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A Simple Method for Preparing Peptide C-Terminal Thioacids and Their Application in Sequential Chemoenzymatic Ligation

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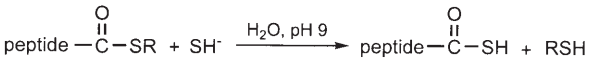
Peptide C^{α}-thioacids are important and versatile building blocks for peptide and protein synthesis. Besides their early applications in segment condensation,[1–3] these compounds have also been used in chemoselective ligation with a bromoacetopeptide to form a pseudopeptidic thioester[4] or in "mini"-thiol capture ligation to form a native peptide bond[5] in the ligated product. More recently, the thioacid functionality has also been shown to react with a suitable azido moiety to form an amide linkage.[6] However, the difficulty in obtaining peptide thioacids synthetically has severely limited their use in peptide and protein chemistry. Unlike peptide thioesters for which many synthetic methods have been developed for both Boc and Fmoc-solid-phase synthesis,[7] only a few methods have been reported so far for the synthesis of peptide C-terminal thioacids.[1,2,8] All of these methods are based on Boc synthesis, and those that have proven practically useful[1,2,8a,b] rely on the same chemistry, which was originally developed by Blake, et al.[1,2] This chemistry requires a special thioester benzhydryl linker that is not commercially available, and the use of hydrogen fluoride for final deprotection and cleavage. The yield is usually not high because, being a supernucleophile, the thioacid group is susceptible to nucleophilic attacks by the cationic electrophiles that are released during the final cleavage. Herein, we report a simple and efficient method to produce peptide C-terminal thioacids through hydrothiolysis of thioesters (Scheme 1). More importantly, because a thioacid group



Scheme 1. Conversion of peptide thioester to thioacid through hydrothiolysis in aqueous buffer.

might not be recognized and hydrolyzed by subtiligase, it would be orthogonal to the subtiligase-catalyzed ligation chemistry.[9,10] This would make it possible to conduct sequential chemoenzymatic ligation through alternate use of mini thiol capture and enzymatic ligations.

We first tested the hydrothiolysis reaction in a simple system with a small peptide thioester, Ac-**HAAPF**-S$(CH_2)_2$-CO_2H, which had been prepared by an enzymatic method.[11] Treatment of this thioester peptide (ca. 0.7 mM) with NaSH ([SH^-]: ca. 120 mM) in 1 M phosphate buffer (pH 9) at 42 °C for 2 h led to near quantitative conversion to the thioacid (Figure 1, trace 2); only a very small fraction was hydrolyzed to the peptide acid (peak c at ~12.5 min, trace 2).

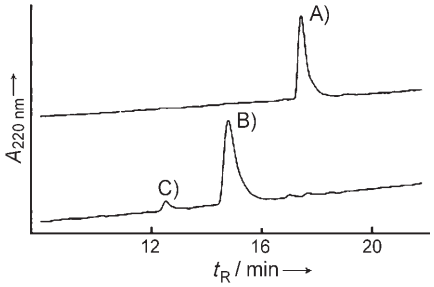


Figure 1. HPLC profile of the starting material, Ac-**HAAPF**-S-$(CH2)_2$-COOH (trace 1) (*m/z* $[M+H]^+$ found 671.7, M_W calcd: 672.5), and of the hydrothiolysis reaction mixture at 2 h (trace 2). peak b is the product, Ac-**HAAPF**-SH (*m/z* calcd.: 599.6 $[M+H]^+$; found: 600.4).

We also conducted the reaction at different pH and temperature. At 23 °C, the hydrothiolysis rate decreased significantly (Figure 2 A). However, the hydrolysis rate also decreased accordingly; for example, at 23 °C and pH 9, the reaction with NaSH for 7.5 h gave ca. 90% thioacid while the hydrolysis product was almost undetected (< 1%) by HPLC. Prolonging the reaction to 10 h led to near complete conversion of the thioester to thioacid (ca. 98%) with only a small amount of hydrolysis product (< 2%). Increasing the pH (from pH 8 to 10) also increased the reaction rate (Figure 2 A). It became practically difficult to conduct the reaction at pH 7 or below because of escape of H_2S from the reaction medium. Therefore for practical reasons, the reaction was performed at pH 8.5–9 in subsequent experiments. Na_2S was also an excellent reagent for this reaction; it gave just a slightly slower reaction than did NaSH (Figure 2 B). $(NH_4)_2S$ was comparable to Na_2S under the same reaction conditions (data not shown). It is not surprising that similar results were obtained with these different sulfide compounds because at these pHs, the hydrosulfide ion HS^- ought to be the effective exchange agent considering the pK_a value of H_2S ($pK_{a1} = 6.9$, $pK_{a2} = 17.1$.[12])

To demonstrate the general utility of this method, we prepared a number of peptide thioesters and tested these in the hydrothiolysis reaction. The results are summarized in Table 1. Peptides 1, 2 and 4 contain a sterically hindered amino acid residue with a β-branched side chain at the C terminus. Yet the hydrothiolysis of these peptide thioesters all proceeded cleanly to give the corresponding thioacids in excellent yields. The C-terminal residues in peptides 3, 5 and 6 are less hindered, and the reactions with these peptides took shorter times to com-

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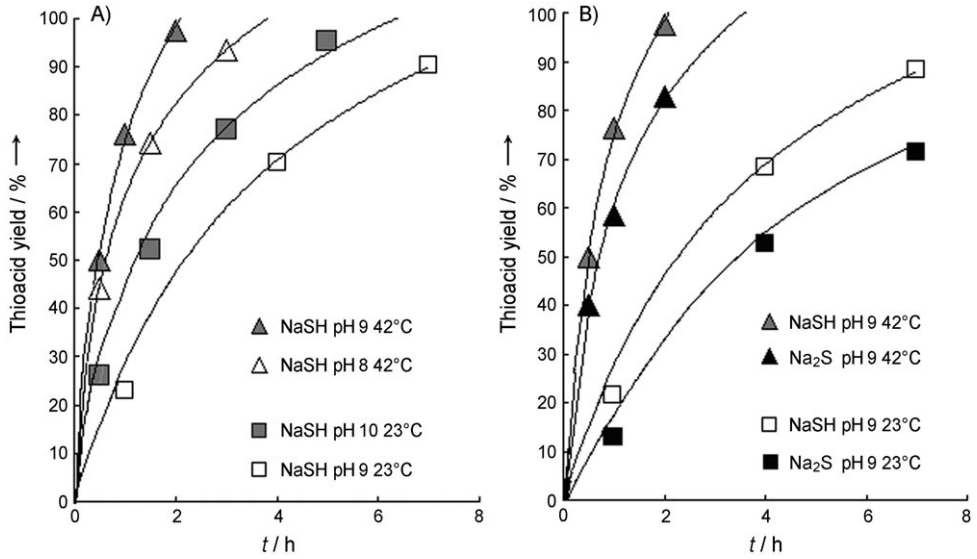


Figure 2. Thioacid formation under different reaction conditions. Reaction was conducted with 0.7 mM Ac-HAAPF-S-$(CH_2)_2$-COOH and NaSH or Na_2S (120 mM effective hydrosulfide ion). Yield was based on HPLC analysis at 220 nm.

plete. It is worth noting that all these peptides had unprotected side chains, and therefore had good solubility in aqueous buffer. Again, as in the model study shown in Figure 1, only a very small amount of hydrolysis was found for all the hydrothiolysis reactions in solution (entries 1–6), and no other side reactions were detected by HPLC. We also found that the reaction could be conducted under denatured conditions, such as in the presence of high concentrations of urea or guanidine·HCl. For instance, hydrothiolysis of peptide 1 was also performed in the presence of 8 M urea, which gave clean formation of the thioacid product (data not shown). This would be useful for long hydrophobic peptides that tend to aggregate in aqueous buffer under native conditions.

A solid-phase hydrothiolysis reaction was also attempted on a resin-bound peptide thioester (Table 1, entry 7). The peptide was synthesized by using Boc chemistry and a mercaptopropionyl linker between the peptide and amino-functionalized NovaSyn TG resin. Final deprotection with TFMSA/TFA removed all side-chain protecting groups without cleaving the peptide from the resin. Treatment of the peptidyl resin with 0.2 M $(NH_4)_2S$ in 1 M phosphate buffer (pH 9) for 3 h at 42 °C cleaved 30–40% of the peptide from the resin based on a quantitative ninhydrin test[13] before and after cleavage. The thioacid product represented approximately 50% of mass in the crude peptide mixture, and an additional ca. 15% was the hydrolysis product (Supporting Information). Longer incubation improved peptide release just appreciably, but led to significantly more hydrolysis. Inclusion of a cosolvent such as DMF or dioxane did not improve the reaction, nor did the use of Na_2S or NaSH. This less satisfactory result is apparently due to the poor water-swelling ability of the resin, which is based on the hydrophobic polystyrene that is grafted with PEG.

An obvious application of the prepared peptide thioacids is the use as the acyl component building blocks for the "mini"-thiol capture ligation strategy,[5] so-named after the template-assisted prior thiol capture ligation strategy that was developed by Kemp.[14] This ligation approach works in two steps: 1) specific capture of the thioacid sulfhydryl of the acyl peptide by an Npys-modified N-terminal Cys residue of the amine component peptide to bring the two peptide components together through a covalent acyl disulfide linkage. The capture step is usually conducted in an acidic buffer (pH 2–4) and finishes almost instantaneously; 2) intramolecular S,N-acyl transfer via a six-member ring intermediate, which leads to the formation of an amide bond upon adjusting the pH to 5–6.[5] A natural Cys residue is regenerated upon addition of a reducing agent such as tris(2-carboxyethyl)phosphine or dithiothreitol at the end of the reaction. We also realized that the hydrothiolysis reaction might also render an otherwise subtiligase-sensitive thioester bond to a subtiligase-insensitive thioacid group because a negatively charged thiocarboxylate is unlikely to be recognized

Table 1. Thiol-acid formation reaction.[a]

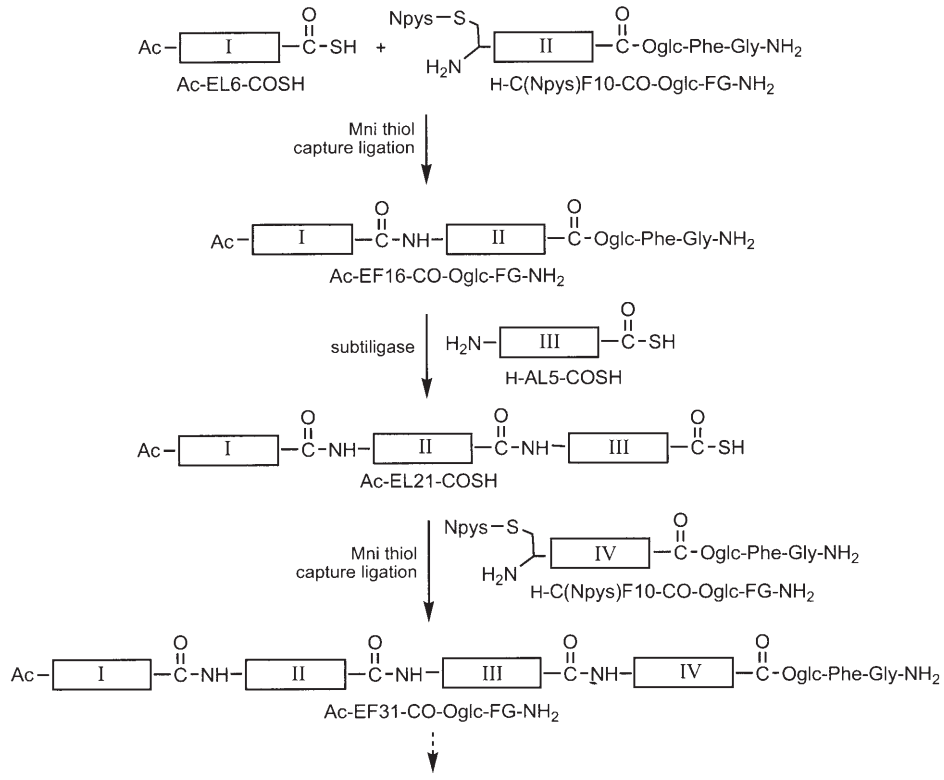
No	Peptide thiolester starting material		Peptide thioacid product		
	Sequence	Thioester component	Yield[b] [%]	M_W calcd	m/z $[M+H]^+$ found
1	H-**FSKLAV**	-S-$(CH_2)_2$-$CONH_2$	96 (4 h)	679.4	680.4
2	H-**FSKLAI**	-S-$(CH_2)_2$-$CONH_2$	90 (4 h)	693.4	694.5
3	H-**AFSKL**	-S-$(CH_2)_2$-$CONH_2$	95 (2 h)	580.3	581.4
4	Ac-**LVKEI**	-S-$(CH_2)_2$-$CONH_2$	85 (8 h)[c]	658.4	659.3
5	Ac-**HAAPF**	-S-$(CH_2)_2$-COOH	92 (2 h)	599.3	600.4
6	H-**TKGSAYSGKLEEFVQ**	-S-$(CH_2)_2$-COOH	94 (3.5 h)	1658.8	1660.0
7	H-**SGEYSKGDVSPNIQA**	-S$(CH_2)_2$CO-NH-NovaSyn TG resin	15-20 (3 h)[d]	1566.7	1567.5

[a] Reaction condition: thioester peptide (0.7-1 mM), NaSH (120 mM effective hydrosulfide ion in 1 M sodium phosphate buffer, pH 9) at 42 °C except for entries 4 and 7. [b] Yield was based on HPLC ananlysis and numbers in the brackets are the reaction time. [c] Na_2S (120 mM effective hydrosulfide ion in 1 M sodium phosphate buffer, pH 9) at 23 °C. [d] $(NH_4)_2S$ (0.2 M in 1 M phosphate buffer, pH 9) at 42 °C.

by subtiligase[9,10] as the substrate for hydrolysis. As a result, a thioacid peptide with a suitably free N terminus can be used as the amine nucleophile substrate for subtiligase-catalyzed peptide ligation while keeping the thioacid functionality intact. This makes it possible to perform sequential chemoenzymatic ligation in alternate mini-thiol capture and enzymatic ligation steps. To demonstrate the feasibility of this sequential chemoenzymatic ligation scheme, a model peptide was synthesized by three consecutive steps of chemical and enzymatic ligation in the N-to-C direction (Scheme 2).

Therefore, when the thioacid peptide **I**, Ac-**EL6**-CO-SH, was ligated with the Npys-modified peptide glycolate ester, H-**C**(Npys)**F10**-CO-Oglc-FG-NH_2 (peptide **II**), ~60% ligation product was obtained after a 20 min ligation reaction (Figure 3, trace 1). Because the newly formed ligation product, Ac-**EF16**-CO-Oglc-FG-NH_2 contains a substiligase-recognizable C-terminal glycolate ester moiety, it could then be used in a second, subtiligase-catalyzed enzymatic ligation step with peptide **III**, H-**AFSKL**-COSH (or H-**AL5**-COSH), which contains a C-terminal thioacid (Figure 3, trace 2). The ligation product, Ac-**EL21**-COSH was obtained in ca. 75% yield after 2 h reaction (trace 2, peak g). Only a small amount of the ligated peptide C^{α}-thioacid product was hydrolyzed to give the peptide C^{α}-carboxylic acid (trace 2, peak f) during the course of the enzymatic reaction. On the contrary, when the corresponding peptide thioester, H-**AFSKL**-CO-$SCH_2CH_2CONH_2$ was used as the amine nucleophile for the enzymatic reaction, almost all the thioester bond in the ligated product was hydrolyzed (data not shown). This result demonstrated the advantage of having a C-terminal thioacid instead of a thioester for such a tandem chemoenzymatic ligation scheme, because the surviving thioacid group would allow another step of mini-thiol capture ligation. Indeed, the new thioacid peptide, Ac-**EL21**-COSH, was ligated again with a fourth peptide segment, H-**C**(Npys)**F10**-CO-Oglc-FG-NH_2, to give Ac-**EF31**-CO-Oglc-FG-NH_2 in 90% yield after a 20 min reaction (Figure 3, trace 3). One should note that the free thiol from the internal Cys residue in Ac-**EL21**-COSH did not interfere with mini-thiol capture ligation because it is much less nucleophilic and less reactive than the thioacid at the acidic operating pH of the capture step. With the presence of the C-terminal glycolate ester bond in the new ligation product, in principle, this sequential chemoenzymatic ligation scheme can still continue to proceed with a new enzymatic ligation step.

As a soft electrophile, a thioester has the unique ability to react with a soft nucleophilic thiol to form a new thioester.[15–21] This thiol–thioester exchange reaction has been exploited in the development of powerful chemoselective ligation methods for protein synthesis.[19–21] What we have demonstrated herein is that when a hydrosulfide ion is used instead as the thiol source, the exchange reaction forms a thioacid as the hydrothiolysis product of the thioester. Since peptide thioesters can now be prepared by the user-friendly Fmoc-SPPS, this method will allow one to prepare peptide thioacids without having to use the Boc-chemistry method that was mentioned earlier, and hence the HF cleavage step, which requires a special apparatus that is not available in many research laboratories. We have further demonstrated that by using peptide thioacids and peptide glycolate esters as building blocks it is possible to conduct alternate mini-thiol capture and enzymatic ligations. Using the two methods in such a combination offers several distinct features. First, the mini-thiol capture ligation is highly efficient and operates at a weakly acidic pH. The ligation reaction usually completes in less than 20 min for peptide thioacids with, for example, a C-terminal Ala or Phe residue. Historically, a drawback of mini-thiol capture ligation has been associated with obtaining the thioacid building block. Now, the hydrothiolysis method reported herein provides a simple method for the preparation of peptide thioacids. Recently, we have applied mini-thiol capture ligation in the synthesis of a histone protein H3 and its analogues.[22] Second, subtiligase is known to have relatively broad substrate specificity.[9,10] This provides one with more flexibility to choose a suitable ligation



Scheme 2. Synthesis of a model peptide by a three-step sequential chemoenzymatic ligation.

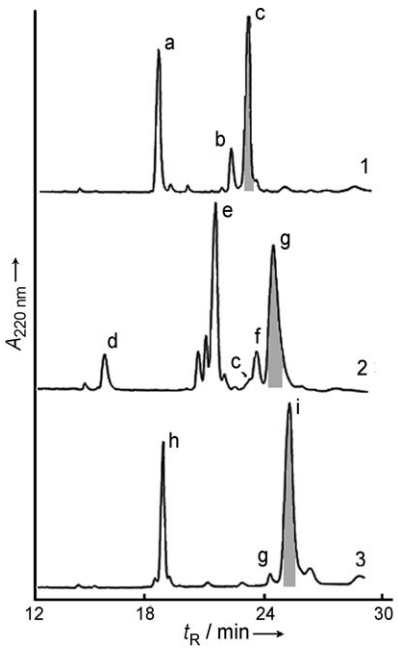


Figure 3. HPLC monitoring of the synthesis of Ac-**EF31**-CO-Oglc-FG-NH_2 (Ac-ELNKLLCSDSEHAAPFAFSKLCSDSEHAAPF-Oglc-FG-NH_2) by three sequential steps of chemical and enzymatic ligation. See the Suporting Information for detailed experimental conditions. Peaks that are highlighted in color correspond to the ligation products from the three steps. Trace 1 (the first ligation step): peak a: H-**CSDSEHAAPF**-Oglc-FG-NH_2 from reduction of remaining H-**C**(Npys)**F10**-CO-Oglc-FG-NH_2; peak b: Ac-**EL6**-COSH or Ac-EKNKLL-SH; peak c: ligation product Ac-**EF16**-CO-Ogly-FG-NH_2 or Ac-ELNKLLCSDSE-HAAPF-Oglc-FG-NH_2 (*m/z* calcd: 2075.9 $[M+H]^+$; found: 2076.9). Trace 2 (the second, subtiligase-catalyzed ligation step): peak d: H-**AFSKL**-COSH; peak e: Ac-ELNKLLCSDSEHAAPF-OH (from the hydrolysis of the glycolate ester substrate Ac-**EF16**-CO-Ogly-FG-NH_2); peak f: Ac-ELNKLLCSDSEHAAPFAFSKL-OH from the hydrolysis of the ligated thioacid product; peak g: ligation product Ac-**EL21**-COSH or Ac-ELNKLLCSDSEHAAPFAFSKL-SH (*m/z* calcd: 2377.1 $[M+H]^+$; found: 2377.9) Trace 3 (the third ligation step): peak h: H-CSDSE-HAAPF-Oglc-FG-NH_2 (identical to peak a); peak g: remaining Ac-**EL21**-COSH after ligation; peak i: ligation product Ac-**EF31**-CO-OglcFG-NH_2 (*m/z* calcd: 3666.7 $[M+H]^+$; found: 3667.6).

site. The utility of subtiligase in enzymatic protein synthesis was highlighted in the synthesis of a ribonuclease analogue.[10] Third, because the thioacid functionality is orthogonal to subtiligase-catalyzed ligation and the glycolate ester is orthogonal to mini-thiol capture ligation, no temporary N or C-terminal protecting groups are needed for the intermediate building blocks in this N-to-C sequential chemoenzymatic ligation strategy, and the newly isolated ligation product from any of the intermediate steps can be used directly for subsequent ligation in the following step. Although our model study demonstrated the synthesis of only a medium-sized peptide, we believe that with the proven value of subtiligase and mini-thiol capture ligation, such combined use of chemical and enzymatic methods holds great promise in synthetic protein chemistry.

In conclusion, the hydrothiolysis reaction of peptide thioesters in aqueous solution provides a convenient and highly efficient method for the preparation of peptide thioacids. Since peptide thioesters can be easily obtained from the various currently available synthetic methods, this reaction can potentially become the method of choice for the preparation of peptide thioacids that are the key building blocks for peptide and protein synthesis by using, for example, mini-thiol capture ligation. Furthermore, the stability of a thioacid group to subtiligase makes it possible to conduct sequential chemical and enzymatic ligations of unprotected peptide segments. This is the first time that an enzymatic ligation method and a chemical ligation method have been used in combination, and the synthesis of a model medium-sized peptide has demonstrated the feasibility of this sequential chemoenzymatic ligation strategy and points to its potential in protein synthesis. The results that were obtained herein are likely to stimulate the development of novel protein synthesis strategies based on the use of peptide thioacids in the future.

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Keywords: peptide ligation · peptide synthesis · subtiligase · thioacid · thioester

[1] J. Blake, *Int. J. Pept. Protein Res.* **1981**, *17*, 273.
[2] J. Blake, C. H. Li, *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 4055.
[3] D. Yamashiro, C. H. Li, *Int. J. Pept. Protein Res.* **1988**, *31*, 322.
[4] M. Schnolzer, S. B. H. Kent, *Science* **1992**, *256*, 221.
[5] C. F. Liu, C. Rao, J. P. Tam, *Tetrahedron Lett.* **1996**, *37*, 933.
[6] R. V. Kolakowski, N. Shangguan, R. R. Sauers, L. J. Williams, *J. Am. Chem. Soc.* **2006**, *128*, 5695.
[7] a) X. Li, T. Kawakami, S. Aimoto, *Tetrahedron Lett.* **1998**, *39*, 8669; b) R. Ingenito, E. Bianchi, D. Fattori, A. Pessi, *J. Am. Chem. Soc.* **1999**, *121*, 11369; c) Y. Shin, K. A. Winans, B. J. Backes, S. B. H. Kent, J. A. Ellman, C. R. Bertozzi, *J. Am. Chem. Soc.* **1999**, *121*, 11684; d) A. Sewing, D. Hilvert, *Angew. Chem.* **2001**, *113*, 3503; *Angew. Chem. Int. Ed.* **2001**, *40*, 3395; e) A. R. Mezo, R. P. Cheng, B. Imperiali, *J. Am. Chem. Soc.* **2001**, *123*, 3885; f) J. Brask, F. Albericio, K. J. Jensen, *Org. Lett.* **2003**, *5*, 2951; g) R. Von Eggelkraut-Gottanka, A. Klose, A. G. Beck-Sickinger, M. Beyermann, *Tetrahedron Lett.* **2003**, *44*, 3551; h) P. Botti, M. Villain, S. Manganiello, H. Gaertner, *Org. Lett.* **2004**, *6*, 4861; i) T. Kawakami, M. Sumida, K. Nakamura, T. Vorherr, S. Aimoto, *Tetrahedron Lett.* **2005**, *46*, 8805; j) J. A. Camarero, A. R. Mitchell, *Protein and Peptide Letters* **2005**, *12*, 723; k) Y. Ohta, S. Itoh, A. Shigenaga, S. Shintaku, N. Fujii, A. Otaka, *Org. Lett.* **2006**, *8*, 467.
[8] a) L. E. Canne, S. M. Walker, S. B. H. Kent, *Tetrahedron Lett.* **1995**, *36*, 1217; b) H. Gaertner, M. Villain, P. Botti, L. Canne, *Tetrahedron Lett.* **2004**, *45*, 2239; c) A. W. Schwabacher, T. L. Maynard, *Tetrahedron Lett.* **1993**, *34*, 1269.
[9] a) L. Abrahmsen, J. Tom, J. Burnier, K. A. Butcher, A. Kossiakoff, J. A. Wells, *Biochemistry* **1991**, *30*, 4151; b) T. K. Chang, D. Y. Jackson, J. P. Burnier, J. A. Wells, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 12544.
[10] D. Y. Jackson, J. Burnier, C. Quan, M. Stanley, J. Tom, J. A. Wells, *Science* **1994**, *266*, 243.
[11] X.-H. Tan, A. Wirjo, C.-F. Liu, *ChemBioChem* **2007**, *8*, 1512.
[12] S. Licht, F. Forouzan, K. Longo, *Anal. Chem.* **1990**, *62*, 1356.
[13] V. K. Sarin, S. B. H. Kent, J. P. Tam, R. B. Merrifield, *Anal. Biochem.* **1981**, *117*, 147.
[14] D. S. Kemp, N. G. Galakatos, B. Bowen, K. Tan, *J. Org. Chem.* **1986**, *51*, 1829.
[15] T. Wieland, E. Bokelmann, L. Bauer, H. U. Lang, H. Lau, *Justus Liebigs Ann. Chem.* **1953**, *583*, 129.
[16] a) G. Pellon, *Biochim. Biophys. Acta Gen. Subj.* **1986**, *883*, 389; b) P. A. Ellison, P. C. Engel, *Biochem. Soc. Trans.* **1987**, *15*, 236.

[17] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent, *Science* **1994**, *266*, 776.
[18] J. P. Tam, Y. A. Lu, C. F. Liu, J. Shao, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 12485.
[19] P. E. Dawson, M. Churchill, M. R. Ghadiri, S. B. H. Kent, *J. Am. Chem. Soc.* **1997**, *119*, 4325.
[20] a) S. Chong, F. B. Mersha, D. G. Comb, M. E. Scott, D. Landry, L. M. Vence, F. B. Perler, J. Benner, R. B. Kucera, C. A. Hirvonen, J. J. Pelletier, H. Paulus, M.-Q. Xu, *Gene* **1997**, *192*, 271–281; b) T. W. Muir, D. Sondhi, P. A. Cole, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6705.
[21] E. C. B. Johnson, S. B. H. Kent, *J. Am. Chem. Soc.* **2006**, *128*, 6640.
[22] X. Zhang, C.-F. Liu, unpublished results.

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