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Design, Synthesis, and Photochemistry of Modular Caging Groups for Photoreleasable Nucleotides

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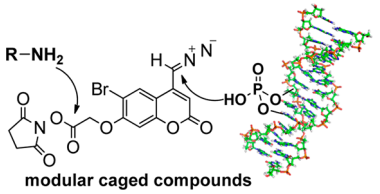
ABSTRACT



A modular approach to preparing caged nucleotides having additional properties has been achieved. The modular caging agent includes three components: an amine reactive NHS ester moiety, a photoactive Bhc group, and tosylhydrazone as a precursor of the diazomethyl group. Various amines including biotin and an Arg-Gly-Asp (RGD) peptide were introduced into the key intermediate via amide linkage. The Bio-Bhc-diazo thus synthesized enables the preparation of a photoreleasable siRNA with additional properties.

Caged compounds are synthetic molecules with biological functions that are masked temporarily by covalently introduced photoremovable protecting groups.[1] Spot illumination of an appropriately designed caged compound enables control of fundamental biological processes with high spatial and temporal resolution. Phosphate-containing molecules are an important class of compounds for biological sciences. Examples are cyclic nucleotides, which are known as second messengers, phosphorylated proteins as intracellular signaling components, and DNAs and RNAs as the carriers of genetic information. Most of the molecules are thought to be activated or produced transiently in a highly localized manner in physiologically relevant circumstances. Therefore, caged compounds of phosphate containing molecules are anticipated for use in the study of fundamental biological processes. Structural variations of caging groups have increased in recent years. Nevertheless, the protecting groups used to produce caged phosphates are limited to a few examples: *o*-nitrobenzyl,[2] *p*-hydroxyphenacyl (includes desyl group),[3] and (coumarin-4-yl)methyl groups.[4] We have developed and used brominated (coumarin-4-yl)methyl groups such as the (6-bromo-7-hydroxycoumarin-4-yl)methyl (Bhc) group as a caging group for carboxylates,[5] amines,[5,6] phosphates,[7] and alcohols.[8] Photochemical and physical properties of the Bhc group have proven to be favorable as a photocaging group. If an additional functional unit such as a specific ligand of a protein of interest and a signal peptide could be installed easily on the Bhc-ring, then the usefulness of the Bhc-caged compounds

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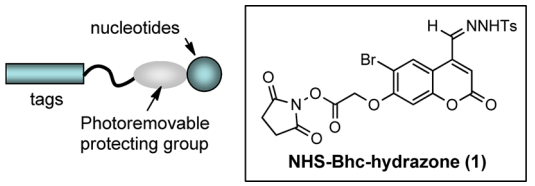


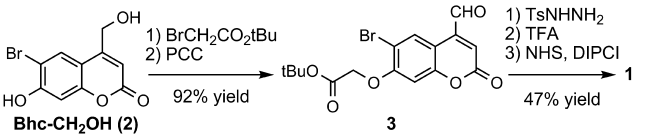
Figure 1. Modular caged compounds.

would be expanded. We report a modular approach to the synthesis of caged nucleotides having additional functionality.

To synthesize caged nucleotides, photoremovable protecting groups can be used either as protecting groups of functional groups in riboses,[9] phosphates,[4,7,10] and nucleobases[11] or as photocleavable linkers of hairpin-like structures.[12] Because free phosphates are known to react with diazomethane derivatives to give the corresponding esters, the synthetic route of phosphate protection is expected to be simpler and to provide easier application for preparing caged compounds having complex structures. Therefore, we designed new precursor molecules of modular caged nucleotides in which an additional functional unit was installed easily. The key compound (**1**) comprises three components: an amine reactive NHS ester moiety, a photoactive Bhc group, and tosylhydrazone as a precursor of a diazomethyl moiety (Figure 1). The precursor molecule NHS-Bhc-hydrazone (**1**) was synthesized from the known alcohol Bhc-CH_2OH (**2**)[5] in five steps (Scheme 1).

The following questions are addressed by this study: (1) which functional units can be added to precursor molecule **1**, (2) whether the precursor molecule can react selectively

Scheme 1. Synthesis of the Key Building Block (**1**)



with free phosphate in the presence of other functional groups, and (3) whether the synthesized phosphate esters can retain their photochemical properties as caged phosphates. To address the first question, the key intermediate **1** was reacted with amine-containing molecules (Figure 2). The NHS ester moiety of **1** reacted quantitatively with a propargyl amine to produce the corresponding propargyl amide. Because the tosylhydrazone moiety of the amide was partly transformed into a diazomethyl group, the crude product was subjected to reaction with triethylamine to give the desired Bhc-diazo derivative propargyl-Bhc-diazo (**4**) in 93% isolated yield. The product is an unexpectedly stable diazomethane derivative and can be stored at ambient temperature for more than a year. After being used in caging nucleotides, the propargyl moiety in **4** can be a chemical handle for additional modification using Cu-catalyzed Huisgen [3 + 2] cyclization.[13]

Using the same reaction conditions, we synthesized other Bhc-diazo derivatives from amine-containing compounds including pentylamine-biotin (Bio-Bhc-diazo **5**) as a ligand of a specific protein and hydrophobic octylamine (Oct-Bhc-diazo **6**). An Arg-Gly-Asp (RGD) peptide, a targeting moiety to cancer cells,[14] was also introduced into **1** to yield the corresponding tosylhydrazone **7**. As far as chemical stability is concerned, we observed decomposition of **7** during storage as a DMF solution. The decomposed products included the 4-hydroxymethyl coumarin derivative **8**, which was generated from hydrolysis of the 4-formyloxymethyl coumarin **9**. A possible explanation of this is the following: during storage, a small amount of the DMF molecules are hydrolyzed to produce diethylamine and formic acid, diethylamine drives the formation of the 4-diazomethyl derivative (RGD-Bhc-diazo) from **7**, and the resulting RGD-Bhc-diazo reacts with formic acid to produce the formic acid ester **9**. Those results raised the next question of whether the coumarinyl diazomethanes react selectively with phosphates in the presence of carboxylates, amines, and alcohols: functional groups that are common among biologically important molecules.

Consequently, two coumarinyl diazomethane derivatives **4** and **6** were chosen to address the second question. Most diazomethane derivatives are known to react with carboxylic acids as well as phosphates with the evolution of nitrogen. As expected, the reaction of **4** with diethyl phosphate produced the corresponding ester **10**. However,

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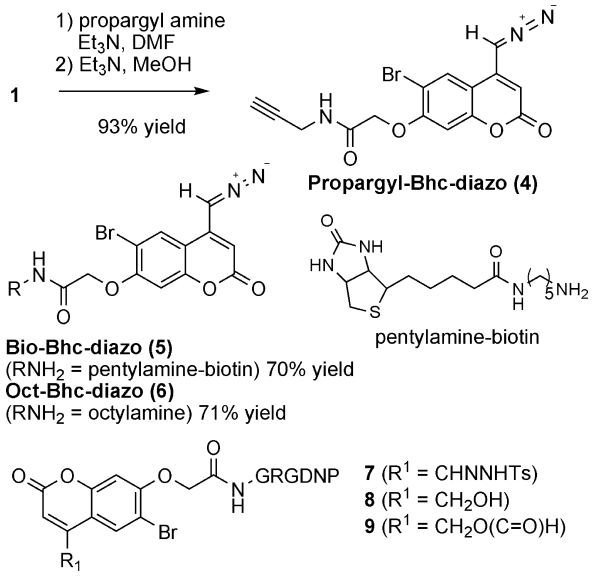

Figure 2. Chemical structures of Bhc-diazo derivatives having additional functionality.

no ester product was obtained in the reaction with acetic acid even in the presence of copper(II) acetate which is known to promote carbene formation from diazomethane derivatives. The results suggest that compound **4** would react with organic acids through a protonation derived S_N2 mechanism, not through the formation of carbenes (Figure 3). Therefore, the reactivity of **4** with organic acids must depend on the acidity of the acid components and nucleophilicity of their conjugated bases. The typical pK_a values of phosphates are 2. Those of carboxylates are 4, indicating that organic acids that are less acidic than carboxylates are unable to react with the coumarinyl diazomethanes in the absence of acid catalysts.[15] Because the acetate anion is the second smallest conjugated base of carboxylic acids, most carboxylic acids cannot react with the coumarinyl diazomethanes. As a consequence, we deduced that the coumarinyl diazomethanes can be caging agents of phosphates but not of carboxylates. Unlike the case with the *o*-nitrophenyl diazomethane derivatives,[10g] an unexpected nucleobase modification reaction must be avoided with the coumarinyl diazomethanes when the compounds are applied to the preparation of caged nucleotides.

To test the assumption, a mononucleotide 3′,5′-cyclic adenosine mononucleotide (cAMP) was mixed with **6** in DMSO (Figure 4). Although the product was formed in trace quantities, the desired ester (**11**) was obtained as the sole product. The remainder was recovered starting cAMP. The free acid form of cAMP has two functional groups other than the cyclic phosphate, a hydroxyl group on the ribose ring and an amino group on the adenine base. Thereby the coumarinyl diazomethane derivatives are useful as a phosphate caging agent with high chemoselectivity.

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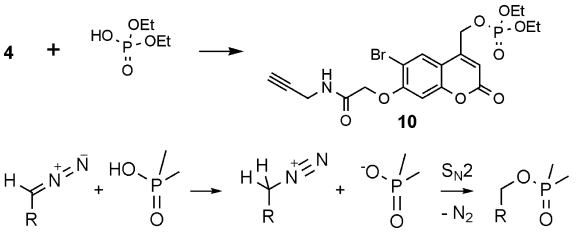

Figure 3. Reaction of the coumarinyl diazomethane derivatives with phosphates.

Photochemical and physical properties of the phosphate ester **10** were investigated to address the third question. The compound **10** has two absorption maxima. The longer maximum, a $\pi-\pi^*$ absorption band that is responsible for the photolysis reaction, lies at 328 nm, which is similar to that of the 7-methoxy analogue of the Bhcmoc group (Bmcmoc group).[6a] The photolysis reaction of **10** was conducted at 350 nm under simulated physiological conditions (10 mM K-MOPS buffer solution at pH 7.2). Photolysis mixtures were analyzed periodically using reversed-phase HPLC. The HPLC traces shown in Figure 5 indicate the almost quantitative production of an expected photobyproduct Propargyl-Bhc-CH_2OH (**12**), and thereby indicate the production of diethyl phosphate. The photolytic consumption of **10** and the production of **12** can be approximated by single exponential equations (Figure 6), suggesting no unexpected secondary effects that interfere with photolysis throughout the photolysis reaction. From the data, the quantum yield (Φ_{350}) for the formation of **12** and diethyl phosphate was calculated as 0.29, which is comparable to those of Bmcmoc-dC (Φ_{350} = 0.30)[6a] and Bhc-cAMP (Φ_{350} = 0.10).[7b] In view of the application to caged nucleotides, photolysis efficiency which can be expressed quantitatively from the product of molar absorptivity (ε) and photolysis quantum yield (Φ) is expected to be the property of practical importance because the value acts as an index for the amount of light intensity required for the uncaging reaction. The $\varepsilon\Phi_{350}$ value for the formation of diethyl phosphate from **10** was 2200 M^{-1} cm^{-1}, which is almost twice as high as those of other coumarinyl methyl caged phosphates.[4,7,10a,10b]

To demonstrate the utility of the compounds, Bio-Bhc-diazo (**5**) was used to test whether the modular caging agents are applicable to the synthesis of caged oligonucleotides. As we described above, direct esterification of

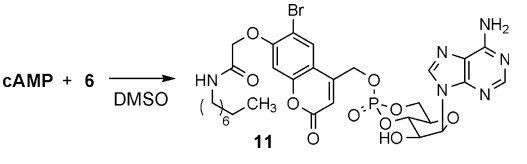

Figure 4. Oct-Bhc caged cAMP (**11**).

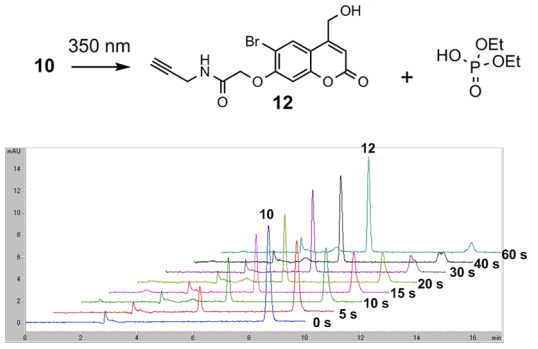

Figure 5. HPLC traces for the photolysis of **10** (measured at 330 nm). Samples were analyzed after the specified irradiation time.

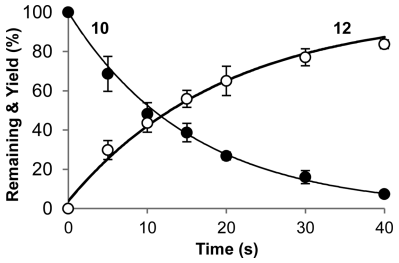

Figure 6. Time course for photolysis of **10**. Samples (10^{-5} M) were irradiated at 350 nm (10 mJ/s) under simulated physiological conditions (10 mM K-MOPS buffer at pH 7.2). Closed circles: consumption of **10**. Open circles: yield of **12**. Solid lines: least-squares curve fit to a simple decaying exponential for **10** and rising exponential for **12**.

oligonucleotides is easy to carry out if an appropriately designed precursor molecule is available. Using these methods, biologists can prepare caged compounds of their own oligonucleotides, for example, plasmid vectors isolated from cultured *E. coli*,[10c] mRNAs prepared by *in vitro* translation,[7a,16] and siRNAs synthesized using automated solid-phase synthesis.[10d−g] We investigated the reaction of Bio-Bhc-diazo (**5**) and a 22-mer siRNA. The precursor molecule **5** has biotin as an affinity tag and the Bhc-diazo group as a caging agent for oligonucleotides. We tried to use biotin as a monitoring tag of the caging reaction. The reaction mixtures were subjected to polyacrylamide gel electrophoresis. The siRNAs were visualized by SYBR Gold staining, whereas the attached Bio-Bhc group was monitored by chemiluminescence-based detection of the biotin tag with an HRP-streptavidin conjugate. The fact that the bands detected by chemiluminescence staining showed the same migration as those by SYBR Gold staining implies that the caging group is attached covalently to the siRNA molecules. The luminescence intensities of each band were

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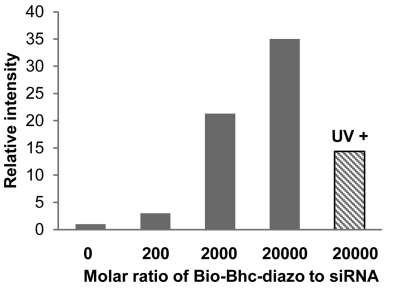

Figure 7. Relative luminescence intensities of biotinylated siRNAs. Luminescence intensity of an untreated siRNA (indicated as 0) was set to 1.

quantified in the reactions of the siRNA with different molar ratios of **5** (Figure 7). The observed intensities of luminescence, and therefore the quantities of the attached caging groups, increased when the larger amount of **5** was used. The synthesized siRNA derivatives were exposed to 350-nm UV light and subjected to electrophoretic analysis. The luminescence intensities of the band correspond to the biotin-labeled siRNA decreased by 60% after 5-min irradiation, clearly indicating that deprotection of the Bio-Bhc group can also be monitored. The biotin moiety of the Bio-Bhc modified nucleotides act not only as a detection tag of the attached caging group described here but also as an affinity tag for purification and a tag for introducing steric bulkiness.[6b]

In summary, compound **1**, a precursor molecule of modular caged nucleotides, was designed and synthesized from commercially available 4-bromoresorcinol. Amine containing functional units including biotin and an RGD peptide were introduced into precursor **1**. After introducing a functional unit, the tosylhydrazone moiety in **1** was transformed into the diazomethyl group to produce the desired coumarinyl diazomethane derivatives. The coumarinyl diazomethanes react chemoselectively with the free acid form of phosphates to yield the corresponding esters in the presence of other functional groups including carboxylates, amines, and alcohols. Propargyl-Bhc-caged diethyl phosphate was photolyzed to produce the parent acid with high photolysis efficiency ($\varepsilon\Phi_{350}$ = 2200 M^{-1} cm^{-1}). Bio-Bhc-diazo (**5**) was used in producing siRNA derivatives having a biotinylated photocaging group. The attached caging group was detected in both caging and uncaging reactions using standard polyacrylamide gel electrophoresis.

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Supporting Information Available. Experimental procedures and spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.