Hypericin, Hypocrellin, and Model Compounds: Steady-State and Time-Resolved Fluorescence Anisotropies

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Steady-state and time-resolved fluorescence anisotropies of hypericin, hypocrellin, and five other analogues have been measured. The steady-state excitation anisotropies for each of these compounds has a broad minimum at ∼400 nm with a negative value. At the blue and red edges of the spectrum the value of the anisotropy is positive. Time-resolved fluorescence anisotropy measurements were performed for both hypericin and hypocrellin at excitation wavelengths of 300 and 570 nm. The limiting anisotropies are in excellent agreement with the corresponding steady-state values. These results are discussed in terms of the directions of the transition dipoles connecting the ground state to various excited states. The role of conformational isomers and tautomers in the ground and excited states is also considered.

Introduction

The naturally occurring polycyclic quinones, hypericin and hypocrellin (Figure 1), have gained considerable attention because of their light-induced antiviral activity, most notably against the human immunodeficiency virus, HIV.[1] In a series of publications it has been established that the main excited-state process in this class of compounds is proton transfer, the rates and behavior of which differ considerably between hypericin and hypocrellin.[2−6,13] The proton-transfer process in hypericin is independent of solvent and exhibits no isotope effect. In hypocrellin, however, the proton transfer has a strong dependence on solvent and there is an isotope effect of 1.4. Given the great structural similarity between these two molecules and their vastly different photophysical behavior, a major problem in understanding their primary photoprocesses is determining whether they can be explained in terms of a clear and unified picture. In this article we address this question by studying the steady-state and time-resolved fluorescence anisotropies of hypericin, hypocrellin, and their analogues with the goal of elucidating elements that are common to their electronic structures and perhaps suggestive of structural inhomogeneities in the ground and excited states. Jardon and Gautron[10] have already provided an initial discussion of the electronic structure of the electronic spectroscopy of hypericin and have presented a fluorescence excitation spectrum of hypericin in Brij 35 micelles, which was a stimulus for the investigation presented here. Song and co-workers[11] have also discussed aspects of the electronic spectroscopy and excited-state properties of hypericin and its analogues.

Experimental Section

Hypericin and hypocrellin (Carl Roth GmbH and Molecular Probes) were used as received. The purity was >98% as determined by supplied thin layer chromatography and NMR measurements. The analogues were prepared as described elsewhere[6a,8] and below.

Synthesis of Compounds I and IV. A solution of anthrone (500 mg, 1.75 mmol) in 15 mL of EtOH was heated to reflux.
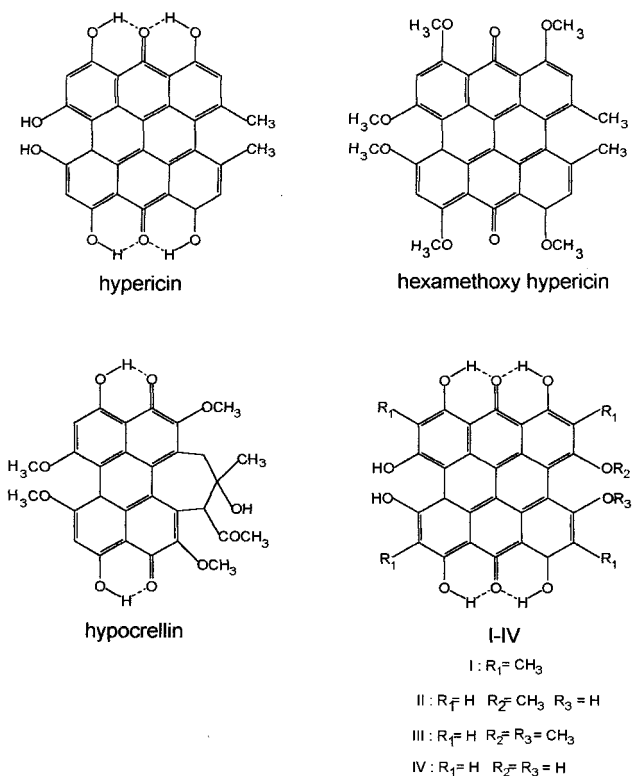

Figure 1. Two-dimensional structures of hypericin, hexamethoxy hypericin, hypocrellin, and compounds **I−IV**.

Then a solution of FeCl$_3$(H$_2$O)$_6$ (550 mg, 2 mmol) in 30 mL of EtOH was added dropwise over 10 min, and the mixture was stirred at refluxing for 1 h. The reaction mixture then was poured into 2% HCl (300 mL) and extracted with AcOEt (50 mL × 3). The AcOEt layer was washed with H$_2$O, brine, and dried over Na$_2$SO$_4$. After the solvent was removed, the residue was purified by flash chromatography eluted with hexane/AcOEt (2:1) to provide bianthraqunoyl 326 mg (81%).
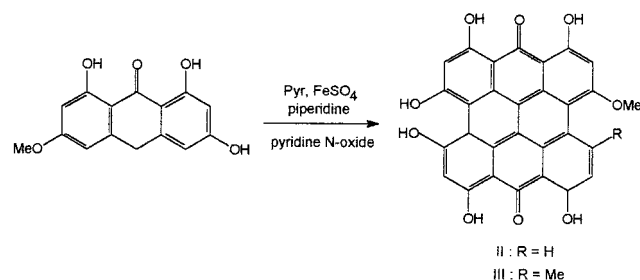
A solution of bianthraqunoyl (50 mg, 0.088 mmol) in 14 mL of AcOH/CH$_2$Cl$_2$ (1:1) was cooled to 0 °C. Then a solution of Pb(OAc)$_4$ (117 mg, 0.26 mmol) in 6 mL of AcOH/CH$_2$Cl$_2$ (1:

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SCHEME 1

I : R = Me
IV : R = H

SCHEME 2

II : R = H
III : R = Me
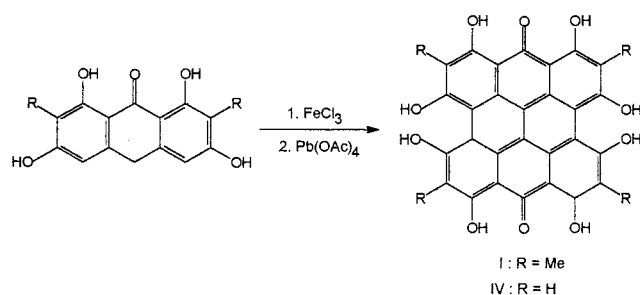
1) was added dropwise at 0 °C. The mixture was stirred at 0 °C for 2 h and was poured into 2 N HCl (100 mL). Then mixture was extracted with AcOEt (30 mL x 3), washed with H$_2$O, and dried over Na$_2$SO$_4$. After the solvent was removed under reduced pressure, the residue was purified by flash chromatography on silica gel eluted with hexane/AcOEt (2:1), AcOet/EtOH (9:1) to provide bisanthraquinone 39 mg (78%) (Scheme 1).

Synthesis of Compounds II and III. To a solution of anthrone (40 mg, 0.14 mmol) in pyridine (1 mL), piperidine (90 mg), pyridine-*N*-oxide, and FeSO$_4$ (5 mg) were added. The mixture was heated to 100 °C for 1 h and poured into 100 mL of 3 N HCl solution. The mixture was extracted with AcOEt (30 mL × 3), and the AcOEt layer was washed with H$_2$O and dried over Na$_2$SO$_4$. After the solvent was removed under reduced pressure, the residue was purified by flash chromatography on silica gel eluted with hexane/AcOEt (2:1), EtOAc, EtOAc/EtOH (7:1). The red fractions were combined to provide bisanthraquinone 25 mg (63%) (Scheme 2).

Steady-state fluorescent measurements were recorded with a Spex Fluoromax; the band-pass for excitation and emission was 2 nm. All spectra are corrected. For the steady-state anisotropy measurements, the sample is dissolved in equal amounts of ethanol and methanol to form a glass at 77 K. The samples are placed in a 5-mm diameter quartz tube, which is inserted inside a Dewar filled with liquid nitrogen. Glan–Thompson polarizers are inserted in the excitation beam used to analyze the emission. The anisotropy, *r*, is determined as follows

$$r = \frac{F_{V,V} - gF_{V,H}}{F_{V,V} + 2gF_{V,H}}$$

$$g = \frac{F_{H,V}}{F_{H,H}} \quad \text{or} \quad g = \frac{F_{V,V}}{F_{V,H}}$$

where the subscripts denote the orientation (horizontal, H, or vertical, V, to the plane of the table) of the excitation and emission polarizers, respectively. *F* is the emission intensity, and *g* is an instrumental correction factor. The value of *g* is calculated in two different ways, each of which yields anisotropy
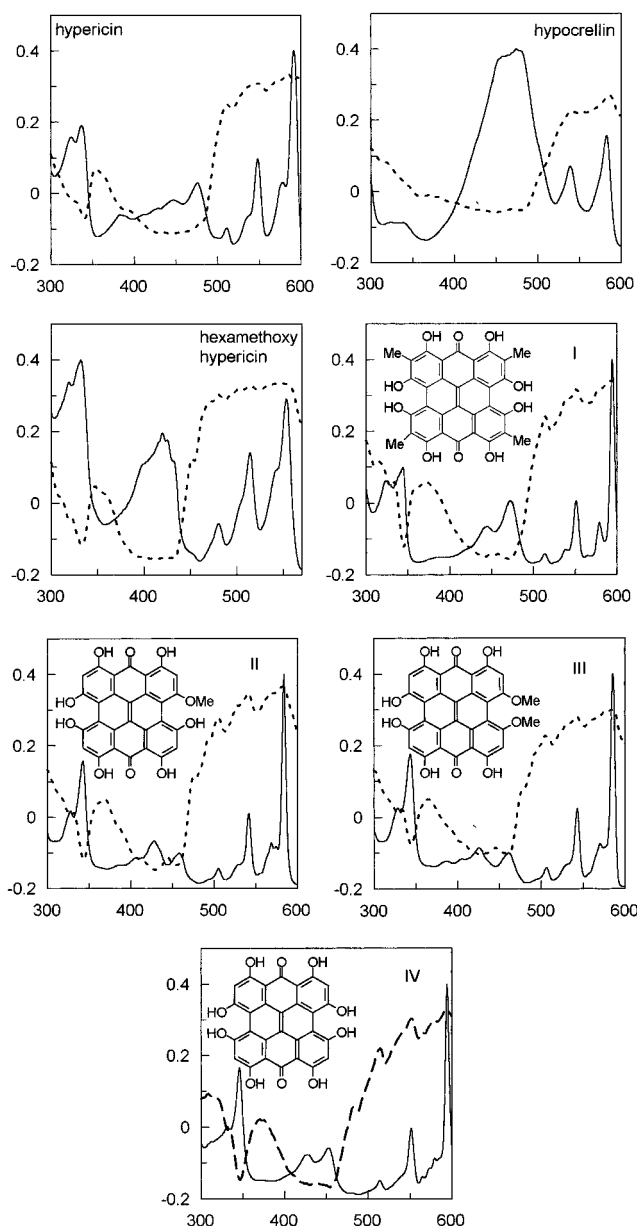

Figure 2. Fluorescence excitation spectra (—) and excitation anisotropy spectra (- - -). The left-hand ordinate gives the anisotropy values. The emission monochromator was set at 610 nm for all measurements.

spectra that are identical within experimental error. For each compound, 8−10 measurements were averaged to construct an anisotropy spectrum.

Time-resolved fluorescence anisotropies were performed using the time-correlated single-photon counting apparatus described elsewhere.[5] For each excitation wavelength a maximum of 10−12,000 counts was collected. A time base of 5.85 ps/channel and 511 channels was employed. Fluorescence was collected over the entire emission band with appropriate filters to block the excitation source. At least three measurements were made for each compound. The criteria for the goodness of fit was judged by the χ^2 values and the residuals. Only data whose χ^2 value was in the range 1.0−1.1 were regarded as acceptable.

Results and Discussion

A. Steady-State Measurements. Figure 2 presents the steady-state fluorescence excitation spectra and the fluorescence excitation anisotropy spectra for hypericin, hypocrellin, hex-
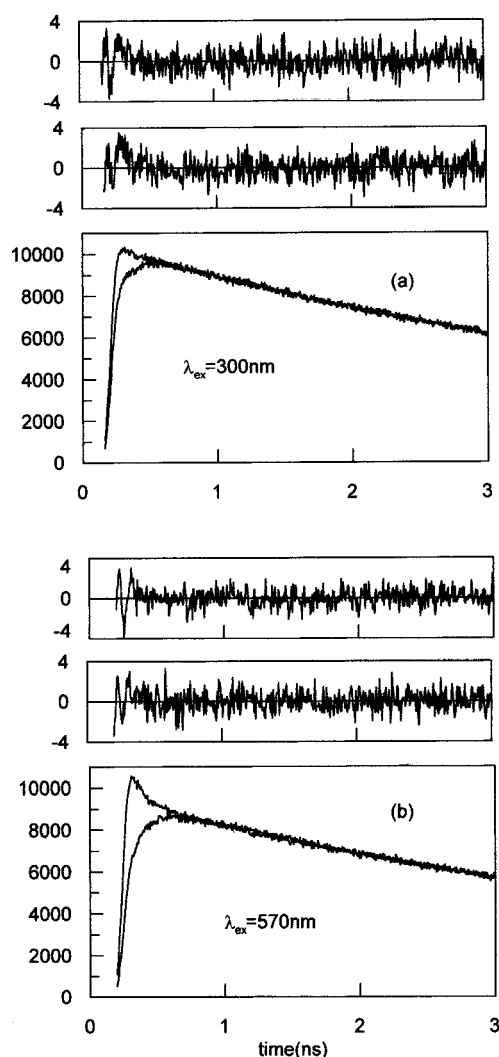
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Figure 3. Polarized fluorescence decay curves and residuals of fits for hypericin. In each panel, the upper trace represents the emission collected parallel to the excitation polarization; and the lower trace, the emission collected perpendicular. (a) $\lambda_{ex} = 300$ nm, $\lambda_{em} > 580$ nm. (b) $\lambda_{ex} = 570$ nm, $\lambda_{em} > 580$ nm.

amethoxy hypericin, and the four analogues denoted **I**−**IV**. There is no significant shift of the peak positions in the excitation spectrum in going from room temperature to 77 K for any of the compounds. The excitation spectrum for hypocrellin is different from that of hypericin and hypericin analogues, **I**−**IV**. For hypocrellin the band centered around 470 nm is the most intense. For the other analogues (aside from the hexamethoxy analogue), the most intense band is that of lowest energy.

The fluorescence excitation anisotropy spectra for all the compounds has a positive value of ~0.10−0.17 around 300 nm (the shortest wavelength at which data were collected). Depending upon the compound, it has one or two minima in the region from ~330−480 nm. At the reddest edge, ~600 nm, the anisotropy rises to a more or less constant value of ~0.32 (0.24 for hypocrellin). In short, the fluorescence excitation anisotropy is positive at the blue and red edges of the spectrum and it is negative near the center of the spectrum. The excitation anisotropy for hypocrellin is significantly different from that of hypericin, hexamethoxy hypericin, and compounds **I**−**IV**. The fluorescence excitation anisotropy spectrum for hypocrellin is broad and rather featureless. The fluorescence emission anisotropy remains unchanged over the entire fluorescence band,
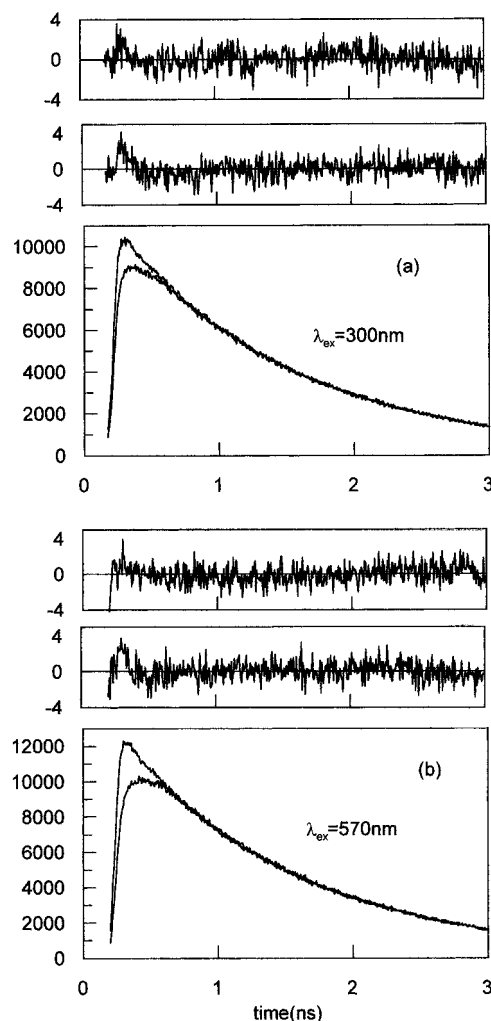
Figure 4. Polarized fluorescence decay curves and residuals of fits for hypocrellin. In each panel, the upper trace represents the emission collected parallel to the excitation polarization; and the lower trace, the emission collected perpendicular. (a) $\lambda_{ex} = 300$ nm, $\lambda_{em} > 580$ nm. (b) $\lambda_{ex} = 570$ nm, $\lambda_{em} > 580$ nm. These results are comparable to those presented for hypericin earlier.[5] In that work, however, a small component of ~7 ns in the anisotropy decay was reported. In our current study, we are unable to resolve such a component, which we now attribute to an impurity or a systematic error in the experiment. We believe that the similarity of the depolarization times with the time constant for excited-state proton transfer in hypocrellin is fortuitous.

and the excitation anisotropy is independent of the fluorescence emission wavelength (measured for hypericin and hypocrellin, data not shown).

B. Time-Resolved Measurements. For hypocrellin and hypericin the fluorescence decay is well described by a single-exponential regardless of excitation wavelength (300 or 570 nm) and regardless of the emission wavelength detected. Fluorescence lifetimes are ~1.3 and ~5.1 ns for hypocrellin and hypericin, respectively. Time-resolved anisotropies of hypericin and hypocrellin performed at two different excitation wavelengths (Figures 3 and 4) yield $r(0)$ values similar to those obtained from the steady-state measurements (Table 1), as expected.

C. Resolution of Fluorescence Excitation Anisotropy Spectra. Following the procedure of Valeur and Weber,[9] we have resolved the anisotropy spectra into two bands. The measured steady-state anisotropy, $r_0(\lambda)$, is considered to be the
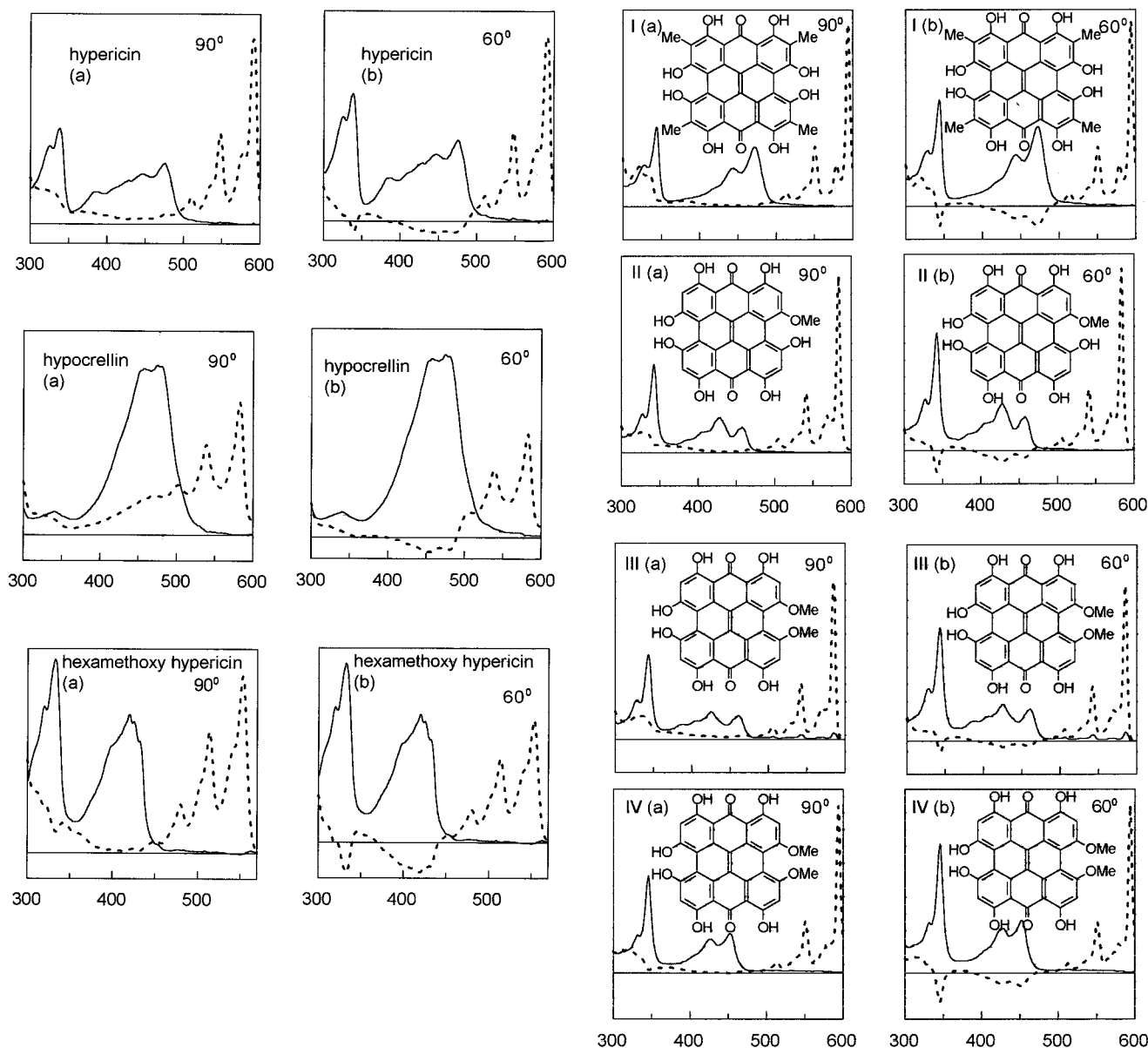
Figure 5. State-resolved excitation spectra. For hypocrellin, r_{0a} was taken as 0.24, while for hypericin and its analogues the value of 0.32 was used. (a) Resolved using an angle of 90° between the two transition dipoles. (b) Resolved using an angle of 60° between the two transition dipoles. The dashed lines (- - -) represent state *a*, and the solid lines (—) represent state *b*.

sum of the contributions of the transitions to two states referred to, for simplicity, as *a* and *b*.

$$r_0(\lambda) = f^a(\lambda)r_{0a} + f^b(\lambda)r_{0b}$$

where r_{0a} and r_{0b} are the anisotropies of each transition and $f^a(\lambda) + f^b(\lambda) = 1$. The fluorescence excitation spectra, $I_a(\lambda)$ and $I_b(\lambda)$, for the states *a* and *b*, respectively, are resolved using the following relations:

$$f^a(\lambda) = \frac{r_0(\lambda) - r_{0b}}{r_{0a} - r_{0b}}$$

$$f^b(\lambda) = \frac{r_{0a} - r_0(\lambda)}{r_{0a} - r_{0b}}$$

$$I_a(\lambda) = f^a(\lambda)\, I(\lambda)$$

$$I_b(\lambda) = f^b(\lambda)\, I(\lambda)$$

TABLE 1: Summary of Fluorescence Parameters for Hypericin and Hypocrellin

sample	λ_{ex} (nm)	steady-state $r(0)$	$r(0)^a$	τ_r (ps)^a	τ_F (ps)^b
hypericin	300	0.11	0.14 ± 0.02	93 ± 8	5132 ± 150
hypericin	570	0.32	0.35 ± 0.01	83 ± 10	5329 ± 240
hypocrellin	300	0.12	0.13 ± 0.02	100 ± 13	1297 ± 40
hypocrellin	570	0.23	0.22 ± 0.04	85 ± 8	1307 ± 30

[a] Obtained from time-correlated single-photon counting measurements. See Figures 3 and 4. [b] Fluorescence lifetime. All fluorescence decays were well described by a single exponential.

The assumptions underlying the spectral decomposition are as follows: (1) At the very reddest excitation wavelengths the steady-state anisotropy is solely due to state *a*. (2) The angle between the absorption and emission dipoles for each state *a* and *b* is assumed to be 0°. (3) The fluorescence quantum yield is independent of excitation wavelength.

Figure 5 presents the resolved excitation spectra. For the resolved spectra, the primary adjustable parameter is the angle between the transition dipoles of the two states. Acceptable

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results are those with positive intensities at all wavelengths. Our results indicate that if we assume an angle between the transition dipoles of 60° or less, the intensity for one state is negative. This implies that the angle must be between 60° and 90°. The resolved spectra have the following characteristics: (1) The spectrum of *a* has sharp vibronic features around 520−600 nm (410−570 nm for hexamethoxy hypericin) and has a weak contribution from 300 to 350 nm. For hypocrellin, however, *a* has a considerable contribution in the middle part of the spectrum. (2) The spectrum of *b* ranges from the ultraviolet to ∼500 nm. It too possesses vibronic structure, but the structure is less pronounced than in the *a* spectrum.

D. The Role of Closely Spaced Electronic States and of Ground-State Inhomogeneity. The spectral decomposition procedure used above has its origins in the investigation of indole carried out by Valuer and Weber[9a] and subsequently by others.[9b,c] In order to explain the observation that the fluorescence anisotropy of indoles varies with excitation wavelength and is never observed in the steady state to be equal to the theoretical limit of 0.40, these workers proposed and demonstrated that the overlapping transitions ($^1A \rightarrow {}^1L_a$ and $^1A \rightarrow {}^1L_b$) responsible for the indole absorption spectrum are oriented at large angles (nearly 90°) to each other. It was subsequently demonstrated by Fleming and co-workers[12] that if sufficient time resolution is employed, limiting anisotropies of 0.4 can indeed be observed. The need for subpicosecond time resolution to observe the limiting anisotropy is a result of the ^1L_a and ^1L_b states being separated by only a few hundred wavenumbers. This results in the upper state being thermally populated by the lower state and reduces the measured anisotropy.

Our results (Figures 2 and 5) indicate strikingly similar behavior for hypericin and its analogues. Insofar as hypericin has C_{2v} symmetry, its electronic transitions are polarized parallel and perpendicular to the axis containing the carbonyl groups. Jardon and Gautron[10] have discussed these aspects of hypericin spectroscopy, including polarized fluorescence in micelles. The most obvious interpretation of our results, then, is that the transition dipoles for the two lowest lying electronic transitions of hypericin and its analogues are at large angles to each other (90° if C_{2v} symmetry is assumed) and that in some cases, these two lowest lying excited states are energetically close to each other, as indicated by the low value of $r(0)$ (always <0.35) even at the reddest excitation wavelengths (e.g., hypocrellin, Figure 5).

The actual situation, however, is necessarily exceedingly more complicated. X-ray data indicate that the aromatic skeletons of hypericin and hypocrellin are twisted significantly, and that this twist angle can be as large as 30°,[7,4b,c] thus destroying the C_{2v} symmetry. Furthermore and more importantly, for hypocrellin the X-ray data and our spectroscopic data[4a] provide persuasive evidence for hypocrellin existing in the ground state, at least partially, in a tautomerized form. And one of our earliest arguments to reconcile the mirror image symmetry of the absorption and emission spectra of hypericin with an excited-state proton-transfer reaction was to postulate the presence of partially tautomerized ground-state species.[5] The existence of such tautomers is consistent with our observation of different excited-state kinetics obtained upon exciting hypericin at different wavelengths.[6] Much of the complexity in the steady-state absorption and anisotropy spectra may also be attributed to such ground-state tautomers. One must therefore regard the above decomposition of the hypericin and analogue fluorescence excitation spectra into two states as being oversimplified and qualitative.
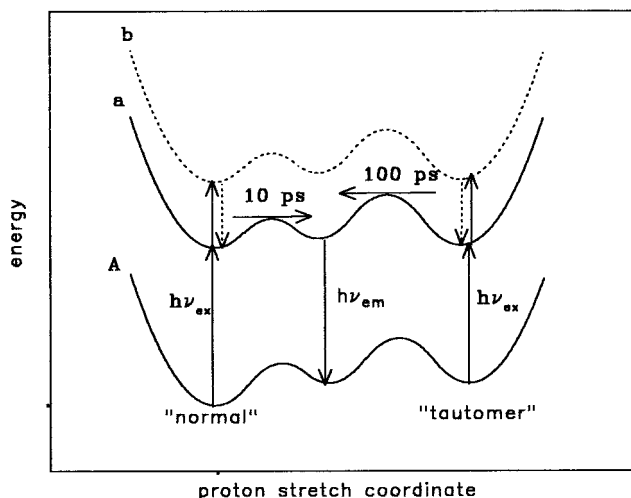

Figure 6. Proposed potential energy surfaces for hypericin, hypocrellin, and their analogues. *A* is the ground-state surface. *a* and *b* are the closely lying excited-state surfaces whose transition dipoles are polarized at large angles to each other. The left side of the upper surface represents the hypericin-like reaction;[5,6] the right side, the hypocrellin-like reaction.[4] We note that this scheme is different from that proposed elsewhere[4a] in which there was no intermediate between the N and T forms.

Conclusions

The fluorescence excitation anisotropy spectra of hypocrellin, hypericin, and five synthetic analogues of hypericin have been presented. The excitation spectrum of hexamethoxy hypericin is a hybrid of the hypericin and hypocrellin excitation spectra. Hypocrellin differs the most from the rest of the compounds studied here in both its limiting steady-state anisotropy at the reddest edge of the absorption spectrum (0.24 as opposed to ∼0.3 for the other compounds) and in the form of its absorption (or fluorescence excitation) spectrum, whose maximum, attributed to state *b*, lies at 470 nm. The hypocrellin absorption spectrum is most similar to that of hexamethoxy hypericin, whose strongest absorption transition is also attributed to state *b* (maxima at 320 and 420 nm). It is intriguing that this similarity exists for these molecules, the former, which very likely is substantially tautomerized in the ground state,[4a] and the latter, which is incapable of tautomerization.

There is also considerable similarity among the hypericin analogues **I**−**IV** and hypericin itself. **I**−**IV**, which are all structurally very symmetrical, have spectra that are comparable. However, **I** differs the most from **II**, **III**, and **IV** regarding the intensity of the 400−500 nm transition (state *b*) and is most like hypericin in this regard. Analysis of excitation spectra for each of those compounds suggests the presence of two closely lying excited states whose transition dipoles lie at an angle greater than 60° to each other.

We observe no difference in fluorescence lifetimes of hypericin or hypocrellin using excitation wavelengths of 300 and 570 nm. Also, the fluorescence quantum yield does not depend upon excitation wavelength for these compounds. This suggests that the fluorescent species in these compounds, regardless of the closely spaced excited states or the degree of inhomogeneity presented by twisted isomers or tautomers, is the same for each compound (or, what is less likely, that the lifetime and quantum yield of all the isomers or tautomers is the same).

A simplified scheme that is consistent with these and our other published data is presented in Figure 6. In this figure, hypericin or hypocrellin exists in either of two tautomers, N

("normal") or T ("tautomer") (as well as in several conformational isomers). Upon optical excitation, an ~10-ps excited-state intramolecular proton transfer that is independent of solvent occurs in hypericin.[5] In hypocrellin, a 50−250-ps excited-state intramolecular proton transfer occurs, this transfer process being strongly dependent upon solvent.[4] There is evidence suggesting, however, that for hypocrellin there is also a faster 10-ps hypericin-like process occurring in the excited state.[4a,14] This is consistent with the idea that hypocrellin can exist in both N and T forms and more generally suggests that the ground- and excited-state potential surfaces of hypericin and hypocrellin are very similar. Regardless of which state, N or T, is excited, the intramolecular proton transfer process forms a common intermediate, approached from either side of the upper surface, whose fluorescence properties are revealed in conventional time-correlated single-photon counting measurements or fluorescence quantum yield measurements. More work is underway to resolve in greater detail the excited-state photophysical processes of the biologically significant compounds.

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