Porphyrin-Tyrosine Conjugate as Synergic Photosensitizer in Photodynamic Therapy

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Photodynamic therapy (PDT) has become an increasingly important method for the chemotherapeutic treatment of some tumors. It involves the systemic, topical, or intraperitoneal administration of a nontoxic drug called photosensitizer, which, to some extent, preferentially localizes in the tumor. When the tumor is illuminated with the wavelength of light that is absorbed by the dye, in the presence of oxygen, toxic oxygen reactive species are produced and they can cause tissue necrosis or apoptosis by a combination of mechanisms. A new compound consisting of multifunctional ligands, such as porphyrin-tyrosine conjugate (5,10,15,20-tetra-amino-phenyl-porphyrin coupled with tyrosine - TAPP-L-TyR), have been reported in this paper for application in photodynamic therapy.

Keywords: Photodynamic therapy, synergic photosensitizers, porphyrin, tyrosine

"Synergy" (from the Greek word *synergos*) is a ubiquitous and fundamentally important aspect of the natural world. Synergy, broadly defined as combined or "cooperative" effect - literally, the effects produced by things that could "operate together" (parts, elements or individuals) [1].

Photodynamic therapy (PDT) is a treatment using photosensitizers in conjunction with light to destroy cancerous cells *via* cytotoxic singlet oxygen. PDT is a viable treatment modality for a variety of tumors, as well as for selected non oncologycal diseases [2]. At present, new photosensitizers with improved spectroscopic, photochemical, and tumor-localizing properties are being tested, which expands the scope of tumor PDT and stimulate clinical applications outside the oncological field [3]. The present research represents a new concept in the development of new photosensitizers that can be called "synergic photosensitizer", i.e. a drug in which the action of one drug aids or enhances the action of another.

In this paper it will be presented a new type of sinergic sensitizer as 5,10,15,20-tetra-amino-phenyl-porphyrin coupled with tyrosine (TAPP-L-TyR), that could be used for photodynamic therapy in the future.

The photocytotoxic properties of this porphyrin were evaluated in vitro with Murine Lymphoma EL-4 cells (as experimental model for photodynamic therapy studies).

Experimental part

Materials

In figure **1**, the first porphyrin-aminoacid conjugate (5,10,15,20-tetra-amino-phenyl-porphyrin coupled with tyrosine - TAPP-L-TyR) is shown, which was synthesized and characterized by R.M.Ion [4].

The synthesis occurs via few pathways, as follows:

Apparatus

To register the absorption UV-Vis wavelengths and calculate the extinction coefficients, a spectrophotometer SPECORD M400 UV-visible was used.

5,10,15,20-tetra-amino-phenyl-porphyrin coupled with tyrosine TAPP-L-TyR

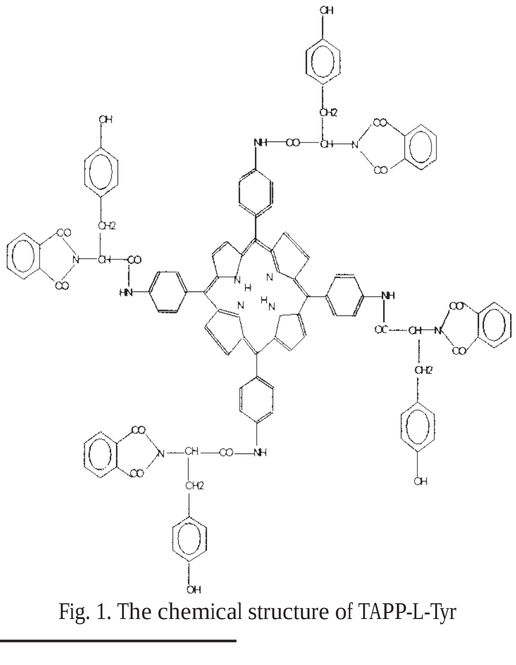

Fig. 1. The chemical structure of TAPP-L-Tyr

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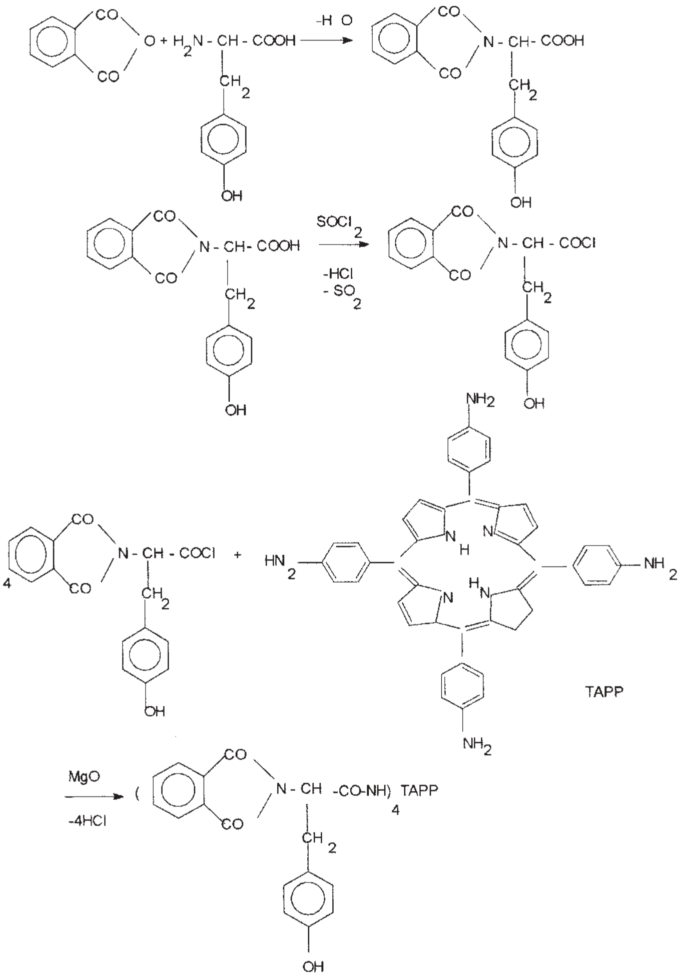


Scheme 1. The pathway of TAPP-L- Tyr synthesis

For IR spectra it has been used a SPECORD M80 spectrophotometer, with KBr pelettes.

Mass spectra have been recorded with a Varian spectrometer GC-MS with silica columns.

For NMR spectra it has been used a Brucker spectrometer.

Fluorescent emission and excitation spectra of solutions of TPP-L-TyR were recorded in a PTI-QM2 (Photon Technology International) spectrofluorometer equipped with a photon counting detector (red extended photomultiplier) in benzene. Quartz cuvettes with 1 cm optical path length were used. The excitation spectra were recorded in the range of 250 and 630 nm for emission at 657 nm, and the fluorescent emission spectra were recorded between 600 nm and 800 nm by excitation at 424 nm.

Triplet states were produced and monitored by laser flash photolysis. The samples were excited with pulses from a nitrogen laser (l=337.1 nm) in nitrogen saturated solutions. Figure 2 shows the experimental array of the flash photolysis equipment. The equipment uses an halogen continue lamp (HLX 150 W) as a monitor beam and a H_2O filter of 10 cm optical path length. The light is collected at right angle to both beams with the photomultiplier after passing through monocromador. The signal from the photomultiplier (Hamamatsu R928) is fed into a digital oscilloscope (Scope Station 140, Lecroy).

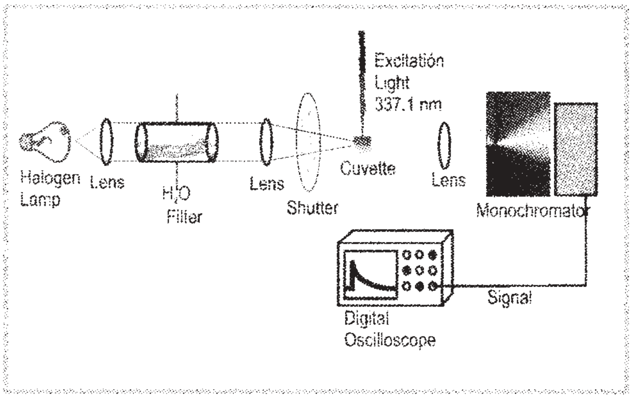


Fig. 2. The experimental array of the flash photolysis equipment

The cell line of standard ascitic limphoma (EL-4) is delivered from European collection of Cellular Cultures (ECACC Nr.85023105). This cell line have been mentained in DMEM with 2mM glutamine and 10% faetal serum cow (SFV) at a concentration of 5 . 10^5 celule/ml in 5% CO_2 and 37^0C.

The cellular viability have been evaluated by LDH test (kit Cytotox96 Non-Radioactive Cytotoxicity Assay).

The results are presented as statistical values of the viability indexes in the presence of sensitizer after the formula:

$$\text{Index effect} = \frac{\text{cellular viability in the presence of TAPP-L-Tyr}}{\text{cellular viability in the presence of control cells}}$$

Their irradiation (after incubation of 48 h with TAPP_L_Tyr), has been achieved with a He-Ne 30 mW, λ=632.8nm [5].

Results and discussions

The absorption spectra and the molar absorption coefficient are shown in table 1.

The proofs of this synthesis have been obtained by IR (CO-NH frequencies from 1400-1700 cm^{-1}), NMR (δ~7 ppm) and mass-spectroscopy data (m/z=1100), as it is shown in figures 3 - 5.

The fluorescence emission spectrum shows maxima at 657 nm and 723 nm after excitation at 424 nm. These spectra are shown in Figure 6. The concentrations used were between 3.489 . 10^{-6} M and 1.075 . 10^{-5} M. The relationship between intensity of the emission bands (λ=657 nm and λ=723 nm) are shown in figure 7.

There are no changes in the spectra or peak shifts (fig. 6), and the relationship between intensity and concentration is linear (fig. 7), so it can be assumed that there are no changes in aggregation of the porphyrin [6].

The acidity of the solution is an important factor affecting the equilibrium between the different ionic species of the

Table 1

THE ABSORPTION SPECTRA AND THE MOLAR ABSORPTION COEFFICIENT FOR TAPP-L-TYR

Wavelength (nm)	Molar absorption coefficient ($M^{-1}.cm^{-1}$) X10^{-3}
420	20.27x10^4
545	3.71x10^4
507	6.8x10^4
643	6.86x10^4
691	8.088x10^4

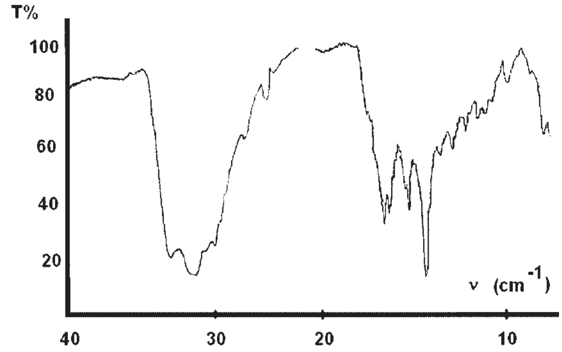


Fig. 3. IR spectrum of TAPP-L-TYR

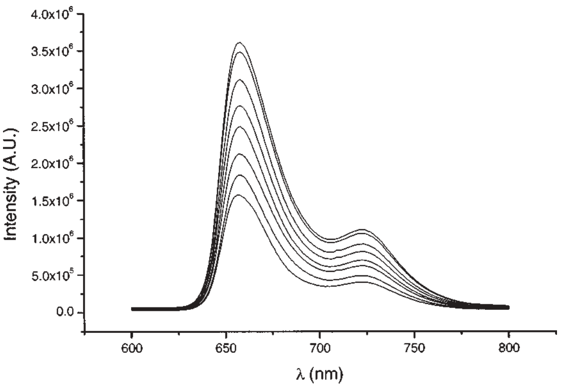


Fig. 6.

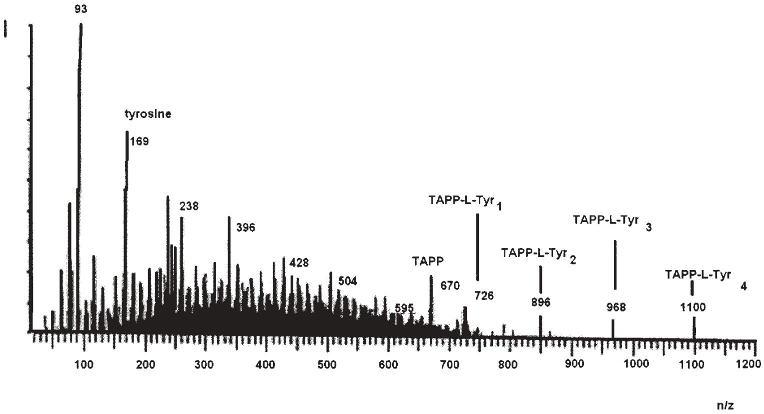


Fig. 4. Mass spectrum of spectrum of TAPP-L-TYR

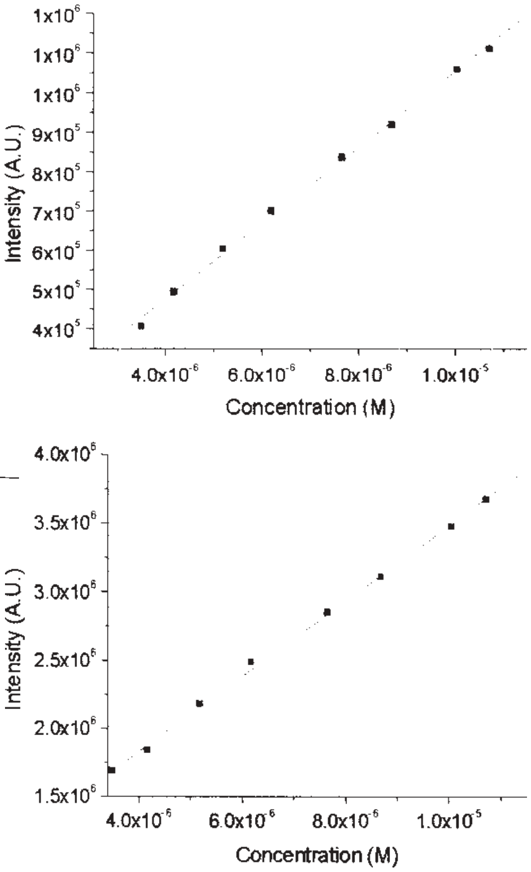


Fig. 7.Relationship between intensity and concentration for the two emission bands: a) λ=657 nm and b) λ=723 nm of TAPP-L-TyR in benzene

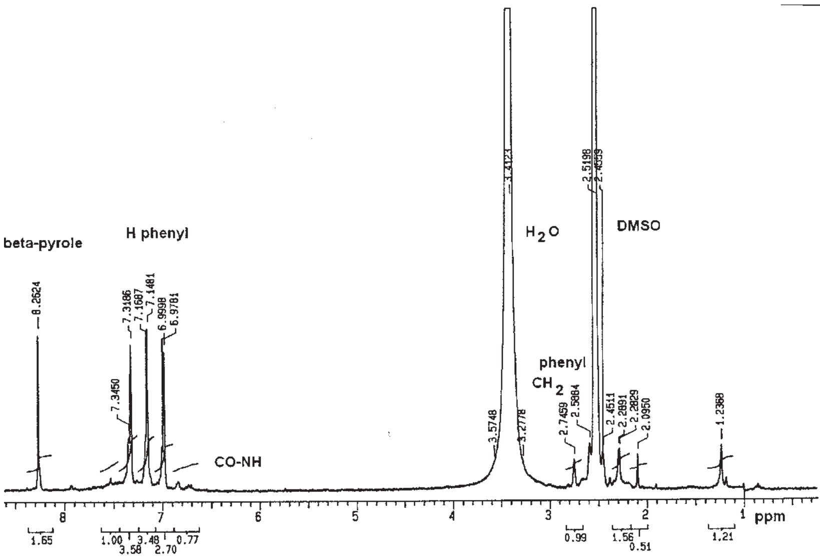


Fig. 5.NMR spectrum of TAPP-L-TYR

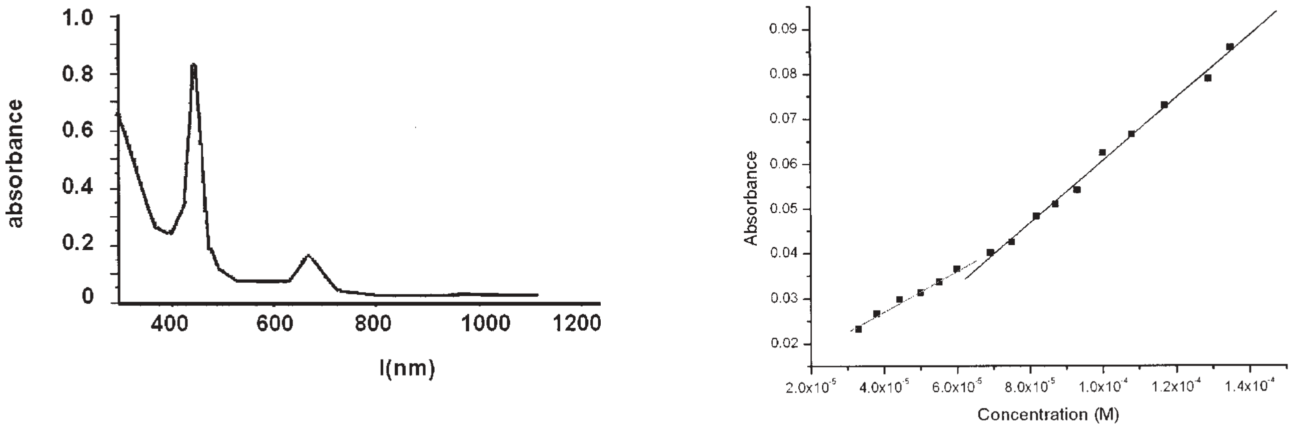
porphyrins, as well as the proportion of monomers, dimers and aggregates of each ionic species [7]. Consequently, the photostability of the photosensitizer may be influenced by a change in the medium acidity (*p*H). This is interesting since tumours are often found to have different (lower) *p*H values than the surrounding tissues[8-11]and many tumours have been reported to have *p*H values in this vicinity. From our assays in H_3PO_4, we have obtained a dicationic porphyrinic form.

For the higher concentrations (c> 6 .10^{-5} M) the cationic form acquired by the porphyrin in this media, form a J aggregate (fig. 8). J-aggregates are formed with the monomeric molecules arranged in one dimension so that the transition moment of the monomers are parallel and the angle between the transition moment and the line joining the molecular centers is zero (End-to-end configurations) [12].

The UV-Vis spectrum of this diacid form is shown in figure 8. The bands are at λ = 446 nm, λ = 668 nm, and at λ = 592 nm. The variation of absorbance vs concentration for the three bands is similar to the found for the emission spectra, and the break in the slope happens at the same concentration. This fact is a prove of the change of aggregation for concentrations around the 6 . 10^{-5} M.

The extinction coefficients are shown in table 2 with the convention that the slope for lower concentration correspond to a monomer, and that the other slope correspond to a dimer.

The fluorescence spectra of TAPP-L-TyR in H_3PO_4 present two peaks at λ =710 mn and at λ = 787 nm. The second band looks like a shoulder. One of these spectra is shown in figure 9. The emission spectrum is not dependent of the wavelength of excitation (λ_{exit}).



Absorption vs concentration for λ=592 nm

Fig. 8. The UV-Vis spectrum of this diacid form

Table 2

THE EXTINCTION COEFFICIENTS FOR MONOMER AND DIMER

λ = 446 nm		λ = 592 nm		λ = 668 nm	
$\varepsilon_{monomer}$	ε_{dimer}	$\varepsilon_{monomer}$	ε_{dimer}	$\varepsilon_{monomer}$	ε_{dimer}
$3.8 * 10^3$	$4.1 * 10^3$	$4.6 * 10^2$	$6.9 * 10^2$	$8.5 * 10^2$	$10.5 * 10^2$

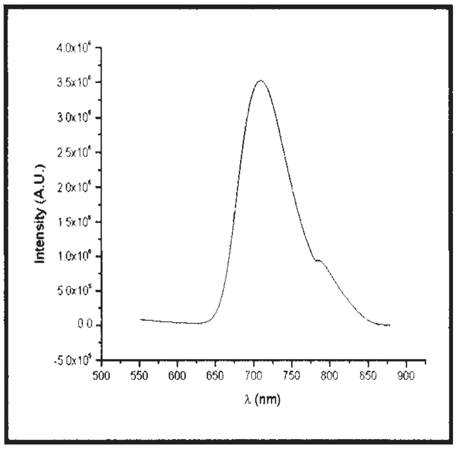


Fig. 9. Fluorescence spectrum of TAPP-L-TyR IN H_3PO_4

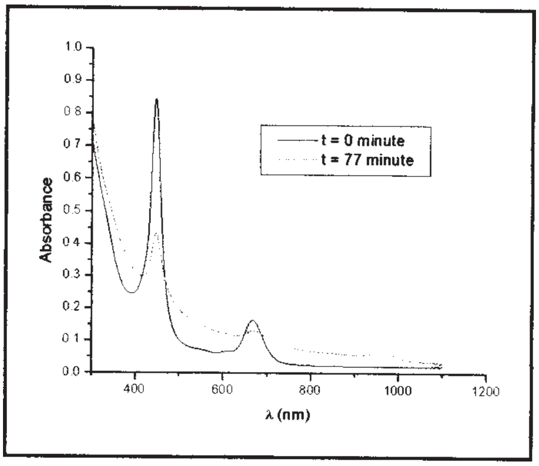


Fig. 11. UV-Vis spectra of TAPP-L-Tyr before and after irradiation

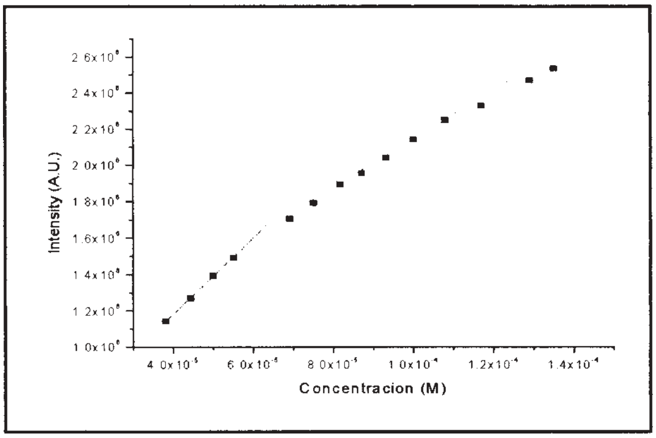


Fig. 10. The relationship between concentration and fluorescence intensity

The only effect of the change in l_{exit} is a difference in the intensity. The emission spectrum is dependent on the concentration. The relationship between concentration and fluorescence intensity is shown in figure 10. There is a break in the slope at a concentration around 6.10^{-5} M. It could be assumed that there is a change in the aggregation of the porphyrin.

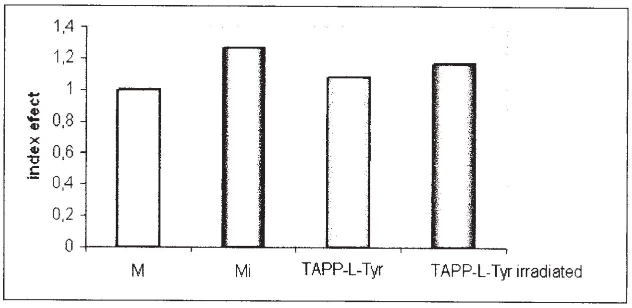


Fig. 12. Viabilitaty of EL-4 incubated with 15μ/mL TAPP-L-Tyr and irradiated (TAPP-L-Tyr irradiated) compared with control cells (M) and irradiated (Mi)

No significative modifications were observed in the fluorescence spectra even at irradiation lasting for hours. The only change between the spectrum recorded before photolysis, and the last one (77 min of photolysis) is a decrease of the intensity without shift or appearance on new bands corresponding to the fluorescence of a photoproduct.

Therefore, we assume that fluorescent photoproducts are not formed. Could be suggested that this behaviour is due to the intermolecular interactions of these tyrosine–porphyrin conjugate, and apart from the π–π interactions, or intermolecular hydrogen bonding with tyrosine. In summary, in the UV-Vis spectrum is clearly visible the appearance of a new band at 962 nm, which correspond to a photoproduct that maybe is not fluorescent.

Some efforts have been made in synthetic procedures to obtain synergic photosensitizers that combine a dual action in one compound. Tyrosine is a nonessential amino acid that is synthesized in the body from phenylalanine. As a building block for several important brain chemicals, tyrosine is needed to make epinephrine, norepinephrine, serotonine, and dopamine. Deficiencies in tyrosine, therefore, have been associated with depression. Tyrosine also aids in the production of melanine (pigment responsible for hair and skin color) and in the function of organs in the body responsible for making and regulating hormones.

Low levels of tyrosine have been associated with low blood pressure, low body temperature, and an under active thyroid.

This new sensitizer synthesized in this paper, has been tested from photodynamic point of view in the tumor cell line EL-4 and evaluated by our group (fig. 12).

The significantly increased action of TAPP-L-Tyr irradiated, is due to the sinergic effect of both components: tyrosine and porphyrin.

Conclusions

A new compound consisting of multifunctional ligands, such as porphyrin-tyrosine conjugate (5, 10, 15, 20-tetra-amino-phenyl-porphyrin coupled with tyrosine - TAPP-L-TyR), have been reported in this paper for application in photodynamic therapy.

In this paper a new synergic sensitizer was obtained, and an icreased photodynamic action of TAPP-L-Tyr irradiated, is due to the sinergic effect of both components: porphyrin and tyrosine.

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