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Research Note

Two-Photon Excitation of 4′-Hydroxymethyl-4,5′,8-Trimethylpsoralen

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ABSTRACT

Psoralens are a class of pharmaceutical agents commonly used to treat several cutaneous disorders. When irradiated with a mode-locked titanium : sapphire (Ti : sapphire) laser tuned to 730 nm, an aqueous solution of 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT) emits blue light. The emission spectrum is centered at 452 nm and is identical to that obtained by one-photon excitation with UVA excitation, and its magnitude depends quadratically on the intensity of laser excitation. These results suggest that two-photon excitation occurs to a potentially photochemically active state. To estimate the two-photon absorption cross section, it was first necessary to measure the emission quantum yield of HMT using 365 nm excitation at room temperature that resulted in a value of 0.045 ± 0.007. The two-photon absorption cross section of HMT at 730 nm is therefore estimated to be 20×10^{-50} cm^4 s (20 Göppert–Mayer). The excited-state photophysics and photochemistry of psoralens suggest potential applications to cutaneous phototherapy in diseases such as psoriasis and dystrophic epidermolysis bullosa.

INTRODUCTION

Psoralens, also known as furocoumarins, are now commonly used in conjunction with 320–400 nm UVA† light to treat a variety of dermatologic disorders such as psoriasis, scleroderma, cutaneous T-cell lymphoma and vitiligo (1). Psoralens associate with double-stranded nucleic acids and, upon irradiation with UVA light, will form interstrand crosslinks with thymidine and other pyrimidine bases, thus disrupting normal replication, transcription and recombination (2–4). Recently, we have shown that it is possible to irradiate psoralen covalently linked to antisense oligonucleotides to inhibit specifically expression of collagenase from cultured human dermal fibroblasts (5). These data suggest a potential use in gene therapy for diseases such as recessive dystrophic epidermolysis bullosa in which collagenase is overexpressed.

While the action spectrum of psoralen photochemotherapy has conventionally been regarded as 320–380 nm, recent investigation has determined that 320–335 nm may be more effective (1,6). Ultraviolet A light penetrates farther into the skin because it is scattered and absorbed less than shorter wavelength UVB light (290–320 nm), and thus UVA may potentially activate psoralens in several layers of tissue, including both the intended target and nontarget cells. Additionally, UVA has biological effects independent of psoralen, such as enhanced collagenase expression in fibroblasts and DNA mutagenesis (7,8). Therefore, a form of therapy in which the specific depth of UVA penetration and psoralen photochemistry could be controlled would be useful and generally applicable to phototherapy.

Two-photon excitation has recently been explored as a method of selectively irradiating a particular volume of sample, with applications in three-dimensional microscopic imaging and optical information storage (9,10). The probability that a molecule absorbs two photons simultaneously depends linearly on its intrinsic two-photon cross section, δ, and quadratically on the incident light intensity (9–11). Although the two-photon cross sections of molecules are typically small compared with those for one-photon absorption, two-photon processes become increasingly important at the high light intensities attainable with laser irradiation. In particular, crossed or focused laser beams make two-photon excitation potentially attractive for depth-specific irradiation of photoactive therapeutic agents in tissues such as skin where the cellular targets may occur in discrete layers. Because longer wavelengths of light in the near-infrared portion of the electromagnetic spectrum can be used, two-photon irradiation may potentially avoid the damaging and toxic effects of UVA light if these processes have different two-photon action spectra. Moreover, the greater depth of cutaneous penetration achievable with near-infrared light compared with

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†*Abbreviations:* CW, continuous wave; G-M, Göppert–Mayer; HMT, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; Rh, rhodamine B; Ti : sapphire, titanium : sapphire; TMP, 4,5′8-trimethylpsoralen; UVA, 320–400 nm radiation.

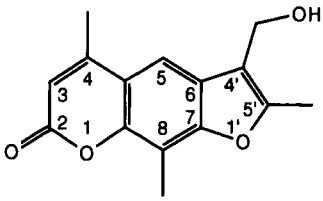

Figure 1. Chemical structure of HMT.

UVA would increase the range of cutaneous and subcutaneous targets accessible to photochemotherapy. As a first step in exploring these possibilities, we have investigated the ability of the psoralen derivative, 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT) (Fig. 1), in aqueous solution to undergo two-photon excitation by irradiating samples with a titanium : sapphire (Ti : sapphire) laser tuned to 730 nm and monitoring the resulting emission. In order to estimate the two-photon absorption cross section quantitatively, it was also necessary to measure the emission quantum yield of HMT using standard techniques.

MATERIALS AND METHODS

HMT and rhodamine B (Rh). HMT synthesized by HRI Associates (Concord, CA) was kindly donated by Dr. Allen Smith and Professor Philip Hanawalt. The powder was dissolved in a 1:3 ethanol : water (vol/vol) mixture and used at concentrations of 0.8–4.3 m*M* for two-photon excitation–emission measurements and 0.1 m*M* for absorption measurements. Solutions were stored at 4°C and manipulated at all times in the dark or under red or yellow light. Rhodamine B (Aldrich Chemical Co., St. Louis) was used as purchased and dissolved in methanol at a concentration of 35 μ*M*. Quinine sulfate (Fluka, New York) was dissolved in 1.0 *N* H_2SO_4 at concentrations of 1 μ*M*. All solvents were spectroscopic grade. No attempt was made to deoxygenate solutions prior to use. All concentrations were confirmed by absorption spectroscopy using known molar extinction coefficients.

Two-photon excitation monitored by emission spectroscopy. The HMT samples were placed in 1 mm pathlength quartz cuvettes for irradiation at room temperature. A mode-locked Ti : sapphire laser (Spectra-Physics Tsunami) tuned to 730 nm with a repetition rate of 80 MHz, pulse width of 150 fs and maximum average power of 760 mW was used to excite samples. The laser beam was focused with a 50 mm focal length lens onto the sample to form a beam diameter of approximately 10 μm at the focus. Conventional emission spectra of HMT were obtained by UV broadband irradiation provided by a deuterium lamp that was filtered by a Hoya U340 filter and then focused onto the sample. For laser excitation, the emitted light was filtered through a homemade $CuSO_4$ solution filter; broadband lamp excitation light was filtered from the emission using a Hoya BG-28 filter. The steady-state emission with either type of excitation was collected by a 50 mm focal length lens before dispersion in a Spex 0.27 m monochromator and detection by a Hamamatsu R955 photomultiplier tube. Emission spectra were uncorrected for the spectral response of the detection system. Absorption spectra were obtained on a Perkin-Elmer Lambda-12 spectrophotometer.

Relative emission quantum yield measurements. Emission spectra were obtained using a Perkin-Elmer LS50B luminescence spectrometer using excitation at 365 nm with 2.5 nm and 5 nm excitation and emission band widths, respectively. Corrections for the wavelength dependence of lamp output, monochromator optics and the detector were generated using the standard lamp spectrum and protocol supplied with the Perkin-Elmer instrument. Samples were excited at room temperature (24.6°C) in a 1 cm cuvette, with detection of emitted light at 90° to the excitation beam.

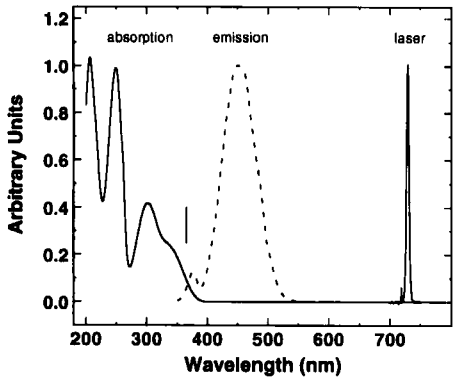

Figure 2. Normalized one-photon absorption and uncorrected emission spectra of HMT and the relative spectral profile of Ti : sapphire laser tuned to 730 nm. The small shoulder on the shorter wavelength side of the emission spectrum represents UVA excitation light that was not completely filtered out. The vertical line at 365 nm represents the expected equivalent one-photon energy for two-photon absorption with the 730 nm laser.

RESULTS

The one-photon absorption and emission spectra of HMT as well as the spectral profile of the Ti : sapphire laser tuned to 730 nm are shown in Fig. 2. The absorption spectrum was obtained to wavelengths as long as 800 nm in order to verify that the psoralen solution had no observable one-photon absorption in the vicinity of the laser emission. The emission peak of HMT appears at 452 nm. The small peak at approximately 375 nm represents a small amount of broadband UVA light that was not rejected by the BG-28 long-wavelength pass filter. The very narrow peak at a slightly shorter wavelength than the broadband laser emission represents a small amount of continuous-wave (CW) lasing. This CW component of the laser emission is of insufficient peak power to cause a significant two-photon excitation.

When a 4.3 m*M* solution of HMT was irradiated with red light (730 nm) from the Ti : sapphire laser, blue light emanated from the irradiated cuvette that was visible to the unaided eye. As shown in Fig. 3, the emitted light had a spectral line shape and emission maximum that were essen-

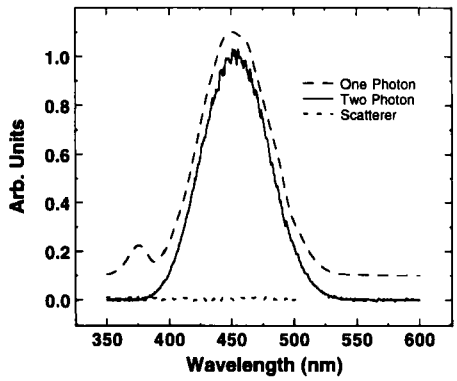

Figure 3. Comparison of the uncorrected emission spectra resulting from irradiation with UVA light and two-photon absorption. The one-photon emission spectrum has been offset from the baseline to allow better visualization of both spectra. No emission is detectable when a generic scatterer (powdered nondairy creamer) in water replaces the HMT solution.

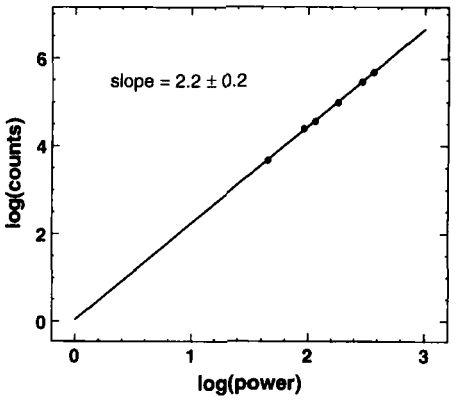

Figure 4. Typical example of the dependence of two-photon-induced emission on laser light intensity from a single experiment (●) with a least-squares fit to a line (——), illustrating a quadratic relationship.

tially identical to the emission spectrum of HMT excited with UVA light. The line shape and maximum did not change when the HMT concentration was lowered to 0.8 m*M*. No detectable emission occurred when a generic light scatterer (coffee creamer) replaced the HMT solution. When the intensity of light at the emission maximum of 452 nm is monitored as a function of the average incident laser power and plotted on a log–log scale, a linear relationship occurs over a range of 5–620 mW (Fig. 4). The slope of this plot from three independent experiments is 2.2 ± 0.2.

We were not able to measure directly the two-photon absorption cross section of HMT, but it is possible to obtain an estimate of this quantity by comparison with a compound such as Rh whose two-photon absorption cross section has been measured at the wavelength of interest. One can then estimate the two-photon cross section using the following formula (Eq. 4 in Birge (11)):

$$\delta = \frac{\sqrt{2}\, N_{abs}}{1.06447 P^2 \Gamma C (n/\lambda)} \qquad (1)$$

N_{abs} is the number of photons absorbed per pulse, P is the peak power of light in photons/s, Γ is the pulse width, C is the concentration of absorber in molecules/m^3, n is the refractive index of the medium and λ is the wavelength of light. If it is assumed that excited-state photodynamics proceed identically regardless of whether one- or two-photon absorption generated the initially excited state, then the quantum yield of emission, Φ_E, will also be identical and N_{em} is the number of photons emitted following two-photon absorption in a single molecule

$$N_{em} = 0.5 N_{abs} \Phi_{em}. \qquad (2)$$

Substituting this expression in Eq. 1, and solving it simultaneously for two compounds, Rh and HMT, measured at identical wavelengths, laser powers and laser pulse widths, as well as refractive indices (12),‡ one obtains the following relationship:

$$\delta_{HMT}\Phi_{em(HMT)} = \frac{N_{em(HMT)}}{N_{em(Rh)}} \frac{C_{Rh}}{C_{HMT}} \delta_{Rh}\Phi_{em(Rh)}. \qquad (3)$$

The emission spectrum of 35 μ*M* Rh in methanol following two-photon excitation at an identical laser wavelength, power and pulse width was obtained (data not shown). The ratio of photons emitted by HMT to Rh, $N_{em(HMT)}/N_{em(Rh)}$, was then estimated by trapezoidal integration of the respective emission spectra. Two-photon excitation spectra and the corresponding cross sections of a variety of fluorophores in the near-infrared region have recently been reported (13). The δ for Rh in methanol at 730 nm appears from the published spectrum to be approximately 50 × 10^{-50} cm^4 s/photon or 50 Göppet–Mayer (G-M). Using Eq. 3, we therefore estimate $\delta_{HMT}\Phi_{em(HMT)} = 1.5\Phi_{em(Rh)}$ G-M; using $\Phi_{em(Rh)} = 0.7$ (17), $\delta_{HMT}\Phi_{em(HMT)} = 1$ G-M.

In order to estimate δ_{HMT} further, it is necessary to know $\Phi_{em(HMT)}$. Because $\Phi_{em(HMT)}$ has not been previously reported, we measured the emission quantum yield of 40 μ*M* HMT in 1:3 ethanol : water (vol/vol) relative to a well-characterized emission standard, 1 μ*M* quinine sulfate in 1 *N* H_2SO_4, whose absorption and emission energies and spectral line shapes are similar to those of HMT (14,15). Absorption spectra and corrected emission spectra with 365 nm excitation were obtained (data not shown) and, taking the emission quantum yield of quinine sulfate in 1 *N* H_2SO_4 at 25°C to be 0.546 (16,17), resulted in $\Phi_{em(HMT)} = 0.045 \pm 0.007$ (18).§ On the basis of this result, δ_{HMT} is estimated to be 20 G-M.

DISCUSSION

Upon excitation with UVA light, free psoralens in the initially excited singlet state may return to the ground state by internal conversion or fluorescence or they may undergo intersystem crossing with fairly high quantum yields to a triplet state; both radiationless decay and phosphorescence can subsequently occur (2–4,19). When psoralen is bound to DNA, both the excited singlet and triplet states are capable of cycloaddition with an appropriately placed thymidine, though the yield from the triplet state most likely predominates (2–4). This photochemistry underlies the major mechanism of action of the psoralens.

The absorption spectrum of HMT does not extend to wavelengths longer than 500 nm. Therefore, the 730 nm light from the Ti : sapphire laser used in our experiments should not result in any one-photon absorption. The presence of emission that is shorter in wavelength than the excitation source, essentially identical to the one-photon emission spectrum, and quadratically dependent on excitation intensity provides strong evidence that two-photon excitation occurs in HMT. The correspondence of the emission maximum and general line shape with published spectra and their independence of concentration indicate that aggregation and excimer formation are not occurring to any appreciable extent, even at millimolar concentrations. Quadratic dependence of the phenomenon on laser power, even at the highest powers, also

‡The refractive indices for methanol and 1:3 (vol/vol) ethanol : water at room temperature are approximately equivalent and will be treated as such in this discussion: n(methanol) = 1.329, n(ethanol) = 1.361, n(water) = 1.333.

§The result also includes a correction for differences in refractive index between solvents that can occasionally be substantial, but in the case of 1 *N* H_2SO_4 (n = 1.338) and 1:3 (vol/vol) ethanol : water (n = 1.347) the correction is minor.

indicates that saturation of the system has not occurred to a significant extent. To our knowledge, this is the first demonstration of two-photon excitation in the psoralen family and indicates that two-photon excitation can occur to the electronic states that should allow psoralens to crosslink with nucleic acids.

Our measurement of HMT emission quantum yield is comparable to that of its parent compound, trimethylpsoralen (TMP) whose values in ethanol and H_2O/ethanol (96%) are 0.044 and 0.087, respectively, based on 355 nm excitation (20,21). Although all of our measurements were on non-deoxygenated solutions, neither the emission of quinine sulfate nor TMP appears to be very sensitive to deoxygenation (16,21), and our value for the emission quantum yield of HMT appears to be reasonable for purposes of estimating the two-photon absorption coefficient.

Our experimental estimate for the two-photon absorption coefficient of HMT ($\delta \approx 20$ G-M) is larger than that measured for Coumarin 138 ($\delta = 5$ G-M), which is structurally related to the psoralens but lacks the furan ring possessed by psoralens (9), although because both measurements were done at a single wavelength, such comparisons are not as meaningful as if both were performed at the maxima of the two-photon spectra. It will thus be of interest to obtain the two-photon excitation spectrum of HMT to determine if it is centered at twice the wavelength of the one-photon absorption spectrum as seen in other molecules without a center of symmetry (13). Because the maxima of the one-photon action spectra of psoralens are thought to be at shorter wavelengths than 365 nm (1,6), irradiation with wavelengths shorter than 730 nm may enhance two-photon excitation as a result of even larger values of δ. The change in permanent electric dipole moment between the ground and excited states ($\Delta\vec{\mu}$) is a factor that governs the magnitude of δ in polar molecules, and one-photon allowed transitions can have significant two-photon cross sections if a large $|\Delta\vec{\mu}|$ exists (11,22). Because psoralen is a very asymmetric molecule (Fig. 1), it is expected to possess a permanent electric dipole moment in its ground state. The magnitude of its two-photon excitation at 730 nm suggests that a significant $|\Delta\vec{\mu}|$ may occur on excitation to the initially excited state. The molecule may also subsequently undergo a further change in dipole moment as the excited state evolves to the triplet state. Theoretical calculations predict $|\Delta\vec{\mu}|$ can be large between the ground and excited triplet states of some psoralens (19). Other systems, such as transition metal complexes, also show disproportionately large measurements of $|\Delta\vec{\mu}|$ in low-energy direct singlet-to-triplet transitions when measured by Stark effect spectroscopy (23). It would be of interest to measure $|\Delta\vec{\mu}|$ for both absorption as well as emission in a series of psoralens to evaluate further which states may possess significant charge transfer character and whether the initially excited state is likely to be the reactive state, or whether the system is initially similar in electronic distribution to the ground state and then evolves to another distorted state that is reactive. Additional studies of both δ and $\Delta\vec{\mu}$ for HMT in the presence of DNA will also provide additional insight into the role of DNA binding in HMT reactivity.

Psoralens constitute an important class of dermatologic drugs. They are used both systemically and topically in conjunction with UVA light to treat psoriasis and a variety of other dermatological diseases (1). The quadratic intensity dependence of two-photon absorption in HMT suggests that it may be explored as a means of selectively irradiating a layer of tissue, such as epidermis or dermis. Two-photon absorption has been previously exploited in other fields to create prototypical optical memory devices and to image biological molecules. Applications in optical memory storage typically initiate a unimolecular photochemical reaction resulting in a molecule with a different absorption spectrum (10); microscopic imaging depends on direct detection of fluorescence (9). Our result that two-photon excitation of psoralens is possible suggests a pharmaceutical application involving photochemical reactions between two noncovalently complexed molecules that may be initiated in a volume-specific manner in tissues or other condensed media; this potential has been noted previously (10) but has not yet been demonstrated. Furthermore, the diminished scattering of the longer (near-infrared) wavelengths used in two-photon excitation relative to one-photon excitation of psoralen is expected to enhance the depth of penetration and range of cellular targets. The ability to conjugate psoralens to antisense oligonucleotides will further enhance both the specificity of the psoralen photochemistry and its general applicability to other diseases where a specific molecular target participates in the pathophysiology (5). Recent experiments with confocal laser scanning microscopy on *in vivo* skin cells also suggest potential applications of two-photon-induced emission and photochemistry in high-resolution imaging and labeling of skin cells for basic research as well as clinical diagnosis and therapy (24).

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