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The Protonation and Deprotonation Equilibria of Hypericin Revisited

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Summary. The protonation and deprotonation behaviour and the assignment of pK_a values of hypericin are reviewed and discussed. Three experiments (electrospray MS, ^{1}H NMR, acid–base indicator equilibria) provided additional evidences for the assignment of pK_a values of -5 and -6 to mono- and diprotonation at the carbonyl groups of hypericin, of $pK_a = 2$ to monodeprotonation at the *bay*-region, and of $pK_a = 11$ to dideprotonation at the *bay*- and *peri*-regions.

Keywords. Hypericin; Indicator; ES-MS; ^{1}H NMR; Acid–base equilibria.

Introduction

Although it is presently clear that hypericin (**1**) is involved in a series of protonation and deprotonation equilibria [1], there is some uncertainty and even disagreement in the literature which species belongs to which characteristic absorption spectrum, and which species prevails under certain experimental conditions, *e.g.* at different *pH* values or in solutions of certain solvents [2–21]. The aim of the present paper is to summarize the experimental and theoretical arguments accumulated so far and to present additional key results to clarify this issue.

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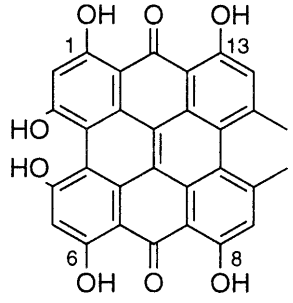

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Results and Discussion

Assignments advanced so far

Before describing new experiments to ultimately settle the question of protonation and deprotonation of hypericin it seems to be appropriate to review experiments and assignments obtained so far, and to critically discuss their pros and cons.

The very first indication that hypericin (**1**) is a rather strong acid has been reported by *Song* more than twenty years ago. He observed that **1** liberated CO_2 from a sodium bicarbonate solution [2], a routine reaction to discriminate phenols from carboxylic acids having pK_a values well below 7 [22] ($pK_a(NaHCO_3) = 6.37$ [23]). This has been later on substantiated by *Mazur* by the preparation of the sodium salt of **1** by directly reacting **1** with an equivalent of aqueous sodium bicarbonate; within this paper, the preparation of the lysine salt of **1** has been also reported [5]. More than ten years later it has been found in our group that the 'soluble form' of **1** occurring in *Hypericum* species consists mainly of its potassium salt [3].

In 1992, pK_a values of **1** and a series of analogous model compounds have been determined by *Falk*'s group yielding values in the range of -6 and in the range of 11 in 80% aqueous *DMSO*. The first value has been assigned to monoprotonation, the second one to monodeprotonation of **1** [4]. A deprotonation step at $pK_a = 11.7$ has been observed for **1** incorporated into aqueous Triton micelles and was also interpreted as monodeprotonation [7]. The 2,5,9,12-tetrabromohypericin derivative has been shown to display titrable groups characterized by pK_a values of 1.2 and 8.8, which were attributed to protonation and deprotonation equilibria [8].

A stable pyridinium salt of **1** ($\mathbf{1}^- \cdot py \cdot H^+$) has been prepared in 1993; its structure was determined by X-ray crystallography [6]. Thereby, the deprotonation site has been unequivocally assigned to the *bay* region (positions 3/4). Moreover, because the pK_a of pyridine amounts to 5.4 [23], a stable salt would only be obtainable if the pK_a of the corresponding acid were equal to or lower than this value. This result has been confirmed and refined later on with a different crystal form of this salt ($\mathbf{1}^- \cdot py \cdot H^+ \cdot py \cdot H_2O$) [9]. In addition, pK_a values for the mono- and dideprotonation steps using 80% ethanol have been measured to amount to 1.7 and 12.5 [9].

Taken together, the results achieved up to this time indicated that in addition to an acidic group characterized by a pK_a value of about 11 there ought to be another much more acidic group present in the molecule. For the latter, one of the *bay*-hydroxyl groups is a proper candidate. This has been complemented by spectrophotometrically titrating fringelite D (**2**), which displays two pairs of *bay*-hydroxyl groups. A two-step titrable system has been observed in this case [9, 11], as well as in the case of stentorin and isostentorin [11], instead of the one-step system in the same region of **1** ($pK_a \approx 2$) [10]. Conductivity measurements also revealed that **2** is dissociated in pure *DMSO* depending on its concentration and that this dissociation is characterized by a mean pK_a value of 3.1, nicely corroborating the spectrophotometric titration experiment. Accordingly, the step in the range of $pK_a = 2$ had to be assigned to monodeprotonation, whereas the step in the region of $pK_a = 11$ had to be reassigned to dideprotonation.

With regard to the question of the protonation of **1**, its parent system, phenanthro[1,10,9,8-*opqra*]perylene quinone, has been investigated [12]. It has been found that it is only protonated at pK_a values as low as -6 and -7, which needs sulfuric acid of at least 70% concentration. Thus, the step in the range of $pK_a = 2$ advanced above could not be attributed to a protonation of the system, but is indeed a deprotonation step. This also made sense in the case of the tetrabromo derivative of **1** mentioned above [8]. In this derivative, the two steps have been found to be shifted to lower pK_a values than those of its parent **1**, which can be attributed to the electron withdrawing property of the bromine substituents.

When titrating **1** incorporated into Brij 35 micelles, *Jardon*'s group found that in addition to the step at $pK_a = 11$ there is also a sigmoid effect observed in the absorption spectra in the range of *pH* 7 [13]. This signal is rather small as compared to the changes within the other buffer ranges. The authors assigned the step at $pH = 7$ to monodeprotonation and the step at $pH = 11$ to dideprotonation. The same conclusions have been drawn from an electrochemical study [15]. However, one should attribute this spurious signal at *pH* 7 to the spectroscopic changes induced by the salt effect, which is experienced by the micelle upon running through the titration of the buffer system. This was inferred from the fact that we could reproduce this experiment, but observed that upon using a weak acid, like acetic acid, for this titration instead of hydrochloric acid, the signal was shifted to about *pH* 5.5 and is therefore characteristic for the buffer system [24]. Accordingly, this obscure 'pK_a value' around 7 should be considered as an artifact.

A water soluble ω,ω'-polyethylene glycol derivative of **1** allowed to determine its pK_a values in pure aqueous solution in 1997 [14]. Aqueous pK_a values for **1** could be extrapolated from a series of aqueous ethanol solutions to -6, 1.8, and 9.2. These values have been assigned to monoprotonation, monodeprotonation, and dideprotonation steps by means of a paper electrophoresis experiment using 90% *DMSO*: between *pH* 2 and 12, the spot of **1** migrated towards the anode, thus corroborating its anionic nature [14].

A more recent confirmation for these assignments has come from an atmospheric pressure electrospray mass spectrometric experiment in which the molecular masses of the anions of **1** were observed in the negative mode depending on the *pH* of the injected solution [16]. At *pH* values up to 3 no negatively charged ions could be detected. Between *pH* 4 and 10.5, the single charged phenolate ion of **1** has been observed, which gradually disappeared at *pH* values up to 13. Concomitantly, the double charged diphenolate ion of **1** appeared in this *pH* region. Further confirmation of these assignments has been achieved by selectively alkylating the hydroxyl groups of **1** [17]. Thus, upon peralkylation of **1** only protonation at pK_a about -2 could be observed, whereas the *peri*-tetraalkylated product displayed a monodeprotonation step at $pK_a = 3$ and the *bay*-dialkylated product was devoid of a deprotonation step in the range of 2–3. This result unequivocally has proven that one of the *bay*-hydroxyl groups displays a very pronounced acidity which was shown to be due to its nature as a vinylogous carboxylic acid; the enhanced stabilization of its phenolate ion results from an exceptionally strong intramolecular hydrogen bond to the remaining *bay*-hydroxyl group. Another strong argument in this direction stems from the observation that in isohypericin (where in both *bay*-regions the hydroxyl group is opposed to the

methyl group in contrast to **1** where the two hydroxyl groups and the two methyl groups are facing each other) the monodeprotonation pK_a is shifted to about 7 [25]. Additional arguments have been achieved by AM1 calculations, which showed that the phenolate $^{(3-)}\mathbf{1}$ is strongly stabilized relative to the corresponding phenolates $^{(1-)}\mathbf{1}$ and $^{(8-)}\mathbf{1}$ [18], and from an ^{1}H NMR investigation of the Li, Na, K, Rb, and Cs salts of **1** [19], which consistently displayed one *bay*-hydroxyl group signal at about 18.5 ppm.

Contrary to these findings and arguments, *Petrich*'s group has assigned the pK_a steps at 1.5 and 12.5 observed for **1** contained in inverse micelles to protonation and monodeprotonation, respectively [21]. The group of *Jardon*, giving recently a short review of the various results, seemed also not very convinced about the nature of the species present between the observed dissociation steps [20].

Additional arguments

To further clarify the assignments reached so far, three additional experiments were designed. First, the mass spectrometric results discussed above [16] were complemented by a search of ions in the positive ion mode. Thus, **1** was dissolved in a *pH* series of 80% ethanol buffers using formic acid or trifluoroacetic acid, and the atmospheric pressure electrospray generated ions in the positive as well as in the negative ion mode were recorded. In these experiments, exclusively the mass of the monodeprotonated phenolate ion $(\mathbf{1}-\mathrm{H})^-$ could be detected above $pH = 2$ in the negative ion mode, whereas in the positive ion mode no ion corresponding to $(\mathbf{1}+\mathrm{H})^+$ could be observed in the full range down to *pH* 1. This experiment unequivocally confirmed the nature of the pK_a around 2, which has to be attributed to monodeprotonation and not to a protonation of **1** as inferred earlier [13, 21].

Second, it could be clarified that **1** is present as the dissociated ion $^{(3-)}\mathbf{1}$ in polar solvents. Thus, we now were able to observe a *bay*-hydroxyl signal at 18.4 ppm in the ^{1}H NMR spectrum of highly purified **1** dissolved in *DMSO*-d_6 which integrated for one proton only, as compared to the double intensity of the respective *peri*-hydroxyl groups at 14.2 and 14.8 ppm. Accordingly, all absorption spectra of **1** recorded so far in polar solvents are characteristic of $^{(3-)}\mathbf{1}$ and not of undissociated **1**. It might be mentioned that only one proton at about 18 ppm has been observed recently for the *bay*-hydroxyl region of an ω-substituted hypericin derivative [26].

Third, we investigated mutual protonation and deprotonation of **1** or its sodium salt with suitable indicator acids or salts. By measuring the absorption spectra of separated and mixed solutions of **1** or $\mathrm{Na}^+ \cdot {}^{(3-)}\mathbf{1}$ and an indicator or an indicator salt it could be shown that under proper relations of the pK_a values **1** could protonate an indicator salt; *vice versa*, $^{(3-)}\mathbf{1}$ could be protonated by an indicator acid. Thus, according to the equation $\mathbf{1} + \mathrm{Na}^+\mathrm{I}^- = \mathrm{Na}^+\mathbf{1}^- + \mathrm{I}$, **1** dissolved in acetone at concentrations of 10^{-3} to $10^{-5}\,\mathrm{mol}\cdot\mathrm{dm}^{-3}$ protonated the sodium salts (10^{-3} to $10^{-5}\,\mathrm{mol}\cdot\mathrm{dm}^{-3}$) of methyl red ($pK_a \approx 5.0$), congo red ($pK_a \approx 4.1$), and methyl orange ($pK_a \approx 3.4$), whereas the sodium salt of eosin G ($pK_a \approx 1.5$) was protonated only at higher concentrations of **1** and those of cresol red or malachite green ($pK_a \approx 1.0$) did not even respond. In addition, it was observed that the bright fluorescence of the sodium salt of eosin G vanished gradually upon addition of more than equimolar amounts of **1**. Of course, the inverse behaviour was observed using

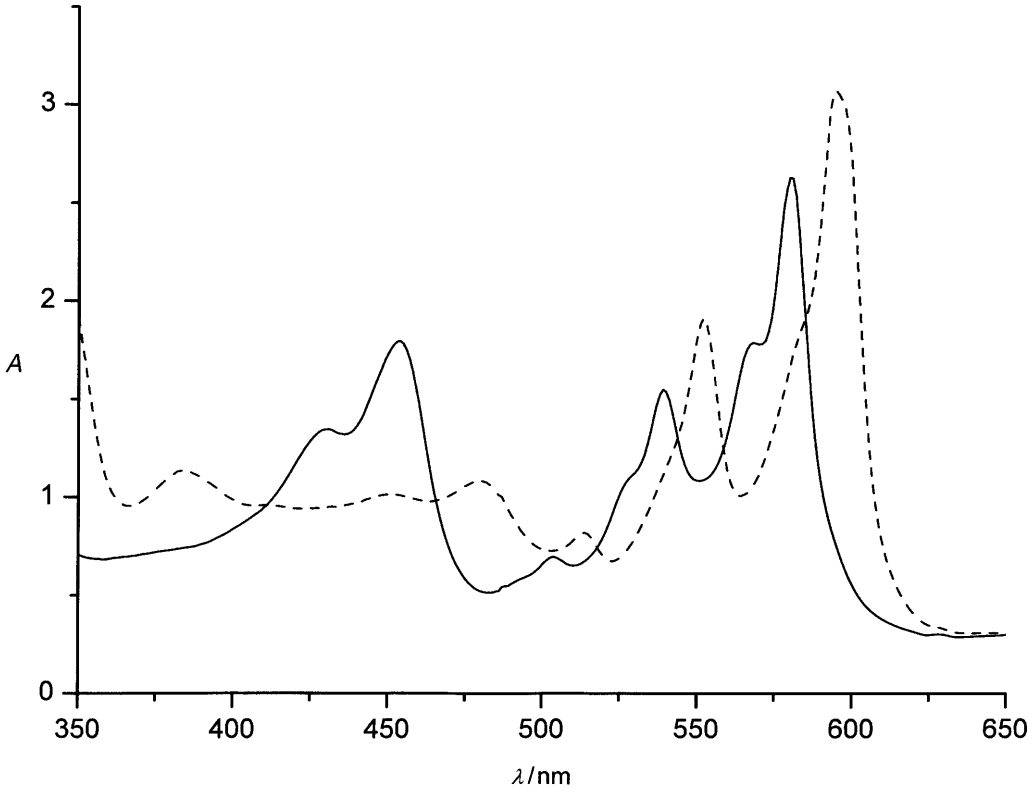

Fig. 1. Absorption spectra of **1** (—) and $^{(-3)}\mathbf{1}\cdot Na^+$ (- - -) in acetone solution at $10^{-4}\,mol\cdot dm^{-5}$

the sodium salt of **1** ($^{(3-)}\mathbf{1}\cdot Na^+$) and the corresponding indicator acids in such experiments. The absorption spectra of the neutral and the deprotonated species of **1** as shown in Fig. 1 correspond to the spectra observed in other solvents. It should be mentioned that this kind of experiment worked only with acetone as the solvent because in protic and aprotic strongly polar solvents the protonation–deprotonation equilibria involving the solvents were dominant. In aprotic apolar solvents, the solubility of the various compounds was too low to allow for reliable results.

According to these observations, **1** displays a monodeprotonation step in the range of the indicator pK_a values of 3.4 to 1.5 in acetone solution, but rather next to the latter value, which is in agreement with all other experimental evidence obtained so far.

In conclusion, we cannot find any reason why one should doubt that hypericin (**1**) is a very strong organic acid, displaying its first deprotonation step around $pK_a = 2$ and the second one in the range of 11. Protonation of **1** to yield $\mathbf{1}\cdot H^+$ and eventually $\mathbf{1}\cdot 2H^+$ with its carbonyl groups as the acceptors affords a strong acid like sulfuric acid of very high concentrations characterized by H_0 values of -6 and -7.

Experimental

Hypericin (**1**) was prepared and purified as described [27, 28]. In addition, it was purified by chromatography on a Sephadex LH20 column using absolute MeOH as the solvent. Its sodium salt

was prepared according to Ref. [28]. The indicators mentioned were of commercial origin. Their absorption spectra in the neutral or deprotonated forms were checked using *TFA* or NaOH.

Solvents were of *p.a.* quality. ^{1}H NMR, UV/Vis, fluorescence, and mass spectra were recorded using Bruker DPX 200, Hewlett Packard 8453 UV/Vis, Hitachi 4010F, and Hewlett Packard 59987 quadrupole instruments.

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