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On the Homo- and Heteroassociation of Hypericin#

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Summary. Hypericin exhibits rather complicated homo- and heteroassociation behavior. Whereas in common polar solvents hypericin dissolves monomolecularly up to concentrations of 10^{-3} mol/l, the presence of water in these solvents leads to homoassociation. As derived by spectroscopic measurements, these homoassociates exhibit a stacking pattern similar to the one observed for the crystalline material. Tetrahydrofuran seems to be an exception, as it is the only solvent which results in 1,6-dioxo tautomer formation. Heteroassociation of hypericin involves two distinct types of behavior. In the majority of cases, hypericin forms homoassociates which then heteroassociate with the co-solvate to yield stabilized solutions of these homoassociates. Only with human serum albumin a specific heteroassociate is formed. By means of competition experiments it could be established that hypericin is binding to the active site of the IIIA subdomain of the protein.

Keywords. Hypericin; Homoassociation; Heteroassociation; Human serum albumin complex; Stacking; Solvent effects.

Zur Homo- und Heteroassoziation von Hypericin

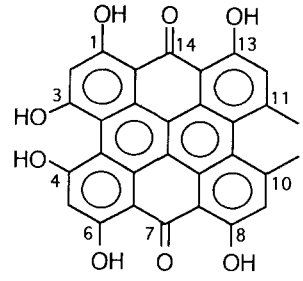
Zusammenfassung. Hypericin zeigt ein kompliziertes Homo- und Heteroassoziationsverhalten. Während Hypericin in den üblichen polaren Lösungsmitteln bis zu einer Konzentration von 10^{-3} Mol/l monomolekular löslich ist, führt die Gegenwart von Wasser zur Ausbildung von Homoassoziaten. Wie aus spektroskopischen Messungen abgeleitet wurde, zeigen diese Homoassoziate ein Stapelungsmuster ähnlich jenem des kristallinen Materials. Tetrahydrofuran scheint eine Ausnahme zu sein, da in diesem Tautomerisierung zum 1,6-Dioxotautomer führt. Für die Heteroassoziation beobachtet man zwei Verhaltenstypen. In der Mehrzahl der Fälle bildet Hypericin Homoassoziate, welche dann durch Heteroassoziation mit dem Kosolvat zu stabilen Lösungen führen. Nur mit Humanserumalbumin wird ein spezifisches Heteroassoziat gebildet. Durch Konkurrenzreaktion konnte abgeleitet werden, daß Hypericin an die aktive Stelle der IIIA-Subdomäne des Proteins bindet.

Introduction

Hypericin (**1**) is the photodynamic principle of certain plants [1], and it recently attracted considerable interest due to its antiviral properties [2]. In the course of our investigations of its chemistry and stereochemistry [3–6] it turned out that **1**

Dedicated to Prof. Dr. *Karl Schlöpl* on the occasion of his 70th birthday

has a pronounced tendency to form aggregates with itself, and also with various other compounds. This behavior of **1** also has been reported occasionally in literature [7].



The phenomenon of association between molecules ($[M_1]_n \cdot [M_2]_m$) of the same kind M_1 ($n = 2, 3, \ldots$; $m = 0$) has been termed homoassociation, whereas those between unlike molecules M_1 and M_2 ($n = 1, 2, \ldots$; $m = 1, 2, \ldots$) including the formation of solvates, has been termed heteroassociation [8]. As the physical and chemical properties of a molecule may be strongly influenced by association, a more systematic investigation of these phenomena of **1** seemed to be of interest. Therefore we will now report on our study of the homo- and heteroassociation of **1**.

Results and Discussion

Homoassociation

The two extreme situations for the homoassociation of molecules are the one of isolated molecules in the gaseous state or in solutions, and the other one of the highly ordered crystalline state. Of course, the most interesting situations are those in between these two extrema.

On dissolving **1** in nonprotic polar solvents, like dimethylsulfoxide, solutions were obtained which obeyed *Lambert–Beer's* law in the concentration range of $1 \cdot 10^{-3}$ to $1 \cdot 10^{-6}$ mol/l. They also did not change their absorption spectrum on varying the temperature between 20° and 80 °C. An estimation of the apparent molecular mass of **1** by means of differential vapor pressure osmometry at 60 °C using the same solvent at a concentration of 10^{-3} mol/l yielded a value of 400 ± 100 (calculated molecular mass 504.4) which corroborated a non-homoassociated state. Similar results were also obtained for other common polar aprotic and polar protic solvents. The band maxima of **1** shifted from the extremal solvents of methanol (long wavelength band at 589 nm) and pyridine (long wavelength band at 602 nm) within a rather small wavelength region. Corresponding shifts were observed for the fluorescence spectra of **1** in these solvents. Moreover, the fluoroscence quantum yields did not change ($\Phi_f \approx 0.2 \pm 0.03$) upon changing the solvent.

Upon gradual addition of water to a dimethylsulfoxide solution of **1** at a certain concentration this picture changed dramatically. Figure 1 shows the absorption spectra of **1** in a series of dimethylsulfoxide–water mixtures. The long wavelength band system remained rather unshifted and the intensity of the long wavelength band decreased to about one fourth of its extinction coefficient for pure dimethylsulfoxide. The spectrum in water containing only traces of dimethylsulfoxide was

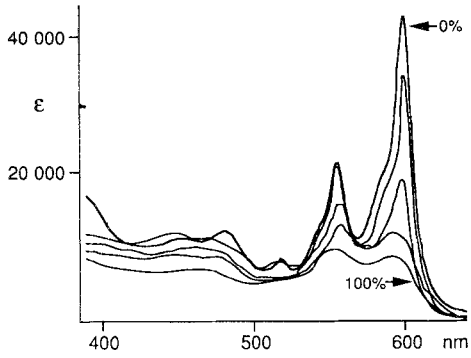


Fig. 1. Absorption spectra of **1** ($c = 1 \cdot 10^{-5}$ mol/l) in dimethylsulfoxide-water mixtures containing 0, 50, 80 and 100% water

found to be similar to the one obtained after suspending **1** in pure water by means of sonication [7]. At a certain concentration of water in dimethylsulfoxide, variation of the concentration of **1** resulted in a change in the absorption spectra which was similar to the changes observed on varying the water concentration. The same effect was found for varying the temperature in the case of a fixed water concentration and a certain concentration of **1** (Fig. 2). These results pointed to a homoassociation of **1** caused by the water content of polar aprotic and polar protic solvents.

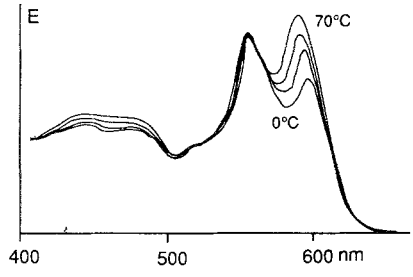


Fig. 2. Temperature dependence (0, 15, 50, 70 °C) of the absoprtion spectrum of **1** ($c = 1 \cdot 10^{-5}$ mol/l) dissolved in a dimethylsulfoxide water mixture containing 80% water

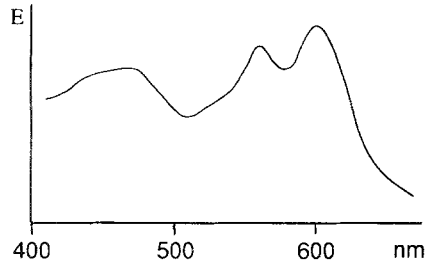


Fig. 3. Absorption spectrum of crystalline **1** dispersed in a KBr disc

To investigate the nature of these homoassociates of **1**, suited spectroscopic techniques were applied. As shown in Fig. 3, the absorption spectrum of crystalline **1** dispersed in a KBr disc was similar to the spectrum of **1** dissolved in water (Fig. 1). As the structure of **1** in the crystalline state is known [4] to involve stacking of the molecules with their mean aromatic planes (together with the crystal solvent pyridine) as shown in Fig. 4, this result indicated that homoassociation of **1** preferentially took place by stacking.

With a continous increase of the water content in dimethylsulfoxide–water mixtures, the fluorescence quantum yield of $\Phi_f = 0.2$ measured for **1** in pure dimethylsulfoxide at a concentration of $1 \cdot 10^{-5}$ mol/l diminished also continuously

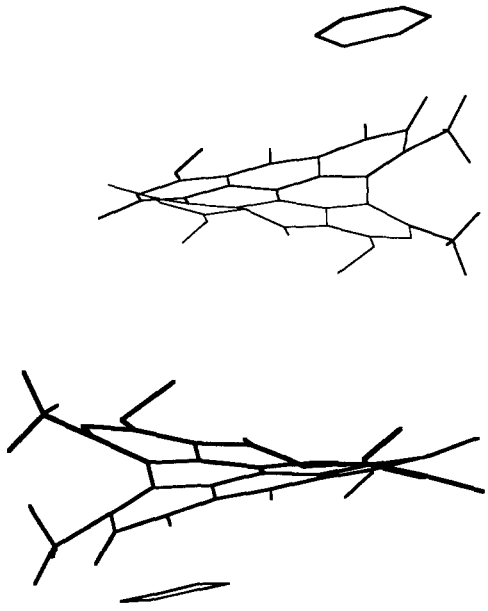


Fig. 4. Wire frame Ball & Stick model [9] of **1** (together with the crystal solvent molecules pyridine) in the crystalline state according to X-ray crystallographic results [4]

until no fluorescence at all was observed for pure water. The fluorescence life times of solutions of **1** in pure dimethylsulfoxide and in a mixture of dimethylsulfoxide and water containing 50% water were measured to decrease from 5.7 ns in the first case to 4.4 ns in the second one. However, the decay was found to be strictly monoexponential in both cases. Thus, the observed fluorescence could not simply originate from the remaining monomers of **1**. Instead, these results pointed to a fluorescence which was also derived from the associated species. Possibly, an ultra fast hyper fluorescence involving the aggregates in the completely associated state (pure water as the solvent) could not be detected by the measuring system used. This hypothesis was corroborated by the observation that the fluorescence life time distribution was narrowed due to the water content of the solvent. Again these data point to species different from monomeric **1** in pure dimethylsulfoxide.

Strictly monoexponential decays of the fluorescence of **1** with life times of 5.4 and 6.3 ns were also obtained for the solvents ethanol and tetrahydrofuran at concentrations of $1 \cdot 10^{-5}$ mol/l. As mentioned above, the fluorescence quantum yield of **1** remained within the same region of about 0.2 ± 0.03 on varying the solvents from protic ones like methanol to aprotic dipolar ones like dimethylsulfoxide. These data pointed to an excited state of **1** which was rather unperturbed by the nature of the solvent.

In the ^{1}H NMR spectra of **1**, dissolved in a series of deuterated dimethylsulfoxide deuterium oxide mixtures, more or less linear high field shifts of about 0.81 (H-9,12), 0.96 (H-2,5). and 0.75 (CH_3-10,11) ppm of the aromatic and aliphatic protons with increasing heavy water content were observed. These shifts could be explained to originate from the magnetic anisotropy of the aromatic system. Accordingly, the aromatic ring current was experienced by the protons as an increased shielding upon increased stacking. In dimethylsulfoxide solutions positive nuclear *Overhauser* effects were measured between the methyl protons in positions 10 and 11 and the aromatic protons in the neighbor positions 9 and 12. At a concentraion of 50%

heavy water in deuterated dimethylsulfoxide an additional negative nuclear *Overhauser* effect between the 10,11-methyl protons and the aromatic protons in positions 2 and 5 was recorded. On the one hand this observation pointed to an apparent molecular mass of the homoassociate of more than 2000 [10]. On the other hand the correlation between protons at opposing ring positions (2,5 ↔ 10,11) indicated an arrangement where subsequent molecules in the stack were rotated against each other by 180°.

Measurement of the spin-lattice relaxation times T_1 of the various protons of **1** in deuterated dimethylsulfoxide and in a deuterated dimethylsulfoxide solution of **1** containing 20% D_2O revealed that due to a slow (with respect to the time necessary for measuring these values) deuterium exchange of the aromatic protons only the values of the methyl protons could be compared. Their T_1 values were found to decrease from 0.57 ± 0.01 s in the first case to 0.40 ± 0.01 s in the second one, indicating a lower mobility of the molecule due to association.

Taking together the results presented above, **1** formed oligomers by stacking of molecules with their mean ring planes. This kind of stacking was obviously due to the hydrophobic effect of the aromatic core and the hydrophilic nature of the perimeter of the molecule. The molecules within the stack were rotated with respect to each other by 180 degrees. They would fit together in an energetically more favorable way if the propeller conformations [4] were of the same configuration. A Ball and Stick model of a stack of **1** built according to these evidences is provided in Fig. 5. No indications could be founed for water containing solvents of an interaction of hydroxy and oxo groups to form homoassociates.

It was interesting that the same kind of homoassociation behavior of **1** was found for the water miscible polar solvents, like the alcohols, mentioned above. However,

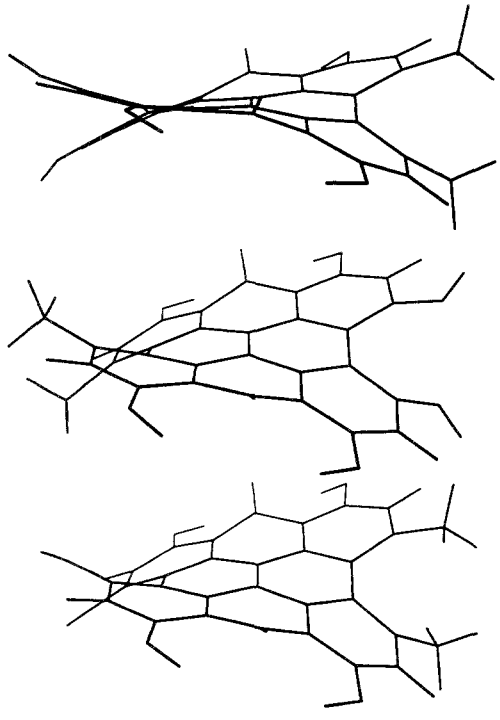


Fig. 5. Wire frame Ball & Stick model [9] of stacked **1** as deduced from experimental evidence

with tetrahydrofuran as the solvent a dramatic concentration dependence of the absorption spectrum of **1** was observed. At concentrations below $5 \cdot 10^{-6}$ mol/l an absorption spectrum which was typical for monomeric **1** was recorded. In the region above $2 \cdot 10^{-5}$ mol/l a well defined new spectroscopic species, characterized by a hypsochromic shift and isosbestic points developed with increasing concentration. It could be characterized by comparison with authentic material and its typical ^{1}H NMR spectrum as the recently described [6] 1,6-tautomer of hypericin. Thus the system tetrahydrofuran – **1** represents an interesting case of a tautomer shift which is due to concentration effects. This effect could result from homoassociation involving the hydroxy and oxo groups of **1** in the more or less hydrophobic solvent tetrahydrofuran. Besides acid catalysis [**6**], homoassociation could thus also catalyze the formation of the hypericin 1,6-tautomer.

Heteroassociation

Of course, on the borderline of heteroassociation one has to deal with specific and oriented solvation. To provide an example, benzene was gradually added to a solution of **1** in dimethylsulfoxide while monitoring the ^{1}H NMR spectra of these mixtures. As shown in Fig. 6, an aromatic solvent induced shift [11] was observed, which was largest for the aromatic protons of **1** at the more hydrophobic side (positions 9, 12) of the molecule. Obviously, benzene displays a tendency to stack with its aromatic plane to the mean molecular plane of **1**.

Another type of heteroassociation would be a donor acceptor interaction. As **1** could be envisaged as a moderate donor especially in the regions bearing the electron donating hydroxyl and methyl groups, it was titrated with the powerful electron acceptor tetracyanoethylene. However, no indication of a charge transfer band in the absorption spectra could be observed up to a hundredfold molar excess of the acceptor. It might also be noted that **1** did not react with typical *Diels–Alder* reagents like 4-phenyl-3*H*-1,2,4-triazolin-2,5-dione and with singlet oxygen.

Experiments to associate **1** with cyclodextrins did not result in a well defined system. Thus, mixing a saturated dimethylsulfoxide solution of **1** and γ-cyclodextrin dissolved in water resulted in a clear solution. Its absorption spectra displayed, however, the characteristics of stacked **1** (compare Fig. 1), and its CD spectrum exhibited only a rather small signal ($\Delta\varepsilon = -2.0$) at 549 nm. The latter observation could indicate that stacked **1** was heteroassociated to γ-cyclodextrin to yield a stable solution of **1** up to 10^{-4} mol/l. Certain neutral or anionic detergents are known [13] to solubilize **1** monomolecularly as judged from the absorption spectra of these micellar solutions, whereas cationic ones obviously tend to stabilize the homoassociate in the same way as observed for cyclodextrin.

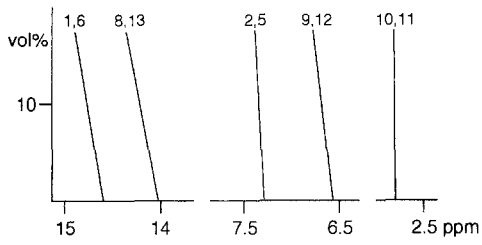


Fig. 6. ^{1}H NMR spectroscopic shifts upon addition of benzene-d_6 to a dimethylsulfoxide-d_6 solution of **1**

Salt formation of **1** with a variety of inorganic and organic bases is well documented [12] and may be envisaged as a reason of heteroassociation with high molecular weight compounds. However, from the polymeric basic systems, neither polysine, nor the lysine-rich hen egg white lysozyme, bovine basic pancreatic trypsin inhibitor, or mellitine, dissolved in water, resulted in defined heteroassociates. This was also the case with apomyoglobin or *DNA*. Instead, the absorption spectra of these solutions displayed the characteristics of stacked **1**, and moreover, their base line CD spectra gave no indication of a specific interaction. Obviously, there was a strong tendency of **1** to form homoassociates in water solutions, which were then stabilized by a heteroassociation with the respective co-solvates in a manner comparable to the case of γ-cyclodextrin mentioned above.

From literature it is known [7, 13], that **1** binds to human serum albumin with a 1:1 complex binding constant of about $7.5 \cdot 10^4 M^{-1}$, however, no information was available about its binding site. This complex has been characterized by its UV-Vis spectrum, which was comparable to the spectrum of **1** in monomolecular solution. Moreover, it displayed fluorescence (612 nm) with a quantum yield similar to its solution value. The fluorescence polarization spectrum of the hypericin human serum albumin complex was found to be similar to the spectra measured for **1** dissolved in ethanol at 77 K and **1** dissolved in glycerol at room temperature [14], indicating a rather rigid positioning of **1** within the complex. A strong chiroptical signal ($\Delta\varepsilon_{600} = +19$) was also observed for complexed **1**.

In order to acquire information on the binding site of **1** in its complex with human serum albumin, substrates of known binding site preference were used to establish the binding site by means of competition experiments. Thus, on the one hand, Diazepam, i.e., 7-chloro-1-methyl-5-phenyl-1*H*-1,4-benzodiazepine-2(3*H*)-one, is known to bind selectively to the most active binding site on the subdomain *IIIA* of human serum albumin [15]. It was found that if Diazepam was bound to the protein it completely inhibited the formation of the hypericin albumin heteroassociate. *Vice versa*, Diazepam effectively displaced **1** from its complex with human serum albumin as was monitored by CD and UV-Vis spectrometry. On the other hand, bilirubin is known to bind selectively to the active site of the subdomain *IIA* of human serum albumin [15, 16] at *pH* 7. As could be extrapolated from the result with Diazepam, **1** was indeed cleanly attached to the bilirubin serum albumin complex, and *vice versa*, bilirubin to the hypericin complex. This was indicated by CD and UV-Vis spectra, which corresponded to superpositions of the respective component spectra. The same result was obtained with Warfarin [3-(α-acetonyl-benzyl)-4-hydroxycoumarin], which is known [15] to bind selectively to the *IIA* subdomain of human serum albumin. From these results it was firmly established that **1** was selectively binding to the active site of the subdomain *IIIA* of human serum albumin. With respect to the binding of **1** to the active site of the subdomain *IIIA* one might infer from the constituents of this site (Pro 384, Leu 387, 407, 423, 426, 430, 453, 457, 460, Ile 388, Phe 395, 488, Val 415, 418, 433, 456, 473, with only Tyr 411 and Arg 410 as polar residues [15]) that hydrophobic interactions play the main part. The active site of the *IIA* subdomain is similarly structured [15], but the presence of lysin and histidin in this pocket would make salt formation more probable. The rather high chiroptical signal obtained for the complex seems to indicate the presence of a non racemic mixture of the enantiomeric propeller

conformers [4] of **1**, and not just an induction of a chiroptical signal in the racemate of **1** by means of the asymmetric environment of the protein. This partial resolution of **1** is corroborated by the time necessary (about 30 min) to develop the chiroptical signal after mixing the components. A rather high inversion barrier [4] had to be surmounted to achieve this enantiomeric enrichment. This phenomenon of partial resolution closely resembles the one observed for bilirubin bound to human serum albumin [8].

Conclusions

Hypericin (**1**) exhibited a rather rich homo- and heteroassociation behavior. Whereas in common solvents **1** was dissolved monomolecularly up to concentrations of 10^{-3} mol/l, the presence of water in these solvents lead to homoassociation. These homoassociates exhibited a stacking pattern similar to the one observed for the crystalline material. Of course, this kind of homoassociation caused dramatic changes in the spectroscopic properties of **1**. Tetrahydrofuran seemed to be an exception, as it was the only solvent which might have led to an association involving the oxygen functions of **1**. This type of homoassociation then resulted in the catalysis of a 1,6-dioxo tautomer formation of **1**. Heteroassociation of **1** produced two distinct types of behavior. In the majority of cases, **1** formed homoassociates which then associated with the co-solvate to yield stabilized solutions of these homoassociates. Only with human serum albumin a specific heteroassociate was formed which involved bonding of **1** to the subdomain *IIIA* of the protein. These results should be kept in mind using **1** as a therapeutic agent.

Experimental Part

^{1}H, ^{13}C, UV-VIS, fluorescence, fluorescence polarization, and CD spectra were recorded using the Bruker-AC-200, and WM-360, Hitachi-U-3210, F-4010, and Jobin-Yvon-Mark-V instruments. Proton spin-lattice relaxation times were derived from measurements of τ by the inversion recovery technique [17] on degassed solutions of **1** ($c = 2 \cdot 10^{-4}$ mol/l) in dimethylsulfoxide-d_6 and dimethylsulfoxide-d_6 containing 20% D_2O at 25 °C using a monoexponential least square fit procedure. Fluorescence life times were measured using the PRA System 3000 of Photochemical Res. Inc., excitation 555 nm, emission 600 nm. Hypericin (**1**) was prepared according to Ref. [5]. Its apparent molecular mass in dimethylsulfoxide solution was estimated using a Knauer vapor pressure osmometer with benzil as the mass standard. Polylysine, human serum albumin, hen egg white lysozyme, mellitine, γ-Cyclodextrin, Diazepam, Warfarin, and bilirubin were obtained from Sigma, 4-phenyl-3*H*-1,2,4-triazolin-2,5-dione and herring *DNA* were obtained from Fluka. Bovine basic pancreatic trypsin inhibitor, "Trasylor®", was a gift from Bayer. Apomyoglobin was prepared from horse myoglobin (Sigma) according to Ref. [18] and purified by gel chromatography on Sephadex G-15. The association experiments involving proteins were performed using phosphate buffer, *pH* 7, with μmolar concentrations of **1**. Tetracyanoethylene was obtained from Du Pont. The solvents used were of spectroscopic quality (Merck).

1: ^{1}H NMR (100% DMSO-d_6, $c = 2 \cdot 10^{-4}$ mol/l; δ: sodium-3-trimethylsilylpropionate-d_4; 200 MHz): 7.55 (H-9, 12), 6.68 (H-2, 5), 2.84 (CH_3-10, 11) ppm. ^{1}H NMR (90% DMSO-d_6, $c = 2 \cdot 10^{-4}$ mol/l; δ: sodium-3-trimethylsilylpropionate-d_4; 200 MHz): 7.52 (H-9, 12), 6.67 (H2, 5), 2.80 (CH_3-10, 11) ppm. ^{1}H NMR (80% DMSO-d_6, $c = 2 \cdot 14^{-4}$ mol/l; δ: sodium-3-trimethylsilylpropionate-d_4; 200 MHz): 7.44 (H-9, 12), 6.57 (H-2, 5), 2.73 (CH_3-10, 11) ppm. ^{1}H NMR (70% DMSO-d_6, $c = 2 \cdot 10^{-4}$ mol/l; δ: sodium-3-trimethylsilylpropionate-d_4; 200 MHz): 7.34 (H-9, 12), 6.44 (H-2, 5), 2.65 (CH_3-10, 11) ppm.

^{1}H NMR (60% DMSO-d_6, $c = 2 \cdot 10^{-4}$ mol/l; δ: sodium-3-trimethylsilylpropionate-d_4; 200 MHz): 7.22 (H-9, 12), 6.29 (H-2, 5), 2.53 (CH_3-10, 11) ppm. ^{1}H NMR (0% DMSO-d_6, $c = 2 \cdot 10^{-4}$ mol/l, linearly extrapolated; δ: sodium-3-trimethylsilylpropionate-d_4; 200 MHz): 6.74 (H-9, 12), 5.72 (H-2, 5), 2.09 (CH_3-10, 11) ppm. ^{1}H NMR (80% DMSO-d_6, $c = 4.5 \cdot 10^{-4}$ mol/l; δ: sodium-3-trimethylsilylpropionate-d_4; 200 MHz): 7.45 (H-9, 12), 6.59 (H-2, 5), 2.74 (CH_3-10, 11) ppm. ^{1}H NMR (80% DMSO-d_6, $c = 9.2 \cdot 10^{-4}$ mol/l; δ: sodium-3-trimethylsilylpropionate-d_4; 200 MHz): 7.40 (H-9, 12), 6.53 (H-2, 5), 2.70 (CH_3-10, 11) ppm. ^{1}H NMR (80% DMSO-d_6, $c = 1.8 \cdot 10^{-3}$ mol/l; δ: sodium-3-trimethylsilylpropionate-d_4; 200 MHz): 7.39 (H-9, 12), 6.51 (H-2, 5), 2.70 (CH_3-10, 11) ppm. ^{1}H NMR (80% DMSO-d_6, $c = 2.3 \cdot 10^{-3}$ mol/l; δ: sodium-3-trimethylsilylpropionate-d_4; 200 MHz): 7.38 (H-9, 12), 6.49 (H-2, 5), 2.69 (CH_3-10, 11) ppm.

NOE (100% DMSO-d_6): H-9, 12 $\leftarrow(+)\rightarrow$ CH_3-10, 11. NOE (50% DMSO-d_6, 50% D_2O): H-9, 12 $\leftarrow(+)\rightarrow$ CH_3-10, 11; H-2, 5 $\leftarrow(-)\rightarrow$ CH_3-10, 11.

UV-Vis (**1**; $c = 1 \cdot 10^{-5}$; methanol): 589 (43 450), 546 (20 860), 509 (7 390), 472 (12 170) nm(ε). UV-Vis (**1**; $c = 1 \cdot 10^{-5}$; ethanol): 591 (37 410), 549 (22 800), 510 (7 760), 476 (12 130) nm(ε). UV-Vis (**1**: $c = 1 \cdot 10^{-5}$; ethyl acetate): 592 (43 450), 548 (17 580), 510 (5 990), 475 (6 700) nm(ε). UV-Vis (**1**; $c = 1 \cdot 10^{-5}$; acetonitrile): 594 (39 560), 550 (18 990), 512 (6 730), 478 (9 890) nm(ε). UV-Vis (**1**; $c = 1 \cdot 10^{-5}$; acetone): 596 (51 190), 552 (23 550), 514 (8 190), 481 (11 260) nm(ε). UV-Vis (**1**; $c = 1 \cdot 10^{-5}$; dimethylformamide): 600 (46 060), 554 (20 730), 516 (6 910), 482 (10 870) nm(ε). UV-Vis (**1**; $c = 1 \cdot 10^{-5}$; dimethylsulfoxide): 600 (48 950), 555 (22 030), 516 (7 340), 482 (11 260) nm(ε). UV-Vis (**1**; $c = 1 \cdot 10^{-5}$; tetrahydrofuran): 601 (55 150), 556 (23 160), 516 (8 280), 481 (13 800) nm(ε). UV-Vis (**1**; $c = 1 \cdot 10^{-5}$; pyridine): 602 (49 410), 557 (22 230), 518 (7 410), 484 (10 870) nm(ε).

Human serum albumin complex with **1**: 30 mg human serum albumin were dissolved in 9.5 ml phosphate buffer (*pH* 7) and 0.2 mg $\mathbf{1}^-K^+$ dissolved in 0.5 ml 0.1 *N* KOH were added under vigorous stirring. After 10 min this solution was chromatographed over a Sephadex G15 column and the resulting orange colored band lyophylized to yield 24 mg of the complex.

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