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Photophysical studies of tetratolylporphyrin photosensitizers for potential medical applications

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Abstract

A group of meso-substituted synthetic tetratolylporphyrins was investigated by the methods of absorption and fluorescence spectroscopies as well as steady-state and time-resolved photothermal spectroscopies. The experiments were carried out in the air, oxygen or nitrogen atmospheres, at two temperatures. The details concerning the fast and slow deactivation processes of excitation energy of the dyes were studied. The estimated quantum yield of fluorescence and the efficiency of (S_1–T_1) intersystem crossing process give a possibility to evaluate the population yield and quenching rate of the triplet state, important for the therapeutic application, mostly mediated by the T_1-state. For tetrapyrrole with the carboxyphenyl group substituted in meso-position, the triplet state yield was the highest, of 0.86–0.94, whereas the porphyrin with aminophenyl group seemed to be efficient singlet oxygen generators (0.81–0.99) with a high rate constant of its excited triplet state quenching by molecular oxygen.

Keywords: Photodynamic therapy; Photothermal spectroscopy; Porphyrin; Singlet oxygen; Triplet state

1. Introduction

Recently, there has been considerable interest in the synthesis and application of tetrapyrrolic photosensitizers such as porphyrinoids: porphyrins, chlorines, bacteriochlorins, phtalocyanines [1–8]. Their photophysical properties can be modified by introducing metal ion at the center of the pyrrole rings and/or by attaching side groups to their skeleton. By these means it is possible to control the influence of the dye molecular structure on dye capability of photodynamic activity. At the first step, the new tetrapyrrole-based photosensitizers are subjected to photophysical and photochemical studies in model systems, which permits the choice of proper ones and their optimization. It is interesting to analyse the properties of dyes in solutions so that to be able to control their properties on the molecular level, that is to study possible aggregation effects, radiative (fluorescence) and non-radiative (internal conversion, intersystem crossing) processes as well as triplet state generation, which are essential or influence the photodynamic treatment and diagnostic efficiency. It is known that dyes characterized by significant intersystem crossing transitions, long-lived triplet states and sufficient excitation energy transfer of their triplets to promote $^1O_2$ formation are able to induce photodynamic reactions leading to the phototherapeutic killing of malignant tumours.

In search for new second-generation photosensitizers or photomarkers useful for medical applications, a group of meso-substituted synthetic tetratolylporphyrins (TTP) has been studied by means of optical and photothermal techniques. The meso-derivatives of tolylporphyrin have been widely applied in various model systems (e.g. for study of photosynthesis, oxygen transport, etc.). They have been chosen for our photophysical study for several reasons: (i) they are relatively easily synthesised in the laboratory, (ii) the introduction of desired functional can be achieved by the Adler–Longo method (or it modifications) with yields varying from a few to about 20% of the total yield (there is a timely separation process of a desired derivative, which makes the yield of a particular derivative about 1–5%, depending on

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Abbreviations: A, absorbance; BCP, bromocresol purple; LIOAS, laser induced optoacoustic spectroscopy; PAS, photoacoustic; PDT, photodynamic therapy; TD, thermal deactivation; TPP, tetraphenylporphyrin; TTP, tetratolylporphyrin.

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the type of substituents applied), (iii) the presence of an acetamide, amine and carboxylic group allows a study of the effect of weakly (–$NHCOCH_3$) or strongly (–NH_2) activating groups and deactivating (–COOH) groups on the porphyrin photophysical properties, and (iv) the presence of an amine or carboxylic groups allow to link, e.g. amino acids, which may influence the dye sensitizing properties (e.g. transport into the cell).

The information on the dye singlet states is obtained from the absorption and fluorescence spectra recorded for dyes in solutions. On the basis of these experiments, the photophysical properties and spectral parameters of dyes, essential for the further analysis of the laser induced optoacoustic spectroscopy (LIOAS) results, were estimated. The time-resolved LIOAS and steady-state photoacoustic (PAS) techniques [4,9–11] detect the fate of the energy absorbed by dyes and its loss through non-radiative channels. The LIOAS signal analysis methods proposed by Marti et al. [12] and Small et al. [13] were applied. The experiments were performed in the air, oxygen or nitrogen atmosphere, at two temperatures. The obtained results give an opportunity to get insight into the mechanisms responsible for molecular processes occurring in dyes upon light illumination. LIOAS results provide the information about the dye triplet state properties, its population and quenching, whereas PAS signals give the global thermal energy release by the dyes.

The aim of our study was to check and to compare the influence of the meso-substituents on the porphyrin photophysical parameters. It was of interest to investigate by means of LIOAS method, the substitution influence on excited porphyrin triplet population and quenching. This study was also supposed to help establish if the time-resolved photothermal method was sensitive enough to differentiate between the porphyrins differing only in the meso-substituted groups. We also wished to find possible correlations between the molecular structure of the dyes and their spectroscopic properties. The estimated properties of the dye-sensitizer can be helpful to determine its photochemical activity, which is of great importance for its successful application for medical purposes such as photodynamic therapy (PDT).

2. Experimental

Meso-substituted tetratolylporphyrins (TTP) (e.g. 5-(4-carboxyphenyl)-10,15,20-tritolylporphyrin, 5-(4′-acetamidophenyl)-10,15,20-tritolylporphyrin, 5-(4′-aminophenyl)-10,15,20-tritolylporphyrin, the so-called Co-TTP, Ac-TTP and Am-TTP, respectively) were studied. Fig. 1 presents molecular structures of the dyes under investigation. Co- and Ac-TTP were synthesized according to the Adler–Longo method, by refluxing in propionic acid, two aromatic aldehydes with pyrrole at the molar ratio of 1:3:4 or 2:2:4, to obtain monosubstituted derivatives. Am-TTP was prepared from 5-(4′-acetamidophenyl)-10,15,20-tritolylporphyrin by heating it in a mixture of trifluoric acid/hydrochloric acid at 80 °C for 20 h [14,15]. Substituted porphyrins were dissolved in toluene and for spectroscopic measurements their concentrations ranged from about 10^{-6} to 10^{-3} M. The organic solvent used in the measurements was of spectroscopic grade.

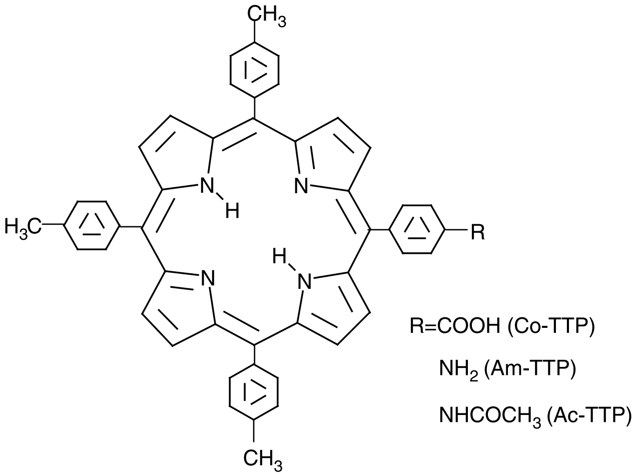

Fig. 1. Molecular structure of porphyrin under investigation.

Electronic absorption spectra were recorded on a Varian Cary 4000 spectrophotometer. The emission spectra were recorded by means of a Hitachi F4500 spectrofluorimeter. The steady-state photoacoustic (PAS) signal measurements were made on a one-beam spectrometer described elsewhere [11]. On the basis of the absorption, fluorescence and PAS spectra, the spectral parameters (position of main bands, extinction coefficient, thermal deactivation (TD) parameter determined as intensity of PAS signal divided by absorbance), the quantum yield of fluorescence (Φ_F) and the natural lifetime (τ_N) of the studied dyes were obtained (Table 1). The Φ_F values were estimated according to the method described in [16] using bacteriochlorophyll *c* in acetone as a reference [17].

Time-resolved photothermal signal was recorded by means of the LIOAS setup [4,9,10,18] in the time range up to 5 μs (limited by the setup used). Before the LIOAS experiments, some of the samples were deaerated (or oxygenated) by bubbling nitrogen (or oxygen). For the LIOAS measurements, at the laser flash wavelengths used (λ_{las}) the absorption intensities of the investigated and the reference samples were adjusted the same. The bromocresol purple (BCP), whose total excitation energy is promptly thermally deactivated (in time shorter than 0.5 μs - the estimated time resolution of the experimental setup [4,18]), was used as a reference in the LIOAS performed measurements. The LIOAS signal analysis method proposed by Marti et al. [12] was applied, based on a comparison of the first maximum of the signal (H_{max}, Eq. (1)) measured for the sample and the reference (Fig. 2). According to [12], the part of the energy exchanged promptly into heat, i.e. in a time shorter than 0.5 μs, is denoted as α (Fig. 3). It can be obtained from the equation:

$$H_{max} = K\alpha E_{las}(1-10^{-A}) \quad (1)$$

where H_{max}, the amplitude of the first maximum of the LIOAS signal; A, the sample absorbance at λ_{las}; E_{las}, the average laser pulse energy; K, the coefficient related to the optical geometry,

Table 1
Comparative spectroscopic properties of porphyrins under investigation in toluene

TTP	λ^{A}_{max} (nm)		$\varepsilon\times10^{4}$ (M^{-1} cm^{-1})		λ^{F}_{max} (nm)	τ_N (ns)	TD (a.u.)	
	Soret	$Q_x(0,0)$	Soret	$Q_x(0,0)$	$Q_x(0,0)$		Soret	$Q_x(0,0)$
Co-	421	648	26.76	1.30	650	17.04	0.43	0.71
Ac-	421	646	57.03	0.92	652	11.61	0.43	0.31
Am-	423	652	31.93	0.90	655	17.54	0.77	0.40

λ^{A}_{max}, λ^{F}_{max}, absorption and fluorescence wavelength at maximum; ε, molar absorption coefficient; TD, thermal deactivation parameter; τ_N, natural lifetime; $(1/\tau_N)=(8\pi\nu^2/c^2)(2303/N_0)\int_\nu \varepsilon(\nu)d\nu$, where $\varepsilon(\nu)$, the molar extinction coefficient as a function of the absorption frequency ν; c, the speed of light; N_0, the Avogadro number.

electronic impedance and thermoelastic properties of the sample solution.

Assuming that, in N_2-saturated samples, the photochemical reactions and other non- or thermal paths of energy deactivation are not realised, the approximate value of the yield of triplet state generation (Φ_T) can be obtained from the equation:

$$\Phi_T=\frac{(1-\alpha)E_{h\nu}-\Phi_F E_F}{E_T} \tag{2}$$

For the calculation, the energy of the dye triplet state (E_T) was taken from literature [6,19], $E_{h\nu}$, the molar energy of the incident photons; E_F, the energy of singlet state and Φ_F, the fluorescence yield of dye were estimated as described above.

The method of LIOAS signal analysis proposed by Small et al. [13] consists in deconvolution of both sample and reference signals, where the sample thermal signal (H) is based on the formula

$$H\sim\sum_i k_i \exp\left(\frac{-t}{\tau_i}\right) \tag{3}$$

where τ_i, the lifetime of the i-th transient; and k_i, a pre-exponential factor (the fraction of thermal energy released in i-th lifetime) [13]. In this procedure one can assume that the k_1 value is related to the decay time $\tau_1\leq0.5$ µs (below the setup time resolution) and describes the fast non-radiative processes. Hence, the values of k_1 and α obtained by deconvolution [13] and Marti et al. [12] methods, respectively, should be similar for a given dye. The contributions from slow thermal deactivation processes occurring in the time range from 0.5 to 5 µs are represented by k_2 and τ_2 values (Fig. 3).

3. Results and discussion

It is known that the porphyrin skeleton is essentially hydrophobic, which facilitates the dye-photosensitizer inclusion into the transformed cells, but it limits they solubility in solutions and physiological liquids. Synthesized meso-substituted TTP can be dissolved only in a few organic solvents. For our investigation toluene was chosen. All studied meso-substituted compounds show typical spectroscopic characteristics of free-base porphyrins (intense Soret band in the violet range of the visible region and four wide Q bands in the 500–680 nm region) (Fig. 4(a), Table 1). As seen from Table 1 the absorption and fluorescence regions for synthetic porphyrins are similar, it means that meso-substituents do not cause significant changes in the positions of the energy bands. It was also established that TTP independently of the groups

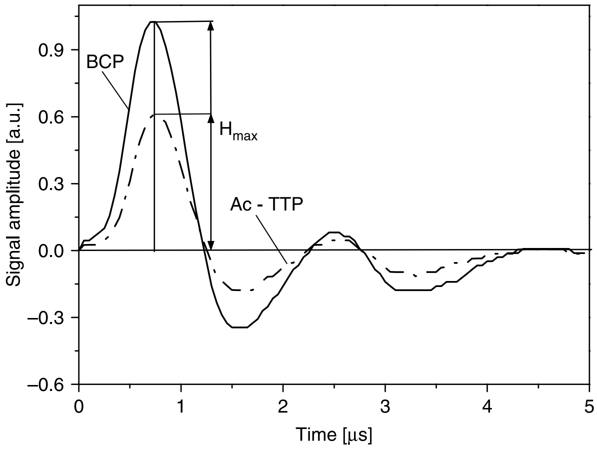

Fig. 2. LIOAS signals of porphyrin (Ac-TTP) and reference (BCP) sample recorded in aerated toluene at room temperature.

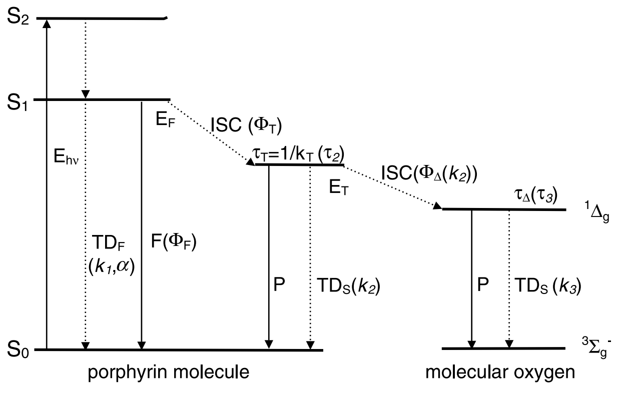

Fig. 3. Schematic diagram of energy levels and photoinduced processes in pigment and oxygen molecules; using LIOAS analysis methods (α, k_i, τ_i - parameters) [12,13], the radiationless processes are described by three sequential exponentials: (i) formation of the triplet state (ISC (Φ_T), Eq. (2)) and internal conversion to the ground state (TD_F), followed by (ii) decay of the triplet ($\tau_T=1/k_T$) by intersystem crossing (TD_S) or energy transfer to oxygen (ISC(Φ_Δ)) and, finally (iii) the decay of singlet oxygen (TD_S, τ_Δ); $E_{h\nu}$, the molar energy of the incident photons; E_F, E_T, the energy of singlet and triplet state, respectively; F, fluorescence; Φ_F, fluorescence quantum yield; P, phosphorescence.

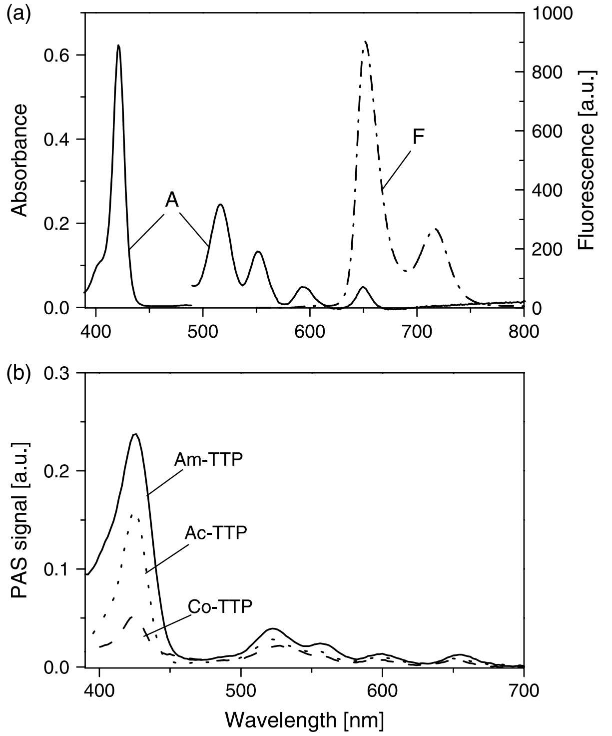

Fig. 4. Exemplary of absorption (*A*) and fluorescence (*F*) ($\lambda_{exc}=421$ nm) spectra of Am-TTP (a) and PAS spectra for all TTP dyes studied (b), the frequency of light modulation, $\nu=15$ Hz.

substituted (Co-, Ac- or Am-) did not show aggregate formation in the concentration range 10^{-6}–10^{-3} M. Aggregation of tetrapyrrolic sensitizers plays a remarkable role in determination of their photophysical properties (e.g. absorption parameters, lifetimes of excited states as well as fluorescence (Φ_F), intersystem crossing (Φ_T) and singlet oxygen (Φ_Δ) quantum yields, etc.). The spectra of the second derivative of absorption and fluorescence signals (not shown) have indicated that at the concentrations used the dyes were in non-aggregated, monomeric forms. The absorption coefficient of the Soret and Q bands ($\varepsilon \leq 10^4$ M^{-1} cm^{-1}) was obtained from the Beer–Lambert law using solutions in the 10^{-6} M concentration range (Table 1). The relative intensity of the Q bands indicates an etio-type spectrum.

The steady-state fluorescence emission spectra of all compounds present two maxima assigned to the Q(0,0) and Q(0,1) transitions (Table 1, Fig. 4(a)). The fluorescence spectra of TTP are roughly the mirror images of the long-wavelength region of the absorption spectra. The Φ_F values of the porphyrins studied were 0.05–0.10 (Table 2) and are typical of porphyrins [6]. The calculated Φ_F values do not depend significantly on the experimental conditions applied (different atmospheres and/or temperatures), i.e. they vary only in the range of accuracy. This suggests no aggregate formation and supports the idea that because of the short lifetime (0.85–1.23 ns, calculated using Φ_F, τ_N from Tables 1 and 2), the quenching of fluorescence by molecular oxygen is not a crucial factor in aerated solutions of these porphyrins. The estimated lifetimes are of an order of nanoseconds as expected. For example Ac-TTP is characterized by the shortest τ_N and by the largest value of Φ_F from among the synthetic porphyrin under investigation (Tables 1 and 2). In general, the spectral parameters of Co-, Ac-, Am-TTP are in good agreement with the available literature data for tetraphenylporphyrin (TPP) and corresponding derivatives [3,6,18]. Furthermore, photophysical parameters of substituted TTP (e.g. Φ_F, τ_N) are comparable with those reported in literature [1,3,6] for TPP and other porphyrin derivatives.

The nanoseconds time range of τ_N for porphyrins (Table 1) could indicate an intensive intersystem crossing transition, which may lead to high population of the their triplet states. To get a deeper insight into this problem we performed steady-state PAS (it gives information on the global TD processes with singlet and triplet states participation) (Fig. 4(b)) and time-resolved LIOAS experiments (give insight into triplet state population) (Fig. 2). The latter was also expected to answer the question about a possibility of triplet state contribution in the photodynamic reaction. The part of the energy absorbed converted into heat is estimated by TD parameters collected in Table 1. For example, Co- and Am-TTP loose much energy in thermal relaxation processes when compared to Ac-TTP, which

Table 2
Results of LIOAS signals analysis of the porphyrin in toluene at 20 and 4 °C (results in parentheses), $\lambda_{las}=421$ nm, $E_{h\nu}=283.9$ kJ mol^{-1}

TTP	Φ_F	α (N_2)	α (air/O_2)	k_2	τ_2 (μs)	Φ_T	Φ_Δ	S_Δ	$k_T \times 10^9$ (M^{-1} s^{-1})
Co-	0.05	0.55 (0.51)	0.59 0.63	0.24	0.53	0.86 (0.94)	0.54 (0.66)	0.63 (0.70)	0.96
Ac-	0.10	0.61 (0.58)	0.67 0.73	0.06	0.76	0.66 (0.73)	0.79 (0.74)	1.19 (1.01)	0.73
Am-	0.07	0.58 (0.52)	0.78 0.85	0.11	0.47	0.78 (0.90)	0.81 (0.99)	1.04 (1.10)	1.18

Φ_F, fluorescence yield; $\Phi_F=\Phi_R(I/I_R)(OD_R/OD)(n^2/n_R^2)$ [16], where Φ_R, the fluorescence quantum yield of the reference; I, I_R, and OD, OD_R and n, n_R, the areas under the fluorescence curves, the absorption intensities, refractive indices of the sample and reference, respectively; α, a fraction of excitation energy exchange into heat promptly (in shorter time than time resolution of apparatus) estimated based on Eq. (1), k_1, pre-exponential factor for $\tau_1 \leq 0.5$ μs, k_2 and τ_2, pre-exponential factor and decay time, for time range: 0.5–5.0 μs; Φ_T, yield of triplet state formation - Eq. (2); Φ_Δ, yield of singlet molecular oxygen production; $\Phi_\Delta=(\Phi_T E_T - E_{h\nu} k_2)/E_\Delta$ [19] where $E_\Delta=94$ kJ mol^{-1}; S_Δ, the fraction of triplet states quenched by oxygen, $S_\Delta=\Phi_\Delta/\Phi_T$, k_T, the rate constant of triplet state quenching by molecular oxygen, estimated taking $[O_2]=1.81\times10^{-3}$ M in toluene at room temperature.

deactivated efficiently its absorbed energy in radiative processes (the high Φ_F value). It means that for porphyrins with acetamidophenyl group there is a competition between the fast TD processes (described as α in Table 2) and fluorescence. For all porphyrins under study, the linear correlation coefficient, estimated on the basis of the experimental dependence of the PAS value signals on light modulation frequency and predicted on the basis of Rosencwaig–Gersho theory [20], does not reach unity (not shown). It indicates that not only prompt internal conversion transitions but also slow relaxation processes, with triplet state participation, are involved.

For the therapeutic application, the yield of dye triplet state and its interactions with molecular oxygen are the most important photophysical properties because the photodynamic activity is mostly mediated by the T_1-state. On the basis of the LIOAS experiments it is possible conclude about the population and quenching efficiency of dye triplet state as well as about singlet oxygen production yield. As follows from Table 2 and LIOAS measurements performed, for all porphyrins a decrease in the O_2 presence in the solution causes an increase in the yield of the slow thermal deactivation. This increase is due to the decrease in quenching of the triplet state by oxygen. For a photochemically stable dye, the Φ_T value can be estimated from the measurement of the slow component of TD of the excitation energy. To evaluate Φ_T for the new compounds the value of E_T was taken the same as for TPP (138 kJ mol^{-1}) [6].

For Ac-TTP the values of Φ_F and α are quite high indicating that a significant fraction of absorbed energy is dissipated in the fast deactivating processes, which is in agreement with the low Φ_T value, while for the other two dyes Co- and Am-TTP the situation is the reverse (Table 2). The latter dyes convert into heat more energy in slow processes taking place above 0.5 μs. For Co- and Am-TTP the obtained Φ_T values are quite high and can be explained by the high probability of intersystem crossing (S_1–T_1) transition. The high yield of the intersystem crossing (S_1–T_1) explains the low value of α related to the fast TD processes (taking place in the time scale up to 5 μs).

Excitation energy transfer between porphyrin derivatives and singlet oxygen has been, in principle, a well-known phenomenon [6,21–23]. Porphyrin in their lowest triplet excited state can transfer their excitation energy to oxygen, resulting in its triplet-singlet transition. Therefore, further analysis by the deconvolution method [13] was made for the LIOAS results recorded in the air. Assuming that the second (exponential) component of the LIOAS signal obtained by the method of Small et al. [13], represents the two possible pathways of the triplet state deactivation involving almost complete energy transfer from this state of porphyrin to the oxygen or radiationless decay to the ground state, the deconvolution implies that the parameters k_2 and τ_2 (Table 2) can be correlated with the dye triplet state quenching by molecular oxygen and generation of singlet oxygen. The values Φ_Δ were determined at two temperatures. As follows from Table 2, the rate constant of triplet state depletion by molecular oxygen (k_T) and singlet oxygen production were higher for Am- than for Ac-TTP. As follows from the Φ_Δ results in Table 2, this efficiency depends strongly on the character of the substituents and does not show a simple correlation with the Φ_T values, as it is confirmed by S_Δ-parameters. The lowest Φ_Δ and S_Δ values were obtained for Co-TTP and the highest for Am-TTP. The Φ_Δ and k_T values imply that a strong quenching of triplets for TTP with aminophenyl-substituted group is observed. But on the other hand, the results indicate that the triplet of Co-TTP is not completely depopulated be energy transfer to O_2 in toluene at ambient and low temperature. The somewhat high values of Φ_Δ estimated at 4 °C are probably due to two effects: the higher Φ_T value and the higher viscosity of toluene, resulting in a lower diffusion coefficient of oxygen in chilled (4 °C) than ambient (20 °C) temperature. It should be also noted here that the change in temperature has not a very strong effect on the yield of (S_1–T_1) transition leading to a slight increase in Φ_T values, which is well correlated with the decrease in α-values at low temperature.

Our results strongly confirm that incorporation of the electron donor amino group (NH_2) in the meso-position of the porphyrin molecule causes an increase in the Φ_Δ and also k_T values, whereas the electron acceptor group (COOH) reduces the singlet oxygen generation quantum yields similarly as it was reported in [6]. So, it seems that the introduction of Co-, Ac- and Am-groups at the meso-position of the phenyl rings does not markedly influence the π-electronic system of the main chromophore and does not cause a significant change in the intersystem crossing probabilities, but it influences the efficiency of the triplet generation and quenching. The LIOAS results in nitrogen atmosphere are not affected by oxygen quenching of the dye triplet state, therefore they give the possibility to calculate Φ_T values, which can be comparable with the data reported by other authors. To the best of our knowledge, the following literature data are available: for unsubstituted TPP in toluene, Φ_T is 0.67–0.83 and Φ_Δ is 0.65–0.68 [6,21,22,24]. The values of Φ_T (0.66–0.86) estimated for meso-substituted TTP dyes are in agreement with that for TPP, while Φ_Δ estimated for Co-TTP is similar as that of TPP.

4. Conclusions

The triplet state generation yield and the photosensitising potential of porphyrins investigated have been estimated on the basis of a study of radiation and thermal depopulation processes of their excited states. The correlation of TTP photophysical parameters and meso-substituted groups has also been examined. The differences in the molecular structure of the dyes lead to distinctly different spectroscopic properties, e.g. the triplets of various dyes are differently populated and quenched by oxygen.

On the basis of the results presented, the following conclusions on the possibility of the dye studied applications in photodynamic therapy or diagnostics of cancerous tissues, can be drawn:

(1) Co-TTP studied exhibits high efficiency of triplet states generation (0.86–0.94) in solution, and it seems that it will also able to trigger directly the photodynamic reaction in biological systems because of their triplet is incompletely depopulated in an access amount of molecular oxygen.
(2) Photophysical properties of meso-substituted TTP dyes depend strongly on the nature of substituents, and only compound with aminophenyl group combines both high yield of triplet (0.78–0.90) and of singlet molecular oxygen production (0.81–0.99), hence it could be used in photodynamic cancer treatment, which involves tumor necrosis by photosensitized $^1O_2$ formation.
(3) The fluorescence quantum yield (0.10) of Ac-TTP seems to be sufficient to suggest that its emission band near 652 nm may be used for diagnostic purposes in cancer therapy but the possibility of aggregation inside human cells in vivo, should be taken into consideration in further clinical examinations.

In the light of our experiments it follows that the time-resolved photothermal method used is characterized by high sensitivity and offers a possibility to detect the influence of substituents on the processes on molecular level. The results of the comprehensive study of optical and photophysical parameters of the compounds under consideration have shown that synthetic derivatives of tolylporphyrins could be interesting molecular materials for medical applications. It is expected that the TTP compounds with strongly activating (amino) groups could be the best for singlet molecular oxygen generation and the most effective in anti-cancer therapy.

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