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Caged Substrates for Protein Labeling and Immobilization

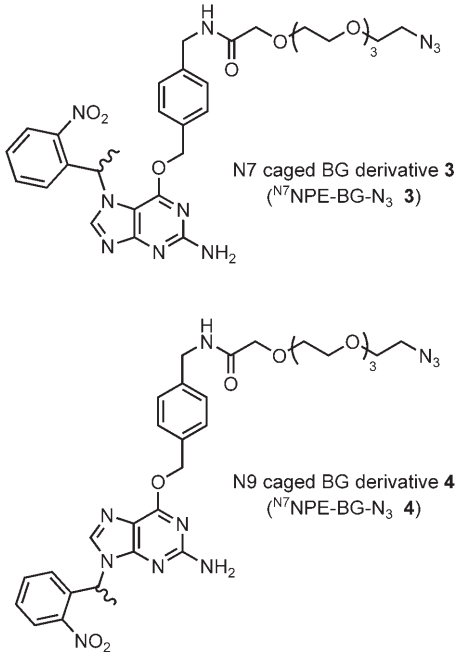
Sambashiva Banala, Anke Arnold, and Kai Johnsson*[a]

The labeling of proteins with synthetic probes in vitro and in living cells has become an important method to investigate and manipulate protein function.[1] Specific labeling is generally achieved through the expression of the protein of interest as a fusion protein with an additional polypeptide that mediates the labeling. One important feature of such an approach is the possibility to control the time of the labeling, and the ability to perform successive labeling experiments with different probes. Successive labeling experiments have allowed the different generations of a fusion protein to be discriminated, and have been shown to be powerful tools to study dynamic processes in living cells.[2] Caged substrates could add further levels of control over time and precise location of labeling.[3,4] Here, we introduce caged substrates for protein labeling that can be efficiently activated with light; these should find applications in biology and nanotechnology.

We have previously developed a general approach for the covalent labeling of proteins that is based on the irreversible reaction of mutants of O^6-alkylguanine-DNA alkyltransferase (AGT or SNAP-tag) with derivatives of O^6-benzylguanine (BG).[5] The labeling is based on the transfer of an appropriately substituted benzyl ring from the BG derivative to a reactive cysteine residue of the SNAP-tag. SNAP-tag fusion proteins can be labeled with a wide variety of different probes both in living cells and in vitro.[6–8] To temporally block the reactivity of the SNAP-tag towards BG, we decided to attach the 1-(2-nitrophenyl)ethyl (NPE) group either at the N7 or N9 position of BG (Scheme 1).

An analysis of the structure of human wild-type AGT suggested that substituents at the N7 of BG should lead to sterically unfavorable interactions with the protein.[9] Similarly, a BG derivative that has a NPE group at the N9 position should show low reactivity towards the SNAP-tag because mutations have been introduced in the protein that obstruct its reaction with N9-substituted BG derivatives.[10]

The synthesis of the NPE derivatives of BG is achieved through direct alkylation of previously described BG-N_3 (**1**) with 1-(1-bromoethyl)-2-nitrobenzene (**2**; Supporting Information). The assignments of the N7 and N9-substituted isomers were based on NMR spectroscopic studies (Supporting Information). Compounds **3** and **4** can either be derivatized by click chemistry,[11] or reduced to the corresponding amines **5** and **6**



Scheme 1. Caged BG derivatives for protein labeling.

and subsequently reacted with an appropriate *N*-hydroxysuccinimide ester (Scheme 2).

To investigate whether both compounds can be uncaged by UV light, **3** and **4** were dissolved in water, irradiated with UV light, and the reaction products were analyzed by HPLC (Figure 1).

Under these conditions, both compounds were cleanly converted into the corresponding BG derivatives through the expulsion of 1-(2-nitrosophenyl)ethanone (Figure 1). To analyze the reactivity of the SNAP-tag towards the caged substrates, the caged fluorescein and digoxigenin derivatives ^{N7}NPE-BG–FL **7**, ^{N9}NPE-BG–FL **8**, ^{N7}NPE-BG–Dig **9** and ^{N9}NPE-BG–Dig **10** were prepared (Scheme 2; Supporting Information).

Caged substrates **7** and **8** were incubated with an excess of a fusion protein of the SNAP-tag with glutathione S-transferase (SNAP–GST) with or without prior illumination, and the protein labeling was examined by SDS-PAGE and fluorescence scanning (Figure 2 A, B). Without prior illumination ^{N7}NPE-BG–FL did not lead to any detectable labeling of SNAP–GST (Figure 2 A and B). Illumination of the N7-caged derivative efficiently liberated the reactive BG derivative: the intensity of the signal that was measured for illuminated ^{N7}NPE-BG–FL was 70% of the observed signal when the protein was directly incubated with an equivalent amount of BG–FL. In contrast to the results with the N-7-caged derivative, incubation of SNAP–GST with ^{N9}NPE-BG–FL led to some labeling without prior illumination (Figure 2 A and B). Similar results were observed for the digoxigenin derivatives **9** and **10**. Incubation of ^{N7}NPE-BG with SNAP–GST with

[a] *S. Banala, Dr. A. Arnold, Prof. Dr. K. Johnsson*
École Polytechnique Fédéral de Lausanne (EPFL)
Institute of Chemical Sciences and Engineering
1015 Lausanne (Switzerland)
Fax: (+41) 21-693-9365
E-mail: kai.johnsson@epfl.ch

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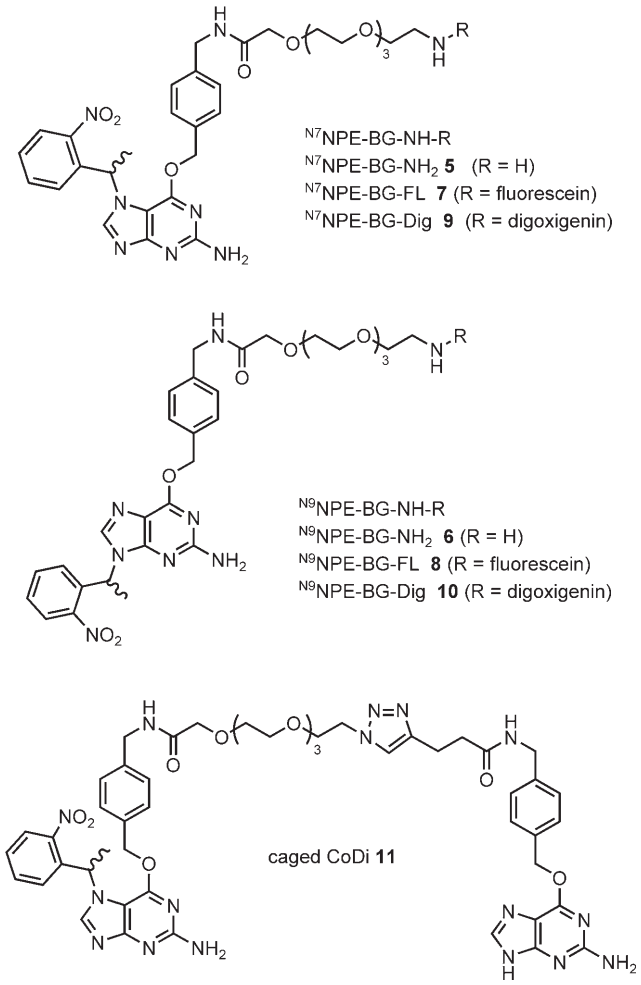

Scheme 2. Caged BG derivatives that were used for protein labeling in this study.

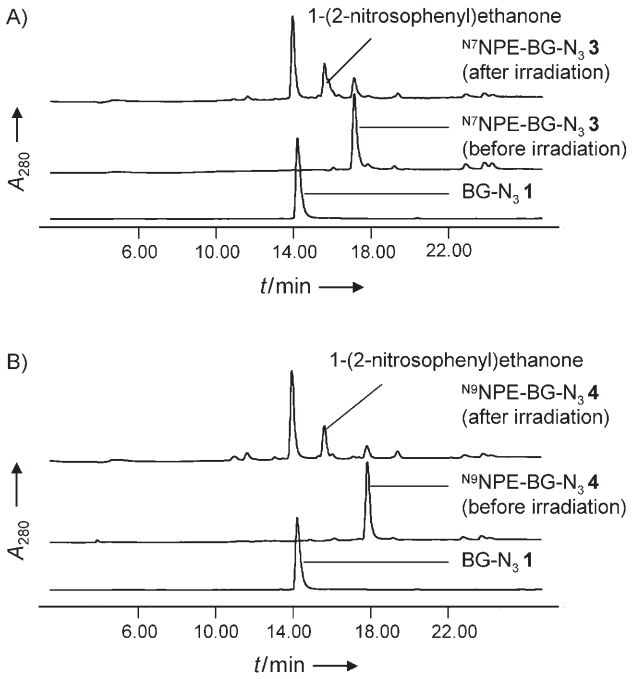

Figure 1. Photolysis of caged BG derivatives **3** and **4**. Samples (20 μM in water, 1% DMSO) were irradiated for 20 sec with 365 nm light from a 100 W Hg lamp. A) HPLC analysis of samples of **3** before and after irradiation. B) HPLC analysis of samples of **4** before and after irradiation.

or without prior illumination and analysis of the reaction through Dot blotting by using an anti-dioxigenin antibody demonstrated that no labeling could be observed without prior illumination, and that illumination led to efficient uncaging of the substrate. The intensity of the signal that was measured for illuminated ^{N7}NPE-BG–Dig was 70% of that observed when the protein was directly incubated with an equivalent amount of BG–Dig. In contrast, ^{N9}NPE-BG–Dig led to some labeling of SNAP–GST, even without prior illumination. In summary, these experiments demonstrate that ^{N7}NPE-BG derivatives are caged SNAP-tag substrates that do not possess any detectable activity prior to illumination, but can be efficiently uncaged with UV light.

As a first application of caged BG derivatives, we investigated the use of these molecules in light-induced protein dimerization and light-directed protein immobilization. We have recently introduced BG derivatives (so-called CoDis) that enable the covalent and irreversible dimerization of SNAP-tag fusion proteins in vitro and in living cells.[12] CoDis are dimers of BG that can be used for the covalent cross-linking of SNAP-tag fusion proteins. In addition to inducing dimerization-dependent phenotypes, the cross-linking efficiency of AGT fusion proteins indicates the proximity and interactions of protein pairs in living cells. We envisioned that caging one of the two BGs in such a CoDi would allow us to trigger the dimerization of a protein through a light pulse. To test the feasibility of this approach, we prepared a fusion protein of the SNAP tag with

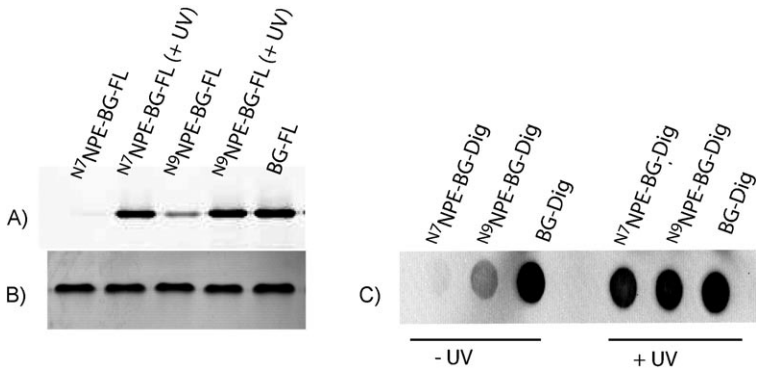

Figure 2. Reactivity of SNAP–GST with caged BG derivatives before and after illumination. A) SNAP–GST (2 μM) was incubated with ^{N7}NPE–BG–FL or ^{N9}NPE–BG–FL (0.5 μM each) with or without prior illumination (365 nm, 1 min) for 3 h at room temperature and the samples were subjected to SDS-PAGE and analyzed by laser-based fluorescence scanning. B) Coomassie blue staining of the gel shown in (A). C) SNAP–GST (0.75 μM) was incubated with ^{N7}NPE–BG–Dig or ^{N9}NPE–BG–Dig (1 μM each) with or without prior illumination (365 nm, 1 min) for 2 h at room temperature and samples subjected to Dot blot analysis using an anti-digoxigenin-peroxidase antibody conjugate and a chemiluminescent peroxidase substrate.

acyl carrier protein (SNAP–ACP) and labeled the protein with Cy3 via ACP.[13] Cy3-labeled SNAP–ACP was then incubated with caged CoDi **11** with or without prior illumination. Aliquots of the reaction mixture were taken at different time points, subjected to SDS-PAGE, and analyzed by laser-based fluorescence scanning. These experiments revealed that dimer formation took place only in illuminated samples (Figure 3A). The efficiency and speed of the dimerization is comparable to that which was reported for uncaged CoDis.[12] Caged CoDis thus offer the opportunity to induce protein dimerization with spatial and temporal resolution.

Another application for the caged BG derivatives might be the light-directed patterning of biomolecules on surfaces or within micro-fluidic channels.[14–16] We have previously shown that SNAP-tag fusion proteins can be selectively and covalently immobilized on surfaces that display BG,[17,18] and caged BG derivatives would offer the opportunity to spatially direct the immobilization through light. N7-caged BG derivative **5** was arrayed on glass slides displaying polymer brushes of poly-[oligo(ethylene glycol)methacrylate] (POEGMA) by using a previously described protocol.[19] The corresponding uncaged $BGNH_2$, **14** and benzylamine were arrayed on the same chip as positive and negative controls, respectively. Part of the glass slide was covered with a mask and the slide was irradiated with 365 nm light. Next, the whole chip was incubated with SNAP–ACP that was labeled with Cy5 via ACP. After several washing steps, the slide was analyzed for fluorescence (Figure 4).

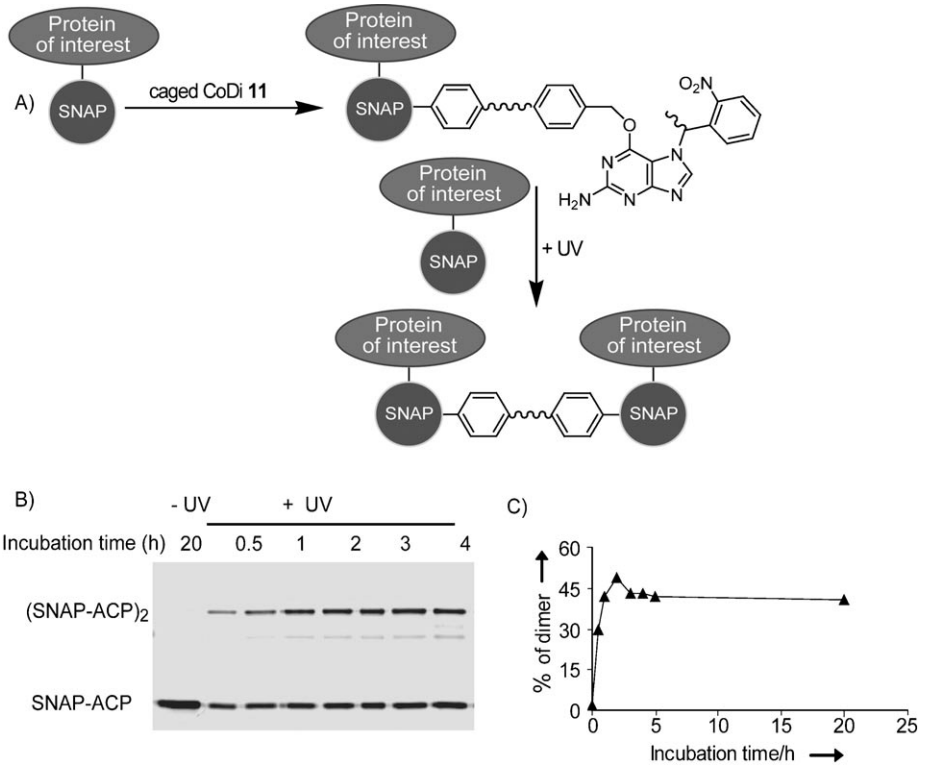

Figure 3. Light-induced covalent dimerization of SNAP-tag fusion proteins. A) General scheme for controlling the dimerization of SNAP-tag fusion proteins by using caged CoDi **11**. B) Cy3-labeled SNAP–ACP (1 μM) was incubated with caged CoDi **11** (0.7 μM) at RT with and without prior illumination. Aliquots of the reaction mixture were taken at the indicated time points, subjected to SDS-PAGE and analyzed by laser-based fluorescence scanning. C) Relative percentage of dimer $(SNAP–ACP)_2$ that was measured at different time points in (B) plotted as a function of time.

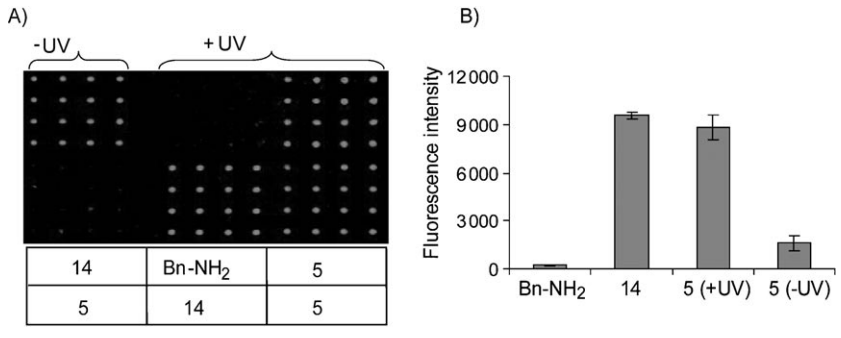

Figure 4. Light-directed immobilization of SNAP-tag fusion proteins. A) Caged BG derivative **5**, BG derivative **14** (as a positive control) and benzylamine ($Bn-NH_2$) were arrayed on an activated, POEGMA-covered glass slide as indicated (for further details see Supporting Information). After illumination at defined locations, the slide was incubated with Cy5-labeled SNAP–ACP (5 μM) for 1 h. The slide was analyzed for fluorescence after several washing steps. Cy5-labeling of SNAP–ACP was achieved by incubation of SNAP–ACP (5 μM) with CoA-Cy5 (10 μM) and AcpS (1 μM) for 30 min. B) Fluorescence intensities measured in (A).

Measurements of the fluorescence intensities on the chip demonstrated that protein immobilization was directed by the uncaging of **5** through light. Immobilization of SNAP–ACP at locations that displayed the N7-caged BG, but that were not illuminated was less than 20% of those that displayed N7-caged BG, and that were illuminated with UV light. Also, the amount of SNAP–ACP that was immobilized after the uncaging of BG derivative **5** was similar to that achieved by displaying BG directly (Figure 4A, B). Because SNAP-tag fusion proteins can be expressed on the surface of mammalian cells,[20] the caged BG derivatives should also find applications in cell patterning and in tissue engineering.[21,22]

In summary, we have reported the synthesis of caged BG derivatives that display no activity towards SNAP-tag fusion proteins,

but which can be efficiently uncaged by light. The caged molecules allow us to control the dimerization and immobilization of SNAP-tag fusion proteins with both spatial and temporal resolution. Considering the breadth of the SNAP-tag technology, the caged BG should furthermore become useful for a variety of other applications.

Experimental Section

Detailed experimental procedures for the synthesis and characterization of all compounds, as well as a detailed description of all experiments are given in the Supporting Information.

Acknowledgements

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Keywords: caged molecules · protein labeling · protein microarrays · protein–protein interactions · proteomics

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