Hypericin, Hypocrellin, and Model Compounds: Primary Photoprocesses of Light-Induced Antiviral Agents

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Abstract
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Disciplines
Chemistry | Organic Chemistry | Other Chemistry | Polymer Chemistry

Comments
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The excited-state photophysics of the light-induced antiviral agents hypericin and hypocrellin are compared with those of the hexa- and tetramethoxy analogues of hypericin. The results are consistent with the interpretation of the primary photoprocess in hypericin and hypocrellin as that of excited-state intramolecular proton or atom transfer.

**Introduction**

Hypericin and hypocrellin are naturally occurring pigments\(^1\) that are remarkable because of their light-induced antiviral activity\(^3\) against enveloped lentiviruses such as the human immunodeficiency virus.\(^4\)\(^5\) Although the requirement of light for this activity in hypericin and hypocrellin is absolute, these two molecules possess very different modes of reactivity.\(^6\) Consequently, we have undertaken the task\(^6\)\(^7\)\(^8\)\(^-\)\(^9\)\(^10\)\(^11\)\(^13\) of unraveling the excited-state primary photophysical processes of hypericin and hypocrellin.

It has been our thesis from the very first that a significant nonradiative process in hypericin and its analogues is intramolecular proton (or atom) transfer.\(^7\)\(^-\)\(^9\)\(^13\) The argument for such a process is the following. The hypericin analogue lacking labile protons, mesonaphthodianthrone (Figure 1g), is significantly fluorescent and has optical spectra that resemble those of hypericin only when its carbonyl groups are protonated. \textit{In hypericin, the fluorescent state grows in on a time scale of several picoseconds, as measured by the rise time of stimulated emission.} 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. The possible role of labile protons in the light-induced antiviral activity of hypericin and its analogues is discussed in detail elsewhere.\(^6\)\(^10\)\(^11\)\(^13\)

In this article, we shall frequently refer to the process in question as a proton transfer, but it should be kept in mind that not enough data are available to determine whether a proton or a hydrogen atom is being transferred.

Two potential arguments against intramolecular excited-state proton transfer in hypericin are the lack of an isotope effect for the process in question\(^8\)\(^-\)\(^9\) and the observation of near mirror-image symmetry between its absorption and emission spectra (Figure 2a). The first of these points is discussed in detail in the companion article. As for the mirror-image symmetry, this is typically taken as a signature of negligible structural changes between the absorbing and the emitting species. Intramolecular excited-state proton transfer usually generates a broad, shifted, and structureless emission spectrum. A classic example of such behavior is given by 3-hydroxyflavone.\(^16\)

The issue of the shape of the spectra may be addressed in several ways. It is possible that the structural changes induced by proton transfer do not significantly affect the electronic structure of the tautomeric species (parts a and b of Figure 1) in such a way as to destroy the mirror-image symmetry. High-level quantum chemical calculations will have much to offer in understanding this problem. It is also possible, as we have argued elsewhere,\(^8\)\(^9\)\(^15\) that the ground state of hypericin is already partially tautomerized and that this ground-state heterogeneity yields the observed mirror-image symmetry between absorption and emission spectra. Another strategy is to study the excited-state photophysics of synthetic analogues that are unable to execute excited-state proton or atom transfer. We have already begun such an examination by comparing the photophysics of hypericin with that of its hexamethoxy analogue (Figure 1f).\(^15\) Here, we pursue this line of investigation more thoroughly by examining the tetramethoxy analogue of hypericin (Figure 1f), which can be considered a methoxy hybrid of hypericin and hypocrellin.

**Experimental Section**

A. Synthesis of the Tetramethoxy Analogue.\(^28\) \textit{Di(3,5-dimethoxy-2-methoxycarbonylphenyl)acetylene.} The compound was obtained as a white solid from the palladium-catalyzed reaction of methyl 2-ethynyl-3,5-dimethoxybenzoate and methyl 2-(trifluoromethanesulfonyloxy)-3,5-dimethoxybenzoate\(^17\) (mp 149 °C). \(^1\)H NMR (CDCl\(_3\))\(\delta \) 3.82 (s, 12H), 3.92 (s, 6H), 6.48 (d, 2H, \(J = 0.9\) Hz), 6.62 (d, 2H, \(J = 0.9\) Hz).

9,10-Bis[2'-methoxy-carbonyl-3',5'-dimethoxy]phenylphenanthrene. To a 2 dram vial equipped with a stir bar is added Pd(OAc)\(_2\) (0.125 mmol), NaOAc (0.50 mmol), LiCl (0.25 mmol), 2-iodobiphenyl (0.25 mmol), di(3,5-dimethoxy-2-methoxycarbonylphenyl)acetylene (0.275 mmol), and 5 mL of DMF. The vial is then flushed with N\(_2\), capped with a screw cap containing a Teflon liner, and placed in an oil bath at 100 °C for 48 h. The vial is then removed from the oil bath, diluted with Et\(_2\)O and EtOAc, washed with saturated NH\(_4\)Cl and water, dried over MgSO\(_4\), and concentrated. This residue was then purified by column chromatography using 4:1 hexanes/EtOAc (49% yield, mp 210–212 °C (toluene)). \(^1\)H NMR (CDCl\(_3\))\(\delta \) 3.18 (s, 6H), 3.63 (s, 6H), 3.82 (s, 6H), 6.39 (d, 2H, \(J = 3.0\) Hz). 13C NMR (CDCl\(_3\))\(\delta \) 51.5, 55.6, 55.9, 98.5, 106.9, 117.2, 122.3, 126.3, 126.5, 127.8, 129.8, 130.5, 134.8, 141.0, 157.7, 161.5, 167.9. IR (CDCl\(_3\)) \(\nu \) 3010, 1493, 1467, 1417, 1307, 1245, 1103, 1047 cm\(^-1\).

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\( ^{1}\) To whom correspondence should be addressed.

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methoxycarbonyl-3',5'-dimethoxyphenyl)phenanthrene with PPA and then concentrated and dried over 4 Å molecular sieves. Care was taken to prevent sieve dust contamination. All experiments were performed at ambient temperature (22 °C). The time-correlated single-photon counting experiments were performed with an apparatus described elsewhere.

Steady-state fluorescent measurements were made using a Spex Fluoromax with 1 nm band-pass, and corrections were made for detector response and excitation lamp spectrum. Steady-state absorbance measurements were made using a Perkin-Elmer Lambda 18 double beam UV–vis spectrophotometer with 1 nm band-pass. Fluorescence quantum yield measurements were made relative to hypericin by exciting the test sample at a given wavelength and determining the relative quantum yield against a standard solution of hypericin. The time-resolved fluorescence data provide a powerful assay of the purity of the tetramethoxy analogue. In addition to a 820 ps decay component, there is a small amount of integrated fluorescence intensity could in principle be given by the ratio of the fluorescence intensity in the ground state, assuming the radiative rate is the same for all the compounds being compared. Such an assumption is probably not warranted given the anomalously low value for the quantum yield of hypocrellin in DMSO as opposed to other solvents.

at >98% purity as determined from the supplied TLC and NMR measurements. The hexamethoxy analogue was prepared as described elsewhere. DMSO was dried over 4 Å molecular sieves. Care was taken to prevent sieve dust contamination. All experiments were performed at ambient temperature (22 °C). The time-correlated single-photon counting experiments were performed with an apparatus described elsewhere. The instrument response function is typically about 70 ps. Samples were prepared for pump–probe experiments with an optical density of 0.4–0.7 at the pump wavelength; for time-correlated single-photon counting, samples were diluted by 10-fold or more. The time-resolved fluorescence data provide a powerful assay of the purity of the tetramethoxy analogue. In addition to a 820 ps decay component, there is a small amount (–5%) of a ~2 ns component.

Steady-state fluorescent measurements were made using a Spex Fluoromax with a 4 nm band-pass, and corrections were made for detector response and excitation lamp spectrum. Steady-state absorbance measurements were made using a Perkin-Elmer Lambda 18 double beam UV–vis spectrophotometer with 1 nm band-pass. Fluorescence quantum yield measurements were made relative to hypericin by exciting the test sample at a given wavelength and determining the relative quantum yield against a standard solution of hypericin. The time-resolved fluorescence data provide a powerful assay of the purity of the tetramethoxy analogue. In addition to a 820 ps decay component, there is a small amount (–5%) of a ~2 ns component.

B. Optical Measurements. Hypericin was used as received from Carl Roth GmbH (distributed by Atomergic Chemetals Corp.). Hypocrellin A (Molecular Probes) was used as received in all other solvents investigated, the quantum yield was 0.07 ± 0.02. This is the major component of the fluorescence decay and comprised at least 95% of the decay intensity. See Figure 7. The steady-state fluorescence quantum yields provide upper limits on the true value because they are contaminated by the few percent of long-lived fluorescent impurity in our samples. A more accurate comparison of integrated fluorescence intensity could in principle be given by the ratio of the fluorescence lifetimes in the table, assuming the radiative rate is the same for all the compounds being compared. Such an assumption is probably not warranted given the anomalously low value for the quantum yield of hypocrellin in DMSO as opposed to other solvents.

<table>
<thead>
<tr>
<th>Table 1: Fluorescence Parameters in DMSO</th>
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<tbody>
<tr>
<td>compound</td>
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<tr>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>hypericin</td>
</tr>
<tr>
<td>hypocrellin</td>
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<tr>
<td>hexamethoxyhypericin</td>
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<td>tetramethoxyhypericin</td>
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</tbody>
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*In all other solvents investigated, the quantum yield was 0.07 ± 0.02. This is the major component of the fluorescence decay and comprised at least 95% of the decay intensity. See Figure 7.*
duration at 2 kHz or of 1–3 ps duration at 30 Hz. Sample preparation for the tetra and hexamethoxy analogue samples required bubbling with argon and filling the spinning sample holder in a glovebag of argon to reduce oxygen content. This was done after observing rapid degradation of the hexamethoxy sample in the presence of oxygen. Deoxygenation sufficiently preserved the samples to withstand pump incidence for several hours. Hypericin is stable for hours without deoxygenation and gives the same experimental results in either case.

Kinetic traces acquired at various wavelengths were fit using a global fitting procedure described in detail elsewhere. The time-resolved absorption/stimulated emission data are displayed in Figures 3–6. The results of the global fits, and the functional forms used to obtain them, are summarized in Table 2. The “spike” that appears at zero time in some of the data arises from the more intense pump pulse modulating the phase of the less intense probe pulse.

Results

A. Steady-State Spectra

The steady-state absorption and emission spectra of hypericin, hexamethoxyhypericin, hypocrellin, and tetramethoxyhypericin are presented in Figure 2. The spectra of hypericin differ significantly from those of the other three analogues. The absorbance maximum of hypericin in DMSO arising from the $S_0 \rightarrow S_1$ transition occurs at 598 nm. Hypericin is unlike the other three compounds investigated...
here in that it lacks an intense transition in the 400–500 nm region, which is most likely due to an $S_0 \rightarrow S_n$ transition. This band is almost as intense as that at 530 nm for the hexamethoxy analogue. In hypocrellin and tetramethoxy hypericin, this band contains the absorbance maximum. These data indicate that methylation of the hydroxyl groups adjacent to the carbonyls (or their removal) provides relative enhancement of the oscillator strength at higher energies. Consequently, these data suggest that interaction of the enol proton with the keto oxygen strongly determines the characteristics of the absorption spectrum. The methoxy analogues are also less fluorescent than their counterparts. This is consistent with our earlier proposals concerning the role of a proton interacting with the keto oxygen for establishing hypericin-like fluorescence.8,9 Note that in Figure 2 we have normalized the emission spectra so that their maxima are identical with those of the peaks (or the shoulder, in the case of hypocrellin) of the lowest energy absorption features. This is done to indicate the mirror symmetry between the absorption and emission spectra. Table 1 provides a summary of fluorescence data.

**B. Excited-State Kinetics.** The excited-state kinetics of hypericin, hexamethoxyhypericin, hypocrellin, and tetramethoxyhypericin are presented in Figures 3–6. The probe wavelengths at which these kinetics were interrogated are indicated in the steady-state spectra. Stimulated emission is observed in hypericin but not in the hexamethoxy analogue. The difference in the kinetics of the tetramethoxy analogue and hypocrellin is even more striking. The tetramethoxy analogue exhibits no stimulated emission and possesses a time constant that is 9 times smaller than that observed in hypocrellin.

In all the data presented there is a component that does not decay on the time scale of the experiment or that is characterized by a long-lived time constant that agrees with the fluorescence decay time (Table 1). Consequently, this long-lived component is attributed to absorption by an excited-state singlet or formation of triplet,23,24 depending on whether the signal is rising or decaying. Figure 7 presents the fluorescence decay of the tetramethoxy analogue in DMSO. Its lifetime can be fit to a time constant of 820 ps, which is consistent with the long-time component extracted from the transient absorption data.

**Discussion**

We have argued in the Introduction and elsewhere8,9 that the signature of proton transfer in hypericin is a rise time in stimulated emission. Such a kinetic feature is absent in the hexamethoxy analogue (Figure 1e), which cannot execute such a nonradiative process. This absence consequently confirms the presence of proton transfer in hypericin. A possible objection to this argument is that the description of the traces
as the fast decaying component at 570 nm. A summary of absorption
DMSO. The rise in absorbance at 500 nm has the same time constant
of 820 ps, which matches the fluorescence decay of the analogue in
The slowly rising transient apparent in the top panel has a time constant
full scale is 320 ps. The fast component has a time constant of 9.9 ps.

in Figure 3 is not unique. For example, instead of attributing
the form of the absorption transients to a rising component
in stimulated emission, they may equally well be described by a
component of stimulated emission that appears instantaneously
and that does not decay on the time scale of the experiment
and a component of transient absorption that decays in 11 ps.
This latter explanation seems unlikely, since the traces obtained
with an excitation wavelength of 588 nm yield much more
complicated kinetics that would be difficult to rationalize by
this alternative. For excitation at 588 nm, two components of
stimulated emission are observed: one rising with a \(\sim\)10 ps
time constant and another that appears instantaneously and that
decays in \(\sim\)1–2 ps.\(^9\)

It is clear that a unique and self-consistent description of the
hypericin photophysics requires a range of experiments. We
have already indicated the utility of varying the excitation
wavelength. Fluorescence upconversion experiments\(^{22}\) will also
provide important complementary information, since only
emission is detected. Comparison of a range of analogues also
proves to be effective in understanding the origin of the photophysics.

The latter approach is taken in this article, the most striking
result of which is the difference in the excited-state kinetics
of hypocrellin and the tetramethoxy analogue. The tetramethoxy
analogue exhibits no stimulated emission and a time constant
that is 9 times smaller than that for hypocrellin (10 ps instead
of 90 ps). The absence of a \(\sim\)100 ps time constant in the
transient absorbance of the tetramethoxy analogue is consistent
with the assignment of this component to excited-state proton
or atom transfer in hypocrellin.

A possible origin of the 2.5 and 10 ps rapid transients in the
hexa- and tetramethoxy analogues is dynamic solvation of an
excited state (or internal conversion). As the solvent dynamical
readjusts itself to the change in the dipole of the solute
upon optical excitation, the solute is lowered in energy. This
is manifested in the transient absorbance. For example, in both
of the methoxy analogues, a decay of the induced absorption at
red wavelengths matches a rise in induced absorption at blue
wavelengths (Figures 3 and 6). These data are consistent with
a picture in which the lowest excited singlet is stabilized relative
to a higher-lying state to which it is optically coupled. The
dynamic solvation time of DMSO is known to be 3 ps.\(^{22}\) It
may, of course, be fortuitous that the observed transients are of
similar magnitude. Ideally, a correlation of decay time in
various solvents with the known dynamic solvation times would
enable us to make this assignment unambiguously. Unfortunately,
DMSO is the only solvent in which we are able to
dissolve the analogues in sufficient quantities to perform these
experiments and at the same time avoid sample degradation.
Another possibility for the origin of this component is prompt intersystem crossing to form triplets.

Conclusions

The comparison of the transient absorption kinetics of the tetramethoxy analogue with those of hypocrellin (taken in the context of the comparison of those of the hexamethoxy analogue and hypericin) is cogent evidence for the identification of the transients in hypericin and hypocrellin with excited-state proton or atom transfer. In the companion article, further evidence will be provided for the assignment of these processes to proton or atom transfer events. In addition, the order of magnitude difference in the time constant between hypericin and hypocrellin will be discussed in detail.

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References and Notes