

# **Investigation of photo-physical properties of selected diaminoacid protoporphyrin derivatives (PP(AA)<sub>2</sub>Arg<sub>2</sub>).**

## **I. Determination of quantum yield of fluorescence**

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An new generation of photosensitizers for photodynamic therapy (PDT) has been investigated. Different amino acids (Ala, Ser, Thr, Phe and Trp) were bound to protoporphyrin (PP). The quantum yield of fluorescence of porphyrin solutions was investigated. Fluorescence spectra and lifetimes of excited porphyrins were determined, too. The purpose of this work was to evaluate the quantum yield of fluorescence of different PP(AA)<sub>2</sub>Arg<sub>2</sub> derivatives, since it is crucial for choosing proper ingredients of photosensitizer for diagnosis and therapy in PDT.

Keywords: photosensitizers, photodynamic therapy, quantum yield of fluorescence.

### **1. Introduction**

Selective destruction of tissues that are sensitized to light with special sensitizers and underwent photochemical reactions found application in a modern method of diagnosis and treatment of tumor diseases. This method is called a photodynamic therapy (PDT) and it has several virtues that distinguish it from currently used therapeutic techniques. In this method, tumor tissues are selectively destroyed but healthy ones remain non-damaged. PDT is relatively low invasive, and additionally photodynamic properties of photosensitizers used in therapy are also useful for diagnosis (photodynamic diagnosis – PDD).

In the photodynamic method, a tumor tissue is destroyed during the process of selective oxidation. Three basic elements are needed to begin such a process in tumor tissues:

- photosensitive dye called photosensitizer that must be selectively retained in a tumor tissue and sensitize it to light,
- oxygen,
- light source of adequate power, emitting light waves coherent with photosensitizer absorption bands.

### 1.1. History

The birth of a photodynamic method dates from the beginning of the last century, *i.e.*, it is connected with the inventions of Raab (1900) and Polcard (1924) [1], [2]. The new era of PDT and PDD methods started with the works of Schwartz and Lipson, who isolated a mixture of protoporphyrine derivatives called HpD (hematoporphyrin derivatives) from the expired erythrocyte mass. Hematoporphyrin derivatives have selectively gathered in a tumor tissue for a few dozen of hours [3] and Dougherty used them for the tests on animals and people, both for diagnosis and therapy [4]. Further works of Dougherty and his co-workers on HpD composition resulted in isolation of dimer and oligomer fractions up to  $n = 8$ . Such a fraction is well retained in a tumor tissue and it can be effectively used for diagnosis and therapy of various types of tumors. From chemical nature, they are dimers and higher mers of hematoporphyrin connected by ether bound and/or ester bound [5]. This fraction is called Photofrin II and it is patented. As the only preparation it obtained a certificate of the Food and Drug Administration (U.S.A.).

Photofrin II is a photosensitizer applied in PDT and PDD methods and it used in many countries; U.S.A., Canada, Japan as well as in the countries of Western Europe [6]. Recently, it has been registered in Poland.

In the 1980's, several photosensitizers with photosensitive properties were elaborated, *i.e.*, various protoporphyrin derivatives, tetrabenzoporphyrin, chlorin, phthalocyanin, texapirine, and 5-aminolevulinic acid (ALA) that is a precursor for creating, in an organism, a protoporphyrin PP IX being a ligand for red dye of blood – heme. All these photosensitizers, except ALA, have a plate ring with the system of coupled double bonds in it. It is much probably that protoporphyrine derivatives are low toxic because mammal organisms developed, in their process of evolution, destruction and utilisation mechanisms of porphyrin ring.

### 1.2. New generation photosensitizers

All the compounds from porphyrinoids group have strong absorption band within the range of 390–405 nm, so-called, Soret band. It is used for diagnosis (PDD) or for treatment of flat lesions of skin or in mucous membrane due to its small penetration into a tissue (~2 mm). For therapeutic purposes, the weaker absorption band of the visible range is used (500–800 nm depending on a derivative). Typical porphyrins have the longest absorption bands with  $\lambda_{\max}$  of ~630 nm and low intensity but chlorins and bacteriochlorins have significantly stronger bands  $\lambda_{\max}$  of ~650 nm and ~710 nm, respectively.

In many laboratories, numerous compounds have been synthesized to have a photosensitizer of properties better than those of Photofrin II. Many derivatives have been obtained by changing the side chains in a porphyrin ring and a number of double bounds inside the ring [7].

An ideal photosensitizer should be non-toxic, selectively and in high concentration retained in a tumor tissue, soluble in water, quickly removed from healthy tissues (also from skin tissues).

A comprehensive review of the latest works on synthesis of new photosensitizers is given in [8]. It results from numerous investigations on photosensitive properties of various dyes that the most advantageous properties have the dyes of amphiphilic nature, *i.e.*, having lipophilic and hydrophilic domains in their structure. Such sensitizers locate well in a tumor cell because they are fixed in the lipidic domains with their lipophilic parts and in an aqueous part of a cell with the side chains of hydrophilic nature.

At the Laboratory of Biochemistry and Spectroscopy of the Institute of Optoelectronics, Military University of Technology (MUT), Warszawa, Poland, the synthesis of new class of photosensitizers, being diaminoacid derivatives of

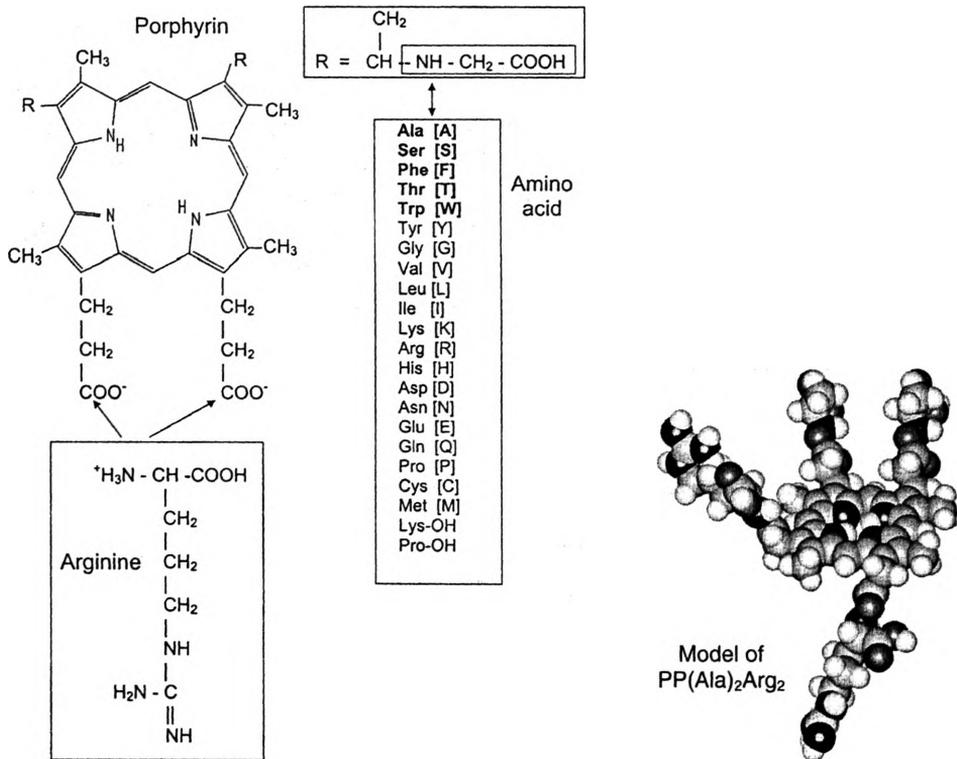


Fig. 1. Diaminoacid derivatives of protoporphyrin PP(AA)<sub>2</sub>Arg<sub>2</sub>.

protoporphyrin PP(AA)<sub>2</sub>Arg<sub>2</sub>, has been developed. Assuming that tumor cells have many amino acid receptors on their surfaces, because they are the cells with faster metabolism and amino acids are the basic elements necessary for their development, two endogenous amino acids were attached to two vinyl bridges in PP IX (from glycine to tryptophan).

To make these compounds soluble in water, two arginine particles were attached to two carboxyl groups in residues of propionic acid being the side chains of PP IX. It results in formation of the system of ionic bounds and making the whole compound perfectly soluble in water and having amphiphilic properties. The group of 23 diaminoacid derivatives PP IX, *i.e.*, PP(AA)<sub>2</sub>Arg<sub>2</sub> was obtained, the chemical structure of which is shown in Fig. 1 and a method of their receiving is described in the patents [9], [10]. Their usefulness for diagnosis and therapy was confirmed by the results of investigations on cell lines of various types of tumors [11], experimental animals [12], and by the results of the first phase of investigations performed in clinics [13].

Usefulness of the given dye as a photosensitizer used for tumor diagnosis and therapy can be initially estimated determining its photophysical parameters; molar absorption coefficient, lifetime in the excited singlet state, and quantum yield of luminescence  $\Phi_L$ . Physical processes proceeding in a sensitizer particle, after absorption of the energy quantum, can be described using the Jabłoński diagram presented for porphyrin sensitizers in Fig. 2.

A photosensitizer irradiated with the light of wavelength adequate to its absorption band, after absorption of the energy quantum  $h\nu$ , is in the excited singlet state  $S_1$  which is the highly-energetic state, *i.e.*, of a short lifetime. Also the higher excited states  $S_2$  and  $S_3$  are partially populated but the population density is low in comparison to the population state  $S_1$  and their lifetimes are significantly shorter. Emptying of the higher singlet states occurs during one tenth of nanosecond and electrons pass to the state  $S_1$ . Thus, the emission which is observed in typical spectrofluorometers is, in fact, only

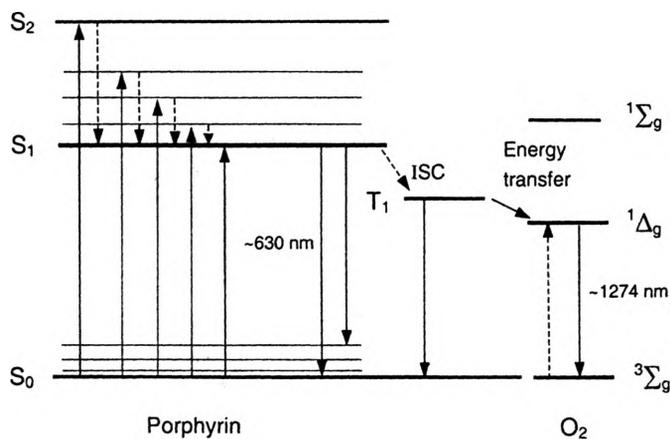


Fig. 2. Jabłoński diagram for photosensitizer and oxygen.

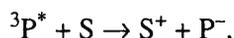
from the state  $S_1$ . The excited state  $S_1$  can be partially emptied due to photon emission and then we observe fluorescence that is used in the PDD. When porphyrin photosensitizers with red fluorescence are used, we can see the shape and size of tumor tissues. This image can be electronically registered and stored in a computer memory for continuous inspection of disease course and treatment monitoring.

Remaining electrons, due to the inter system crossing mechanism, pass from the state  $S_1$  and populate the excited triplet state  $T_1$  that is the lower energetic state but of significantly longer lifetime (of microseconds and even milliseconds) because it is a metastable state. A sensitizer, being in the excited triplet state, can react with medium components in which sensitizer is present or it can populate the basic state through quantum emission. This is a fluorescence phenomenon.

From theoretical point of view, a transition from the excited state  $T_1$  to the basic singlet state  $S_0$  is a forbidden transition because a change in multiplicity of electron spin is required. Such transitions occur but with a low efficiency, so phosphorescence spectra are of low intensity and it is difficult to register them [14]. Moreover, the excited particle of a photosensitizer can lose its energy through internal conversion during collision with other particles [15].

In the oxygenated medium, the photosensitizer particles, being in the excited state  $T_1$ , transfer easily their energy to the oxygen particle, the basic state of which is a triplet one. In this case, spins are of the same multiplicity, according so to the selection rules, energy transfer from triplet state of porphyrin  $P(T) \rightarrow$  to basic triplet state of oxygen particle  $O_2(T_0)$  is not forbidden [16]. Oxygen particle has low-situated excited states because transition energy from the base, triplet state of oxygen particle  $O_2(T_0) \rightarrow$  to excited singlet state of oxygen particle  $O_2(S_1)$  is only 22 kcal/mol, which corresponds to the wavelength of 1274 nm (Fig. 2). Because low energy is necessary for oxygen production in the singlet state  $^1O_2$ , this reaction is privileged in the oxygenated medium and the main cytotoxic factor is the singlet oxygen  $^1O_2(\Delta)$ . Generation of  $^1O_2$  in tumor tissue is described as type II photoreaction and its efficiency depends, first of all, on concentration of oxygen in reaction medium and photophysical properties of a photosensitizer.

In oxygen-free medium or hypoxia medium, the excited photosensitizer  $P^*$  can react directly with organic substrates S (tissue elements) through electron exchange, producing as a result an oxidated substrate



The reduced photosensitizer particle  $P^-$  can react with oxygen giving peroxide anion  $O_2^-$  and the excited photosensitizer  $^3P^*$  can react with peroxide radicals  $O_2^*$  giving peroxide anions  $O_2^-$  that in specified conditions can produce highly reactive hydroxylic radicals  $OH^*$  destroying each living tissue in which they will be generated. This kind of reaction is called type I photoreaction [17]. Figure 3 shows fundamental photoreactions of types I and II [18].

In both mechanisms, I and II, the substrate, *i.e.*, tumor tissue is destroyed during selective oxidation process. At the beginning of the irradiation process, when large

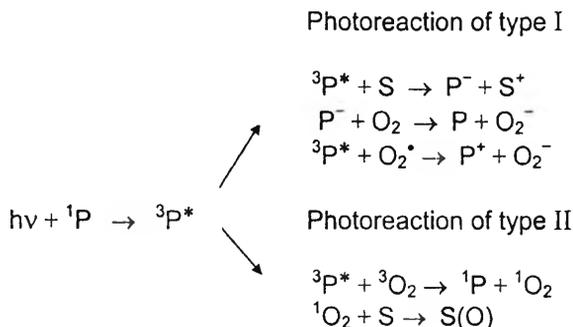


Fig. 3. Fundamental photoreactions of types I and II (after [17]).

amount of oxygen is in a reaction medium, it is type II of photochemical reaction that most frequently dominates and when oxygen amount decreases mechanism I of photodynamic reaction begins. Usually, in PDT method, both mechanisms are observed and their intensity changes with time.

To characterise diagnostic usefulness of the given photosensitizer, one should determine quantum yield of fluorescence  $\Phi_f$ . As results from the Jabłoński diagram the sensitizers of high  $\Phi_f$  value will be less capable of generating singlet oxygen  $^1\text{O}_2$  because they will populate the triplet state to a lower degree. There is no simple inversely proportional relationship between quantum yield of fluorescence and quantum efficiency lower  $\Phi_{\Delta\text{O}_2}$  for the given photosensitizer because not all particles that emitted the excess energy in emission process populate the triplet state as a result of the inter system crossing transition. Some particles loose their energy as a result of intermolecular collisions or the energy is dissipated in the processes of aggregation and formation of dimers [19], [20]. These photosensitizers that *in vitro* have high values of  $\Phi_f$  will be more useful for diagnostics than for therapy.

In this part of the work, quantum yield of luminescence was determined for five chosen diaminoacid derivatives of protoporphyrin which significantly differ in structure from amino acid ligands.

## 2. Material and method

For investigations, five derivatives were used from  $\text{PP}(\text{AA})_2$  and  $\text{PP}(\text{AA})_2\text{Arg}_2$  group, namely,  $\text{PP}(\text{Ala})_2$ ,  $\text{PP}(\text{Ser})_2$ ,  $\text{PP}(\text{Thr})_2$ ,  $\text{PP}(\text{Phe})_2$ ,  $\text{PP}(\text{Trp})_2$  and  $\text{PP}(\text{Ala})_2\text{Arg}_2$ ,  $\text{PP}(\text{Ser})_2\text{Arg}_2$ ,  $\text{PP}(\text{Thr})_2\text{Arg}_2$ ,  $\text{PP}(\text{Phe})_2\text{Arg}_2$ ,  $\text{PP}(\text{Trp})_2\text{Arg}_2$ , the general formulae of which are shown in Fig. 4a and b, respectively. The structure of the attached amino acid group (R) is shown in Fig. 4c.

All the compounds were synthesized at the Biochemistry and Spectroscopy Laboratory of the Institute of Optoelectronics, MUT, according to the method described in [9] and [10]. Amino acids were attached to vinyl bridges of PP and, in the next step, the arginine was attached to this modified porphyrin. The derivatives obtained from the synthesis were purified using column chromatography and their purity was controlled

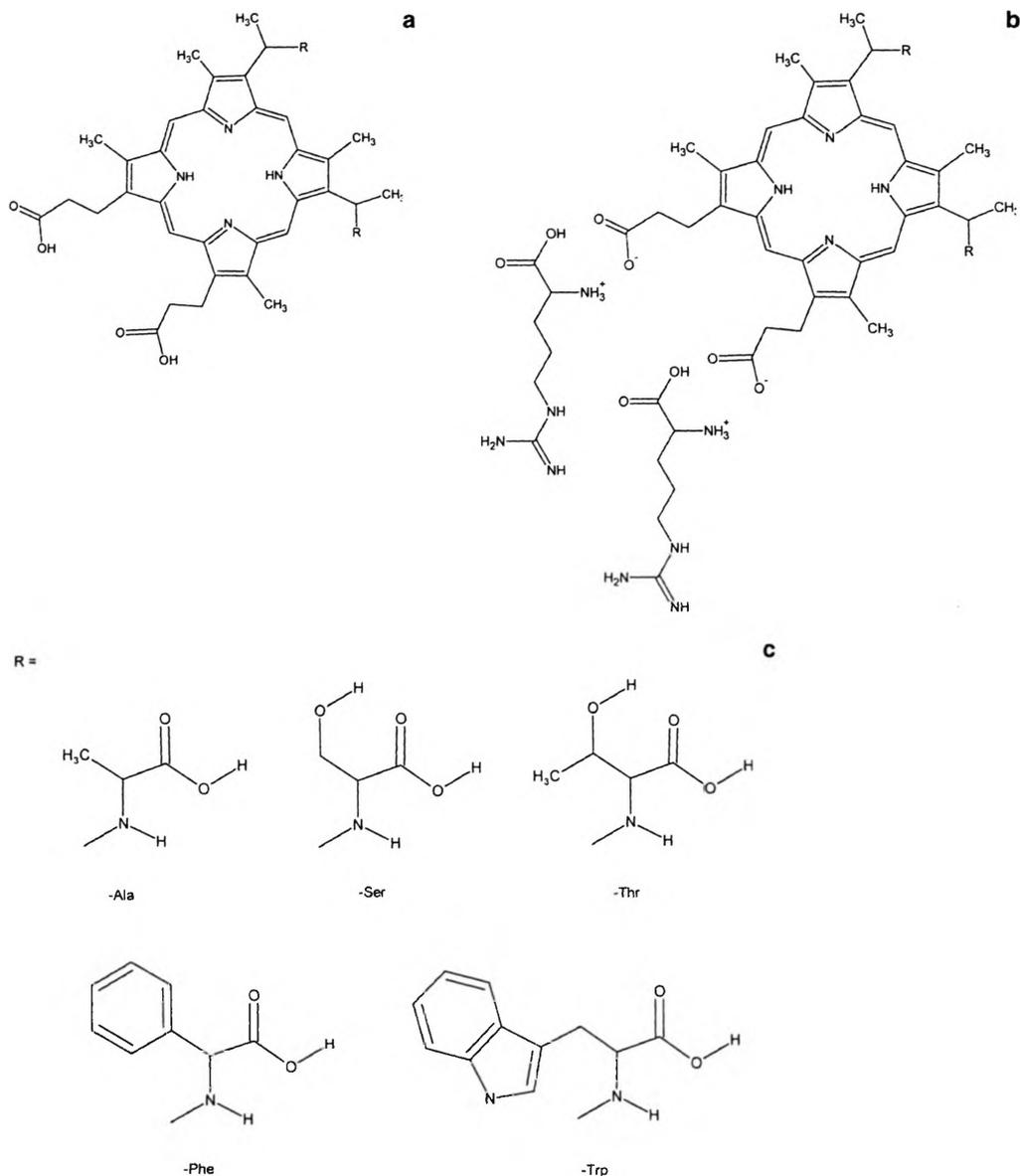


Fig. 4. Structure of PP(AA)<sub>2</sub> (a), structure of PP(AA)<sub>2</sub>Arg<sub>2</sub> (b), structure of amino acid (-AA) groups (c). Some hydrogen atoms are omitted for clarity.

with the high performance liquid chromatography (HPLC). The post-reaction mixture was separated using fractional elution technique in the column for extraction on solid state phases and with the high thin-layer chromatography (HTLC) method and highly efficient HPLC method. Correctness of mixture separation was estimated through determination of the retention  $R_F$  and the separation coefficient  $R_s$ , [21].

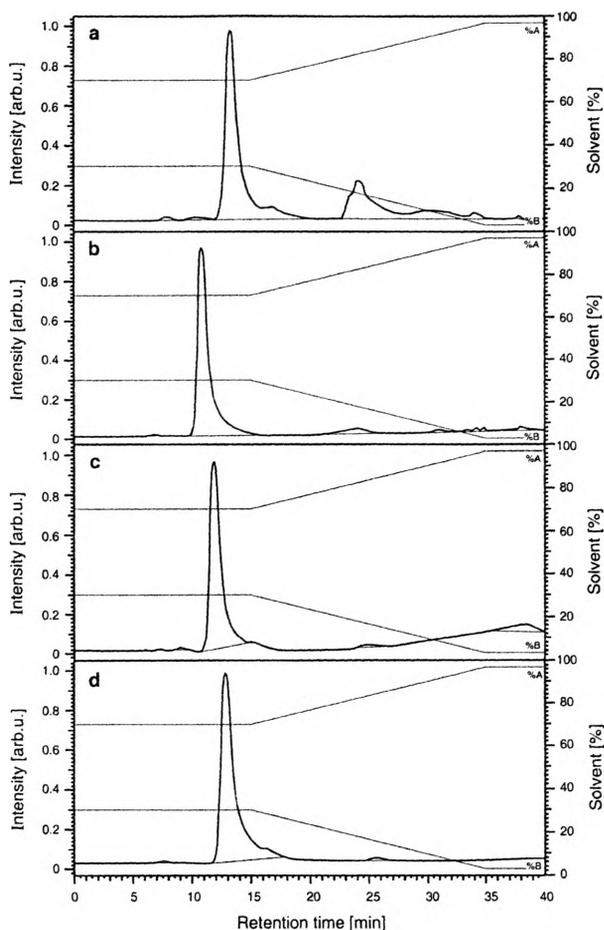


Fig. 5. PP(Ala)<sub>2</sub> chromatographs obtained after its additional purification in HPLC preparatory column. PP(Ala)<sub>2</sub> purity: 65.2% without elution (a), 88.3% after single elution (b), 90.1% after double elution (c), and 98.0% after triple elution (d).

In order to obtain a substance of standard purity (concentration >98%), purification was continued using preparative column of HPLC as shown in Fig. 5a–d.

Expensive purification of samples to the standard purity forced us to use mid-purity samples (~65%) both for investigations on cellular lines, experimental animals, and in phase I of clinical investigations. Such samples, besides PP(Ala)<sub>2</sub>Arg<sub>2</sub>, also contain dimers and multimers produced as a result of reaction between the various hydroxy protoporphyrin derivatives that are chromatographically and spectrally similar to the compounds in Photofrin II.

The quantum yield of luminescence  $\Phi_f$  and lifetimes in the excited state  $S_1$  were determined for the same series of samples that were used for clinical investigations.

Table 1. Determined values of molar absorption coefficient  $\epsilon$  for  $\lambda_{\max}$  of Soret band for PP(AA)<sub>2</sub> in MeOH and PP(AA)<sub>2</sub>Arg<sub>2</sub> in PBS of pH 7.2.

Compound	$\epsilon$ [M <sup>-1</sup> cm <sup>-1</sup> ]	Compound	$\epsilon$ [M <sup>-1</sup> cm <sup>-1</sup> ]
PP(Ala) <sub>2</sub>	30 428	PP(Ala) <sub>2</sub> Arg <sub>2</sub>	13 256
PP(Ser) <sub>2</sub>	32 850	PP(Ser) <sub>2</sub> Arg <sub>2</sub>	14 504
PP(Thr) <sub>2</sub>	60 333	PP(Thr) <sub>2</sub> Arg <sub>2</sub>	25 843
PP(Phe) <sub>2</sub>	98 821	PP(Phe) <sub>2</sub> Arg <sub>2</sub>	23 135
PP(Trp) <sub>2</sub>	41 109	PP(Trp) <sub>2</sub> Arg <sub>2</sub>	13 671

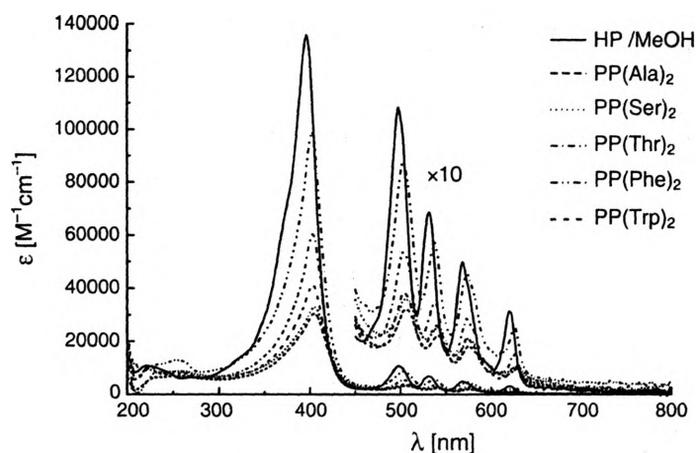


Fig. 6. Dependence of molar absorption coefficient for solution of PP(AA)<sub>2</sub> in methanol vs. wavelength.

One of the parameters describing photosensitizer purity is a molar absorption coefficient, especially in the Soret band (Tab. 1). Its spectra were shown in Fig. 6 [22].

### 2.1. Determination of fluorescence quantum yield

Emission spectra of the porphyrins under investigation are shown in Figs. 7 and 8. They were normalised to maximum for comparison. Determination of absolute value of fluorescence quantum yield  $\Phi_f$  does not belong to the category of photo-physical measurements that can be made without special apparatus usually used in highly specialised photo-physical laboratories. In our work, we took advantage of the method of relative measurements. It is easy to use and the quantum yield of fluorescence  $\Phi_f$  can be determined with accuracy up to  $\pm 5\%$  [23]. At present, the method of relative measurements for  $\Phi_f$  determination is the most frequently used method in laboratory practice.

To determine  $\Phi_f$  for five derivatives of PP(AA)<sub>2</sub>, methanol was used as a solvent and hematoporphyrin (HP) of the Porphyrin Products firm, of purity >98%, was used as a standard. According to KWAŚNY [24] and SMITH [25], the quantum yield of

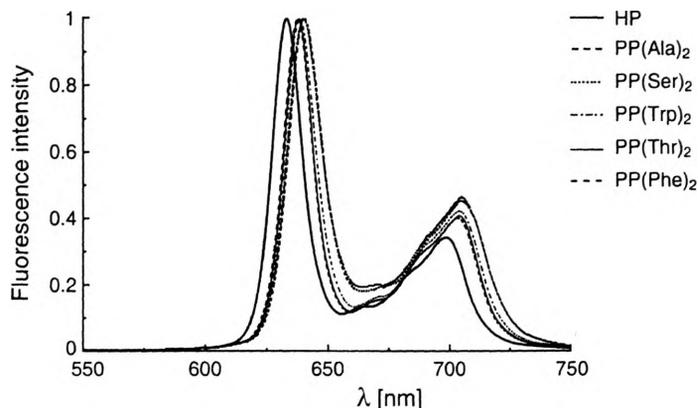


Fig. 7. Normalised emission spectra for  $PP(AA)_2$  and HP and PP IX in methanol.

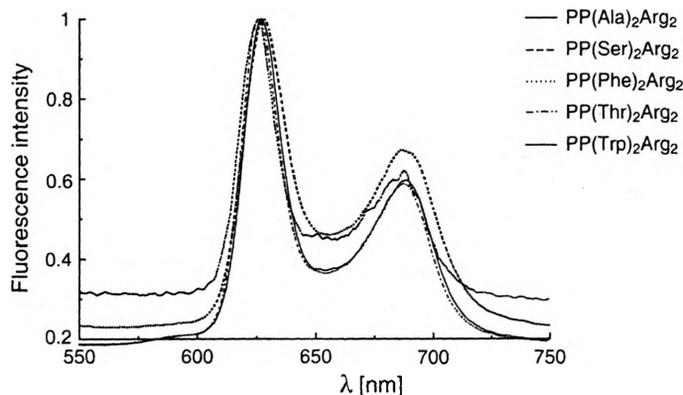


Fig. 8. Normalised emission spectra for  $PP(AA)_2Arg_2$  in PBS at pH 7.2.

luminescence for HP monomer was 0.08–0.09. In our measurements, HP concentration was  $2 \times 10^{-6}$  M, which in an organic solvent, such as methanol, causes that the major amount of the sensitizer is in a monomeric form. Concentration of the compounds investigated was chosen so that they have the same number of absorbed photons for each  $PP(AA)_2$  derivative. A cuvette for fluorescence measurement of the optical path  $l = 1$  cm was used and filled in with  $2.5 \text{ cm}^3$  solution. Prior to measurement, the solvents under investigation were deoxidised and blew out with nitrogen purified from oxygen. After deoxidation, the cuvette was closed with a plug and placed in a chamber of the Perkin–Elmer LS-5B spectrofluorometer, next the emission and excitation spectra were measured. The emission spectra were excited with the light of  $\lambda_{\text{exc}} = 400$  nm. Excitation and emission monochromator slots were set at  $S_{\text{exc}} = 5$  and  $S_{\text{em}} = 5$ , respectively.

## 2.2. Calculations of luminescence quantum yield

Intensity of excitation monochromatic light incident on the measured layer of the solvent, from which radiation was emitted, has been determined from the formulae

$$I_0/I_t = 10^A, \quad (1)$$

$$I_0 = I_a + I_t, \quad (2)$$

$$I_a = I_0(1 - 10^{-A}) \quad (3)$$

where  $I_0$  is the intensity of incident radiation,  $I_t$  – the intensity of radiation after its passing through the layer of the solvent,  $A$  – the absorbance of light, and  $I_a$  – the intensity of radiation absorbed in the layer investigated. To determine luminescence quantum yield, total number of photons (photon flux) emitted by the particles emitting fluorescence radiation in the whole range of emission spectrum was determined and next compared with the number of absorbed photons

$$\Phi_f = \frac{\Psi_f}{\Psi_a} \approx \frac{I_f}{I_a} = \frac{\int_{\lambda} I_{f,\lambda} d\lambda}{\int_{\lambda} I_{a,\lambda} d\lambda} = \frac{\int_{\lambda} I_{f,\lambda} d\lambda}{I_a} \quad (4)$$

where:  $\Phi_f$  – the quantum fluorescence efficiency,  $\Psi_f$  – the photon fluorescence flux,  $\Psi_a$  – the flux of photon absorbance,  $I_f$  – the integral radiant fluorescence intensity,  $I_a$  – the integral radiant absorbance intensity, and  $I_{f,\lambda}$  – the spectral radiation intensity at the wavelength  $\lambda$ .

Luminescence intensity is proportional to the intensity of incident light and the quantum yield of fluorescence  $I_f = I_a \times \Phi_f$ . The samples under investigation and the standard ones were irradiated in similar conditions. Dividing the equations of fluorescence of the sample  $I_f^s$  by the equation for the standard sample  $I_f^w$  we have

$$\frac{I_f^s}{I_f^w} = \frac{\Phi^s (1 - 10^{-A_w})}{\Phi^w (1 - 10^{-A_s})} \quad (5)$$

where  $I_f^s$  is the value  $I_f$  for the substance investigated,  $I_f^w$  is the value  $I_f$  for the standard sample,  $A_w$  and  $A_s$  are the absorbances for the standard sample and the one under investigation, respectively.

The ratio  $I^s/I^w$  is equal to the ratio of area under the curves of spectral emission of luminescence  $I_{f,\lambda}$  of the sample investigated to that of the standard sample.

Table 2. Values of quantum yield of luminescence  $\Phi_f$  for selected PP(AA)<sub>2</sub> in methanol, calculated in relation to the henatoporphyrin (HP) standard ( $\Phi_w = 0.09$ ) at a temperature of 20°C.

Compound	$\Phi_f$	Concentration C [M]	Molar absorption coefficient $\epsilon_{400}$ [M <sup>-1</sup> cm <sup>-1</sup> ]
HP	0.09	2.00×10 <sup>-6</sup>	128 000
PP IX Na <sub>2</sub>	0.091	1.90×10 <sup>-6</sup>	132 000
PP(Ala) <sub>2</sub>	0.024	6.10×10 <sup>-6</sup>	30 428
PP(Ser) <sub>2</sub>	0.024	5.64×10 <sup>-6</sup>	32 850
PP(Thr) <sub>2</sub>	0.061	3.13×10 <sup>-6</sup>	60 333
PP(Phe) <sub>2</sub>	0.063	1.93×10 <sup>-6</sup>	98 821
PP(Trp) <sub>2</sub>	0.037	4.57×10 <sup>-6</sup>	41 109

The final equation expressing quantum yield of fluorescence of the compounds under investigation takes the form

$$\Phi_f^s = \Phi_f^w \frac{F^s (1 - 10^{-A_w})}{F^w (1 - 10^{-A_s})} \quad (6)$$

where  $F^s$  is the photon fluorescence flux for the compounds investigated  $\approx \sum_{\lambda} I_{f,\lambda}^s$  and  $F^w$  is the photon fluorescence flux for the standard compound  $\approx \sum_{\lambda} I_{f,\lambda}^w$ .

The values of the quantum yield of luminescence  $\Phi_f$  of the compounds under study are listed in Tab. 2.

### 2.3. Determination of fluorescence lifetime

Also the time of luminescence decay for emission band with  $\lambda_{\max}$  of nearly 628 nm was determined. Figure 9 illustrates decays of fluorescence for three types of porphyrins. Table 3 shows the lifetimes of the excited singlet state that were determined from the times of emission decay,  $\lambda_{\text{exc}} = 416$  nm according to the equation

$$I(t) = I_1 \exp\left(\frac{-t}{\tau_1}\right) + I_2 \exp\left(\frac{-t}{\tau_2}\right) \quad (7)$$

where  $I(t)$  – the luminescence intensity in the time  $t$ ;  $I_1$  and  $I_2$  – the initial luminescence intensities of fractions 1 and 2, respectively;  $\tau_1$ ,  $\tau_2$  – the luminescence lifetimes in a solution, respectively.

A more detailed analysis of luminescence decays, using Simpleks method, showed that besides 95% fraction of monomer also several-percentage fraction of lifetimes of the order of 2–5 ns is present which most probably consists of the aggregates of a dimer type. The fluorescence rate  $k_f = \Phi_f/\tau_f$  shows high dispersion of PP(AA)<sub>2</sub> similarly as

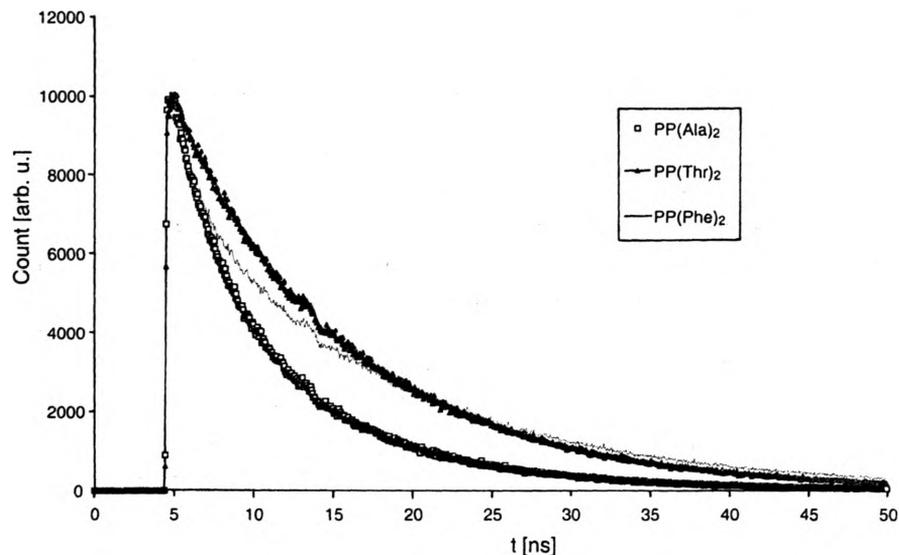


Fig. 9. Changes of fluorescence emission of PP(AA) solvents in MeOH after excitation with picosecond laser pulses of  $\lambda_{\text{exc}} = 416$  nm vs. time for: PP(Ala)<sub>2</sub> at emission wavelength 626 nm, PP(Thr)<sub>2</sub> at emission wavelength 626 nm, and PP(Phe)<sub>2</sub> at emission wavelength 616 nm,.

Table 3. Parameters of luminescence decay for selected PP(AA)<sub>2</sub> derivatives in methanol, for  $\lambda_{\text{exc}} = 416$  nm, at ambient temperature.

Compound	Concentration [M]	$\epsilon_{400}$ [M <sup>-1</sup> cm <sup>-1</sup> ]	$\lambda_{\text{em}}$ [nm]	$\tau_1$ [ns]	$\tau_2$ [ns]	$k_f$ [Hz]	Portion of 1 and 2 fraction*
PP(Ala) <sub>2</sub>	$5.95 \times 10^{-6}$	30 428	626	9.51	3.58	$2.58 \times 10^6$	0.72/0.27
PP(Thr) <sub>2</sub>	$3.2 \times 10^{-6}$	60 333	626	11.76	4.23	$5.19 \times 10^6$	0.96/0.03
PP(Phe) <sub>2</sub>	$7.82 \times 10^{-7}$	98 821	616	14.14	1.73	$4.46 \times 10^6$	0.95/0.04

\* at presume that  $\epsilon_1 = \epsilon_2$  and  $\Phi_{f1} = \Phi_{f2}$ .

absorbance molar coefficient, which can testify to the various purities of the porphyrin under examination.

### 3. Discussion of results

The quantum yield of fluorescence  $\Phi_f$  is one of two important parameters characterising photosensitizer. Fluorescence is the process of emission of short lifetimes from 1 ns to 1  $\mu$ s that depend on sensitizer and medium conditions.

According to the Kasha rule, despite many electron excited states of porphyrin compounds, the whole fluorescence observed originates practically only from the

lowest excited state of the given multiplicity. It is caused by the very high values of constants of non-radiant deactivation velocities within the states of the same multiplicity, in comparison with the velocity constants of radiant transitions. Internal conversion between the states of the same spin multiplicity is significantly faster than between the states of various multiplicities. When, as a result of excitation, a sensitizer particle passes to the excited state and populates not only  $S_1$  but also  $S_2$  and  $S_3$ , the higher singlet states disappear due to transition to  $S_1$  but not to  $T_2$  and  $T_3$ .

The quantum yield of fluorescence is used in medical practice as a diagnostic parameter. The higher the quantum yield of fluorescence, the more efficiently can the photosensitizer be used for diagnosis to determine the shape and size of tumor lesion and the earlier changes of tumor tissues can be observed, which is very important under treatment. It is also, or even first of all, used for operation diagnosis and for treatment monitoring. The quantum yields of fluorescence  $\Phi_f$  for diaminoacid derivatives, presented in Tab. 2, vary depending on aminoacid substituent. Of course, it cannot be assumed that the values  $\Phi_f$  obtained in the model investigations *in vitro* will not be exactly reproduced under *in vivo* conditions. Many factors of living organism affect positively and negatively the value of the quantum yield of luminescence. In living organism, an aqueous medium is present, which shortens the lifetimes of porphyrin in the excited state. On the other hand, the reports are known that aggregated photosensitizer particles undergo dissociation after being introduced into a cell. This increases the concentration of particles being in the form of monomer, which then causes an increase in the value  $\Phi_f$  [26], [27]. Moreover, photosensitizers penetrating the surroundings of cell structures cause the change in pH of the nearest surroundings, which results in a change, most often an increase (due to pH increase) in quantum yield of fluorescence due to the increase in monomerisation degree [28].

From the five diaminoacid derivatives of protoporphyrin PP(AA)<sub>2</sub>Arg<sub>2</sub>, the highest quantum yields of fluorescence  $\Phi_f$ , for measuring conditions described here, have been obtained for PP(Thr)<sub>2</sub> and PP(Phe)<sub>2</sub>, *i.e.*, 0.061 and 0.063, respectively. PP(Phe)<sub>2</sub> was tested during clinical investigations both for diagnosis and treatment applications and the results were satisfactory.

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