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Two-Photon Induced Uncaging of a Reactive Intermediate. Multiphoton In Situ Detection of a Potentially Valuable Label for Biological Applications

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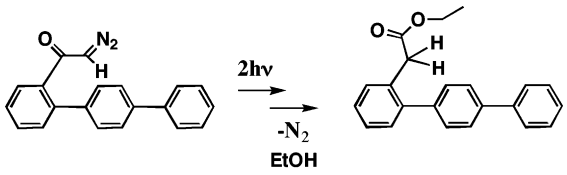

Two-photon induced Wolff rearrangement of a terphenyl diazoketone **1** was achieved by using focused laser pulses of 532 nm from a Q-switched Nd:YAG laser. The nonfluorescent terphenyl diazoketone **1** was transformed into a fluorescent ester derivative **4**, which can be detected in situ using the focused laser pulses at 532 nm. Laser power dependence studies show that the Wolff rearrangement is induced by two-photon absorption of the terphenyl diazoketone **1**, but suggests that more than two photons of 532 nm are involved (a multiphoton process) in excitation of the ester derivative **4**.

Introduction

Multiphoton absorption is a higher order, nonlinear optical process in which two or more photons are simultaneously absorbed such that the sum of the energy of the individual photons is equal to the energy of the excited atom or molecule. The phenomenon of two-photon absorption was theoretically predicted by Goppert-Meyer in 1931,[1] but since the probability of such a process depends on both a spatial and temporal overlap of a very high density of the incident photons, it was not observed experimentally until the advent of pulsed laser sources three decades later.[2] The significance of this nonlinear dependence on the incident light intensity with respect to the potential applications of two-photon absorption was immediately recognized. In contrast to single-photon absorption processes, where substantial absorption occurs along the path of the beam of light employed, two-photon or multiphoton absorption is negligible outside the immediate vicinity of the focal volume of the beam. This allows spatial and radial resolution about the beam axis, which provides a means of activating photochemical processes with high spatial resolution in 3D, leading to applications ranging from fluorescence imaging[3] and optical data storage[4] to photoinitiated microlithography[5] and polymerization.[6] The biological applications[7−9] of two-photon absorption have been especially highlighted, since two-photon absorption can occur at wavelengths well beyond that at which the majority of organic substances absorb under single-photon absorption conditions. The use of these longer wavelengths couples a greater depth of penetration with less overall damage to the host, and is thus particularly useful in the presence of biological substrates.

Recently, photochemical transformations resulting from two-photon absorption,[10] have become of interest in their own right and analogies have been drawn between our current understanding of two-photon processes and that of single-photon organic chemistry 50 years ago. These investigations are driven in part by the possibility of alternative or improved reaction pathways as a result of

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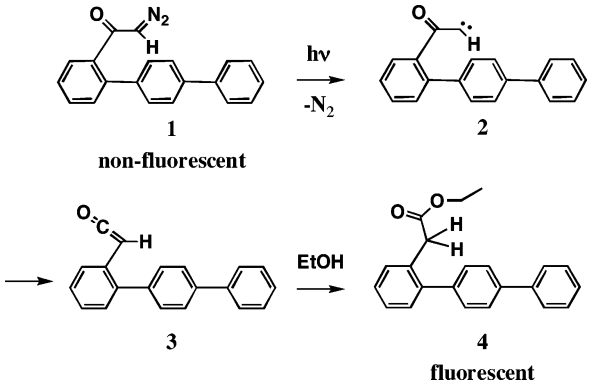
SCHEME 1. Photoreaction of the Nonfluorescent Terphenyl Diazoketone (1) to the Fluorescent Ester Derivative (4)



two-photon absorption. The availability of readily obtainable two-photon excitation sources is also an important factor in such studies. In this work, a Q-switched Nd:YAG laser is used in the investigation of the two-photon absorption properties of a terphenyl diazoketone, **1** (Scheme 1). Currently, mode-locked titanium:sapphire lasers are generally favored for two-photon absorption studies,[11] particularly those carried out in the so-called "phototherapeutic window", the region of relative tissue transparency between 650 and 950 nm, due to the ability of such lasers to generate femtosecond pulses with very high peak powers (100 kW) in this region.[12] The major disadvantage of mode-locked titanium:sapphire lasers is that the currently available lasers are relatively complicated to operate and are generally limited to expert users. On the other hand, Q-switched Nd:YAG lasers are easy to operate and, therefore, widely used in medicine[13] and industry.[14]

The photochemical "decaging" of organic molecules (or the generation of fluorescent organic species via a photochemical transformation) has been widely exploited in single cell biology.[15,16] Analysis of uncaged molecules in single cells places stringent requirements of (1) spatial resolution because of the small size of single cells and (2) ultrasensitivity of detection because of the small number of relevant molecules in the single cell under examination. A laser can be focused to a volume of the order of a single cell very precisely in space to meet requirement (1) above. Fluorescence analysis is the method of choice for convenient ultrasensitivity and is capable of single molecule detection under favorable circumstances, thus meeting requirement (2). The advantages of focused laser excitation in the UV range may be compromised by the strong competing single-photon excitation of proteins and DNA in cells. A possible means of overcoming the latter problem is to use a focused laser to decage an organic molecule contained in a single cell by *multiphoton absorption of visible or infrared photons*.

Terphenyl diazoketones have been demonstrated to be a new fluorogenic probe that can be transformed from a nonfluorescent substrate to yield strongly fluorescent products as the result of thermal atom-transfer reactions.[17] The photolysis of diazoketones results in the formation of ketenes which are rapidly converted to esters in the presence of alcohols.[18] Photolysis of the terphenyl diazoketone **1** (Scheme 1) yields the ester **4** through the intermediacy of the carbene **2** and the ketene **3**. The photolysis of a similar diazoketone, 2-diazo-1,2-naphthoquinone, using two-photon excitation, has been reported.[19] With the use of a titanium:sapphire laser (800 nm; 94 fs pulse width), it was demonstrated that the two-photon excitation of 2-diazo-1,2-naphthoquinone initiates a photo-Wolff reaction analogous to that shown in Scheme 1. In this paper we report an investigation of the multiphoton photochemical uncaging of nonfluorescent **1** to produce **4**, whose strong fluorescence can be analyzed in situ by multiphoton excitation. The results discussed in the following sections highlight the value of convenient Nd:YAG lasers as a practical tool in the study of (1) two-photon induced uncaging of diazoketones such as **1** and (2) the in situ multiphoton detection of the fluorescence of the uncaged product **4**. The terphenyl diazoketone **1** is proposed as an exemplar of a family of diazoketones that have TPA characteristics which are not only of interest in terms of the photochemical transformations induced but which also show promise in the fluorescent labeling of biological substrates such as proteins.[20]

Results and Discussion

The terphenyl diazoketone (**1**) shows a strong absorbance in the UV region ($\lambda_{max} = 269$ nm; $\epsilon_{269} = 26500$ dm^3 mol^{-1} cm^{-1}), but practically no absorbance in the visible region (>400 nm) (Figure 1a). Excitation into the absorption band centered at 269 nm showed no detectable fluorescence, indicating that the singlet excited states of **1** are deactivated very rapidly. Diazoketones are known to efficiently and rapidly undergo N_2 elimination from the excited state to generate carbenes.[18] It is therefore likely that as shown in Scheme 1, such a carbene (**2**), produced from photochemical excitation of **1**, undergoes the Wolff rearrangement generating a ketene (**3**), which reacts with the solvent, ethanol, to produce the terphenyl alkyl ester (**4**) (see the Supporting Information).[18] We determined the quantum yield of the photoreaction ($\lambda_{irr} = 266$ nm) using the ferrioxalate actinometer (see the Supporting Information for details).[21] In ethanol solution, a quantum yield of $\phi = 0.5 \pm 0.1$ was observed, which is consistent with quantum yields of similar diazoketones.[22] The absorption spectrum of **4**, shown in Figure 1b, exhibits an absorbance in the UV region ($\lambda_{max} = 262$ nm;

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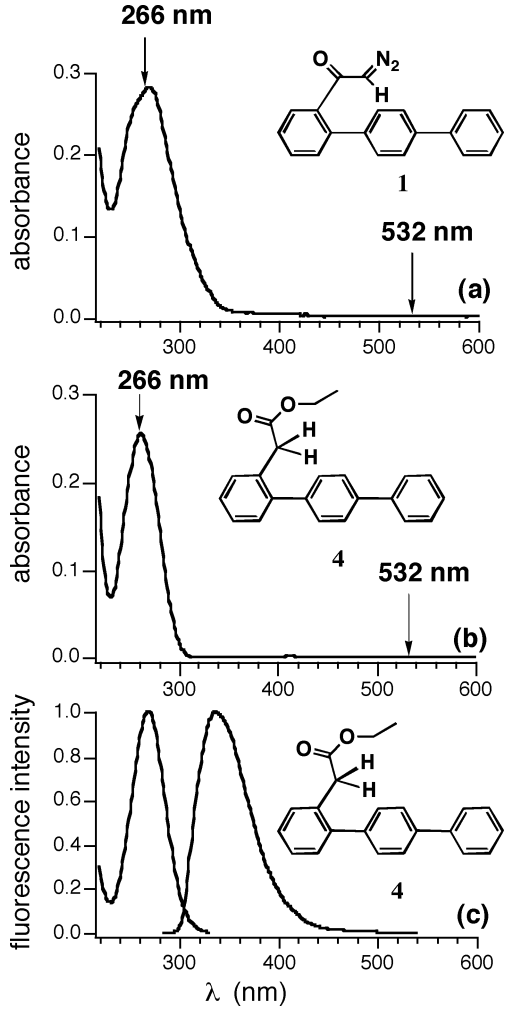

FIGURE 1. Absorption spectra of **1** (a) and **4** (b) in ethanol solution (10^{-5} M). Fluorescence emission (c, right, λ_{ex} = 266 nm) and excitation (c, left, λ_{em} = 340 nm) spectra of **4** in ethanol solution.

ϵ_{262} = 28 000 dm^3 mol^{-1} cm^{-1}) similar to that of the terphenyl diazoketone, **1**. Excitation into the UV absorption band (e.g., at 266 nm) generates a strong fluorescence with a maximum at 337 nm (Figure 1c, right). The excitation spectrum (Figure 1c, left) matches the absorption spectrum (Figure 1b) of **4**.

UV irradiation with a low-pressure Hg lamp (254 nm) or a Xe lamp in conjunction with a monochromator (269 nm), or a frequency quadrupled Nd:YAG laser (266 nm; 5 ns pulses), generated the fluorescent terphenyl ester, **4**, from the nonfluorescent precursor terphenyl diazoketone, **1**. The production of **4** can be conveniently monitored by conventional fluorescence spectroscopy. For practical applications in biochemistry, irradiation at wavelengths below 300 nm is unfavorable, because of the competitive absorption of most biomaterials and their potential damage of biological samples at these wavelengths. **1** was excited with unfocused laser pulses at 532 nm (frequency doubled Nd:YAG laser; 7 ns pulses). Even after prolonged irradiation (100 000 pulses) at 532 nm, no terphenyl ester was generated. However, if the laser beam (532 nm) was focused to a single point inside the sample solution using identical laser power, generation of **4** was observed by fluorescence spectroscopy. Figure 2

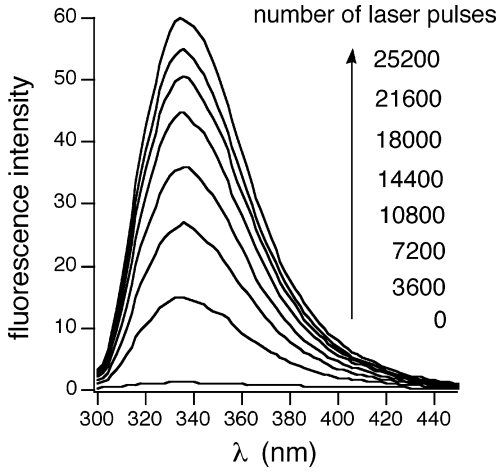

FIGURE 2. Fluorescence spectra (λ_{ex} = 266 nm) of solutions of **1** (0.01 mM in ethanol) before and after irradiation with focused laser pulses (532 nm; 30 Hz; 30 mJ/pulse). The fluorescence spectra correspond to **4**, which is generated from **1** during laser irradiation.

shows an example of how the production of **4** was monitored by fluorescence spectroscopy. Solutions of **1** (0.01 mM) were analyzed by fluorescence spectroscopy (λ_{ex} = 266 nm), using a standard steady-state fluorimeter. Following irradiation by a certain number of focused laser pulses of 532 nm, the sample solution was placed into the steady-state fluorimeter to monitor the generation of **4** (Figure 2).

The rate of production of **4** was determined from the slope in the linear region of a plot of the intensity of the fluorescence signal of **4** vs laser-irradiation time (Figure 3, left). To ascertain whether two-photon or multiphoton absorption is involved, the laser intensity (532 nm) was decreased using neutral density filters. Figure 3 (left) shows that the rate of production of **4** decreases with decreasing laser power. The plot of the laser power vs the rate of formation of **4** shows a quadratic dependence[23] (Figure 3h, right) demonstrating that two-photon absorption occurred.

In control experiments, solutions of **1** (0.01 mM) were irradiated with laser pulses of 266 nm (5 ns pulse width) and analyzed for the formation of **4** by fluorescence spectroscopy, as described above. As expected for one-photon absorption (**1** absorbs strongly at 266 nm), a linear dependence of the rate of formation of **4** was observed (Figure 3i, right).

Having demonstrated that **1** undergoes photochemical transformation into **4** by two-photon absorption using focused laser pulses at 532 nm, we next investigated the photoproduct **4**, to establish whether it could be analyzed in situ by fluorescence after two-photon or multiphoton absorption using focused laser pulses at 532 nm. Solutions of **4** were irradiated with focused laser pulses (532 nm), and the emitted light from the sample was analyzed with a CCD detector. Figure 4 (left) shows the emission spectrum, which is similar to the fluorescence spectrum of **4** after 266 nm excitation (one-photon excitation) (Figure 1c). On the basis of this spectral similarity, the emission spectrum (Figure 4, left) was assigned as due

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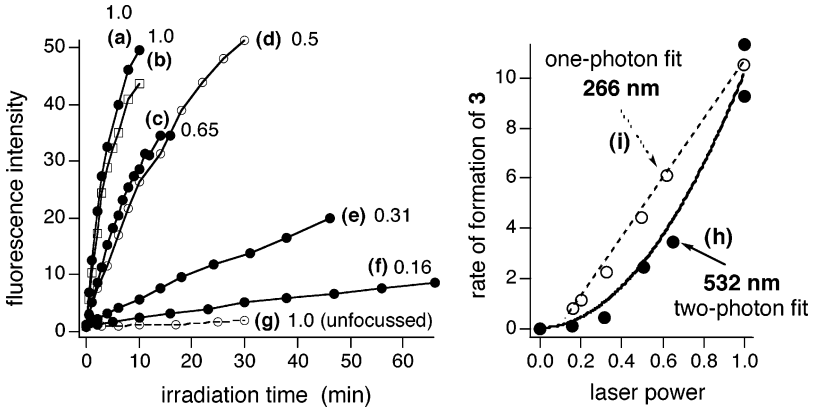


FIGURE 3. Left: Fluorescence intensity of **4** vs irradiation time using focused (a−g) laser pulses at 532 nm, 30 Hz at different relative laser power (100% (a, b); 65% (c); 50% (d); 31% (e); 16% (f)). Slope (a) derived from data shown in Figure 2. Slope (g) corresponds to equivalent experiments as (a) but using unfocused laser pulses. Right: Rate of formation of **4** vs relative laser power of focused laser pulses at 532 nm (h) and laser pulses at 266 nm (i). The relative rates for (h) were obtained from the slopes (a−f). The experimental data at 266 nm (i) were fitted to a linear equation ($y = n + mx$) corresponding to an one-photon absorption. The experimental data at 532 nm (h) were fitted to a quadratic equation ($y = mx^2$) corresponding to two-photon absorption.

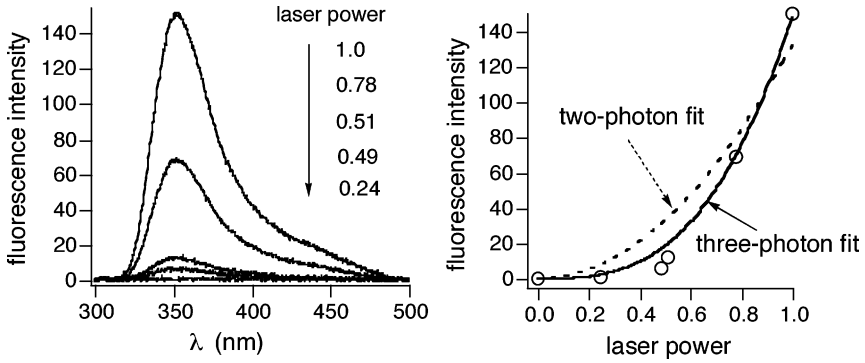
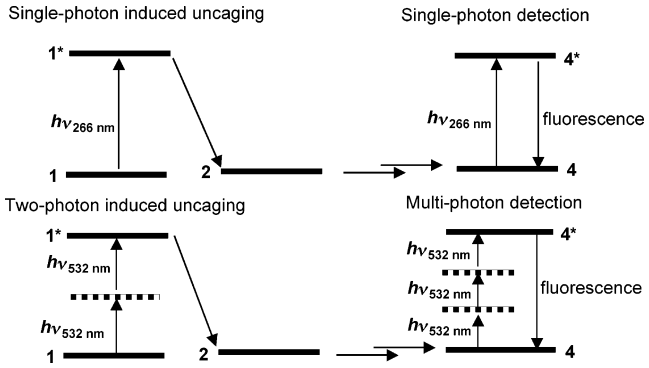


FIGURE 4. Left: Fluorescence observed during irradiation of ethanol solutions of **4** (0.02 mM) with focused laser pulses at 532 nm (30 Hz; 22 mJ/ pulse and reduced power). Right: Fluorescence intensity vs relative laser power. The experimental data were fitted to a quadratic equation ($y = mx^2$) corresponding to two-photon absorption and to a cubic equation ($y = mx^3$) corresponding to three-photon absorption.

to fluorescence from **4**. To determine how many photons of 532 nm were involved, the fluorescence intensity was investigated at various laser powers. The dependence of the fluorescence intensity on the relative laser power (Figure 4, right) is clearly not quadratic, but of a higher order. This suggests that more than two photons of 532 nm are involved (multiphoton process) in the excitation of **4**. Figure 4 (right) shows that it is likely that three photons are absorbed by **4**, since the data display a better fit to a three-photon model. A representation of this three-photon absorption process is shown in Scheme 2. This implies that for compound **4** the cross section for three-photon absorption is higher than that for two-photon absorption at the excitation wavelength of 532 nm.

SCHEME 2. Uncaging and Fluorescence Detection by Conventional One-Photon Absorption or Novel Multiphoton Absorption



Conclusion

We have demonstrated that the uncaging of the fluorescence of diazoketones can be achieved by two-photon excitation. The nonfluorescent diazoketone **1** generated the fluorescent ester derivative **4** after excitation with focused laser pulses of 532 nm. The quadratic power dependence for this reaction shows that two-photon excitation is involved in the transformation of **1** to **4**. Furthermore, we have shown that the fluorescent ester derivative **4** can be detected in situ using the same focused laser pulses at 532 nm. Laser power dependence studies suggest that more than two photons of 532 nm are involved (a multiphoton process) in the excitation of **4**. These findings are summarized in Scheme 2.

In our studies presented here, the photoreactions were carried out in ethanol solution, which leads to the

formation of fluorescent ethyl esters (**4**). However, if the photoreaction is carried out in the presence of biomolecules, the carbene (**2**) and/or the ketene (**3**), which are generated in the first step of photolysis of **1**, can react with such substrates leading to a fluorophore labeled biomolecule. Since, two-photon chemistry occurs only in the focal point of the laser light, fluorophore labeling can be achieved with high spatial resolution. In addition, we have shown that two-photon induced uncaging can be achieved with a Nd:YAG laser, which is much easier to operate and less expensive than the mode-locked titanium:sapphire lasers generally favored for two-photon studies. The potential of two-photon induced production and detection of this new class of fluorescent probes is yet to be "uncaged" with respect to in vivo investigations. However, studies to employ this as a label in biological applications are currently underway.

Experimental Section

The terphenyl diazoketone, **1**, was synthesized and characterized following a previously published procedure.[17] The ester derivative **4** was synthesized as follows.

Ag_2O (48 mg, 0.21 mmol) was added to a solution of **1** (50 mg, 0.17 mmol) in EtOH−CH_2Cl_2 10:1 (5 mL). The mixture was stirred at room temperature until the reaction was complete (5 h). The reaction mixture was diluted with $CHCl_3$ and filtered through Celite, and the solvent was removed in vacuo. Purification by column chromatography on silica gel (hexanes−EtOAc 95:5) afforded pure **4** (49 mg, 92%). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.66−7.59 (m, 4H); 7.49−7.27 (m, 9H); 4.09 (q, 2H, J = 7.1 Hz); 3.63 (s, 2H); 1.20 (t, 3H, J = 7.1 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 172.0; 142.0; 140.7; 140.1; 139.9; 131.9; 130.4; 130.2; 129.7; 128.8; 127.6; 127.3; 127.2; 127.1; 126.9; 60.8; 39.0; 14.1. IR (NaCl, cm^{-1}) 3024; 2980; 1732; 1480; 1366; 1330; 1243; 1208; 1154; 1029; 1007; 843; 756; 697. LRMS (FAB) 317 ($C_{22}H_{21}O_2$, M + H).

The fluorescence spectra were recorded as follows. The sample solution (3 mL) was placed in a 2 × 1 cm^2 fluorescence cell and stirred continuously. For the two-photon excitation experiments, the pulses of a Spectra Physics GCR-150-30 Nd:YAG laser (532 nm; 30 Hz; 30 mJ/pulse; pulse width ca. 10 ns) were focused in the center of the sample cell. For one-photon excitation studies, the sample solution (3 mL) in the 2 × 1 cm^2 fluorescence cell was irradiated with the pulses of a Spectra Physics GCR-150-30 Nd:YAG laser (266 nm; 30 Hz; ca. 10 mJ/pulse). To determine the rate of conversion of **1** into **4** during one- and two-photon excitation, the fluorescence cell containing the sample solution was placed into a Fluorolog-3 spectrometer (Jobin Yvon Inc.) to determine the amount of **3** by fluorescence spectroscopy.

The fluorescence spectra after multiphoton excitation of **4** were recorded as follows. The solution of **4** in ethanol (3 mL) was placed in a 2 × 1 cm^2 fluorescence cell and stirred continuously during irradiation with focused pulses of a Spectra Physics GCR-150-30 Nd:YAG laser (532 nm; 30 Hz; 10 mJ/pulse; pulse width ca. 10 ns). The emitted light was collected with a series of lenses and focused on a fiber optic, which couples the fluorescence light into the CCD detector system (USB2000; Ocean Optics, Inc.).

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Supporting Information Available: Analysis of the product obtained from steady-state photolysis of **1** and experimental details for quantum yield determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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