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Concerning Regioselective Photochemical Intermolecular Proton Transfer from Hypericin

Roland A. Obermüller[1], **Gerhard J. Schütz**[2], **Hermann J. Gruber**[2], and **Heinz Falk**[1,*]

[1] Institut für Chemie, Johannes Kepler Universität, A-4040 Linz, Österreich
[2] Institut für Biophysik, Johannes Kepler Universität, A-4040 Linz, Österreich

Summary. Using epifluorescence microscopy on lipid vesicles containing hypericin or several of its O-alkylated derivatives together with a fluorescence *pH* indicator it was shown that upon excitation of the respective hypericinate ion an excited state derived proton is transferred to the indicator molecule. In addition, it could be also unequivocally derived that this proton originates from one of the *peri*-hydroxyl groups of the pigment.

Keywords. Hypericin; O-Alkyl hypericines; Intermolecular proton transfer; Epifluorescence microscopy.

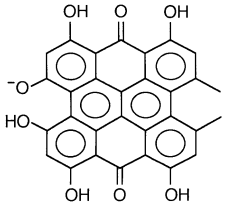
Zum regioselektiven photochemischen intermolekularen Protonentransfer von Hypericin

Zusammenfassung. Mit Hilfe der Epifluoreszenzmikroskopie an Lipidvesikeln, die Hypericin oder O-alkylierte Hypericinderivate sowie einen Fluoreszenz-*pH*-Indikator enthielten, konnte nachgewiesen werden, daß nach Anregung des Hypericinations ein aus dem angeregten Zustand resultierendes Proton auf den Indikator übertragen wird. Darüber hinaus konnte gezeigt werden, daß dieses Proton zweifelsfrei von einer der *peri*-Hydroxylgruppen herrührt.

Introduction

Hypericin, or more specifically spoken, its *bay*-phenolate ion (**1**) is found mostly in *Hypericum* species [1] and is of interest for a variety of physiological effects [1, 2]. For its mode of action intermolecular proton transfer as a consequence of photochemical excitation, leading to an acidification of the surrounding medium and, consequently, to cell death or apoptosis, has been advocated [3–6]. Indeed, intermolecular proton transfer from the *bay*-hypericinate ion has been deduced from fluorescence studies of hypericinate embedded in lipid vesicles with an encapsulated *pH* indicator solution [3]. Based on quenching experiments and electron paramagnetic resonance studies, it has been argued that a cation radical formed by electron transfer from the hypericin triplet state could serve as the strongly acidic proton source [6, 7].

* Corresponding author



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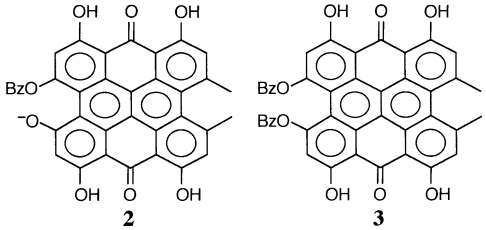
With respect to the fundamental interest in this physiological relevant reaction it seemed to be worthwhile to extend these investigations to the regional aspect of proton transfer. Since there are five to six more or less acidic hydroxyl groups present in the hypericinate (**1**) or hypericin molecule, there is a number of possibilities to abstract a proton. Fortunately, the hydroxyl protons of **1** can be divided into two subgroups of different ground state acidity, with the *bay*-region protons more acidic than the *peri*-region hydroxyl protons (those close to the carbonyl groups) by several orders of magnitude [8].



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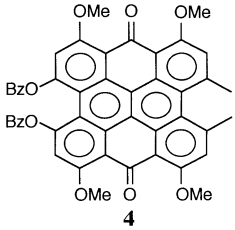

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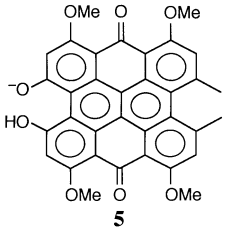

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To differentiate at least between the proton expulsion from the *bay*- and the *peri*-regions as a consequence of excitation, proton transfer experiments with the recently prepared and characterized hypericin derivatives **2**–**5** [9] seemed to be promising. In these derivatives, the two regions have been selectively and mutually blocked by alkyl residues.

Results and Discussion

Experiments to reproduce the steady state results of the proton transfer from **1** contained in a lipid vesicle wall to the fluorescence *pH* indicator 2′,7′-*bis*-(2-carboxyethyl)-5(6)-carboxy-fluorescein (**6**) dissolved in the encapsulated water phase of the vesicles as described in Ref. [3] were unsuccessful. This was obviously due to the an extreme sensitivity to experimental parameters, making the access of an ideal setup somewhat fortuitous. Since also experiments to use inverse micelles for this purpose did not yield significant effects, the methodology of epifluorescence microscopy was chosen as a simpler and more direct means to monitor proton transfer from **1** to the fluorescence indicator molecule **6**.

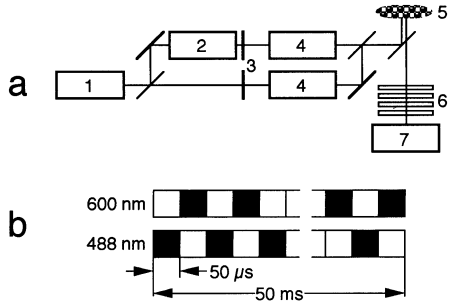


Fig. 1. Epifluorescence experimental setup ; a) 1: Ar^+ ion laser (488 nm), 2: dye laser (600 nm), 3: shutter, 4: acousto-optic modulators, 5: vesicle suspension, 6: optical filters, 7: charge coupled device camera; b) pulse trains from the two lasers reaching the vesicles (white squares: light, black squares: dark)

In these experiments, the hypericin pigments **1**–**5** were embedded into dipalmitoylphosphatidylcholine vesicles. An aqueous buffer solution ($pH = 7.1$; as was estimated from a spectrofluorimetric titration, this was well within the region of highest indicator sensitivity) of the indicator **6** was encapsulated into them and surrounded them as well. Using the experimental setup illustrated in Fig. 1a, the hypericin derivatives and the indicator were alternatively excited with light of 600 (**1**–**5**) and 488 nm (**6**), and the resulting fluorescence F of **6** was detected (Fig. 1b). These measurements were then normalized by means of the indicator fluorescence F_0 obtained without excitation of the pigments **1**–**5**. The resulting ratio F/F_0 was then measured under variation of the pigment excitation power. The results of these measurements together with that for vesicles containing only **6** for comparison are illustrated in Fig. 2. It should be noted that upon simultaneous excitation an artifact effect (*i.e.* an apparent *pH* decrease) was observed which was due to a depopulation of the excited indicator singlet into a higher state. Moreover, a μs excitation time domain had to be applied in the experiment, since upon a ms duration of excitation or observation the effect vanished. This observation would also nicely point to the triplet state involved into the proton ejection, since the triplet lifetime of **1** in ethanol has been measured to amount to about 40 μs [7]. However, a cation radical formed by electron transfer from the excited singlet state [6] could also display the observed time characteristics.

Since it has been shown [3] that there is a decrease of indicator fluorescence caused by the *pH*-decrease upon acidification of the inner bulk phase of the vesicle, enhancement of the pigment excitation power in the present experiment should lead to a contiguous diminution of this fluorescence. This was nicely demonstrated by the experiment for **1**+**6** in Fig. 2. The fluorescence images of the sample without and with excitation of **1** are shown in Fig. 3. It should be emphasized that this kind of experiment, in contrast to that described in Ref. [3], measured the protons injected to the bulk water phase within *and* outside of the vesicle after excitation of the pigment. Nevertheless, the result of the present experiment was found to be in agreement with the factual findings on **1** given in Ref. [3].

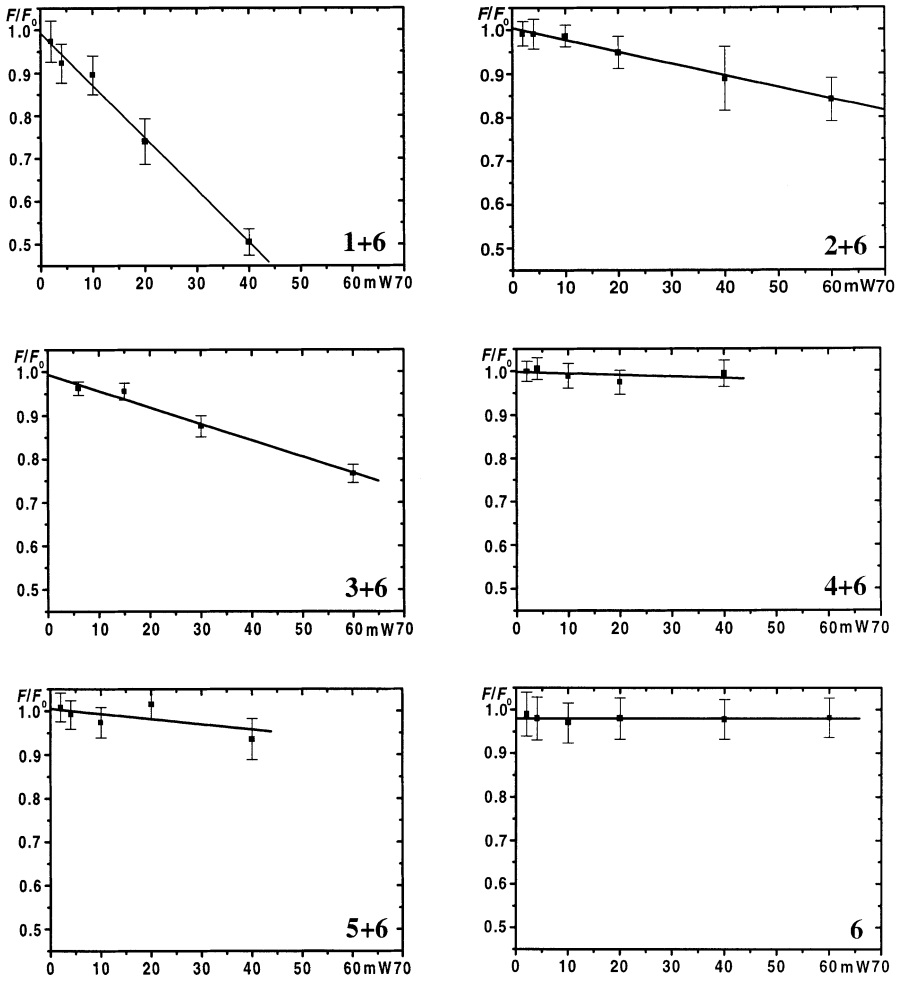


Fig. 2. Normalized fluorescence intensity F/F_0 of indicator (**6**) fluorescence (excitation at 488 nm) after excitation of the hypericin pigments present (**1**+**6**, **2**+**6**, **3**+**6**, **4**+**6**, **5**+**6**) or absent (**6**) with varied power at 600 nm

As could be speculated from the behavior of the *bay*-deprotonated hypericin **1**, alkylation of the *bay*-hydroxyl groups as exemplified by derivatives **2** and **3** exerted no qualitative influence on the proton expulsion potency of the system (Fig. 2). With respect to a quantification of the effect, one has to keep in mind that it is also dependent on the relative light absorption properties and various quantum yields of the different pigments at the excitation wavelength (cf. Ref. [7]) as well as on their respective pK_a values. Thus, one should not compare different slopes of the power dependence, but instead derive from these experiments only the information of occurring proton transfer: yes or no. From these three experiments, the *peri*-hydroxyl protons became evident as the proton source. This was corroborated by experiments on derivatives **4** and **5** which did not display any significant proton transfer reaction (Fig. 2). When the vesicles were prepared without the hypericin pigments and contained only the indicator **6** for comparison, no effect within

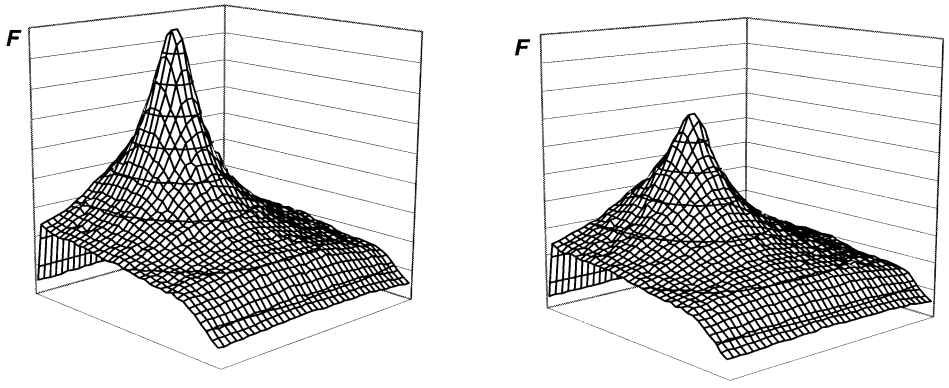
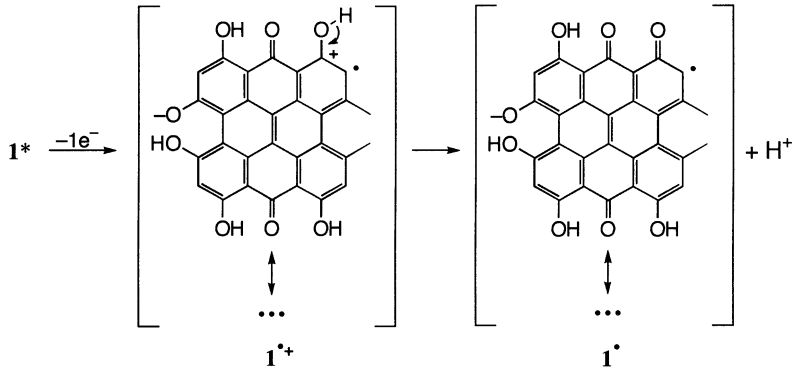


Fig. 3. Fluorescence intensity (F; scale length corresponds to about $2 \cdot 10^4$ counts) images of the sample containing **1**+**6** excited at the indicator (**6**) wavelength 488 nm without (left side) and with (right side) prior pigment (**1**) excitation at 600 nm

experimental error was observed. Accordingly, the results on proton transfer were clearly caused by pigment excitation.

As mentioned above, the μs time window of the experiments could point to an ejected proton derived from a triplet state as has been inferred recently [3]. However, there is no reason why a triplet state should exhibit the prominent acidification of *peri*-hydroxyl protons with a ground state pK_a in the order of 12 [8] (with respect to the $pH = 7$ region of the experiment, this amounted to an acidification by at least five orders of magnitude). However, as pointed out by *Song* recently, electron transfer from the singlet state to one of the several possible acceptors, including the ground state of **1** present in the rather high local concentrations of the experiment, would result in a cation radical which could be of dramatically enhanced acidity [6]. Such a behavior has been observed for the phenolic proton of the tyrosin cation radical ($pK_a^{\text{tyrosin}} \approx 10$, $pK_a^{\text{tyrosin radical cation}} \approx -1$[10]). Thus, one could hypothesize that the reaction takes place (Scheme 1) starting from either the singlet state $^1\mathbf{1}^*$ or following internal conversion to the triplet state, $^3\mathbf{1}^*$. Since, from the present state of knowledge, either state could be responsible for the electron transfer step, we simply assigned



Scheme 1

it to be an excited state $\mathbf{1}^*$. It should be noted that **1** actually is already a *bay*-phenolate. However, since it is extremely stabilized by hydrogen bonding [8], it might not interfere with the generation and transformation of the cation radical $\mathbf{1}^{\bullet+}$.

In conclusion, this study has corroborated that upon excitation of hypericinate an excited state derived proton is transferred to an acceptor; moreover, it could be demonstrated unequivocally that this proton was derived from one of the *peri*-hydroxyl groups of the pigment.

Experimental

Hypericin derivatives **1** [11] and **2**–**5** [9] were prepared as described recently; the indicator **6** was of commercial origin (Fluka). To 1 ml of a solution (10 µmol/ml) of dipalmitoylphosphatidylcholine (Sigma) in $CHCl_3$ (p.A. Merck, percolated over basic Al_2O_3 before use), 2 ml of a 0.95 µmol/ml solution of **1** in ethanol, or 1 ml of a 1.9 µmol/ml solution of **2**–**5** in $CHCl_3$ were added, and the solvent was evaporated in a slow stream of N_2. The residue was again dissolved in 1 ml $CHCl_3$ and dried once more by means of an N_2 stream. This procedure was repeated for another time. Finally, the residual solvent was removed by evaporation at 10^{-3} mm Hg for 30 min. The resulting film was mixed with 100 µl of a solution of 1 g/l (1.92 µmol/ml) **6** in 30 m*M* NaN_3/120 m*M* NaCl buffer ($pH \approx 7.1$). This heterogeneous mixture was then sonicated (Elma, Transsonic T 460) for 5 min at 56–57°C resulting in the vesicle solution. Vesicles prepared by means of this procedure have been shown to consist of a double membrane encapsulating a H_2O phase of about 12 nm diameter and an outer diameter of about 20 nm [12]. The vesicle solution was diluted with another 100 µl of the buffer solution mentioned above and then applied to the sample holder of the epifluorescence microscope.

The epifluorescence experimental setup is illustrated in Fig. 1a. The samples were observed while illuminated alternately for 50 µs with 600 nm light from a dye laser (SP-375 B, Spectra Physics, Mountain View, CA, loaded with the dye Rhodamine 6G) by respective power settings up to 60 mW to excite **1**–**5** and for 50 µs by 10 µW with a wavelength of 488 nm from an Ar^+ ion laser (C306, Coherent Radiation, Mountain View, CA) to excite the monitor fluorescence of **6** (Fig. 1b) using a ×100 objective (Plan-Neofluar, NA = 1.3, Zeiss, Jena, D) in an epifluorescence microscope (Axiovert 135TV, Zeiss. Jena, D). The full width of the irradiation at half maximum was measured to amount to 2.16 µm. The observed volume was set to $2.2 \times 2.2 \times 1.6\ \mu m^3$. The sample fluorescence was effectively separated from scattered light by means of a filter combination (custom made dichroic and emission filters: HQ 700/75, Chroma, Brattleborough, OR; twice BP 515–564, Zeiss, Jena, D; and OG 515, Schott, Mainz, D). Crosstalk between both wavelength channels upon simultaneous illumination was < 5%, as given by the ratio of $2 \cdot 10^4$ counts for the signal from **6** and a background noise of about 1000 ± 500 counts for red illumination at 600 nm only. The shutters (3) produced pulses of 50 ms which were split into 50 µs pulse trains by means of acousto-optic modulators (4). The sample fluorescence was collected on a liqu.-N_2 cooled slow scan charge coupled device camera system (AT200, Photometrics, Tuscon, AZ) equipped with a TK512CB chip (Tektronix) and evaluated on a PC [13]. Images with and without prior excitation of **1**–**5** at 600 nm were acquired. The latter represent the reference fluorescence of **6** only. Subsequently, the mean value from each image was determined for the central part of the image yielding the fluorescence value, F, and its respective reference F_0. Comparison of both signals permitted the calculation of the normalized signal F/F_0. For every experiment, 35 independent image pairs were acquired.

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