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Kohei Sato,*[a] Shoko Tanaka,[a] Kazuki Yamamoto,[a] Yosuke Tashiro,[ab] Tetsuo Narumi[abc] and Nobuyuki Mase[abc]

We report a simple and promising synthetic method to oxidize peptide hydrazides containing N-terminal thiazolidine as a protected cysteine. This yields the corresponding thioester *via* a peptide azide without decomposition of the thiazolidine ring. The newly developed protocol was validated by the synthesis of the bioactive peptide LacZα.

Chemical synthesis of proteins has attracted considerable attention as an important tool in the field of structural biology,[1] drug discovery[2] and chemical biology.[3] Although the total- or semi-synthesis of a protein remains challenging, recent advances, including chemoselective ligations and use of related protecting groups are bringing the chemical synthesis of proteins into the mainstream in synthetic chemistry. In particular, native chemical ligation (NCL), developed by Kent et al.[4,5] is a powerful technique for the chemoselective ligation between a peptide thioester and an N-terminal cysteinyl peptide, yielding the ligated product with high efficiency. A key to the success of the chemical synthesis of a large protein is how more than two peptide segments can be assembled in a sequential and convergent manner.[6] In sequential NCL strategy, the N-terminal Cys residue of the middle segment must be protected in order to prevent cyclization caused by intramolecular reactions with the thioester moiety (Figure 1). Among the protecting groups that have been developed,[7] the 1,3-thiazolidine-4-carboxo (Thz) group is a reliable choice because of its facile convertibility to Cys and its commercial availability, and it has been applied extensively to the synthesis of various proteins.[6,7c]

Recently, peptide hydrazides, developed by Liu et al.[8] as peptide thioester precursors, are beginning to replace traditional thioesters. In this protocol, a peptide hydrazide is

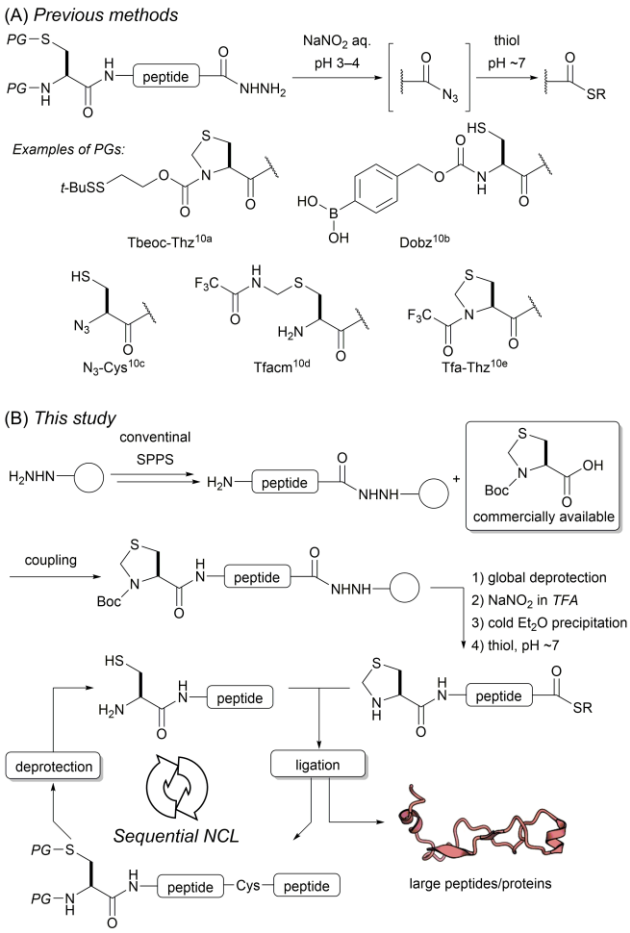

Fig. 1 (A) Conversion of peptide hydrazide bearing protected cysteine to the corresponding thioester. (B) Direct conversion of Thz-peptide hydrazide to the corresponding thioester using TFA as a solvent and subsequent sequential NCLs. PG: protecting groups; Tbeoc: 2-(*tert*-butyldisulfanyl)ethyloxycarbonyl; Dobz: *p*-dihydroxyborylbenzyloxycarbonyl; Tfacm: trifluoroacetamidomethyl; Tfa: trifluoroacetyl.

[a] *Department of Engineering, Graduate School of Integrated Science and Technology, Shizuoka University, 3-5-1 Johoku, Hamamatsu, Shizuoka 432-8561, Japan. Email: sato.kohei@shizuoka.ac.jp*
[b] *Graduate School of Science and Technology, Shizuoka University, 3-5-1 Johoku, Hamamatsu, Shizuoka 432-8561, Japan*
[c] *Research Institute of Green Science and Technology, Shizuoka University, 3-5-1 Johoku, Hamamatsu, Shizuoka 432-8561, Japan*

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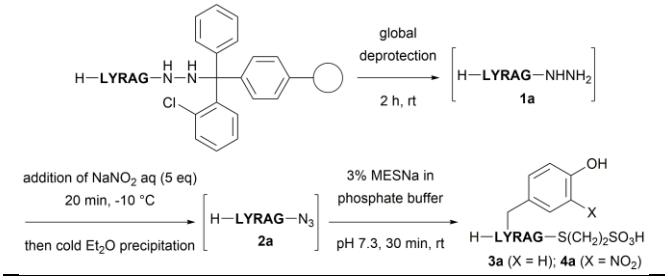
prepared by conventional 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) on hydrazine-incorporated resin and can be converted into a thioester by treatment with $NaNO_2$ under acid conditions, followed by thiolysis of the resulting peptidyl azide (Figure 1A). This technique has seen widespread use and the number of ligations carried out using hydrazides as an acyl donor represents 30% of the total ligations reported in 2016.[9] This method however has not been used widely in the synthesis of N-terminal Thz-peptide thioesters due to the decomposition of the Thz moiety in the hydrazide in the acidic activation step.[10a] To circumvent this problem, alternative protections for Cys have been examined. The functionalized Cys derivatives that are called for by these methods must be prepared (Figure 1A).[10] Thus, there is an increasing demand for the development of practical methods for the direct synthesis of Thz-containing peptide thioesters from peptide hydrazides (Figure 1B).

Peptides with a Thz group are stable during cleavage reactions mediated by HF or TFA, and the moiety can be converted to the corresponding Cys residue under acidic aqueous conditions with $MeONH_2$.[7c] Based on these facts, we speculated that Thz-containing peptide hydrazides would be compatible with the $NaNO_2$-mediated activation step using a TFA-based cocktail. Since TFA is usually used as a cleavage reagent in Fmoc SPPS, we also envisioned that the cleavage and conversion of the hydrazide could be achieved in a one-pot reaction.

In our initial attempts, the peptide hydrazide H-**LYRAG**-$NHNH_2$ (**1a**) lacking a Thz group at N-terminus was used as a substrate (Table 1). The peptide hydrazide was elongated on 2-chlorotrityl chloride resin using a previously reported protocol.[11] The peptidyl resin was treated with TFA-triisopropylsilane (TIS)-H_2O (95:2.5:2.5) to give the peptide hydrazide **1a**. Aqueous $NaNO_2$ solution (approximately 5 equivalents relative to the hydrazide) was added to the resulting mixture, affording the corresponding azide intermediate **2a**. After a 20 min reaction at -10 °C followed by trituration with Et_2O, the formed precipitate was dissolved into a buffer containing sodium 2-mercaptoethanesulfonate (MESNa).

Table 1 Conversion of peptide hydrazide to thioester during global deprotection[a]



entry	global deprotection (*v*/*v*)					HPLC area ratio [%]		
	TFA	TIS	H_2O	*m*-cresol	thioanisole	**1**	**3**	**4**
1	95	2.5	2.5	0	0	58	20	17
2[b]	95	2.5	2.5	0	0	61	16	12
3	90	2.5	2.5	5	0	54	39	0
4	90	2.5	2.5	0	5	0	77	13
5[c]	80	2.5	2.5	5	10	0	87	0
6[de]	80	2.5	2.5	5	10	0	93	0

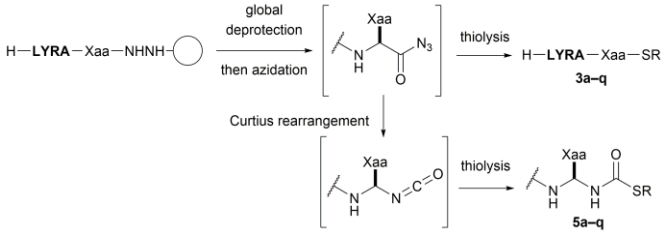
[a] All reactions were performed as follows: the peptidyl resin was treated under global deprotection conditions at rt for 2 h. The reaction mixture was then incubated at -10 °C for 20 min after addition of 10% aqueous $NaNO_2$. The peptide azide precipitated by the addition of cold Et_2O was subjected to thiolysis with MESNa (3% *w*/*w*) in 6 M Gn·HCl, 0.2 M sodium phosphate buffer (pH 7.3) at rt for 0.5 h. The product was analysed by HPLC. [b] The activating step with $NaNO_2$ was performed at 0 °C. [c] 69% isolated yield. [d] N-Terminal Thz-containing peptide (H-**Thz**-**LYRAG**-$NHNH_2$) was used as a substrate. [e] 68% isolated yield. HPLC charts: see ESI.†

HPLC analysis indicated that the desired thioester **3a** had been formed, while the hydrazide **1a** and the nitrated thioester **4a** were also observed (Table 1, entry 1). Higher temperatures during the azidation were not effective (entry 2). The nitration occurs in the phenol ring of Tyr residue, but was not observed during the reaction of peptide hydrazides with $NaNO_2$ in aqueous media.[12] This side reaction was suppressed by the addition of *m*-cresol which is commonly used as an additive in global deprotection of peptides (entry 3). Surprisingly, the addition of thioanisole, which is also used as an additive for peptide deprotection, accelerated the conversion of the hydrazide (entry 4).[13] Use of the both additives resulted in complete conversion and no nitrated product was formed (entry 5). This condition was applied to the conversion of the peptide hydrazide with a Thz group at the N-terminus, successfully producing the N-terminal Thz-containing peptide thioester in 68% isolated yield. Side reactions, including opening of the thiazolidine and subsequent intramolecular or intermolecular ligations were not observed (entry 6). To the best of our knowledge, this is the first example of the direct synthesis of an N-Thz peptide thioester from the peptide hydrazide without any additional protecting groups.

With the optimal conditions in hand, we evaluated the effects of the C-terminal amino acids with the exception of Asp, Asn and Gln, of which thioesters are applicable to NCL reactions[17b] but the corresponding hydrazides cannot be prepared due to intramolecular cyclization between the hydrazide and side-chain during SPPS.[8a] As summarized in Table 2, in all cases, a peptide hydrazides was successfully converted to the corresponding thioester with a modest to high purity (63–95%). Sterically hindered amino acids (Val and Ile) afforded relatively low 70% and 63% yields, respectively, as a result of Curtius rearrangement of the azide to an isocyanate, affording thiocarbamate derivatives (entries 3 and 4).[14] This side reaction also occurred at a higher level than 5% with Glu, Arg and Trp (entries 11, 18 and 19). This probability of formation of a Curtius rearrangement product is consistent with a previous report.[14] The epimerization levels of C-terminal amino acids (Ala, Phe, Ser, Cys, Tyr, and His) were assessed. Peptides containing Ser and His were more sensitive to epimerization whereas the epimerization was suppressed under more acidic thiolysis conditions (entries 8 vs 9 and 15 vs 16).

The synthetic applicability of our protocol was confirmed through the synthesis of the LacZ alpha subunit (LacZα) as shown in Figure 2.[15] Since LacZα has no Cys residue, use of an NCL-desulfurization strategy, formally enabling the assembly of peptides at Xaa-Ala junctions, was envisioned.[16] Peptide

Table 2 Scope of the conversion of peptide hydrazides to peptide thioesters[a]



entry	Xaa	product	HPLC area ratio [%] 3	HPLC area ratio [%] 5[b]	epimerization (L:D) [%][c]
1	Gly	**3a**	87	n.d.	-
2	Ala	**3b**	90	<1	>99:1
3	Val	**3c**	70	8	-
4	Ile	**3d**	63	13	-
5	Leu	**3e**	89	3	-
6	Phe	**3f**	90	2	>99:1
7	Pro	**3g**	89	n.d.	-
8	Ser	**3h**	67	n.d.	88:12
9[d]	Ser	**3h**	90	n.d.	>99:1
10	Thr	**3i**	72	n.d.	-
11	Glu	**3j**	76	7	-
12	Cys	**3k**	79	n.d.	>99:1
13[e]	Met	**3l**	95	<1	-
14	Tyr	**3m**	82	2	>99:1
15	His	**3n**	72	n.d.	84:16
16[d]	His	**3n**	82	n.d.	>99:1
17	Lys	**3o**	93	n.d.	-
18	Arg	**3p**	78	5	-
19	Trp	**3q**	81	10	-

[a] All reactions were performed under similar conditions to that of Table 1 except for global deprotection conditions (TFA-TIS-H_2O-*m*-cresol-thioanisole = 80:2.5:2.5:5:10). [b] n.d. = not detected. [c] Standard material as an epimerized peptide was separately synthesized using the corresponding D-isomer, and epimerization ratio was determined by HPLC analysis. [d] Buffer solution containing 10% MESNa (pH 4) was used. [e] TFA-TIS-H_2O-*m*-cresol-thioanisole = 75:2.5:2.5:5:15 was used as a global deprotection cocktail.

segments **6**–**9** covering the entire sequence of LacZα were prepared as peptide hydrazide for segments **6**–**8** or peptide acid for **9** by the conventional Fmoc-SPPS (Figure 2B). After construction of the peptide segments, the peptide hydrazides were converted to the corresponding thioesters by the established protocol. This procedure afforded the desired peptide thioesters in 31% isolated yield for **6**, 34% for **7** or 64% for **8**. Notably, no decomposition of N-terminal Thz group in segments **7** and **8** during the activating step was observed.

The first NCL between **9** bearing a Cys residue at the N-terminus and **8** with a Glu-thioester at the C-terminus in 6 M guanidine hydrochloride (Gn·HCl), 0.2 M sodium phosphate buffer (pH 7.3) in the presence of 4-mercaptophenylacetic acid (MPAA) (100 mM) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) (50 mM) at 37 °C was complete within 7 h but HPLC analysis of the products of the ligation followed by conversion of Thz to Cys by the addition of $MeONH_2$·HCl showed two peaks with the expected molecular weight of the product **10** (Figure 3A, denoted by **10'**). The formation of γ-linked side products had been observed previously in an NCL at the Glu-Cys

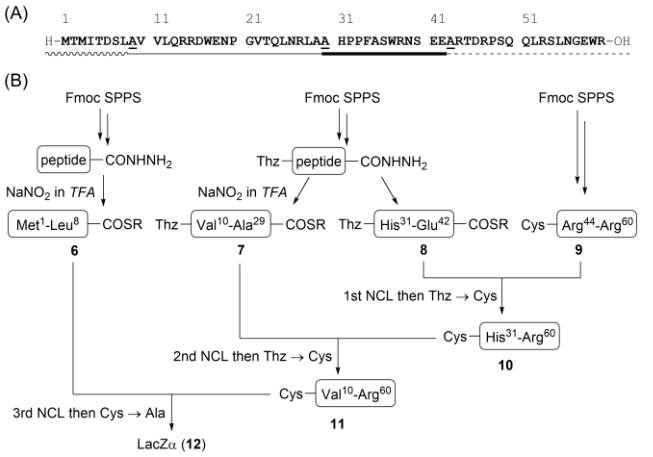


Fig. 2 (A) Primary sequence of synthesized LacZα. (B) Synthetic scheme for LacZα by sequential NCLs-desulfurization.

site.[17] Kent et al. demonstrated that the use of an increased concentration of MPAA and/or high pH can prevent such by-product formation,[17b] but although we tried to optimize ligation conditions, pH or concentration of peptides and MPAA, suppression of the side reaction could not be achieved.

After being purified by HPLC, the ligated peptide **10** was subjected to the next NCLs. We initially envisioned a one-pot reaction consisting of sequential ligations between peptides **7**, **10** and **6** followed by desulfurization, yielding the desired product **12**. Peptides **7** and **10** in 6 M Gn·HCl, 0.2 M sodium phosphate, 40 mM methyl thioglycolate as a non-aryl thiol additive,[18] 20 mM TCEP·HCl (pH 7.2) were coupled smoothly. The coupled material was converted to the corresponding Cys form with the aid of $MeONH_2$·HCl, and the peptide thioester **6** was added to the resulting mixture to yield the full-length polypeptide. Howev er, the thioester **6** reacted preferentially with the residual $MeONH_2$ and no ligated product was obtained. We examined the use of $[Pd(allyl)Cl]_2$ as a reagent to convert Thz to a free Cys,[19] but this did not give the desired product. These results led us revise our synthetic design, which is based on an NCL-deprotection between **7** and **10** followed by removal of $MeONH_2$ and subsequent one-pot NCL-desulfurization between peptide **6** and **11** using imidazole-aided NCL conditions (Figure 2B).[20]

The reaction sequence for the synthesis of LacZα is shown in Figure 3A–C. Ligation between the Thz-containing thioester peptide **7** and Cys-peptide **10** followed by deprotection of the Thz ring was conducted under the optimum conditions, affording the three-component ligated product **11** (Figure 3B). After purification by HPLC, the ligated peptide was subjected to the next NCL reaction with thioester **6** under imidazole-aided NCL conditions, allowing us to conduct subsequent free-radical-based desulfurization of three Cys residues in a one-pot reaction which proceeded efficiently to afford LacZα (**12**) in 85% isolated yield (Figures 3C and D).

Finally, β-galactosidase activity of the complex between the synthesized LacZα and expressed LacZ omega subunit (LacZω) in the conversion of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was evaluated (Figure 4A). Incubation of ONPG with the

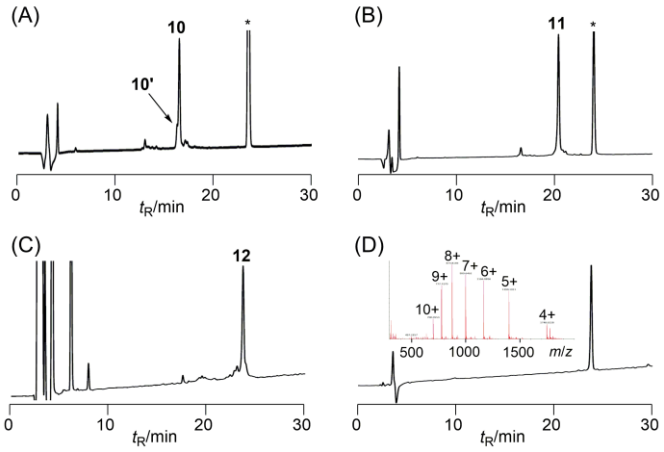


Fig. 3 HPLC analyses of reactions for **12**: (A) ligation of **8** and **9** followed by deprotection of Thz (t = 7 h and 11 h); (B) ligation of **7** and **10** followed by deprotection of Thz (t = 5 h and 3 h); (C) ligation of **6** and **11** followed by desulfurization (t = 11 h and 16 h). (D) HPLC analysis and mass spectrum of purified **12**. The calculated mass is 6990.83 Da. Deconvolution of the mass spectrum yielded an observed mass of 6991.98 Da as a proton adduct. Reaction and HPLC conditions: see ESI.† * MPAA.

synthetic material and bacterial homogenate of *Escherichia coli* DH5α as a source of LacZω resulted in a colour change resulting from the formation of *o*-nitrophenol through the enzymatic reaction, whereas absence of LacZα or LacZω did not affect the colour of the reaction solution (Figure 4B). This result clearly indicated that the synthesized LacZα can exhibit α-complementation.[15]

In summary, we have developed the simple and practical protocol for converting N-Thz-peptide hydrazides into the corresponding thioesters. A key to our developed protocol is the use of TFA as a reaction medium for $NaNO_2$-mediated activation in the presence of thioanisole and *m*-cresol, which can accelerate the desired reaction and suppress the side reaction, respectively. Application of the developed protocol to the synthesis of more-complex proteins will be presented in due course.

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Conflicts of interest

There are no conflicts to declare.

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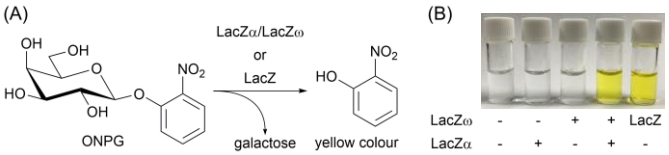

Fig. 4 (A) Enzymatic hydrolysis of ONPG by β-D-galactosidase. (B) α-Complementation assay using ONPG with synthesized LacZα and crude proteins of *E. coli* DH5α expressing LacZω. Full length of LacZ expressed by *E. coli* W3110 was used as a positive control.