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Spectroscopic Investigation of the Molecular Structure of Hypericin and its Salts

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Summary. Based on the interpretation of UV/Vis, IR, and NMR spectra of hypericin as well as of its salts and the products of total O-methylation, it was concluded that hypericin transfers from solid state to low or moderate polar organic media as its 1,6-dioxo-tautomer, thereby conserving the molecular structure characteristic for the crystalline state of the material. In concentrated hypericin solutions, the 1,6-dioxo isomer converts slowly into the 7,14-dioxo isomer. Dilution of the solution or addition of pyridine *DMSO*, or other polar compounds accelerates the reaction. The metastable hypericin 1,6-dioxo-tautomer is stabilized in the solid phase and concentrated solutions by intermolecular hydrogen bonds. Salts of hypericin with alkali metals retain the structure of the 7,14-dioxo-tautomer both in solutions and crystalline state.

Keywords. Hypericin; Molecular structures; 7,14-Dioxo-isomer; 1,6-Dioxo-isomer; Tautomerization.

Spektroskopische Untersuchung der molekularen Struktur von Hypericin und seinen Salzen

Zusammenfassung. Basierend auf der Interpretation von UV/Vis-, IR- und NMR-Spektren von Hypericin und seinen Salzen sowie den Produkten aus der vollständigen O-Methylierung wird gezeigt, daß Hypericin aus dem Festzustand in Lösungsmittel niederer oder geringer Polarität in sein 1,6-Dioxotautomeres übergeht, wobei die molekulare Struktur des Festzustandes erhalten bleibt. In konzentrierten Hypericinlösungen isomerisiert das 1,6-Dioxotautomere langsam zum 7,14-Dioxotautomeren. Verdünnen solcher Lösungen oder Zusatz von Pyridin beschleunigt diesen Vorgang. Das metastabile 1,6-Dioxotautomere wird im Festzustand und in konzentrierten Lösungen durch intermolekulare Wasserstoffbrücken stabilisiert. Hypericinsalze mit Alkalimetallen weisen sowohl in Lösung als auch in kristallinem Zustand die Struktur des 7,14-Dioxotautomeren auf.

Introduction

Hypericin is an acidic photodynamic pigment which has been derived from plants of the genus *Hypericum* [1] and produced synthetically [2, 3]. Recent interest in hypericin has been triggered by observations of its antiviral, antiretroviral [4, 5], and photodynamic properties [6].

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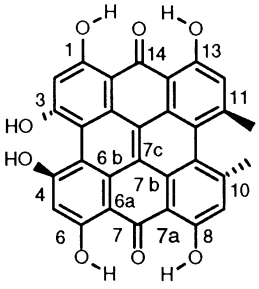
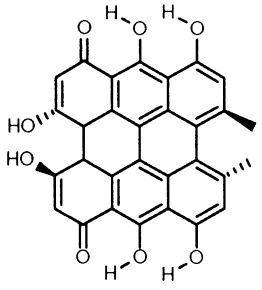
The molecular structure of hypericin is still imperfectly understood. According to a fundamental analysis [6, 7], ten tautomers of hypericin are principally possible. The 7,14-dioxo isomer is the most stable structure among all possible hypericin tautomers.



7,14-Dioxo isomer



1,6-Dioxo isomer

X-Ray analysis demonstrated that the hypericin monoanion possesses the 7,14-structure [8, 9]. However, other hypericin tautomers may be also stable owing to rather strong intermolecular forces. As illustrated in the present paper, hypericin may be present as the 7,14- or 1,6-dioxo tautomer both in solutions and in the crystalline state.

Results and Discussion

Absorption spectra of hypericin and its salts

The absorption spectrum of hypericin is solvent dependent; in *DMSO* it shows an intensive band at about 600 nm and less intensive bands at 550, 516, 481, 454, 388, and 335 nm (Fig. 1, insert). The absorption spectrum is invariant to hypericin concentration between 5×10^{-6} and $5\times10^{-3}M$, with the exception of a small (*ca.* 1.5 nm) hypsochromic shift as the hypericin concentration increases.

In the presence of HCl, the absorption spectrum of hypericin in *DMSO* differs essentially from that in neutral solution, containing bands at 587, 545, 461, 437, and 322 nm. The distinctive characteristics of the hypericin absorption spectra in acidic solution are fairly intensive bands between 400 and 500 nm (Fig. 1, insert). Similar transformations of absorption spectra occur also upon acidification of hypericin solutions in other solvents. In the following, HyH_I will denote the species having an absorption spectrum like spectrum 2 (Fig. 1, insert) and HyH_{II} the species which shows an absorption spectrum similar to spectrum 1 of Fig. 1.

In solution, the absorption spectra of hypericin salts are of the HyH_I type. The positions of their absorption bands depend only very slightly on the nature of the alkali metal. Addition of *Lewis* acids like J_2 or JCl to solutions of the hypericin salts produces a $HyH_I \rightarrow HyH_{II}$ transformation as evident from changes of the absorption spectra. The molecular compounds between hypericin salts and *Lewis* acids are sparingly soluble and precipitate from solutions containing both hypericin salt and J_2 (or JCl).

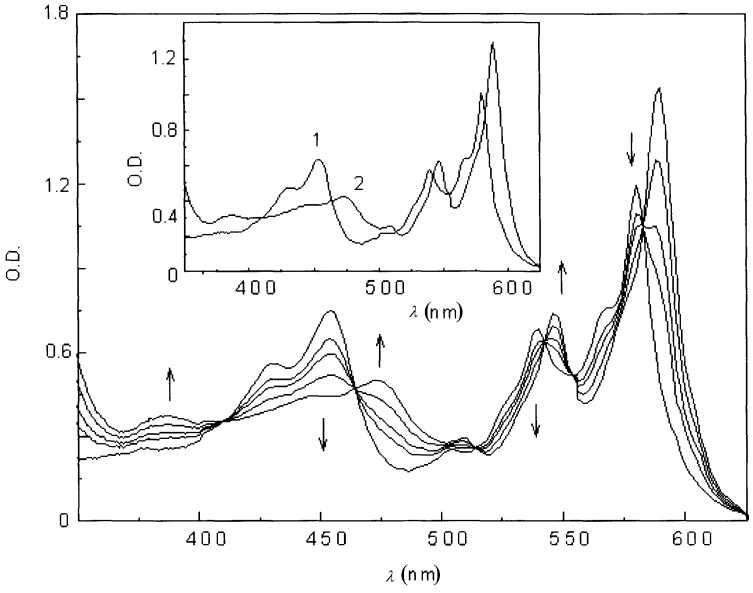
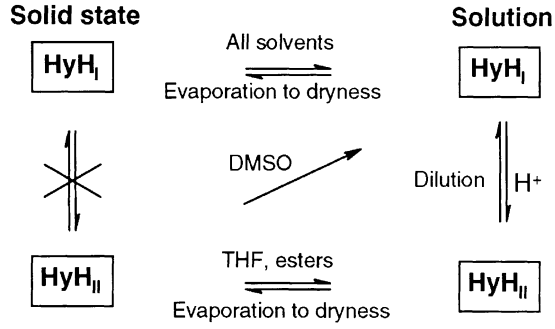


Fig. 1. Changes in the absorption spectra of a hypericin solution in butyl acetate at room temperature; spectra were recorded after 0, 4, 12, 30, and 48 h after solution preparation: insert: absorption spectra of hypericin in *DMSO* (1) and in *DMSO* containing 10% HCl (2)

Interconversions between HyH_I and HyH_{II}

Saturated solutions (about 5×10^{-3} *M*) of hypericin in neutral *THF*, esters, and acetone afford absorption spectra, similar to that of hypericin in acidic *DMSO* characteristic for $Hy\mathrm{H_{II}}$ (spectrum 1 in Fig. 1). The absorption spectrum of hypericin in *THF* or butyl acetate does practically not change in the course of a few days except for a small decrease of optical density because of precipitation of sparingly soluble material. However, the absorption spectrum of a diluted hypericin solution (*ca.* 10^{-5} *M*) is altered through time, converting into the spectrum of the $Hy\mathrm{H_I}$ form. At room temperature this reaction proceeds over a period of several days until a complete transformation of $Hy\mathrm{H_{II}}$ into $Hy\mathrm{H_I}$ is achieved. Its rate tends to increase with a rise in temperature or with dilution of the hypericin solution. Thus, at 90°C complete transformation of $Hy\mathrm{H_{II}}$ into $Hy\mathrm{H_I}$ takes about 5 hours. High dilution (up to 10^{-6} *M*) results in immediate conversion of the $Hy\mathrm{H_{II}}$ spectrum into the $Hy\mathrm{H_I}$ one. The $Hy\mathrm{H_{II}} \rightarrow Hy\mathrm{H_I}$ transformation is accelerated by addition of pyridine, *DMSO*, or water to the *THF* solution. In acetone, the described reaction proceeds even in saturated hypericin solution. Interestingly enough, filtration of a solution of hypericin in *THF* or butyl acetate through cotton or filter paper also induces the $Hy\mathrm{H_{II}} \rightarrow Hy\mathrm{H_I}$ reaction. This conversion can also be provoked by irradiation with visible light.

Evaporation of the $Hy\mathrm{H_I}$ solution derived from the above conversions to dryness results in a dark-gray sparingly soluble material. Contrary to the source hypericin, solutions of this material, even in *THF* or butyl acetate, show only the $Hy\mathrm{H_I}$ absorption spectrum. Acidification of such solutions results in the conversion



Scheme 1

of *Hy*H$_I$ into *Hy*H$_{II}$. When acidic solutions of the dark-gray sparingly soluble material are evaporated to dryness washing of the obtained solid material with water produces hypericin which does not differ in its properties from an authentic sample.

It should be stressed that evaporation of a freshly prepared hypericin solution in *THF* (>10^{-5} *M*) to dryness or dilution of this solution with hexane results in a sample of hypericin which is also identical to the initial substance. So, according to their absorption spectra, the above two hypericin forms. *Hy*H$_I$ and *Hy*H$_{II}$, can occur both in solid material and in solution. Transformations between these two states proceed only in solutions. Thus, we failed to detect any *Hy*H$_{II}$ → *Hy*H$_I$ conversion in solid hypericin at a temperature of about 80°C for 5 hours. Most likely, the solid material prepared by the procedures reported in Refs.[2, 3], has been obtained in the *Hy*H$_{II}$ form. Mutual relations between the two hypericin forms are presented in Scheme 1.

IR spectra of hypericin and its monosodium salt

Hypericin and its monosodium salt diverge considerably in their IR spectra. In the region of the carbonyl and aromatic stretching vibrations, the IR spectrum of Na*Hy* in KBr pellets shows a strong band at 1592 cm^{-1} and a weaker one at 1558 cm^{-1} as well as a shoulder at *ca.* 1620 cm^{-1} (Fig. 2, a and b). In the hypericin IR spectrum, there is an intensive band at 1064 cm^{-1} and two shoulders at 1560 cm^{-1} and at *ca.* 1620 cm^{-1}. Differences between hypericin and its sodium salt are present also in other parts of the IR spectra. In the IR spectrum of Na*Hy*, as an example, the bands at 1501, 1465, 1422, 1026, and 988 cm^{-1} are missing from the IR spectrum of hypericin.

However, the distinctions between the IR spectra of hypericin and its sodium salt can be explained by different molecular structures of these compounds to only a small extent as is demonstrated with the IR spectrum of the dark-gray material obtained in the *Hy*H$_{II}$ → *Hy*H$_I$ conversion mentioned above. As may be inferred from Fig. 2c, the IR spectrum of this species is very similar (but not identical) to that of hypericin, although these two substances display different UV/Vis spectra as well as different solubilities in organic solvents.

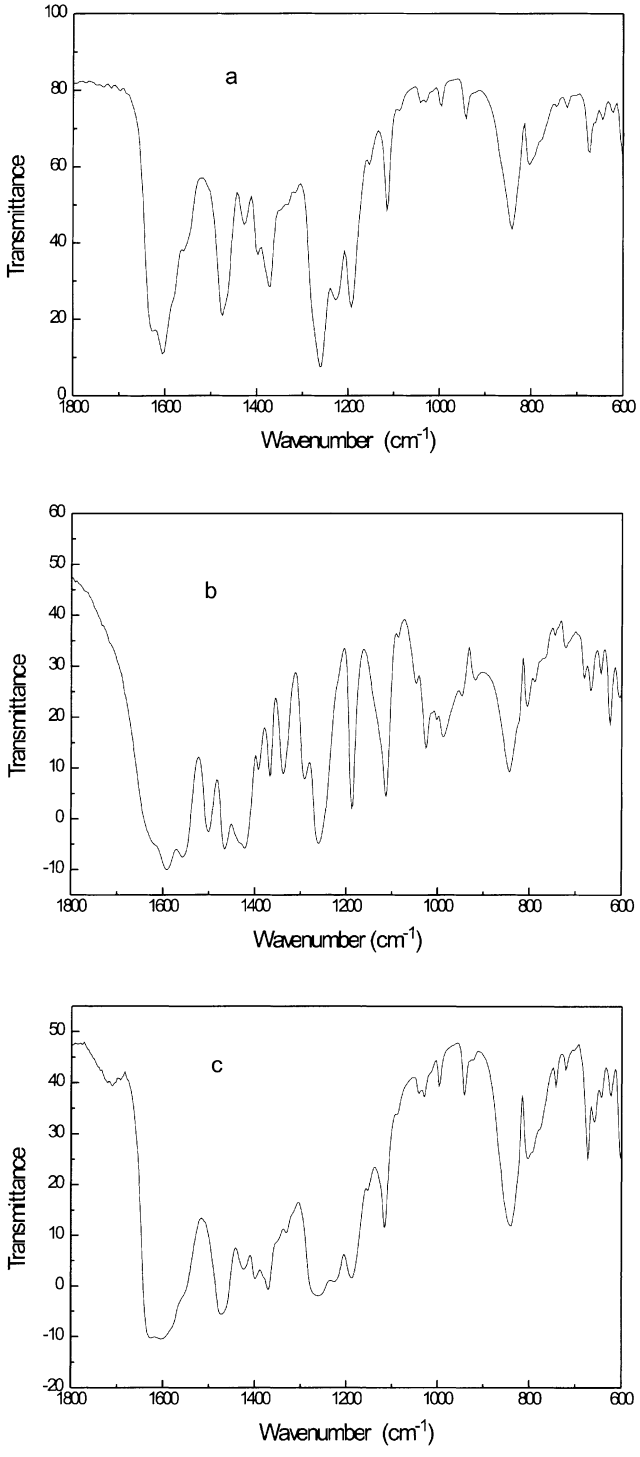


Fig. 2. IR spectra of hypericin (a), hypericin monosodium salt (b), and HyH_{II} (c) in KBr

^{1}H NMR spectra of hypericin and its salts

Following the interpretation of the NMR spectra of hypericins proposed in Refs. [8, 11, 12], the ^{1}H NMR spectra of hypericin and its salts in *DMSO*-d_6 display almost the same chemical shifts (Table 1). When hypericin is compared to its salts with alkali metals, however, an additional signal at 18.4–18.5 ppm, which was assigned to the OH proton at C(3) or C(4), is observed. Probably, this signal is not observed for hypericin because of an isotope exchange with D_2O which is usually present in deuterated solvents. According to Refs. [9, 12], protons of the *bay*-OH-groups are much more acidic as compared to hydroxyls in the *peri*-positions. In *THF*-d_8, all hypericin salts have almost identical ^{1}H NMR spectra.

Two pairs of signals were found in the area of the *peri*-hydroxyls in the ^{1}H NMR spectrum of hypericin dissolved in *THF*-d_8. For each pair, the shift difference is about 10 Hz at a frequency of 200 MHz. In these pairs, the intensity of the high field components decreases with time or as a consequence of adding water. Therefore, the described four signals are observed only in freshly prepared solutions of hypericin in dry *THF*-d_8.

Chemical shifts of hypericin salts depend only slightly on the solvent (Table 1). For the *peri*-OH groups of hypericin salts, the displacement of the signals does not exceed 0.075 ppm when going from *DMSO*-d_6 to *THF*-d_8. However, for hypericin the displacement amounts to 0.5 ppm. The solvent influence on signals of the CH and CH_3 groups is almost the same for hypericin and its salts.

For all hypericin monosalts, the signals of the *bay*-hydroxyls are narrow (half width $\leq$ 10 Hz). The line widths of the *peri*-hydroxyl protons are alkali metal dependent. However, there is no correlation between the line width and the atomic number of the cation. The line width for the sodium and rubidium salts does not exceed 10 Hz. The corresponding line widths for lithium, potassium, and cesium salts range from 20 to 40 Hz. Addition of water to solutions of Rb*Hy* or Na*Hy* in *DMSO*-d_6 does not influence the line widths. In *THF*-d_8, the signals of all studied hypericin salts were observed to be rather narrow as compared to other derivatives measured in this solvent ($\leq$ 10 Hz).

Table 1. ^{1}H NMR spectra of hypericin and its salts (δ in ppm)

	Solvent	OH (*bay*)	OH-1,6	OH-8,13	CH-9,12	CH-2,5	CH_3
*Hy*H	*DMSO*	–	14.66	14.0	7.42	6.57	2.73
*Hy*H	*THF*	–	14.17	14.12	7.29	6.75	2.66
			13.67	13.62			
Li*Hy*	*DMSO*	18.46	14.76	14.12	7.45	6.59	2.76
Na*Hy*	*DMSO*	18.47	14.75	14.11	7.48	6.61	2.77
Na*Hy*	*THF*	18.14	14.73	14.14	7.33	6.64	2.77
K*Hy*	*DMSO*	18.39	14.83	14.16	7.46	6.61	2.77
K*Hy*	*THF*	18.39	14.76	14.17	7.32	6.61	2.77
Rb*Hy*	*DMSO*	18.48	14.76	14.11	7.48	6.62	2.77
Cs*Hy*	*DMSO*	18.40	14.75	14.14	7.42	6.56	2.74
Cs*Hy*	*THF*	18.53	14.78	14.18	7.33	6.61	2.78

^{13}C NMR spectra of hypericin and its salts

Hypericin dissolved in *DMSO* displays a ^{13}C NMR spectrum identical to the ^{13}C NMR spectrum of the hypericin monosodium salt both in *DMSO*-d_6 and *THF*-d_8 (Fig. 3, Table 2). This leads to the conclusion that the ^{13}C NMR spectrum of the *Hy*H_I form depends only slightly on the solvent. However, solutions of the *Hy*H_I

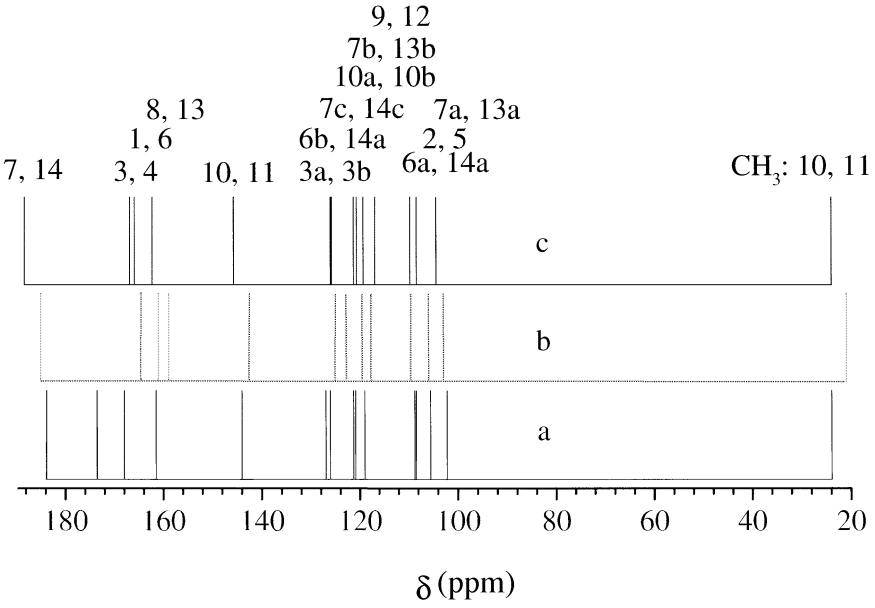


Fig. 3. Schematic ^{13}C NMR spectra of hypericin in *DMSO*-d_6 (a), *THF*-d_8 (b), and the 1,6-isomer (c); chemical shifts of the 1,6-isomer were extracted from Ref. [8]

Table 2. ^{13}C NMR spectra of hypericin and its salts (δ in ppm)

*Hy*H/*DMSO*	*Hy*H/*THF*	Na*Hy*/*DMSO*	Na*Hy*H/*THF*	Cs*Hy*/*DMSO*	Carbon atom[a]
184.9	184.9	184.4	185.6	184.2	7, 144
173.6		175.5	175.4	175.3	3,4
168	164.5	169.0	170	169	1,6
161.5	161.1	162.2	163.3	162.3	8, 13
144	142.5	144.7	144	144.5	10, 11
127	125	128.5	128.5	127.8	3a, 3b
126.1	123	127.8	127.9	127	6b, 14a
121.3		123	122.9	122.2	7c, 14c
120.9	119.4	122	122	121.6	10a, 10b
119	117.6	120.6	120.6	120.2	7b, 13b
118.4		119.5	119.5	119.7	9, 12
108.7	109.5	110	110	109.3	6a, 14a
105.5	105.8	106.8	106.8	106.5	2, 5
102.2	103	103.6	103.6	102.9	7a, 13a
23.8	21.1	24.5	24.9	24.5	10, 11

[a] From Ref. [8]

form in *DMSO*-d_6 and of the *Hy*H_{II} form in *THF*-d_8 differ essentially in their ^{13}C NMR spectra (Fig. 3). In contrast to the minor shift differences observed for the carbon signals of the C(7)–C(14) peripheral region of the hypericin molecule, those in the C(1)–C(6) peripheral region were up to 40 ppm. The differences between the hypericin solutions in *DMSO*-d_6 and *THF*-d_8 indicate that the molecular structures of *Hy*H_I and *Hy*H_{II} diverge considerably. The ^{13}C NMR spectrum of hypericin in *THF* is similar to that of the hypericin 1,6-dioxo tautomer described in Ref. [8].

Hexamethoxy derivatives of hypericin

Exhaustive O-methylation of the hypericin forms *Hy*H_I and *Hy*H_{II} with diazomethane could in principle lead to a blocking of these two forms, prohibiting further interconversion. The product of O-methylation of sodium hypericin shows absorption bands at 513 and 416 nm. The absorption spectrum of this material is almost identical with the absorption spectrum of hexamethoxyhypericin [13]. The material is soluble in chloroform and insoluble in hexane. The ^1H NMR spectrum of the O-methylated hypericin shows signals at 7.23 and 7.51 ppm which belong to protons at C(2), C(5), C(9), and C(12), and three signals (4.05, 4.15, and 4.18 ppm) of the methoxy groups. There are no signals from hydroxyls which points to complete O-methylation. The ^{13}C NMR spectrum of hexamethoxyhypericin (*DMSO*-d_6) is characterized by signals at 161.6, 141, 128, 121.8, 121.5, 115, 110.5, 97, 69.7 (OCH_3), 56.6 (OCH_3), and 56.25 (OCH_3) ppm.

In order to provide unequivocal structural proof for this product, two-dimensional NMR experiments were performed. The NOESY spectrum displayed in Fig. 4 allowed the correlation of the OCH_3 protons and hence permitted an assignment of the ^1H NMR signals of the O-methylated hypericin. From this assignment, the 7,14-dioxo structure of the compound under study is evident. The latter conclusion is based upon the fact that in the NOESY experiment the signal at 7.23 ppm of OCH_3 at C(2) and C(5) was found to correlate with the signals at 4.15 and 4.18 ppm which were assigned above to the protons of OCH_3 groups at C(1), C(6) and C(3), C(4). The signal at 7.51 ppm (protons at C(9), C(12)) correlates with the signals at 4.05 ppm (OCH_3 at C(8) and C(13)) and with 2.66 ppm (CH_3 at C(10) and C(11)). This correlation pattern is only possible and consistent with the symmetry of the 7,14-dioxo system.

As pointed out, in acidic solutions hypericin is present in the *Hy*H_{II} form. Thus, we made an effort to fix this structure using O-methylation with diazomethane for further ^1H NMR investigations of the product. O-Methylation of hypericin itself in acidic or neutral solutions proceeds much slower than that of *Hy*Na. It has been proposed occasionally (see Ref. [14] for instance) that O-methylation with diazomethane is accelerated by HBF_4. We discovered that it was most convenient to use a *THF* solution of 0.2% HCl for this purpose. However, even under this conditions the reaction results in a complex mixture of products. Within three weeks, the majority of the reaction products still contained hydroxyl groups. A substance which did not show signals of hydroxyl protons in the ^1H NMR spectrum was isolated from the reaction mixture using chromatography on sephadex LH-20. The ^1H NMR spectrum of the completely O-methylated hypericin obtained in

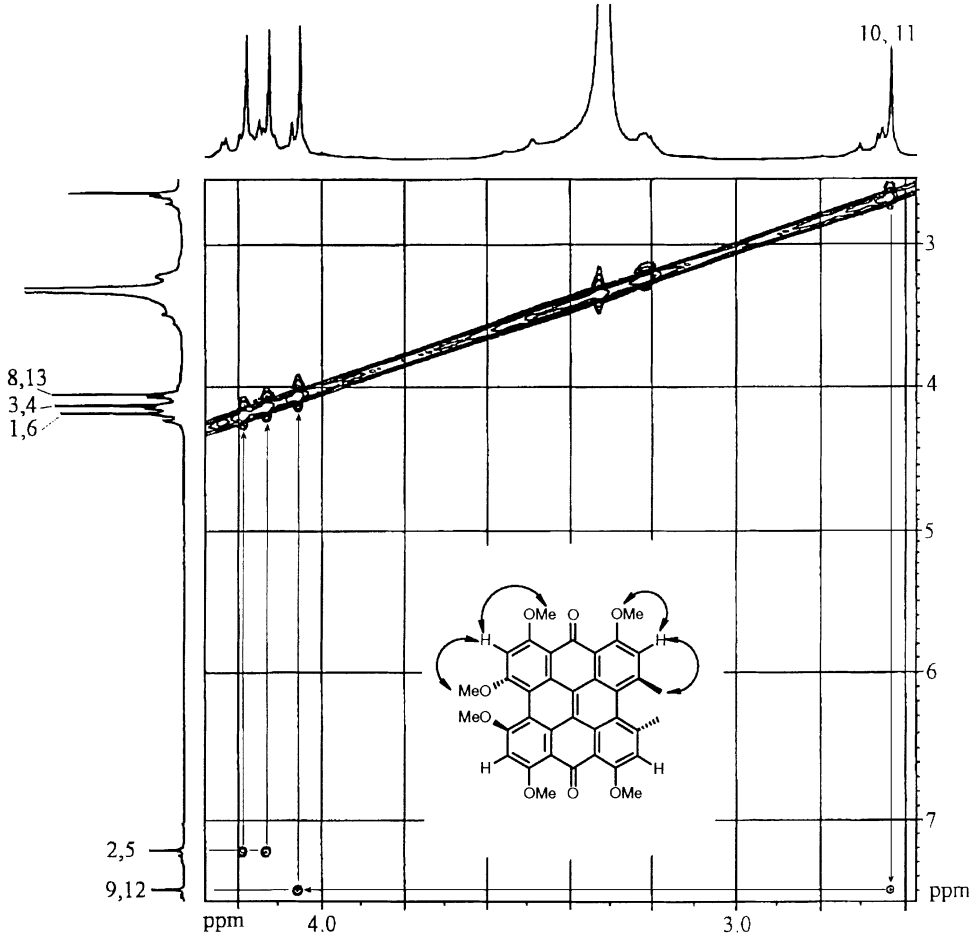


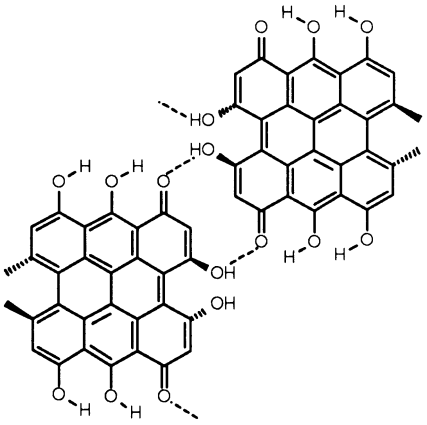
Fig. 4. NOESY spectrum of O-methylated hypericin (*DMSO*-d_6)

acidic *THF* showed eleven signals in the aromatic region (8.23, 8.23, 7.82, 7.78, 7.52, 7.3, 6.77, 6.48, 6.40, 6.38, 6,32, and 6.27 ppm). This suggests that the product comprises a mixture of isomers of hexamethoxyhypericins. Besides some O-methylated 7,14-dioxo isomer (^{1}H NMR signals at 7.52 and 7.30 ppm), this mixture probably contained at least two unsymmetric isomeric hexamethoxy-hypericins, perhaps hexamethoxy derivatives of hypericin with carbonyl groups at C(1), C(7) and C(7), C(13).

As a conclusion, results obtained from O-methylation of the hypericin forms *Hy*H_I and *Hy*H_{II} by means of diazomethane demonstrated that they exhibit different molecular structures.

Conclusions

The results obtained in the present work show unequivocally that *Hy*H_I possesses the structure of the 7,14-dioxo tautomer which has been demonstrated to be the most stable hypericin isomer [7–9, 17]. This tautomeric structure is also

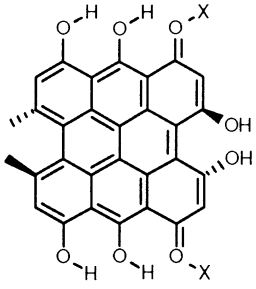


Scheme 2

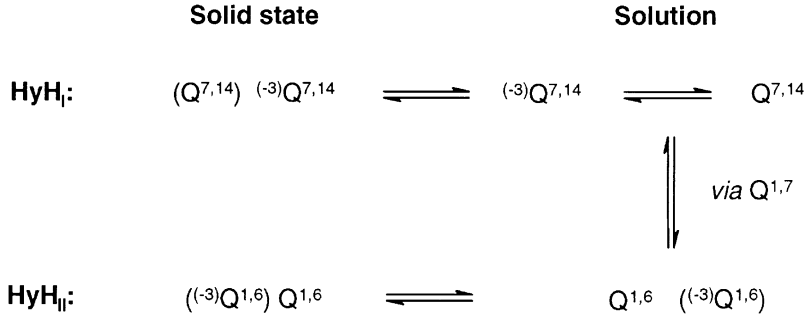
characteristic for the hypericin salts as can be seen from an X-ray analysis of the hypericin monopyridinium salt [8, 9]. This structure is most likely conserved also in solutions of the hypericin salts as corroborated by the above NOESY spectrum of the O-methylated hypericin. In polar solvents, this form will be present as the *bay* dissociated phenolate ion [12]. So, HyH_I may be thought of as the 7,14-isomer in an ionized or unionized state which is thus the most stable molecular structure for hypericin and its salts.

HyH_{II} probable has the 1,6-dioxo tautomer structure. We believe that the solid hypericin, prepared according to the commonly used procedure [3], exists as the 1,6-dioxo isomer. This structure does not change during dissolving hypericin in solvents of low polarity like *THF* or butyl acetate. The 1,6-dioxo tautomer of hypericin incorporates two highly acidic *bay*-hydroxyls [9, 12] that can be responsible for strong intermolecular hydrogen bonds in homoassociates and for the stabilization of the structure with carbonyls at positions 1 and 6 as indicated in Scheme 2. As has been recently shown by *ab initio* calculations [17], the difference between the acidities of the *bay*-hydroxyl group of the 7,14- and 1,6-tautomers is negligible. Thus, the observed stabilization of the 1,6-form may be due to the efficient intermolecular hydrogen bonding shown in Scheme 2. However, even a small difference could lead to selective precipitation upon lowering the solutions *pH* value. The homoassociates of HyH_{II} exhibit the properties of *J*-aggregates which, in contrast to the H-aggregates found for hypericin in water or water-ethanol mixtures [18], show high extinction coefficients and narrow absorption bands as well as rather high quantum yields of luminescence [16].

Hypericin salts with alkali metals possess exclusively the 7,14-structure because they do not contain an acidic group like the 1,6-dioxo isomer which is able to donate a hydrogen bridge. However, J_2, JCl, and some other *Lewis* acids like BF_3 can also initiate the isomerization of the 7,14-tautomer of hypericin salts into the 1,6-tautomer. It is well known that *Lewis* acids can form strong *EDA* complexes with carbonyl compounds (see *e.g.* Ref. [15]). The isomerization due to the formation of an *EDA* complex occurs because the carbonyls of the 1,6-isomer are more easily accessible for *Lewis* acids than those of the 7,4-dioxo isomer



Scheme 3



Scheme 4

(Scheme 3). *EDA* complexes of the 1,6-isomer should be more stable than that of the 7,14-isomer.

In solution, isomerization of hypericin occurs as a result of dissociation of homoassociates:

$$(Hy\mathrm{H_{II}})_{\mathrm{n}} \overset{K}{\rightleftharpoons} (Hy\mathrm{H_{II}})_{n-1} + Hy\mathrm{H_{II}} \xrightarrow{\text{fast}} (Hy\mathrm{H_{II}})_{n-1} + Hy\mathrm{H_{I}}$$

That is why dilution of hypericin solutions in low or moderate polar solvents accelerates the isomerization. The transformation is favoured also by addition of pyridine, *DMSO*, and other polar compounds which can solvate hypericin molecules and destroy the hypericin homoassociates. As is evident from the O-methylation experiment, the 1,7-tautomer should be an intermediate in the tautomerization path.

In conclusion, this investigation sheds light on a particular part of the complex equilibrium system of hypericin as given in Scheme 4 ($^{(-3)}Q^{7,14}$ denotes the *bay*-phenolate of the 7,14-hypericin tautomer, *etc.*).

Experimental

The NMR, IR, and UV/Vis spectra were recorded using Bruker DRX 500 and DPX 200, Biorad FT-IR 45, and Hitachi U-3210 instruments. Chemical shifts are reported in ppm relative to *TMS* as internal standard. The NOESY experiment was conducted using standard software of the Bruker company.

Hypericin and its salts

Hypericin was prepared according to Ref. [3]. For the synthesis of the hypericin salts, a solution of 50 mg purified hypericin in 30 ml of freshly distilled ethyl acetate was mixed with 20 ml of a saturated aqueous solution of lithium, sodium, potassium, rubidium, or cesium bicarbonate. The mixture was sonicated for 30 min at room temperature. The ethyl acetate solution was then separated from the aqueous phase and evaporated to about 3 ml. A precipitate was separated from the liquid by centrifugation, and the liquid phase was diluted with hexane (spectral grade). The precipitated hypericin salt was washed with a mixture of ethyl acetate/hexane (1:3) and dried at 40°C in vacuum over P_2O_5.

Pure hypericin was prepared by means of acidification (HCl) of the sodium hypericin solution in 70% aqueous ethanol, washing the precipitated material with water, and drying the product in vacuum at 50°C over P_2O_5.

O-methylation of hypericin and its sodium salt

Diazomethane was prepared from *p*-tolylsulfonyl-nitrosamine [10]. For the O-methylation, a solution of diazomethane in ethyl ether was added to a solution of hypericin (about 10^{-4} *M*) in *THF*; a 50-fold excess of diazomethane was used. The reaction was conducted at room temperature. The reaction took four days for completion in the case of the sodium salt; in acidified (*TFA*) or neutral *THF* three weeks were necessary for the O-methylation. However, according to the ^{1}H NMR spectrum of the reaction product there were still free hydroxyl groups present in the constituents of the product mixture. For the isolation of the reaction products, the *THF* solutions were evaporated to dryness, and the mixtures of the products were separated on sephadex LH-20 using methanol as eluent. The ^{1}H spectroscopic investigations were then performed directly on the evaporation residues of the appropriate fractions.

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