-3-carboxylic acid {Tic(OH)} [12] have revealed that its heterocyclic ring interconversion is faster than fluorescence rate constant, and thus the lack of hydroxyl group in aromatic ring of Tic should not influence the dynamicity of the ring interconversion. Therefore, we can state that also for Tic the observed decay time is average value of the conformational isomers.

Conformational constrain of phenylalanine by cyclization (Tic and (5-Me)Tic) caused decrease of k_{nr}, a constant associated with nonradiative decay and increase in fluorescence rate constant k_f, especially for Tic (Table 1). The cyclization of Phe via Pictet–Sprengler reaction is also a reason of less conformational freedom for amino group — one of factors making a favourable condition for quenching of fluorescence by the protonated or amidated carboxylic group trough its close proximity. The influence of an incorporation of methyl substituents into phenyl ring seems to be interesting. The incorporation of one methyl is causing increase of fluorescence rate constant k_f but introduction of the second methyl is decreasing k_f and increasing simultaneously k_{nr} as compared to (2′-Me)Phe and Tic. This kind of photophysical behaviour can be associated with the change of vibrational energy levels of phenyl ring resulting in changed vibrational coupling between ground end and excited state, as can be seen for (p-F)Phe (Table 1).

4. Conclusions

In our work we prepared and studied constrained analogues of phenylalanine. The cyclization to isoquinoline ring yielded compounds with longer fluorescence lifetime and higher quantum yield {Tic, (5-Me)Tic}. The photophysical properties of obtained compounds are pH-dependent, e.g. protonation of carboxylate (experiment at pH=1) caused lower quantum yield and shorter lifetime (Table 1). It is interesting that the protonated carboxylic group is a stronger quencher of tyrosine and phenylalanine fluorescence than the amide group [41].

The influence of amino group status in the studied analogues indicates that this group is not directly involved in the quenching process but increases quenching efficiency by better hydration of the whole molecule, which is consistent with the previous suggestions [16,48].

In the final conclusion of our work, we would like to state that prepared and studied compounds — constrained analogues of phenylalanine should be very useful as elements increasing conformational stability of peptides, especially small peptides [31,32]. This kind of modification, e.g. an incorporation of the constrain amino acid residues into peptide chain have interesting impact on biological activity, and also should be more often used in fluorescence conformational studies of the biopolymers because the compounds prepared by us have interesting photophysical parameters (monoexponential fluorescence decay, longer fluorescence lifetime and higher quantum yield). Moreover, rotational restrictions of the chromophores make them more suitable for conformational studies using Förster type energy transfer, because estimation of space location of such modified chromophore is more reliable which results in better interchromophoric distance or distance distribution estimation [49].

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