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A New Method for Synthesis of Peptide Thioesters via Irreversible N-to-S Acyl Transfer

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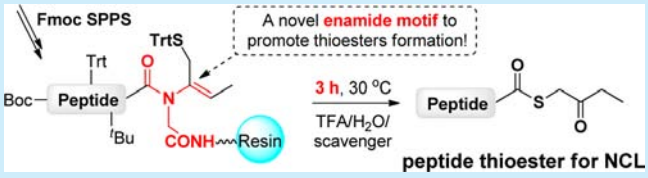
Supporting Information

ABSTRACT: A new synthetic method for peptide thioesters is described using Fmoc solid-phase peptide synthesis (Fmoc-SPPS). This method employs a novel enamide motif to facilitate irreversible intramolecular N-to-S acyl migration, which can efficiently afford the desired peptide thioesters (3 h, 30 °C) under the final trifluoroacetic acid (TFA) cleavage conditions. The acyl-transfer-mediated approach for synthesis of peptide thioesters tolerated different C-terminal residues and was used to synthesize human C−C motif chemokine 11 (hCCL11) via native chemical ligation.

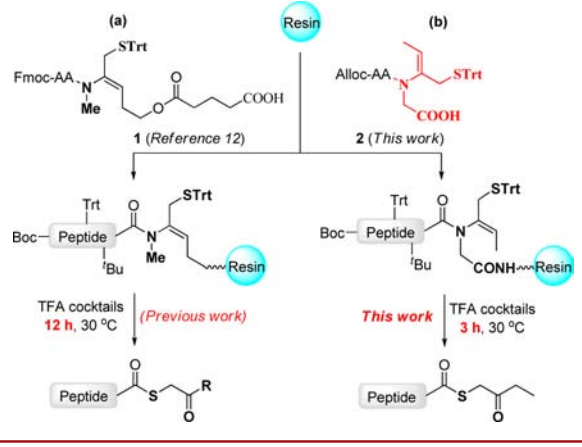


The native chemical ligation (NCL) developed by Kent et al. is the most robust method for protein chemical synthesis.[1] As indispensible intermediates for NCL, peptide thioesters can be synthesized by Boc/Bzl protocols on a thioester-linked resin.[2] However, the repetitive use of TFA and the harsh cleavage conditions (usually HF) have limitations, especially when acid-sensitive modifications such as glycosylation[3] or phosphorylation[4] are present. On the other hand, the Fmoc-SPPS strategy cannot be directly used for the synthesis of peptide thioesters due to the susceptibility of thioester to piperidine. Considerable efforts have been made to develop Fmoc methods for the synthesis of peptide thioesters,[5−12] such as a sulfonamide safety-catch linker,[6] an activated *N*-acylurea linker,[7] a backbone pyroglutamyl imide linker,[8] and peptide hydrazides.[9] There have been a number of methods utilizing the N-to-S acyl-transfer strategy for synthesis of peptide thioesters.[10−12]

In the N-to-S acyl-transfer strategy, a relatively inert amide undergoes an intramolecular N-to-S acyl shift to give a more reactive thioester. However, this reaction is reversible and unfavorable for the thioester formation. As a result, an intermolecular transthioesterification with excess external thiols is usually needed.[10] Alternatively, one may design a scaffold to cause an irreversible N-to-S acyl shift.[11] Notwithstanding these advances, the efficiency of the N-to-S acyl-transfer method remains to be improved, particularly for the synthesis of peptide thioesters with encumbered C-termini.

We recently reported another Fmoc-compatible method for preparing peptide thioesters relying on an enamide moiety.[12] In this approach, an intramolecular N-to-S acyl transfer can proceed with concomitantly irreversible hydrolysis of an enamine intermediate such that the thioester is produced (Scheme 1a) in the final TFA cleavage step. One drawback of our previous method was the tedious synthesis of enamide-containing amino acids. Besides, the N-to-S acyl transfer speed was relatively slow (12 h even for Gly) as compared to the typical cleavage time of peptide (2−4 h). These limitations hampered the application of the method for chemical protein synthesis.

Scheme 1. Previous (a) *versus* Present (b) Synthesis of Peptide Thioesters via Enamide-Based N-to-S Acyl Shift



Here we describe a new enamide-based method for Fmoc-compatible synthesis of peptide thioesters (Scheme 1b). The new enamide amino acids can be easily synthesized in large quantities. An enamide containing peptide chain is assembled by a standard Fmoc-SPPS procedure. The target peptide thioester can be generated through an irreversible N-to-S acyl transfer within 3 h under the TFA cleavage conditions at 30 °C. The new method possesses the potential to increase the

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efficiency and practicability of synthetic peptide thioesters. This strategy was employed for the "one-pot" synthesis of bioactive hCCL11.

The N-to-S acyl shift in the enamide linker produces a thioester with a carbonyl group. To explore whether this carbonyl group interferes with the NCL, we synthesized a peptide thioester **3** and an N-Cys peptide **4**. **4** (1.5 equiv) was dissolved in ligation buffer [1:9 v/v DMF/0.2 M phosphate buffer containing 6 M guanidine hydrochloride (Gn·HCl), 10 mM 4-mercaptophenylacetic acid (MPAA), and 50 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl), pH 3.0]. This solution was transferred to an eppendorf tube containing **3** (1 equiv) and adjusted to pH 5.3 providing the conditions under which the thiaproline ligation would tend to occur.[13] The ligation mixture was incubated at 30 °C with gentle mixing. The ligation product was monitored by HPLC and confirmed by ESI-MS. Fortunately, no thiazolidine-thioester intermediates were observed and only a single product **5** (>92% HPLC yield) was formed within 2 h (Figure 1). Furthermore, **3** and **4** can

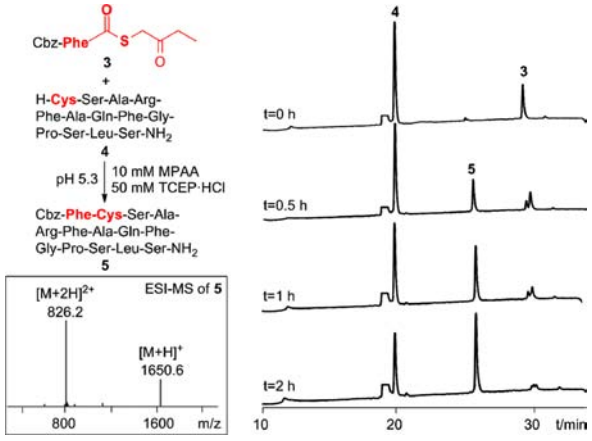
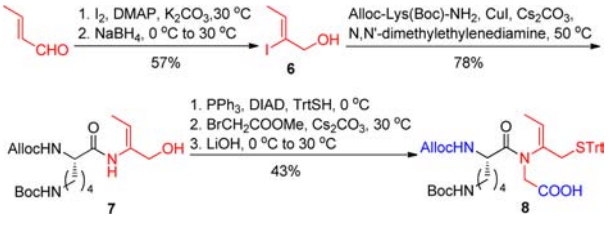


Figure 1. Native chemical ligation of peptide thioesters **3** bearing carbonyl groups with N-Cys peptides **4**. Right: RP-HPLC traces (t = 0, 0.5, 1, and 2 h); inset: ESI-MS of **5**. Cbz: carbobenzoxy group.

also be coupled quantitatively under standard neutral ligation conditions (Supplementary Figure 1). The data confirmed that the carbonyl groups of thioesters had no interference with NCL.

Encouraged by the above results, we synthesized enamide-containing amino acids used in Fmoc-SPPS. Using the enamide-containing Lys amino acid **8** as an example (Scheme 2), commercially available 2-butenal was converted to a vinyl iodide **6**.[14] The formation of the enamide **7** was carried out via Cu(I) catalyzed C−N cross-coupling between **6** and an allyloxycarbonyl (Alloc)-protected amino amide.[12,14] The desired product **8** was generated by introduction of the thiol unit employing a Mitsunobu reaction, followed by the

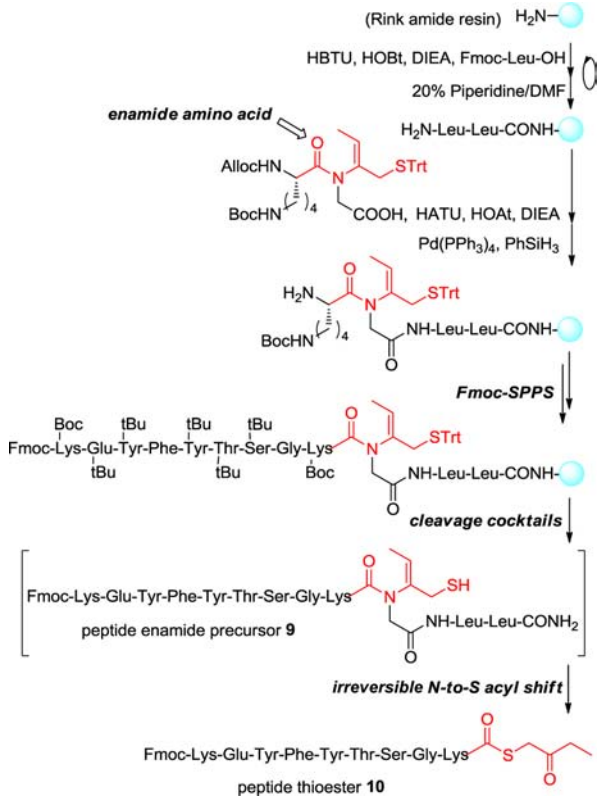
Scheme 2. Synthetic Route for Enamide Amino Acids



hydrolysis of ester with lithium hydroxide. Thus, **8** was generated in 6 steps (19% overall yield) from 2-butenal. The efficiency is much higher than that of **1** in 12 steps (~3% overall yield) from 3-butyn-1-ol.[12] The gram-scale synthesis of enamide amino acid **2** should greatly extend the practicality for the synthesis of peptide thioesters by the enamide-based N-to-S acyl shift strategy.

Model peptide thioesters Fmoc-Lys-Glu-Tyr-Phe-Tyr-Thr-Ser-Gly-Lys-COSR (R = $CH_2COC_2H_5$) **10** were synthesized based on the new enamide structure (Scheme 3). The enamide

Scheme 3. Synthesis of Model Peptide Thioesters 7 Using the New Enamide Amino Acids by Fmoc-SPPS



Lys amino acid **8** was introduced into Leu-incorporated Rink amide resin (0.1 mmol) using HATU/HOAt/DIEA coupling. After completion of the coupling of **8**, the resin was treated with tetrakis(triphenylphosphine)Pd(0) ($Pd(PPh_3)_4$)/phenylsilane ($PhSiH_3$) to remove Alloc. The peptide-chain assembly was elongated by standard Fmoc-SPPS. The peptide was cleaved from the resin with TFA/triisopropylsilane (TIPS)/H_2O (95:2.5:2.5, v/v/v) cocktails at 30 °C. During this step, the desired peptide thioester was obtained by in situ N-to-S acyl transfer followed by hydrolysis of the enamine intermediate. The formation process of peptide thioesters was monitored by an analytical RP-HPLC at 214 nm and MALDI-TOF-MS (Figure 2). Over 50% of **9** was converted to **10** after 0.5 h while the conversion was almost quantitative after 3 h. The isolated yield of **10** was 56% (80 mg) after HPLC purification and lyophilization. Note that the acyl transfer rate for thioesters is much higher than the previous enamide motif (12 h, 30 °C). The fast conversion rate could avoid the generation of peptide side products due to long-time TFA treatment.[15] The favorable transfer is attributed to the electron-withdrawing β-CONHR group which may partially activate the

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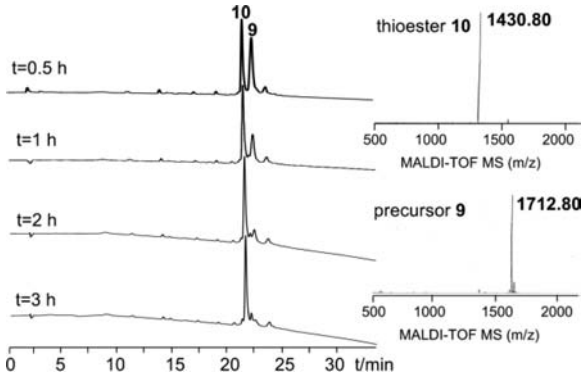


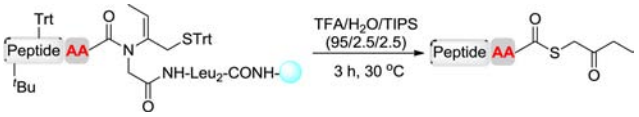
Figure 2. Formation process of peptide thioesters **10** using new enamide motifs for N-to-S acyl shift. Left: RP-HPLC traces (t = 0.5, 1, 2, and 3 h). Right: MALDI-TOF MS of **9** and **10**.

scissile amide bond and make the amide bond prone to intramolecular thiolysis.[16] Besides, a key step for synthesis of peptide thioesters is the introduction of two Leu residues as a spacer (Supplementary Schemes 3−5).

To evaluate the racemization at the C-terminal amino acid during thioesterification, we made the enamide amino acids corresponding to L-Phe and D-Phe. With the two compounds in hand, we then prepared the peptide thioesters Fmoc-Val-Lys-His-(**D-**)**Phe**-COSR **11** and Fmoc-Val-Lys-His-(**L-**)**Phe**-COSR **12** (R = $CH_2COC_2H_5$) according to the above Fmoc-SPPS protocols. RP-HPLC analysis at 214 nm of peptides **11** and **12** (Supplementary Figure 7) reveals that the epimerization ratio of the C-terminal Phe is less than 1.3%. We also synthesized a series of peptide thioesters corresponding to Gly, Ser, His, Tyr, Lys, Leu, and Thr with good purities and high yields within 3 h at 30 °C (Table 1 and Supplementary Figure 8). Note that the conversion for sterically hindered amino acids such as Thr, Val, or Ile requires more time (12 h, 30 °C).

To test the practical use of the new method, hCCL11 was synthesized (Figure 3a). hCCL11 is a 74-mer chemokine with two disulfide bridges and is chemo-attractive toward eosinophils and basophils through its receptors CCR2,

Table 1. Fmoc SPPS Synthesis of Peptide Thioesters



entry	target peptides	purity %[a]	yield %[b]
1	H-TVQARQL**L**-SR[c]	76	38
2	Fmoc-RRGKKKS**G**-SR	57	25
3	H-WTPEVKH**(D-)F**-SR	92	51
4	H-WTPEVKH**(L-)F**-SR	91	60
5	Fmoc-KEYFYTSG**K**-SR	88	56
6	H-KDQFPEV**Y**-SR	91	46
7	H-HPGSIPT**S**-SR	62	39
8	H-WVQDATK**H**-SR	71	50
9	H-GDVACGK**T**-SR[d]	55	20
10	H-SDITASV**N**-SR	64	42

[a]Purity of crude peptide thioesters based on HPLC at 214 nm. [b]Isolated yield based on the loading of the enamide-containing amino acid. [c]R = $CH_2COC_2H_5$. [d]Cleavage for 12 h at 30 °C.

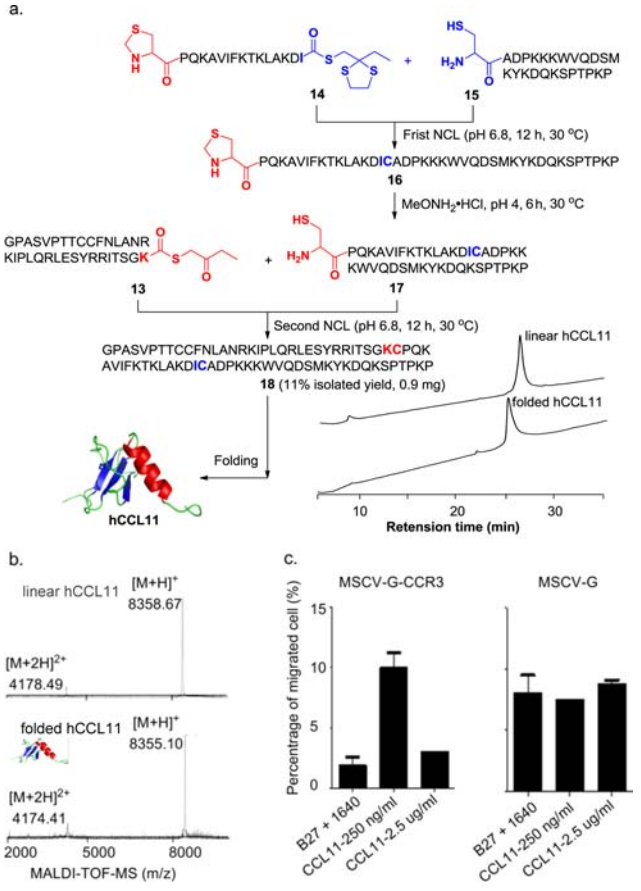


Figure 3. "One-pot" chemical synthesis of hCCL11. (a) HPLC traces, (b) MALDI-TOF-MS, and (c) chemotactic activity of chemical synthetic hCCL11.

CCR3, or CCR5.[17] hCCL11 was assembled by the "one-pot" strategy[18] with three segments: 1−33 (**13**), 34−49 (**14**), and 50−74 (**15**). The peptide thioester **13** (37% isolated yield) and **14** (11% isolated yield) were prepared using the similar Fmoc method described in Scheme 3. The third N-Cys peptides **15** (19% isolated yield) was also obtained by Fmoc SPPS. The reagent K was used as the peptide cleavage reagent because it was maximally efficient in increasing the yield of **14**. It is noted that the 1,3-dithiolane-protected peptide thioesters **14** were obtained owing to the reduction of the carbonyl group by EDT with the long cleavage time (12 h) (Supplementary Figures 5 and 11). Assembly of the hCCL11 began with ligation of 2.0 mg of **14** with 2.9 mg of **15** (ligation buffer: 6 M Gn·HCl, 0.2 M phosphate buffer, 200 mM MPAA, 50 mM TCEP·HCl, pH 6.8). The reaction led to the formation of Thz-peptide **16** after 12 h at 30 °C. 0.2 M methoxyamine·HCl was then added to the crude reaction mixture to quantitatively covert the Thz in **16** to Cys in **17** with the pH ≈ 4 for 6 h at 30 °C. Intermediates **16** and **17** were verified by MALDI-TOF-MS ($[M + H]^+$ for **16**: calcd 4714.4 Da, found 4714.2 Da; $[M + H]^+$ for **17**: calcd 4702.4 Da, found 4702.5 Da). The thioester **13** (5.6 mg) was added to the reaction, and the pH was adjust to 7.0 for 12 h. The one-pot process affords full-length **18** after purification by RP-HPLC (11% isolated yield, 0.9 mg). HPLC and MALDI-TOF-MS analysis demonstrated the high quality of the ligation product ($[M + H]^+$ for **18**: calcd 8358.7 Da, found 8360.5 Da). **18** was then folded under the cystine/cysteine redox system to give the folded hCCL11 **19** with 57% isolated yield ($[M + H]^+$

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for **19**: calcd 8355.1 Da, found 8356.5 Da). The chemotactic activity was tested by treatment of CCR3-expressing cells with the folded hCCL11 in a 96-well-plate format (5 mm pore, Corning). A strong chemotactic activity was triggered at the 250 ng/mL concentration (Figure 3c).

In conclusion, we present an improved method for Fmoc-SPPS synthesis of peptide thioesters using an *N*-CH_2CONHR-substituted enamide linker. The method relies on the introduction of an enamide motif which renders the desired peptide thioesters through a rapid and irreversible N-to-S acyl shift under the TFA cleavage conditions (3 h, 30 °C). Straightforward formation of peptide thioesters could extend the application of native chemical ligation and provides an additional tool for the chemical synthesis of cyclic peptides/proteins.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization data, NMR spectra, HPLC traces and MS spectra are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776. (b) Nilsson, B. L.; Soellner, M. B.; Raines, R. T. *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 91. (c) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338.

(2) Schnolzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Res. Ther.* **2007**, *13*, 31. (b) Izumi, M.; Murakami, M.; Okamoto, R.; Kajihara, Y. *J. Pept. Sci.* **2014**, *20*, 98.

(3) (a) Yamamoto, N.; Tanabe, Y.; Okamoto, R.; Dawson, P. E.; Kajihara, Y. *J. Am. Chem. Soc.* **2008**, *130*, 501. (b) Izumi, M.; Makimura, Y.; Dedola, S.; Seko, A.; Kanamori, A.; Sakono, M.; Ito, Y.; Kajihara, Y. *J. Am. Chem. Soc.* **2012**, *134*, 7238. (c) Wang, P.; Dong, S.; Shieh, J. H.; Peguero, E.; Hendrickson, R.; Moore, M. A.; Danishefsky, S. J. *Science* **2013**, *342*, 1357.

(4) (a) Huse, M.; Holford, M. N.; Kuriyan, J.; Muir, T. W. *J. Am. Chem. Soc.* **2000**, *122*, 8337. (b) Goguen, B. N.; Hoffman, B. D.; Sellers, J. R.; Schwartz, M. A.; Imperiali, B. *Angew. Chem., Int. Ed.* **2011**, *50*, 5667. (c) Hejjaoui, M.; Butterfield, S.; Fauvet, B.; Vercruysse, F.; Cui, J.; Dikiy, I.; Prudent, M.; Olschewski, D.; Zhang, Y.; Eliezer, D.; Lashuel, H. A. *J. Am. Chem. Soc.* **2012**, *134*, 5196.

(5) (a) Okamoto, R.; Morooka, K.; Kajihara, Y. *Angew. Chem., Int. Ed.* **2012**, *51*, 191. (b) Camarero, J. A.; Hackel, B. J.; de Yoreo, J. J.; Mitchell, A. R. *J. Org. Chem.* **2004**, *69*, 4145.

(6) Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369.

(7) Blanco-Canosa, J. B.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2008**, *47*, 6851.

(8) Tofteng, A. P.; Sorensen, K. K.; Conde-Frieboes, K. W.; Hoeg-Jensen, T.; Jensen, K. J. *Angew. Chem., Int. Ed.* **2009**, *48*, 7411.

(9) (a) Fang, G. M.; Li, Y. M.; Shen, F.; Huang, Y. C.; Li, J. B.; Lin, Y.; Cui, H. K.; Liu, L. *Angew. Chem., Int. Ed.* **2011**, *50*, 7645. (b) Fang, G. M.; Wang, J. X.; Liu, L. *Angew. Chem., Int. Ed.* **2012**, *51*, 10347. (c) Zheng, J. S.; Tang, S.; Qi, Y. K.; Wang, Z. P.; Liu, L. *Nat. Protoc.* **2013**, *8*, 2483. (d) Li, Y.-M.; Li, Y.-T.; Pan, M.; Kong, X.-Q.; Huang, Y.-C.; Hong, Z.-Y.; Liu, L. *Angew. Chem., Int. Ed.* **2014**, *53*, 2198. (e) Zheng, J.-S.; Yu, M.; Qi, Y.-K.; Tang, S.; Shen, F.; Wang, Z.-P.; Xiao, L.; Zhang, L.; Tian, C.-L.; Liu, L. *J. Am. Chem. Soc.* **2014**, *136*, 3695.

(10) (a) Kawakami, T.; Sumida, M.; Nakamura, K.; Vorherr, T.; Aimoto, A. *Tetrahedron Lett.* **2005**, *46*, 8805. (b) Ollivier, N.; Behr, J. B.; El-Mahdi, O.; Blanpain, A.; Melnyk, O. *Org. Lett.* **2005**, *7*, 2647. (c) Nagaike, F.; Onuma, Y.; Kanazawa, C.; Hojo, H.; Ueki, A.; Nakahara, Y.; Nakahara, Y. *Org. Lett.* **2006**, *8*, 4465. (d) Ohta, Y.; Itoh, S.; Shigenaga, A.; Shintaku, S.; Fujii, N.; Otaka, A. *Org. Lett.* **2006**, *8*, 467. (e) Hojo, H.; Onuma, Y.; Akimoto, Y.; Nakahara, Y.; Nakahara, Y. *Tetrahedron Lett.* **2007**, *48*, 25. (f) Kang, J.; Richardson, J. P.; Macmillan, D. *Chem. Commun.* **2009**, 407. (g) Tsuda, S.; Shigenaga, A.; Bando, K.; Otaka, A. *Org. Lett.* **2009**, *11*, 823. (h) Hou, W.; Zhang, X.; Li, F.; Liu, C. F. *Org. Lett.* **2011**, *13*, 386. (i) Ollivier, N.; Vicogne, J.; Vallin, A.; Drobecq, H.; Desmet, R.; El Mahdi, O.; Leclercq, B.; Goormachtigh, G.; Fafeur, V.; Melnyk, O. *Angew. Chem., Int. Ed.* **2012**, *51*, 209. (j) Yang, R.; Hou, W.; Zhang, X.; Liu, C. F. *Org. Lett.* **2012**, *14*, 374. (k) Eom, K. D.; Tam, J. P. *Org. Lett.* **2011**, *13*, 2610. (l) Taichi, M.; Hemu, X.; Qiu, Y.; Tam, J. P. *Org. Lett.* **2013**, *15*, 2620. (m) Sato, K.; Shigenaga, A.; Kitakaze, K.; Sakamoto, K.; Tsuji, D.; Itoh, K.; Otaka, A. *Angew. Chem., Int. Ed.* **2013**, *52*, 7855. (n) Sharma, R. K.; Tam, J. P. *Org. Lett.* **2011**, *13*, 5176.

(11) (a) Kawakami, T.; Aimoto, S. *Chem. Lett.* **2007**, *36*, 76. (b) Dheur, J.; Ollivier, N.; Melnyk, O. *Org. Lett.* **2011**, *13*, 1560.

(12) (a) Zheng, J. S.; Chang, H. N.; Wang, F. L.; Liu, L. *J. Am. Chem. Soc.* **2011**, *133*, 11080. (b) Zheng, J. S.; Tang, S.; Huang, Y. C.; Liu, L. *Acc. Chem. Res.* **2013**, *46*, 2475.

(13) (a) Miao, Z. W.; Tam, P. J. *J. Am. Chem. Soc.* **2000**, *122*, 4253. (b) Tam, J. P.; Yu, Q.; Yang, J. L. *J. Am. Chem. Soc.* **2001**, *123*, 2487. (c) Li, X.; Lam, H. Y.; Zhang, Y.; Chan, C. K. *Org. Lett.* **2010**, *12*, 1724. (d) Zhang, Y.; Xu, C.; Lam, H. Y.; Lee, C. L.; Li, X. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 15698.

(14) Hu, T. S.; Li, C. Z. *Org. Lett.* **2005**, *7*, 2035.

(15) (a) King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Pept. Protein Res.* **1990**, *36*, 255. (b) Fields, C. G.; Fields, G. B. *Tetrahedron Lett.* **1993**, *34*, 6661.

(16) (a) Zheng, J. S.; Cui, H. K.; Fang, G. M.; Xi, W. X.; Liu, L. *ChemBioChem* **2010**, *11*, 511. (b) Zheng, J. S.; Chang, H. N.; Shi, J.; Liu, L. *Sci. China Chem.* **2012**, *55*, 64.

(17) (a) Vogel, B.; Klinder, A.; Sittig, D.; Aust, G. *Vet. Immunol. Immunopathol.* **2005**, *107*, 67. (b) Yates, D. *Nat. Rev. Neurosci.* **2011**, *12*, 547.

(18) Bang, D.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2004**, *43*, 2534.