ORGANIC LETTERS

2010
Vol. 12, No. 22
5238−5241

Bis(2-sulfanylethyl)amino Native Peptide Ligation

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Received September 22, 2010

ABSTRACT

The reaction of a peptide featuring a bis(2-sulfanylethyl)amino (SEA) group on its C-terminus with a cysteinyl peptide in water at pH 7 and 37 °C leads to the chemoselective and regioselective formation of a native peptide bond. This method called SEA ligation enriches the native peptide ligation repertoire available to the peptide chemist. Preparation of an innovative solid support which allows the straightforward synthesis of peptide SEA fragments using standard Fmoc/*tert*-butyl solid phase peptide synthesis procedures is also described.

Peptide ligation methods such as native chemical ligation (NCL),[1] Staudinger ligation,[2] or the decarboxylative condensation of *N*-alkylhydroxylamines and α-ketoacids[3] lead to the formation of a native peptide bond at the ligation site.[4] In particular, NCL is a powerful method for synthesizing native or modified proteins of moderate size (∼150 amino acids). NCL is based on the reaction of a peptide thioester with a cysteinyl peptide. A first transthioesterification step is followed by an intramolecular *S*,*N*-acyl shift that results in the formation of a native X−Cys peptide bond. Thiol amino acid derivatives combined with a methylation, desulfurization, or saponification step[5] or the use of N-linked thiol-containing removable auxiliaries[6] were used to extend the principle of NCL to sites other than Cys residues.

Recently, the use of the reverse *N*,*S*-acyl shift has emerged as a promising strategy for peptide thioester synthesis or for designing novel native ligation methods relying on the in situ generation of peptide thioesters. The key idea within these approaches is to facilitate peptide thioester synthesis[7] or find alternatives to these useful peptide derivatives.[8] For example, the elegant work of Aimoto et al. showed the

(1) (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776. (b) Hackeng, T. L.; Griffin, J. H.; Dawson, P. E. *Proc. Natl. Acad. Sci.* **1999**, *96*, 10068.

(2) (a) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007. (b) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2000**, *2*, 1939. (c) Nilsson, B. L.; Hondal, R. J.; Soellner, M. B.; Raines, R. T. *J. Am. Chem. Soc.* **2003**, *125*, 5268.

(3) Bode, J. W.; Fox, R. M.; Baucom, K. D. *Angew. Chem., Int. Ed. Engl.* **2006**, *45*, 1248.

(4) For a recent review: Hackenberger, C. P.; Schwarzer, D. *Angew. Chem., Int. Ed. Engl.* **2008**, *47*, 10030.

(5) Selenocysteine: (a) Gieselman, M. D.; Xie, L.; van Der Donk, W. A. *Org. Lett.* **2001**, *3*, 1331. (b) Quaderer, R.; Hilvert, D. *Chem. Commun. (Cambridge, U. K.).* **2002**, *21*, 2620. Methionine: Tam, J. P.; Yu, Q. *Biopolymer (Pept. Sci.)* **1998**, *46*, 319. Selenomethionine: Roelfes, G.; Hilvert, D. *Angew. Chem., Int. Ed.* **2003**, *42*, 2275. Recent review on desulfurization-based ligation methods: Rohde, H.; Seitz, O. *Biopolymers* **2010**, *94*, 551. Alanine: Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526. (g) Wan, Q.; Danishefsky, S. J. *Angew. Chem., Int. Ed. Engl.* **2007**, *46*, 9248. (h) Phenylalanine: Crich, D.; Banerjee, A. *J. Am. Chem. Soc.* **2007**, *129*, 10064. Valine: Haase, C.; Rohde, H.; Seitz, O. *Angew. Chem., Int. Ed. Engl.* **2008**, *47*, 6807. Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J.; Danishefsky, S. J. *Angew. Chem., Int. Ed. Engl.* **2008**, *47*, 8521. Leucine: Harpaz, Z.; Siman, P.; Kumar, K. S.; Brik, A. *ChemBioChem* **2010**, *11*, 1232. Glycopeptide synthesis: Brik, A.; Yang, Y. Y.; Ficht, S.; Wong, C. H. *J. Am. Chem. Soc.* **2006**, *128*, 5626. Side-chain auxiliaries for Asp, Glu, and Ser: Lutsky, M. Y.; Nepomniaschiy, N.; Brik, A. *Chem. Commun. (Cambridge, U. K.)* **2008**, *10*, 1229. Kumar, K. S.; Harpaz, Z.; Haj-Yahya, M.; Brik, A. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3870. Hojo, H.; Ozawa, C.; Katayama, H.; Ueki, A.; Nakahara, Y.; Nakahara, Y. *Angew. Chem., Int. Ed. Engl.* **2010**, *49*, 5318.

(6) (a) Canne, L. E.; Bark, S. J.; Kent, S. B. H. *J. Am. Chem. Soc.* **1996**, *118*, 5891. (b) Offer, J.; Dawson, P. E. *Org. Lett.* **2000**, *2*, 23. (c) Botti, P.; Carrasco, M. R.; Kent, S. B. H. *Tetrahedron Lett.* **2001**, *42*, 1831.

(7) (a) Hojo, H.; Onuma, Y.; Akimoto, Y.; Nakahara, Y.; Nakahara, Y. *Tetrahedron Lett.* **2007**, *48*, 25. (b) Nakamura, K.; Mori, H.; Kawakami, T.; Hojo, H.; Nakahara, Y.; Aimoto, S. *Int. J. Pept. Res. Ther.* **2007**, *13*, 191. (c) Tsuda, S.; Shigenaga, A.; Bando, K.; Otaka, A. *Org. Lett.* **2009**, *11*, 823.

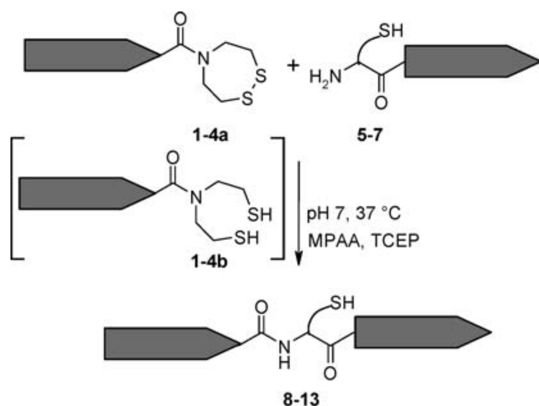
(8) (a) Kawakami, T.; Aimoto, S. *Chem. Lett.* **2007**, *36*, 76. (b) Kawakami, T.; Aimoto, S. *Adv. Exp. Med. Biol.* **2009**, *611*, 117. (c) Kawakami, T.; Aimoto, S. *Tetrahedron* **2009**, *65*, 3871. (d) Kawakami, T.; Aimoto, S. *Tetrahedron Lett.* **2007**, *48*, 1903.

10.1021/ol102273u © 2010 American Chemical Society
Published on Web 10/21/2010

capacity of cysteinyl prolyl ester peptides to form in situ peptide thioesters through an intramolecular *N*,*S*-acyl shift.[8]

We describe hereinafter the chemoselective and regioselective reaction of a peptide featuring a bis(2-sulfanylethyl)amino (SEA) group on its C-terminus with a cysteinyl or homocysteinyl peptide in the presence of 4-mercaptophenylacetic acid[9] (MPAA) and tris(2-carboxyethyl)phosphine (TCEP). This reaction occurs in water at pH 7 and leads to the formation of a native peptide bond in good yield (Scheme 1).

Scheme 1. SEA Ligation between a Bis(2-sulfanylethyl)amino Peptide **1−4b** and a Cysteinyl or Homocysteinyl Peptide Leading to the Chemoselective and Regioselective Formation of a Native Peptide Bond[a]

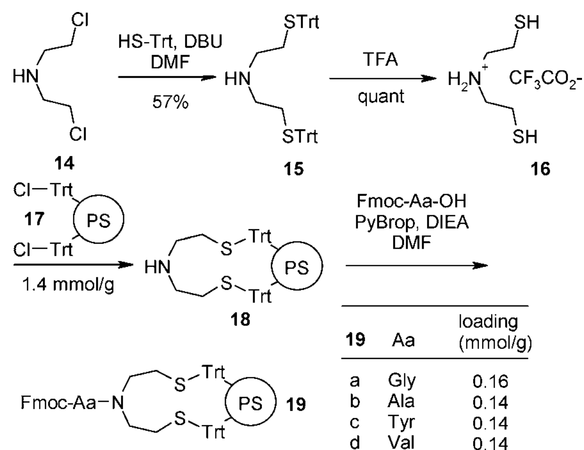


peptide	sequence
1	H-ILKEPVHG**G**-
2	H-ILKEPVHG**A**-
3	H-ILKEPVHG**Y**-
4	H-ILKEPVHG**V**-
5	H-**C**ILKEPVHGV-NH_2
6	H-**Hcy**ILKEPVHGA-NH_2
7	H-**C**ILKEPCVHGV-NH_2
8	H-ILKEPVHG**GC**ILKEPVHGV-NH_2
9	H-ILKEPVHG**AC**ILKEPVHGV-NH_2
10	H-ILKEPVHG**YC**ILKEPVHGV-NH_2
11	H-ILKEPVHG**VC**ILKEPVHGV-NH_2
12	H-ILKEPVHG**GHcy**ILKEPVHGA-NH_2
13	H-ILKEPVHG**GC**ILKEPCVHGV-NH_2

[a] Peptides **1−4b** are formed in situ by TCEP reduction of peptides **1−4a**.

We first examined the synthesis of peptides **1−4a** starting from the innovative solid support **18**, which features a bis(2-sulfanylethyl)amino moiety linked to a trityl polystyrene resin through both sulfur atoms (Scheme 2). For this, trityl-protected bis(2-sulfanylethyl)amine **15** was prepared by reacting bis(2-chloroethyl)amine hydrochloride with triphenylmethyl mercaptan in the presence of DBU.[10] To minimize the oxidation of **16** into the cyclic disulfide, **16** was immediately reacted with chlorotrityl polystyrene resin **17** under an inert atmosphere after deprotection in TFA.

Scheme 2. Synthesis of Solid Support **19**



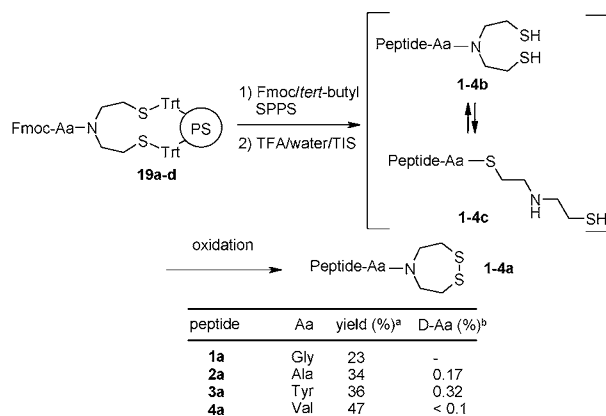
Chlorotrityl resin **17** (1.4 mmol/g) was used in excess to amine **16** (molar ratio **17**/**16**: 10/1) to lower the loading of the solid support to a value compatible with standard Fmoc/*tert*-butyl solid phase peptide synthesis protocols,[11] as well as to favor the bridging of polystyrene with amine **16**. The reaction was performed in the absence of base to allow amine protection by protonation.[12] A negative Ellman test for free thiol groups showed the successful bridging of amine **16** through both thiol groups.[13] Moreover, a positive chloranil test ascertained the presence of a secondary amine on the solid support.[14] High resolution magic angle spinning ^{1}H NMR of solid support **18** was consistent with the proposed structure (see Supporting Information). The loading was determined by coupling Fmoc-protected amino acids to solid support **18**, followed by removal of the Fmoc group with piperidine and UV quantification of the dibenzofulvene-piperidine adduct. Amino acid activation using PyBOP or HBTU was unsuccessful in accord with the low reactivity of secondary amines in amide bond-forming reactions. Alternately, Fmoc-amino acid fluorides[15] or PyBrOP[16] activation permitted the efficient acylation of solid support **18** to give resin **19**. The loadings obtained for resin **19** (0.14−0.16 mmol/g) correspond to a nearly quantitative immobilization of amine **16**.

We next examined the synthesis of peptides **1−4a**. For this, resin **19** was submitted to standard automated Fmoc/*tert*-butyl SPPS (Scheme 3). Deprotection and cleavage of the peptidyl resin furnished successfully peptides **1−4b** in excellent purity, which equilibrated with the thioester forms **1−4c** in acidic solution. This equilibration occurred in about 1 h for the Gly analogue **1b** but required up to 25 h for the Val analogue **4b**. The relationship between the aqueous solution pH and the ratio between thioester **2c** and amide

(9) Johnson, E. C.; Kent, S. B. *J. Am. Chem. Soc.* **2006**, *128*, 664.
(10) Adapted from Banerjee, S. R.; Maresca, K. P.; Stephenson, K. A.; Valliant, J. F.; Babich, J. W.; Graham, W. A.; Barzana, M.; Dong, Q.; Fischman, A. J.; Zubieta, J. *Bioconjugate Chem.* **2005**, *16*, 885.

(11) Fields, G. B.; Noble, R. L. *J. Pept. Protein Res.* **1990**, *35*, 161 Int.
(12) Conrad, R. A.; Cullinan, G. J.; Gerzon, K.; Poore, G. A. *J. Med. Chem.* **1979**, *22*, 339.
(13) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.
(14) Vojkovsky, T. *Pept. Res.* **1995**, *8*, 236.
(15) Carpino, L. A.; Mansour, E.-S. M. E.; Sadat-Aalaee, D. *J. Org. Chem.* **1991**, *56*, 2611.
(16) Coste, J.; Frerot, E.; Jouin, P. *J. Org. Chem.* **1994**, *59*, 2437.

Scheme 3. Synthesis of Peptides **1−4a**[a,b]



peptide	Aa	yield (%)[a]	D-Aa (%)[b]
1a	Gly	23	-
2a	Ala	34	0.17
3a	Tyr	36	0.32
4a	Val	47	< 0.1

[a] Isolated yields. [b] D-Aa content was determined by chiral GC-MS analysis after acid hydrolysis.

2b is shown in Figure 1. This equilibrium complicated the RP-HPLC purification of peptides **1−4b** using an acidic

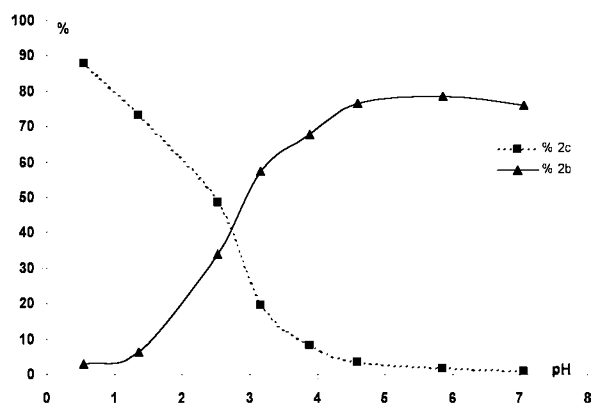


Figure 1. Influence of the aqueous solution pH on the equilibrium between thioester **2c** and amide **2b**. C18 RP-HPLC (detection at 215 nm).

water/acetonitrile linear gradient containing 0.5‰ TFA (pH 1.8). To avoid this problem, peptides **1−4b** were oxidized by air into the cyclic disulfides **1−4a** prior to the purification step.

With peptides **1−4a** in hand, we investigated their reaction with cysteinyl peptide **5** in the presence of TCEP and MPAA. TCEP was used to reduce in situ peptides **1−4a** into peptides **1−4b**. TCEP is still necessary when the reduced form **1−4b** is used directly in the ligation experiment, due to the ease of oxidation of the bis(2-sulfanylethyl)amino moiety by traces of molecular oxygen. Gratifyingly, ligation between peptide **1−3a** and cysteinyl peptide **5** proceeded cleanly within 27 h at 37 °C and pH 7 to give the native peptides **8−10** (Table 1). Figure 2 shows the LC-MS analysis of the ligation reaction for **3a**. After 27 h, only cysteinyl peptide **5** used in slight excess and native peptide **10** could be detected.

Table 1. Ligation of Peptides **1−4a** with Peptides **5−7**

peptide	peptide SEA	aminothiol fragment	yield[a] (%)
8	**1a**	5	32[b]
9	**2a**	5	77[b]
10	**3a**	5	54[b]
11	**4a**	5	58[c]
12	**1a**	6	29[b]
13	**1a**	7	42[b]

[a] Isolated yields. [b] 37 °C. [c] 45 °C.

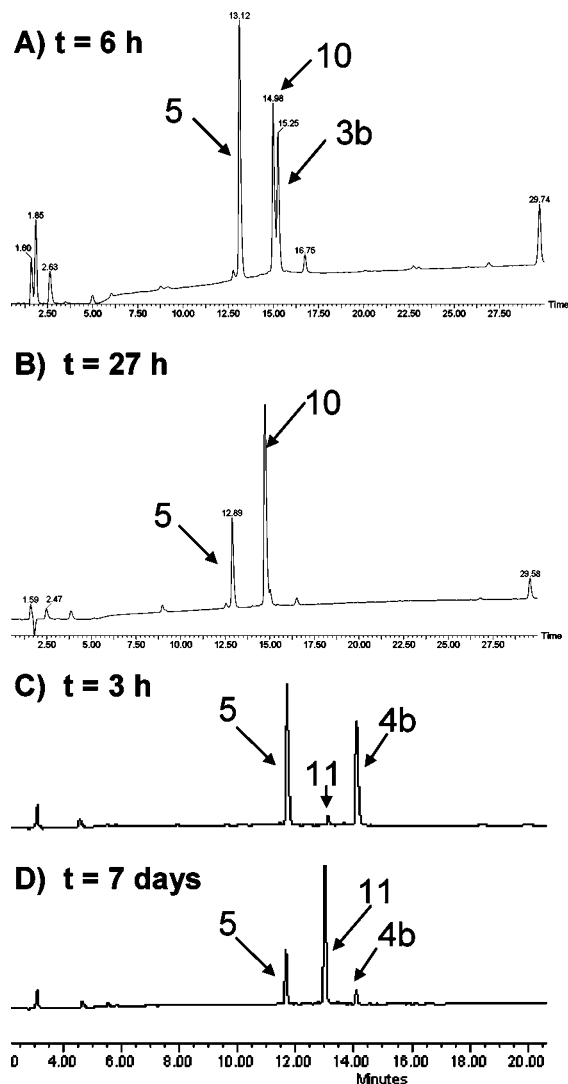


Figure 2. RP-HPLC monitoring of the ligation reaction (100 mM, pH 7.05, sodium phosphate buffer, TCEP 200 mM, MPAA 200 mM; peptide SEA: 7 mM; peptide **5**: 10 mM). (A,B) **3a** + **5** at 37 °C. (C, D) **4a** + **5** at 45 °C. C18 Nucleosil column (detection at 215 nm). More data (LC-MS) can be found in the Supporting Information.

Importantly, chiral GC-MS analysis of peptide **10** after acid hydrolysis showed that racemization of the Tyr residue was minimal (1.2% D-Tyr). Similar reactivities were observed for Gly analogue **1a** or Ala analogue **2a** (1.7% D-Ala by

chiral GC-MS for peptide **9**). NMR analysis of peptide **9** confirmed the presence of a native Ala-Cys peptide bond (see Supporting Information). Ligation of Val analogue **4a** with Cys peptide **5** led also successfully to native peptide **11** (0.18 D-Val by chiral GC-MS, Figure 2C,D), but this required 7 days at 45 °C. However, the purity of the crude product must be highlighted and illustrates the high stability of SEA fragments which do not hydrolyze even for prolonged reaction times.

Reaction of peptide **1a** with peptide **5** in the absence of TCEP failed to give any ligation product **8**. No reaction was also observed when peptide **1a** was incubated with the Ser analogue of peptide **5** (data not shown). Moreover, reaction of peptide **1a** with peptide **7**, which features two Cys residues within its sequence, furnished successfully peptide **13**. The reaction is thus selective for the N-terminal Cys residue. These experiments show the importance of both bis(2-sulfanylethyl)amino and free β-aminothiol groups for the ligation to proceed. Importantly, ligation of peptide **1a** with Hcy peptide **6** led successfully to native peptide **12**. The compatibility of SEA ligation with the Hcy residue extends potentially the methodology to the assembly of native peptides at X−Met junctions. Finally, MPAA is not absolutely required for the ligation to proceed. The ligation rate of peptide **2a** with peptide **5** in the absence of MPAA was reduced by a factor of 2.5 only.

The reactivity of peptide SEA fragments is unprecedented. At pH 7, peptide thioesters **1**−**4c** or transthioestherification products with MPAA were not detected by RP-HPLC. Only the reduced SEA fragments **1**−**4b** are observed (Figure 2). This is in contrast with 2-sulfenylethylamido peptide derivatives reported to date, which show an amide−thioester equilibrium at acidic pH too but do not ligate at pH 7 with Cys peptides. Other systems such as the cysteinyl−prolyl ester peptides (CPE) ligate with Cys peptides at pH 8.2.[8] In this case, the *N*,*S*-acyl shift equilibrium is displaced toward the thioester form by an intramolecular amide bond forming reaction involving the α-amino group of the Cys residue and ester group. The corresponding cysteinyl−prolyl amide peptides do not ligate showing the critical role played by the ester functionality and thus the intramolecular locking mechanism. Another strategy for favoring thioester formation through an intramolecular acyl shift is to use more electrophilic carbonyl derivatives.[17] In particular, Botti et al. reported that peptide-C$^{\alpha}$oxy-(2-mercapto-1-carboxyamide) ethyl ester undergoes an *O*,*S*-acyl shift at neutral pH. The resulting thioester reacted in situ with a cysteinyl peptide in a NCL-like reaction, but hydrolysis of the starting ester was found to be significant.

The structure of peptide SEA fragments does not permit a priori an intramolecular locking reaction as in the CPE strategy. Moreover, the peptide carbonyl group which participates in the *N*,*S*-acyl shift is not activated compared to *N*-alkyl cystein derivatives already reported.[7a,b] In this context, a novel mechanism may operate that could involve the participation of neighboring amino and sulfenyl groups within thioesters **1**−**4c** (Scheme 3). Few reports have described a significant acceleration of the rate of ester solvolysis by an intramolecular bifunctional general base−general acid catalysis mechanism.[18] In such a catalysis, one functional group can activate the incoming nucleophile, whereas another group stabilizes the transition state by hydrogen bonding or ion pair formation. In this context, the efficiency of the ligation reaction might be due to a significant acceleration of the transthioesterification step due to an intramolecular bifunctional catalysis, thereby overcoming the low concentration of the thioester at pH 7.

In conclusion, SEA ligation is a chemoselective, regioselective, and racemization free native ligation method that enriches the existing native peptide ligation tool box. This method can potentially be used in various chemical or biochemical applications thanks to the ease of synthesis of SEA peptide fragments and to the efficiency and simplicity of this ligation method. The elucidation of the SEA ligation mechanism as well as its application to the total synthesis of proteins is in progress.

Acknowledgment. We acknowledge financial support from Cancéropôle Nord-Ouest, Région Nord Pas de Calais, and the European Community. We thank Emmanuelle Boll (CNRS) for NMR experiments.

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.

OL102273U

(17) (a) Ollivier, N.; Behr, J. B.; El-Mahdi, O.; Blanpain, A.; Melnyk, O. *Org. Lett.* **2005**, *7*, 2647. (b) Botti, P.; Villain, M.; Manganiello, S.; Gaertner, H. *Org. Lett.* **2004**, *6*, 4861.

(18) (a) Kupchan, S. M.; Eriksen, S. P.; Shen, Y.-T. *J. Am. Chem. Soc.* **1963**, *85*, 350. (b) Maugh II, T.; Bruice, T. C. *J. Am. Chem. Soc.* **1971**, *93*, 3237.