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Peptidyl *N,N*-Bis(2-mercaptoethyl)-amides as Thioester Precursors for Native Chemical Ligation†

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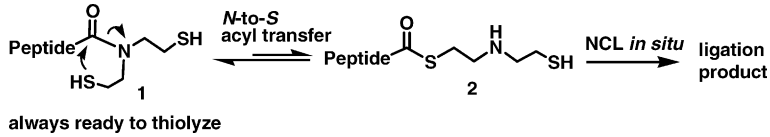
ABSTRACT



With two β-mercaptoethyl groups on the N, a tertiary amide of structure 1 is always poised for intramolecular thioesterification however it flips about the C−N bond. It is shown that a peptide with such a C-terminal *N,N*-bis(2-mercaptoethyl)-amide (BMEA) can be used directly for native chemical ligation (NCL). These BMEA peptides are easily prepared with standard Fmoc-solid phase peptide synthesis protocols, thus giving a very convenient access to the thioester components for NCL.

Peptide C^{α}-thioesters are key building blocks for a number of protein synthesis strategies,[1] including notably native chemical ligation.[2] This has stimulated considerable interest in developing new methods to prepare these important compounds in recent years.[3] Traditionally, thioester peptides are prepared with the Boc-solid phase synthesis method whereby the peptide chain is assembled directly on a thioester linker.[1a,4] Although this method is very effective, the need for a highly hazardous strong acid such as HF at the final cleavage step represents a deterring element to many research laboratories. Direct Fmoc-solid phase synthesis of peptide thioesters on a thioester linker is also possible using a modified Fmoc-deprotection protocol; however its use is restricted to relatively small peptides and racemization of the C-terminal amino acid residue is a non-negligible problem.[5] Considerable efforts have been devoted to developing alternative strategies to obtain thioester peptides indirectly from nonthioester precursors, the synthesis of which is compatible with standard Fmoc chemistry.[6−8] For

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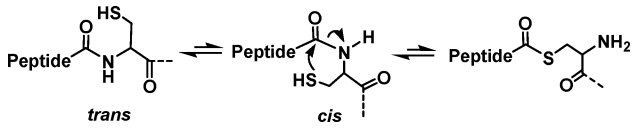
example, certain activated amide systems based on Kenner's safety-catch sulfonamides,[6a−c] acylureas,[6d] and pyroglutamyl imides[6e] have been developed. Other systems utilize *N*→*S* acyl transfer to produce thioesters,[9,10] which in a way are mechanistically reminiscent of protein splicing.[11] Most of these systems require acidic conditions to catalyze amide-to-thioester conversion which is followed by transthioesterification with a free thiol compound to generate a thioester. Aimoto's group reported the use of an autoactivating C-terminal Cys-Pro ester (CPE) to mediate amide-to-thioester conversion at neutral or slightly basic pH, which is driven by diketopiperazine formation to trap the transiently exposed α-amine of Cys through intramolecular aminolysis of the prolyl ester.[10a] Despite an inconvenience in loading the first amino acid to the CPE linker and the need for a relatively reactive glycolic ester at the C-terminus, this method is appealing for its clever design.

In our efforts to develop new and convenient methods for peptide thioester synthesis, we have paid particular attention to the *N*→*S* acyl transfer reaction. Mechanistically, in order for the *N*→*S* acyl transfer to take place, the planar amide bond must be in the conformation where the thiol-bearing *N*-substituent is *anti* to the carbonyl oxygen, as shown in Scheme 1 for an Xaa-Cys peptide bond. This requires an

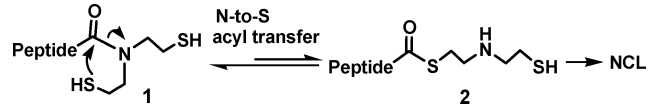
Scheme 1. Mechanism of *N*→*S* Acyl Transfer Involving a Peptidyl-Cys Amide Bond



energetically unfavorable *cis* isomer of the secondary amide, a conformation it almost never adopts. And *trans*−*cis* isomerization of a secondary amide peptide bond requires a significant amount of activation energy ($\Delta H^{\ddagger}$ ∼20 kcal/mol).[12] Furthermore, to drive the reaction equilibrium toward thioester formation, there must be a trapping mechanism, e.g., protonation, for the newly exposed amine. In protein splicing, *trans*−*cis* amide isomerization is catalyzed presumably by the intein which also serves as a general acid−base catalyst for thioesterification.[11] N^{α}-Cys alkylation (e.g., methylation and ethylation) would increase the ratio of the productive *cis* form to the nonproductive *trans* form in the resultant tertiary amide bond, but the conversion of the *trans* isomer to the *cis* isomer would still need to overcome a significant energy barrier no less than in the case of a normal secondary amide peptide bond.[12] For example, even an Xaa-Pro tertiary amide has a high rotational barrier ($\Delta G^{\ddagger}$ = 18−21.5 kcal/mol) and a low *cis*/*trans* ratio of ∼0.05/1.0 in unstructured polypeptide chains.[12] For this reason, the *N*-alkyl Cys systems, which require acidic conditions for thioesterification, are usually very inefficient and give very low yields of thioester products.[9d,3b]

These considerations have led us to propose a new *N*→*S* acyl transfer system to generate thioesters for NCL, as shown in Scheme 2. This system is based on the use of a peptide

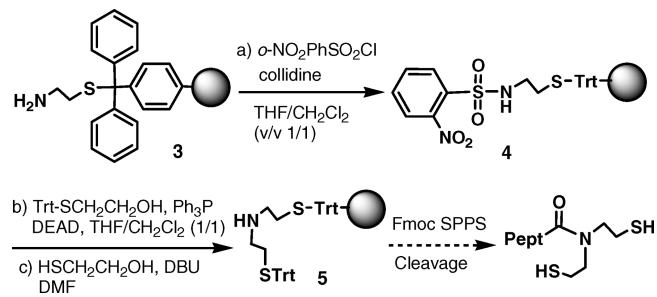
Scheme 2. *N*-to-*S* Acyl Transfer Using an *N,N*-Bis(2-mercaptoethyl)-Substituted Tertiary Amide To Generate a Thioester Peptide for NCL



C-terminal tertiary amide **1**, namely *N,N*-bis(2-mercaptoethyl)-amide or BMEA. With this system, the *N*→*S* acyl transfer reaction is twice as likely. It also obviates the need for *trans*−*cis* amide isomerization prior to the *N*→*S* acyl transfer reaction step because, with two β-mercaptoethyl (HS-Et) *N*-substitutions, the BMEA amide will always have one HS group correctly positioned for the intramolecular thiolysis reaction (Scheme 2), and the relatively high basicity of the secondary amine in the thioesterification product **2** would also make its trapping easier via protonation, which might help drive the formation of **2** at an NCL-operable pH. Coupling thioesterification with native chemical ligation would then ultimately lead the overall reaction in its forward direction (Scheme 2).

For the synthesis of a C-terminal BMEA peptide **1** shown in Scheme 2, we designed a bis(2-mercaptoethyl)amine-derived trityl resin **5** which was prepared in straightforward reaction steps (Scheme 3). Thus, (2-aminoethyl)sulfanyl-trityl

Scheme 3. Synthesis of C-Terminal BMEA Peptides



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resin **3** was reacted with *o*-nitrobenzenesulfonyl chloride to afford the sulfonamide resin **4**. Alkylation of the sulfonamide with Trt-SCH_2CH_2OH by Mitsunobu reaction and subsequent thiolytic removal of the sulfonyl group yielded the desired dialkylamine resin **5**, ready for use in Fmoc SPPS.

Loading the first Fmoc-amino acid onto resin **5** was achieved using DIC/HOAt, a coupling protocol known to be effective for a sterically hindered secondary amine with minimum risks of racemization.[13] Subsequent assembly of the peptide chain was carried out using standard Fmoc SPPS procedures. Because of its inactivated nature, the tertiary amide linkage is completely stable during Fmoc SPPS.

To test the BMEA-mediated ligation method, we first prepared a small BMEA peptide with a Gly as the C-terminal residue, H-**LKSFG**-*BMEA*. Interestingly, HPLC analysis of the peptide showed the coexistance of the amide form **1** and thioester form **2** (Scheme 2) as two peaks at a ratio of about 1:5 which gave the same molecular ion (Figure 1). The

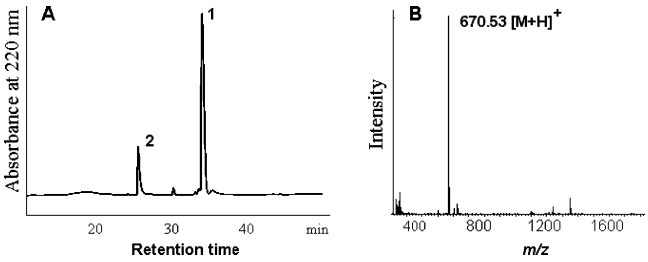

Figure 1. HPLC (A) and MS (B) analyses of **LKSFG**-*BMEA*. Peak 1, amide form; peak 2, thioester form. HPLC gradient: 0 − 45% buffer B in 45 min.

smaller peak (Figure 1A, peak 2) appeared to be the thioester form which, with a free amine in the BMEA portion, was expected to be more hydrophilic and would elute out earlier in HPLC, and the larger peak (peak 1) was the amide form. This result was very encouraging as it clearly showed that *N*→*S* acyl transfer with this BMEA peptide could readily take place under these relatively acidic conditions (pH of HPLC buffer: ∼2). In fact, when this peptide was treated with 20% mercaptopropionic acid (MPA) in H_2O, the MPA thioester was obtained in ∼85% yield after 8 h at RT (Supporting Information). In previous studies with the *N*-alkyl-cysteine method, thiol exchange with MPA yielded ∼33% of the thioester product after 2−3 days of reaction.[9d] Our results show that the presence of two HS-Et groups on the amide nitrogen significantly increased the thioesterifying capability of BMEA as compared with *N*-alkyl cysteine systems.

We next wanted to test whether the BMEA peptide could be used directly for NCL, which would be ideal and is also the focus of our present study. We first performed ligation of H-**LKSFG**-*BMEA* (5 mM) with H-**CLKFA**-NH_2 (15 mM) at 37 °C using benzylmercaptan (1%) as the thiol additive. A very clean ligation reaction was observed at pH 4 or 5 with a yield of ∼75% after 24 h of reaction (Supporting Information). pH 6 also gave a relative clean reaction with just a slight increase in side products. The rate of ligation was about the same at pH 4, 5, or 6 (Supporting Information). We also found MESNa ($HSCH_2CH_2SO_3Na$) to be a better thiol additive for the ligation reaction presumably owning to its excellent solubility in aqueous buffer. As we can see from Figure 2A, when the reaction was conducted at 37 °C

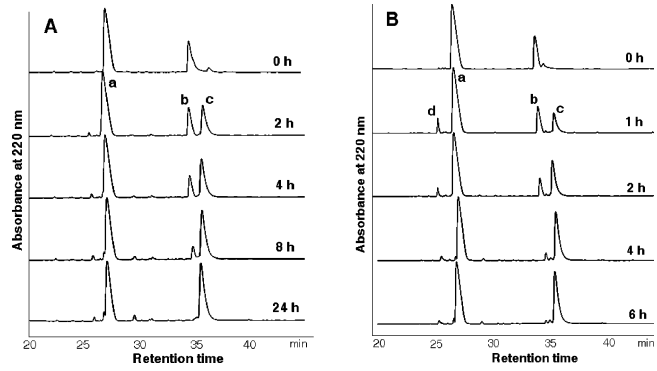

Figure 2. Ligation between H-**LKSFG**-*BMEA* and H-**CLKFA**-NH_2 in the presence of MESNa (2% w/v) and 50 mM TCEP and at pH 5. HPLC gradient: 0−50% buffer B in 50 min. (A) Reaction at 37 °C. (B) Reaction under microwave irradiation. Peak a, H-**CLKFA**-NH_2; peak b, H-**LKSFG**-*BMEA*; peak c, ligation product; peak d, H-**LKSFG**-*MES* thioester.

and pH 5, the yield was ≥80% after 8 h in the presence of 2% (w/v) MESNa. Interestingly, low-power microwave irradiation could significantly accelerate the reaction, which gave >90% yield after just 4 h (Figure 2B). *N*→*S* acyl transfer appeared to be the rate-limiting step, as only a very small amount of the MES thioester, but not the BMEA thioester **2** (Scheme 2), was seen in the HPLC (Figure 2), suggesting that, once formed, the BMEA thioester was quickly reacted with MESNa and/or the cysteinyl peptide H-**CLKFA**-NH_2.

The above data clearly show that a peptide with a C-terminal BMEA can be used as a thioester equivalent for ligation with a cysteinyl peptide through a transiently formed thioester. To investigate the scope of this BMEA-mediated reaction system, we synthesized six other small BMEA peptides with six representative C-terminal amino acid residues: Ala, Phe, Ser, Leu, Asn, and Val, respectively (Table 1). These peptides were then used in the ligation reaction with H-**CLKFA**-NH_2 at pH 5 and under microwave irradiation. The results were summarized in Table 1. One can see that excellent yields were obtained after 6 h of reaction for almost all of these BMEA peptides with H-**LKSFV**-*BMEA* being the only exception. The presence of the Val β-branched side chain seemed to hinder the ability for this BMEA peptide to undergo *N*→*S* acyl transfer. In spite of this, the results show that BMEA peptides can tolerate most amino acids at the C-terminal position for ligation with a cysteinyl-peptide. Very clean ligation reactions were observed for all of these BMEA peptides (Supporting Information). This

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Table 1. Conversion (%) of H-**LKSFXaa**-*BMEA* Peptides in Forming Ligation Products with H-**CLKFA**-NH_2[a]

Xaa/time (h)	Gly	Ala	Ser	Phe	Leu	Asn	Val
2	65	56	58	50	52	57	
4	90	82	77	78	75	78	
6	100	100	90	92	86	92	
8			93	96	95	99	
10			99		98		~15

[a] Reaction conditions: H-**LKSFXaa**-*BMEA* (5 mM) and H-**CLKFA**-NH_2 (15 mM) in 100 mM acetate buffer (pH 5) containing 2% (w/v) MESNa and 50 mM TCEP under microwave irradiation. Yields were based on HPLC analysis and on the consumption of the BMEA peptides. See Supporting Information for experimental details.

therefore demonstrates the broad application scope of the tertiary amide BMEA system in native chemical ligation.

Having confirmed that BMEA peptides can be directly used for NCL in the model studies, we then applied this method to protein synthesis. The semisyntheses of a histone H3 protein as well as an analog of it were successfully carried out. The overall synthetic strategy involved the ligation between an H3 N-terminal BMEA peptide which was synthesized by Fmoc-SPPS on the BMEA linker-derived resin and the H3 C-terminal globular domain which was prepared by recombinant DNA technology. So, a 13-residue BMEA peptide, **H3(1−13)**-*BMEA* (~6 mM), was ligated with the recombinant H3 globular domain, **H3(14−135)/K14C** (~2 mM), in a denaturing buffer (6 M Gdn-HCl, pH 5) containing 2% (w/v) MESNa and 50 mM TCEP and under microwave irradiation. The HPLC analysis and the mass spectral data of the ligation reaction were shown in Figure 3 (top half). One can see that a significant amount of ligation product **H3/K14C** was formed after 1 h and that the yield reached about 55−65% after 16 h as estimated from HPLC analysis. Similarly, an H3 analog containing a methylated lysine at position 4, **H3/K4me**, was also prepared through ligation between **H3(1−13)/K4me**-*BMEA* and **H3(14−135)/K14C** (Figure 3, bottom half). The ligation reaction was conducted at 37 °C (no microwave) under otherwise identical conditions. As one can see from HPLC analysis, about 70% of the ligation product was formed after 2 days of reaction (Figure 3A′). MALDI-TOF MS analysis clearly confirmed the identity of the two purified ligation products (Figure 3B and B′). These results indicated that the BMEA method can also be applied to the synthesis of large polypeptide molecules such as the histone proteins. These synthetic histones are valuable reagents for studying histone epigenetics.[14] A preliminary bioassay showed that the synthetic H3 proteins were fully functional to form histone octamers with the other three core histone proteins (Supporting Information).

It is interesting to note that a very recent paper by Melnyk et al. described the same system for peptide ligation.[15] So our two teams have independently shown that a seemingly inert tertiary amide of the BMEA nature can undergo facile

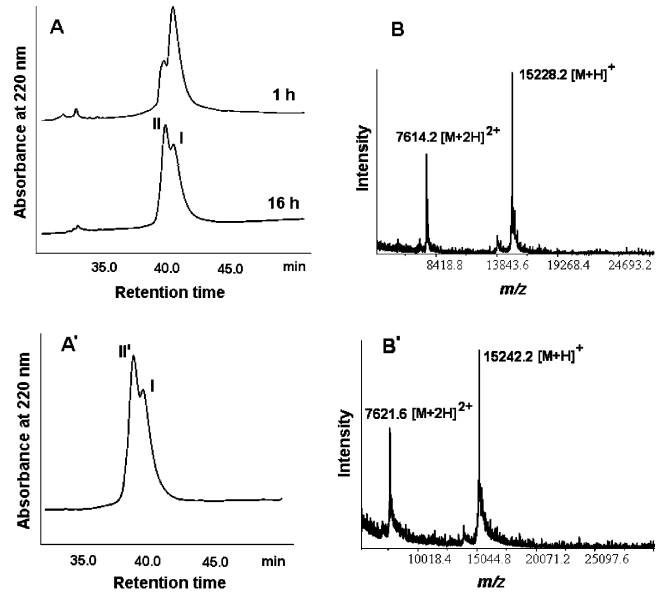

Figure 3. (Top half) Synthesis of histone **H3/K14C** through ligation between **H3(1−13)**-*BMEA* and **H3(14−135)/K14C** under microwave irradiation. (A) HPLC monitoring of the ligation reaction; (B) MALDI MS of the isolated ligation product H3/K14C (MW calcd 15227.7). (Bottom half) Synthesis of a histone H3 analog **H3/K4me-K14C** through ligation between **H3(1−13)/K4me**-*BMEA* and **H3(14−135)/K14C** at 37 °C. (A′) HPLC monitoring of the ligation reaction at 2 days; (B′) MALDI MS of the isolated ligation product (MW calcd 15241.7). Peak I, unreacted **H3(14−135)/K14C**; peak II or II′, ligation product. The *BMEA* peptide eluted out much earlier before the HPLC range shown here.

intramolecular thiolysis (or *N*-to-*S* acyl transfer) to form a thioester under very mild conditions. Although formed transiently, the thioester has a lifetime that is long enough to allow its capture for direct reaction with a cysteinyl peptide at weakly acidic (or neutral[15]) pH. Notably, we show that low-power microwave irradiation can significantly accelerate BMEA-mediated ligation. The reaction system is applicable to a wide range of C-terminal amino acid residues, pointing therefore to its broad utility. The successful synthesis of histone H3 proteins in our work further demonstrates the practical value of this BMEA methodology in synthetic protein chemistry. Compared to most other existing methods, this system does not need a preactivation step or an intrinsic driving device for the thioester formation reaction, making it one of the simplest and most straightforward Fmoc-based systems for the synthesis of thioester components for NCL.

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Supporting Information Available: Experimental procedures and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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