A bromocoumarin-based linker for synthesis of photocleavable peptidoconjugates with high photosensitivity†

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A new bromocoumarin-based bi-functional linker was developed for preparing photocleavable peptides and proteins with high photolytic efficiency, which have many potential applications in the study and engineering of biological systems.

Peptides, proteins, or their conjugates whose function can be controlled by light are powerful tools for cell biology and biotechnology.[1] Light can be projected into samples, *e.g.* in test tubes, on solid surfaces, or in live cells and tissues, at a desired time and area, allowing us to trigger molecular and cellular events with high spatiotemporal precision. Typically, they are prepared by introducing a photoremovable protecting (caging) group at the side chain of essential amino acid residue(s) to abolish the function. The activity is then regained by releasing the caging group with a flash of light. Another important approach involves the incorporation of a photocleavable site within the polypeptide backbone, so that a single peptide/protein chain can be self-split into two fragments upon photoillumination. This "backbone-photocleavage" strategy is particularly useful for designing various types of light-controlled molecules.[2–6] For example, Bosques and Imperiali synthesized a light-activatable self-assembling peptide by connecting an amyloidogenic sequence and a fibril-inhibitory unit *via* a photocleavable linker.[2] As well as activation,[2,3] light-induced inactivation of proteins[4] and peptides[5] has also been demonstrated. In recent notable applications, Pellois and Muir[6] and Otaka *et al.*[7] used the approach to control subcellular localization of a protein and a peptide, respectively, by light.

The choice of photocleavable linker is the key that determines photochemical properties of such peptides and proteins. Although a number of new caging molecules with improved efficiency of photolysis have been developed in the last decade,[8] currently available linkers are limited to classical 2-nitrobenzyl (NB) derivatives with relatively low photolytic efficiencies. A photoreaction that proceeds as efficiently as possible with UV light (ideally over 350 nm) is desired, particularly for cell biological applications to minimize photodamage of cells. Thus, the development of new photocleavable linkers with improved photolysis efficiency is of crucial importance to expand the applicability of light-controlled peptides/proteins. Here, we report the design, synthesis, and photochemistry of a new photocleavable linker which is based on a 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) group.

Bhc is a recently developed caging agent which has a photosensitivity superior to that of NB-based photolabile groups.[9,10] It has been previously used for preparing caged analogues of various biologically active molecules, such as glutamic acid,[10a] mRNA,[10b] cyclic nucleotides,[10c] protein synthesis inhibitors,[10d] and phosphotyrosine peptides.[10e] Unfortunately, the original Bhc is mono-functional and thus needs to be modified to be introduced into polypeptides by conventional methods. Furuta and coworkers recently reported that the 7-hydroxyl moiety of the Bhc group can be alkylated without sacrificing the overall photosensitivity to UV light.[10f] Based on this finding, we decided to attach an additional functionality at the 7-position of Bhc and designed a new 6-**b**romo-7-**a**minoethoxy**c**oumarin-4-ylmethoxycarbonyl (Bac) linker building block **1** which is suitable for Fmoc-based solid-phase peptide synthesis (SPPS) (Fig. 1). As shown in Fig. 1, during SPPS the Bac linker **1** can be incorporated within a polypeptide chain at any position *via* stable amide (the 7-position side) and carbamate (the 4-position side) bonds. Upon light irradiation, the Bac linker-containing

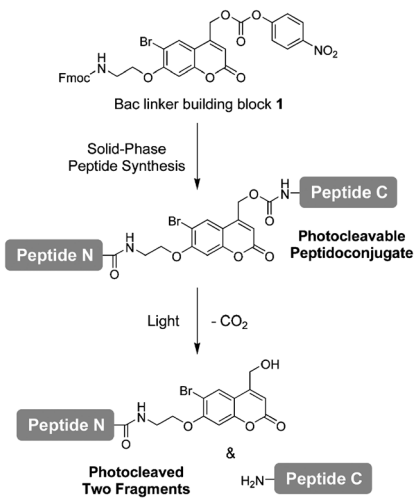

Fig. 1 Schematic illustration of synthesis and photochemistry of photocleavable peptidoconjugates containing the Bac linker.

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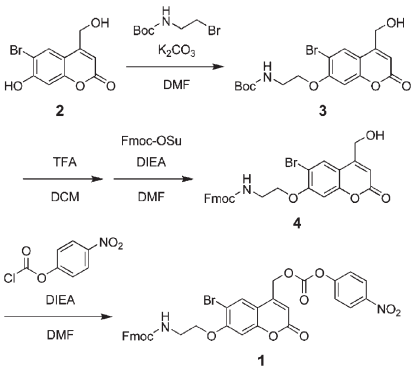
† Electronic supplementary information (ESI) available: Full experimental procedures, UV/vis spectrum of **5**, and HPLC traces of photolysis of **6** before and after photolysis. See DOI: 10.1039/b812058g

peptides undergo self-cleavage into two fragments through the photochemical decomposition of the carbamate linkage[10a,f] with concomitant release of a bromocoumarin-4-ylmethanol (Bac_{OH})-attached N-terminal half peptide and another C-terminal half peptide with a free amine.

The synthesis of **1** was accomplished as shown in Scheme 1 (see ESI† for detailed procedures). The starting material 6-bromo-7-hydroxycoumarin-4-ylmethanol **2** was prepared in two steps as previously reported.[10a] The 7-hydroxyl group of **2** was selectively *O*-alkylated using 2-(*tert*-butoxycarbonylamino)ethyl bromide and potassium carbonate in dimethylformamide (DMF), producing the compound **3** (50%). After removal of the Boc protecting group in dichloromethane (DCM) containing 10% trifluoroacetic acid (TFA), the amine group was re-protected by an Fmoc group using 9-fluorenylmethyl succinimidyl carbonate (Fmoc-OSu) and *N*,*N*-diisopropylethylamine (DIEA) in DMF to yield compound **4** (93% over two steps). Finally, the building block **1** was obtained by reaction of **4** with 4-nitrophenyl chloroformate and DIEA in DCM (91%). It should be noted that compound **1** is solid and stable and can be stored for long periods at 4 °C without significant decomposition.

To investigate the versatility of building block **1**, we synthesized two model photocleavable peptidoconjugates, a linear form **5** and a branched form **6**, based on a FLAG epitope (DYKDDDDK) (Fig. 2). The linear peptidoconjugate **5** contained the Bac linker incorporated between a flexible GSGS sequence and the FLAG motif, and a biotin tag was also introduced at the N-terminus. In the branched peptidoconjugate **6**, a heptaarginine sequence, which is known as a cell-penetrating peptide,[11] was attached to the side-chain of the lysine residue in the FLAG motif *via* the Bac linker. They were built on a Rink amide resin using a standard Fmoc-based SPPS procedure according to the scheme shown in Fig. 2. In the synthesis of **5**, after assembly of protected FLAG resin **7** and Fmoc deprotection, the Bac linker was installed by treating the resin with **1** and DIEA in DMF. Complete incorporation of the linker was confirmed by the Kaiser test.[12] Next the Fmoc group was removed by a standard method using 20% piperidine in DMF, which was followed by subsequent coupling/deprotection cycles. In the synthesis of **6**, following Fmoc deprotection and acetylation of protected FLAG resin **7**, the acid-sensitive methoxytrityl (Mtt) group on the side chain of the lysine residue on position 3 was selectively removed with 2% TFA and 5% triisopropylsilane (TIS) in DCM. Subsequent incorporation of **1** and chain elongation was achieved using a similar procedure



Scheme 1 Route for synthesis of the Bac linker building block **1**.

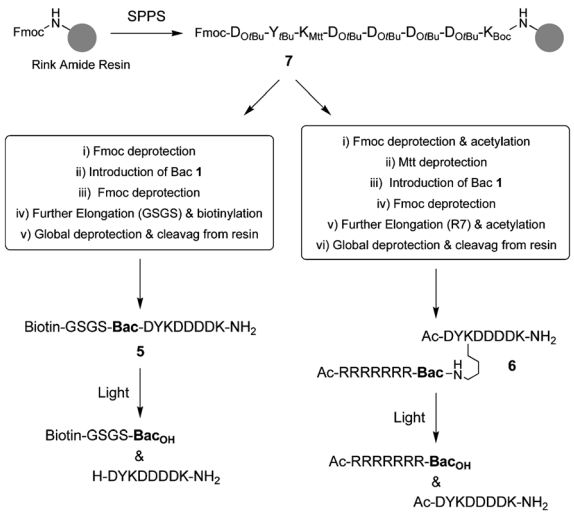


Fig. 2 Synthesis and photoreaction of Bac-containing peptidoconjugates **5** and **6**.

to that described above. Upon complete assembly of the peptidoconjugates, global deprotection and cleavage from the resin was performed with TFA containing 5% ethanedithiol (EDT) and 2.5% H_2O. Both of the products were precipitated by Et_2O and purified to homogeneity by reversed-phase HPLC (RP-HPLC). The new building block **1** proved resistant to the basic conditions used for coupling and Fmoc removal reactions and was also resistant to the acidic treatment necessary for the final cleavage and deprotection steps.

Next, we investigated the photochemical properties of **5** and **6** in K-MOPS solution (10 mM MOPS, 100 mM KCl, pH 7.2). Both conjugates showed a characteristic absorption band with a maximum at 330 nm (ε_{330} = 10 080 $M^{-1}\cdot cm^{-1}$), which is ascribed to the Bac moiety (Fig. S1, ESI†). The absorption maximum was almost identical to that of the (6-bromo-7-methoxycoumarin-4-yl)methoxycarbonyl (Bmcmoc) group,[10f] indicating that the replacement of a methyl group with an amidoethyl group does not affect the absorption properties of the 6-bromocoumarin photo-trigger. The conjugate solutions were irradiated with UV light at 350 nm and analyzed by RP-HPLC as a function of time. **5** was photocleaved into the corresponding two fragments, biotin-GSGS-Bac_{OH} and FLAG motif with an N-terminus amine, which were characterized by MALDI-TOF-MS analyses (Fig. 3). **6** also underwent photocleavage to produce Ac-R_7-Bac_{OH} and an Ac-FLAG sequence as expected (Fig. S2, ESI†). The photolytic consumption of the peptidoconjugates **5** and **6** followed a single exponential decay (Fig. 4). The quantum yields for the photocleavage reaction were determined to be 0.26 for **5** and 0.16 for **6** using the previously reported method.[10a] In addition, the overall photosensitivities, which can be expressed as the product of the quantum yield of the photolysis (Φ) and the molar absorption coefficient (ε), were calculated to be 1445 M^{-1} cm^{-1} for **5** and 1139 M^{-1} cm^{-1} for **6**. These values are almost comparable with those of Bmcmoc-caged nucleobases, thus also indicating that the photosensitivity of Bac is markedly higher than that of other NB-based linkers (see ref. 10*f*). Moreover, we confirmed that the peptidoconjugates were sufficiently stable from hydrolysis in the absence of light (less than 10% of hydrolysis at 37 °C even after one day incubation in K-MOPS solution).

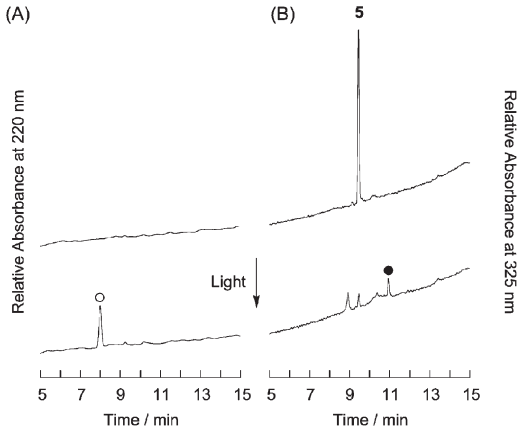


Fig. 3 HPLC traces of **5** before (top) and after (bottom) UV photolysis (350 nm, 2 min). Conditions: 9 μM in K-MOPS buffer. HPLC was performed on an ODS column with a linear gradient of acetonitrile containing 0.1% TFA (solvent A) and 0.1% aqueous TFA (solvent B). (A) Detection at 220 nm. Gradient: A/B = 4 : 90 to 10 : 60 for 15 min. Peak ○ denotes FLAG motif with an N-terminus free amine (H-DYKDDDDK-NH_2). (B) Detection at 325 nm. Gradient: A/B = 10 : 90 to 40 : 60 for 15 min. Peak ● denotes Biotin-GSGS-Bac_{OH}.

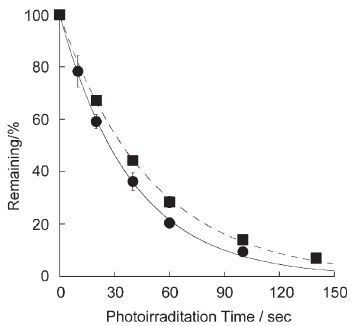


Fig. 4 Time courses of photolysis of peptidoconjugates **5** (●, 9 μM) and **6** (■, 8 μM) in K-MOPS buffer.

In summary, we have developed a new bromocoumarin-based linker for the synthesis of photocleavable peptido-conjugates with high photosensitivity. The building block **1** is versatile and can in principle be incorporated into any peptide sequence at any position by SPPS.[13] It will also be feasible to integrate Bac-containing peptides with larger proteins using protein ligation[14] or chemical modification techniques[15] to yield photocleavable semisynthetic proteins. This work will enhance the applications of light-controlled peptide/protein tools for investigating and/or artificially manipulating biological processes. Moreover, the strategy described here to design the Bac linker may also be used to create new photocleavable linkers with different reactive functionalities such as maleimide, which could be used in preparing photoactivatable gene-targeting compounds.[16]

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13 We have also successfully prepared a cyclic analogue of 16-residue peptide derived from a Bcl-2 homology 3 (BH3) domain of Bak (GQVGRQLAIIGDDINR) by connecting the N- and C-terminus through the Bac linker. The Bak peptide is known to interact with Bcl-x_L and induce apoptosis (M. Sattler, H. Liang, D. Nettesheim, R. P. Meadows, J. E. Harlan, M. Eberstadt, H. S. Yoon, S. B. Shuker, B. S. Chang, A. J. Minn, C. B. Thompson and S. W. Fesik, *Science*, 1997, **275**, 983–986). In preliminary experiments, it was confirmed that the cyclic form has no affinity toward Bcl-x_L, but recovers its binding ability upon light-induced conversion to a linear peptide. These results demonstrate the feasibility of controlling the function of peptides by constraining the structure with photocleavable linkers, which should become a new design strategy for light-activatable peptides/proteins. More details and cell biological applications of the cyclized Bak peptide will be reported elsewhere in due course.

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