

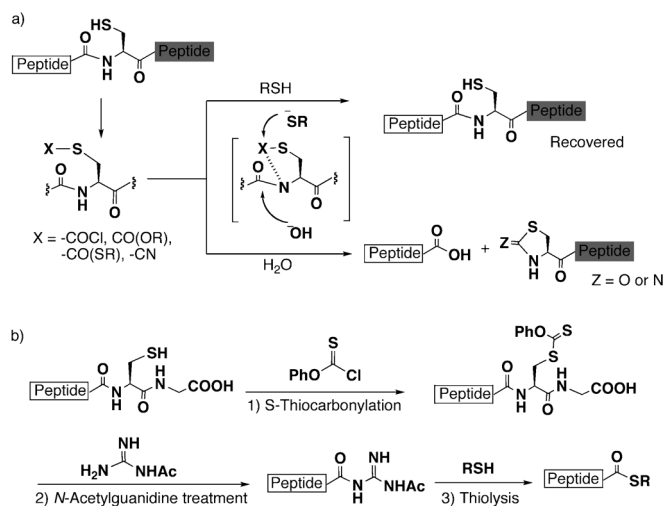
Peptide Synthesis

A Synthetic Approach to a Peptide α -Thioester from an Unprotected Peptide through Cleavage and Activation of a Specific Peptide Bond by *N*-Acetylguanidine

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In modern procedures for total chemical protein synthesis, the concept of chemical ligation plays an essential role in the assembly of target protein polypeptide chains.^[1] The peptide α -thioester is the key component for chemical ligation such as native chemical ligation (NCL), direct segment coupling methods, or traceless Staudinger ligation.^[2] Thus, substantial effort has been expended on the establishment of peptide- α -thioester synthesis based on conventional Boc or Fmoc solid-phase peptide synthesis (SPPS, Boc = *tert*-butoxycarbonyl, Fmoc = 9-fluorenylmethoxycarbonyl).^[3] However, because of the inherent limitations of SPPS, synthesis of peptide α -thioesters with more than 50 amino acids (aa) is still challenging. For the preparation of such a polypeptide α -thioester, an expression method using the intein system has come to be recognized as a robust technology with the capacity to provide polypeptide α -thioesters of more than 50 aa.^[4] Inspired by the biological system, we explored an intein-like chemical methodology for preparing a long peptide α -thioester by using a native (unprotected) peptide as the starting material. Some groups recently reported thioesterification of *E. coli*-expressed peptides using acid treatment, but these intriguing methods lead to epimerization of the C-terminal amino acid residue and still have sequence limitations.^[5] Thus we set out to find a widely usable new methodology.

The key point was the manipulation of an unprotected peptide to install a C-terminal α -thioester. This task appears to require the selective activation of a native amide bond and subsequent thiolysis. Recently, several groups reported elegant methods of activating the peptide backbone to install an α -thioester at the peptide C terminus.^[6] In these methods, the activation of the peptide bond was performed by a selective acylation strategy of an *N* α -amide nitrogen atom at a specific amino acid residue. To examine the thioesterification of unprotected peptides, we focused on the cysteine (Cys) residue, because Cys possesses a thiol group, which might be more easily modified than the other amino acid side chains and thus selectively induce *N*-acylation. In fact, selective peptide-cleavage methods at the Cys residue employing an *N*-



Scheme 1. a) Conventional strategy for the cleavage of a peptide bond based on a selective S-carbonylation of the Cys residue. b) General scheme for the new synthetic strategy for the synthesis of peptide α -thioesters through selective activation of the Cys residue and subsequent treatment with *N*-acetylguanidine.

acylation strategy have been reported for protein sequencing (Scheme 1 a).^[7] In these methods, *N*-acylation is induced by an electrophilic auxiliary group introduced on a Cys side chain (e.g. S-carbonyl group), and this auxiliary group provides the activation of the peptide bond and subsequent hydrolysis. These reports encouraged us to hypothesize the feasibility of the selective activation of a native amide bond at the Cys residue and subsequent thiolysis, instead of hydrolysis, to obtain a peptide α -thioester. However, our preliminary examination of thiolysis for the activated Cys residue did not proceed and regenerated unprotected peptide substrate or hydrolyzed product instead of the desired peptide α -thioester (Scheme 1 a). Therefore, we have explored a new nucleophile as an alternative to a thiol for the cleavage of the native peptide bond and fortuitously discovered the utility of *N*-acetylguanidine. Guanidine is known as a very strong base and a good nucleophile, so its monoacetylated form was expected to possess mild nucleophilicity. Furthermore, it has already been shown that one of the acyl groups on a diacylated guanidino group is ready to deprotect under mild basic conditions, while this is not the case for a monoacylated guanidino group.^[8]

We hypothesized that *N*-acetylguanidine would provide a peptide derivative having *N*-acetylguanidine at the C terminus (peptidyl-*N*-acetylguanidine) after activation of the amide bond at the Cys residue. The resultant peptidyl-*N*-

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acetylguanidine, which possessed a similar diacylated guanidino group, would be converted to a peptide α -thioester under suitable thiolysis conditions. Herein, we report a new synthetic method of peptide- α -thioester formation by using peptidyl-*N*-acetylguanidine and discuss the details of the synthesis of peptidyl-*N*-acetylguanidine by activation of a specific amide bond based on an *N*-acylation strategy at the Cys residue. As a consequence, we also discuss the unique ability of *N*-acetylguanidine to serve as a new leaving group for a thiolysis reaction, as well as for NCL.

As shown in Scheme 1b, we designed a new thioester synthesis consisting of three steps: the selective S-thiocarbonylation of a β -thiol group on a cysteine residue to provide a phenyl xanthate group (Scheme 1b1), treatment of the resultant peptide with *N*-acetylguanidine (Scheme 1b2), and a subsequent thiolysis reaction (Scheme 1b3). As shown in Scheme 1b1, we introduced the thiocarbonyl group as an electrophilic auxiliary group by using *O*-phenyl chlorothionoformate. Because the electron density on a thiocarbonyl group tends to localize around a sulfur atom, we envisioned that the modification would enable the $N\alpha$ -amide nitrogen atom to interact with the thiocarbonyl carbon atom.

At first, we undertook the synthesis of peptide α -thioesters having a variety of C-terminal amino acids to verify the strategy (Table 1). The sequence of the model peptide was Ac-VYAXCG-OH, where the X was Ala, Ser, Phe, Leu, or Lys. We thought if the peptide had a free Cys residue at the

Table 1: Yield of isolated product of each reaction step of the new peptide- α -thioester synthesis using the model peptide Ac-VYAXCG-OH.^[d]

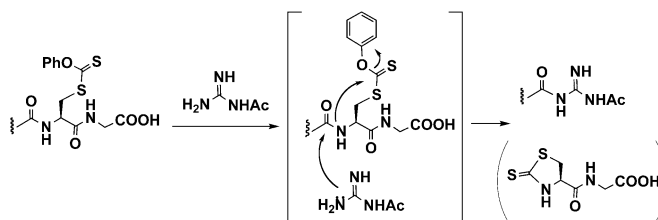
X	Ala	Ser	Phe	Leu	Lys
Step 1 ^[a]	88% (1a)	92% (1b)	84% (1c)	92% (1d)	94% (1e)
Step 2	70% (2a)	65% (2b)	70% (2c)	73% (2d)	71% (2e) ^[b]
Step 3	72% (3a)	63% (3b) ^[c]	66% (3c)	82% (3d)	72% (3e)

[a] Each step 1–3 corresponds to the numbers shown in Scheme 1b. Reagents and conditions: 1) *O*-phenyl chlorothionoformate, pH 5.0 buffer (6 M Gu-HCl, 0.2 M sodium phosphate), acetonitrile, 2) 0.5 M *N*-acetylguanidine in DMSO, 3) pH 7.3 buffer (6 M Gu-HCl, 0.2 M sodium phosphate), MESNa (5 % wt/wt) at 37 °C; [b] Yield after the two steps of Boc protection and treatment with *N*-acetylguanidine. [c] The product was unexpectedly obtained as mixture of diastereomers; see the Supporting Information for details. [d] V = Val = valine, Y = Tyr = tyrosine, A = Ala = alanine, C = Cys = cysteine, G = Gly = glycine, S = Ser = serine, F = Phe = phenylalanine, L = Leu = leucine, K = Lys = lysine.

C terminus, then two intramolecular reactions would compete. The desired intramolecular reaction is the reaction between the $N\alpha$ -amide nitrogen atom of the Cys residue and the Cys β -thiol. The undesired intramolecular reaction is the competitive cyclization between a carboxylate of the Cys residue and the same thiocarbonyl group. Thus, we installed a Gly residue at the C terminus next to the Cys residue to avoid this undesired reaction.

The selective S-thiocarbonylation of a Cys residue was successfully performed by using *O*-phenyl chlorothionoformate in pH 5.0 buffer solution containing acetonitrile

(Scheme 1b1). These conditions afforded S-thiocarbonylated peptide in excellent yield, even in the case of X = Lys, which had a potentially reactive amino group (Table 1, **1e**, 94 % yield of isolated product, see the Supporting Information Figure S-1 for details). Subsequent treatment with *N*-acetylguanidine (0.5 M) was performed in DMSO. Monitoring the reaction by reversed-phase (RP) HPLC revealed that this reaction afforded the peptidyl-*N*-acetylguanidine within several hours (ca. 70 % yield of isolated product, Table 1, **2a–2e**). In the case of X = Lys, we found that selective *N*-Boc protection is essential to avoid side reaction of the amino group (see Figure S-2 in the Supporting Information for details). The selective *N*-Boc protection was carried out by using Boc-*O*-succinimide (Boc-OSu), as reported by Aimoto and Hojo.^[9] This step enabled us to perform *N*-Boc protection of the Lys- ϵ -amino group of Ac-VYAKC(C(=S)OPh)G and the subsequent *N*-acetylguanidine treatment, and we obtained the peptidyl-*N*-acetylguanidine in good yield (71 % yield of isolated product in two steps, Table 1 **2e**). Because we did not observe an activated intermediate having a cyclic structure at the Cys position provided by intramolecular *N*-acylation during these *N*-acetylguanidine treatment, we speculated that this reaction is carried out by the concerted reaction of a nucleophilic attack of *N*-acetylguanidine and an intramolecular *N*-acylation as suggested in Scheme 2. To date



Scheme 2. Presumable reaction mechanism of the *N*-acetylguanidine treatment.

we could not perform the reaction in aqueous solutions but only in organic solvents such as DMSO or DMF. We hypothesized that the nucleophilicity of *N*-acetylguanidine was suppressed in the aqueous solution (e.g. by hydrogen bonds or protonation). In terms of stability of peptidyl-*N*-acetylguanidines, these compounds are stable in neutral and slightly basic solution but exhibit slow decomposition in acidic aqueous conditions (0.1 % trifluoroacetic acid solution). Therefore, the peptidyl-*N*-acetylguanidine needs to be lyophilized immediately after HPLC purification.

Then, the afforded peptidyl-*N*-acetylguanidine was subjected to a thiolysis reaction (Scheme 1b3). The reaction was performed in a buffer solution (pH 7.3) containing guanidine hydrochloride (6 M, Gu-HCl), sodium phosphate (0.2 M) and a high concentration of sodium 2-mercaptoethanesulfonate (MESNa, 5 % wt/wt). In this step, all of the peptides afforded the corresponding peptide α -thioester in good yield (Table 1, **3a–3e**), although a few side reactions were observed, such as deacetylation of *N*-acetylguanidine by the nucleophilic attack of MESNa or H₂O and hydrolysis of the *N*-acetylguanidine group. We speculate that the regioselectivity of this thiolysis reaction comes from the electrophilic difference between

corresponding carbonyl groups in peptidyl-*N*-acetylguanidine and the acetyl group of *N*-acetylguanidine.

Because the synthesis of the peptide α -thioester was demonstrated with the corresponding peptidyl-*N*-acetylguanidine, we estimated the degree of epimerization afforded during this new reaction by a comparison with authentic peptides having a D-amino acid at position X. These authentic peptides were synthesized according to a reported peptide- α -thioester synthetic method,^[10] which is a simple coupling reaction of a fully side-chain-protected peptide α -carboxylic acid and a nucleophile (*N*-acetylguanidine, in this case). As shown in Table 2, in the case of X = Ala, there was no epimerization and the maximum epimerization ratio was 2.5% (X = Phe). Indeed, Phe is known as an amino acid relatively prone to epimerization, and thus these results indicate that our new

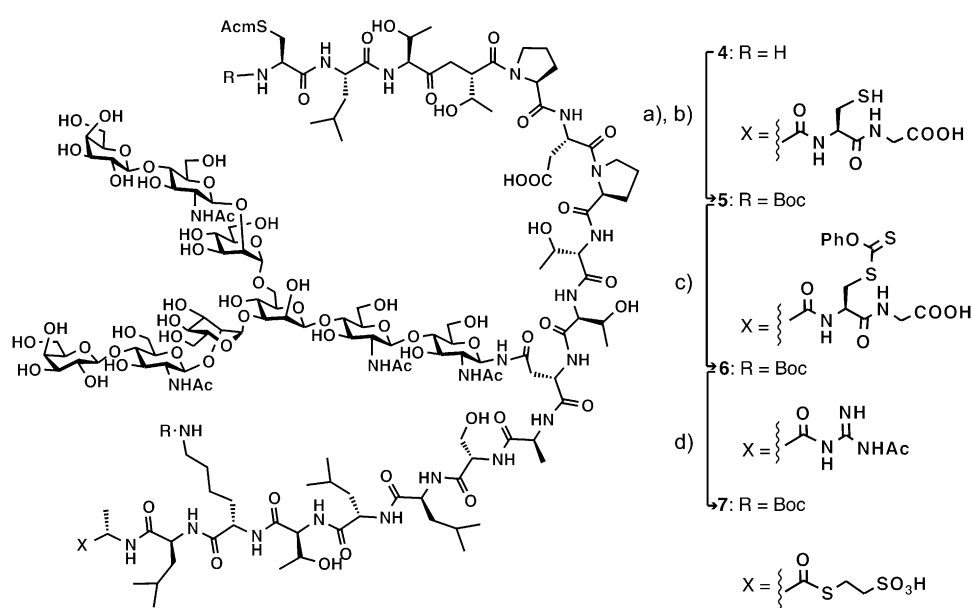
Table 2: Confirmation of the degree of epimerization after the *N*-acetylguanidine treatment step in the obtained Ac-VYAX-NACGu.

X	Ala	Ser	Phe	Leu
Epimerization [%] ^[a]	none	1.1	2.5	0.67

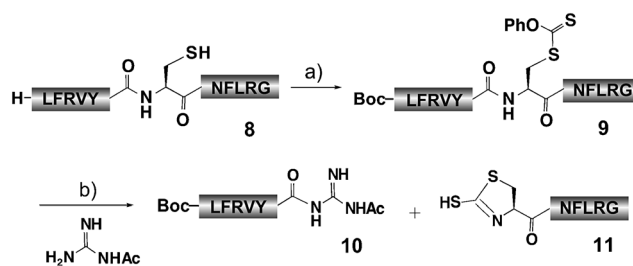
[a] The ratios were obtained from the peak area in the HPLC chromatogram.

methodology did not afford substantial epimerization. Because our new methodology could be applied to unprotected peptides, we also synthesized a glycopeptide α -thioester having an asialo-complex-type nonasaccharide.^[11] Sequential Boc-protection, S-thiocarbonylation, and *N*-acetylguanidine treatment of glycopeptide **4** successfully afforded the desired glycopeptidyl-*N*-acetylguanidine **6** (24% yield of isolated product after three steps, Scheme 3 a–c, see the Supporting Information for details). This compound was easily converted into corresponding glycopeptide α -thioester **7** (Scheme 3 d, see the Supporting Information for details).

To obtain insight into the reaction of the *N*-acetylguanidine treatment step, we applied the reaction for 11-aa peptide **9**, which has an S-thiocarbonylated Cys residue in the middle of the peptide sequence. This reaction provided peptidyl-*N*-acetylguanidine **10** as well as N-terminal cyclized peptide **11** (Scheme 4). NMR spectroscopy analysis revealed that the structure of **11** was in a 2-mercaptothiazolidine form rather than in a 2-thioxothiazolidine form (for details see the Supporting Information). On the other hand, we also



Scheme 3. Synthesis of a *N*-glycopeptide α -thioester through the corresponding *N*-glycopeptidyl-*N*-acetylguanidine. a) *N*-Boc protection, b) S-thiocarbonylation, c) *N*-acetylguanidine treatment, and d) trans-thioesterification by MESNa. Ac = acetamidomethyl.



Scheme 4. Synthesis of peptidyl-*N*-acetylguanidine **10** and presumable 2-mercaptothiazolidinyl peptide **11** from an 11-aa peptide having a Cys residue in the middle of the peptide: a) *N*-Boc protection and subsequent S-thiocarbonylation, b) *N*-acetylguanidine treatment. R = Arg = arginine, N = Asn = asparagine.

performed NMR spectroscopy analysis of the peptidyl-*N*-acetylguanidine product. We observed a methyl signal of the acetyl group on the *N*-acetylguanidino group but did not observe any evident proton peaks of the C-terminal guanidine moiety. We speculated that the protons might move between several positions, indicating that the product exists in a mixture of several forms (details are discussed in the Supporting Information).

At this point, because the peptidyl-*N*-acetylguanidine could be converted to the corresponding peptide α -thioester, we anticipated the direct use of the peptidyl-*N*-acetylguanidine for NCL. We used peptidyl-*N*-acetylguanidine (Ac-VYAS-NACGu) or peptide α -thioester (Ac-VYAS- α -thioester) to evaluate the reactivity of the *N*-acetylguanidine as a leaving group for the ligation with the peptide CFRA. The results are shown in Figure 1. It is known that NCL usually requires a thiol additive for the conversion of a low reactive thioester such as 3-mercaptopyriononyl-Leu (MPAL) thioester^[3a] into a reactive thioester, such as 4-mercaptophenyl-

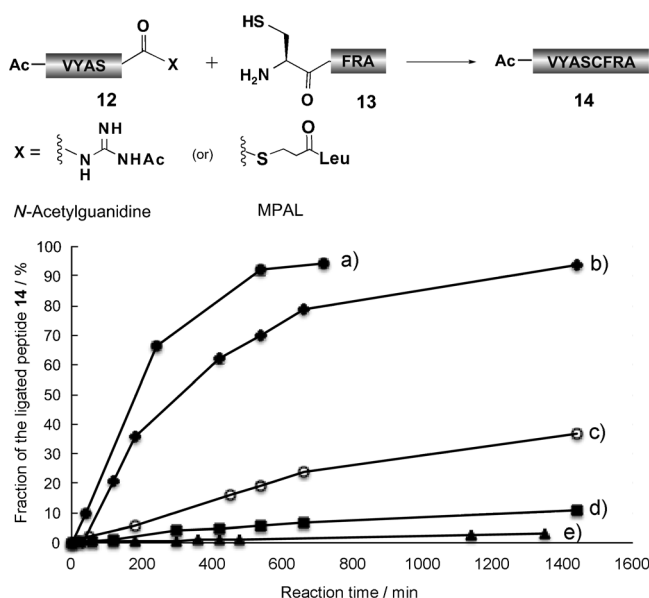


Figure 1. Comparison of reactivity of peptidyl-*N*-acetylguanidine and a conventional peptide α -thioester in NCL. NCL between CFRA and Ac-VYAS-X, where X = *N*-acetylguanidine or X = SCH₂CH₂CO-Leu, was performed at pH 7.0–7.1 in buffer with thiol additive as shown below. At each time point, the fraction of the ligated product was determined by analytical RP-HPLC monitored by UV absorbance at 220 nm as a fraction of the sum of starting materials and ligation product; a) MPAL in the presence of 20 mM MPAA (●), b) *N*-acetylguanidine in the presence of 200 mM MESNa (◆), c) MPAL without thiol (○), d) *N*-acetylguanidine in the presence of 200 mM MPAA (■), e) *N*-acetylguanidine in the presence of 20 mM MPAA (▲).

acetic acid (MPAA) thioester.^[12] Thus, we performed the ligation reaction between Ac-VYAS-*N*AcGu and CFRA under three conditions using buffer solutions of 20 or 200 mM MPAA or with no thiol additive (Figure 1). We found that the *N*-acetylguanidine group exhibited very slow ligation with CFRA. To our surprise, even in the case of 20 or 200 mM MPAA, the reaction afforded a ligation product of only 3 or 11 % after 24 h, respectively (Figure 1 e, d).

These results were quite impressive, because usually the peptide α -thioester is ready for trans-thioesterification by an exogenous thiol. Indeed, when we performed this reaction using MPAL thioester, which is one of the common peptide α -thioesters for NCL, the reaction was essentially completed within 9 h in the presence of MPAA (20 mM, Figure 1 a) and afforded the ligated peptide even without thiol additive (33 % after 24 h, Figure 1 c).

Since we had already found that the peptidyl-*N*-acetylguanidine could be converted into the corresponding MESNa thioesters, we also performed the ligation between the peptides Ac-VYAS-*N*AcGu and CFRA in the presence of MESNa (200 mM). In contrast to the result obtained with MPAA (Figure 1 d), the reaction was essentially completed in 24 h (Figure 1 b). Thus, we concluded the peptidyl-*N*-acetylguanidine is much less reactive than the corresponding thioester but is suitable for thiolysis under certain conditions.

At last, this finding prompted us to utilize the peptidyl-*N*-acetylguanidine for kinetically controlled ligation (KCL), reported by Bang et al.^[13] KCL is a chemoselective ligation

between the peptide 1 α -thioester and a Cys-peptide 2 α -thioester affording a peptide 1–peptide 2 α -thioester utilizing the intrinsic difference in reactivity between an aryl thioester (e.g. thiophenylester) for peptide 1 and an α -alkyl thioester (e.g. an MPAL thioester) for Cys-peptide 2. Durek et al. reported that the KCL reaction can generate peptide α -thiolactone or branched peptide thioester by the reaction between an internal cysteine and a C-terminal thioester moiety.^[14] This reaction takes place because generally KCL is performed without addition of exogenous thiol, and thus these peptides cannot be resolved and reversed into the reactive thioester derivatives. As we mentioned, because the peptidyl-*N*-acetylguanidine is much less reactive than the α -thioester (Figure 1), we thought the peptide MPAL thioester would ligate selectively with Cys-peptidyl-*N*-acetylguanidine even in the presence of MPAA (20 mM) without side reactions.

To test this hypothesis, we carried out the ligation between an 11-aa-peptide MPAL thioester and 24-aa Cys-glycopeptidyl-*N*-acetylguanidine (Figure 2). In this case we prepared the latter glycopeptide derivative according to our reported method,^[10] as mentioned in the synthesis of the D-amino-acid-containing peptidyl-*N*-acetylguanidine. The ligation reaction was performed in sodium phosphate (0.2 M) buffer solution containing Gu-HCl (6 M), tris(2-carboxyethyl)phosphine (20 mM, TCEP), and MPAA (20 mM) at pH 7.0. As expected, we could perform the selective ligation reaction as well as the subsequent trans-thioesterification of the *N*-acetylguanidine moiety by treatment with a high concentration of MESNa (10 % wt/wt), and we obtained 35-aa-peptide MESNa α -thioester **18** (see the Supporting Information for details). Thus, we demonstrated the utility of peptidyl-*N*-acetylguanidine for a new type of KCL reaction, which can be performed in a buffer containing exogenous thiol additive.^[15]

In conclusion, we report a new method for activating the C terminus of a peptide and the new functional group *N*-acetylguanidine as an alternative to the thioester group. The synthesis of peptidyl-*N*-acetylguanidine was performed through selective S-thiocarbonylation and subsequent treatment with *N*-Boc protection of both amino groups of the Lys side chains and of the N terminus prior to the *N*-acetylguanidine treatment step are necessary to avoid side reaction during this new reaction sequence. It is noteworthy that the resultant peptidyl-*N*-acetylguanidine is much less reactive for NCL than a corresponding thioester, even in the presence of a thiol (e.g. 20 mM MPAA, Figure 1 e). This reactivity enabled us to perform NCL between a peptide alkyl thioester and Cys-peptidyl-*N*-acetylguanidine and to obtain selectively ligated peptide at the alkyl thioester with the other Cys moiety. Finally, considering the successful demonstration of the utility of this methodology, we envision that this method can be applied to a polypeptide prepared by an *E. coli* expression system as the starting material. These investigations are now underway.

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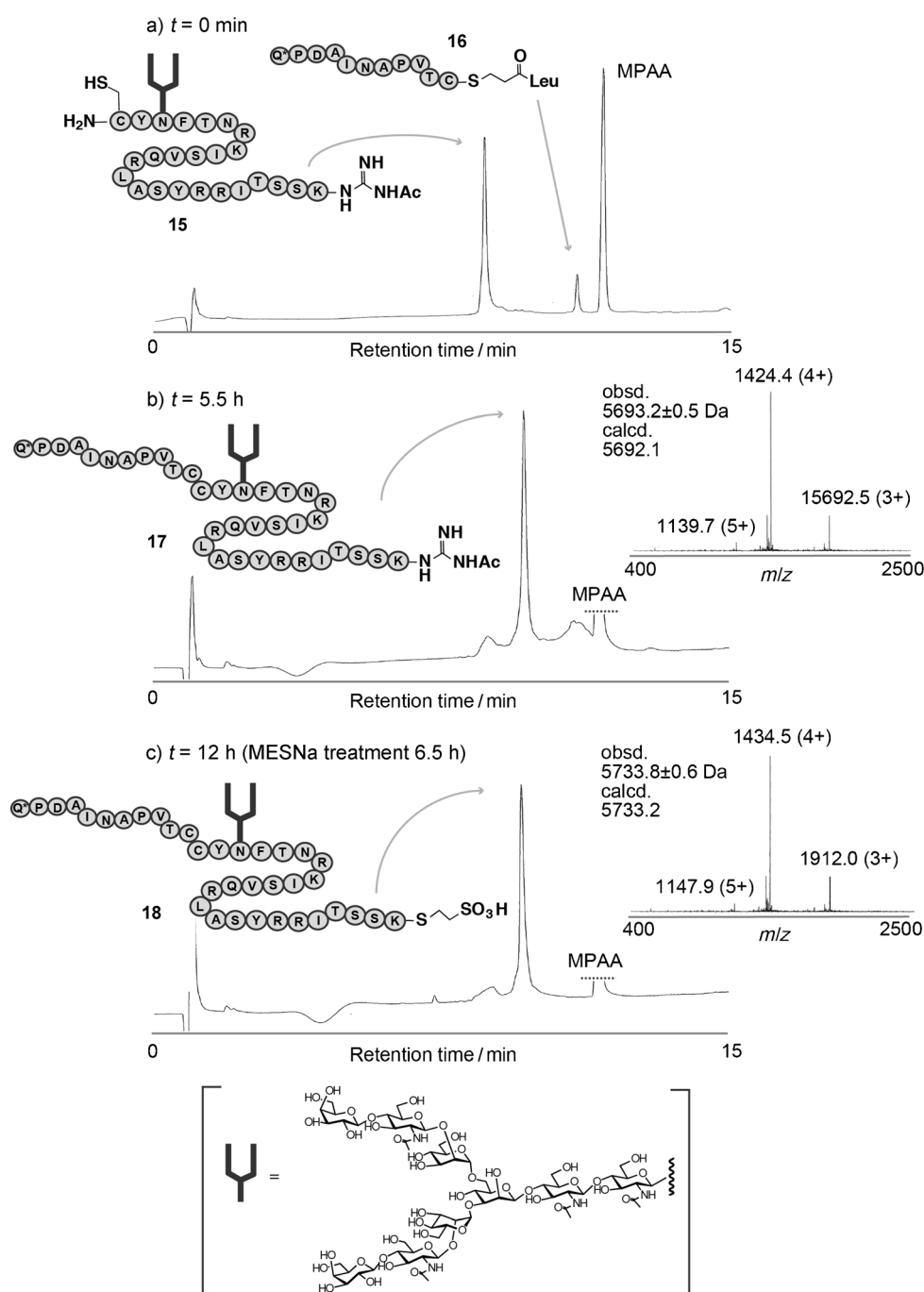


Figure 2. KCL between the peptide MPAL thioester and Cys-glycopeptidyl-*N*-acetylguanidine. The reaction was monitored by RP-HPLC: a) $t = 0$ min, b) $t = 5.5$ h, and c) $t = 12$ h in the total reaction time. After completion of the KCL (b) $t = 5.5$ h, MESNa was added to the reaction (10% wt/wt) and the 35-aa glycopeptidyl-*N*-acetylguanidine **17** was efficiently converted into the corresponding MESNa thioester **18** in 6.5 h (c), total reaction time was 12 h. Each inset is the ESI mass spectrum of each peak. Q* = pyroglutamine, Q = Gln = glutamine, P = Pro = proline, D = Asp = aspartic acid, I = Ile = isoleucine, T = Thr = threonine.

Keywords: glycopeptides · kinetically controlled ligation · native chemical ligation · peptides · thioesters

- [3] a) M. Schnölzer, P. Alewood, A. Jones, D. Alewood, S. Kent, *Int. J. Pept. Res. Ther.* **2007**, *13*, 31–44; b) F. Mende, O. Seitz, *Angew. Chem.* **2011**, *123*, 1266–1274; *Angew. Chem. Int. Ed.* **2011**, *50*, 1232–1240.
- [4] T. W. Muir, D. Sondhi, P. A. Cole, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6705–6710.
- [5] For recent advance of the thioesterification of *E. coli*-expressed peptide see: a) J. Kang, D. Macmillan, *Org. Biomol. Chem.* **2010**, *8*, 1993–2002; b) J. Masania, J. Li, S. J. Smerdon, D. Macmillan, *Org. Biomol. Chem.* **2010**, *8*, 5113–5119; c) T. Kawakami, S. Shimizu, S. Aimoto, *Bull. Chem. Soc. Jpn.* **2010**, *83*, 570–574; d) G.-M. Fang, Y.-M. Li, F. Shen, Y.-C. Huang, J.-B. Li, Y. Lin, H.-K. Cui, L. Liu, *Angew. Chem.* **2011**, *123*, 7787–7791; *Angew. Chem. Int. Ed.* **2011**, *50*, 7645–7649.
- [6] a) J. B. Blanco-Canosa, P. E. Dawson, *Angew. Chem.* **2008**, *120*, 6957–6961; *Angew. Chem. Int. Ed.* **2008**, *47*, 6851–6855; b) T. Kawakami, S. Aimoto, *Tetrahedron* **2009**, *65*, 3871–3877; c) A. P. Tofteng, K. K. Sørensen, K. W. Conde-Frieboes, T. Hoeg-Jensen, K. J. Jensen, *Angew. Chem.* **2009**, *121*, 7547–7550; *Angew. Chem. Int. Ed.* **2009**, *48*, 7411–7414.
- [7] a) Y. Degani, A. Patchornik, J. A. Maclaren, *J. Am. Chem. Soc.* **1966**, *88*, 3460–3461; b) G. R. Stark in *Methods in Enzymology*, Vol. 47, Academic Press, New York, **1977**, pp. 129–132; c) S. Nakagawa, Y. Tamakashi, T. Hamana, M. Kawase, S. Taketomi, Y. Ishibashi, O. Nishimura, T. Fukuda, *J. Am. Chem. Soc.* **1994**, *116*, 5513–5514; d) M. Tanaka, K. Kajiwarra, R. Tokiwa, K. Watanabe, H. Ohno, H. Tsutsumi, Y. Hata, K. Izumi, E. Kodama, M. Matsuoka, S. Oishi, N. Fujii, *Bioorg. Med. Chem.* **2009**, *17*, 7487–7492.
- [8] L. Zervas, T. T. Otani, M. Winitz, J. P. Greenstein, *J. Am. Chem. Soc.* **1959**, *81*, 2878–2884.
- [9] H. Hojo, S. Aimoto, *Bull. Chem. Soc. Jpn.* **1991**, *64*, 111–117.
- [10] Y. Kajihara, A. Yoshihara, K. Hirano, N. Yamamoto, *Carbohydr. Res.* **2006**, *341*, 1333–1340.

- [1] S. B. H. Kent, *Chem. Soc. Rev.* **2009**, *38*, 338–351.
- [2] C. P. R. Hackenberger, D. Schwarzer, *Angew. Chem.* **2008**, *120*, 10182–10228; *Angew. Chem. Int. Ed.* **2008**, *47*, 10030–10074.

- [11] a) N. Yamamoto, A. Takayanagi, A. Yoshino, T. Sakakibara, Y. Kajihara, *Chem. Eur. J.* **2007**, *13*, 613–625; b) N. Yamamoto, Y. Tanabe, R. Okamoto, P. E. Dawson, Y. Kajihara, *J. Am. Chem. Soc.* **2008**, *130*, 501–510.
- [12] E. C. B. Johnson, S. B. H. Kent, *J. Am. Chem. Soc.* **2006**, *128*, 6640–6646.
- [13] a) D. Bang, B. L. Pentelute, S. B. H. Kent, *Angew. Chem.* **2006**, *118*, 4089–4092; *Angew. Chem. Int. Ed.* **2006**, *45*, 3985–3988; b) J. Lee, Y. Kwon, B. L. Pentelute, D. Bang, *Bioconjugate Chem.* **2011**, *22*, 1645–1649.
- [14] T. Durek, V. Y. Torbeev, S. B. H. Kent, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 4846–4851.
- [15] For the latest new KCL method, see reference [5d].
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