Photoactivatable Compounds

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Coumarinylmethyl Esters for Ultrafast Release of High Concentrations of Cyclic Nucleotides upon One- and Two-Photon Photolysis**

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Biologically inert, photoactivatable precursors ("caged" compounds) of cyclic nucleoside monophosphates (cNMPs) are powerful tools for studying the spatiotemporal dynamics of cyclic nucleotide dependent processes. Among these compounds, (coumarin-4-yl)methyl esters of cNMPs are most useful because they show no background bioactivity, are stable to solvolysis, and can be photolyzed efficiently and extremely quickly.[1,2] Recently, we introduced [7-(diethylamino)coumarin-4-yl]methyl (DEACM) esters of cNMPs as caged compounds.[3,4] Compared with other coumarinylmethyl-caged cNMPs, the DEACM esters photorelease cNMPs with higher photosensitivity at long-wavelength irradiation (up to 436 nm), thus minimizing or even preventing damage to cellular components and chromophores by photobleaching.

Herein, we describe the development of the 7-[bis(carboxymethyl)amino]-substituted coumarinylmethyl building blocks **1** and **2** (structures in Scheme 1) for the caging of phosphates and other functionalities. With the axial and the equatorial diastereomers of the {7-[bis(carboxymethyl)amino]coumarin-4-yl}methyl (BCMACM) esters of cAMP, cGMP, 8-Br-cAMP, and 8-Br-cGMP **3**–**6** (Scheme 1), we present new variants of the DEACM-caged cNMPs, which maintain their favorable properties and additionally have much higher aqueous solubility by virtue of their anionic

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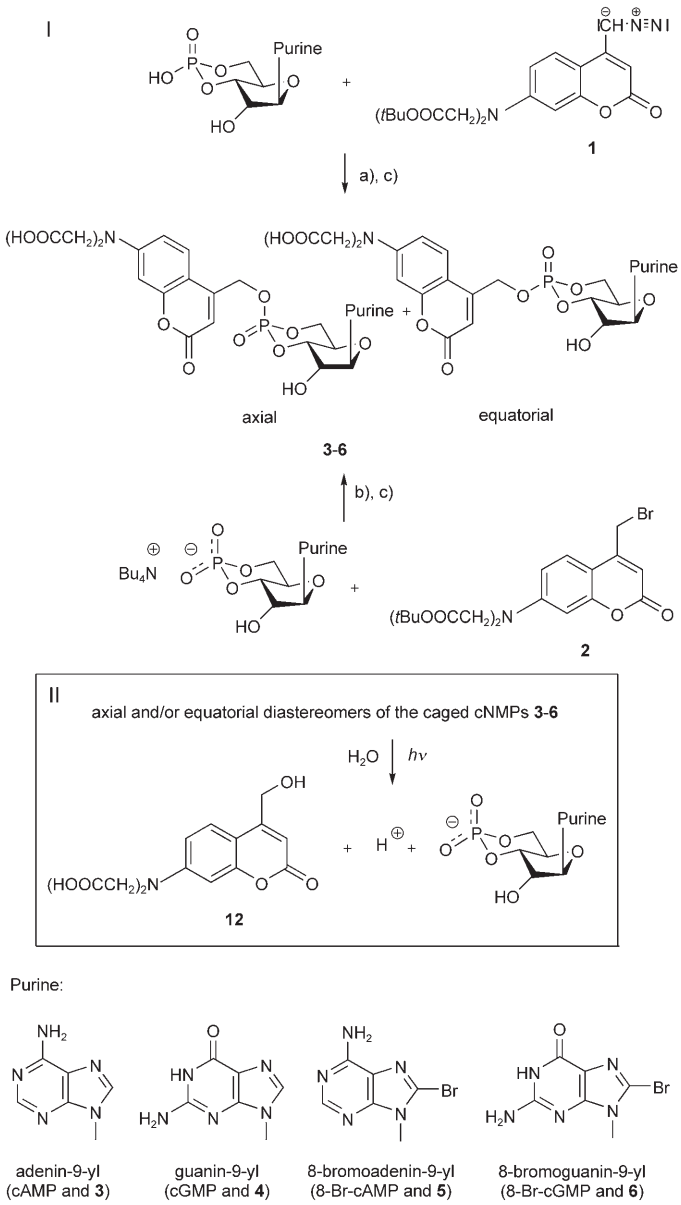


Scheme 1. Synthesis (I) and photolysis (II) of **3**–**6**: a) DMSO/CH_3CN (1:4), 60 °C, 24 h; b) CH_3CN, 85 °C, 5 h; c) TFA/CH_2Cl_2/H_2O (75:24:1), room temperature, 20 min.

centers. Furthermore, we show that the new caged compounds photorelease cNMPs upon one- as well as two-photon excitation and that it is possible to determine quantitatively the amount of cyclic nucleotide photoreleased inside a cell by fluorescence measurements.

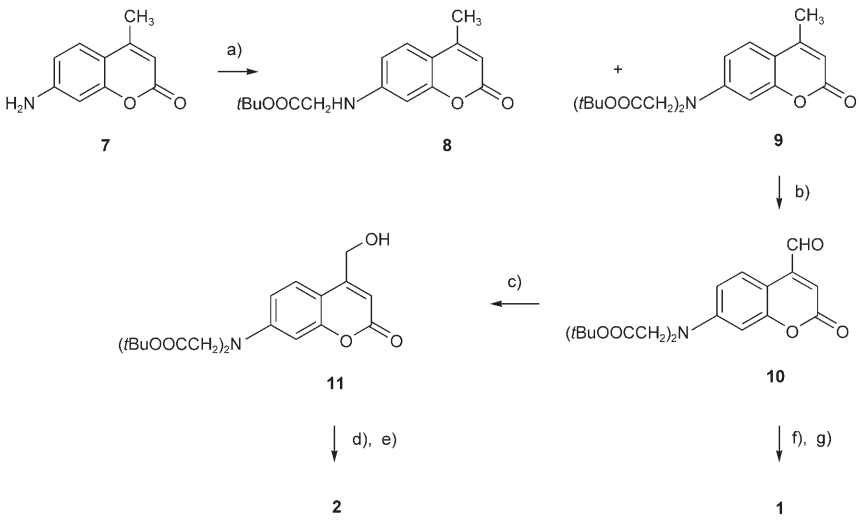
The compounds **3**–**6** were prepared as shown in Scheme 1 I by alkylation of the free acid form of the respective cNMP with the 4-(diazomethyl)coumarin **1** (Method A) or by reaction of the tetra-*n*-butylammonium salts of the cNMPs with the 4-(bromomethyl)coumarin **2** (Method B) according to procedures described earlier for other coumarinylmethyl esters of cNMPs[5,6] and by subsequent removal of the *tert*-butoxy protecting groups with trifluoroacetic acid (TFA).

The synthesis of the key compounds **1** and **2** is outlined in Scheme 2. 7-Amino-4-methylcoumarin (**7**) was treated with an excess of bromoacetic acid *tert*-butyl ester in the presence of diisopropylethylamine to yield a mixture of **8** and **9**, which was separated by flash chromatography to provide the two products in yields of 27% and 31%, respectively. SeO_2 oxidation of **9** gave the corresponding aldehyde **10** in a yield of 80%. Condensation of **10** with tosylhydrazide furnished the corresponding hydrazone (84% yield), which was transformed into **1** by triethylamine-mediated Bamford–Stevens reaction (87% yield). Compound **2** was best prepared (81% yield) from the alcohol **11**, which was synthesized in 74% yield from **10** by reduction with $NaBH_4$, by mesylation and subsequent displacement of the mesyl group using LiBr in THF. Our attempts to convert **9** directly into **2** by utilizing *N*-bromosuccinimide were not successful as they resulted mainly in bromo substitution in position 3 of the coumarin ring.

Alkylation of the cNMPs using Method A gave relatively low product yields for the *tert*-butoxy-protected derivatives of **3** (12%) and **6** (7%) but an acceptable yield for the corresponding analogue of **4** (25%). The reaction of **1** with 8-Br-cAMP yielded only traces of **5**. However, the alternative esterification by Method B gave relatively high product yields for the *tert*-butoxy-protected derivatives of **3** (38%) and **5** (49%), an acceptable yield for the corresponding derivative of **6** (18%), and only negligible amounts of the analogue of **4**. Compared with the syntheses of other caged cNMPs,[2,3,7] **3**–**6** can be prepared with good yields by Method A for the synthesis of **4** and Method B for the preparation of **3**, **5**, and **6**, particularly because the product yields represent typical rather than optimal conditions.

Both synthetic procedures resulted in diastereomeric mixtures of the *tert*-butoxy-protected caged compounds. The ratios of the axial to the equatorial diastereomers were approximately 2:3 (Method A) and from 2:1 to 4:1 (Method B). The diastereomers were separated by reversed-phase HPLC on a preparative scale. In all cases, the axial isomers showed lower retention times than the equatorial isomers. TFA deprotection of the *tert*-butoxy-protected diastereomers of the esters resulted in the pure axial and equatorial isomers of **3**–**6** with approximately quantitative yields (see the Supporting Information for preparative details and analytical characterization). The isomers were assigned by ^{31}P NMR spectroscopy.[8,9]

Like other coumarinylmethyl-caged cNMPs, compounds **3**–**6** are highly resistant to spontaneous hydrolysis in the dark. HPLC monitoring of the pure diastereomers of the caged compounds in aqueous HEPES buffer at pH 7.2 during a 24-h period revealed no measurable formation ($<0.5\%$) of the free cNMPs at room temperature (HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid). All the compounds are readily soluble in aqueous HEPES buffer at pH 7.2 (Table 1), which is a prerequisite for the administration of large concentrations of phototriggers in cells or around membrane patches at the tip of glass pipettes when the patch–clamp technique is used. As expected, the hydrophilic caged cNMPs do not measurably partition into model lipid membranes at pH 7.2. This was demonstrated by the lack of reaction heats when compounds **3** and **5** were titrated with



Scheme 2. Synthesis of **1** and **2**: a) $BrCH_2COOtBu$ (3 equiv), iPr_2EtN, NaI, CH_3CN, reflux, 24 h; b) SeO_2, *p*-xylene, reflux, 6 h; c) $NaBH_4$, MeOH, room temperature, 2 h; d) CH_3SO_2Cl, Et_3N, CH_2Cl_2, 5 °C, 20 min; e) LiBr, THF, room temperature, 1.5 h; f) *p*-tosylhydrazide, EtOH, room temperature, 1.5 h; g) Et_3N, MeOH, room temperature, 3 h.

Table 1: Long-wavelength absorption maxima (λ_{abs}^{max}), extinction coefficients, (ε^{max}), photochemical quantum yields (ϕ_{chem}), fluorescence maxima (λ_f^{max}), fluorescence quantum yields (ϕ_f), lifetimes of the lowest excited singlet state (τ), and solubilities (*s*) of the BCMACM esters **3**–**6** and of **12** in CH_3CN/HEPES-KCl buffer (5:95), pH 7.2.

Compound	λ_{abs}^{max} [nm]	ε^{max} [$M^{-1}cm^{-1}$]	ϕ_{chem}[a]	λ_f^{max} [nm]	ϕ_f[b]	τ [ns]	*s* [mM]
3 (axial)	384	17000	0.26	483	0.007	0.07[c]	>2
3 (equatorial)	383	17900	0.30	484	0.007	0.06[c]	>1
4 (axial)	386	18000	0.25	482	0.008	0.07[c]	>2
4 (equatorial)	383	18700	0.30	480	0.011	0.10[c]	>1
5 (axial)	384	18500	0.23	486	0.012	0.11[c]	≈1
5 (equatorial)	383	18100	0.26	483	0.025	0.23[c]	≈1
6 (axial)	387	18100	0.25	479	0.015	0.13[c]	≈1
6 (equatorial)	282	18400	0.29	487	0.023	0.21[c]	≈1
12	376	18400	–	479	0.17	1.59[d]	–

Estimated average uncertainties: [a] 15%, [b] 8%. [c] Calculated as $\tau = \tau_{12} \times \phi_f/\phi_f^{12}$,[13] τ_{12} = fluorescence lifetime of **12**, ϕ_f^{12} = fluorescence quantum yield of **12**. [d] Measured fluorescence lifetime of **12**.

lipid vesicles at different temperatures (see the Supporting Information for details). Nevertheless, the caged compounds rapidly enter live cells, probably using transport proteins or by endocytosis, and HEK293 cells could be loaded with **6** by incubation with a 50-μM buffered (pH 7.2) solution.

Photoactivation of the axial or equatorial diastereomers of the (coumarin-4-yl)methyl esters **3**–**6** by irradiation with wavelengths of 330–430 nm in aqueous buffer leads to the 7-[bis(carboxymethyl)amino]-4-(hydroxymethyl)coumarin **12**, a proton, and the respective "free" cNMP anion (Scheme 1 II). The photoreaction is efficient and clean and gives about 90–95% of the cNMP. Indications of a significant triplet-state population were not found. In fact, the lifetimes of the lowest excited singlet states S_1 are extremely short, about 0.1 ns (see Table 1). We assume that the photochemical conversion proceeds by means of a photochemical S_N1 mechanism (solvent-assisted photoheterolysis) from S_1 via a singlet ion pair. This ion pair decays mainly by recombination to the singlet ground state.[10] Competitively, separation of the ion pair by the polar solvent (formation of the cNMP anion) and subsequent hydrolysis of the coumarinylmethyl carbocation or, alternatively, direct reaction of a water molecule from the solvent cage of the ion pair with its carbocation followed by a very fast deprotonation step may take place.[10,11] Such a photolysis pathway implies that product formation is very fast. Indeed, time-resolved fluorescence measurements upon single-pulse excitation (0.5 ns half-width, 337 nm) of the axial diastereomers of **3** and **4** revealed fluorescence contributions from **12**. Because pure solutions of the caged compounds were irradiated, **12** must have been formed and excited during the photolysis pulse. Actually, deconvolution of the fluorescence signals with a biexponential decay function yielded rise times of the fluorescence of **12** of about 0.7 ns and 0.4 ns for the axial diastereomers of **3** and **4**, respectively. The rate constants of cNMP formation are the quotients of the efficiencies of product formation from the ion pairs and the fluorescence rise times of **12**.[10] Considering the corresponding efficiencies of product formation of 0.27 for **3** and 0.26 for **4**, we obtain for the rate constants values of $4 \times 10^8\ s^{-1}$ and $6 \times 10^8\ s^{-1}$, respectively. As the quantum yields, ϕ_{chem} and ϕ_f, do not vary much (Table 1), it is expected that the equatorial diastereomers of **3** and **4** as well the diastereomers of **5** and **6** photorelease the cNMPs with similar rates.

The photochemical characteristics of the BCMACM esters **3**–**6** in HEPES buffer (pH 7.2) are summarized in Table 1. The absorption and the fluorescence spectra show maxima at 382–387 nm and 479–487 nm, respectively, and correspond to those of the DEACM esters. The single-photon photochemical quantum yields, ϕ_{chem}, as well as the extinction coefficients, ε^{max}, are high for caged compounds, which results in excellent photosensitivity at long-wavelength irradiation. As a consequence, even moderate UV/Vis irradiation is sufficient to obtain large amounts of free cNMP.

The combination of the high photosensitivity and the good solubility of **3**–**6** also permits the generation of large instantaneous cNMP concentration jumps. This could be

demonstrated by means of cyclic nucleotide gated (CNG) ion channels of the rod photoreceptor, which are activated by cGMP. The CNGA1 subunit of these channels was expressed heterologously in *Xenopus* oocytes, and the current conducted by these channels was measured in inside-out patches with the patch–clamp technique. For example, with 400 μM **4** in the bath solution, a 150-μs light flash generated concentration jumps of free cGMP from zero to 72 μM. Figure 1

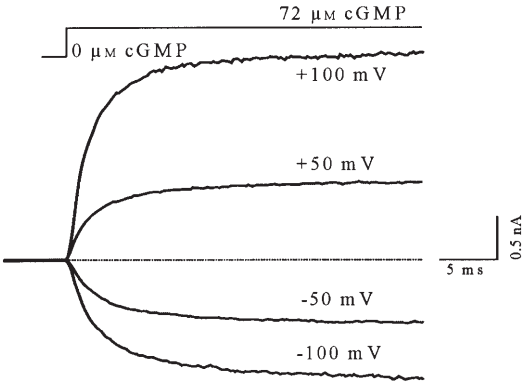


Figure 1. Activation of homotetrameric CNGA1 channels by jumps in cGMP concentration upon photolysis of **4** (axial isomer). The channels were expressed heterologously in *Xenopus* oocytes, and the recordings were performed in inside-out patches containing a high number of channels. The concentration of free cGMP was determined from the steady-state current of the traces referred to the steady-state current at saturating cGMP and the concentration–response relationship for these channels.[15] Before the flash, no current was present. The four traces at the indicated voltages were recorded from the same patch and were corrected for small leak currents by subtracting the respective traces recorded in the absence of cyclic nucleotide.

illustrates the resulting activation time courses at four transmembrane voltages. The time courses are highly resolved and are therefore most appropriate to give information on the molecular mechanisms underlying the activation gating of these channels. At maximum concentrations, **4** allowed us to elicit larger cGMP concentration jumps than the DEACM ester of cGMP.

BCMACM-caged cNMPs are highly sensitive not only to one-photon but also to two-photon excitation. Images obtained by confocal laser scanning microscopy (Figures 2 and 3) indicate that upon photolysis of **6**, 8-Br-cGMP mediates the entry of Ca^{2+} ions into HEK293 cells expressing the cGMP-gated ion channel of the cone photoreceptor (CNGA3). UV (argon laser, 364 nm) as well as IR light (Cameleon diode-pumped laser, 745 nm) evoked a saturable increase in the fluorescence intensity of the Ca^{2+} indicator FLUO-4, which indicates Ca^{2+}-ion influx through CNG channels. Addition of excess Mg^{2+} to the used Ca^{2+} buffer blocked Ca^{2+} influx, and neither UV nor IR irradiation in the absence of **6** led to changes in the fluorescence signal of the cells (not shown). High sensitivity to two-photon photolysis has been described earlier for (6-bromo-7-hydroxycoumarin-4-yl)methyl-caged compounds[2,12] and recently for (6,7-dimethoxycoumarin-4-yl)methyl diethyl phosphate as well as sodium [7-(dimethylamino)coumarin-4-yl]methyl sul-

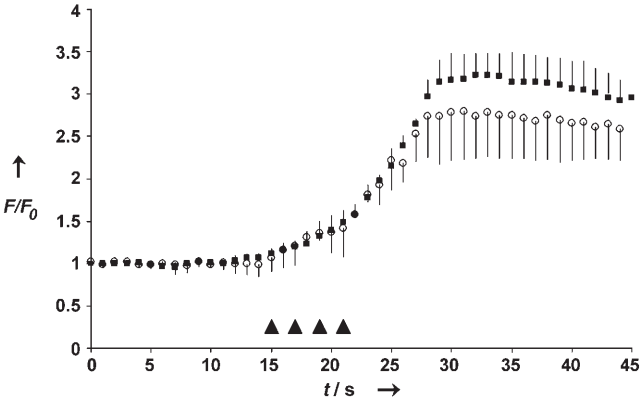


Figure 2. Time course of the Ca^{2+} influx through CNG channels into HEK293 cells expressing the CNGA3 channel after photorelease of 8-Br-cGMP from **6** (axial isomer) by exposure to UV light (364 nm, one-photon excitation, ■) and to IR light (745 nm, two-photon excitation, ○), respectively. The cells were preincubated with 4 μM FLUO-4/AM (30 min) and 50 μM **6** (20 min). The fluorescence intensity F of the Ca^{2+} indicator FLUO-4 was recorded before and after irradiation (▲). There was no significant difference between the two curves. F/F_0 = relative fluorescence intensity (mean values ± standard deviations obtained from three cells).

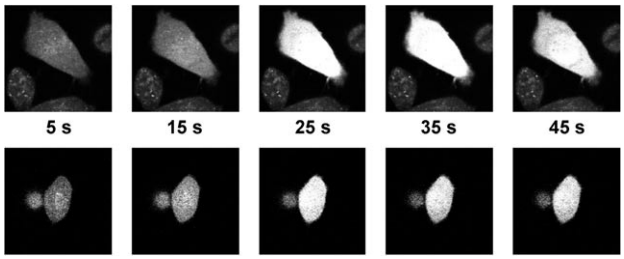


Figure 3. Fluorescence intensity of the Ca^{2+} indicator FLUO-4 in two CNGA3-transfected HEK293 cells before and after photorelease of 8-Br-cGMP from **6** (axial isomer) by exposure to UV (364 nm, upper row) or to IR light (745 nm, lower row). The cells were preincubated with 4 μM FLUO-4/AM (30 min)and 50 μM **6** (20 min). The indicated times correspond to those given in Figure 2. The fluorescence intensity is represented in an 8-bit gray scale.

fate.[13] Direct comparison of the time course for two-photon uncaging of **6** (axial isomer) with that of *N*-[(6-bromo-7-hydroxycoumarin-4-yl)methoxycarbonyl]-L-glutamic acid (Bhc-glu)[12] reveals that **6** photodecomposes initially 1.3 times faster than Bhc-glu under identical conditions (Figure 4). Because Bhc-glu displays a two-photon uncaging action cross-section, δ_u, at 740 nm of about 10^{-50} $cm^4 s\,photon^{-2}$, the two-photon action cross-section of **6** should be slightly larger than 10^{-50} $cm^4 s\,photon^{-2}$. Similar δ_u values are expected also for the equatorial isomer of **6** as well as for the diastereomers of **3**–**5**.

The fluorescence quantum yields ϕ_f of **3**–**6** are one order of magnitude smaller than that of the photoreleased alcohol **12** (Table 1). Compound **12** fluoresces also inside cells (see the Supporting Information), in contrast to 7-(diethylamino)-4-(hydroxymethyl)coumarin whose fluorescence is totally quenched inside HEK293 cells.[4] Thus, fluorescence spectro-

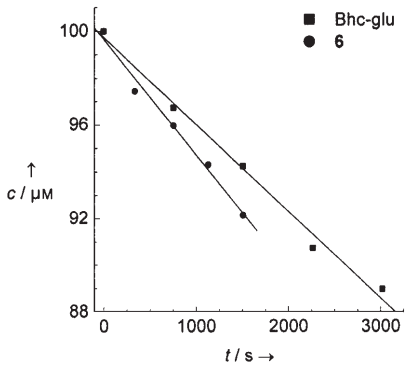


Figure 4. Initial time course of the two-photon photolysis of **6** (axial isomer) and Bhc-glu upon 740-nm irradiation in HEPES buffer, pH 7.2. The concentrations of **6** and Bhc-glu (starting concentrations 100 μM) were determined by RP-HPLC. Solid lines are linear regression lines. **6**: $c/\mu m = -0.0049t/s + 99.65$ ($R^2 = 0.987$); Bhc-glu: $c/\mu m = -0.0037t/s + 99.74$ ($R^2 = 0.991$). c = concentrations of the caged compounds.

scopic visualization of the progress of cNMP photorelease from BCMACM-caged cNMPs within cells becomes possible, as demonstrated in Figure 5. The generation of 8-Br-cGMP

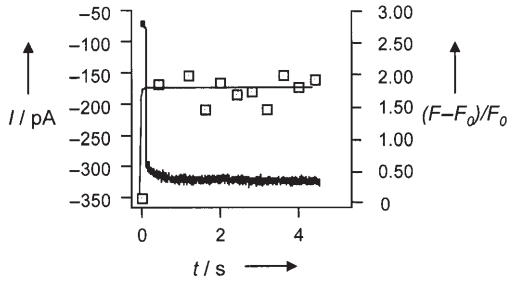


Figure 5. Monitoring the photorelease of 8-Br-cGMP from **6** within a CNGA3-transfected HEK293 cell by measuring the total whole-cell current and the fluorescence emitted from liberated **12** using excitation at 360 nm. The cell was loaded in the whole-cell configuration of the patch–clamp technique with 80 μM **6** and irradiated. The change in current amplitude and the fluorescence intensity increase (□) show photolytic liberation of 8-Br-cGMP.

from **6** in CNGA3-transfected HEK293 cells was monitored simultaneously by current recordings resulting from CNG-channel activation and by the fluorescence emitted from the liberated photoproduct **12** upon excitation at 360 nm. Since the fluorescence signal can be calibrated,[14] it is possible to quantify cNMP release from BCMACM esters of cNMPs using fluorescence spectroscopy. In addition to the high solubility of BCMACM-caged cNMPs in aqueous buffer, the possibility of visualizing and quantifying the release process within cells is a considerable advantage of BCMACM-caged cNMPs over the DEACM-caged analogues.

In conclusion, we have developed a photolabile coumarinylmethyl protecting group bearing a dianionic substituent and used this protecting group for the caging of cNMPs. The caged cNMPs exhibit high photosensitivities at long-wavelength irradiation and allow large and instantaneous cNMP concentration jumps upon one- and two-photon excitation within cells under physiological conditions. This approach can extend decisively the tools available for the study of space- and time-dependent aspects of cNMP-triggered processes. Finally, the novel protecting group should be useful in the caging and uncaging of other biomolecules with phosphate, carboxylate, and other functionalities. Exemplarily, the BCMACM ester of phenylacetic acid has already been prepared. The caged compound shows high solubility in aqueous buffer and can be photolyzed rapidly and efficiently ($\phi_{chem} = 0.02$, $\varepsilon^{max} = 20000$) in the visible-wavelength region.

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