Bhc-cNMPs as either Water-Soluble or Membrane-Permeant Photoreleasable Cyclic Nucleotides for both One- and Two-Photon Excitation

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*Cyclic nucleoside monophosphates (cNMPs) play key roles in many cellular regulatory processes, such as growth, differentiation, motility, and gene expression. Caged derivatives that can be activated by irradiation could be powerful tools for studying such diverse functions of intracellular second messengers, since the spatiotemporal dynamics of these molecules can be controlled by irradiation with appropriately focused light. Here we report the synthesis, photochemistry, and biological testing of 6-bromo-7-hydroxycoumarin-4-ylmethyl esters of cNMP (Bhc-cNMP) and their acetyl derivatives (Bhc-cNMP/Ac) as new caged second messengers. Irradiation of Bhc-cNMPs quantitatively produced the parent cNMPs with one-photon uncaging efficiencies ($\Phi\varepsilon$) of up to one order of magnitude better than those of 2-nitrophenethyl (NPE) cNMPs. In addition, two-photon induced photochemical release of cNMP from Bhc-cNMPs (**7** and **8**) can be observed with the two-photon uncaging action cross-sections (δ_u) of up to 2.28 GM (1 GM = 10^{-50} cm^4 s photon^{-1}), which is the largest value among those of the reported Bhc-caged compounds. The wavelength dependence of the δ_u values of **7** revealed that the peak wavelength was twice that of the one-photon absorption maximum. Bhc-cNMPs showed practically useful water solubility (nearly 500 μM), whereas 7-acetylated derivatives (Bhc-cNMPs/Ac) were expected to have a certain membrane permeability. Their advantages were demonstrated in two types of biological systems: the opening of cAMP-mediated transduction channels in newt olfactory receptor cells and cAMP-mediated motility responses in epidermal melanophores in scales from medaka fish. Both examples showed that Bhc and Bhc/Ac caged compounds have great potential for use in many cell biological applications.*

Introduction

Cyclic nucleoside monophosphates (cNMPs) are an important class of compounds that regulate several cellular functions.[1] The known main targets of cNMPs are distributed in either the cytosol (protein kinases[2] and phosphodiesterases,[3] for example) or the plasma membrane (cyclic nucleotide-gated ion channels[4]), and so highly localized regulation of the intracellular concentrations of cNMPs should be needed for live cells to respond to various stimuli.[5] To study the regulatory functions of cNMPs, the use of active agonist or inhibitor molecules might be helpful. Since unmodified cNMPs can hardly penetrate the intact plasma membrane, several membrane-permeant derivatives have been reported;[6] examples include 8-Br-cNMP, 8-pCPT-cNMP, Bt_2cNMP, and their acetoxymethyl (AM) esters,[7] which can diffuse across the plasma membrane and activate intracellular targets. This approach is especially effective when the compound needs to be applied to a group of cells or tissue samples simultaneously, but would not be suitable for an experiment that required the localized activation of a target with sub-cellular resolution or kinetically resolved analysis, since activation of the target molecule would occur simultaneously everywhere inside the cells.

Caged compounds that can be activated by photoirradiation may offer an ideal method for controlling the intracellular concentration of a signaling molecule and should provide an opportunity to investigate the molecular mechanisms underlying signaling processes with high temporal and spatial resolution.[8] Various caged cNMPs with different caging groups have been reported by us[9] and by others[10–13] since the first report of the 2-nitrobenzyl ester of cAMP by Engels and Schlaeger in

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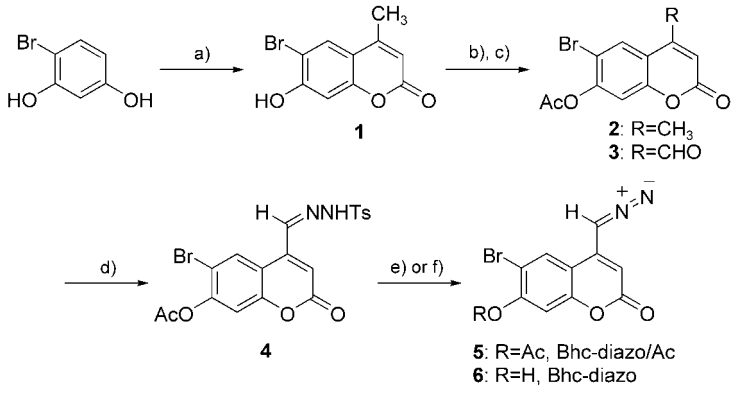
1977.[14] However, most of these were either too lipophilic for appropriately concentrated aqueous solutions to be prepared or too hydrophilic to pass through the plasma membranes of intact live cells, which might limit their broader application. We reported 7-alkoxycoumarin-4-ylmethyl groups as an alternative to the conventional 2-nitrobenzyl cages in 1995,[9a] and found that a hydroxycoumarin cage could satisfy both criteria: that is, appropriate membrane permeability and a certain hydrophilicity. The Bhc (6-bromo-7-hydroxycoumarin-4-ylmethyl) group is our latest version of the hydroxycoumarin cage and has several advantages over those reported previously: high photolysis efficiency upon one-photon irradiation, biologically useful cross-sections upon two-photon excitation, and improved stability in the dark.[15]

To extend the utility of Bhc-caged compounds, here we report the synthesis, photochemistry, and biological testing of Bhc-caged cyclic nucleotides. Bhc-caged cNMPs were synthesized via the corresponding diazomethane derivatives Bhc-diazo (**6**) and Bhc-diazo/Ac (**5**, Scheme 1). In vitro uncaging of the Bhc-cNMPs quantitatively produced the parent cyclic nucleotides with particularly useful one- and two-photon uncaging efficiencies. A greater photosensitivity of the Bhc cage than of the 2-nitrobenzyl cage was also seen for the activation of cAMP-gated ion channels in olfactory receptor cells. Furthermore, the potency of the membrane-permeant Bhc/Ac cage in terms of its application to live cells is demonstrated in the activation of motile responses of melanin pigments in fish melanophores. The results revealed that Bhc-cAMP/Ac can cross the intact plasma membrane, is accumulated inside live melanophores, and repeatedly releases the parent cyclic nucleotides with high efficiency upon irradiation.



Scheme 1. *Synthesis of Bhc-diazo (**5**) and Bhc-diazo/Ac (**6**): a) ethyl acetoacetate,* p*-TsOH, toluene, reflux, 17 h, 90%; b) acetic anhydride, 110°C, 3 h, 95%; c) SeO_2, chlorobenzene, reflux, 5 d, 88%; d)* p*-tosylhydrazide, EtOH, 40°C, 82%; e) for **5**, Et_3N (1 equiv), MeOH, RT, 1 h, 85%; f) for **6**, Et_3N (2 equiv), MeOH, RT, 2 d, 79%.*

Results

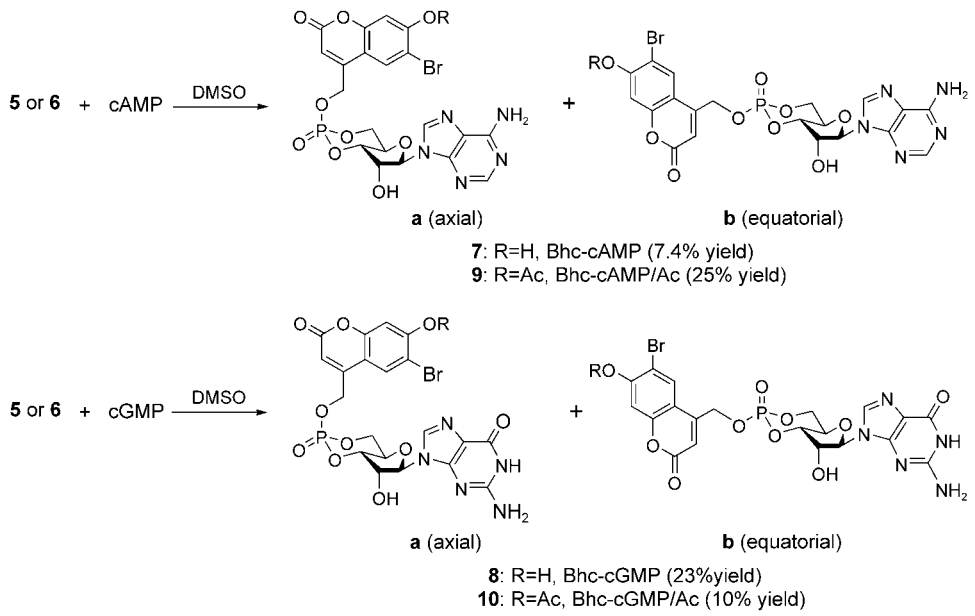
Synthesis

To mask the biological functions of cyclic nucleotides (cNMPs), the cyclic phosphate moiety must be protected as an ester that can later be deprotected by photolysis. Thus, Bhc esters of cNMPs were synthesized by esterification of the free acid moieties of cNMPs by 6-bromo-7-hydroxy-4-diazomethylcoumarin (Bhc-diazo, **6**). Since a preliminary synthetic method and the application of Bhc-diazo (**6**) have already been reported,[15a,b] here we describe its synthetic details with some improvements (Scheme 1). An acid-catalyzed condensation of 4-bromoresorcinol with ethyl acetoacetate gave 4-methylcoumarin derivative **1** in good yield. In our original reports we used conc. H_2SO_4 as an acid catalyst as well as as a solvent, but the reaction time was lengthy (>5 days) and the product yields varied from 50 to 80%. A slight improvement was achieved by replacing sulfuric acid (best case: 5–6 days, 83.4% yield) with a catalytic amount of *p*-toluenesulfonic acid in toluene at reflux (17 h, 89.7% yield).[16] Allylic oxidation of the 4-methyl group by selenium dioxide in chlorobenzene, followed by condensation with *p*-tosylhydrazide, gave the corresponding hydrazone **4**. Hydrazone **4** was transformed into Bhc-diazo/Ac (**5**) by treatment with a methanolic triethylamine solution. Simultaneous deprotection of the 7-acetyl moiety was achieved by prolonged exposure to a basic methanol solution, which led to the desired Bhc-diazo (**6**). Esterification of the free acid forms of cyclic AMP and cyclic GMP with Bhc-diazo (**6**) in DMSO gave Bhc-cAMP **7** (7.4% isolated yield) and Bhc-cGMP **8** (23% isolated yield), respectively (Scheme 2). Each compound exists as a mixture of axial and equatorial isomers with respect to the six-membered cyclic phosphate ring. The axial/equatorial stereochemistry was assigned by ^{31}P NMR measurements as previously reported.[14] Alternatively, it can be estimated by comparison of the retention times in reversed-phase HPLC as previously described.[9c] In a similar manner, membrane-permeant derivatives Bhc-cAMP/Ac (**9**) (25% isolated yield) and Bhc-cGMP/Ac (**10**) (10% isolated yield) were prepared by use of the corresponding diazo compound Bhc-diazo/Ac (**5**) (Scheme 2).

The hydrolytic stability of the Bhc-cNMPs was measured in K-MOPS solution (pH 7.2) and expressed as a half-life ($t_{1/2}$) in the dark. The results are summarized in Table 1. For the Bhc-caged cyclic nucleotides, the axial isomers are always more stable than the equatorial isomers. This is consistent with the results for other coumarin-caged cAMPs as we described previously. In **9** and **10**, hydrolysis of the 7-acetoxy group to a 7-hydroxy group occurred to produce still-caged Bhc-cAMP and Bhc-cGMP with half-lives of 13.4 h and 17 h, respectively (Table 1), which are comparable to the values reported for other 7-acyloxycoumarin-caged cAMPs.[9c] Solubility in an aqueous buffer solution (K-MOPS, pH 7.2, containing 1% DMSO) were determined as nearly 500 μM for both isomers of Bhc-cAMP and cGMP (Table 1).

Photochemical properties

The photochemical properties of Bhc-cyclic nucleotides were examined in K-MOPS solution (10 μM, pH 7.2) at 350 nm



Scheme 2. *Synthesis of Bhc-cNMPs.*

Table 1. *Selected photochemical and photophysical properties of coumarin-caged cyclic nucleotides.*

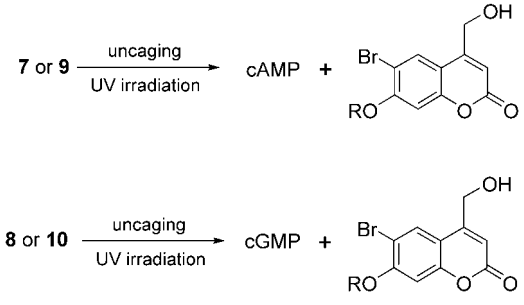
Substrate	λ_{max}[a] (ε)[b]	Φ_{dis}[c]	Φ_{app}[d]	$\Phi_{app}\varepsilon$[e]	s[f]	$t_{1/2}$[h]
Bhc-cAMP (ax) (**7a**)	375 (14550)	0.081			440	260
Bhc-cAMP (eq) (**7b**)	371 (17000)	0.107	0.100	1120	490	90
Bhc-cGMP (ax) (**8a**)	377 (14800)	0.075			580	1240
Bhc-cGMP (eq) (**8b**)	372 (15360)	0.117	0.116	1260	430	420
Bhc-cAMP/Ac (**9**)	320 (6000)	0.080	0.074	141	nd[g]	13.4[i]
Bhc-cGMP/Ac (**10**)	320 (5120)	0.161	0.146	175	nd[g]	17[i]

[a] Absorption maximum [nm] measured in K-MOPS (pH 7.2). [b] Molar absorptivity [$M^{-1}cm^{-1}$]. [c] Quantum yields for the disappearance of the starting materials upon irradiation (350 nm). Samples (10 μM) in K-MOPS (pH 7.2) were photolyzed with two RPR 350 nm lamps. [d] Quantum yields for the appearance of the cyclic nucleotides from both isomers upon irradiation (350 nm). [e] The product of quantum yields for the appearance and molar absorptivity at 350 nm. [f] Solubility [μM] in K-MOPS (pH 7.2) containing 1% DMSO. [g] Not determined. [h] Half-life [h] in the dark. Samples (10 μM) in K-MOPS (pH 7.2) were placed in the dark at room temperature. [i] Half-life for the deacetyl reaction.

(≈10 mW). Figure 1 shows the time course of the consumption of the starting materials and the production of the parent cyclic nucleotides. After irradiation for about 1 min, almost quantitative production of cAMP and cGMP was observed. Selected photophysical and photochemical properties of Bhc-cAMP and Bhc-cGMP are summarized in Table 1. A slight difference in the quantum yields of photolysis between the axial and equatorial isomers was observed; equatorial isomers are more reactive than axial isomers. The overall photosensitivity, which can be expressed as the product of the quantum yield of photolysis (Φ) and molar absorptivity (ε), was calculated to be greater than 1000 $M^{-1}cm^{-1}$ for both Bhc-caged nucleotides, which is almost 10 times greater than those of conventional 2-nitrobenzyl-type cages (110 for 1-(2-nitrophenyl)ethyl cAMP, NPE-cAMP, for example[14]). Furthermore, the $\Phi\varepsilon$ values of **7** and **8** were two to three times greater than those for HCM- (310 for $\Phi\varepsilon_{eq}$ and 450 for $\Phi\varepsilon_{ax}$) and MCM-caged cAMP (697 for $\Phi\varepsilon_{eq}$ and 606 for $\Phi\varepsilon_{ax}$), the previously reported prototypes of Bhc-cage.[9]

We next examined the effect of 7-acetyl protection on the photochemistry. The absorption maxima were shifted to shorter wavelengths by about 50 nm, and the molar absorptivities at 350 nm were therefore five to ten times lower than those of Bhc-cNMPs. Photolysis of **9** and **10** also produced cAMP and cGMP (Scheme 3), respectively, with quantum yields comparable to those with a Bhc-cage (see Table 1); however, the overall efficiencies of photorelease from the acetylated compounds were almost ten times lower than with a Bhc-cage. These lower efficiencies are of little biological relevance because the acetyl groups will normally hydrolyze before photolysis.

Two-photon uncaging action cross-sections (δ_u) of **7** and **8** were measured by use of a femtosecond-pulsed, mode-locked Ti-sapphire laser by the previously described method.[15a] The wavelength dependence of the δ_u values of **7** from 720 to 800 nm shown in Figure 2 indicates that the peak wavelength of the δ_u value appears at around 740 nm. The values of δ_u (740 nm excitation) determined for **7** were 2.06 ± 0.18 GM ($\times 10^{-50}$ cm^4 s photon^{-1}) for the axial isomer and 2.28 ± 0.22 GM for the equatorial isomer, and the values for **8** were 1.68 ± 0.09 GM for the axial and 1.95 ± 0.01 GM for the equatorial isomers.



Scheme 3. *Photolysis of Bhc-cNMPs.*

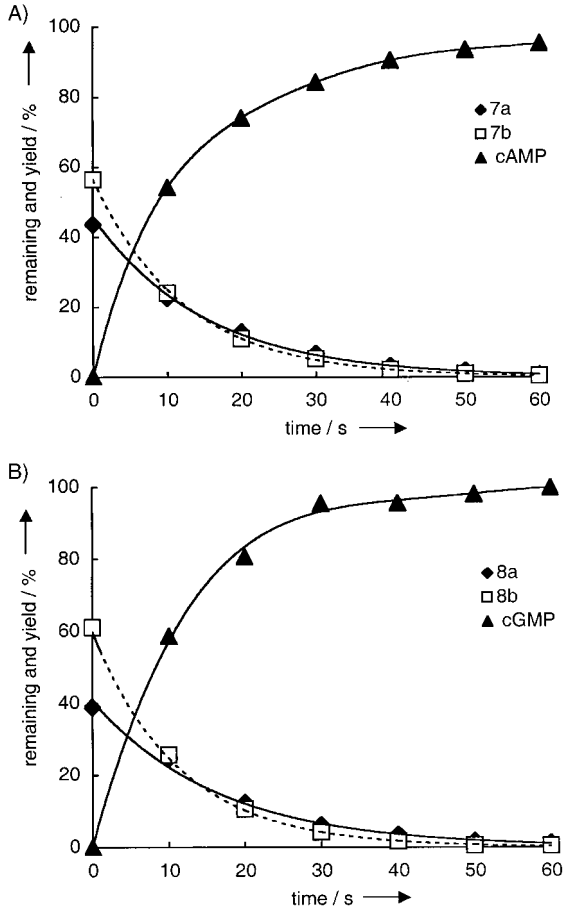


*Figure 1. Time course of photolysis of the Bhc-cNMPs at 350 nm: A) photolytic consumption of Bhc-cAMP (equatorial), **7a** (◆), Bhc-cAMP (axial), **7b** (□), and production of cAMP from both isomers (▲). Solid or dotted lines, least-squares curve fits to a simple decaying or rising exponential; B) photolytic consumption of Bhc-cGMP (equatorial), **8a** (◆), Bhc-cGMP (axial), **8b** (□), and production of cGMP from both isomers (▲).*

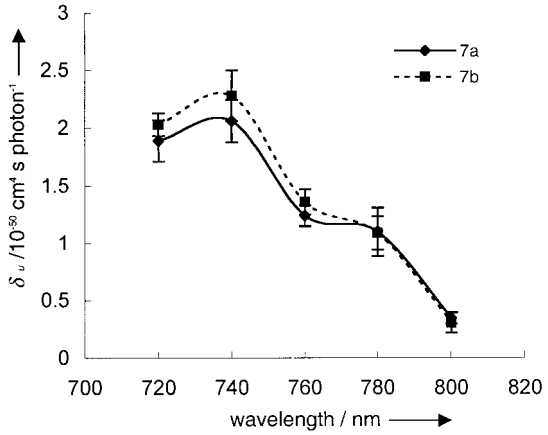


*Figure 2. Two-photon uncaging action cross-sections (δ_u) of Bhc-cAMP (**7**). Solutions of **7** (100 μM in 10 mM K-MOPS solution, pH 7.2) were irradiated at 720 nm (average power: 400–440 mW, 54 fs pulse width), 740 nm (average power: 800 mW, 72 fs pulse width), 760 nm (average power: 780 mW, 61 fs pulse width), 780 nm (average power: 600–700 mW, 64–80 fs pulse width), and 800 nm (average power: 840 mW, 56 fs pulse width). The values are averages ± S.D. of the means.*

Biological tests

Cyclic nucleotide-induced olfactory signal transduction: The properties of Bhc-cAMP were tested in newt olfactory receptor cells. Bhc-caged cAMP was useful for patch clamp experiments, when the nucleotide was introduced from the whole-cell pipette. Figure 3 shows membrane responses of the olfactory receptor cell, which is now well known to utilize cAMP-mediated transduction machinery on its sensory cilia.[17] When the ciliary region of a solitary olfactory receptor cell containing caged cAMP was illuminated by UV light, an inward current was immediately induced. The amplitude and initial slope of the inward current were dependent on the intensity of uncaging light, reflecting that the amount and rate of cAMP production could be experimentally controlled.

The intensity-response relationship obtained from the photolysis of Bhc-cAMP was fitted by the Hill equation with very high cooperativity ($n_H = 4.3 \pm 2.2$, $n = 3$) as has been previously shown with NPE-cAMP ($n_H = 6.3 \pm 0.9$, $n = 4$). The response with very high cooperativity is triggered by the elevation of cytoplasmic cAMP levels within olfactory cilia by sequential opening of cAMP-gated channels and calcium-activated chloride channels.[18] It is therefore reasonable to conclude that the responses do reflect elevation of cytoplasmic cAMP. The efficiency for flash photolysis was measured by obtaining the $K_{1/2}$ value, a relative photolysis intensity that causes a half-maximum response, from the intensity-response relationship. The $K_{1/2}$ value of maximum responses induced by Bhc-cAMP was 63.6 ± 19.0, and that obtained from NPE-cAMP was 622.8 ± 77.3. It is thus likely that the uncaging efficiency of Bhc-cAMP was an order of magnitude greater than that of NPE-cAMP even in an olfactory receptor cell, and quite consistent with the value observed in the cuvette experiments described above.

Motile responses in medaka fish melanophores: It is a challenge to deliver membrane-impermeant molecules into live intact cells without activating or deactivating any biological systems, and then to achieve locally controlled activation with high temporal resolution. To show that the intracellular targets of cAMP can be activated by photoirradiation of extracellularly applied Bhc-cAMP/Ac, we used the motility response of pigment granules in fish melanophores. Pigment motility in fish melanophores is regulated by the intracellular concentration of cAMP, which activates its intracellular target enzyme, cAMP-dependent protein kinase (PKA), and can be easily monitored by an optical microscope. A scale was isolated from wild-type medaka fish and the overlaying epiderm was removed. Bhc-cAMP/Ac was extracellularly applied to the melanophores in the scale for 1 h, and extracellular caged molecules were then washed out by perfusion with Ringer's solution. Most of the melanin pigment granules were fully dispersed at this stage and photographed for quantitative measurement. To minimize the contribution from endogenous cAMP, the scale was treated with an α-adrenergic agonist, norepinephrine, which caused

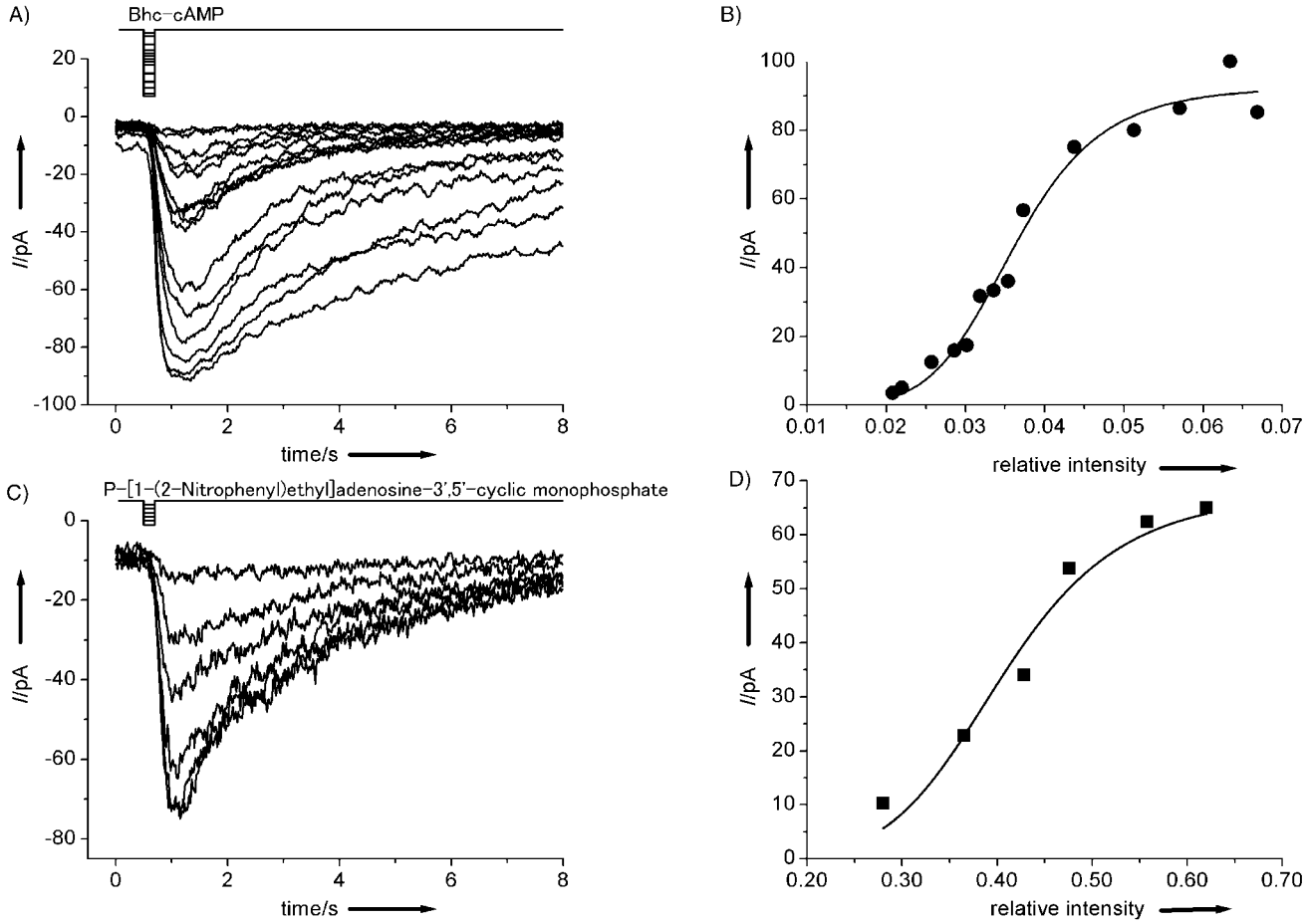


Figure 3. *The current responses induced by flash photolysis of 1 mM caged cAMP in olfactory receptor cells. A) Membrane currents were recorded from a cell loaded with 1 mM Bhc-cAMP (**7**). Light intensity was varied, while the duration was kept constant (200 ms). Downward deflections of the upper trace indicate the timing and duration of the light stimulation. B) Intensity-response relationship of the light-induced current. Peak amplitudes of responses obtained in A were plotted against the relative intensity of light. The solid line—a least squares fitting of the data points by the Hill equation—gave* $I_{max} = 90$ *pA,* $K_{1/2} = 63.6$*, and* $n_H = 4.3$*. C) Membrane currents induced by photolysis of 1 mM NPE-cAMP. D) Intensity-response relationship of the light-induced current. Peak amplitudes of responses obtained in (C) were plotted against the relative intensity of light. The solid line—a least square fitting of the data points by the Hill equation—gave* $I_{max} = 65$ *pA,* $K_{1/2} = 622.8$*, and* $n_H = 6.0$*.*

complete aggregation of the melanin pigments. The effect of uncaging upon UV irradiation was quantified by measuring the degree of dispersion with an optical microscope. Irradiation of 10 μM Bhc-cAMP/Ac for 30 s was enough to cause maximum dispersion. Even at a concentration of 100 nM Bhc-cAMP/Ac, irradiation caused a measurable degree of dispersion (Figure 4A). One potential application for caged molecules is repetitive stimulation that could cause artificial oscillation of a cytosolic effector molecule.[19] The scale was incubated with 10 μM Bhc-cAMP/Ac and repeatedly irradiated for 30 s at 60 s intervals, and the dispersing responses were recorded (Figure 4B). Almost full dispersion was obtained 30 s after each irradiation, and this could be repeated at least five times.

Discussion

Synthesis

We had previously reported a simple method for the preparation of the alkyl triesters of some phosphates by use of silver(I) oxide, and had used this method to prepare the (7-methoxycoumarin-4-yl)methyl ester of cAMP (MCM-cAMP).[9a,20] However, the silver(I) oxide method did not give phosphate triesters with free phenolic hydroxy groups on the coumarin ring. A simple alternative by which to introduce photoremovable protecting groups onto phosphate is the esterification of free phosphoric acid with appropriately designed diazo compounds. We thus designed Bhc-diazo (**6**), which is a stable, non-volatile, and solid diazo compound and can be stored for at least six months at room temperature without any detectable decomposition. The syntheses of Bhc-diazo (**6**) and Bhc-diazo/Ac (**5**) are based on a previously described method.[15a,b] Unlike other 4-methylcoumarins,[21] oxidation of the solvent *m*-xylene to *m*-methylbenzaldehyde competes with oxidation of the 4-methyl group of **2**, and therefore an excess amount of selenium dioxide should be needed to improve the yield of the desired product. The use of fresh and finely powdered selenium dioxide (colorless solid) is also necessary to achieve reproducible product yields. With chlorobenzene, which lacks a benzylic methyl group, we avoided competing solvent oxida-

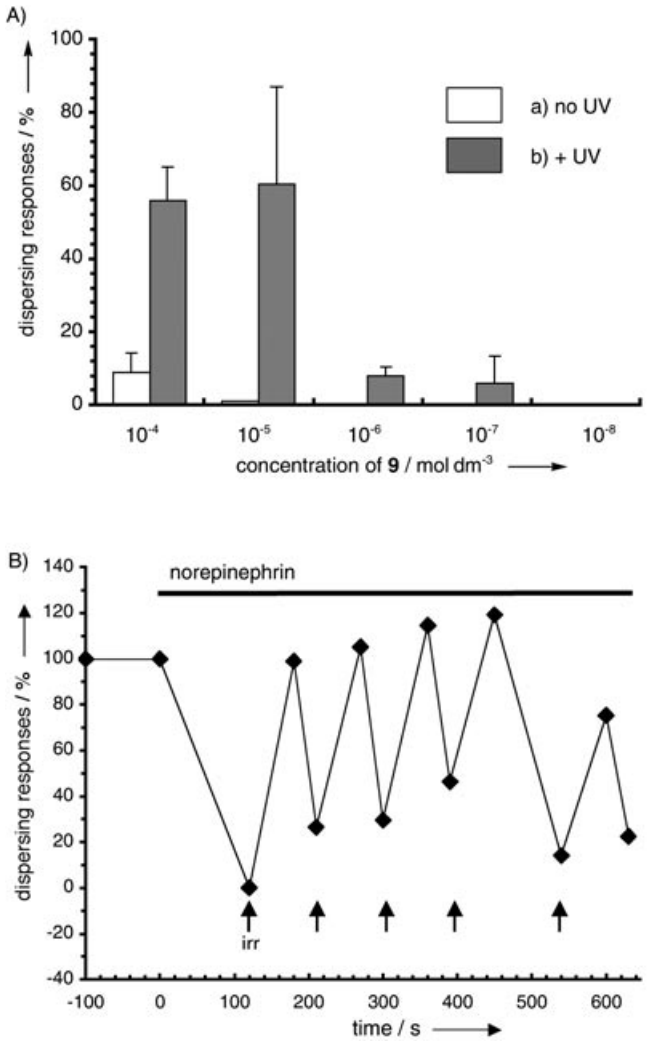


Figure 4. *Dispersion of pigment granules in medaka fish melanophores on scales by the photolysis of Bhc-cAMP/Ac. A) Dose responses of Bhc-cAMP/Ac (**9**). Melanophores on scales were incubated for 60 min with various concentrations of **9** in Ringer's solution (pH 7.4); norepinephrine (500 nM) was present throughout to suppress endogenous production of cAMP. Dispersing response of 100% and 0% mean full dispersion and full aggregation, respectively. The values are the mean ± S.D. of three separate experiments: a) no UV: magnitude of dispersing responses in the absence of irradiation, b) + UV: scales were irradiated through an objective lens of a fluorescent microscope equipped with a band-pass filter (pass wavelength 334–365 nm) for 30 s. Magnitude of the dispersing responses was quantified at 60 s after the irradiation. B) Responses of repetitive irradiation of melanophores on a single scale incubated with 10 μM Bhc-cAMP/Ac (**9**). The same experimental conditions as for (A) were used.*

tion and achieved better product yields (67% yield with *m*-xylene against an 89% yield with chlorobenzene).

Photochemical properties

An advantage of Bhc-caged compounds is their higher efficiency in uncaging reactions upon one- and two-photon irradiation. Typically, the efficiency of a photochemical reaction can be expressed by the quantum yield or quantum efficiency (Φ) of the product. This is true if unlimited and continuous irradiation can be applied to a reaction system. However, in a cell biological application, the incident light intensity for an uncaging reaction has to be severely limited so as to avoid significant cell damage. Both the quantum efficiency of photolysis and the absorption cross-section of the molecule should be considered. We are therefore in favor of using the product of the photolysis quantum yield (Φ) and molar absorptivity (ε) instead of the quantum yield alone to compare the overall efficiency of an uncaging reaction quantitatively. According to our comparison of $\Phi\varepsilon$ values in vitro, Bhc-cNMP is nearly ten times as photosensitive as NPE-cAMP, and we can reduce the intensity of the uncaging light to 10% with Bhc-cNMPs to achieve the same magnitude of activation. For two-photon irradiation, the efficiency of a photochemical reaction can be quantitatively compared by uncaging action cross-sections (δ_u), the product of the photolysis quantum yield (Q_{u2}) and the two-photon absorption cross-sections (δ_a).[15a,d,f] Values of δ_u larger than 0.1 GM are desirable for cell biological experiments. The 2-nitrobenzyl groups, the most commonly used phototriggers, fall considerably short of this criterion (0.01 GM for 4,5-dimethoxy-2-nitrobenzyl is the highest value reported).[22] We have reported δ_u values of 0.89–0.95 GM (at 740 nm) for Bhc-caged glutamates[15a] and 0.51–1.23 GM (at 740 nm) for Bhc-diol-caged aldehydes and ketones.[15f] The observed δ_u values of **7** and **8** were 1.74–2.28 GM at 740 nm and are the largest values obtained for other Bhc-caged compounds.

An action spectrum of two-photon uncaging reactions of **7** (Figure 2) indicates that the peak wavelength of two-photon excitation (740 nm) is approximately twice the one-photon absorption maximum (375 nm for **7a** and 371 nm for **7b**). This suggests that the same excited states are generated regardless of the number of absorbed photons. The observation is consistent with the results of two-photon excitation spectra of the fluorophores that have no center of symmetry, such as coumarin 307 dye, reported by Xu and Webb.[23]

Biological tests

The introduction of a bromo substituent on the coumarin ring not only accelerates the rate of intersystem crossing, but also enhances the acidity of the phenol moiety of a hydroxycoumarin. The *pKa* value of the Bhc-cage (Bhc-OH, 6-bromo-7-hydroxycoumarin-4-yl methanol) is 6.2, which is lower than that of the HCM-cage (HCM-OH, 7-hydroxycoumarin-4-ylmethanol) by 1.7 units (data not shown). As a result, more than 90% of the Bhc cage is ionized to give a negative charge at the 7-position of the coumarin ring at physiological pH, while less than 30% of the HCM cage might be ionized. This negative charge decreases the inherent lipophilicity of coumarins, so as to give a certain hydrophilicity to the Bhc-cage. Solubilities of 500 μM in an aqueous buffer observed with Bhc-cNMPs were approaching the values observed for CMCM- and BCMCM-caged cNMPs designed to have high water solubility. On the other hand, we can add substantial lipophilicity to the Bhc-cage by masking the C7 phenolic hydroxy group with an acyl substituent. The lipophilicity of the 7-acylated Bhc-cage would enable the introduction of the otherwise membrane-impermeant caged-cNMPs into cells by simple diffusion. The acyloxy group

is unmasked by intrinsic and non-specific esterases to release the more hydrophilic Bhc-caged compounds, which can scarcely escape from cells and have higher uncaging efficiency. This amphiphilic nature of hydroxycoumarins is another advantage of the Bhc-cage in cell biological applications, and the two examples provided in this paper demonstrate how this might work.

We have demonstrated that the combined use of the chemistry of caged compounds with an electrophysiological technique is quite useful to dissect a complex signaling cascade in living cells.[9d,18] Caged compounds useful for such experiments should have substantial water solubility so as to be incorporated into cells by patch pipette and be able to diffuse freely through the cytoplasm. They should possess a high uncaging efficiency to reduce cellular damages upon irradiation. In this study, Bhc-caged cAMP was intracellularly applied through a whole-cell patch pipette in solitary olfactory receptor cells. Giga-seal formation and cell functions were retained even after addition of Bhc-cAMP. Olfactory signal transduction is carried out at the ciliary membrane, which comprises very narrow cylinders (0.2 μm in diameter). Since the whole-cell pipette is always placed far from the cilia (either on the dendrite or on the cell body), caged substances have to travel for several tens of micrometers through the cytoplasm. In this study we were able to obtain consistent and highly efficient responses of the receptor cells with photolysis of Bhc-cAMP. The result indicates that Bhc-cAMP diffuses through the cytoplasm and releases the parent cAMP with high efficiency and faster kinetics than that of the channel responses upon UV irradiation.

The fish melanophore experiments showed that Bhc-cAMP/Ac could penetrate through the plasma membrane, be concentrated in a group of intact melanophores, and release the parent cAMP molecules with high efficiency upon UV irradiation. The advantage of membrane-permeant caged compounds is obvious. There is no need for laborious microinjections to treat large numbers of cells or for models membrane-permeabilized by lysis, which could cause severe damage to membrane micro-domain structures. This advantage could also be achievable by use of simple membrane-permeant derivatives of cNMPs, such as AM esters, that are simpler to apply and have been shown to be effective for many types of cells. However, almost no temporal or spatial resolution would be expected, since membrane-permeant cNMPs might spread by diffusion through the cells with the simultaneous activation of target proteins. This would limit the usefulness of these derivatives for investigation of intracellular signaling processes, since the activation or deactivation of a signaling molecule should occur in a highly localized manner in a physiological environment. Bhc/Ac-caged compounds should enable us to mimic a physiological environment as closely as possible. In addition, we can choose the location, area (or volume), and magnitude of activation simply by controlling the location, area (or volume), and dose of irradiated light, which can be easily achieved with a microscope fitted with a laser or a Xe lamp. Hagen and Bendig synthesized other coumarin-caged cNMPs with a certain water solubility—BCMCM and DEACM-cNMPs, for example—and reported that they also had high uncaging efficiency and were able to activate cyclic nucleotide-gated ion channels expressed in cultured HEK293 cells.[12,24] All of these results clearly demonstrate that coumarinylmethyl-caged cNMPs can be a good alternative to conventional 2-nitrobenzyl-type caged cNMPs. An application of the membrane-permeant Bhc-cNMPs/Ac to tackle a biological problem by dissecting a signaling cascade in a sea urchin sperm will be reported elsewhere.[25]

Experimental Section

Synthesis: All reagents and solvents were purchased from commercial sources and were used without further purification. Flash column chromatography was carried out on 43–60 mesh silica gel. NMR spectra were recorded (JEOL GSX270) at 270 MHz for ^{1}H and at 67 MHz for ^{13}C with $CDCl_3$ as a solvent and TMS as an internal standard unless otherwise specified. IR spectra were recorded on a Thermo Nicolet Avatar 320 instrument in ATR mode. Analytical HPLC was run on an Agillent HP 1100 system with DAD detection and preparative HPLC on a JASCO PU9800 system with UV detection.

6-Bromo-7-hydroxy-4-methylcoumarin (1): A mixture of 4-bromoresorcinol (1.9850 g, 10.4 mmol), ethyl acetoacetate (2.0 mL, 15 mmol), and *p*-toluenesulfonic acid (200.8 mg, 1.06 mmol) in toluene (25 mL) was heated at reflux for 17 h with azeotropic removal of water and ethanol by use of a Dean–Stark trap. The reaction mixture was cooled, water was added (25 mL), and the stirring was continued for 30 min at 0 °C to provide a finely powdered precipitate. The precipitate was collected by filtration, washed with cold water, and dried under vacuum over P_2O_5 to yield compound **1** (2.3697 g, 9.3 mmol, 90% yield) as a solid. mp 267–269 °C; ^{1}H NMR ($CDCl_3$ + 1%CD_3OD): $\delta = 7.73$ (s, 1H), 6.88 (s, 1H), 6.13 (s, 1H), 2.40 (s, 3H) ppm; ^{13}C NMR ([D$_6$]DMSO): $\delta = 159.8$, 157.2, 153.6, 152.8, 129.1, 113.4, 111.2, 105.9, 103.0, 18.1 ppm; IR (neat): $\tilde{\nu}$ = 3300–3000, 1693, 1674, 1600, 1387, 1364, 1270, 1234, 1224, 1161, 1078, 843 cm^{-1}; MS (ESI) *m/z* 252.9 $[C_{10}H_7{}^{79}BrO_3-H]^+$, 254.9 $[C_{10}H_7{}^{81}BrO_3-H]^+$.

7-Acetoxy-6-bromo-4-methylcoumarin (2): A mixture of **1** (1.0203 g, 4.0 mmol) and acetic anhydride (3 mL, 20 mmol) was heated at 110 °C for 3 h. The reaction mixture was cooled and evaporated to give the crude **2** (1.1301 g, 3.8 mmol, 95% yield). mp 175–176 °C; ^{1}H NMR: $\delta = 7.82$ (s, 1H), 7.16 (s, 1H), 6.30 (s, 1H), 2.42 (s, 3H), 2.40 (s, 3H) ppm; ^{13}C NMR $\delta = 167.8$, 159.7, 153.0, 150.8, 150.3, 128.6, 119.4, 115.4, 112.6, 11.6, 20.7, 18.6 ppm; IR (neat): $\tilde{\nu}$ = 1749, 1712, 1395, 1385, 1364, 1201, 1145, 1042, 917, 894, 853 cm^{-1}.

7-Acetoxy-6-bromo-4-formylcoumarin (3): A mixture of **2** (1.8939 g, 6.374 mmol) and selenium dioxide (855 mg, 7.7 mmol) in chlorobenzene (60 mL) was stirred at reflux temperature for 5 d. The reaction mixture was cooled, filtered, and evaporated. Purification by recrystallization from toluene gave compound **3** (1.7401 g, 5.594 mmol, 88% yield). mp: 148–149 °C; ^{1}H NMR: $\delta = 10.01$ (s, 1H), 8.93 (s, 1H), 7.23 (s, 1H), 6.90 (s, 1H), 2.41 (s, 3H) ppm; ^{13}C NMR (67.80 MHz; $CDCl_3$): $\delta = 20.76$ (q), 112.60 (s), 112.99 (d), 113.75 (d), 126.39 (s), 130.57 (d), 142.06 (s), 151.13 (s), 153.93 (s), 159.16 (s), 167.69 (s), 190.82 (d) ppm; IR (neat): $\tilde{\nu}$ = 3107, 3065, 1764, 1742, 1710, 1189, 1143, 1028 cm^{-1}.

7-Acetoxy-6-bromo-4-formylcoumarin *p*-tosylhydrazone (4): *p*-Toluenesulfonyl hydrazine (218 mg, 1.17 mmol) was added to a stirred solution of **3** (342.0 mg, 1.10 mmol) in EtOH (2 mL). After 19 h

of stirring at 40 °C, the resulted precipitate was collected by filtration, washed with EtOH, and dried under vacuum to yield **4** (431.9 mg, 0.90 mmol, 82 % yield). mp 173 °C (decomp.); ^{1}H NMR ([D$_6$]DMSO): δ = 8.93 (s, 1H), 7.98 (s, 1H), 7.82 (d, J = 7.6 Hz, 2H), 7.51 (s, 1H), 7.5 (s, 1H), 7.47 (d, J = 7.6 Hz, 2H), 6.77 (s, 1H), 2.45 (s, 3H), 2.38 (s, 3H) ppm; ^{13}C NMR ([D$_6$]DMSO): δ = 167.9, 159.1, 153.4, 149.8, 144.2, 143.8, 141.9, 135.5, 130.7, 127.3, 119.3, 115.4, 112.8, 111.0, 21.1, 20.5 ppm; IR (neat): $\tilde{\nu}$ = 3239, 1767, 1709, 1603, 1401, 1365, 1268, 1186, 1169, 1152, 1086, 1027, 928, 918, 883, 845, 819 cm^{-1}; MS (ESI) *m/z* 500.90 [$C_{19}H_{15}{}^{79}BrN_2O_6S$+Na]$^+$, 502.95 [$C_{19}H_{15}{}^{81}BrN_2O_6S$+Na]$^+$.

When the reaction was performed below 30 °C, the dehydration step was sometimes incomplete and the addition product α-hydroxy-hydrazine **4′** was obtained. ^{1}H NMR: δ = 8.89 (s, 1H), 8.74 (s, 1H), 7.92 (d, J = 8 Hz, 2H), 7.70 (s, 1H), 7.37 (d, J = 8 Hz, 2H), 7.16 (s, 1H), 6.42 (s, 1H), 2.42 (s, 3H), 2.41 (s, 3H) ppm; ^{1}H NMR ([D$_6$]DMSO): δ = 9.13 (br s, 1H), 8.54 (s, 1H), 7.66 (d, J = 8 Hz, 2H), 7.44 (s, 1H), 7.25 (d, J = 8 Hz, 2H), 6.79 (d, J = 4.5 Hz, 1H), 6.53 (s, 1H), 2.49 (s, 3H), 2.37 (s, 3H) ppm.

7-Acetoxy-6-bromo-4-diazomethylcoumarin, Bhc-diazo/Ac (5): Et_3N (63 μL, 0.45 mmol) was added to a stirred suspension of **4** (206.5 mg, 0.43 mmol) in MeOH (2 mL). After 1 h, the resulted yellowish precipitate was collected by filtration and washed and dried under vacuum to yield **5** (118.0 mg 0.37 mmol, 85 % yield). ^{1}H NMR: δ = 7.54 (s, 1H), 7.17 (s, 1H), 5.87 (s, 1H), 5.27 (s, 1H), 2.40 (s, 3H) ppm; ^{13}C NMR ([D$_6$]DMSO): δ = 167.9, 158.8, 152.7, 150.2, 145.9, 127.8, 115.6, 112.9, 110.9, 99.7, 46.2, 20.5 ppm; IR (neat): $\tilde{\nu}$ = 3087, 2089, 1748, 1696, 1593,1422, 1387, 1371, 1203, 1141, 1022, 927, 905, 822 cm^{-1}.

6-Bromo-7-hydroxy-4-diazomethylcoumarin, Bhc-diazo (6): Et_3N (948 μL, 6.80 mmol) was added to a stirred suspension of **4** (1.6298 g, 3.40 mmol) in MeOH (7 mL). After 2 days, the resulting precipitate was collected by filtration, washed, and dried under vacuum to yield **6** (751.1 mg, 2.67 mmol, 79 % yield).

Compound 6: (CD_3OD): δ = 7.81 (s, 1H), 6.81 (s, 1H), 5.68 (s, 1H), 4.56 (s, 1H); ^{1}H NMR ([D$_6$]DMSO): δ = 7.97 (s, 1H), 6.85 (s, 1H), 6.62 (s, 1H), 5.71 (s, 1H) ppm; ^{13}C NMR ([D$_6$]DMSO): δ = 159.4, 157.6, 153.5, 146.5, 127.7, 109.5, 105.9, 103.2, 96.8, 45.7 ppm; IR (neat): $\tilde{\nu}$ = 3300–3000, 3099, 2101, 1653, 1618, 1537, 1394, 1274, 1230, 1166, 1153, 940, 836, 810 cm^{-1}.

6-Bromo-7-hydroxycoumarin-4-ylmethyl adenosine cyclic 3′,5′-monophosphate, Bhc-cAMP (7): A mixture of cAMP (19.9 mg, 60.5 μmol) and **6** (28.1 mg, 100 μmol) in DMSO (0.5 mL) was stirred at room temperature for 39 h. The reaction mixture was evaporated. Purification by flash column chromatography (10 g of SiO_2, 10 % MeOH in $CHCl_3$) yielded **7** (2.6 mg, 4.5 μmol, 7.4 % yield) as a stereoisomeric mixture (axial/equatorial 44:56). The axial and equatorial isomers were separated by semi-preparative reversed-phase HPLC (Column: Cosmosil 5C18 AR-II, 250×20, eluent: 50 % MeOH/H_2O, 3 mL min^{-1}). The more rapidly eluted isomer was the axial isomer (retention time: 30–40 min) and the more slowly eluted isomer the equatorial (retention time: 40–50 min).

Compound 7a (axial isomer): ^{1}H NMR ([D$_6$]DMSO): δ = 8.33 (s, 1H), 8.10 (s, 1H), 7.55 (s, 1H), 7.36 (s, 2H), 6.41 (s, 1H), 6.04 (s, 2H), 5.81 (s, 1H), 5.35 (m, 1H), 5.28 (d, J = 7 Hz, 2H), 4.65–4.55 (m, 2H), 4.34 (ddd, J = 10, 10, and 2 Hz, 1H,), 4.18–4.17 (m, 2H) ppm; ^{31}P NMR ([D$_6$]DMSO): δ = −4.98 ppm.

Compound 7b (equatorial isomer): ^{1}H NMR ([D$_6$]DMSO): δ = 8.39 (s, 1H), 8.18 (s, 1H), 7.58 (s, 1H), 7.37 (s, 2H), 6.37 (s, 1H), 6.07 (s, 2H), 5.80 (s, 1H) ppm; ^{31}P NMR ([D$_6$]DMSO): δ = −3.62 ppm.

HRMS (FAB$^+$) Calcd for $C_{20}H_{18}O_9N_5BrP$: 582.0026 and 584.0006; found 582.0027 and 584.0010.

6-Bromo-7-hydroxycoumarin-4-ylmethyl guanosine cyclic 3′,5′-monophosphate, Bhc-cGMP (8): A mixture of cGMP (17.4 mg, 50.4 μmol) and **6** (28.9 mg, 103 μmol) in DMSO (0.5 mL) was stirred at room temperature for 76 h. The reaction mixture was directly purified by flash column chromatography (5 g of SiO_2, 10 % MeOH in $CHCl_3$, then 25 % MeOH in $CHCl_3$) to yield **8** (7.0 mg, 11.7 μmol, 23 % yield) as a stereoisomeric mixture (axial/equatorial 38:62). The axial and equatorial isomers were separated by semi-preparative reversed-phase HPLC (column: COSMOSIL 5C18 AR-II, 250×20, eluent: 50 % MeOH/H_2O). HRMS (FAB$^+$) calcd for $C_{20}H_{18}O_{10}N_5{}^{79}BrP$: 597.9975; found: 598.0023.

Compound 8a (axial isomer): ^{1}H NMR ([D$_6$]DMSO): δ = 10.52 (s, 1H), 7.90 (s, 1H), 7.60 (s, 1H), 6.44 (s, 2H), 6.32 (s, 1H), 6.19 (s, 1H), 5.90 (s, 1H), 5.79 (s, 1H), 5.25 (d, J = 7 Hz, 2H), 4.8–4.5 (m, 2H), 4.2–4.0 (4H, m) ppm; ^{31}P NMR ([D$_6$]DMSO): δ = −5.06 ppm.

Compound 8b (equatorial isomer): ^{1}H NMR ([D$_6$]DMSO): δ = 10.53 (s, 1H), 7.94 (s, 1H), 7.78 (s, 1H), 6.66 (s, 1H), 6.58 (s, 2H), 6.27 (s, 1H), 6.14 (s, 1H), 5.84 (s, 1H), 5.32 (d, J = 7 Hz, 2H), 5.15 (m, 1H), 4.70 (m, 1H), 4.56–4.40 (m, 3H) ppm; ^{31}P NMR ([D$_6$]DMSO): δ = −3.90 ppm.

7-Acetoxy-6-bromocoumarin-4-ylmethyl adenosine cyclic 3′,5′-monophosphate, Bhc-cAMP/Ac (9): A mixture of cAMP (36.0 mg, 109 μmol) and **5** (104 mg, 323 μmol) in DMSO (0.6 mL) was stirred at room temperature for 20 h. The reaction mixture was evaporated. Purification by flash column chromatography (24 g of SiO_2, 7.7 % MeOH in $CHCl_3$) yielded **9** (17.3 mg, 27.8 μmol, 25.4 % yield). The axial/equatorial stereochemistry was estimated by comparison of the reversed-phase HPLC retention times (COSMOSIL 5C18 AR-II, 250×4, eluent: 50 % MeOH/H_2O containing 0.1 % TFA). The more rapidly eluted isomer, with a retention time of 13.6 min, was assigned as the axial isomer and the more slowly eluted isomer (16.5 min) as the equatorial. ^{1}H NMR (CD_3OD) (axial isomer): δ = 8.20 (s, 1H), 8.19 (s, 1H), 8.06 (s, 1H), 7.37 (s, 1H), 6.72 (s, 1H), 6.09 (s, 1H), 5.55–5.45 (m, 3H), 4.65–4.55 (4H, m), 2.36 (s, 3H) ppm; ^{1}H NMR (CD_3OD) (equatorial isomer): δ = 8.24 (s, 1H), 8.23 (s, 1H), 8.05 (s, 1H), 7.36 (s, 1H), 6.64 (s, 1H), 6.11 (s, 1H), 5.55–5.45 (m, 3H), 4.65–4.55 (4H, m), 2.37 (s, 3H) ppm; HRMS (FAB$^+$) calcd for $C_{22}H_{20}O_{10}N_5BrP$: 624.0132 and 626.0112; found 624.0087 and 626.0072.

7-Acetoxy-6-bromocoumarin-4-ylmethyl guanosine cyclic 3′,5′-monophosphate, Bhc-cGMP/Ac (10): A mixture of cGMP (25.0 mg, 72.4 μmol) and **5** (35.0 mg, 109 μmol) in DMSO (0.5 mL) was stirred at room temperature for 24 h. The reaction mixture was evaporated. Purification by flash column chromatography (10 g of SiO_2, 10 % MeOH in $CHCl_3$) yielded **10** (4.1 mg, 7.2 μmol, 10 % yield). The axial/equatorial stereochemistry was estimated by comparison of the reversed-phase HPLC retention times (COSMOSIL 5C18 AR-II, 250×4, eluent: 50 % MeOH/H_2O containing 0.1 % TFA). The more rapidly eluted isomer was assigned as the axial isomer and the more slowly eluted isomer as the equatorial. ^{1}H NMR (CD_3OD) (axial isomer): δ = 8.04 (s, 1H), 7.82 (s, 1H), 7.36 (s, 1H), 6.63 (s, 1H), 5.92 (s, 1H), 5.55–5.45 (m, 3H), 4.64–4.50 (4H, m), 2.37 (s, 3H) ppm; ^{1}H NMR (CD_3OD) (equatorial isomer): δ = 8.05 (s, 1H), 7.83 (s, 1H), 7.35 (s, 1H), 6.69 (s, 1H), 5.95 (s, 1H), 5.48 (2H, d, J = 7.3 Hz), 4.64–4.50 (5H, m), 2.36 (s, 3H) ppm; HRMS (FAB$^+$) calcd for $C_{22}H_{20}O_{11}N_5BrP$: 640.0081 and 642.0061; found 640.0101 and 642.0104.

Photochemical properties

Quantum efficiency measurement: Substrate solution in K-MOPS solution (pH 7.2, 10 μM, 2 mL) containing DMSO (1%) was placed in a Pyrex test tube of 12 mm diameter. The solution was irradiated at 350 nm with two RPR 350 nm lamps ($\approx$10 mJ s^{-1}). Aliquots of 10 μL were removed periodically and analyzed by HPLC. The light output for the quantum efficiency measurement was performed by ferrioxalate actinometry.[26]

Measurements of two-photon uncaging action cross sections: Measurements of cross sections for two-photon uncaging were performed with a femtosecond-pulsed, mode-locked Ti/sapphire laser (Tsunami pumped by a Millenium Va; both from Spectra-Physics) with fluorescein as an external standard as previously described.[15a]

Biological tests

Cyclic nucleotide-induced olfactory signal transduction: Olfactory receptor cells were dissociated enzymatically from the olfactory epithelium of the newt *Cynops pyrrhogaster* as described previously.[27] Isolated cells were plated on a concanavalin A-coated glass cover slip. Cells were maintained at 4°C until use. In this study, olfactory receptor cells possessing more than five cilia were selected.

Membrane currents were recorded by a patch clamp technique in the whole-cell recording configuration.[28] The recording pipette was filled with a solution containing CsCl (pH was adjusted to 7.4, HEPES buffer, 119 mM) to suppress background K^+ conductance. Normal Ringer's solution (in mM: 110 NaCl, 3.7 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 15 glucose, 1 pyruvate) was used for all recordings as an external solution. The pipette resistance was 10–15 MΩ. Experiments were performed at room temperature (23–25°C).

Caged cNMP was dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C in complete darkness. Under these conditions, caged cNMP was stable up to 30 days without degradation. However, after this period, the success rate for giga-Ohm seal formation declined considerably. The stocks were diluted into a cesium-containing pipette solution prior to each experiment. After the establishment of whole-cell recording, caged compounds entered the cell interior by free diffusion. The ultraviolet (UV) component from a xenon lamp (100 W, XPS-100, Nikon, Japan) was used as a light source for photolysis. The stimulus light was focused on the cell undergoing recording (objective, 60 ×). By adjustment of the diaphragm, the illuminated area was set to 40 μm in diameter, and only the ciliary region where olfactory transduction is locally carried out was illuminated. Light stimuli were applied with >20 s intervals to avoid adaptation of the system and depletion of caged substances.

Intracellular motile responses in medaka fish melanophores: Wild-type medaka (*Oryzias latipes*), each 2.5–3.5 cm in total length, were used. Scales isolated from the dorsal trunk were rinsed in CMF (Ca^{2+}- and Mg^{2+}-free) Ringer's solution for 20 min at 4°C. The overlaying epidermal layer was removed with forceps. The scale thus prepared was immersed in the experimental solution (containing an appropriate concentration of Bhc-cAMP/Ac) for 60 min at room temperature, placed on a holder with a glass fiber, both sides of which had been attached to a coverslip, and rinsed by perfusion with normal Ringer's solution. The mostly dispersed melanophores were photographed (100% dispersion) and treated with 500 nM norepinephrine to lower endogenous cAMP and to cause aggregation of pigment granules (0% dispersion). UV irradiation (334–365 nm light was selected by U-filter) was applied for 30 s through an objective lens of an epifluorescent microscope (Olympus BHT-RFC type fluorescent microscope fitted with a BH2-RFC). Motile responses of melanophores were photographed at 30 s after the irradiation had been completed. To perform quantitative analysis, the area occupied by the melanin pigments within a defined area (0.09 mm^2) in individual scales was measured and compared as previously described.[9a] In repetitive stimulation experiments, a scale was incubated in Ringer's solution containing Bhc-cAMP/Ac (10 μM) for 60 min at room temperature. The same irradiation procedure as above was followed.

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