[7-(Dialkylamino)coumarin-4-yl]methyl-Caged Compounds as Ultrafast and Effective Long-Wavelength Phototriggers of 8-Bromo-Substituted Cyclic Nucleotides

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[7-(Dimethylamino)coumarin-4-yl]methyl (DMACM) and [7-(diethylamino)coumarin-4-yl]methyl (DEACM) esters of 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) and 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP) are described as novel caged compounds for 8-bromo-substituted cyclic nucleotides. Synthesis is accomplished by treatment of the free acids of the cyclic nucleotides with the corresponding 7-(dialkylamino)-substituted 4-(diazomethyl)coumarins. Irradiation of the DMACM- and DEACM-caged cyclic nucleotides with UV light stimulates the release of the cyclic nucleotides within roughly a nanosecond. The new caged compounds are resistant to hydrolysis in aqueous buffers and exhibit long-wavelength absorption properties with maxima at 400 nm, high extinction coefficients, and high quantum yields (0.15 – 0.31). Their favorable properties render these compounds the most efficient and rapid phototriggers of 8-bromo-substituted cyclic nucleotides known. The usefulness of the compounds for physiological studies under nondamaging light conditions was examined in HEK293 cells expressing the α subunit of the cyclic-nucleotide-gated (CNG) channel of cone photoreceptors (CNGA3) and of olfactory neurons (CNGA2) by using confocal laser scanning microscopy and the patch clamp technique.

KEYWORDS:

caged compounds · ion channels · nucleotides · photolysis · protecting groups

Introduction

The light-induced release of 8-bromo-substituted adenosine 3′,5′-cyclic monophosphate (8-Br-cAMP, **1a**) and guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP, **1b**) from caged compounds is a powerful tool to generate concentration jumps of cyclic nucleotides for studies of cyclic-nucleotide-gated (CNG) channels.[1–9] In contrast to cAMP and cGMP, 8-Br-cAMP and 8-Br-cGMP are poorly hydrolyzed by phosphodiesterases[10, 11] and often display a higher biological efficacy.[10–12] Therefore, activation of CNG channels requires lower concentrations of the cyclic nucleotide, that is, less-intensive light flashes, which minimizes cell damage caused by irradiation with UV light.

Recently, we introduced the (7-methoxycoumarin-4-yl)methyl (MCM)[6] and (6,7-dimethoxycoumarin-4-yl)methyl (DMCM)[13] esters of 8-Br-cAMP and 8-Br-cGMP as novel caged compounds. Compared to other caged compounds of the 8-bromo-substituted cyclic nucleotides[1, 2] the MCM esters show a higher photoefficiency for irradiation with light at 337 nm (nitrogen laser), and the DMCM esters at 364 nm (argon-ion laser). Important properties of coumarinylmethyl esters of cyclic nucleotides are their exceptional solvolytic stability and very rapid photorelease.[6, 14]

Herein, we report the synthesis and characterization of new photolabile cyclic nucleotide analogues, the [7-(dimethylamino)coumarin-4-yl]methyl (DMACM) and [7-(diethylamino)coumarin-4-yl]methyl (DEACM) esters of 8-Br-cAMP and 8-Br-cGMP **4a,b** and **5a,b** (Scheme 1). DMACM and DEACM have been previously described as novel caging groups for caging cAMP.[14, 15] We expected that the DMACM- and DEACM-caged compounds would combine the high solvolytic stability and rapid photorelease of the MCM and DMCM esters with long wavelength absorption and high extinction coefficients. Long-wavelength irradiation allows photorelease inside cells under nondamaging light conditions.

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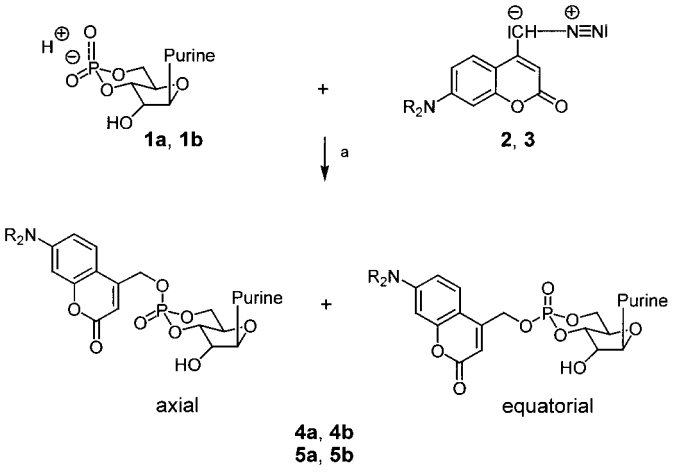
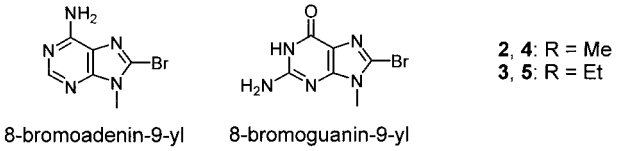


a: 8-Br-cAMP and caged 8-Br-cAMPs: Purine = 8-bromoadenin-9-yl
b: 8-Br-cGMP and caged 8-Br-cGMPs: Purine = 8-bromoguanin-9-yl



Scheme 1. *Synthesis of the axial and equatorial isomers of* ***4a,b*** *and* ***5a,b****. a) Acetonitrile/DMSO (4:1), 60 °C, 24 h.*

We demonstrate that the properties of **4a,b** and **5a,b** compare favorably with those of known phototriggers for 8-Br-cAMP and 8-Br-cGMP, and successfully apply these compounds in studies of CNG channels with confocal laser scanning microscopy (CLSM) and the patch clamp technique.

Results and Discussion

Synthesis and properties

4a,b and **5a,b** were prepared by reaction of the free acids of the respective cyclic nucleotides with the corresponding 7-dialkylamino-substituted 4-(diazomethyl)coumarin **2** or **3** in acetonitrile/dimethylsulfoxide (DMSO) at 60 °C (Scheme 1) by using a procedure[16, 17] adapted for the preparation of MCM-caged cAMP[18] and cGMP.[19] Compound **2** was synthesized by SeO_2 oxidation of (7-dimethylamino)-4-methylcoumarin to the corresponding coumarin-4-carbaldehyde followed by triethylamine-mediated Bamford – Stevens reaction of its tosylhydrazone by a procedure described previously for **3**.[20]

The diastereoisomeric mixtures of the caged compounds (for structures see Scheme 1) were separated into the axial and equatorial isomers by using preparative HPLC. The axial isomers showed lower retention times compared to the equatorial isomers. The total yields varied between 18% and 21%. The synthesis led to the preferential formation of the equatorial isomers, however, all isomers could be isolated in acceptable yields. Isomeric species were assigned by ^{31}P NMR as reported for the axial and equatorial isomers of known cAMP and cGMP esters.[21, 22] The ^{31}P NMR signals at higher field correspond to the axial isomers, and those at lower field correspond to the equatorial isomers. Comparison of the ^{1}H NMR spectra of the axial and equatorial isomers of **4a,b** and **5a,b** revealed a significant downfield shift of the H-4′[23] signal for the equatorial isomers.

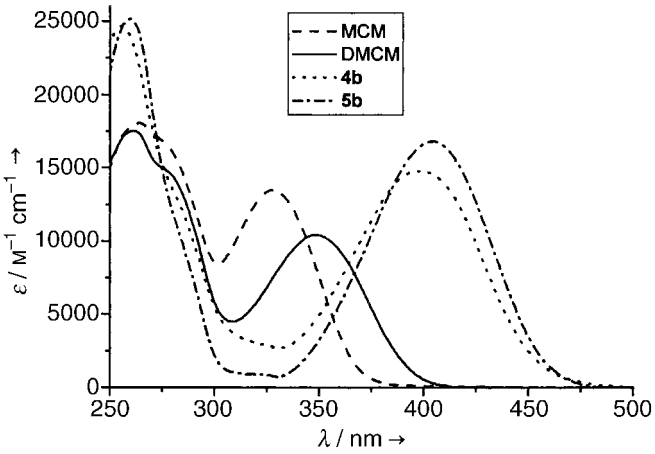


Figure 1. *UV/Vis spectra of the axial diastereomers of* ***4b***, ***5b****, and MCM- and DMCM-caged 8-Br-cGMP.*

Like other coumarinylmethyl-caged cyclic nucleotides,[6, 13, 18, 24] the two isomers of **4a,b** and **5a,b** are highly resistant to spontaneous hydrolysis. HPLC monitoring of the caged compounds in aqueous buffers at pH 7.2 during a 24 h period revealed no measurable formation of the "free" 8-bromo-substituted cyclic nucleotide.

The solubility of the axial isomer of **5b** in 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer is relatively high, whereas the solubilities of the other diastereomer of **5b** and of both diastereoisomers of the other three products are relatively low (Table 1). However, the high biological activity of 8-Br-cAMP and 8-Br-cGMP means that the lower solubility will be sufficient for most studies.

Figure 1 shows the absorption spectra of the axial isomers of **4b** and **5b** compared to those of the axial isomers of MCM- and DMCM-caged 8-Br-cGMP.[6, 13] The absorption spectra of the DMACM-caged cyclic nucleotides show maxima at 386 – 398 nm with molar extinction coefficients of 16000 – 17000 $L\,M^{-1}\,cm^{-1}$; the absorption maxima of the DEACM-caged cyclic nucleotides were 395 – 406 nm with molar extinction coefficients of 17000 – 20000 $L\,M^{-1}\,cm^{-1}$ (Table 1). These values (absorptions caused by the $\pi - \pi^*$ transition)[15] are characteristic for the DMACM and the DEACM chromophore. Caging groups with such long-wavelength maxima and strong absorptivities are unique.

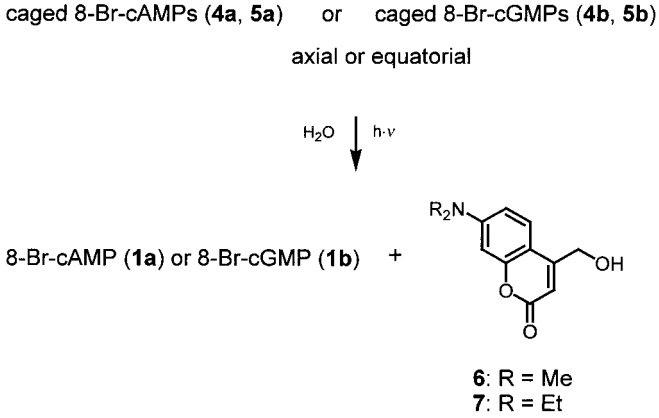
Photolysis and quantum yield

The photochemistry of the DMACM and DEACM esters **4a,b** and **5a,b** is shown in Scheme 2. Irradiation of the axial and/or equatorial diastereomers with 330 – 440 nm light in aqueous buffer resulted in the liberation of only "free" cyclic nucleotides and 7-dimethylamino- (DMACM-OH, **6**) or 7-diethylamino-4-(hydroxymethyl)coumarin (DEACM-OH, **7**), as judged by HPLC

Table 1. *Characteristics of the DMACM and DEACM esters* ***4a,b*** *and* ***5a,b*** *and of* ***6*** *and* ***7****.*[a]

Compound	λ_{abs}^{max} [nm] (ε^{max} [$M^{-1}cm^{-1}$])[b]	φ_{chem}[f,d]	λ_f^{max}[f] [nm]	φ_f[e,f]	τ_f[b] [ns]	s[c] [μM]
4a (axial)	393 (16100)	0.16	481	0.006		12
4a (equatorial)	386 (16800)	0.15	481	0.006		6
5a (axial)	401 (20100)	0.23	485	0.007		3
5a (equatorial)	395 (19600)	0.24	487	0.006		10
4b (axial)	398 (16000)	0.31	481[b]	0.008[b]	< 0.2	20
4b (equatorial)	386 (15800)	0.30	480[b]	0.007[b]	< 0.2	10
5b (axial)	406 (16800)	0.27	487	0.007	< 0.2[f]	120
5b (equatorial)	396 (17300)	0.28	486	0.007		4
6	378 (17800)		491	0.21	3.42	
7	387 (20900)		484	0.08	3.35	

[a] Long-wavelength absorption maximum, λ_{abs}^{max}; extinction coefficient, ε^{max}; photochemical quantum yield, φ_{chem}, at $\lambda_{exc} = 333$ nm; fluorescence maximum, λ_f^{max}; fluorescence quantum yield, φ_f; fluorescence life time, τ_f; solubility, s. [b] In MeOH-HEPES-KCl buffer (1/4), pH 7.2. [c] In acetonitrile/HEPES-KCl buffer (5:95), pH 7.2. [d] Error limit ± 0.01. [e] Error limit ± 0.002. [f] In MeOH/HEPES-KCl-buffer (1/1), pH 7.2.



Scheme 2. *Photolysis of* ***4a,b*** *and* ***5a,b****.*

measurements. At high concentrations of the caged compounds and with extensive photolysis (> 60%), the 7-dialkylamino-4-methylcoumarins were the only byproducts formed (< 1%). The ability of the caged compounds to fluoresce is very poor (Table 1), which indicates that both the photochemical reaction (ester cleavage) and the nonradiative deactivation (internal conversion) represent main deactivation pathways of the excited state.

The quantum yields, φ_{chem}, for the consumption of the axial and equatorial isomers of **4a,b** and **5a,b** are relatively high (Table 1). The caged cGMP derivatives display higher quantum yields compared to the respective caged cAMPs. Compared to the MCM- and DMCM-caged cyclic nucleotides,[6, 13] quantum yields were increased by up to threefold. The combination of high quantum yields and large absorptivities (350 – 440 nm) yields very high efficiencies of photocleavage under nondamaging light conditions. This outcome is demonstrated by comparison of the efficiencies of photocleavage of **4b** and **5b** with those of other caged 8-Br-cGMPs at different wavelengths (Table 2). The results show that **4b** and **5b** are efficient phototriggers for 8-Br-cAMP and 8-Br-cGMP at excitations of 365, 405, and even 436 nm, and that the coumarinylmethyl-caged compounds are superior to 1-(2-nitrophenyl)ethyl- (NPE) or 4,5-dimethoxy-2-nitrobenzyl (DMNB)-caged derivatives. Furthermore, the highest release efficiency for 8-Br-cGMP at 333 nm is achieved with MCM-caged 8-Br-cGMP.

We propose that the mechanism underlying the photochemical conversion involves a photo S_N1 reaction (solvent-assisted photoheterolysis) in analogy to the conversion of MCM-caged cyclic nucleotides.[6, 19] In accordance with previous results,[6, 19] no indications of a significant triplet state population were found. Instead, the observation that hardly any hydrogen abstraction products (< 1%) are produced during photolysis and that no appreciable phosphorescence was detected for any of the photolabile DMACM- or DEACM-caged cyclic nucleotides indicates the involvement of a singlet-state upon electronic excitation.

Fluorescence properties and kinetics of the uncaging process

Fluorescence quantum yields, φ_f, of **4a,b** and **5a,b** as well as those of **6** and **7** are given in Table 1. The fluorescence intensity (at the maximum emmission wavelength, λ_{max}) of **6** is 25 –

Table 2. *Comparison of the efficiency (%) of the photorelease of 8-Br-cGMP from 25-μM solutions of caged compounds (axial isomers) in MeOH:HEPES (1/4) with various irradiation times at different wavelengths.*

Compound	t ($\lambda_{exc} = 333$ nm)			t ($\lambda_{exc} = 365$ nm)			t ($\lambda_{exc} = 405$ nm)			t ($\lambda_{exc} = 436$ nm)		
	6 s	60 s	600 s	6 s	60 s	600 s	6 s	60 s	600 s	6 s	60 s	600 s
NPE-caged 8-Br-cGMP	< 1	3	12	< 1	< 1	1	< 1	< 1	< 1	< 1	< 1	< 1
DMNB-caged 8-Br-cGMP	< 1	4	30	< 1	3	20	< 1	1	2	< 1	< 1	< 1
MCM-caged 8-Br-cGMP	17	77	> 98	4	30	96	< 1	2	10	< 1	< 1	< 1
DMCM-caged 8-Br-cGMP	10	46	97	10	60	91	1	3	19	< 1	1	3
4b	4	32	93	15	64	> 98	20	90	> 98	7	75	98
5b	1	7	60	12	60	90	20	91	> 98	12	80	> 98

35 times larger, and that of **7** about 11 times larger than those of the corresponding DMACM- or DEACM-caged derivatives. Figure 2 illustrates the differences in emission spectra and fluorescence intensities between the axial isomer of **4a** and **6**.

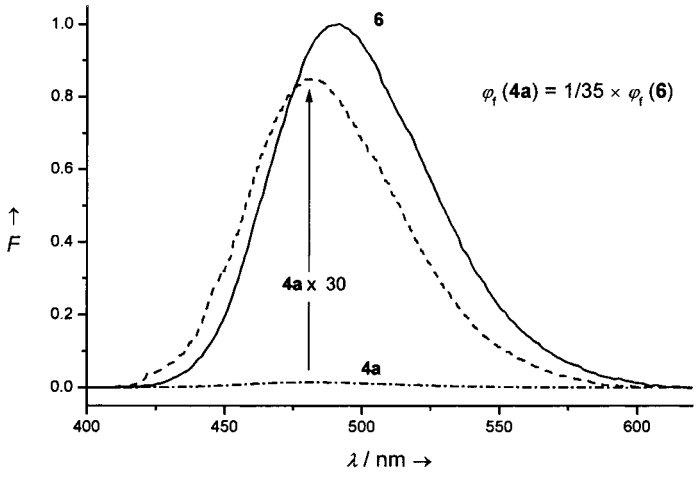


Figure 2. *Fluorescence spectra of 25-μM solutions of the axial isomer of **4a** and of **6** in 20% methanol/0.01 M HEPES-KOH buffer containing KCl (0.12 M), at pH 7.2. F = relative fluorescence intensity.*

A linear relationship exists between the fluorescence intensity and the extent of photolysis for the axial isomers of **4a** and **5b** in MeOH-HEPES buffer (1/4), as shown in Figure 3. A linear relationship is also observed for the other isomers of the caged compounds as well as for **4b** and **5a** (data not shown). Therefore, measurement of the increase of fluorescence intensity allows an estimate of the amount of liberated coumarinylmethanol and thereby the amount of liberated cyclic nucleotide.

The magnitude of the rate constants of the release process can be obtained from time-resolved fluorescence spectroscopy[15] of the liberated coumarinylmethanol. The rate constant is about $10^9\ s^{-1}$ for the DMACM- and about $1.4 \times 10^9\ s^{-1}$ for the DEACM-caged compounds, that is, the cyclic nucleotides are liberated within a nanosecond. The high rate of photorelease also supports the proposed photolytic reaction mechanism.

CNG channel activation

To test the usefulness of the novel caged compounds we investigated the activation of different CNG channels in HEK293 cells upon release of 8-Br-cGMP and 8-Br-cAMP by long-wavelength illumination. CNG channels are highly permeable to Ca^{2+} ions.[25] Cyclic-nucleotide-mediated entry of Ca^{2+} ions into CNGA3-expressing HEK293 cells was measured with CLSM upon flash photolysis of **4a** and **5b** ($\lambda = 364$ nm). Figure 4 shows the fluorescence intensity of the Ca^{2+} indicator Fluo-3 before and after UV irradiation. Photolysis of both **4a** and **5b** evoked an increase of the fluorescence intensity (Figure 4), which indicates Ca^{2+} ion influx through CNG channels. In the absence of the caged compounds, no change in the fluorescence was observed. Figure 4 also shows that the increase of the fluorescence intensity with 8-Br-cGMP is stronger than that with

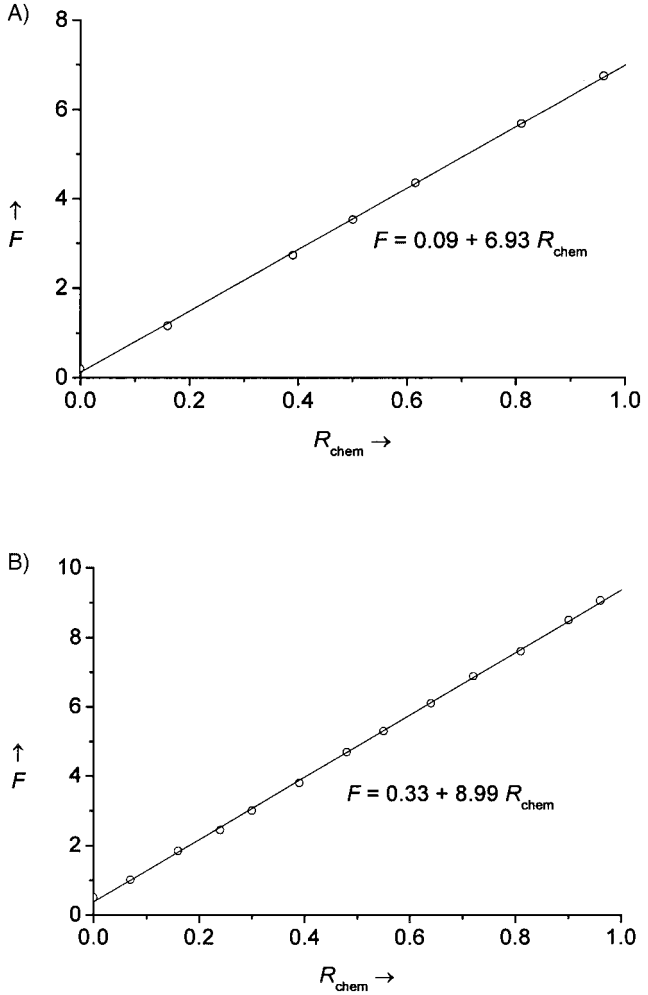


Figure 3. *Increase in relative fluorescence intensities of 25-μM solutions of the axial isomers of **4a** (A) and **5b** (B) as a function of the extent of photolysis (R_{chem}) in 20% methanol/0.01 M HEPES-KOH buffer containing KCl (0.12 M), at pH 7.2. Excitation was at 333 nm, XBO 150 W. Emission wavelength: 480 nm.*

8-Br-cAMP. This result is consistent with the higher sensitivity of the CNGA3 channel for 8-Br-cGMP compared to cAMP.[25]

Figure 5 shows the increase in fluorescence intensity with time (400 s, 100 images). CNGA3-transfected HEK293 cells were loaded with **5b** and illuminated with UV light ($\lambda = 364$ nm) from the 5th scan to the 40th scan. This procedure allows stepwise photolysis of the caged compound. The stepwise photolysis of **5b** (Figure 5) evoked a stepwise increase of the fluorescence intensity.

The use of **4b** and **5b** for electrophysiological experiments was tested in patch clamp experiments on HEK293 cells expressing the CNGA3 channel ($K_{1/2} = 1.8 \pm 0.28$ μM for 8-Br-cGMP, $n = 9$, Hill coefficient $= 2.36 \pm 0.17$, $V_m = -50$ mV).[26] Long-wavelength light flashes (405 nm) induced the photorelease of 8-Br-cGMP (Figure 6). The DMACM-caged compound **4b** was about twofold more effective than **5b**, whereas no photorelease was observed with the DMNB-caged analogue under these conditions. Thus, the novel caged compounds are suitable for electrophysiology experiments at excitation wavelengths greater than 400 nm.

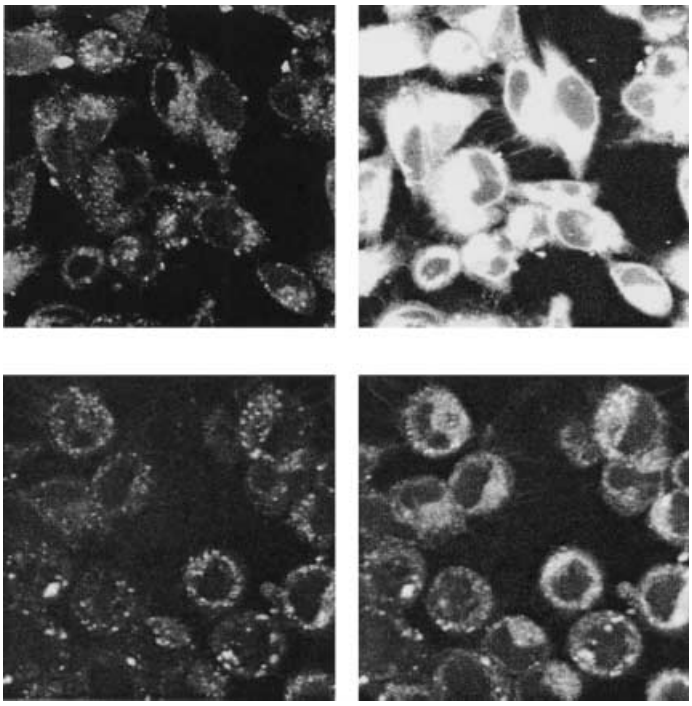


*Figure 4. Fluorescence intensity of the Ca^{2+} indicator Fluo-3 in HEK293 cells expressing the CNGA3 channel before (left) and after (right) UV illumination ($\lambda_{exc} = 364$ nm). The increase of the intracellular Ca^{2+} signal was induced by photolysis of the caged compounds. The cells were preincubated with **5b** (upper panel) or **4a** (lower panel).*

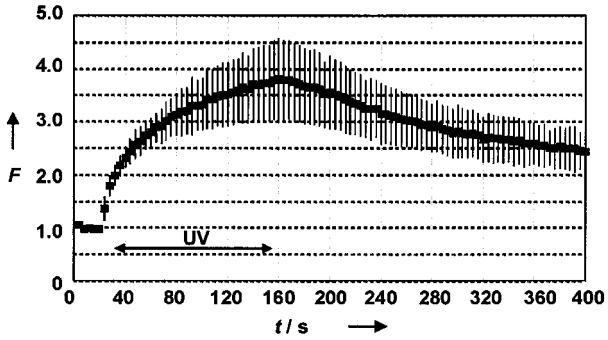


*Figure 5. Time course of the fluorescence intensity (mean ± standard deviation (SD), n = 20) of Fluo-3-loaded HEK293 cells expessing the CNGA3 channel. The cells were preincubated with **5b**. From the 5th scan (t = 20 s) to the 40th scan (t = 160 s) the cells were illuminated with UV light flashes (λ = 364 nm, 8,7 μW/cm², two scans each) after each image was taken. The increase of the intracellular Ca^{2+} ion concentration was induced by photolysis of the caged compound.*

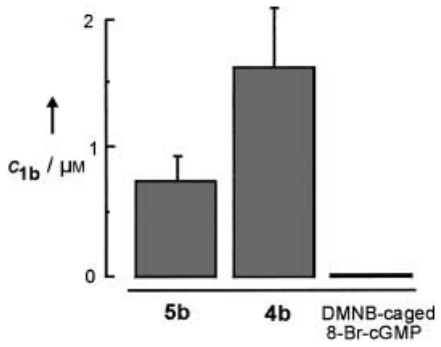


*Figure 6. Photorelease of 8-Br-cGMP from the axial isomers of the indicated caged compounds (20 μM) by 5-ms flashes (λ = 405 ± 20 nm). Mean quantities released (± SD) by 30 – 70 flashes collected from 6 – 9 cells for each compound were 1.6 ± 0.5 μM[6] for **4b** and 0.7 ± 0.2 μM[9] for **5b**. No release was detectable for the DMNB-caged compound. Concentrations were determined from the current increment caused by each flash and the known dose – response relation of 8-Br-cGMP for CNGA3 channels.[26] c_{1b} = concentration of liberated **1b**.*

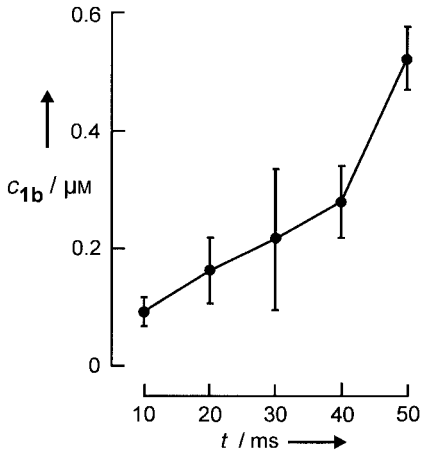


*Figure 7. Photorelease of 8-Br-cGMP from **4b** in HEK293 cells expressing the rat olfactory CNGA2 channel. Cells were filled with pipette solution containing **4b** (5 μM) and photolysis was induced by 405-nm flashes of the indicated duration. Each point represents the mean (± SD) concentration of photoreleased 8-Br-cGMP in 6 different cells determined through activation of CNGA2 channels at $V_m = -70$ mV. V_m = membrane voltage.*

Figure 7 shows that the concentration of 8-Br-cGMP released from **4b** is roughly proportional to the duration of the light flash. This experiment was performed with HEK293 cells expressing the rat olfactory CNGA2 channel. About 10% of the caged substance was liberated by a single 50-ms flash at 405 nm. By adding the appropriate concentration of caged compound to the pipette solution, one can determine the size of the concentration step produced by a light flash. Thus, with solubilities between 20 and 120 μM for the axial isomers of the DMACM- and DEACM-caged compounds (Table 1), concentration steps of 2 – 12 μM 8-Br-cGMP can be generated by a 50-ms flash of 405-nm light under our experimental conditions.

To investigate whether the fluorescence of the photolysis products **6** and **7** could be used to monitor photorelease in patch clamp experiments, we filled cells with **7**. However, as reported earlier for MCM-OH,[6] no fluorescence signal could be recorded from HEK293 cells loaded with 100 μM **7** (Figure 8). Apparently, the fluorescence was completely quenched by unkown cellular constituents. Thus, the photolysis of [7-(dialkylamino)coumarin-4-yl]methyl esters of cyclic nucleotides inside cells cannot be followed by fluorescent measurements.

These studies on CNG channels confirm that the novel coumarinylmethyl-caged 8-bromo-substituted cyclic nucleotides are indeed highly efficient phototriggers for 8-Br-cAMP and 8-Br-cGMP at long-wavelength excitation. The CLSM experiments demonstrate that a rapid increase in the intracellular concentrations of 8-Br-cAMP or 8-Br-cGMP can be achieved at

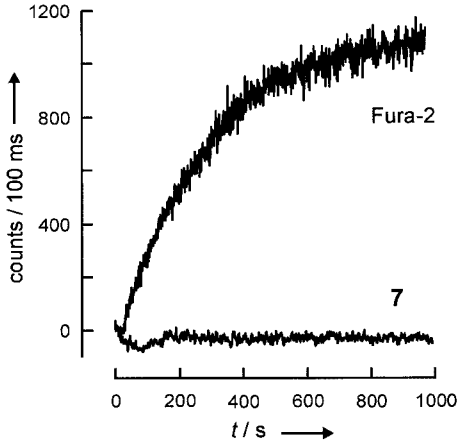


Figure 8. *Increase in fluorescence intensity during loading of an HEK293 cell with Fura-2 (100 μM, upper trace), and suppression of fluorescence in another cell during loading with* ***7*** *(100 μM, lower trace). Both solutions show similar fluorescence in cell-free solution (not shown), but only Fura-2 fluorescence persists within cells (excitation: 380 ± 5 nm, emission: > 460 nm).*

$\lambda = 364$ nm. The investigations carried out with the patch clamp method illustrate a very efficient photorelease of 8-Br-cGMP from **5b** at $\lambda = 405$ nm. Long-wavelength photorelease of biomolecules from caged compounds inside cells has not been described before and our studies in solution show that efficient photorelease is possible even at $\lambda = 436$ nm. The fact that no measurable release could be detected from DMNB-caged 8-Br-cGMP at $\lambda = 405$ nm agrees with the results obtained in buffer solutions.

Conclusions

Our results demonstrate that **4a,b** and **5a,b** represent a class of phototriggers with favorable properties. The caged compounds are resistant to hydrolysis, sufficiently soluble in aqueous buffer solutions for most studies and photocleavage is very rapid and efficient under long-wavelength excitation. Activation of the caged compounds by light at more than 400 nm minimizes or even prevents damage of cellular components and chromophore bleaching by light.

In summary, these novel caged compounds will extend the repertoire of tools available for the study of spatial- and time-dependent aspects of cyclic-nucleotide-dependent cellular processes.

Experimental Section

Materials: The free acid of 8-Br-cAMP and the sodium salt of 8-Br-cGMP were purchased from Biolog (Germany). SeO_2 was obtained from Sigma (Germany). 7-(Diethylamino)-4-methyl-coumarin, p-toluensulfonyl hydrazide, and triethylamine were obtained from Lancaster (Germany). 7-(Dimethylamino)-4-methyl-coumarin were purchased from ICC (USA). Pluoronic F-127, Fura-2, and the acetomethoxy ester of Fluo-3 were obtained from MoBiTec (Germany). The sodium salt of 8-Br-cGMP was converted to the acid form as described previously.[1] 4-(Diazomethyl)-7-(diethylamino)coumarin (**3**) was prepared according to Ito et al.[20] DMNB- and NPE-caged 8-Br-cGMP were synthesized by employing published procedures.[1, 2] 7-(Dimethylamino)-4-(hydroxymethyl)coumarin (**6**) and 7-(Diethylamino)-4-(hydroxymethyl)coumarin (**7**) were prepared according to published procedures.[15, 27] Silica gel for flash chromatography was from J. T. Baker (The Netherlands). All solvents from J. T. Baker (USA) were HPLC grade. All other chemicals were reagent grade. Water was purified with a Milli Q system (Millipore, Germany).

Instrumentation: ^{1}H and ^{31}P NMR spectra were recorded on a Bruker DRX 600 spectrometer at 600 MHz and 243 MHz, respectively. ^{1}H chemical shifts are given in parts per million (ppm) relative to tetramethylsilane as an internal standard. ^{31}P NMR chemical shifts are reported in ppm referenced to external 85 % H_3PO_4.

Mass spectra were measured by electrospray ionization mass spectrometry in the positive ionization mode by using a TSQ 700 (Finnigan MAT) spectrometer. UV spectra were recorded with a U-3410 spectrophotometer (Hitachi, Japan).

General procedure for the preparation of DMACM- or DEACM-caged 8-Br-cAMPs and 8-Br-cGMPs (4a,b and 5a,b): A mixture of the free acid of 8-Br-cAMP (204.5 mg, 0.5 mmol) or the free acid of 8-Br-cGMP (dihydrate, 230.1 mg, 0.5 mmol) and **2** (114.6 mg, 0.5 mmol) or **3** (128.65. mg, 0.5 mmol) in acetonitrile (16 mL) and DMSO (4 mL) was stirred at 60 °C in the dark for 8 h. An additional quantity (0.5 mmol) of the respective (4-diazomethyl)-7-(dialkylamino)coumarin was added and the mixture was stirred at 60 °C for a further 16 h. Acetonitrile was evaporated under reduced pressure and DMSO was removed by repeated extraction with ether/pentane. The residue was dissolved in a small volume of chloroform/methanol (1:1, v/v) and separated by flash chromatography on a silica gel column. Elution with chloroform (100 %), chloroform/methanol (48:1, v/v), chloroform/methanol (24:1, v/v), and chloroform/methanol (4:1, v/v) gave fractions containing mixtures of the axial and and equatorial diastereomers of the caged compounds. The fractions were dried on a rotary evaporator. Lyophilization gave mixtures of the two isomers of **4a** (63 mg, 20.1 %), **5a** (68 mg, 21.2 %), **4b** (57 mg, 17.8 %), or **5b** (63 mg, 18.8 %) in approximately 45:55 ratios (axial/equatorial) as yellow solids. The axial and equatorial isomers were separated from each other by preparative reversed phase HPLC (RP-HPLC). The separated isomers purified by HPLC were not contaminated by 8-Br-cAMP or 8-Br-cGMP (< 0.05 %).

Data for axial 4a: TLC: $R_f = 0.80$ (chloroform/methanol, 5:1 v/v); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled $\delta = -5.03$ ppm; ^{1}H NMR (DMSO-d_6): $\delta = 3.02$ (s, 6H; $N(CH_3)_2$), 4.29 – 4.31 (m, 2H; H-4′ and H-5′), 4.68 (dd, $J = 22.0$ and 4.0 Hz, 1H; H-5″), 4.97 (t, $J =$ 4.5 Hz, 1H; H-2′), 5.42 (d, $J = 6.0$ Hz, 2H; coumarin CH_2), 5.56 (q, $J =$ 3.0 Hz, 1H; H-3′), 5.89 (s, 1H; H-1′), 6.24 (s, 1H; coumarin H-3), 6.41 (m, 1H; OH), 6.61 (d, $J = 2.1$ Hz, 1H; coumarin H-8), 6.70 (dd, $J = 9.0$ and 2.2 Hz, 1H; coumarin H-6), 7.54 (s, 2H; NH_2), 7.55 (d, $J = 9.0$ Hz, 1H; coumarin H-5), 8.06 (s, 1H; H-2) ppm; ESI MS: 609.1 and 611.3 $[M+H]^+$; elemental analysis calcd (%) for $C_{22}H_{22}BrN_6O_8P \times 1.5\,H_2O$ (636.36): C 41.52, H 3.96, N 13.21; found: C 41.93, H 3.81, N 12.89.

Data for equatorial 4a: TLC: $R_f = 0.74$ (chloroform/methanol, 5:1 v/v); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: $\delta = -3.31$ ppm; ^{1}H NMR (DMSO-d_6): $\delta = 3.02$ (s, 6H; $N(CH_3)_2$), 4.40 (q, $J = 9.0$ Hz, 1H; H-5′), 4.51 (dt, $J = 10.0$ and 6.0 Hz, 1H; H-4′), 4.72 – 4.77 (m, 1H; H-5″), 5.06 (d, $J = 5.0$ Hz, 1H; H-2′), 5.34 – 5.42 (m, 2H; coumarin CH_2), 5.49 (dd, $J = 9.5$ and 5.5 Hz, 1H; H-3′), 5.91 (s, 1H; H-1′), 6.15 (s, 1H; coumarin H-3), 6.40 (br s, 1H; OH), 6.61 (d, $J = 2.2$ Hz, 1H; coumarin H-8), 6.77 (dd, $J = 9.0$ and 2.2 Hz, 1H; coumarin H-6), 7.52 (d, $J =$ 9.0 Hz, 1H; coumarin H-5), 7.55 (br s, 2H; NH_2), 8.21 (s, 1H; H-2) ppm; ESI MS: 609.3 and 611.3 $[M+H]^+$; elemental analysis calcd (%) for

$C_{22}H_{22}BrN_6O_8P \times H_2O$ (627.35): C 42.12, H 3.86, N 13.40; found: C 41.81, H 3.81, N, 13.34.

Data for axial 5a: TLC: $R_f = 0.74$ (chloroform/methanol, 5:1 v/v); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: $\delta = -5.03$ ppm; ^{1}H NMR (DMSO-d_6): $\delta = 1.10$ (t, $J = 7.0$ Hz, 6H; $2 \times CH_3$), 3.43 (q, $J = 7.0$ Hz, 4H; $2 \times CH_2$), 4.29–4.31 (m, 2H; H-4′ and H-5′), 4.69 (dd, $J = 22.0$ and 4.0 Hz, 1H; H-5″), 4.97 (t, $J = 4.5$ Hz, 1H; H-2′), 5.39 (d, $J = 6.0$ Hz, 2H; coumarin CH_2), 5.57 (q, $J = 5.0$ Hz, 1H; H-3′), 5.89 (s, 1H; H-1′), 6.20 (s, 1H; coumarin H-3), 6.41 (d, $J = 4.0$ Hz, 1H; OH), 6.57 (d, $J = 2.0$ Hz, 1H; coumarin H-8), 6.65 (dd, $J = 9.0$ and 2.0 Hz, 1H; coumarin H-6), 7.52 (s, 2H; NH_2), 7.52 (d, $J = 9.0$ Hz, 1H; coumarin H-5), 8.08 (s, 1H; H-2) ppm; ESI MS: 637.3 and 639.4 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{26}BrN_6O_8P$ (637.38): C 45.23, H 4.11, N 13.13; found: C 45.44, H 3.93, N 12.99.

Data for equatorial 5a: TLC: $R_f = 0.69$ (chloroform/methanol, 5:1 v/v); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: $\delta = -3.33$ ppm; ^{1}H NMR (DMSO-d_6): $\delta = 1.13$ (t, $J = 7.0$ Hz, 6H; $2 \times CH_3$), 3.44 (q, $J = 7.0$ Hz, 4H; $2 \times CH_2$), 4.39 (q, $J = 9.0$ Hz, 1H; H-5′), 4.50–4.51 (m, 1H; H-4′), 4.74–4.75 (m, 1H; H-5″), 5.06 (t, $J = 4.5$ Hz, 1H; H-2′), 5.34–5.36 (m, 2H; coumarin CH_2), 5.49 (dd, $J = 9.5$ and 5.5 Hz, 1H; H-3′), 5.91 (s, 1H; H-1′), 6.11 (s, 1H; coumarin H-3), 6.37 (d, $J = 5.0$ Hz, 1H; OH), 6.56 (d, $J = 2.0$ Hz, 1H; coumarin H-8), 6.73 (dd, $J = 9.0$ and 3.0 Hz, 1H; coumarin H-6), 7.49 (d, $J = 9.0$, 1H; coumarin H-5), 7.54 (br s, 2H; NH_2), 8.20 (s, 1H; H-2) ppm; ESI MS: 637.3 and 639.4 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{26}BrN_6O_8P \times 0.5\,H_2O$ (646.39): C 44.60, H 4.21, N 13.00; found: C 44.73, H 3.85, N 12.61.

Data for axial 4b: TLC: $R_f = 0.59$ (chloroform-methanol, 5:1 v/v); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: $\delta = -4.93$ ppm; ^{1}H NMR (DMSO-d_6): $\delta = 3.02$ (s, 6H; $N(CH_3)_2$), 4.22 (dt, $J = 10.0$ and 5.0 Hz, 1H; H-4′), 4.33 (t, $J = 10.0$ Hz, 1H; H-5′), 4.66 (ddd, $J = 22.0$, 9.0 and 4.0 Hz, 1H; H-5″), 4.99 (t, $J = 4.5$, 1H; H-2′), 5.08 (dd, $J = 10.0$ and 5.0 Hz, 1H; H-3′), 5.37 and 5.43 ($2 \times$ dd, $J = 14.5$ and 8.0 Hz, 2H, coumarin CH_2), 5.75 (s, 1H; H-1′), 6.22 (s, 1H; coumarin H-3), 6.35 (d, $J = 4.6$ Hz, 1H; OH), 6.52 (s, 2H; NH_2), 6.58 (d, $J = 2.1$ Hz, 1H; coumarin H-8), 6.69 (dd, $J = 9.0$ and 2.2 Hz, 1H; coumarin H-6), 7.57 (d, $J = 9.0$ Hz, 1H; coumarin H-5), 10.84 (s, 1H; NH) ppm; ESI MS 625.1 and 627.3 $[M+H]^+$; elemental analysis calcd (%) for $C_{22}H_{22}BrN_6O_9P \times 0.5H_2O$ (634.34): C 41.66, H 3.65, N 13.25; found: C 41.93, H 3.28, N 12.96.

Data for equatorial 4b: TLC: $R_f = 0.55$ (chloroform-methanol, 5:1 v/v); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: $\delta = -4.15$ ppm; ^{1}H NMR (DMSO-d_6): $\delta = 3.02$ (s, 6H; $N(CH_3)_2$), 4.37 (q, $J = 11.0$ Hz, 1H; H-5′), 4.48 (dt, $J = 10.5$ and 6.0 Hz, 1H; H-4′), 4.74 (q, $J = 6.0$ Hz, 1H; H-5″), 4.87 (t, $J = 5.0$ Hz, 1H; H-2′), 5.34–5.37 (m, 3H; H-3′ and coumarin CH_2), 5.74 (s, 1H; H-1′), 6.17 (s, 1H; coumarin H-3), 6.27 (d, $J = 4.0$ Hz, 1H; OH), 6.61 (d, $J = 2.2$ Hz, 1H; coumarin H-8), 6.74 (s, 2H; NH_2), 6.76 (dd, $J = 9.0$ and 2.2 Hz, 1H; coumarin H-6), 7.52 (d, $J = 9.0$ Hz, 1H; coumarin H-5), 10.85 (1H, s; NH) ppm; ESI MS: 625.1 and 627.1 $[M+H]^+$; elemental analysis calcd (%) for $C_{22}H_{22}BrN_6O_9P \times 1.5\,H_2O$ (652.35): C 40.51, H 3.86, N 12.88; found: C 40.57, H 3.66, N 12.46.

Data for axial 5b: TLC: $R_f = 0.59$ (chloroform/methanol, 5:1 v/v); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: $\delta = -4.93$ ppm; ^{1}H NMR (DMSO-d_6): $\delta = 1.10$ (t, $J = 7.0$ Hz, 6H; $2 \times CH_3$), 3.42 (q, $J = 7.0$ Hz, 4H; $2 \times CH_2$), 4.22 (dt, $J = 10.0$ and 5.0 Hz, 1H; H-4′), 4.30 (t, $J = 10.0$ Hz, 1H; H-5′), 4.64 (ddd, $J = 22.0$, 9.0 and 4.0 Hz, 1H; H-5″), 4.99 (t, $J = 5.0$ Hz, 1H; H-2′), 5.08 (dd, $J = 10.0$ and 5.0 Hz, 1H; H-3′) 5.35 and 5.41 ($2 \times$ dd, $J = 14.5$ and 8.0 Hz, 2H; coumarin CH_2), 5.74 (s, 1H; H-1′), 6.18 (s, 1H; coumarin H-3), 6.35 (d, $J = 4.0$ Hz, 1H; OH), 6.55 (m, 3H; coumarin H-8 and NH_2), 6.66 (dd, $J = 9.0$ and 3.0 Hz, 1H; coumarin H-6), 7.56 (d, $J = 9.0$ Hz, 1H; coumarin H-5), 10.85 (s, 1H; NH) ppm; ESI MS: 653.2 and 655.1 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{26}BrN_6O_9P \times 1.5\,H_2O$ (680.41): C 42.37, H 4.30, N 12.35; found: C 42.72, H 3.97, N 11.98.

Data for equatorial 5b: TLC: $R_f = 0.52$ (chloroform/methanol, 5:1 v/v); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: $\delta = -4.17$ ppm; ^{1}H NMR (DMSO-d_6): $\delta = 1.13$ (t, $J = 7.0$ Hz, 6H; $2 \times CH_3$), 3.44 (q, $J = 7.0$ Hz, 4H; $2 \times CH_2$), 4.37 (q, $J = 10.0$ Hz, 1H; H-5′), 4.47 (dt, $J = 10.5$ and 6.0 Hz, 1H; H-4′), 4.74 (q, $J = 6.0$ Hz, 1H; H-5″), 4.87 (t, $J = 5.0$ Hz, 1H; H-2′), 5.32–5.37 (m, 3H; H-3′ and coumarin CH_2), 5.73 (s, 1H; H-1′), 6.13 (s, 1H; coumarin H-3), 6.27 (d, $J = 4.0$ Hz, 1H; OH), 6.57 (d, $J = 2.0$ Hz, 1H; coumarin H-8), 6.72 (dd, $J = 9.0$ and 2.0 Hz, 1H; coumarin H-6), 6.75 (s, 2H; NH_2), 7.49 (d, $J = 9.0$ Hz, 1H; coumarin H-5), 10.89 (s, 1H; NH) ppm; ESI MS: 653.4 and 655.3 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{26}BrN_6O_9P \times H_2O$ (671.40): C 42.93, H 4.20, N 12.52; found: C 43.16, H, 3.90, N, 11.98.

7-(Dimethylamino)-4-formylcoumarin: A stirred mixture of 7-(dimethylamino)-4-methylcoumarin (4.06 g; 20 mmol) and selenium dioxide (3.33 g; 30 mmol) in p-xylene (150 mL) was refluxed for 7 h. The mixture was filtered hot to remove black selenium, and the filtrate was concentrated under reduced pressure. Purification of the resulting precipitate by recrystallization from *i*propanol gave the aldehyde (2.68 g, 61.9%) as yellow crystals. M.p.: 215–20 °C; ^{1}H NMR (DMSO-d_6): $\delta = 3.03$ (s, 6H; $N(CH_3)_2$), 6.62 (d, $J = 2.1$ Hz, 1H; H-8), 6.66 (s, 1H; H-3), 6.79 (dd, $J = 9.2$ and 2.2 Hz, 1H; H-6), 8.22 (d, $J = 9.1$ Hz, 1H; H-5), 10.07 (s, 1H; CHO) ppm; ESI MS: 218.02 $[M+H]^+$; elemental analysis calcd (%) for $C_{12}H_{11}NO_3$ (217.22): C 66.35, H 5.10, N 6.45; found: C 66.36, H 5.16, N 6.30.

7-(Dimethylamino)-4-formylcoumarin tosylhydrazone: A mixture of 7-(dimethylamino)-4-formylcoumarin (2.17 g; 10 mmol) and p-tosylhydrazine (2.05 g; 11 mmol) in ethanol/tetrahydrofuran (THF) (1:1, v:v; 100 mL) was stirred at room temperature for 6 h. THF was removed in vacuo and the solution was allowed to stand in a refrigerator. The resulting precipitate was collected by filtration and washed with ethanol to give the desired product (2.54 g, 65.9% yield) as brown-orange solid. M.p: 128–33 °C; ^{1}H NMR (DMSO-d_6): $\delta = 2.36$ (s, 3H; CH_3), 3.02 (s, 6H; $N(CH_3)_2$), 6.17 (s, 1H; H-3), 6.53 (d, $J = 2.5$ Hz, 1H; H-8), 6.71 (dd, J = 9.1 and 2.2 Hz, 1H; H-6), 7.44 (d, $J = 8.5$ Hz, 2H; H-3′ and H-5′), 7.78 (d, $J = 7.3$ Hz, 2H; H-2′ and H-6′), 7.96 (s, 1H; CH=N), 8.02 (d, $J = 9.7$ Hz, 1H; H-5), 12.11 (s, 1H; NH) ppm; ESI MS: 386.2 $[M+H]^+$; elemental analysis calcd (%) for $C_{19}H_{19}N_3O_4S$ (385.44): C 59.21, H 4.97, N 10.90; found: C 59.60, H 5.01, N,10.57.

4-(Diazomethyl)-7-(dimethylamino)coumarin (2): Triethylamine (556.5 mg; 5.5 mmol) was added to a stirred suspension of the tosylhydrazone of 7-(dimethylamino)-4-formylcoumarin (1.93 g; 5 mmol) in THF/methanol (25 mL). The mixture was stirred at room temperature for 3 h, THF was removed in vacuo, and the resulting brown solid was collected by filtration and washed with methanol/ether to give the diazo compound **2** (2.22 g, 96.8%). M.p.: > 210 °C (dec.); ^{1}H NMR (DMSO-d_6): $\delta = 3.03$ (s, 6H; $N(CH_3)_2$), 5.48 (s, 1H; CH—N_2), 6.40 (s, 1H; H-3), 6.50 (d, $J = 2.2$ Hz, 1H; H-8), 6.68 (dd, J = 8.9 and 1.8 Hz, 1H; H-6), 7.46 (d, $J = 8.8$, 1H; H-5) ppm; ESI MS 230.2 $[M+H]^+$; elemental analysis calcd (%) for $C_{12}H_{11}N_3O_2$ (229.24): C 62.87, H 4.84, N 18.33; found: C,62.56, H, 4.65, N 18.42.

Reversed-phase HPLC: Characterization of the diastereoisomers of **4a,b** or **5a,b** by analytical RP-HPLC was carried out on a PLRP-S column, 250 × 4.6 mm internal diameter, 8 μm (Polymer Laboratories Ltd., UK) with a Shimadzu LC-6A system equipped with a UV detector operating at 254 nm. The sample concentration was 20 μM, with an injection volume of 20 μL. Runs were performed at 20 °C and at an eluent flow rate of 1 mL min^{-1}. The **4a**, **4b**, and **5b** isomers were eluted by using a linear gradient of 5–45% B in 35 min (eluent A, water; eluent B, acetonitrile). A linear gradient of 20–60% B over 60 min was used for the elution of **5a**. The retention times (min) for

each compound were determined as follows: **4a**: 25.8 (axial), 27.3 (equatorial); **5a**: 24.5 (axial), 28,8 (equatorial); **4b**: 21.2 (axial), 27.5 (equatorial); **5b**: 26.8 (axial), 32.8 (equatorial). In hydrolysis experiments the fractions were eluted by using a two-step gradient of 0–5% B in 5 min (in order to seperate HEPES buffer from 8-Br-cAMP or 8-Br-cGMP) followed by 5–95% B in 15 min (eluent A, water; eluent B, acetonitrile).

Separation and purification of the caged 8-Br-cAMPs and 8-Br-cGMPs were carried out by preparative HPLC on Nucleogel RP 100–10 C_{18}, 300 × 25 mm internal diameter, 10 μm (Machery-Nagel, Germany) by using a Shimadzu LC-8A system. Separations of the caged cyclic nucleotides were performed at an eluent flow-rate of 10 mL min^{-1} with a linear gradient of 15–45% B (**4a** as well as **4b** and **5b**) or 20–60% (**5a**) over 105 min (eluent A, water; eluent B, acetonitrile).

Photolysis and quantum yield determination: Photolysis was carried out using a high pressure mercury lamp (HBO 500, Oriel, U.S.A.) with controlled light intensity and metal interference filters of 333, 365, 405, and 436 nm transmission (Schott-Glaswerke, Germany). For the kinetic investigations the irradiated solutions were analyzed by analytical HPLC. Quantum yields φ (Table 1) were determined as described previously[2] with the modification that 25 μM solutions of the isomers of **4a,b** and **5a,b** in 20 or 50% methanol /0.01 M HEPES-KOH buffer (pH 7.2) were photoirradiated in steps of 10 s.

Fluorescence measurements: The fluorescence spectra were measured on a MPF-2A fluorescence spectrometer (Hitachi-Perkin Elmer). In the case of the highly reactive caged compounds (10 μM) the excitation intensity was very low and the registration time was very short (10 s) to minimize photolysis. The fluorescence quantum yields were determined at 298 K by the relative method[28] with quinine sulfate as a standard ($\varphi_f = 0.545$ in 0.1 N H_2SO_4). At the excitation wavelength used (333 nm) the absorbance values of the solutions of the standard and the investigated compound were identical. The different refractive indices of the solutions were considered.

Time-resolved fluorescence decay measurements (pulse sampling method) were performed by using a nitrogen laser ($\lambda = 337$ nm) as the excitation source and a transient recorder for the decay registration ($\lambda_{em} = 500$ nm). Details of the equipment and the deconvolution procedure of the experimental decay curve are described elsewhere.[29] The time resolution achieved was about 200 ps.

Stability measurements in H_2O: Freshly prepared solutions of the axial and the equatorial forms of the DMACM- and DEACM-caged compounds (axial **4a**: 10 μM; equatorial **5a**: 10 μM; axial **5b**: 50 μM; axial and equatorial **4b**: 10 μM) in 5% acetonitrile/0.01 M HEPES-KOH buffer (pH 7.2) containing KCl (0.12 M) were maintained in the dark at ambient temperature for 24 h and monitored by HPLC.

Solubility determination: The diastereoisomers of **4a,b** and **5a,b** were examined for their solubilities in 5% acetonitrile/0.01 M HEPES-KOH buffer (pH 7.2) containing KCl (0.12 M), at room temperature. After sonication to disperse solid material in the buffer solution and subsequent centrifugation, the concentrations of the axial and the equatorial isomers of the caged compounds were measured by HPLC.

Optical Ca^{2+} measurements by confocal laser scanning microscopy: HEK293 cells expressing the α subunit of the CNG channel from bovine cone photoreceptor cells (CNGA3),[30] were loaded with the Ca^{2+} indicator Fluo-3/AM (4.4 μM) in the presence of 0.01% Pluronic F-127 for 30 min at 37 °C. The cells were then incubated in the dark for 15 min with **4a** (20 μM; axial isomer) or **5b** (100 μM; axial isomer). The bath solution contained (mM): 140 NaCl, 4.6 KCl, 2 $CaCl_2$, 10 glucose; pH 7.4 (NaOH).

Ca^{2+}-dependent changes in the fluorescence intensity of Fluo-3 were detected with an LSM 510 invert confocal laser scanning microscope (Carl Zeiss Jena GmbH, Germany) with an oil immersion objective (× 63/1.4) and an argon-ion laser (488 nm) as excitation source. The excitation wavelength was selected by a dichroic mirror (FT 490). Fluorescence was detected at wavelengths above 505 nm by using a cut-off filter (LP 505) in front of the detector. The experimental chamber consisted of a culture dish (35 × 10 mm, Costar, Tecnomara Germany GmbH, Germany) with a glass base (22 × 22 mm, thickness 0.1 mm). Fluorescence images were usually scanned and stored as a time series. For rapid measurements of the Ca^{2+}-induced fluorescence changes, regions of interest were selected. The caged compounds were photolyzed with an argon-ion laser (Coherent, Germany) at $\lambda = 364$ nm. The UV light was guided onto the sample by fiber optics, as described in detail elsewhere.[8]

Electrophysiological experiments: Patch clamp experiments with the axial isomers of **4b**, **5b** and DMNB-caged 8-Br-cGMP were conducted as described previously.[1] HEK293 cells were transfected with the rat olfactory CNG channel subunit (CNGA2) or the bovine cone CNG channel (CNGA3). Stock solutions of the caged compounds in DMSO (10 mM and 1 mM) were added to the pipette solution, which contained (mM): 140 KCl, 5 NaCl, 1 $MgCl_2$, 1 ethylene glycol tetraacetic acid (EGTA), 10 HEPES; pH 7.2 (KOH). The bath solution contained (mM): 140 NaCl, 3 KCl, 50 glucose, 10 EGTA, 10 HEPES; pH 7.4 (NaOH). Divalent cations were omitted from the solutions to avoid blockage of CNG channels. Light flashes were generated by a 100-W Hg lamp (AMKO, Tornesch, Germany) equipped with an infrared and a UV filter (transmission > 320 nm) and an electronic shutter. For photolysis, the flash light was passed through a bandpass filter (405 ± 20 nm). The fluorescence of Fura-2 and of **7** was recorded for single cells by using an excitation filter at 380 ± 5 nm and an emission filter at more than 460 nm. Current and fluorescence signals were recorded by using a patch clamp amplifier (List, Darmstadt, Germany) and an integrating photon counter (Life Science Resources, Cambridge, UK).

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