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(Coumarin-4-yl)methyl Ester of cGMP and 8-Br-cGMP: Photochemical Fluorescence Enhancement

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We investigated the spectroscopic, photophysical, and photochemical properties of novel caged cyclic nucleotides, the axial and equatorial (7-methoxycoumarin-4-yl)methyl esters of cGMP and of 8-Br-cGMP. The competition between fluorescence and photochemical ester cleavage results in a strong quenching of the coumarin fluorescence. This fluorescence quenching is enhanced additionally by the donor–acceptor interaction between the coumarin unit and the nucleic base, pronounced in the axial configuration. On photochemical decaging the fluorescence intensity increases up to 48-fold and more. This photochemical fluorescence enhancement can be used for the description of the temporal and local distribution of biologically active cGMP derivatives.

KEY WORDS: Caged nucleotide; fluorescence quenching; photochemical ester cleavage; configuration.

INTRODUCTION

Photolysis of caged compounds using near-UV light has been used by many biological investigators as a means to release biological effector molecules within living systems.[1-5] A variety of caging groups and caged compounds exists, including caged nucleotides, neurotransmitters, chelators, and drugs.[4-9] Photolabile (caged) compounds which rapidly release cGMP and 8-Br-cGMP are widely used for *in situ* studies of signaling pathways inside cells.[4] Our goal was to develop caged cGMP and 8-Br-cGMP derivatives which show a strong change in fluorescence properties, especially in fluorescence intensity, during the decaging photo reaction.

We present novel caged compounds [MCM-cGMP, (7-methoxycoumarin-4-yl)methyl ester of cGMP; and MCM-8-Br-cGMP, (7-methoxycoumarin-4-yl)methyl ester of 8-Br-cGMP] which release biologically active cGMP or hydrolysis-resistant analogous 8-Br-cGMP by photochemical cleavage of the phosphoric acid ester bond.

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The liberation of 4-hydroxymethyl-7-methoxy-coumarin ($HOCH_2$-7-MC) on photolysis is combined with a strong increase in the fluorescence ability of the solution caused by the extreme difference in the fluorescence quantum yields of the investigated caged compounds MCM-cGMP and MCM-8-Br-cGMP and $HOCH_2$-7-MC. Here we investigate the fluorescence properties of the corresponding coumarin derivatives and discuss the reasons for fluorescence quenching in the caged compounds.

EXPERIMENTAL

The synthesis of MCM-cGMP and MCM-8-Br-cGMP is described previously.[4,10] The axial and equatorial isomers are separated using preparative HPLC.[4] All preparation and measurements were done under yellow light.

The absorption spectra were recorded using a U-3410 spectrophotometer (Hitachi, Japan) combined with a computer and the software package SPECTRACALC. The fluorescence spectra were measured using a MPF-2A fluorescence spectrometer (Hitachi–Perkin Elmer) combined with a correction and digitalization unit. The

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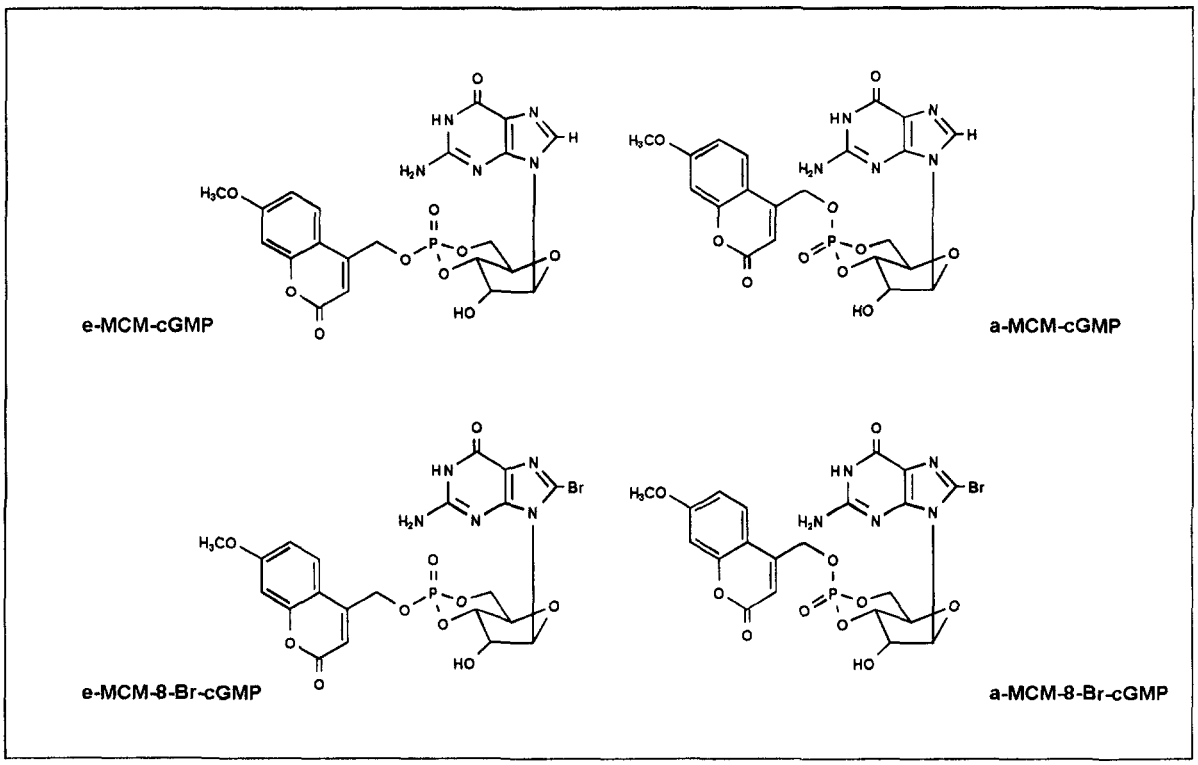


Scheme I.

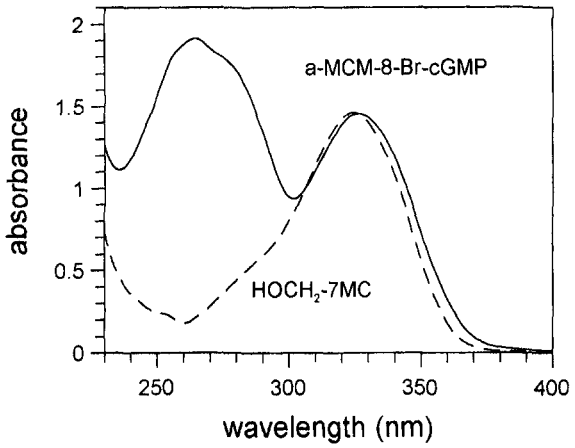


Fig. 1. Absorption spectra in MeOH/HEPES (2:8); c = 25 μM.

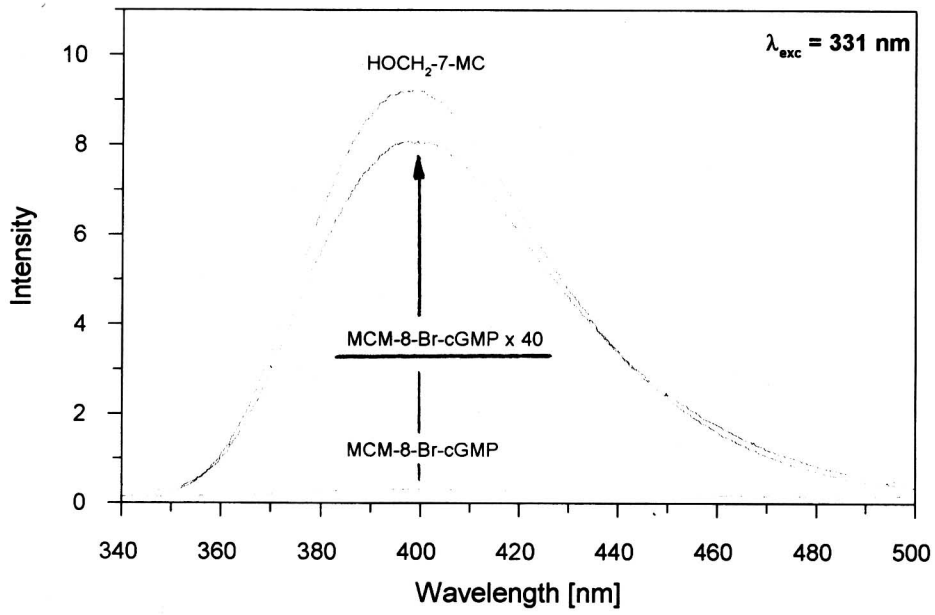
fluorescence quantum yields were determined at 298 K by the relative method using quinine sulfate as a standard (ϕ_f = 0.545 in 0.1 N H_2SO_4).

Photolysis was carried out using a high-pressure mercury lamp (HBO 500, Oriel, USA) with controlled light intensity and a metal interference filter of 333 nm (Carl Zeiss, Germany). For determination of the photochemical quantum yields and for identification of the photoproducts (absorption spectra), the irradiated solutions were analyzed using reversed-phase HPLC (isocratic; RP 18; diode array detector Kontron DAD 440; λ_{det} = 254 nm; mobile phase, methanol/water, 60:40). The photochemical quantum yields were determined by the relative method using the potassium iron(III) oxalate actinometer.[12]

Fluorescence Behavior

4-Hydroxymethyl-7-methoxy-coumarin ($HOCH_2$-7-MC) is a strong fluorescing compound (MeOH/HEPES 1:4): ϕ_f = 0.65, τ_f = 3.5 ns, k_f^n = $1.86 \cdot 10^8$ s^{-1}, and k_f = $2.86 \cdot 10^8$ s^{-1} (absorption spectrum, see Fig. 1; fluorescence spectrum, see Fig. 2).

On photolysis, the phosphoric acid esterfied derivative $(OEt)_2P(O)OCH_2$-7-MC (Fig. 4) undergoes ester cleavage via a photochemical S_N1 mechanism.[10] In the first excited singlet state of the phosphoric acid ester derivative $(OEt)_2P(O)OCH_2$-7-MC, photochemistry competes successfully with photophysical deactivation,



φ_f (MCM-8-Br-cGMP) = **1/48** x φ_f (4-$HOCH_2$-7-MC)

Fig. 2. Fluorescence spectra in MeOH/HEPES (2:8); c = 25 μM.

reducing the fluorescence quantum yield: ϕ_f = 0.052, and ϕ_{chem} = 0.049. The phosphoric acid esters are thermally stable within the investigated time scale.

The caged compounds are bichromophoric systems (Fig. 1). At long-wavelength excitation (λ_{exc} = 320. . .350 nm), only the coumarin chromophor is locally excited. The fluorescence spectra of the caged compounds are very similar to those of $HOCH_2$-7-MC (Fig. 2) and the phosphoric acid ester $(OEt)_2P(O)OCH_2$-7-MC.

The fluorescence quantum yield of the caged nucleotides is decreased compared with the "nucleic base free" phosphoric acid ester derivative $(OEt)_2P(O)OCH_2$-7-MC (Fig. 3). The nucleic base guanine acts as an intramolecular fluorescence quencher. Fluorescence quenching by the nucleic base goes on side by side with an increase in the photochemical quantum yield. Both effects, the fluorescence quenching and the acceleration of the photochemical ester cleavage, are stronger in the axial configuration (*a*-MCM-cGMP, *a*-MCM-8-Br-cGMP) than in the equatorial configuration (*e*-MCM-cGMP, *e*-MCM-8-Br-cGMP), respectively: *a*-MCM-cGMP, ϕ_f = 0.014 and ϕ_{chem} = 0.21; *e*-MCM-cGMP, ϕ_f = 0.030 and ϕ_{chem} = 0.092; *a*-MCM-8-Br-cGMP, ϕ_f = 0.014 and ϕ_{chem} = 0.20; and *e*-MCM-8-Br-cGMP, ϕ_f = 0.020 and ϕ_{chem} = 0.098.

On the addition of guanine or cGMP to a solution of $(OEt)_2P(O)OCH_2$-7-MC in MeOH/HEPES (2:8), the fluorescence of the phosphoric acid ester derivative is quenched. The rate constant of this intermolecular fluorescence quenching determined by a Stern–Volmer plot is $k_q = 7.8 \cdot 10^9$ L mol^{-1} s^{-1} ($k_{diff} \approx 10^{10}$ L mol^{-1} s^{-1}) for the system $(OEt)_2P(O)OCH_2$-7-MC/cGMP.

The nucleotides of 8-Br-cGMP and cGMP exist in the syn conformation.[11] This molecular structure favors the through-space interaction between the nucleic base guanine (donor) and the methylene bridge in the ester unit forming the carbonium cation intermediate (acceptor) during the S_N1 reaction. The stronger stabilization due to the smaller donor–acceptor distance in the case of the axial configuration should be the reason for the higher photochemical and lower fluorescence quantum yield compared with the corresponding values of equatorial isomer.

A heavy atom effect of the bromo substituent is not observed explained by the large distance between the locally excited coumarin chromophore and the bromine in the syn configuration.

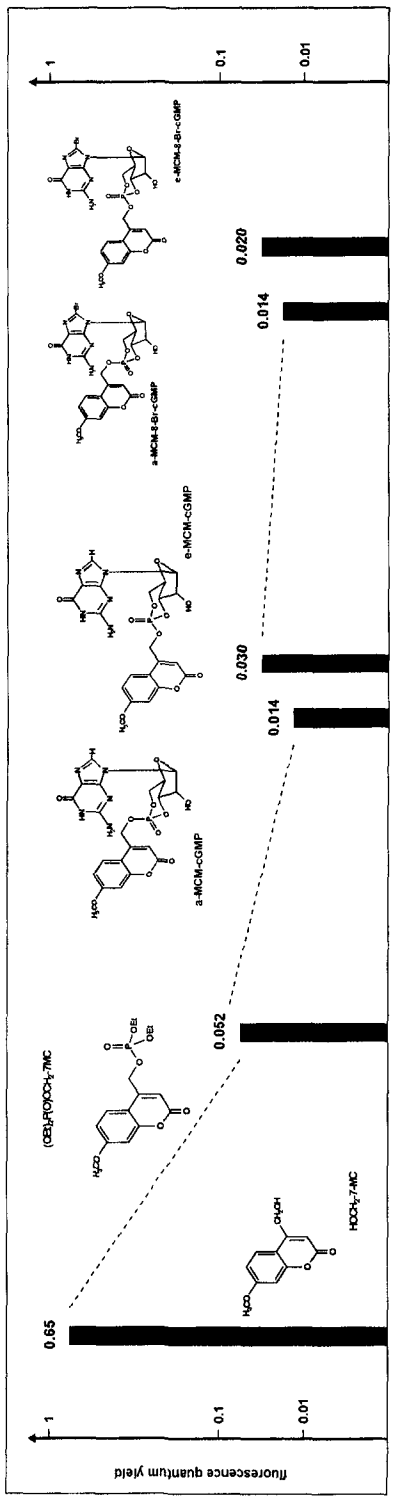


Fig. 3. Fluorescence quantum yields of the investigated compounds in MeOH/HEPES (2:8).

Photochemical Fluorescence Enhancement

The bromo substituted caged compounds show the highest photochemical and the lowest fluorescence quantum yields (axial isomers), which is interesting for the photochemical decaging hand in hand with fluorescence enhancement. In all investigated cases the fluorescence quantum yields of the caged compounds differ extremely from the corresponding value of the photochemical liberated 4-hydroxymethyl-7-methoxy-coumarin (Fig. 3). During photolysis of the caged nucleotides the fluorescence increases (Fig. 5). The local and temporal distribution of the fluorescence intensity correlate with the formation of the observed fluoresceing $HOCH_2$-7-MC. Under the investigated conditions (solvent, MeOH/HEPES; concentration $c = 10...50\ \mu M$; thickness $d \leq 10$ mm), which are similar to those in native cells, the fluorescence intensity depends linearly upon the cGMP concentration (Fig. 6).

CONCLUSION

The photochemical fluorescence enhancement on photolysis of the coumarin-yl ester of caged nucleotides is an excellent method for (temporal, local and kinetic) monitoring the decaging process in native materials. Using the fluorescence data of $HOCH_2$-7-MC formed in parallel, description of the concentration distribution of the biologically active cyclic nucleotides depending upon time and place is possible.

ACKNOWLEDGMENTS

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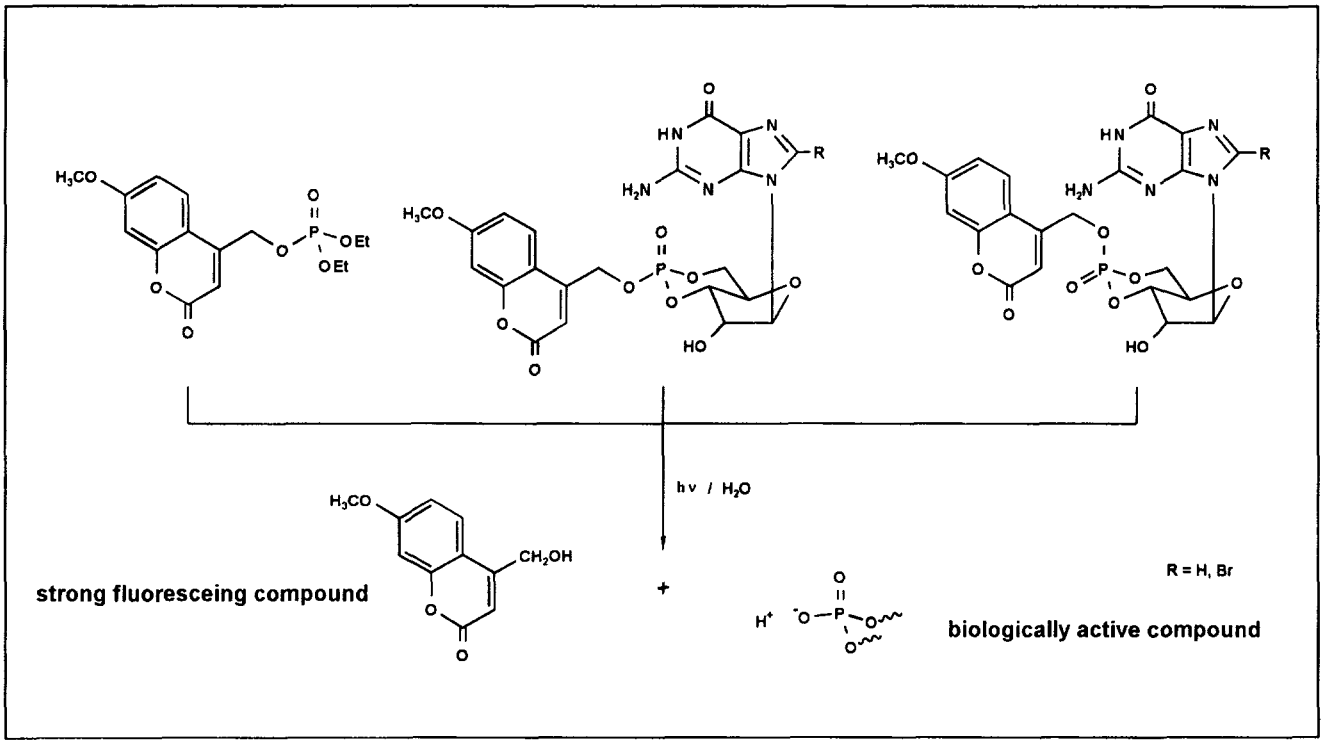

Fig. 4. Photochemical ester cleavage of phosphoric acid (coumarin-yl)methyl ester.

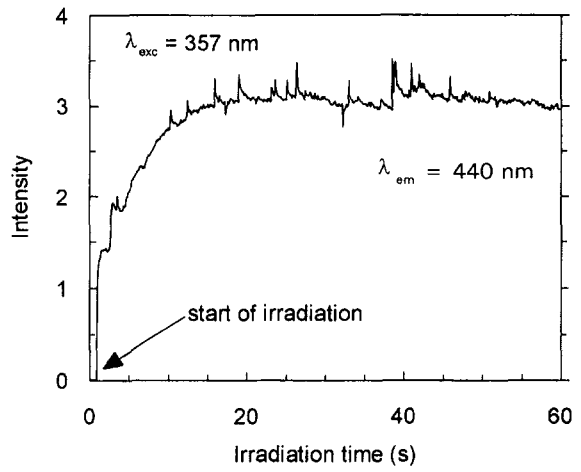

Fig. 5. Fluorescence intensity during photolysis of *a*-MCM-8-Br-cGMP; c = 25 μ*M*; XBO, 150 W.

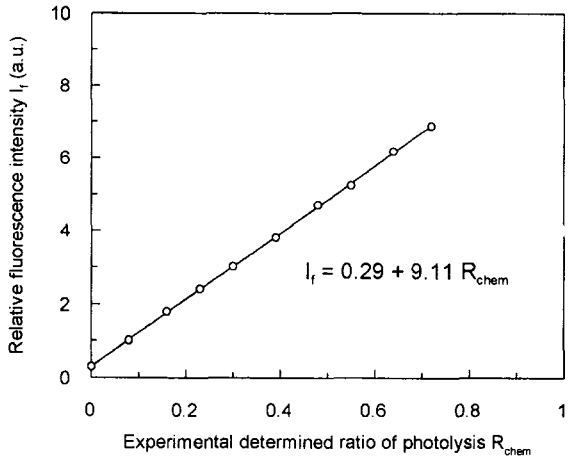

Fig. 6. Relative intensity of fluorescence I_f depending upon the ratio of photolysis R_{chem} determined using HPLC. *a*-MCM-8-Br-cGMP in MeOH/HEPES (2:8); c = 20 μ*M*; λ_{exc} = 333 nm (photochemistry and fluorescence excitation).

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