Substituent Effect on the Photobleaching of Pyrylium Salts under Ultrashort Pulsed Illumination

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Received: July 12, 2005; In Final Form: November 12, 2005

The current article presents the photobleaching properties of a group of pyrylium salts under ultrashort pulsed illumination. These pyrylium salts have the same basic chemical structure and differ only by a specific substituent. It is proven experimentally that two different mechanisms are simultaneously present to the photobleaching of all molecules studied (independently of their specific chemical structure). However, the particular parameters of each mechanism are influenced significantly by the substituent change. The experimental investigation of these parameters showed the presence of multiphoton interactions in the photobleaching of pyrylium salts depending essentially on the specific substituent.

Introduction

Laser-induced fluorescence of organic materials is widely used in the fields of physics, chemistry, biology, and medicine, especially in sensitive applications. Over the past 50 years a wealth of information has been accumulated, and the interest in this subject continuously increases. Molecules with diverse optical properties are required for special applications, but in general the suitability of a molecule for every application depends on its absorption and emission coefficients as well as on its photostability. The importance of photostability is based on the fact that its absence leads to the irreversible loss of emitted photons decreasing the sensitivity and the accuracy of measurements.

A special process that reduces the photostability of a molecule is photobleaching. This term includes all the photochemical reactions that permanently remove a molecule from the absorption−emission cycle. It is the main limiting factor in single-molecule spectroscopy.[1−3] More specifically, when the time scale of the process being studied is comparable with the survival time (photobleaching time) of the molecule no useful information for this process can be collected. Furthermore, photobleaching is a destructive parameter in laser scanning microscopy since the quality of the obtained images depends on the amount of collected fluorescence (which is limited by photobleaching).[4,5]

Until recently, the fluorescence induced by a continuous wave laser and thereafter photobleaching was occurring in the whole illuminated area. Nowadays, infrared pulsed lasers are commonly used for this purpose. The high photon densities provided from these laser sources allow molecules to absorb simultaneously two or more photons.[6] The main advantage of this procedure is that the absorption is restricted to a small volume. Thus, photobleaching is confined to this small volume suppressing (but not eliminating) the above-mentioned problems. Indeed, this property of two-photon excitation is used construc-

tively in applications such as fluorescence recovery after photobleaching (FRAP)[7] and three-dimensional optical data storage.[8−13] The highly confined, in three dimensions, photobleaching is used for measuring diffusion processes of chromophore molecules in liquids and polymer films as well as for recording irreversibly photobleached patterns in polymer films. Therefore, photobleaching is not always an undesirable effect.

From the above-mentioned discussion the need for a better understanding of the photobleaching mechanism of molecules is obvious. A large amount of work has been done for the better understanding of this process in the case of one-photon excitation.[14−16] Unlike that case, only a few data are available for photobleaching in the case of two-photon excitation.[17−19] The photophysics of photobleaching in the last case is not well-known yet. As the intensity level increases the contribution of higher-order nonlinear phenomena becomes significant. Thus, at high intensities, the mechanism of photobleaching may change fundamentally.

In a previous work of ours[20] the photobleaching of a pyrylium salt[21] was used as the recording mechanism for three-dimensional optical data storage. An experimental investigation of the photophysics involved in this photobleaching process was followed.[22] It was proven that photobleaching was occurring through two diverse mechanisms. One of the mechanisms was major and caused a fast bleaching, whereas the other was minor and caused a slow bleaching. Furthermore, it was shown that both mechanisms were three- or more-photon processes. In the present article, the investigation of the photobleaching behavior is extended to a group of pyrylium salts. This group consists of molecules with the same basic chemical structure and differs only by a specific substituent. The effect of substitution on the photobleaching properties of these pyrylium salts is discussed.

Experimental Section

Materials. Our experiments were conducted on polymer films of PMMA doped with pyrylium salts. The pyrylium salts that were investigated are 4-methoxyphenyl-2,6-bis(4-methoxyphen-

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TABLE 1: Comparison between Two-Photon Excitation and Photobleaching Parameters

	substituent	$\varphi\delta$ (GM)[a,b]	A_1 (photons/s)[c]	k_1 (s^{-1})[c]	A_2 (photons/s)[c]	k_2 (s^{-1})[c]
C_1	OCH_3	182	2×10^2	2.9	37	0.06
C_2	CH_3	513	6.8×10^3	26.8	5×10^2	0.5
C_3	H	767	1.5×10^6	130	3.3×10^4	7
C_4	Cl	390	4.7×10^3	38	1.4×10^2	0.5

[a] φ: quantum yield. δ: TPA cross section. 1 GM = 10^{-50} cm$^4\cdot$s/(photon molecule). [b] Ref 20. [c] Irradiation power ~5 mW.

yl)pyrylium tetrafluoroborate (C_1), 4-methylphenyl-2,6-bis(4-methoxyphenyl)pyrylium tetrafluoroborate (C_2), 4-phenyl-2,6-bis(4-methoxyphenyl)pyrylium tetrafluoroborate (C_3), and 4-(4-chlorophenyl)-2,6-bis(4-methoxyphenyl)pyrylium tetrafluoroborate (C_4). In parentheses are shown their abbreviated names. These molecules differ only by a specific substituent, which is OCH_3, CH_3, H, and Cl for C_1, C_2, C_3, and C_4, respectively (Table 1). A detailed characterization of these pyrylium salts regarding one- and two-photon excitation is available in our previous publication.[20] To prepare the dye-doped polymer films, salt (purified 99%) and PMMA (Aldrich) were mixed together with a weight ratio of 1%. The mixture was diluted in tetrahydrofuran (THF) and cast over a microscope slide glass to form the film. The evaporation of the solvent was made in ambient air and at room temperature (25 °C). The same procedure was followed for all pyrylium salts. It is noted that the photobleaching process of C_3 was studied previously.[22]

Experimental. The laser system used in our experiments consisted of a mode-locked Ti:Sapphire laser (Tsunami, Spectra Physics) pumped with a CW, solid-state, Nd:YLF laser (Millenia Vs, Spectra Physics) (Figure 1). This system was tunable from 740 to 850 nm and provided ultrashort laser pulses in the femtosecond regime (duration ~100 fs). A 5-fold expanding telescope was used to expand the laser beam in order to uniformly illuminate the back-aperture of an oil immersion objective lens (100×; N.A., 1.2). This tight focusing geometry made available the high intensities required for the nonlinear excitation of the samples. The irradiation power was controlled with a combination of a motorized $\lambda/2$ plate and an ultrafast polarizer (Melles Griot, 16PPB200) and measured at the back-aperture of the lens. The fluorescence signal was collected with the same lens used for excitation and separated from the input beam by a cold mirror reflecting from 400 to 600 nm (Thor-Labs, FM03). The remaining infrared irradiation was rejected with a sequence of three short pass filters. A photomultiplier tube (PMH-100-4) and a single-photon counting system (Becker and Hickl, PMS300) were used for the measurement of fluorescence. To record the sharp decay in the fluorescence caused by photobleaching a fast shutter system (rise time 3 ms, Newport 845HP) was synchronized with the single-photon counting electronics. The time resolution of the experimental setup was 250 ns which is much smaller than the resolution needed in our measurements. Each decay curve consists of 1500–12000 points depending on the time resolution selected. The choice of time resolution was made with respect to the initial fluorescence signal and in every case was as small as needed for recording correctly this signal.

Results and Discussion

To investigate the photobleaching properties of pyrylium salts the samples were continuously irradiated with ultrashort laser pulses while the resulting fluorescence was recorded at the same time. The results regarding C_2 are shown in Figure 2. A decrease in fluorescence is observed as irradiation time increases revealing the presence of photobleaching. Specifically, as irradiation time increases more molecules are removed from the normal absorption–emission cycle. The large amount of molecules stops to emit photons at the very beginning of irradiation leading to the steep decrease in fluorescence shown in the figure. The photobleaching process continues as irradiation time increases but with a much slower rate in comparison with that at the beginning. This behavior, with small variations, is common for all pyrylium salts investigated. The only function that fits to our experimental data for fluorescence signal versus irradiation time is a double-exponential decay.

$$F(t) = F_0 + A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$$

In this expression F is the fluorescence photon flux (photons/s), k_1 and k_2 are the decrease rates of fluorescence, called here
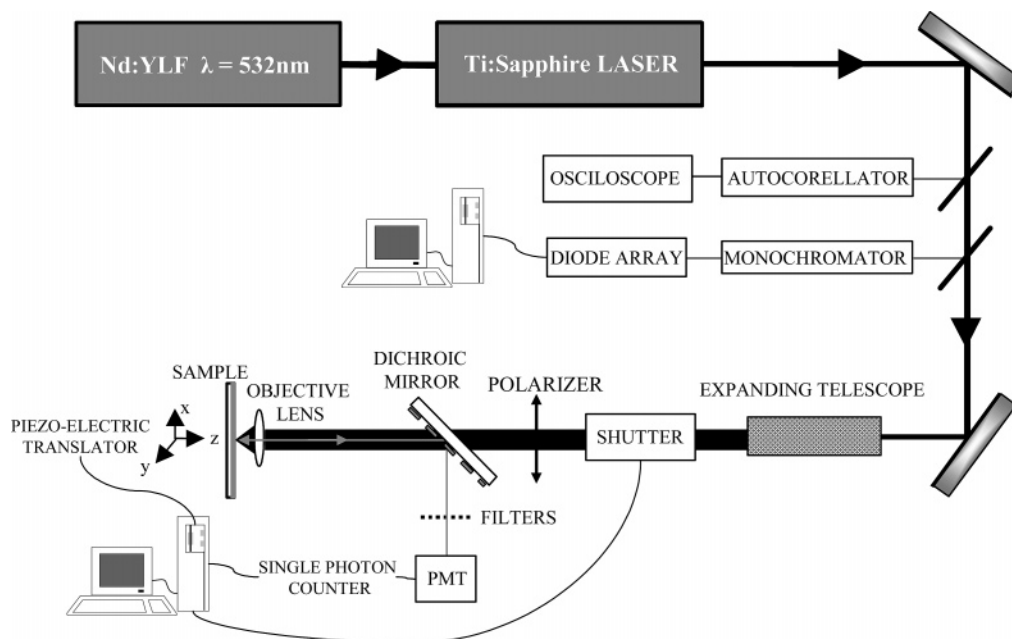

Figure 1. Experimental setup.
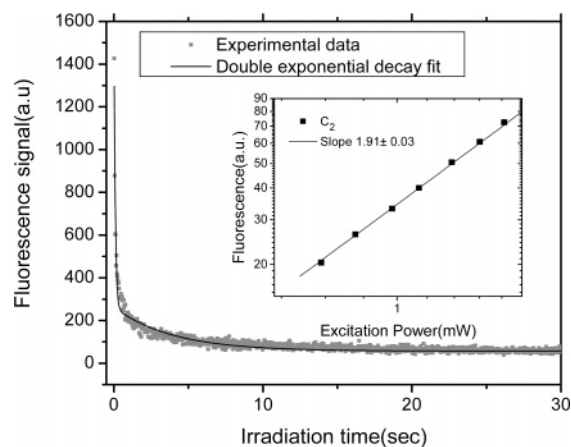
Figure 2. Fluorescence decay for pyrylium salt C_2 (wavelength λ_{exc} = 750 nm, irradiation power P = 3.8 mW, pulse duration $\tau \approx$ 100 fs). Data are fitted to a double-exponential decay (R^2 = 0.93). Inset: log to log plot of fluorescence vs excitation power.
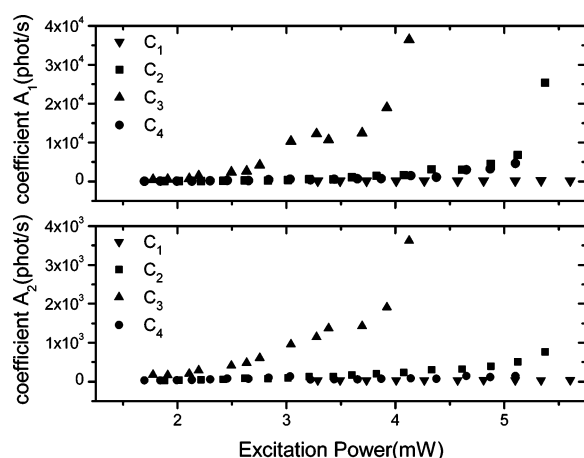
Figure 3. Contribution from each photobleaching mechanism to total fluorescence, $A_{1,2}$, for C_1, C_2, C_3, and C_4 (λ_{exc} = 750 nm, $\tau \approx$ 100 fs). The uncertainty of the calculated values ranges from 1% to 5%.

photobleaching rates (1/s), and F_0 is a noise constant (photons/ s). A_1 and A_2 are the initial contributions to the fluorescence signal from each exponential decay (photons/s) since at the beginning of irradiation ($t = 0$) the fluorescence photon flux is $F(0) = F_0 + A_1 + A_2$. The existence of two independent exponential decays denotes that the photobleaching process is occurring through two different mechanisms. The simultaneous existence of both mechanisms at the first few milliseconds of irradiation must be underlined. This is critical since a delay in the appearance of one of the mechanisms could lead to the conclusion that it originates from a photoproduct. This does not happen in our measurements, and photobleaching occurs from the pyrylium salt itself. Furthermore, these two photobleaching mechanisms are present in all pyrylium salts studied, independently of the specific chemical structure. The influence of substituent change is therefore restricted only to the specific parameters of photobleaching. These parameters will be presented in detail afterward.

Figure 3 shows the initial contribution to the fluorescence signal from each photobleaching mechanism. Regarding the first mechanism, it is clear that C_3 is the most fluorescent molecule. This may be attributed partially to its large two-photon excitation cross section (Table 1). The fluorescence signal resulting from C_2 is higher than the corresponding one of C_4 and lower than that of C_3 in agreement with their cross section values. Finally, C_1 is the less fluorescent molecule since it has the smallest two-
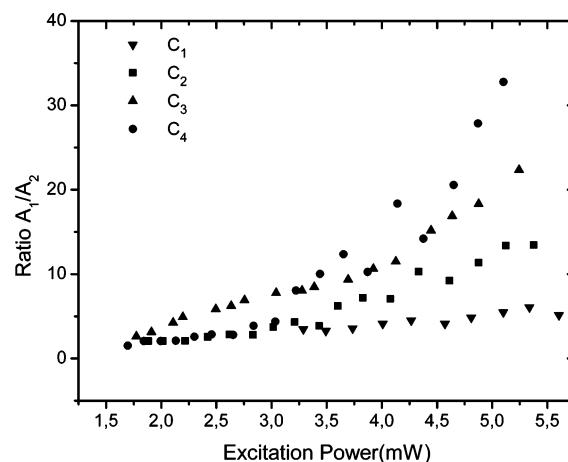

Figure 4. Relative contribution to initial fluorescence from the two mechanisms vs irradiation power for C_1, C_2, C_3, and C_4.

photon excitation (TPE) cross section. Similar observations stand for the second mechanism. The strong dependence of the initial fluorescence on the excitation power, observed in all pyrylium salts, is an indication that higher-order processes take place during photobleaching. The above rather qualitative analysis reveals in a preliminary way how the photobleaching parameters, A_1 and A_2, are affected by the changes in the basic chemical structure.

Figure 4 shows the relative initial contribution to the fluorescence signal from the two mechanisms. It can be seen that for all pyrylium salts the ratio A_1/A_2 is much greater than 1. This means that the first mechanism contributes much more to the fluorescence signal in comparison with the second one. Furthermore, this ratio is not constant but increases with irradiation power. Hence, at large power values the molecules show a preferred excitation via the first mechanism. This characteristic is more intense for C_4 in comparison with those of the other salts. The ratio A_1/A_2 of C_4 increases more quickly for irradiation powers above 3 mW than the corresponding ratio of the other molecules. This indicates a further confinement of the second mechanism, relative to the first one, caused by the specific substituent (Cl). As depicted in Figure 4, at a power value of about 5 mW the ratio between A_1 and A_2 becomes 6, 13, 22, and 33 for C_1, C_2, C_3, and C_4, respectively. The domination of the first mechanism is more than obvious.

In our previous work[22] the photobleaching rates of C_3 were found to depend on the third or even the forth power of the irradiation intensity. The behavior of photobleaching rates $k_{1,2}$ for the group of pyrylium salts we worked on will be exhibited below. The dependence of k_1 versus irradiation power for C_1, C_2, C_3, and C_4 is shown in Figure 5. The slopes of the logarithmic plots, which are shown in the graph, are 2 for C_1 and about 3 for the other three salts. Hence, our measurements show a square dependence on excitation power for C_1 and a cubical one common for the other three pyrylium salts. However, there is a quantitative divergence in the values of the photobleaching rates. More specifically, C_3 has the higher photobleaching rate in comparison with those of the other pyrylium salts. C_4 and C_2 are following, respectively. It would be expected that C_2 would be photobleached more efficiently than C_4 according to the two-photon excitation cross sections (Table 1). As mentioned above the initial contributions to the fluorescence signal from each molecule follow this "rule". Nevertheless, this is not resulted from our measurements for k_1. This discrepancy is important since it reveals the fact that photobleaching and fluorescence are occurring either from different
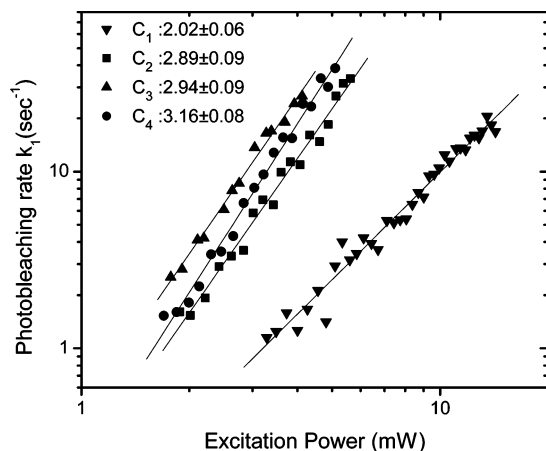
Figure 5. Log−log plots of the photobleaching rates k_1 vs irradiation power for each pyrylium salt. The fitted slopes are shown in the graph. The uncertainty of the k_1 values ranges from 1% to 5%. The power range of each data set was selected with respect to the starting point of bleaching.
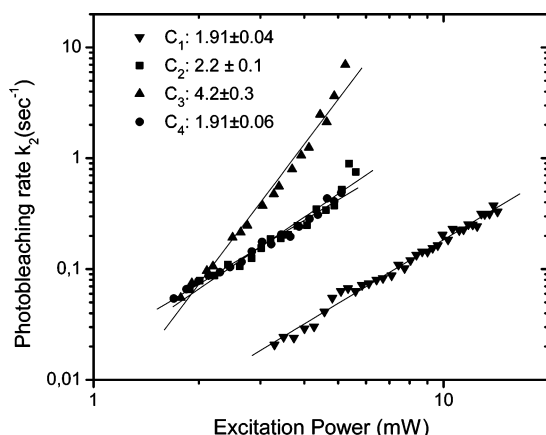
Figure 6. Log−log plots of the photobleaching rates k_2 vs irradiation power for each pyrylium salt. The fitted slopes are shown in the graph. The uncertainty of the k_2 values ranges from 1% to 5%. The power range of each data set was selected with respect to the starting point of bleaching.

electronic states of the molecule or from the same electronic states but with different efficiencies.

The photobleaching rate k_2 versus irradiation power is depicted in Figure 6. It can be easily seen that there is a significant difference in the behavior of C_3 and the other pyrylium salts. k_2 depends on the square of irradiation power for C_1, C_2, and C_4, whereas it depends on the forth power for C_3. The small change in the chemical structure caused a significant change in the nature of the second photobleaching mechanism. Furthermore, C_3 has the higher photobleaching rate k_2 (because of its higher-order power dependence), C_2 and C_4 have lower ones but comparable with each other, and finally C_1 has the lowest one.

The group of investigated pyrylium salts are found to have large two-photon absorption cross sections, ranging from 267 to 1722 GM.[20] Hence, it was expected that, at very low power values (where photobleaching is negligible), fluorescence would depend on the square of the incident power. This was confirmed experimentally for all pyrylium salts (data for a representative salt are shown in the inset of Figure 2). The meaning of this result is that the molecule absorbs two photons to reach the first excited state and then relaxes by emitting one photon. *Unwanted* effects such as self-quenching, saturation, etc. are insignificant. At higher power levels photobleaching makes the

situation more complicated. The cubical dependence of the photobleaching rate k_1 on irradiation power for the three pyrylium salts shows that the bleaching state is reached with the absorption of three photons. The analysis followed in our previous work[22] proved that this process is not a simultaneous three-photon absorption but a stepwise one. In more detail, two photons are absorbed simultaneously to reach the first excited state, and one more photon is absorbed from this state to reach the bleaching state. Thus, photobleaching through this mechanism is an excited-state absorption process. In the case of C_1 the square dependence of k_1 on irradiation power indicates that only two photons are involved in the bleaching procedure. However, at the wavelength of our measurements C_1 can be excited only with a two-photon excitation process leading to the conclusion that photobleaching is occurring from the two-photon excited state. As we noticed above the substituent change affects fluorescence and photobleaching in a different way. For example, the CH_3 (C_2) as a substituent raises fluorescence in comparison with that of Cl (C_4). In the case of the photobleaching rate k_1 the situation is reversed, namely, the substituent change lowers the photobleaching efficiency. This observation shows that the substituent change causes considerable modifications not only on the two-photon absorption properties but also on the excited-state ones. Indeed, these modifications are realized in a different manner.

The substitution effect on the other mechanism is more decisive. When the substituent is H (C_3) the mechanism is a stepwise four-photon process.[22] Two photons are absorbed simultaneously to reach the excited state, and from this state with the simultaneous absorption of two more photons the molecule is bleached. Photobleaching is therefore again an excited-state absorption. For the other three pyrylium salts, where the substituents are OCH_3 (C_1), CH_3 (C_2), and Cl (C_4), photobleaching occurs with the simultaneous absorption of only two photons. In this case photobleaching is not caused by an excited-state absorption.

Many times the irradiation of a molecule with ultrashort laser pulses causes the generation of photoproducts affecting significantly the photobleaching mechanisms. The slow nature of the second mechanism may lead someone to assume that it occurs from a fluorescent photoproduct and not from the pyrylium salt. A discussion on this assumption is following. As shown in Figure 4 for pyrylium salt C_3 at the low-power value $P = 1.88$ mW the ratio of the initial fluorescence signals, A_1/A_2, is 2.6. This means that the contribution to the fluorescence signal from the two mechanisms is comparable, and thus, the first mechanism does not overlap the second one. The photobleaching rates k_1 and k_2 are 2.53 s⁻¹ and 0.055 s⁻¹, respectively (Figures 5 and 6). These values correspond to a decrease of the fluorescence signal at the 1/e of the maximum fluorescence at ∼400 ms ($1/k_1$) and ∼16.5 s ($1/k_2$) for mechanisms 1 and 2, respectively. The slow mechanism is therefore more than 40 times slower than the fast one. If we assume that the second mechanism occurs from a photoproduct at the first 400 ms of irradiation an accumulation of fluorescent photoproducts should be generated (due to the very slow photobleaching of these photoproducts). This accumulation would lead to an increase of fluorescence through this mechanism. Therefore, at the beginning of irradiation (0−400 ms) a significant deviation from the double-exponential decay should be observed. The time scale of the phenomenon described above is large enough to be recorded accurately from our experimental setup (time resolution of only a few milliseconds is sufficient). However, such a deviation was not observed in our measurements. In every case

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a double-exponential decay was recorded. Thereafter, we conclude that the second mechanism is not generated from some fluorescent photoproduct but from the pyrylium salt itself. We have no indication for a divergence from this behavior at the whole power range of the photobleaching measurements. Similar results stand for the other pyrylium salts C_1, C_2, and C_4. The above discussion makes the reliability of our measurements and results more evident.

According to Eggeling et al. and Mertz photobleaching may occur from an excited-state absorption in a singlet or a triplet manifold.[16,23] The excited singlet state is reached with the simultaneous absorption of two photons, whereas the triplet state is reached with the intervention of intersystem crossing. The presence of two mechanisms in the photobleaching of pyrylium salts with different contributions to the fluorescence signal as well as with much different photobleaching rates is a strong indication that both singlet and triplet states are involved. The small contribution to the fluorescence signal from the slow mechanism is an indication that it is a triplet state absorption since fluorescence is mainly related to the first excited state S_1. This assumption is in agreement with its slow nature in comparison with the other mechanism. On the other hand, the fast mechanism is responsible for the majority of the generated fluorescence. Thus, we suggest that the fast and dominant photobleaching mechanism is a singlet to singlet process, whereas the minor and slow one is a triplet to triplet process. In the cases where the slow mechanism is not an excited-state absorption (as mentioned above) the molecule is bleached in the triplet state without the extra absorption of one photon. This proposal is enforced from the very low measured photobleaching rates k_2 for these salts (Table 1).

Conclusions

In conclusion, an experimental investigation of the photobleaching properties of a group of pyrylium salts under ultrashort pulsed illumination was realized. It was confirmed that two photobleaching mechanisms are simultaneously present in all the salts studied. In addition, it was proved that these mechanisms are multiphoton processes. The involvement of two, three, or even four photons in these processes was observed depending on the specific chemical structure. Furthermore, the contribution of each mechanism to the total photobleaching process as well as the efficiency of photobleaching was found to depend essentially on the substituent changes in the basic chemical structure. Finally, it was shown that photobleaching and fluorescence are mainly not occurring from the same electronic states.

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