Kinetics Study of the Photocleavage of (Coumarin-4-yl)methyl Esters

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Received: February 2, 2005; In Final Form: April 11, 2005

Photolabile coumarinylmethyl esters of biomolecules (caged compounds) are new tools for studying spatial and time-dependent aspects of signal transduction in living cells. Herein we describe a fluoresence spectroscopic method for the determination of the rate constants of the photolysis steps of such caged compounds using (6.7-dimethoxycoumarin-4-yl)methyl diethyl phosphate (DMCM-DEP) and sodium (6,7-dimethoxycoumarin-4-yl)methyl sulfate (DMCM-S). DMCM-DEP and DMCM-S are caged compounds which photorelease a proton, the corresponding acid anion, and the strongly fluorescent alcohol DMCM-OH upon excitation. The results of stationary and time-resolved measurements of the photochemistry and the luminescence of both caged compounds indicate that DMCM-OH is produced already during the excitation pulse. The quantitative analysis of the data demonstrates that the first step of the reaction—heterolytic bond cleavage of the coumarinylmethyl ester leading to the ion pair of a DMCM cation and an acid anion—is very fast with a rate constant of $k_1 \approx 2 \times 10^{10}$ s^{-1}. Recombination of the ion pair occurs with a rate constant of $k_{rec} \approx 2.3 \times 10^9$ s^{-1} and is about 10 times faster than the competing hydrolysis reaction of the DMCM cation yielding DMCM-OH and a proton. Thus, both caged compounds belong to the fastest phototriggers known.

Introduction

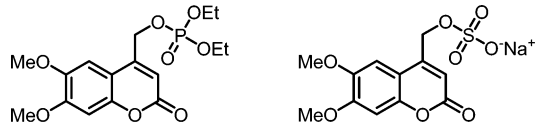
The photorelease of biomolecules from inactive precursors (caged compounds) is a powerful tool for studying the fast kinetics or spatial heterogeneity of biochemical responses in cell or tissue culture.[1−3] In caged compounds, the biological recognition or activity has been disabled by chemical modification at an essential functionality by introducing a photosensitive protecting group (caging group) until light of a specific color uncages and activates the probe.

(Coumarin-4-yl)methyl derivatives are newly developed caging groups and have been successfully applied to protect biological activity in phosphates,[4−13] carboxylates,[6,14] sulfates,[13] sulfonates,[13] diols,[15] and carbonyl compounds.[16] Amino and hydroxyl functionalities were also protected via carbamate,[14,17−19] or carbonate linker.[20]

The rate of the photorelease of the biomolecule from caged compounds is a very important parameter. It should be as fast as possible. (Coumarin-4-yl)methyl derivatives belong to the fastest systems; however, accurate results are still rare. In this paper, we present results of stationary and time-resolved investigations of the photochemistry and the luminescence of (6,7-dimethoxycoumarin-4-yl)methyl diethyl phosphate (DMCM-DEP) and sodium (6,7-dimethoxycoumarin-4-yl)methyl sulfate (DMCM-S) which allow a closer view on the uncaging processes (Scheme 1).

DMCM-DEP and DMCM-S are recently developed weakly fluorescent caged protons which photorelease protons and the strongly fluorescent 6,7-dimethoxy-4-(hydroxymethyl)coumarin (DMCM-OH) in the nanosecond time scale.[13] Earlier, we found also very high rate constants for the release of cyclic nucleotides and of ATP, respectively, from the corresponding coumarinylmethyl esters.[6,7,10]

SCHEME 1



Experimental Details

DMCM-OH, DMCM-DEP, and DMCM-S were prepared as previously described, purified by preparative HPLC, and stored in the dark.[9,13] Contamination of the caged compounds with the product alcohol could not be detected by fluorescence spectroscopy, indicating an upper limit of ≪0.1%. The methods for determination of fluorescence quantum yields and photochemical quantum yields have already been reported earlier.[7,9] The photophysical and photochemical experiments have all been carried out in CH_3CN/H_2O (HEPES buffer, pH 7.2) 5:95 vol/vol at about 23 °C under yellow light. Time-resolved fluorescence rise and decay curves have been recorded in right-angle arrangement. As an excitation source, we used a MSC 1600 N_2 laser from LTB (337 nm, pulse width 0.5 ns, maximum pulse energy 0.7 mJ). The fluorescence was excited in 1 cm × 1 cm fluorescence cells, observed through suited interference filters with a wide aperture collection lens and detected by an amplified AD 110 silicon avalanche diode from opto-electronics with a rise time of 600 ps. All signals were digitized and fed to a Tektronix TDS 620A storage oscilloscope. Variation of the laser pulse energy was possible by use of suited glass filters attenuating the laser beam.

The apparatus function AF(t) was obtained by irradiating a layer of MgO with N_2 laser pulses and recording the reflected

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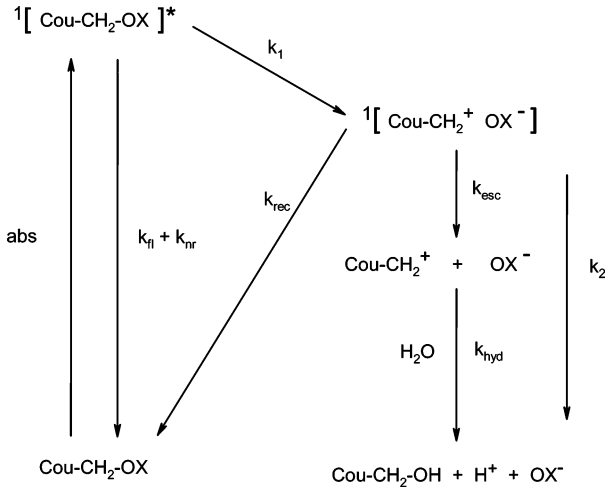
10.1021/jp050581k CCC: $30.25
Published on Web 05/21/2005

light by the setup. Hereby, the signal was averaged over 50 laser shots. The AF(t) curve comprises the deformation of an instantaneously decaying signal due to the finite laser pulse profile and the time response of the detection system. Therefore, convolutions of suited kinetic equations $Y(t)$ with AF(t) can be fitted to experimentally recorded florescence rise and decay curves $I(t)$. In the case of photostable fluorescing compounds, the signals were also averaged to further improve the signal-to-noise ratio, whereas, with photolabile caged compounds, only single-shot experiments were allowed. The evaluation of the decay time of the fluorescing alcohols was performed by nonlinear least-squares fitting routines convoluting the apparatus function with a monoexponential decay function.[21,22]

Results and Discussion

The photoreaction of (coumarin-4-yl)methyl esters Cou-CH_2-OX produces in aqueous solvents a mixture of the (coumarin-4-yl)methyl alcohol Cou-CH_2-OH and the acid HOX or its acid anion and a proton. After light absorption of Cou-CH_2-OX, relaxation takes place to the lowest excited singlet state S_1 which is denoted as ^{1}[Cou-CH_2-OX]* in Scheme 2.

SCHEME 2



In previous works, we have shown that the reaction proceeds from the lowest excited singlet ^{1}[Cou-CH_2-OX]* via heterolytic bond cleavage, forming with rate constant k_1 the singlet ion pair ^{1}[Cou-CH_2^+ OX^-] in the first step.[6] Competitively, ^{1}[Cou-CH_2-OX]* decays by fluorescence and nonradiative deactivation processes with rate constants k_{fl} and k_{nr}. Recombination of the ion pair leads back to the ground state of Cou-CH_2-OX with rate constant k_{rec}. Product formation was proposed to occur in two steps.[6,9] Escape from the common solvent cage affords the solvent separated ions Cou-CH_2^+ and OX^-. The cation Cou-CH_2^+ reacts then with water followed by a very fast deprotonation to yield the product alcohol Cou-CH_2-OH and a proton. The respective first-order and pseudo-first-order rate constants are k_{esc} and k_{hyd}.

Since our experiments allow no distinction between these subsequent reactions, it is reasonable to combine both. For the general reaction sequence A $\rightarrow$ B, B $\rightarrow$ C with the corresponding rate constants k_1' and k_2', the concentration growth of the final product C is well approximated by a single-exponential rise with rate constant $k' = k_1' \times k_2'/(k_1' + k_2')$; see eq 1.

$$[\mathrm{C}] \approx [A]_0 \times \{1 - \exp(-k' \times t)\} \qquad (1)$$

Thus, the two-step reaction from the ion pair to the product alcohol can likewise be approximated by an exponential rise kinetic with rate constant $k_2 = k_{esc} \times k_{hyd}/(k_{esc} + k_{hyd})$. The smaller of both rate constants determines principally the value of k_2.

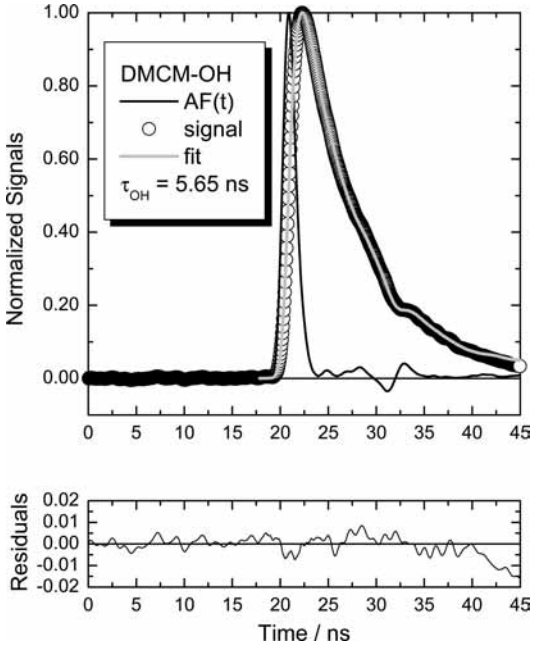


Figure 1. Normalized fluorescence decay of DMCM-OH in CH_3CN/H_2O (HEPES buffer) 5:95 vol/vol. Emission wavelength 433 nm. The fit is a convolution of AF(t) with $Y(t) = A \times \exp(-t/\tau_{OH})$.

The long wavelength absorption band of the caged compound, the coumarinylmethyl ester, and of the product, the coumarinylmethyl alcohol, corresponds for both to the excitation of the coumarin chromophore. The strength and location of this absorption band varies only insignificantly between both species, as has been demonstrated for six differently substituted coumarinylmethyl ester/alcohol pairs.[9] According to basic photophysics, the rate constants of radiative deactivation of the lowest excited singlet state S_1 of the corresponding ester and alcohol are therefore almost equal. Because of the effective electronic decoupling of the coumarin chromophore and the O-bound substituent X by the $-CH_2-$ linker, it is reasonable to assume that also the rate constants of nonradiative physical deactivation of S_1 are the same for the caged compound and the corresponding alcohol. Hence, we set $k_{fl} = k_{fl}^{OH} = k_{fl}^{C}$ and $k_{nr} = k_{nr}^{OH} = k_{nr}^{C}$.

According to Scheme 2, eqs 2 to 5 describe the fluorescence quantum yields φ_{fl}^{OH} and φ_{fl}^{C} of the product alcohol and the caged compound, the fluorescence lifetime τ_{OH} of the product alcohol, and the chemical quantum yield φ_{ch}^{C} of the caged compound, which is the product of the quantum yield φ_1 of ion pair formation and the efficiency f_2 of alcohol formation in the decay of the ion pair.

$$\varphi_{fl}^{OH} = k_{fl}/(k_{fl} + k_{nr}) \qquad (2)$$

$$\varphi_{fl}^{C} = k_{fl}/(k_{fl} + k_{nr} + k_1) \qquad (3)$$

$$\tau_{OH} = 1/(k_{fl} + k_{nr}) \qquad (4)$$

$$\varphi_{ch}^{C} = k_1/(k_{fl} + k_{nr} + k_1) \times k_2/(k_{rec} + k_2) = \varphi_1 \times f_2 \qquad (5)$$

Figure 1 displays the experimental fluorescence decay curve of the product alcohol DMCM-OH and the corresponding fit obtained by convolution of the apparatus function AF(t) with the monoexponential decay kinetic $Y(t) = A \times \exp(-t/\tau_{OH})$. The residuals are additionally given.

$\tau_{OH} = 5.65 \pm 0.3$ ns results for the fluorescence lifetime of DMCM-OH. With that value and $\varphi_{fl}^{OH} = 0.57 \pm 0.05$, we

TABLE 1: Photophysical and Photochemical Data of (6,7-Dimethoxycoumarin-4-yl)methyl Caged Compounds (Solvent CH_3CN/H_2O (HEPES Buffer) 5:95 vol/vol)

caged compound	φ_{fl}^C	φ_{ch}^C	$k_1/10^9\ s^{-1}$	τ_C/ns	φ_1	f_2	k_{rec}/k_2	τ_M/ns	τ_{OH}/ns
DMCM-DEP	0.005[a]	0.08[b]	20[c]	0.05[d]	0.99[e]	0.08[f]	11[f]	0.16[g]	4.87[h]
DMCM-S	0.006[a]	0.09[b]	17[c]	0.06[d]	0.99[e]	0.09[f]	10[f]	0.21[g]	5.83[h]

[a] Average uncertainty: ±15%. [b] Average uncertainty: ±10%. [c] Average uncertainty: ±25%. [d] Average uncertainty: ±27%. [e] Average uncertainty: ±10%. [f] Average uncertainty: ±13%. [g] Average results of time-resolved single-pulse experiments: ±40%. [h] Average results of time-resolved single-pulse experiments: ±15%.

calculate via eqs 2 and 4 the rate constants $k_{fl} = (1.01 \pm 0.08) \times 10^8\ s^{-1}$ and $k_{nr} = (0.76 \pm 0.06) \times 10^8\ s^{-1}$. Combining these numbers with the experimental values of φ_{fl}^C and φ_{ch}^C for DMCM-DEP and DMCM-S, we obtain via eqs 3 and 5 the rate constants k_1, the decay times $\tau_C = 1/(k_{fl} + k_{nr} + k_1)$ of the S_1 excited caged compounds, as well as values of φ_1 and f_2, and finally the ratios $k_{rec}/k_2 = 1/f_2 - 1$ listed in Table 1. This evaluation demonstrates that the primary reaction of both caged compounds is very fast with rate constants k_1 of up to $2 \times 10^{10}\ s^{-1}$ and decay times τ_C down to about 50 ps.

It seems possible that the product alcohol is even already formed during the laser pulse used for excitation for these caged compounds. In this case, the fluorescence of the just formed DMCM-OH could eventually be excited by a second photon of the same laser pulse and would add to the direct fluorescence of the caged compound to yield the overall fluorescence decay signal $I(t)$. Considering the pulse width of the laser of $\tau_{1/2} = 0.5$ ns, this would require a product rise time of $\tau_A \approx 1$ ns.

Since intermediates have to be passed on the way to the product alcohol, $\tau_A > \tau_C$ holds true. According to Scheme 2, the product alcohol is formed in a sequential reaction with branching, which is written in general form as A → B, B → C, B → D with rate constants k_1', k_{2C}', and k_{2D}'. The concentration growth of final product D can again well be approximated by a single-exponential rise with rate constants $k_2' = k_{2C}' + k_{2D}'$ and $k' = k_1' \times k_2'/(k_1' + k_2')$; see eq 6.

$$[D] \approx [A]_0 \times (k_{2D}'/k_2') \times \{1 - \exp(-k' \times t)\} \qquad (6)$$

Thus, the rise time of the product alcohol after excitation of the caged compound is according to Scheme 2 given by eq 7

$$\tau_A \approx \{k_1 + (k_2 + k_{rec})\}/\{k_1 \times (k_2 + k_{rec})\} \qquad (7)$$

The question of whether for both caged compounds DMCM-OH is already formed during the laser pulse can be answered by analysis of the time-resolved overall fluorescence decay. The increase of the product alcohol concentration follows the sigmoidal rise function sig(t), which is normalized to a unity amplitude, and whose width and location on the time scale depend on the true laser pulse profile LP(t, $\tau_{1/2}$) and on τ_A according to the convolution integral of eq 8.

$$\mathrm{sig}(t) = \mathrm{LP}(t, \tau_{1/2}) \otimes \{1 - \exp(-t/\tau_A)\} \qquad (8)$$

The actual amplitude of the rise curve of the product alcohol depends on the product $N_p \times F^C \times \varphi_{ch}^C$ of the number N_p of photons entering the irradiated sample volume, the fraction F^C of photons absorbed by the caged compound, and the photochemical quantum yield φ_{ch}^C. Estimations based on data of N_p, F^C, and φ_{ch}^C and considering the irradiated sample volume as well as the concentration of the caged compound demonstrate that the conversion of the caged compound amounted even at maximum applied pulse energy only to about 0.3%. Since the long wavelength absorption bands of the caged compound and product alcohol are the same, the fraction F^{OH} of photons absorbed by the product alcohol was at maximum $F^{OH} = 0.003 \times F^C$.

If the product alcohol is already formed during the single excitation pulse, both the caged compound and the product alcohol are excited and contribute to the overall fluorescence kinetics $Y(t)$ according to eq 9.

$$Y(t) = N_p \times F^C \times \varphi_{fl}^C \times \exp(-t/\tau_C) + N_p \times F^C \times \varphi_{ch}^C \times \mathrm{sig}(t) \times N_p \times F^{OH} \times \varphi_{fl}^{OH} \times \exp(-t/\tau_{OH}) \qquad (9)$$

sig(t) could not be calculated, since the exact form of LP(t, $\tau_{1/2}$) was unknown. Furthermore, the number of variables (F^C, F^{OH}, τ_C, τ_A, and τ_{OH}) of eq 9 is too large for evaluation. Thus, simplifications have to be introduced. To this end, we substitute the product of the sigmoidal rise curve sig(t) with the exponential decay curve $\exp(-t/\tau_{OH})$, which represents the rise and decay of the fluorescence caused by excitation of the just produced alcohol by a second photon of the same laser pulse, by the biexponential rise (τ_A) and decay (τ_{OH}) function $1/(1 - \tau_A/\tau_{OH}) \times \{\exp(-t/\tau_{OH}) - \exp(-t/\tau_A)\}$. The error introduced by this simplification is not large, since the weighting factor of the fluorescence of the caged compound is always larger than that of the product alcohol due to $F^{OH} \lesssim 0.003 \times F^C$. Thus, the contribution of the fluorescence of the caged compound to the overall fluorescence signal dominates, particularly in the rising part because of $\tau_C < \tau_A$. Furthermore, we set

$$A' = N_p \times F^C \times \varphi_{fl}^C \qquad (10)$$

$$B' = N_p^2 \times F^C \times \varphi_{ch}^C \times F^{OH} \times \varphi_{fl}^{OH} \qquad (11)$$

and obtain

$$Y(t) = A' \times \exp(-t/\tau_C) + B'/(1 - \tau_A/\tau_{OH}) \times \{\exp(-t/\tau_{OH}) - \exp(-t/\tau_A)\} \qquad (12)$$

τ_C is for both caged compounds much shorter than the minimum possible time resolution of our setup of about 0.2 ns. Therefore, $\tau_C = 50$ ps can be taken as constant. Efforts to fit the experimental fluorescence curves $I(t)$ by convolutions of AF(t) with $Y(t)$ according to eq 12 with four variables (A', B', τ_A, and τ_{OH}), however, did not lead to meaningful results. Therefore, we simplified the kinetics further, setting for both τ_C and τ_A the common time constant τ_M, which can roughly be interpreted as the mean value of both. This leads to eq 13.

$$Y(t) = (A' - B'/(1 - \tau_M/\tau_{OH})) \times \exp(-t/\tau_M) + B'/(1 - \tau_M/\tau_{OH}) \times \exp(-t/\tau_{OH}) \qquad (13)$$

$B = B'/(1 - \tau_M/\tau_{OH})$ is positive, since $\tau_{OH} > \tau_M$. Since A' is much larger than $B'/(1 - \tau_M/\tau_{OH})$, vide supra, $A = (A' - B)$ is also positive and we obtain finally eq 14 as an approximation for the overall fluorescence kinetics $Y(t)$.

$$Y(t) = A \times \exp(-t/\tau_M) + B \times \exp(-t/\tau_{OH}) \qquad (14)$$

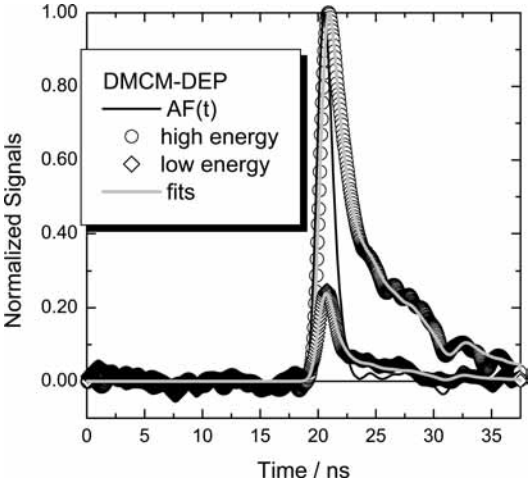


Figure 2. Normalized fluorescence decays of DMCM-DEP in CH_3CN/H_2O (HEPES buffer) 5:95 vol/vol at different laser pulse energies. Emission wavelength 433 nm. The fits are convolutions of AF(t) with eq 14. High laser pulse energy: $A = 9.0 \times 10^8$, $\tau_M = 0.11$ ns, $B = 2.9 \times 10^7$, and $\tau_{OH} = 4.7$ ns. Low laser pulse energy: $A = 2.0 \times 10^9$, $\tau_M = 0.18$ ns, $B = 2.0 \times 10^8$, and $\tau_{OH} = 5.2$ ns.

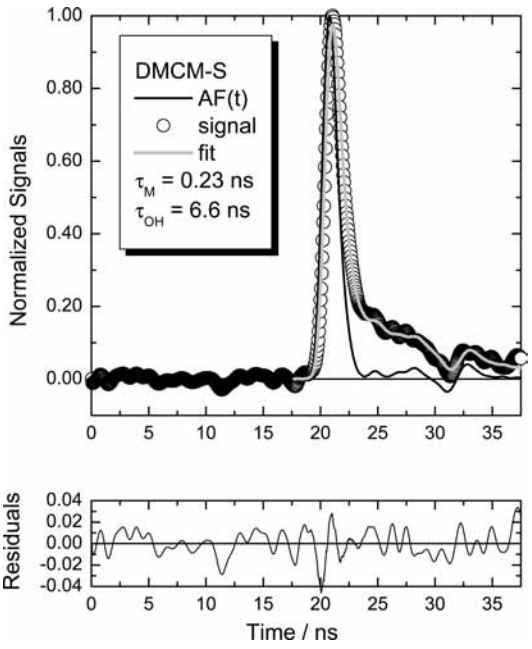


Figure 3. Normalized fluorescence decay of DMCM-S in CH_3CN/H_2O (HEPES buffer) 5:95 vol/vol. Emission wavelength 433 nm. The fit is a convolution of AF(t) with eq 14.

Convolutions of AF(t) with the function $A \times \exp(-t/\tau_M) + B \times \exp(-t/\tau_{OH})$ with four variables (A, B, τ_M, and τ_{OH}) could well be fitted to the experimental fluorescence curves $I(t)$. The quality of the fits obtained is very good, as is illustrated by Figures 2 and 3, although the $I(t)$ curves are in part noisy results of single laser pulse experiments. Moreover, the values resulting for τ_M and τ_{OH} listed in Table 1 are reasonable. The fluorescence lifetime of $\tau_{OH} = 5.65 \pm 0.3$ ns obtained with pure solutions of DMCM-OH agrees in the limits of mutual uncertainty very well with the mean values of τ_{OH} determined with pure solutions of DMCM-DEP and DMCM-S; see Table 1. Very short time constants τ_M of about 0.2 ns have been found for both caged compounds.

Variation of the laser pulse energy influences the preexponential B corresponding to the product alcohol fluorescence much stronger than the preexponential A of the fluorescence of the caged compound; see Figure 2. Since two photons are required for excitation of the product alcohol and only one is required for excitation of the caged compound, A' should linearly and B' should quadratically depend on N_p and thus on the laser pulse energy; see eqs 10 and 11. Since the fits according to eq 14 yield $B = B'/(1 - \tau_M/\tau_{OH})$ and $A = (A' - B)$, B should vary with A even stronger than quadratically. B depends for DMCM-DEP as well as DMCM-S approximately quadratically on A in the low energy region, as is shown by the double-logarithmic plot of Figure 4.

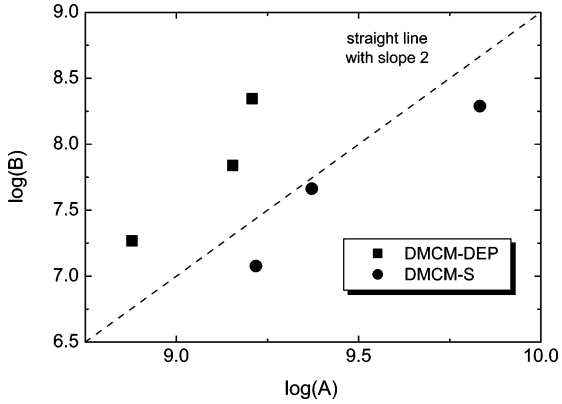


Figure 4. Double-exponential plot of average preexponential factors B and A. The straight line indicates quadratical dependence of B with A. For details, see text.

The deviation from the expected stronger dependence could well be caused by the inner filter effect of the ion pair, which is the precursor of the product alcohol and probably also absorbs the laser light. In any case, the dependence of B on A is much stronger than linear for DMCM-DEP and DMCM-S, in accordance with the fast formation of DMCM-OH during the excitation laser pulse.

The analysis of the photokinetic data demonstrates that the primary reaction is very fast for both caged compounds. Rate constants k_1 of up to 2×10^{10} s^{-1} have been found for the heterolytic bond cleavage of DMCM-DEP and DMCM-S leading to ^{1}[Cou-CH_2^+ OX^-] ion pair formation; see Table 1. In fact, fast reactions can be expected, since the diethyl phosphate anion and the sulfate anion are very weak bases and known as excellent leaving groups.

The time-resolved experiments yielded very short time constants τ_M of about 0.2 ns for DMCM-DEP and DMCM-S. Considering τ_M as the mean value of τ_C and τ_A, rise times of DMCM-OH of $\tau_A \approx 0.4$ ns can be estimated which indicate a sub-nanosecond photorelease of the protons. Hence, caged compounds based on the coumarin caging group are the most rapidly photolyzing caged compounds known.

The above result allows also the investigation of the processes competing in the decay of the ion pair. The ratio $k_1/(k_2 + k_{rec})$ determines the magnitude of τ_A; see eq 7. If k_1 were much smaller than $k_2 + k_{rec}$, then $\tau_A \approx 1/k_1$ and $\tau_A \approx 0.05$ ns would hold true for both ($k_1 \approx 2 \times 10^{10}$ s^{-1}) caged compounds. In that case, however, the primary reaction forming the ion pair would be the rate-determining step and the subsequent decay of ^{1}[Cou-CH_2^+ OX^-] would have to be significantly faster; that is, $k_2 + k_{rec} > 2 \times 10^{10}$ s^{-1} should be valid. Since the efficiency of alcohol formation in the decay of ^{1}[Cou-CH_2^+ OX^-] amounts to $f_2 \approx 0.1$ for both caged compounds, the unreasonably large rate constant $k_2 > 2 \times 10^9$ s^{-1} would be the consequence for ion pair dissociation and Cou-CH_2^+ hydrolysis. Furthermore, $\tau_A \approx 0.05$ ns is much smaller than the above derived estimate $\tau_A \approx 0.4$ ns from time-resolved experiments.

Thus, the inequality $k_1 > (k_2 + k_{rec})$ can be assumed, leading to a product rise time of $\tau_A \approx 1/(k_2 + k_{rec})$ which is controlled

by the decay of the ion pair. $\tau_A \approx 0.4$ ns corresponds to $k_2 + k_{rec} \approx 2.5 \times 10^9\ s^{-1}$ which indicates a fast decay of the ion pair ^{1}[Cou-CH_2^+ OX^-] of DMCM-DEP and DMCM-S which is dominated by ion pair recombination to the ground states of DMCM-DEP and DMCM-S with $k_{rec} \approx 2.3 \times 10^9\ s^{-1}$. Possibly, it is the only weak stabilization of the (6,7-dimethoxycoumarin-4-yl)methyl carbocation combined with the high excess energy which leads to that fast recombination reaction. The overall rate constant of product formation from the ion pair (cage escape and hydrolysis of the Cou-CH_2^+ ion) is significantly smaller but with $k_2 \approx 2.5 \times 10^8\ s^{-1}$ still rather large. This is probably also the consequence of the high water content of 95 vol % of the solvent mixture. Most probably, H_2O molecules are present in the inner surface of the cage surrounding the ion pair which could act as potential reaction partners of Cou-CH_2^+.

Therefore, product formation could occur in competition with recombination directly in the reaction of the Cou-CH_2^+ cation with a neighboring water molecule followed by a very fast deprotonation step; that is, the distinction between cage escape and hydrolysis reaction still made in Scheme 2 could well be meaningless.

The diffusion-controlled rate constant amounts to $k_{diff} \approx 7 \times 10^9\ M^{-1}\ s^{-1}$ in water at 23 °C. Neglecting a possible effect of preferential solvation of the ion pair by CH_3CN, the concentration of H_2O in the direct surroundings of the ion pair amounts to 53 M. Assuming that only half of the inner surface of the cage is accessible for the reaction of Cou-CH_2^+ with H_2O, an upper limit of the rate constant of $2 \times 10^{11}\ s^{-1}$ could hold true, indicating that the actual hydrolysis reaction occurs not barrierless. This rough estimation also demonstrates that the reaction of Cou-CH_2^+ with OH^- is not important for DMCM-OH formation. The concentration of OH^- is in HEPES buffered water (pH 7.2) approximately 9 orders of magnitude smaller than the concentration of H_2O, leading to a correspondingly smaller upper limit of the rate constant of the reaction of Cou-CH_2^+ with OH^- being much smaller than the actual value of k_2.

Acknowledgment. Financial support by the Adolf Messer Stiftung and the Fonds der Chemischen Industrie is gratefully acknowledged.

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