ORGANIC LETTERS

2005
Vol. 7, No. 22
5003−5006

Peptide Ligation by a Reversible and Reusable C-Terminal Thiol Handle

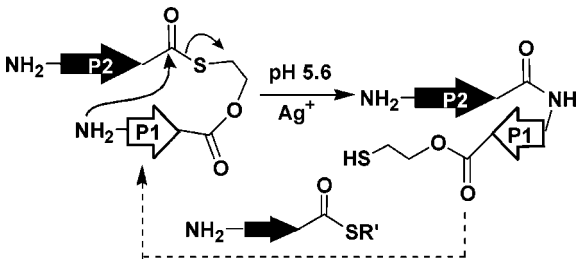
Yi-An Lu[†] and James P. Tam*[,†,‡]

Department of Biomedical Research, The Scripps Research Institute, Jupiter, Florida 33458, and School of Biological Sciences, Nanyang Technological University, Singapore 637551

jamestam@scripps.edu

Received August 18, 2005

ABSTRACT



Peptide ligation of noncysteinyl residues can be achieved conveniently by a reversible C-terminal thiol handle together with a Ag^+ ion-assisted S,N-acyl migration. The regenerated C-terminal handle permits tandem ligation of multiple segment.

Native or cysteine (Cys) ligation affording a cysteine at the ligation site has been widely employed to synthesize peptides and proteins.[1−6] An advantage of this method is its use of unprotected peptides as building blocks. However, the presence of an N-terminal (NT) Cys in the ligating pair is essential. In recent years, NT-Cys mimetics[7−9] and azido acids (Staudinger ligation)[10−13] have successfully extended the repertoire of peptide ligation for noncysteinyl ligation.

A common design of NT-Cys mimetics uses a reversible thiol handle attached to the α-amine as an NT-thiol,[7−9] mimicking an N-terminal Cys (Figure 1). Similar to Cys

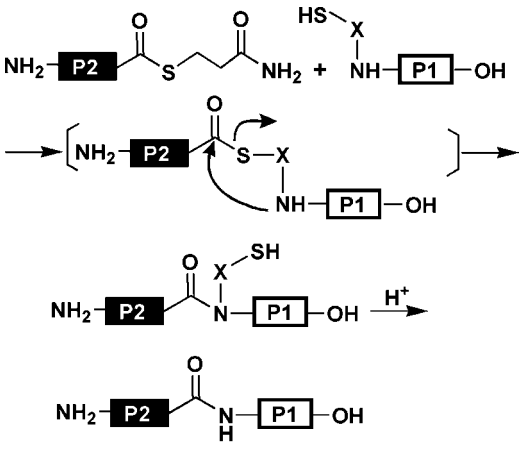


Figure 1. General scheme of N-terminal Cys-mimetic ligation.

[†] Scripps Florida.
[‡] Nanyang Technological University.

(1) Tam, J. P.; Lu, Y.-A.; Liu, C.-F.; Shao, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12485−12489.
(2) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779.
(3) Eom, K. D.; Miao, Z.; Yang, J.-L.; Tam, J. P. *J. Am. Chem. Soc.* **2003**, *125*, 73−82.
(4) Tam, J. P.; Lu, Y.-A. *Protein Sci.* **1998**, *7*, 1583−1592.
(5) Kochendoerfer, G. G.; Chen, S.-Y.; Mao, F.; Cressman, S.; Traviglia, S.; Shao, H.; Hunter, C. L.; Low, D. W.; Cagle, E. N.; Carnevail, M.; Gueriguian, V.; Keogh, P. J.; Porter, H.; Stratton, S. M.; Wiedeke, M. C.; Wilken, J.; Tang, J.; Levy, J. J.; Miranda, L. P.; Crnogorac, M. M.; Kalbag, S.; Botti, P.; Schindler-Horvat, J.; Savatski, L.; Adamson, J. W.; Kung, A.; Kent, S. B. H.; Bradburne, J. A. *Science* **2003**, *299*, 884−887.
(6) Muir, T. W. *Annu. Rev. Biochem.* **2003**, *72*, 249−289.
(7) Canne, L. E.; Bark, S. J.; Kent, S. B. H. *J. Am. Chem. Soc.* **1996**, *118*, 5891−5896.
(8) Offer, J.; Dawson, P. E. *Org. Lett.* **2000**, *2*, 23−26.
(9) Botti, P.; Carrasco, M. R.; Kent, S. B. H. *Tetrahedron Lett.* **2001**, *42*, 1831−1833.

10.1021/ol052000f CCC: $30.25
Published on Web 09/27/2005

ligation, NT-Cys ligation proceeds by a two-step reaction: a thiol-thioester exchange reaction by the NT-thiol to form a branched thioester linking both segments to enable an intramolecular S,N-acyl migration to form an amide bond.[1,2] The thiol handle is then removed, usually under acidic conditions. Apart from the synthetic challenges in preparing NT-Cys mimetics, NT-thiol handles are not generally reusable. Here we describe the use of C-terminal mercaptoethyl ester as a reversible and reusable thiol handle for peptide ligation of noncysteinyl residues. For comparison, we also prepared the corresponding mercaptopropyl ester as a thiol handle. Our ligation scheme (Figure 2) makes use of the

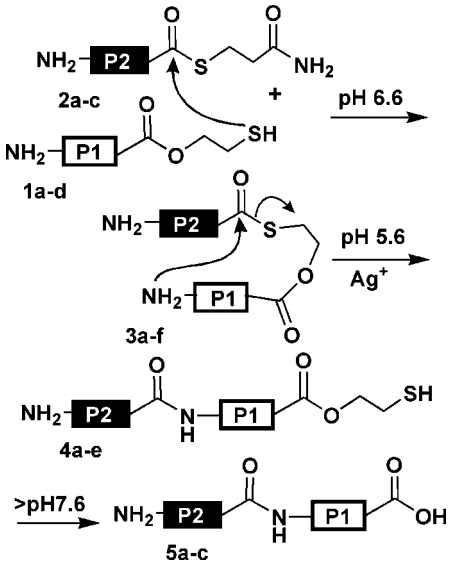


Figure 2. General scheme of C-terminal thiol ligation using unprotected peptides in aqueous solution. Peptide sequence shown in Table 1.

C-terminal thiol from mercaptoethyl ester **1** for thiol-thioester exchange reaction with thioester **2**, affording a single-chain diester, **3**. After activation by AgTfa (silver trifluoroacetate), diester **3** then undergoes a S,N-acyl migration to form a peptide bond and concurrently regenerates the C-terminal mercaptoethyl ester **4**, which could be used for another ligation with a new thioester or removed to afford peptide **5** with a C-terminal carboxylic acid. Removal of β-mercaptoethyl ester under mildly basic conditions is facile as a result of the anchimeric assistance of its terminal thiol.

Our scheme required two types of unprotected peptide esters, mercaptoethyl ester **1** and thioester **2**. Both were assembled by a stepwise solid-phase method[14] using an in situ neutralization and benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate (BOP) activation protocol.[15]

Peptide mercaptoethyl esters **1a**−**c** were prepared by the base-labile 9-fluorenyl-methoxycarbonyl (Fmoc) chemistry on mercaptoethyl chlorotrityl resin (Figure 3) that was

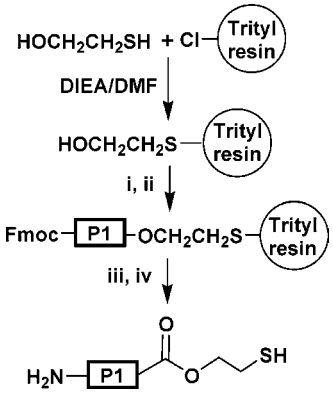


Figure 3. Scheme for preparing C-terminal mercaptoethyl ester peptides **1a**−**d**. Trityl resin: 2-chlorotrityl chloride resin. (i) Fmoc-amino acid, DCC/DMAP/DMF, (ii) *n* cycles of Fmoc-amino acid, BOP/DIEA/DMF, (iii) 20% piperidine/DMF, (iv) TFA, room temperature, 1 h.

generated on-resin by a displacement reaction to the commercially available 2-chlorotrityl chloride resin[16] using a 15-fold excess of mercaptoethanol/diisopropylethylamine (ME/DIEA) in dried dimethylformamide (DMF) for 24 h. Mercaptopropyl ester **1d** was also prepared similarly on a mercaptopropyl-chlorotrityl resin. The first Fmoc-amino acid was attached on resin by dicyclohexylcarbodiimide (DCC) and a catalytic amount of (dimethylamino)pyridine (DMAP) in DMF. Subsequent amino acids were coupled on-resin by Fmoc chemistry using BOP/DIEA protocol to afford peptide mercaptoethyl esters **1a**−**c** or mercaptopropyl ester **1d** in >50% yields after cleavage from resin by TFA and HPLC purification (Table 1). Attempts to prepare mercaptoethyl ester **1a** by Boc chemistry on the hydrogen fluoride (HF)-labile mercaptoethyl-benzyl resin (prepared by a 15-fold excess of ME/DIEA in DMF for 24 h on a commercially available benzyl chloride resin) were unsuccessful because the mercaptoethyl ester handle released from the HF-cleavage reaction was unstable to HF.

For peptide thioesters **2a**−**c**, acid-labile *tert*-butyloxycarbonyl (Boc)-benzyl chemistry (Figure 4) was used, starting with a Boc-amino acid thioester resin.[17,18] For reasons that will be discussed later, we used longer peptide thioesters **2a**−**c** (10 and 16 residues) compared to mercaptoethyl ester

(10) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007−2010.
(11) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R. *Org. Lett.* **2000**, *2*, 2141−2143.
(12) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2000**, *2*, 1939−1941.
(13) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2001**, *3*, 9−12.
(14) Merrifield, R. B. *Science* **1986**, *57*, 1619−1625.
(15) Castro, B.; Dormoy, J. R.; Evin, G.; Selve, C. *Tetrahedron Lett.* **1975**, *14*, 1219−1222.
(16) Barlos, K.; Gatos, D.; Kallitsis, J.; Papaphotiu, G.; Sotirus, P.; Yao, W.; Schafer, W. *Tetrahedron Lett.* **1989**, *30*, 3943−3946.
(17) Hojo, H.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 111−117.
(18) Zhang, L. S.; Tam, J. P. *J. Am. Chem. Soc.* **1999**, *121*, 3311−3320.

Table 1. Sequences of Peptide **1**−**5**

	sequences
	Peptide Mercaptoethyland Mercaptopropyl Ester **1**
1a	**AEVSYG**-*OCH_2CH_2-SH*
1b	**SAEVSYG**-*OCH_2CH_2-SH*
1c	**GYGGFLG**-*OCH_2CH_2-SH*
1d	**GYGGFLG**-*$OCH_2CH_2CH_2$-SH*
	Peptide Thioester (-$SCH_2CH_2CONH_2$) **2**
2a	**Ac-GASLRRSFGG**
2b	**FTQGVGNPVG**
2c	**VPDTYIGASLRRSFGG**
	Peptide Diester **3**
3a	**Ac-GASLRRSFGG**-*SCH_2CH_2O*-**GYSVEA**[a]
3b	**Ac-GASLRRSFGG**-*SCH_2CH_2O*-***GYSVEAS***[a]
3c	**Ac-GASLRRSFGG**-*SCH_2CH_2O*-***GLFGGYG***[a]
3d	**FTQGVGNPVG**-*SCH_2CH_2O*-***GLFGGYG***[a]
3e	**FTQGVGNPVG**-*$SCH_2CH_2CH_2O$*-***GLFGGYG***[a]
3f	**VPDTYIGASLRRSFGG**-*SCH_2CH_2O*-***GLFGGYG***[a]
	Ligated Peptide Mercaptoethyl and Mercaptopropyl Ester **4**
4a	**Ac-GASLRRSFGG-AEVSYG**-*OCH_2CH_2SH*
4b	**Ac-GASLRRSFGG-SAEVSYG**-*OCH_2CH_2SH*
4c	**Ac-GASLRRSFGG-GYGGFLG**-*OCH_2CH_2SH*
4d	**FTQGVGNPVG-GYGGFLG**-*OCH_2CH_2SH*
4e	**FTQGVGNPVG-GYGGFLG**-*$OCH_2CH_2CH_2SH$*
	Ligation Peptide-COOH **5**
5a	**Ac-GASLRRSFGG-AEVSYG**
5b	**Ac-GASLRRSFGG-SAEVSYG**
5c	**FTQGVGNPVG-GYGGFLG**

[a] Peptide chain from C to N with N^{α}-amine.

1a−**c** (6−8 residues) or mercaptopropyl ester **1d** in model ligation reactions. All peptides were confirmed by MS (SI S1).

To initiate peptide ligation, thiol-thioester exchange to form diesters **3a**−**f** by mercaptoethyl or mercaptopropyl ester **1** (1.2 equiv) and thioester **2** (1 equiv) was performed in an aqueous solution buffered by sodium phosphate at pH 6.6. The exchange reaction was >85% complete in 2−3 h as monitored by C_{18} reverse-phase HPLC. Mercaptoethyl esters

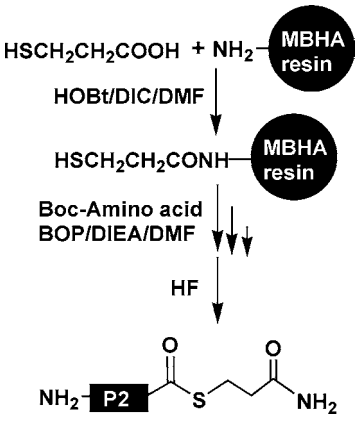


Figure 4. Scheme for preparing peptide thioesters **2a**−**c**.

1a−**c** were susceptible to hydrolysis, and product analysis of the reaction mixture by HPLC from pH 5.6 to 7.6 showed that pH 6.6 was suitable. At pH >7, mercaptoethyl esters **1a**−**c** were hydrolyzed at a rate >10%, whereas the thiol-thioester exchange reaction was sluggish at pH <6. In contrast, mercaptopropyl ester **1d** was less susceptible than mercaptoethyl ester **1a**−**c** to hydrolysis, and the exchange reaction with thioester **2b** could be performed at pH 7 to afford diester **3e** in 90% yield. Once formed, both S- and O-esters in **3a**−**f** were relatively stable to the reaction conditions and for purification under acidic conditions. Diesters **3a**−**f** were purified by HPLC, and their identities were confirmed by MS prior to the Ag^+-mediated S,N-acyl rearrangement (SI S2, S3).

Enthalpic activation of thioesters by Ag^+ ion has been used effectively for intra- and intermolecular aminolysis, as well as macrocyclization.[19,20] Previously, we have successfully exploited Ag^+-assisted cyclization of peptide thioester through long-range S,N-acyl migrations.[21] For transforming diester **3** to peptide product **4**, we envisioned that activation by a Ag^+ complex in bridging the N^{α}-amine and diester linker could favor an intra- rather than intermolecular acylation. Indeed, purified diesters **3a**−**f** underwent S,N-acyl migration to form **4** with AgTfa (3−5 equiv) at pH 5.6 buffered by sodium acetate. However, the S,N-acyl migration was slow. After 20−30 h, peptides **4a**−**e** were obtained in 30−45% yield. HPLC and MS analysis of the reaction mixture of **3a** after 20 h showed three major products: the ligated peptide **4a** (43%), the starting material **3a** (15%), and hydrolyzed thioester **2** to the corresponding free carboxylic acid (17%).

Diesters **3** contains two N^{α}-amines that can give, after S,N-acyl migration, a cyclic peptide (derived from thioester **2**) as side product or **4** as the desired ligation product. To deter cyclization, we examined three factors to gain selectivity for intramolecular acylation of the desired N^{α}-amine on the mercaptoethyl ester chain over the N^{α}-amine on the thioester chain in diester **3**. First, a protecting group was placed on the N-terminal of peptide thioester **2a** to avoid cyclization side reaction. Although a reversible protecting group on N^{α}-amine would be desirable, we selected N^{α}-acetyl for the peptide thioester **2a**. Indeed, no cyclic peptides of **1a**−**1c** were found in the product mixtures of **3a**−**c**. Second, we used longer peptide thioesters **2b,c** compared to mercapto-ethyl ester **1a**−**c**. In the diester form **3d**−**f**, entropy favors ligation of the shorter-length peptide **1** rather than cyclization of the longer-length peptide **2**. Finally, we considered the affinity of Ag^+ ion by the respective N-terminal amino acids of peptides **1a**−**d** and **2b,c**. Previously, we have found a 20-fold faster cyclization rate of four amino acids, Gly, Ser, Asn, and His, compared to other amino acids.[18] Thus, the precedent for selectivity for N-terminal amino acids in the Ag^+-assisted S,N-acyl migration provides guidance for selecting N-terminal amino acids for the present ligation scheme. Under the proposed ligation reaction conditions, no

(19) Blake, J.; Li, C. H. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4055−4058.
(20) Hojo, H.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 3055−3063.
(21) Zhang, L. S.; Tam, J. P. *Tetrahedron Lett.* **1997**, *38*, 4375−4378.

cyclic peptide, c(FTQGVGNPVG) was foundon the basis of the HPLC profile of synthetic cyclic peptide of **2b**.

Finally, the ligated mercaptoethyl esters **4a,b** were converted quantitatively to **5a,b** with a free carboxylic acid under basic conditions at pH 8.0 with sodium phosphate buffer (Table 2). Hydrolytic removal of the mercaptoethyl ester handle of **4a,b** was pH-dependent in the tested range of pH 6.6−8.0. At pH 6.6, <10% yield of **5a,b** was obtained after 60 h. The yield increased to 90% at pH 7.6 and the reaction time could be shortened substantially at pH >8. In contrast, removal of the mercaptopropyl ester handle in **4e** to **5c** required pH >9 for satisfactory results.

Table 2. HPLC and MS Data for **5a**−**c**

	HPLC t_R (min)	MS $[M + H]^+$	calcd
5a	18.41[a]	1655.82	1655.73
5b	18.68[a]	1672.34	1671.35
5c	20.92[b]	1626.83	1626.34

[a] HPLC using linear gradient 0−85% B/30 min. [b] HPLC using linear gradient 10−90% B/30 min.

In the present work, we selected ligation sites containing two small amino acids to produce Gly-Gly, Gly-Ala, and Gly-Ser in **4a**−**e**. The C-terminal Gly in thioester **2** avoids racemization that may accompanyAg$^+$ ion-assisted rearrangement. The choice of N$^\alpha$-Gly, Ala, and Ser at the N-terminal of mercaptoethyl esters **1a**−**c** favors ligation over cyclization by a steric-hindered N$^\alpha$-amino acid on thioester **2b,c**. Our proposed method may not be compatible with Cys-containing peptide segments because of the strong affinity of cysteinyl thiol toward Ag$^+$ ion. Previously, we have found that the S,N-acyl rearrangement mediated by Ag$^+$ ion is compatible with peptide segments containing unprotected Lys when N-terminal Gly, Ser, or Asn is used because the large pK_a difference between the N$^\alpha$- and N$^\epsilon$-amines. However, this and other aspects regarding compatible ligation sites need further exploration.

In summary, we show a peptide ligation scheme using a reversible C-terminal thiol handle that is convenient because the building blocks, peptide ester **1** and thioester **2**, can be directly prepared by a solid-phase method. The reusable feature of the C-terminal thiol handle also holds promise for tandem ligation of multiple segments.

Acknowledgment. This work was supported in part by NIH EB 001986.

Supporting Information Available: Additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

OL052000F