Confirmation of Excited-State Proton Transfer and Ground-State Heterogeneity in Hypericin by Fluorescence Upconversion

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Abstract: Fluorescence upconversion measurements of hypericin and its methylated analog, *O*-hexamethoxyhypericin, which possesses no labile protons, confirm excited-state proton (or hydrogen atom) transfer as the primary photophysical event in hypericin. The presence of a rising component in the time-resolved fluorescence of hypericin and the absence of such a component for the hexamethoxy analog are consistent with our assignment of excited-state proton or atom transfer as the primary photophysical process in the light-activated antiviral compound, hypericin. The results using the fluorescence upconversion technique, *which detects only emission from the excited state*, are in good agreement with our previous transient absorbance measurements. The results are also consistent with a heterogeneous ground state of hypericin.

Introduction

Interest in the polycyclic quinone, hypericin (see ref 1 for reviews, Figure 1a), was spawned by the discovery that it possesses extremely high toxicity toward certain viruses, including HIV, and toward tumors[2−4] and that this toxicity absolutely requires light.[5] The interaction of light with hypericin and hypericin-like chromophores is clearly of fundamental biological importance. Previously we have used ultrafast time-resolved transient absorption spectroscopy as a tool to understand the excited-state processes of hypericin (and its analog hypocrellin).[6−13] We have concluded that the primary nonradiative
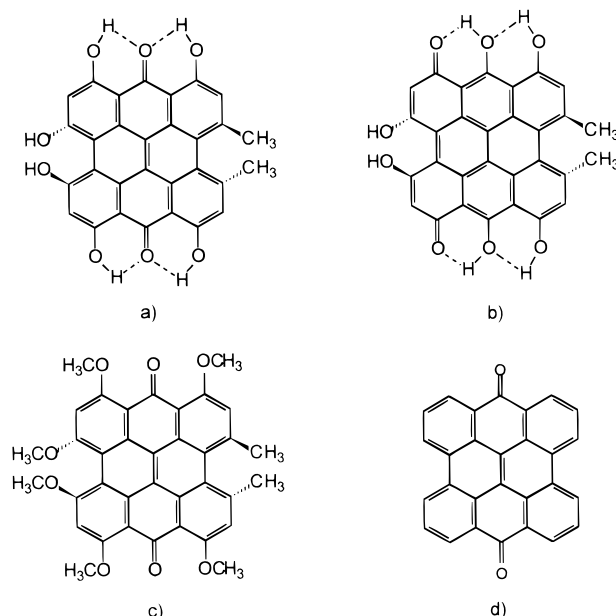
Figure 1. Structures of (a) hypericin, (b) one of the three possible hypericin double tautomers[7] (there are two possible monotautomers), (c) *O*-hexamethoxyhypericin, and (d) mesonaphthobianthrone.

process[38] in hypericin is excited-state intramolecular proton (or atom) transfer.

In this work, we make use of a complementary technique, fluorescence upconversion, to measure the excited state dynamics. In fluorescence upconversion spectroscopy, an ultrashort laser pulse populates the excited state of the sample. The molecular emission is collected efficiently and imaged into a nonlinear optical or gating crystal. The spontaneous emission is gated or "upconverted" with a replica of the excitation pulse: the sum of the emission light frequency and the gating pulse is detected as a function of gate pulse delay time. The

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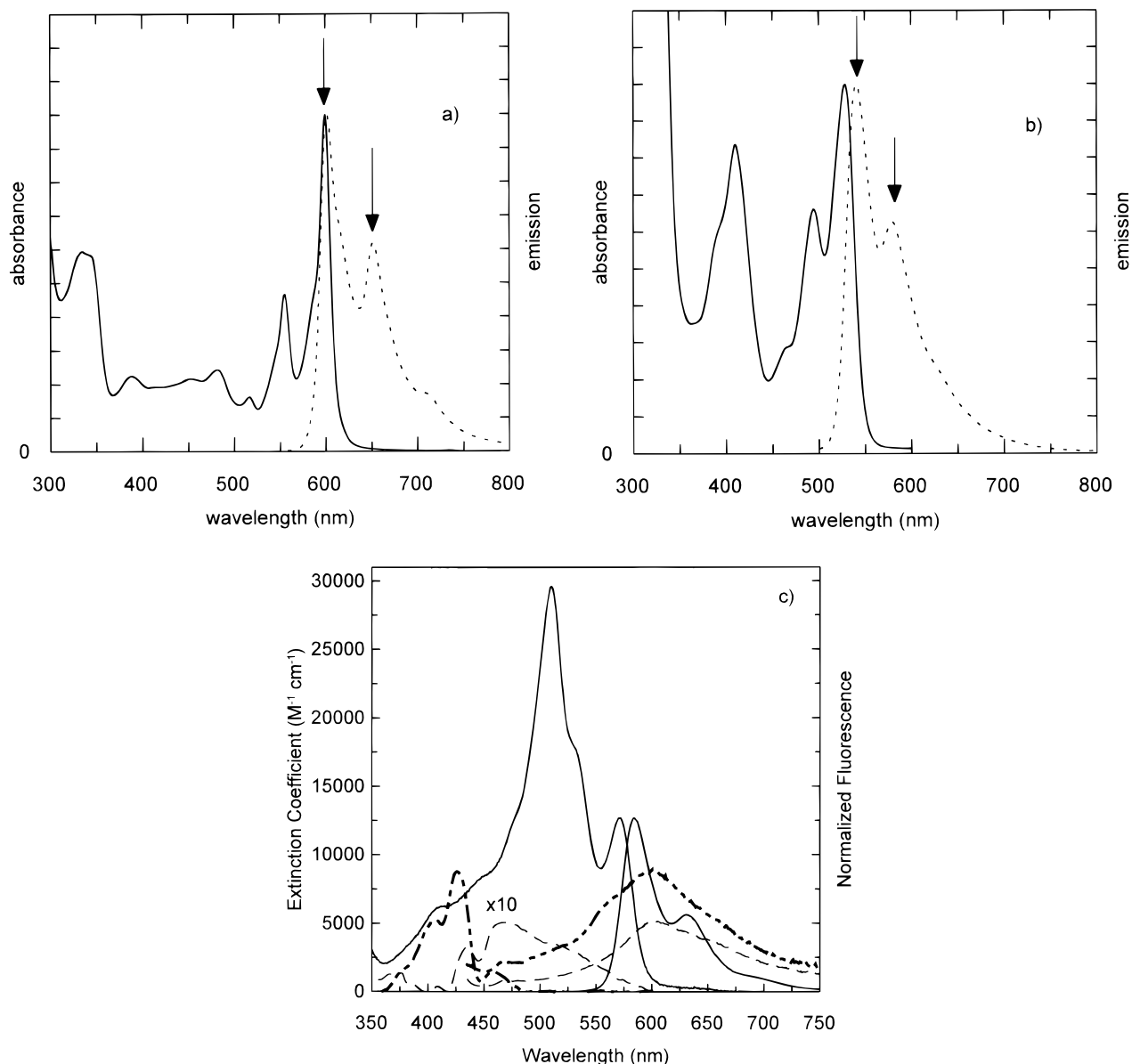(9) English, D. S.; Zhang, W.; Kraus, G. A.; Petrich, J. W. *J. Am. Chem. Soc.* **1997**, *119*, 2980−2986.
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Figure 2. Steady-state absorbance (—) and emission (- - -) spectra of (a) hypericin in DMSO and (b) *O*-hexamethoxyhypericin in DMSO. Also presented are emission and absorption spectra for mesonaphthobianthrone (mnb (c)) in DMSO (- - —), methanol (- - -), and sulfuric acid (—). In panels a and b, the arrows denote the detection wavelengths selected for the transient absorbance and upconversion experiments. Mesonapthobianthrone (mnb) was prepared[1d] according to the procedure of Koch et al.[39] A synthesis of this compound has also been reported by Falk and Vaisburg.[14] The absorption spectra of mnb in DMSO and methanol are of higher quality than those presented earlier[1d,7] where the very weak features were hidden by scattering and other artifacts. Note that the absorption spectra in these solvents are multiplied by a factor of 10 in order to compare them with those in sulfuric acid. In all cases, the emission spectra are normalized to have the same intensity as the corresponding absorption spectra. In order to compare the absorption and emission of hypericin with that of mnb, we cite their extinction coefficients and the fluorescence quantum yields of mnb *relative to that of hypericin in DMSO*. Hypericin/ethanol: $\epsilon(590\ nm) = 40\ 000\ cm^{-1}\ M^{-1}$;[40] $\phi_F = 1.00$ (the absolute value is conventionally taken as 0.3).[19] Mnb/DMSO: $\epsilon(428\ nm) = 850\ cm^{-1}\ M^{-1}$; $\phi_F = 0.056$. Mnb/methanol: $\epsilon(468\ nm) = 510\ cm^{-1}\ M^{-1}$; $\phi_F = 0.11$. Mnb/H$_2$SO$_4$: $\epsilon(510\ nm) = 3.0 \times 10^4\ cm^{-1}\ M^{-1}$; $\phi_F = 1.5$. The extinction coefficients we have obtained for mnb in DMSO and methanol are subject to considerable uncertainty. Mnb is very insoluble in these solvents; light scattering from undissolved material, as well as the presence of impurities, contributes to the poor quality of the absorption spectra. Falk and Vaisburg[14] report $\epsilon(423\ nm) \approx 7000\ M^{-1}\ cm^{-1}$ in DMSO. The spectra they obtain, especially in methanol, bear a qualitative similarity to those presented here. The relative fluorescence quantum yields of mnb in DMSO and methanol must be considered to be upper limits, since even a small amount of highly fluorescent (i.e., long-lived) impurity can contribute to the measured value.[10] The value obtained in H$_2$SO$_4$ is more certain than those obtained in DMSO or methanol, since in H$_2$SO$_4$ mnb is much more fluorescent and has a relatively long-lived fluorescence lifetime of 15 ns.[7]

excited-state dynamics, as manifested by the spontaneous emission transients, can be observed with <25 fs time resolution in this way. This technique provides the shortest possible time resolution for spontaneous emission dynamics, at least an order of magnitude better than available with direct detection techniques using streak cameras.

Our argument for intramolecular excited-state proton transfer in hypericin is as follows. The hypericin analog lacking labile protons, mesonaphthobianthrone[14] (Figure 1d), is significantly fluorescent and has optical spectra that resemble those of hypericin only when its carbonyl groups are protonated[6,7] (Figure 2). Previously, we have argued that the fluorescent state of hypericin grows in on a time scale of several picoseconds, based

(14) This compound is more correctly referred to as phenanthro[1,10,9,8-*opqra*]perylene-7,14-dione. Falk, H.; Vaisburg, A. F. *Monatsh. Chem.* **1995**, *126*, 361−364.
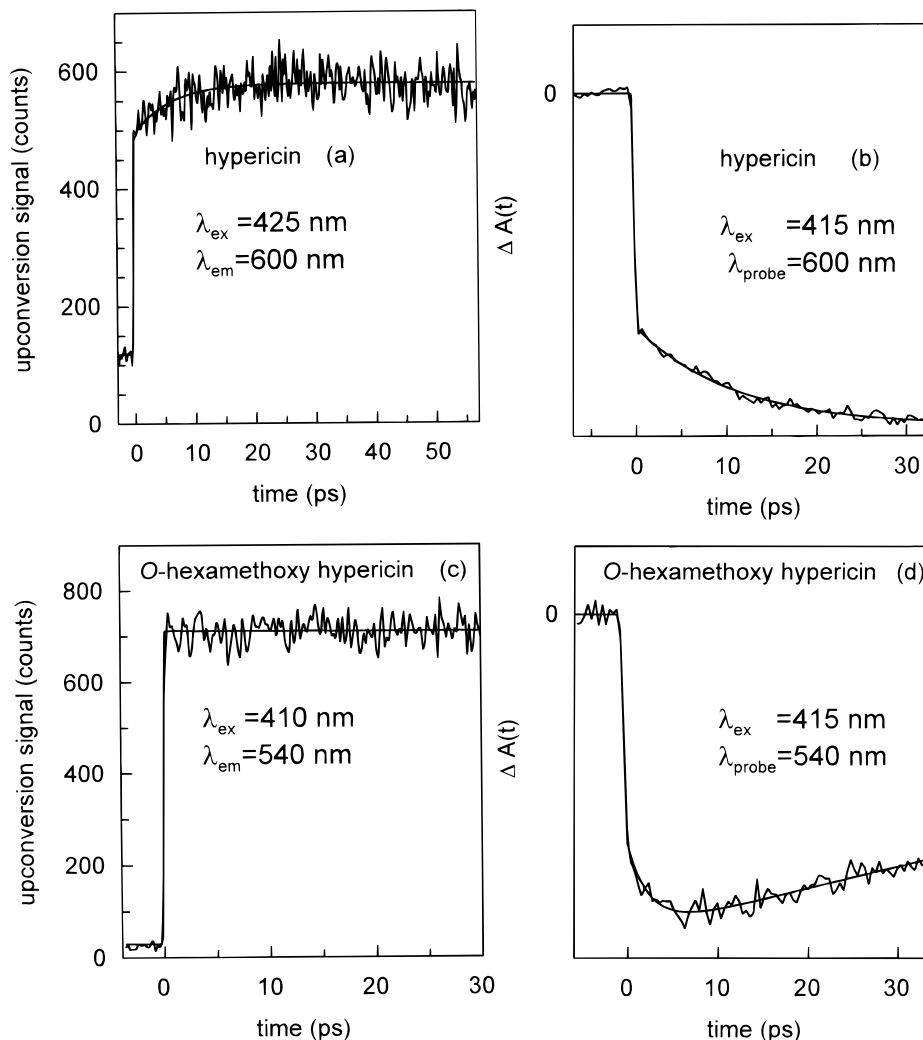
Figure 3. Comparison of fluorescence upconversion and transient absorbance traces for hypericin in DMSO at $\lambda_{em} = \lambda_{probe} = 600$ nm and for *O*-hexamethoxyhypericin in DMSO at $\lambda_{em} = \lambda_{probe} = 540$ nm. These wavelengths correspond to the maxima in the steady-state emission spectra of the respective compounds. In the absorption transients of *O*-hexamethoxyhypericin, there is evidence for a rising component which is not present in the upconversion data. We have assigned this as a rise in the bleach that is attributed to a decay of excited-state absorbance with a time constant of 2.6 ps. The data are fit to the following functions: (a) $F(t) = -0.19 \exp(-t/7.3 \text{ ps}) + 1.0 \exp(-t/\infty)$; (b) $\Delta A(t) = 0.30 \exp(-t/11.6 \text{ ps}) - 1.00 \exp(-t/\infty)$; (c) $F(t) = 1.00 \exp(-t/\infty)$; (d) $\Delta A(t) = 0.11 \exp(-t/2.6 \text{ ps}) - 1.20 \exp(-t/480 \text{ ps}) + 0.89 \exp(-t/\infty)$.

on transient absorption measurements interpreted in terms of the rise time of stimulated emission of one species and the concomitant decay of transient absorption of another species. Therefore, the combined observations of the requirement of protonated carbonyls for strong hypericin-like fluorescence and the rise time of fluorescence in hypericin were taken as evidence for intramolecular excited-state proton transfer in hypericin.[7,8] This conclusion was further strengthened by a comparison of the transient absorbance of hypericin and its methylated analog, *O*-hexamethoxyhypericin.[9,10] The assignment of proton transfer also was supported by the observation of a deuterium isotope effect of 1.4 in the excited-state transients of the hypericin analog, hypocrellin.[13]

The interpretation of transient absorbance data, however, can be subject to complications because they measure ground-state bleaching, absorption of all excited states present (both singlet and triplet), and stimulated emission.[9,10] Because fluorescence upconversion monitors emission only from the fluorescent singlet state, it is not subject to these complications and hence provides complementary information not subject to the same ambiguities. The fluorescence upconversion measurements presented here clearly reveal a rising component of ~7 ps in the emission of hypericin and the absence of such a component

in the emission of hexamethoxyhypericin, which cannot execute excited-state proton transfer.

Of special relevance to the role of labile protons for light-induced antiviral activity is the observation that hypericin acidifies its surroundings upon light absorption[15–17] and that it retains its toxicity in the absence of oxygen.[18] The retention of toxicity in the absence of oxygen excludes unique assignment of antiviral activity to the trivial generation of singlet oxygen—even though hypericin does generate triplets in high yield (~70%).[19–21] Thus, the role of photogenerated protons takes on additional significance, especially in the context of the growing body of literature implicating pH decreases with

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pharmacologically important functions, such as virucidal activity,[22] antitumor activity,[23,24] apoptosis (a form of cell death associated with DNA fragmentation and chromatin condensation),[25−27] and the subcellular distribution of hexokinase.[28] We have proposed a chemiluminescent means of activating the pharmacological activity of hypericin and its analogs.[29]
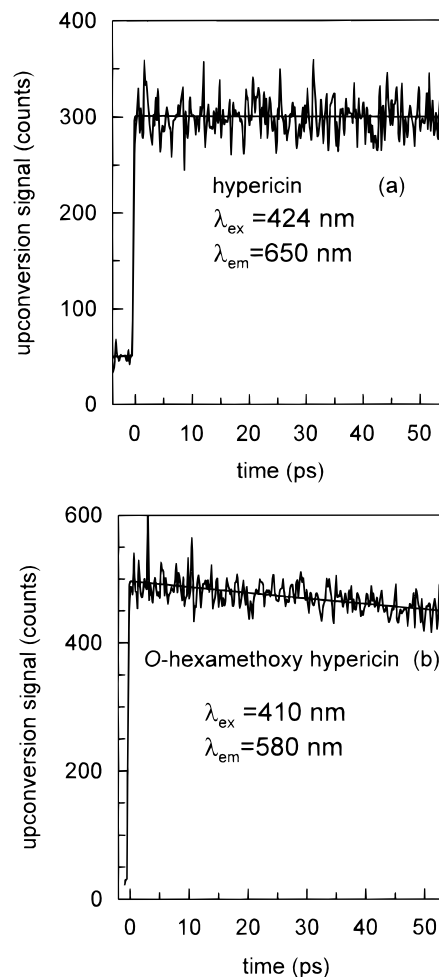
Experimental Section

Hypericin [Carl Roth GmbH (for transient absorption measurements) or Molecular Probes (for upconversion measurements)] was used as received. Anhydrous DMSO from Aldrich was used (freshly opened) without further purification. *O*-Hexamethoxyhypericin was prepared as described elsewhere,[9] dissolved in DMSO, and kept under argon for both the upconversion and transient absorbance experiments. (Falk and Mayr have reported the synthesis of an *O*-octamethoxyhypericin.[30]) Storage under argon was necessary to prevent the hexamethoxy sample from degrading, possibly from singlet oxygen formation. Steady-state absorbance spectra were obtained on a Perkin Elmer Lambda 18 double-beam UV−vis spectrophotometer with 1 nm resolution. Steady-state fluorescence spectra were obtained on a Spex Fluoromax with a 4 nm bandpass and corrected for detector response.

The apparatus for transient absorbance measurements is based on an amplified, homemade Ti:sapphire laser system producing pulses of less than 200 fs fwhm at a variable repetition rate as high as 10 kHz. This system is described in detail elsewhere.[9]

The fluorescence upconversion apparatus is described in detail elsewhere[31,32] and is based on a Ti:sapphire oscillator (Spectra Physics, Tsunami) producing tunable (750−900 nm) 50−65 fs pulses. Frequency-doubled pulses are used to excite the sample. The residual fundamental wavelength is used as the gate pulse to upconvert the fluorescence, which is collected and focused into a 0.4 mm β barium borate (BBO) crystal with an ellipsoidal reflector. The upconverted signal is separated from the gate beam, from the second and third harmonic beams, and from the fluorescence; it is focused into a monochromator coupled to a Hamamatsu R760 photomultiplier selected for near-zero dark counts. The instrument response function is obtained by collecting a cross-correlation function of the second harmonic and the fundamental: the resulting third harmonic is plotted against delay time. Cross-correlation functions typically have a fwhm of 140−144 fs. All curves were fit and deconvoluted from the instrument function using an iterative convolute-and-compare nonlinear least-squares algorithm.

Results and Discussion

Steady-state absorption and emission spectra for hypericin and *O*-hexamethoxyhypericin are given in Figure 2. Also included in Figure 2 are absorption and emission spectra for the simplest hypericin analog that we have investigated, mesonaphthobianthrone. As we noted in the Introduction, this compound has played a pivotal role in our early investigation

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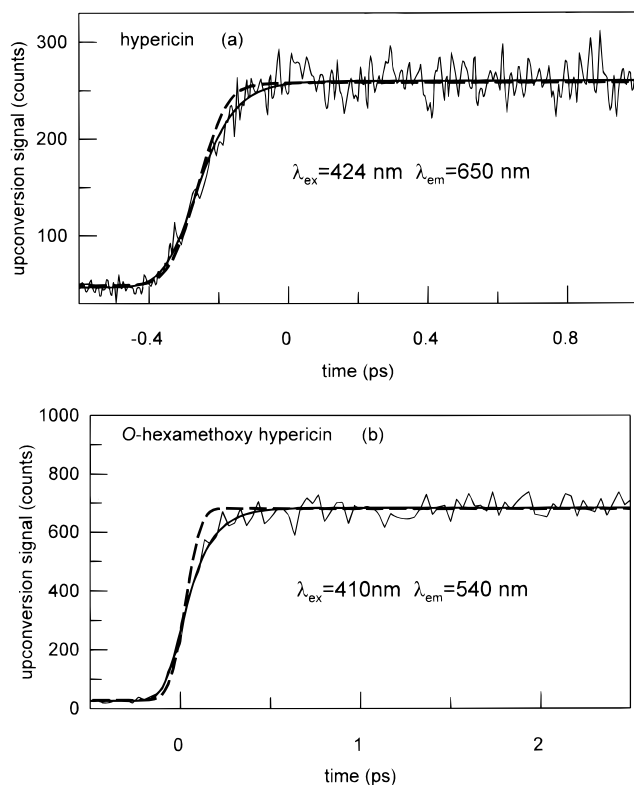
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Figure 4. Fluorescence upconversion traces of hypericin at 650 nm and *O*-hexamethoxyhypericin at 580 nm. These wavelengths (Figure 2) correspond to the second maxima of the respective fluorescence spectra. The data are fit to the following functions: (a) $F(t) = 1.00 \exp(-t/\infty)$; (b) $F(t) = 1.00 \exp(-t/503 \text{ ps})$.

of the hypericin photophysics and in our identification of intramolecular proton (or atom) transfer as the primary photoprocess. The spectra presented here are of higher quality than those we have previously reported.[1d,7]

Figure 3 presents a comparison of the fluorescence upconversion and transient absorption traces for hypericin and hexamethoxyhypericin in DMSO. The fluorescence upconversion signal for hypericin clearly shows a rising component (~7 ps), which is not present in hexamethoxyhypericin. (Preliminary data indicate an ~2.5 ps rising component in ethanol.) This result is consistent with results obtained using a pump wavelength of 415 nm, in which the rise time is fit to an ~11 ps component.

The fluorescence upconversion signal of hypericin reveals significantly different kinetics in the first 40 ps of the fluorescence decay at the two emission wavelengths investigated, 600 and 650 nm (compare Figures 3 and 4). At 650 nm, the ~7 ps rising component is completely absent. As we discuss in the Conclusions, these results support our previous suggestions that the ground state of hypericin is heterogeneous.

Figure 5 presents data collected on a shorter time scale. For both hypericin and hexamethoxyhypericin at both emission wavelengths, there is evidence of an ~100 fs rise time in the upconversion signal. These ultrafast 100 fs rise times are not instrumental artifacts. The same upconversion spectrometer has been used to observe fluorescence decays for various photoinduced electron transfer systems with lifetimes of <50 fs[32] as

Figure 5. Observation of an ~100 fs rise time in the fluorescence upconversion signal. The dashed lines are single exponential fits corresponding to an instantaneous rise time in the fluorescence signal. Fitting the rise of the fluorescence signal to a rise time yields (solid lines) the followingt: (a) $F(t) = -0.91 \exp(-t/64 \text{ fs}) + 1.00 \exp(-t/\infty)$; (b) $F(t) = -0.83 \exp(-t/114 \text{ fs}) + 1.00 \exp(-t/\infty)$.
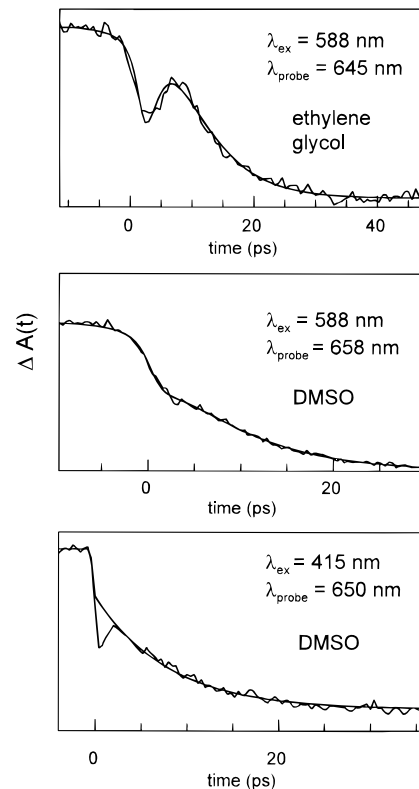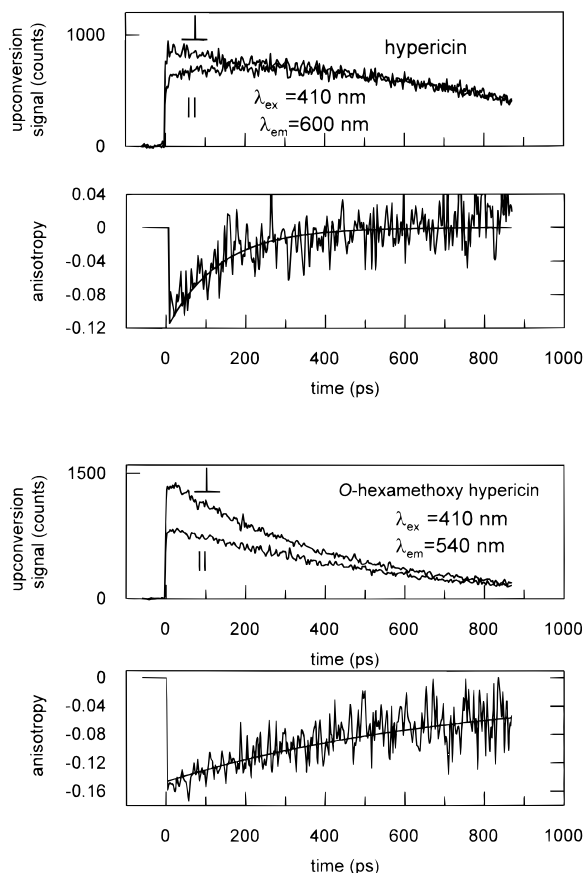

Figure 6. Reconsideration of previously obtained transient absorption signals for hypericin in the light of upconversion data presented here. Top: $\lambda_{ex} = 588$ nm, $\lambda_{probe} = 645$ nm, ethylene glycol;[7,8] $\Delta A(t) = -0.56 \exp(-t/2.0 \text{ ps}) + 0.43 \exp(-t/6.4 \text{ ps}) - 0.19$. Middle: $\lambda_{ex} = 588$ nm, $\lambda_{probe} = 658$ nm, DMSO;[7,8] $\Delta A(t) = -0.19 \exp(-t/2.0 \text{ ps}) + 0.27 \exp(-t/6.4 \text{ ps}) - 0.30$. Bottom: $\lambda_{ex} = 415$ nm, $\lambda_{probe} = 650$ nm, DMSO;[9] $\Delta A(t) = 0.21 \exp(-t/8.0 \text{ ps}) - 0.29$. The spike at "zero time" is not considered in this fit because it is attributed to a cross-phase modulation artifact resulting from the ~150 fs pulses used in the experiment.[9,10,41] It is unlikely that this is the case for the top trace, however, since the pulses used for this experiment were no shorter than 1 ps. In each case, the last term in the fit corresponds to a component that does not decay on the time scale of the experiment and that is attributed to the long-lived fluorescent species.

well as a series of subpicosecond to several-picosecond decays which have a definite rise time.[31a] In the case of rise times observed for fluorescent coumarin excited singlet states being reductively quenched by aromatic amine solvents, there is good reason to assign these ~100 fs rise times to inertial solvation dynamics, as the polar solvents librate about the very polar coumarin excited state.[31b] Solvation dynamics cannot, however, be invoked to explain the rise times for the hypericins, as the emission displays only a small amount of solvatochromism, indicating a negligible difference between ground- and excited-state dipole moments for the hypericins. A likely assignment for the observed rise times in the hypericins is internal conversion from a higher-lying singlet state, pumped by the ~415 nm laser pulses, to the lowest singlet excited state near 600 and 540 nm for hypericin and *O*-hexamethoxyhypericin, respectively. Chudoba et al. have also assigned such 100 fs transients to internal conversion for a different excited-state proton-transfer molecule, 2-(2′-hydroxy-5′-methylphenyl)benzotriazole.[35]

Both in the Introduction and the Conclusions of this paper, we note the potential ambiguities to which transient absorption measurements are subject. In this context, Figure 6 presents fits to previous transient absorption data in which the component previously described as a rise in stimulated emission is now attributed to a decay of one species whose excited-state absorption spectrum overlaps the emission spectrum of at least one other species.

Fluorescence anisotropy decays were constructed from up-conversion signals polarized parallel and perpendicular to the excitation beam. These are displayed in Figure 7 for hypericin and hexamethoxyhypericin. Of significance is that excitation at 410 nm gives rises to a negative anisotropy, i.e., a negative prefactor, $r(0)$. Elsewhere[33] we have presented a detailed investigation of the dependence of the steady-state fluorescence−excitation anisotropy and have observed similar behavior: depending on the excitation wavelength, the prefactor can be positive or negative. This results from the transition dipoles connecting the ground electronic state to the first two excited electronic states being at large angles to each other. If hypericin (which actually has a very twisted aromatic skeleton[13,34]) had C_{2v} symmetry, these transition dipoles would be orthogonal. Also of interest is that the time constant for the anisotropy decay for hexamethoxyhypericin is roughly 3 times greater than that for hypericin and does not decay to zero on the same time scale.

Conclusions

The fluorescence upconversion technique with 100 fs resolution was applied to hypericin and its synthetic analog, *O*-

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Figure 7. Polarized fluorescence traces and anisotropy curves. Hypericin: $r(t) = -0.10 \exp(-t/192 \text{ ps})$. *O*-Hexamethoxyhypericin: $r(t) = -0.10 \exp(-t/621 \text{ ps}) - 0.05 \exp(-t/2.1 \text{ ns})$.

hexamethoxyhypericin. The results of these studies were compared with previous results obtained by means of transient absorption spectroscopy. Fluorescence upconversion is a powerful technique that is complementary to transient absorption. Transient absorption monitors all absorbing and emitting species (via stimulated emission), given an adequate signal-to-noise ratio. It is difficult, if not impossible, to distinguish ground-state bleaching from stimulated emission in regions where the emission spectrum overlaps the absorption spectrum. Also, emitting species may often be obscured by the presence of absorbing species (singlets and triplets) present in higher concentration or with a larger extinction coefficient. We have discussed and addressed these complications in previous work.[7,9,10] *Because the upconversion technique detects only emission from the excited state, the potential ambiguities in the transient absorption experiment referred to above are obviated.* The fluorescence upconversion results presented here are in complete agreement with the major results and conclusions we have already presented: namely, that excited-state intramolecular proton (or atom) transfer is a primary photophysical event for hypericin and its analogs with labile protons. The upconversion

results also validate the transient absorption technique as a means of monitoring proton or atom transfer rates in hypericin, assuming that the transient absorption spectrum is well understood.

In addition, the results presented here support our previous suggestions that the ground state of hypericin is heterogeneous,[7,9,10] existing as either various tautomers or conformational isomers, and implies that the same argument can be made for hypocrellin and other hypericin analogs.[11-13,33] We originally suggested that the ground state of hypericin is heterogeneous in order to rationalize the mirror-image symmetry between its steady-state absorption and emission spectra.[7,8] Such symmetry is atypical of molecules whose excited states undergo nuclear rearrangements, such as proton or atom transfer. The archetypal excited-state proton-transfer system, 3-hydroxyflavone, provides a good example of the absence of such symmetry.[36] The transient absorption data obtained using different excitation wavelengths (415 and 588 nm) presented in Figure 6 yield different excited-state kinetics and are also suggestive of such heterogeneity. The upconversion results provide the most direct evidence to date for ground-state heterogeneity in hypericin at ambient temperatures.[37] Not only are the fluorescence profiles for hypericin different at the emission wavelengths of 600 and 650 nm but even at 600 nm there are, in addition to the ~100 fs component, two components to the fluorescence rise time with 7 ps and "instantaneous" time constants. These may be attributed to untautomerized and tautomerized (or at least partially tautomerized) species.

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