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Bis(2-sulfanylethyl)amido Peptides Enable Native Chemical Ligation at Proline and Minimize Deletion Side-Product Formation

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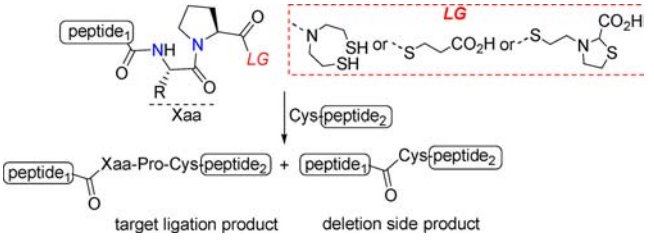
ABSTRACT



Native chemical ligation of C-terminal peptidyl prolyl alkylthioesters with N-terminal cysteinyl peptides usually exhibits poor kinetic rates compared to other C-terminal amino acid residues. It is shown here that the reaction is accompanied by the formation of a deletion side product which is minimized by using a bis(2-sulfanylethyl)amido (SEA) thioester surrogate at a mildly acidic pH.

Modern protein chemical synthesis usually involves the sequential ligation of unprotected peptide segments in aqueous solution.[1] Several chemoselective reactions enable the formation of a peptide bond between two unprotected peptide segments.[2] Among these reactions, native chemical ligation (NCL),[2a] which is based on the coupling of a C-terminal peptide thioester with an N-terminal cysteinyl peptide, is undoubtedly the most popular reaction for protein total synthesis.[3]

The extensive use of NCL for protein total synthesis over almost 20 years has shed some light on the scope and limitations of this reaction.[4] In particular, early studies identified leucine, threonine, valine, isoleucine, and proline as the slowest reacting C-terminal amino acid residues.[4a] Among these, proline displayed the slowest kinetic rates and poorest ligation yields (~10%) after 48 h. Recent studies suggest that the poor reactivity of peptidyl prolyl thioesters could be due to electronic effects rather than to steric effects. Indeed, in a trans Xaa-Pro bond, the π*-orbital of the prolyl thioester carbon can interact with the n-orbital of the preceding $^{\alpha}$N carbonyl oxygen.[5] This interaction might reduce the electrophilicity and accessibility of prolyl thioesters and, thus, explain the difficulty of forming a Pro-Cys bond using the NCL reaction.

Besides the work of Alewood and co-workers on C-terminal peptidyl prolyl selenoesters,[6] previous studies in the field examined mainly the reactivity of peptidyl prolyl thioesters derived from simple alkyl thiols such as 3-mercaptopropionic acid (MPA). We thus explored the

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(5) Such an interaction is significant for Xaa-Pro bonds in proteins; see: Bartlett, G. J.; Choudhary, A.; Raines, R. T.; Woolfson, D. N. *Nat. Chem. Biol.* **2010**, *6*, 615–620.

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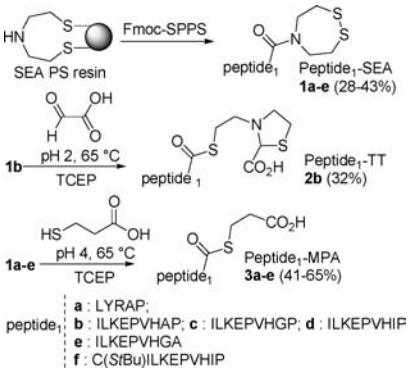
reactivity of other types prolyl thioesters in NCL reactions with the aim to solve this difficult problem.

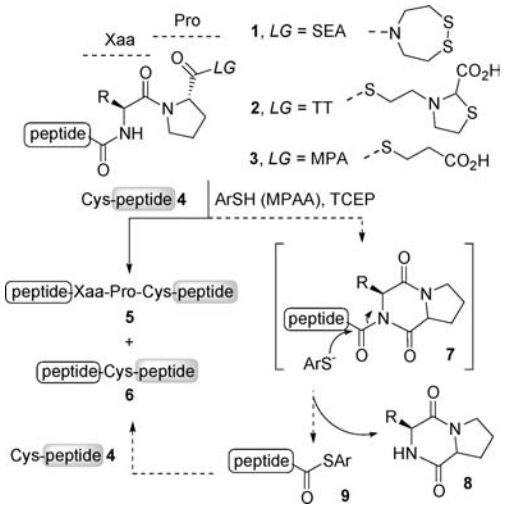
In this study we examined the reactivity of bis(2-sulfanylethyl)amido (SEA)[7] peptides **1**, thiazolidine thioester (TT) peptides[8] **2**, and MPA-derived peptide thioesters **3** (Scheme 1). Peptides **1**–**3** were produced starting from SEA polystyrene resin using Fmoc-SPPS (Scheme 1). Previous work showed that SEA peptides **1** featuring a C-terminal residue other than proline could be converted efficiently into thiazolidine thioester peptides[8] or MPA thioesters[9] at 37 °C using an excess of MPA or glyoxylic acid respectively at acidic pH. However, these conditions were unsuccessful with proline as the C-terminal residue. We reasoned that this lack of reactivity might be due to a slow *N,S*-acyl shift of the prolyl SEA group at 37 °C compared to other amino acid residues. Interestingly, heating the mixture at 65 °C to accelerate *N,S*-acyl shift solved the problem and cleanly yielded thiazolidine thioester peptide **2b** or MPA thioesters **3a**–**d** in good yield.

Scheme 1. Synthesis of Peptide Prolyl Thioesters **1**–**3**



We first examined the ligation of MPA thioesters **3** with N-terminal cysteinyl peptides **4** since no detailed kinetic data are available in the literature for the NCL reaction with peptidyl prolyl alkylthioesters (Scheme 2). The use of standard conditions for the NCL reaction yielded the target peptide **5** but also unexpectedly two amino acid deletion side-product **6** sometimes in significant amounts (Scheme 2). In particular, formation of peptide LYRCFRANK **6a** (~8.8% by HPLC; see Figure S8 in the Supporting Information) was observed with model thioester LYRAP-MPA **3a** in reaction with CFRANK **4a** (Table 1, entry 1). Similar kinetic rates and the amount of deletion side-product ILKEPVHCILKEPVHGV-NH_2 **6b** were observed during the ligation of thioester peptide ILKEPVHAP-MPA **3b** with Cys peptide CILKEPVHGV-NH_2 **4b**, showing little influence of the peptide length or composition on the occurrence of this side reaction (Table 1, compare entries 1 and 3; see also Figure 1).

Scheme 2. NCL with Peptide Prolyl Thioesters **1**–**3**



The formation of deletion side-product **6** might proceed through the transient peptidyl diketopiperazine intermediate **7** (Scheme 2). Displacement of the diketopiperazine moiety by 4-mercaptophenylacetic acid (MPAA) used in excess during the NCL reaction might furnish the arylthioester **9**, which is expected to react with Cys peptide **4** to yield the deletion side-product **6**. This mechanism is supported by (i) the detection of the diketopiperazine **8** by LC-MS in the ligation mixtures (see Figures S5, S6 in the Supporting Information), (ii) the ability of imides of type **7** to react with thiols,[10] and (iii) the propensity of Xaa-Pro dipeptidyl esters to form diketopiperazines.[11]

The other experiments described in Table 1 show that with MPA thioesters **3** the formation of the deletion side-product **6** (i) decreases with the bulkiness of the Xaa residue (Table 1, entry 3 for Ala, entry 9 for Gly, entry 11 for Ile), Gly being particularly prone to deletion side-product **6** formation, and (ii) increases with the pH of the reaction since the amount of side-product **6b** formed at pH 7.8 is twice those observed at pH 6.8 (Table 1, entries 2–4).

We next examined the reactivity of SEA peptides **1b**–**d** (Xaa = Ala, Gly, Ile). The study of the effect of the pH on the rate of ligation of prolyl SEA peptide **1b** with Cys peptide **4b** reveals that SEA ligation is *accelerated* significantly by decreasing the pH from 7.1 to 5.5 (Figure 1d).[12] We thus reasoned that decreasing the pH of the ligation with prolyl SEA peptides would increase the rate of the

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(12) Decreasing the pH from 7.1 to 5.5 accelerated also the ligation of peptide ILKEPVHGA-SEA **1e** with Cys peptide **4b**, but reduced the rate of NCL reaction between peptide ILKEPVHGA-MPA **3e** and **4b** (see Figure S9 in the Supporting Information).

Table 1. Kinetic Data for the Ligation of Peptidyl Prolyl Thioesters **1**–**3**[a]

entry		peptide prolyl thioester	Cys peptide$_2$	temp (°C)	pH	HPLC yield for **5** (%)	HPLC yield for **6** (%)	ligation product **5**: $t^{1/2}$ (h)	deletion product **6**: $t^{1/2}$ (h)
1	**3a**	LYRAP-MPA	**4a**: CFRANK	37	7.1	67.6	8.8	**5a**: 20.2	**6a**: 25.7
2	**3b**	ILKEPVHAP-MPA	**4b**: CILKEPVHGV-NH_2	37	6.8	65	4.2	**5b**:17.1	**6b**: 14
3	**3b**	ILKEPVHAP-MPA	**4b**	37	7.1	60	6.8	**5b**: 16.9	**6b**: 16.1
4	**3b**	ILKEPVHAP-MPA	**4b**	37	7.8	66	8.6	**5b:** 18.3	**6b**: 12.8
5	**3b**	ILKEPVHAP-MPA	**4b**	65	5.5	62.6	16.9	**5b**: nd[c]	**6b**: nd
6	**3b**	ILKEPVHAP-MPA	**4b**	65	7.1	28	48	**5b**: nd	**6b**: nd
7	**2b**	ILKEPVHAP- TTX	**4b**	37	7.1	68.4	3.25	**5b**: 2.84	**6b:** 2.63
8	**1b**	ILKEPVHAP- SEA	**4b**	65	5.5	76.5 (36[b])	3.3	**5b**: 12.4	**6b**: 19.8
9	**3c**	ILKEPVHGP-MPA	**4b**	37	7.1	38	22.4	**5c**: nd	**6b**: nd
10	**1c**	ILKEPVHGP- SEA	**4b**	65	5.5	63.7	15	**5c**: nd	**6b**: nd
11	**3d**	ILKEPVHIP-MPA[d]	**4b**	37	7.2	75.2	<1	**5d**: 15.2	**6b**: nd
12	**1d**	ILKEPVHIP- SEA[d]	**4b**	65	5.5	78.3	<1	**5d**: 12.7	**6b**: nd
13	**1d**	ILKEPVHIP- SEA[d]	**4a**	65	5.5	85.8 (42[b,d])	<1	**5e**: nd	**6c**: nd[e]

[a] All the ligation reactions showed typical first-order kinetic profiles (see Figure 1). [b] HPLC purified. [c] nd: not determined. [d] D-Pro content: 0.19% for **1d**, 0.58% for **3d**, and 1.1% for peptide ILKEPVHIPCFRANK **5e** (see Supporting Information). [e] **6c** corresponds to peptide ILKEPVHCFRANK.

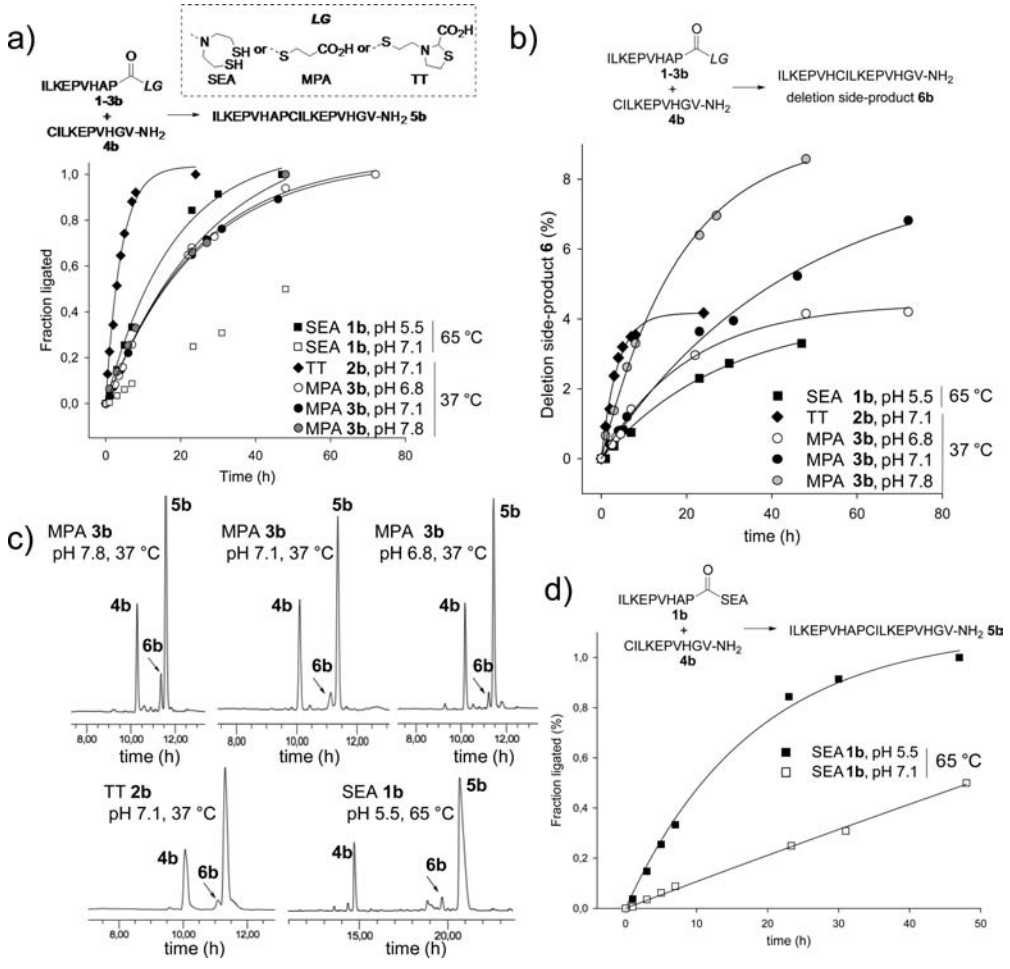


Figure 1. Reaction of SEA peptide **1b**, TT peptide **2b**, or MPA peptide **3b** with Cys peptide **4b**. (a, b, d) Kinetic data (HPLC, UV detection at 215 nm). The data were modeled using the first-order rate law (continuous curves); see also Figure S7 in the Supporting Information. (c) HPLC profiles (C18 column, UV detection at 215 nm).

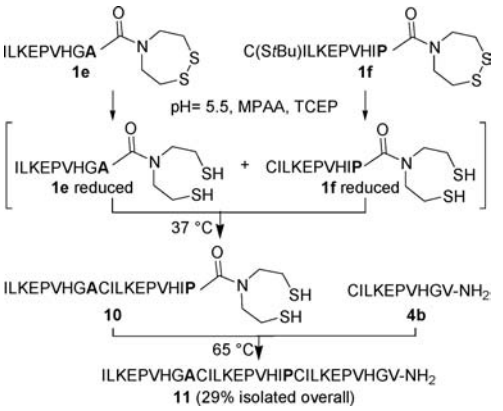
ligation, while minimizing the base-catalyzed process leading to deletion side-product **6** formation. Since ligation of prolyl SEA peptides **1** with Cys peptide **4** showed poor kinetic rates at 37 °C, the ligations were performed at 65 °C to accelerate the *N,S*-acyl shift process as in the case for the synthesis of TT and MPA thioesters **2** and **3**. Satisfactorily, the reaction of prolyl SEA peptides **1b,c** (Xaa = Ala, Gly) with Cys peptide **4b** resulted in higher ligation yields and lower deletion side-product levels in comparison with MPA analogs **3b,c** (Table 1, compare entries 3,9 and 8,10). The data in Table 1 show that, as in the case for MPA thioesters **3**, the amount of deletion side-product **6** with prolyl SEA peptides **1** increased in the order Ile < Ala < Gly.

In a control experiment, we reacted MPA thioester **3b** with Cys peptide **4b** at pH 5.5 or 7.1 at 65 °C, the temperature used for prolyl SEA peptides. At pH 7.1, the deletion side-product **6b** (48%) dominated over the ligation product **5b** (28%, Table 1, entry 6). At pH 5.5, it still represented a 17% yield (Table 1, entry 5) compared to only a 3.3% yield with the SEA peptide analog **1b** (Table 1, entry 8). Overall, these data show that SEA peptides **1** are significantly more resistant to deletion side-product formation than MPA thioesters **3** and therefore constitute a potential alternative to the formation of Pro-Cys peptide bonds.

Finally, the NCL reaction of thiazolidine thioester peptide **2b** with Cys peptide **4b** proceeded 6 times faster ($t^{1/2}$ 2.8 h) than for MPA thioester **3b** ($t^{1/2}$ 16.9 h). The fact that the yield of the deletion side-product was only 3.3% suggests that thioesters of type **2** are also potentially useful derivatives for the formation of Pro-Cys peptide bonds.

In the last part of this work, we sought to exploit the fact that prolyl SEA peptides are slow reacting systems at 37 °C in comparison with other C-terminal residues for designing a kinetically controlled one-pot three-segment assembly process (Scheme 3).[13] The first ligation step between alanyl SEA peptide **1e** and Cys peptide **1f** was performed at 37 °C and cleanly yielded prolyl SEA peptide **10** (see Figure S11 in the Supporting Information). In particular, cyclization of peptide segment **1f** was not observed. Then, the third

Scheme 3. SEA Kinetically Controled Ligation



Cys peptide segment **4b** was added to the mixture and the temperature was raised to 65 °C to trigger the second ligation step and the formation of target peptide **11** (29% overall yield).

In conclusion, we report here that a deletion side product is formed during the NCL reaction of peptidyl prolyl thioesters with cysteinyl peptides. This side reaction is significant for peptide thioesters derived from MPA which are often used in the field of protein total synthesis. It can be minimized with MPA thioesters by lowering the pH down to 6.8. The level of side-product formation and the rate of ligation vary also significantly with the peptide thioester structure. Thiazolidine thioester peptides are significantly more reactive then MPA thioesters at 37 °C, but significantly less susceptible to deletion side-product formation. Prolyl SEA peptides are slow reacting systems at 37 °C but ligate efficiently at pH 5.5 and 65 °C. In this case, the level of deletion side product is reduced compared to MPA-derived thioesters. The dependence of prolyl SEA peptide reactivity on the temperature opens novel synthetic opportunities as illustrated by the design of a kinetically controlled one-pot three-segment assembly process.

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

The authors declare no competing financial interest.

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