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Concerning the Dissociation of Hypericin: An Atmospheric Pressure Ionization Mass Spectrometric Study

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Summary. The dissociation of hypericin (**1**) was investigated using atmospheric pressure ionization mass spectrometry of 80% ethanolic solutions under variation of their *pH* values. Thus, it could be unequivocally established that the species predominant within the *pH* region of 3–10 and 11–13 are the mono- and dideprotonated compounds $\mathbf{1}^{(-)}$ and $\mathbf{1}^{(2-)}$. These results nicely corroborate the hitherto accumulated mostly spectrophotometric evidence for the first deprotonation step occurring at a pK_a value in the order of 2 and the second one at about 12. Moreover, this example demonstrates the potential of atmospheric pressure ionization mass spectrometry as a valuable tool to investigate dissociation equilibria.

Keywords. Deprotonation; Hypericin; Atmospheric pressure ionization mass spectrometry; pK_a values.

Zur Dissoziation des Hypericins: Eine massenspektrometrische Untersuchung mit Atmosphärendruck-Ionisierung

Zusammenfassung. Die Dissoziation von Hypericin (**1**) wurde mit Hilfe der Elektrospray-Massenspektrometrie von Lösungen in 80% Ethanol unter Variation des *pH* Wertes untersucht. Damit konnte eindeutig abgeleitet werden, daß die vorherrschenden Spezies innerhalb der Bereiche von *pH* 3–10 und 11–13 mono- und dideprotoniert sind. Dieses Ergebnis bestätigt die bislang erzielte vornehmlich spektrophotometrische Evidenz für einen ersten Deprotonierungsschritt im pK_a-Bereich von 2 und eines zweiten bei etwa 12. Darüber hinaus demonstriert dieses Beispiel das Potential der Atmosphärendruckionisations-Massenspektrometrie für die Untersuchung von Dissoziationsgleichgewichten.

Introduction

The natural pigment hypericin (**1**), contained mostly in *Hypericum* species [1], displays a variety of physiological effects [1, 2]. Several of them seem to depend critically upon its unique acid-base properties [3]. Since the protonation-deprotonation behavior of **1** is thought to be of fundamental importance for its

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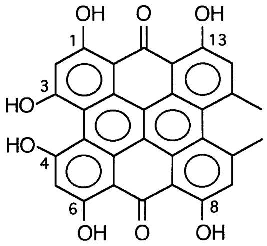
physiological effects, there is a need to characterize the various species present in aqueous systems.



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Investigations have been undertaken to derive pK_a values of **1** by means of ^1H NMR and UV/Vis spectroscopy and electrophoresis, especially with respect to its physiologically meaningful deprotonation steps [4, 5]. However, such studies have been severely limited by the notorious solubility problems encountered with **1**. Remedies have been sought by using certain derivatives, like ω,ω'-oligoethyleneglycol conjugates, for such measurements [5]. However, the results of these studies as shown in Scheme 1 for water as the solvent (*bay* denotes the 3/4- and *peri* the 1/6/8/13-positions), mainly with regard to the species involved, have not yet been generally accepted. Therefore, any additional methodologically independent experimental evidence brought about, could be of value.

$$\mathbf{1}{=}OH^+ \xrightleftharpoons{pK_a = -6} \mathbf{1} \xrightleftharpoons{pK_a = 1.8} \mathbf{1}_{bay}\text{–}O^- \xrightleftharpoons{pK_a = 9.2} \mathbf{1}_{bay\text{–}O^-,\,peri}\text{–}O^-$$

Scheme 1

Ionization in the course of atmospheric pressure ionization mass spectrometry forms intact, low internal energy, even-electron molecular ions by protonation or deprotonation of neutral molecules having acidic and/or basic functional groups, depending also on the acid or base properties of the medium [6]. Thus, it could be envisaged that upon spraying **1** from solvents of different *pH* values, molecular ions would result that are characteristic for their state of dissociation in the bulk medium. This report will describe such experiments.

Results and Discussion

Recording the atmospheric pressure ionization mass spectrum of **1** dissolved in an 80% ethanol water mixture ($pH \approx 5$) yielded a spectrum with essentially the molecular ion peak of the monodeprotonated system $\mathbf{1}^{(-)}$ at $m/z = 503$ ($M_{1\text{calc.}} = 504$). Upon lowering the *pH* value of the solution this ion persisted down to about *pH* 3. From *pH* 3.5 downwards, **1** started to precipitate, and no ions involving **1** but exclusively ions characteristic of the acid used for acidification (HCl, H_3PO_4) were observed, *e.g.* $3H_3O^+ \cdot 3Cl^-$, $m/z = 162$; $3H_3O^+ \cdot 3Cl^- \cdot HCl$, $m/z = 198$; $H_3PO_4 \cdot H_2PO_4^-$, $m/z = 195$; $2H_3PO_4 \cdot H_2PO_4{}^-$, $m/z = 293$; $3H_3PO_4 \cdot H_2PO_4^-$, $m/z = 391$; $4H_3PO_4 \cdot H_2PO_4^-$, $m/z = 489$; $5H_3PO_4 \cdot H_2PO_4^-$, $m/z = 587$. The relative abundance of the $\mathbf{1}^{(-)}$ ion varied with the solution's *pH* value as illustrated in Fig. 1. This behavior is consistent with a pK_a value for the first deprotonation step of 1.7 of **1** dissolved in 80% ethanol [7] and of 1.5 in 80%

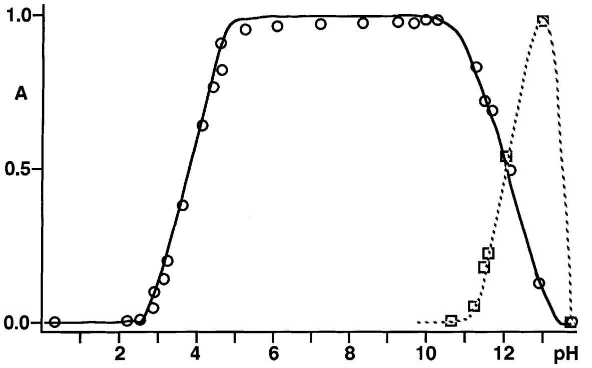

Fig. 1. Atmospheric pressure ionization mass spectrometric relative abundances (normalized to their maximum peak intensity) of the hypericinate ions $\mathbf{1}^{(-)}$ ($m/z = 503$;—) and $\mathbf{1}^{(2-)}$ ($m/z = 251$; · · · · ·) in dependence of the bulk *pH* values of a solution of **1** in 80% ethanol

DMSO/water [8], provided that the extremely low solubility of **1** in these acid systems is properly taken into account. Accordingly, rising precipitation in this *pH* range would lead (from an elaboration of Fig. 1) to an apparent pK_a value of about 4. This phenomenon of substantially higher apparent pK_a values is commonly observed for dissociation equilibria coupled to solubility equilibria, like in the case of the bilirubin pK_a values [9].

Of course, one has to be also aware of a recent study which showed that there could be a difference between the acidity of the bulk solution and the acidity within the atmospheric pressure ionization droplets [10]. However, this effect has been found to be large for neutral solutions, with an 10^3–10^4 fold increase in acidity, but it has been found to be more or less negligible for already acidic solutions [10]. Thus, this effect could also contribute to the higher apparent pK_a value of **1**, but the main reason for the difference between apparent and spectrophotometric pK_a values has to be blamed to the insolubility of **1**. Nevertheless, the present atmospheric pressure ionization mass spectrometric experiment proved nicely, and beyond any doubt, that the species present in the *pH* range above 3 was the mono-deprotonated hypericin $\mathbf{1}^{(-)}$. With respect to the position of deprotonation, a variety of experiments have shown it to be located at the *bay* region *i.e.* at position 3 [4, 5].

Upon increasing the *pH* value of the solution of **1** the relative abundance of the sole ion at $m/z = 503$ kept its intensity at a plateau value and then started to decrease at a *pH* value of about 10 to vanish around *pH* 14. Within this latter range a new ion was observed which was characterized by $m/z = 251$ (cf. Fig. 1). Its abundance peaked at about *pH* 13, and it had to be attributed ($m/z = 502/2 = 251$) to the doubly charged dideprotonated species $\mathbf{1}^{(2-)}$. The corresponding apparent pK_a value of about 12 as deduced from Fig. 1 was in accordance with the spectrophotometric titration values of 12.5 and 13 observed for 80% ethanol and 80% dimethylsulfoxide solutions [7, 8]. The species pre-dominant at *pH* values in the region of 13 was thus found from the mass spectroscopic data to be the dideprotonated hypericin ion $\mathbf{1}^{(2-)}$, corroborating recent assignments [5, 8]. With respect to its structural details it has been deduced that the second deprotonation step takes place at a *peri* position [5, 8]. It should also be stressed that from the difference between apparent and spectrophotometric pK_a values there seemed to be no significant difference between the bulk and spray droplet acidities in the basic region.

It should be mentioned that in the range of decreasing abundance of the $\mathbf{1}^{(2-)}$ ion, *i.e.* at pH values of about 13–14, the trideprotonated hypericin ($\mathbf{1}^{(3-)}$) at $m/z = 167$ could not be observed with certainty, since in contrast to the acidic and slightly basic conditions, where the molecular peak dominated the spectra, basic conditions led to a variety of mass fragments. However, the sharp decrease in the $\mathbf{1}^{(2-)}$ ion abundance between *pH* 13 and 14 suggests that there is a further deprotonation step (cf. Fig. 1). This trideprotonation step has been hitherto observed only in the case of certain hypericin analogous compounds [8] to occur in the range characterized by pK_a values above 13.

Conclusions

Atmospheric pressure ionization mass spectrometry of solutions of hypericin (**1**) involving a series of *pH* values unequivocally demonstrated that the species present within the *pH* ranges of 3–10 and 11–13 are the mono- and dideprotonated ions $\mathbf{1}^{(-)}$ and $\mathbf{1}^{(2-)}$. These results nicely corroborate the hitherto accumulated evidence for the first deprotonation occurring at a pK_a value in the order of 2 and the second one at about 12 depending on the solvents used. With respect to the methodological aspect, the example of hypericin served to illustrate that atmospheric pressure ionization mass spectrometry under variation of *pH* constitutes a valuable tool to clarify the nature of species involved in dissociation equilibria.

Experimental

Hypericin (**1**) was prepared by means of the recently described semi-synthetic procedure from emodin which was extracted from the commercially available bark, *Cortex frangulae* [11].

Samples were prepared by saturating **1** in 80% ethanol water mixtures and adjusting the *pH* values by means of HCl or H_3PO_4 on the one hand, and by means of NaOH or NH_3 on the other hand. The *pH* values of the resulting samples were measured using a Metrohm-654 pH-meter equipped with a glass electrode.

Atmospheric pressure ionization mass spectrometry was performed in the negative ion mode using a quadrupole instrument (HP 5989B, Hewlett Packard, Palo Alto, CA) equipped with an Atmospheric Pressure Ionization interface (HP 59987A). Sample introduction was performed by continuous flow injection using a syringe pump (model 22, Harvard Apparatus, South Natick, MA) at a flow rate of 15 µl/min. The spraying gas (N_2 5.0) was delivered at a pressure of 60 psi, the drying gas (N_2 5.0) was delivered at a flow of approximately 7 l/min and at a temperature of 150 °C. The voltages of the interface were set to $V_{cyl} = 3350\,V$, $V_{end} = 2580\,V$, and $V_{cap} = 3350\,V$. The electron multiplier voltage was adjusted to 1795 V.

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