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A new photolabile carboxyl protecting group for native chemical ligation

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Abstract—Native chemical ligation (NCL) at Glu–Cys sites requires carboxyl protection that is compatible with the chemistry used for assembling the peptides. In the case of Fmoc-chemistry our novel BCMACM-group is particularly suitable for NCL because of the good solubility in aqueous solvent systems and mild conditions of photolytic removal.

Native chemical ligation (NCL) is a powerful tool for the synthesis of small to medium-size proteins, which has found a widespread use because of the possibility to incorporate non-proteinogenic amino acids, labels or post-translational modifications.[1–4] NCL involves the coupling of two unprotected peptide segments in aqueous solution: a peptide segment bearing an N-terminal cysteine and a C-terminal thioester peptide segment. After an initial thioester exchange, a subsequent intramolecular *S*/*N*-acyl shift results in an amide bond formation between the two segments.

The 20 natural amino acids are compatible in a Xaa–Cys ligation[5] but it has been reported that ligation at Asp–Cys and Glu–Cys sites are prompted to side reactions since the β- or γ-carboxyl group of the side chain can react with the α-thioester to produce an anhydride, which can be opened, forming the α- and γ-isomers.[6] Therefore, the side chain of aspartic and glutamic acid has to be protected during NCL at Asp–Cys and Glu–Cys sites. Thus far, such protecting groups have been used in NCL in combination with Boc/benzyl strategy[6] for solid-phase peptide synthesis but no application of the widespread Fmoc/*tert*-butyl strategy[7] has been described.

For use in NCL, the side chain protecting group of the aspartic and glutamic acid has to fulfil several requirements. Firstly, it must be compatible with the peptide synthesis using Fmoc/*tert*-butyl strategy, for example, it has to be stable during the cleavage of the Fmoc group (20% piperidine/DMF) and the final TFA treatment for deprotection. Secondly, this protecting group must be stable towards aqueous, weakly basic conditions used for the NCL reaction and must also be highly soluble to achieve the high concentration required for the NCL. Thirdly, the cleavage of this protecting group has to be accomplished under mild conditions, for example, without any addition of chemical reagents which could possibly react with the ligation product.

Among the orthogonal carboxyl protecting groups (CPGs) commercially available which could fulfil these requirements we decided to use the 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl (Dmab) protecting group[8] (Fig. 1). This protecting group is compatible with Fmoc/*tert*-butyl strategy and is not cleaved during the final TFA treatment. It is easily removed on solid phase or in solution using hydrazine. However, this hydrophobic protecting group has poor solubility in aqueous solution which can be a drawback for achieving high concentrations required for the NCL.

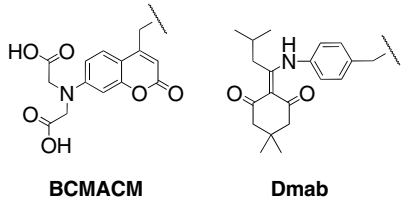

Figure 1. BCMACM and Dmab carboxyl protecting groups.

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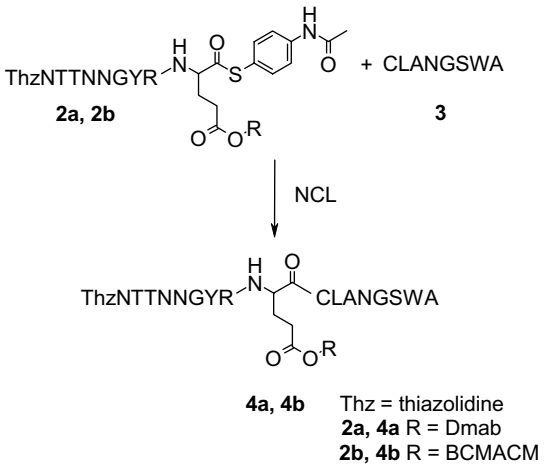
To solve this hindrance we decided to develop a new CPG accomplishing an increased water solubility and an orthogonal cleavage. Use of photolabile protecting groups is an appealing solution because their removal requires only light and no chemical reagents, allowing therefore a very mild cleavage.[9] Among the different photolabile protecting groups the coumarinylmethyl system has received increased attention in recent years and (coumarin-4-yl)methyl caged glutamates have been reported as efficient caged compounds.[10,11] We therefore decided to use the {7-[bis(carboxymethyl)amino]coumarin-4-yl}methyl (BCMACM) group (Fig. 1) which was previously reported by our group for the synthesis of caged compounds.[12,13] Herein, we describe the use of the BCMACM-chromophore for carboxylic protection and NCL and compare it with the commercially available Dmab-group.

We started with the synthesis of the corresponding Fmoc glutamic acid. The *tert*-butyl protected 7-[bis(*tert*-butoxycarbonylmethyl)amino]-4-(hydroxymethyl)coumarin was synthesised using the previously described protocol[12] and introduced into the Fmoc-Glu(OH)-OAll in DCM with DIC/DMAP. The allyl ester group was then cleaved by adding Pd/PPh_3 directly to the reaction mixture[14] to give the *tert*-butyl and Fmoc protected derivative Fmoc-Glu(γ-OBBCMACM)-OH **1** quantitatively. Purification by flash chromatography and lyophilisation afford **1** in a 61% yield (two steps). The differential photochemical quantum yield (ϕ) of the *tert*-butyl deprotected **1** is $\phi = 0.01$ at 365 nm in 5% CH_3CN/0.01 M HEPES/KOH buffer (pH 7.2).[15,16]

To compare the two CPGs, we synthesised a model peptide by NCL (Scheme 1). At first the two peptide thioesters **2a** and **2b** were synthesised according to our previously described procedure.[17] For this, Fmoc-Glu(ODmab)-OH and Fmoc-Glu(γ-OBBCMACM)-OH, respectively, were coupled as the first amino acid onto a 2-chlorotrityl resin. In the case of the BCMACM protecting group, the first Fmoc cleavage had to be carried out with care to avoid the formation of pyroglutamate.



Scheme 1. Native chemical ligation between peptide **3** and thioesters **2a** and **2b**.

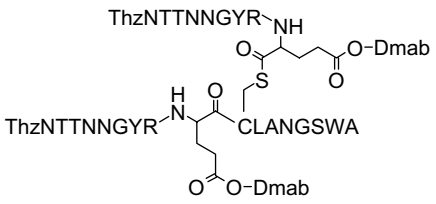


Figure 2. Intermediate thioester formed between **4a** and **2a**.

Pyroglutamate formation could be reduced to 10% with short cleavage (1 min) using 10% piperidine/DMF. Pyroglutamate formation was also observed with Dmab but to a lesser extent of only 5% using the same cleavage procedure. After assembling the whole sequences, the two peptides were cleaved from the resin as fully protected peptides using AcOH/TFE/DCM. After lyophilisation these protected peptides were reacted with *p*-acetamidothiophenol (Aatp) and DIC in DCM to form the protected C-terminal thioesters. The side chain protecting groups were then cleaved using TFA and the two C-terminal thioesters were purified by HPLC.

We investigated NCL between peptide **3** bearing an N-terminal cysteine and the two C-terminal thioesters **2a** and **2b**. NCL requires a high concentration to proceed fast; usually a concentration of at least 1 mM is used. In the case of the Dmab-group this concentration was difficult to achieve because the C-terminal thioester **2a** was poorly soluble in aqueous buffer solution (6 M GnHCl/0.2 M Na_2HPO_4/(TCEP, 10 equiv)) and **2a** was reacted in suspension with peptide **3** dissolved in buffer solution. After 1 h the reaction went to comple-

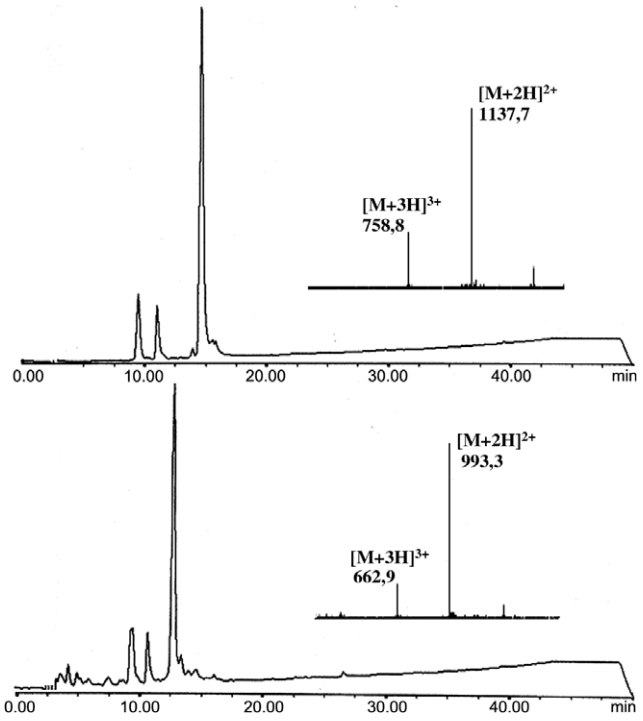


Figure 3. HPLC profile at 220 nm together with ESI/MS data for products **4b** and **5**.

tion. We observed the formation of a side product that was identified using LC–MS. Its mass corresponds to the intermediate thioester formed by thioester exchange between the internal cysteine of the ligation product **4a** and the C-terminal thioester **2a** (Fig. 2). This intermediate thioester is normally not observed or isolated during NCL because it should further react with other thiols present in the mixture, such as the N-terminal cysteine of peptide **3** or Aatp. Two Dmab protecting groups in the corresponding intermediate thioester resulted in very poor solubility and its subsequent precipitation. Thus, it was no longer available for reaction even after the addition of an excess of Aatp.

This was contrasted starkly with our new water soluble protecting group BCMACM. Here, the C-terminal thioester **2b** was high soluble in buffer solution. **2b** was added to the segment **3** and after 20 min the reaction was complete (Fig. 3). We could also observe the formation of the above-mentioned side product but only to a very minor extent. The BCMACM protecting group was finally cleaved from the ligation product **4b** by irradiation at 405 nm for 20 min giving the final peptide **5** ThzNTTNNGYRECLANGSWA which was then purified and characterised.

In summary, the {7-[bis(carboxymethyl)amino]coumarin-4-yl}methyl group has shown to be an efficient carboxyl protecting group for NCL at Glu–Cys sites, which is compatible with the widely used Fmoc/*tert*-butyl strategy. Its solubility in aqueous solution and its mild removal upon UV irradiation at 405 nm makes it very suitable for NCL at Glu–Cys sites.

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Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.11.028.

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