Resin-bound crypto-thioester for native chemical ligation

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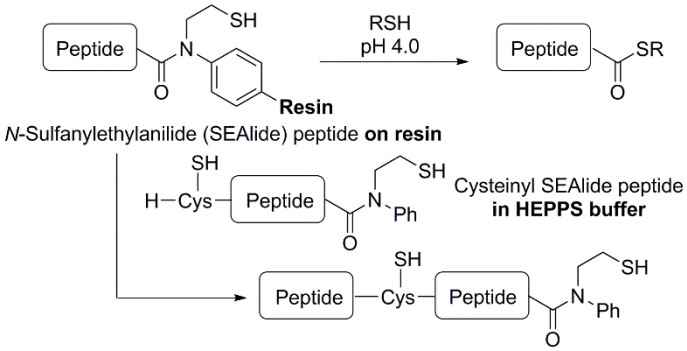
Supporting Information Placeholder



ABSTRACT: Resin-bound *N*–sulfanylethylanilide (SEAlide) peptide was found to function as a crypto-thioester peptide. Exposure of the peptide resin to an aqueous solution under neutral conditions in the presence of thiols affords thioesters without accompanying racemization of C-terminal amino acids. Furthermore, the resin-bound SEAlide peptides react with N-terminal cysteinyl peptides in the absence of phosphate salts to afford ligated products, whereas soluble SEAlide peptides do not. This unexpected difference in reactivity of the SEAlide peptides allows for a one-pot/three-fragment ligation using resin-bound and unbound peptides.

N–Sulfanylethylanilide (SEAlide) peptide **1** as a 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry compatible crypto-thioester was developed, and its utility in native chemical ligation (NCL)[1] has been extensively examined by our group.[2] In the early examination,[2a] N–S acyl-transfer-mediated conversion of the amide-type SEAlide peptide **1** to the corresponding thioester-type SEAlide **2** was found to be effected by acidic treatment such as 4 M HCl in DMF (Fig. 1). This early finding was applied to the thiolytic release of an alkylthioester peptide from resin-bound SEAlide peptide, in which treatment with 4 M HCl in DMF, followed by thiolytic release using sodium sulfanylethanesulfonate under neutral conditions was used. However, racemization of C-terminal amino acids was accompanied by the acidic treatment involved in this protocol.

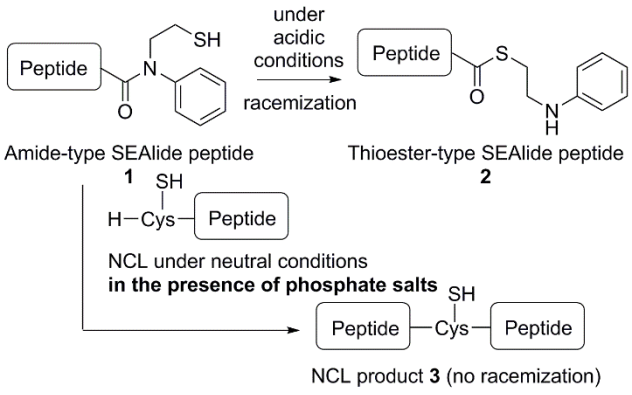
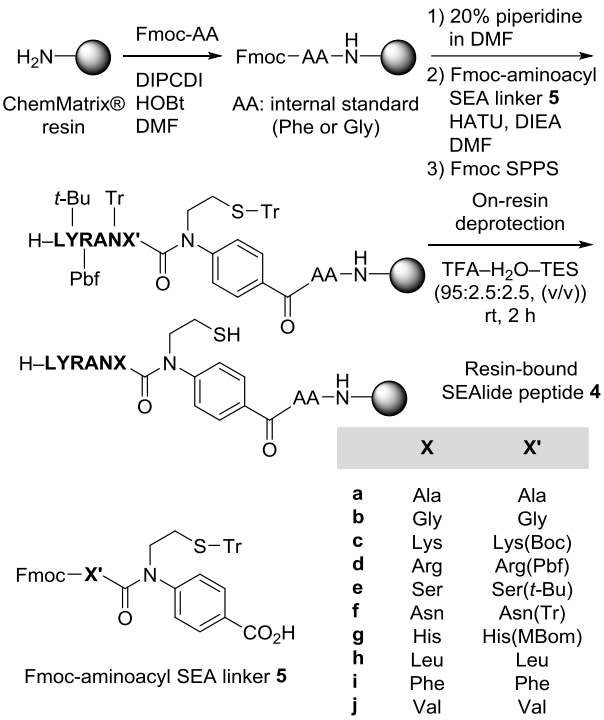


Figure 1. Chemical behavior of SEAlide peptides.

Close investigation into the utilization of SEAlide peptide in NCL disclosed that the amide-type SEAlide peptide can participate in NCL to yield ligated NCL product **3** without racemization in the presence of phosphate salts, but not in the absence of phosphate salts.[2b-f]

Scheme 1. Synthesis of Resin-bound SEAlide Peptides



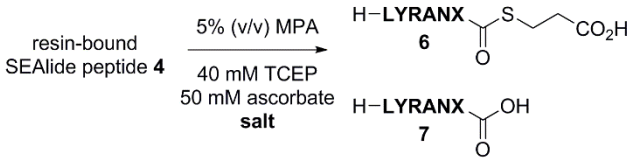
	X	X'
a	Ala	Ala
b	Gly	Gly
c	Lys	Lys(Boc)
d	Arg	Arg(Pbf)
e	Ser	Ser(*t*-Bu)
f	Asn	Asn(Tr)
g	His	His(MBom)
h	Leu	Leu
i	Phe	Phe
j	Val	Val

Here, phosphate salts are likely to function as an acid–base catalyst for the N–S acyl transfer and allow the amide-type SEAlide to work as a thioester in NCL without pre-converting to the thioester. Having been inspired by the role of phosphate salts in NCL using SEAlide peptide, we reexamined the thiolytic release of peptide thioesters from resin-bound SEAlide peptides for the establishment of a facile preparation of the thioesters from SEAlide peptides.

Reexamination began with the preparation of resin-bound model SEAlide peptides **4** using standard Fmoc solid-phase

peptide synthesis (SPPS) (Scheme 1). Aminomethyl ChemMatrix® resin[3] as an aqueous-solution-swelled resin was used for a solid support. Internal standard amino acids (Gly or Phe) and Fmoc aminoacyl *N*–sulfanylethylaniline (SEA) likers[4] **5** were successively coupled on the amino group of the resin by the action of *N,N'*–diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt) and *O*–(7-azabenzotriazol-1-yl)–*N,N,N',N'*–tetramethyluroniumu hexafluorophosphate (HATU) and *N,N'*–diisopropylethylamine (DIEA), respectively, in DMF. Resulting resins were brought to the chain elongation step to give requisite resin-bound peptides **4**.

Table 1. Examination of Conditions for Thiolytic Release



entry	salt (M, pH)	content of amine[a] (mmol/g)	ratio[b] (**6a**:**7a**)	yield[c] (%)	yield[d] (%)
1	phosphate (0.2, 6)	0.43	89:11	45	60
2	phosphate (0.2, 5)	0.43	90:10	55	76
3	phosphate (0.2, 4)	0.43	93:7	53	66
4[e]	phosphate (0.2, 4)[d]	0.43	100:0	3.5	7.9
5[f]	phosphate (0.2, 4)[e]	0.43	95:5	49	—
6	phosphate (0.5, 4)	0.43	92:8	47	—
7	phosphate (1.0, 4)	0.43	92:8	46	—
8	HEPPS (0.1, 4)	0.43	92:8	51	67
9	HEPPS (0.1, 4)	0.64	93:7	55	—
10	HEPPS (0.1, 4)	0.27	93:7	31	—

[a]Determined by quantitative spectrophotometric monitoring following piperidine deprotection of the internal standard-incorporated resin; [b]Peak ratio on HPLC analysis; [c]Isolated yield; [d]Determined by amino acid analysis; [e]25 °C; [f]In the presence of 3 M Gn·HCl

Initially, resin **4a** (X = Ala, initial content of NH_2: NH_2 0.43 mmol/g) was treated with 3-mercaptopropionic acid[5] (MPA, 5% (v/v)) in the presence of 40 mM tris(2-carboxyethyl)phosphine (TCEP) and 50 mM sodium ascorbate[6] in an 0.2 M phosphate buffer at 50 °C for 12 h under several pH conditions. Gratifyingly, MPA-thioester peptide **6a** was released from the resin in 45–55% isolated yields. The ratio of hydrolyzed peptide **7a** to **6a** decreased in proportion to decrease of pH and optimal pH for thiolytic release was fixed at 4.0 (Table 1, entries 1–3). Efficacy of the release depended on reaction temperature. Although the release at 25 °C did not accompany the formation of **7a**, isolated yield remained low (3.5%) (Table 1, entries 3 and 4). On the basis of our previous finding that SEAlide peptide in solution functions as a thioester in NCL only in the presence of phosphate salts, we speculated that the release efficacy should depend on the concentration of phosphate (Table 1, entries 3, 6 and 7). However, unequivocal dependency was not observed at all. More surprisingly, a reaction in 0.1 M 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid (HEPPS) at pH 4.0 at 50 °C proceeded efficiently to afford desired thioester **6a** in 51% isolated yield (Table 1, entry 8).

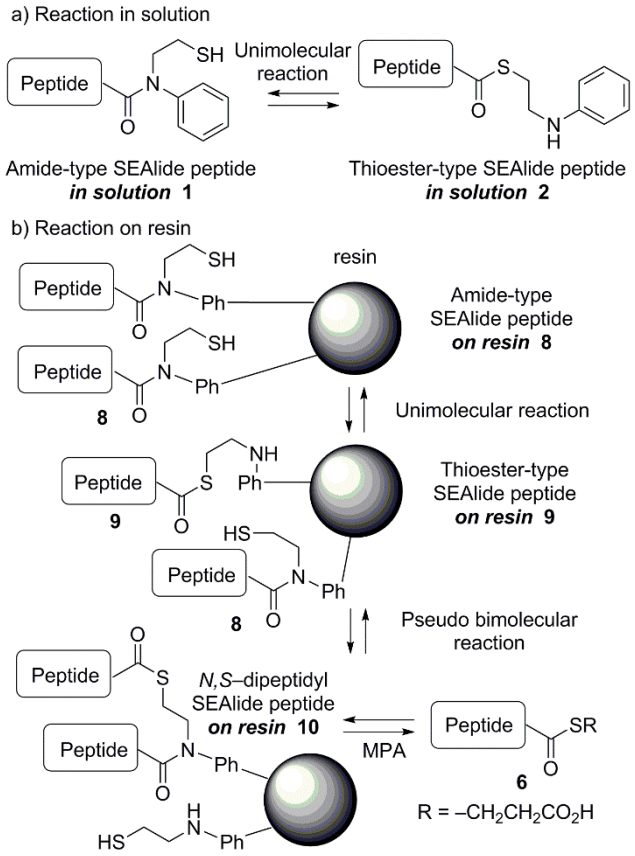


Figure 2. N–S Acyl transfer of SEAlide peptide in solution or on resin.

Here, of note, is the fact that the resin-bound SEAlide peptide could behave in a fashion different to that of resin-unbound SEAlide peptide. We assume that the reason for the difference results from the "concentration effect," as shown in Fig. 2. Compared with SEAlide peptide in solution (**1** and **2**), it seems the peptide on resin generally exists under highly concentrated conditions. Thiolytic release of thioester peptides from amide-type SEAlide peptide on resin **8** initially requires the N–S acyl-transfer-mediated conversion of **8** to the corresponding thioester-type SEAlide peptide on resin **9**. Under diluted solution conditions in HEPPS buffer, unimolecular reverse reaction of the thioester-type SEAlide peptide **2** to the amide form **1** preferentially occurs, thereby inhibiting the formation of thioester **6**. On the other hand, a pseudo bimolecular reaction between amide-type and thioester-type SEAlide peptides on resin (**8** and **9**) potentially proceeds to give *N,S*-dipepeptidyl SEAlide peptide on resin **10** under concentrated resin-bound conditions. The resulting dipeptidyl peptide **10** remains intact, and a reaction with thiol yields the desired thioester **6**. Formation of the resin-bound dipeptidyl SEAlide peptide **10** under concentrated con-

ditions is provably indispensable for the thiolytic release of thioester from the resin. This hypothesis was partly confirmed by the experimental results in an HEPPS buffer that the higher content resin (highly concentrated) more efficiently gave the desired thioester than the lower content resin (Table 1, entries 8–10)

Next, applicability of the thiolytic release protocol to various C-terminal amino acids was confirmed (Table 2). Except for Asn and His, peptide thioesters were obtained in reasonable isolated yields. Low yield of Asn peptide **6f** is provably attributable to the facile imide cyclization of carbonyl activated Asn residue. In terms of His, the reaction was stopped (1 h) before completion due to the suppression of racemization. Since intrinsically optically unstable Ser thioester peptide was obtained along with 3% epimerized peptide after 12 h reaction,[7] we concluded that attempted thiolytic release protocol using resin-bound SEAlide peptide should be of use for preparation of the thioesters. Therefore, we tried to synthesize 28-residue thioester corresponding to the C-terminal region of chemokine CXCL14.

Table 2. Thiolytic Release of Various Thioester from SEAlide Resins

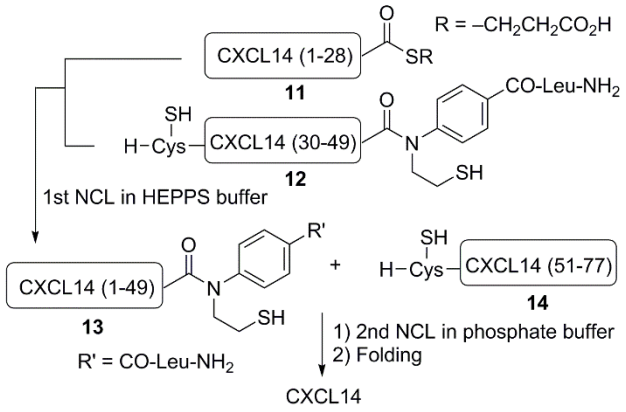
entry	X	rt[a] (h)	ratio[b] (**6**:**7**)	yield[c] (%)	epimerization (%)
1	Ala (**4a**)	12	93:7	53	1.5
2	Gly (**4b**)	6	91;9	57	—
3	Lys (**4c**)	12	96:4	58	—
4	Arg (**4d**)	12	95:5	63	—
5	Ser (**4e**)	12	92:8	72	4.8
6	Asn (**4f**)	12	89:11	33	—
7	His (**4g**)	1	91:9	33	3.0
8	Leu (**4h**)	24	96:4	43	—
9	Phe (**4i**)	24	89:11	52	—
10	Val (**4j**)	72	96:4	43	—

[a]rt = reaction time; [b]Peak ratio on HPLC analysis; [c]Isolated yield

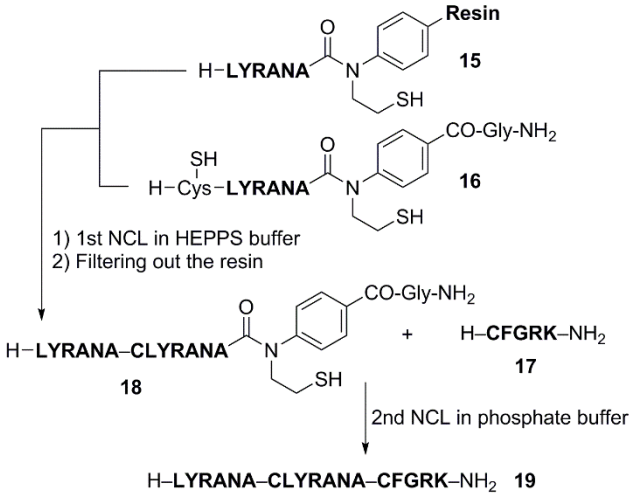
During the course of our study on CXCL14,[8] we have utilized NCL for preparation of various CXCL14 analogues. For the facile introduction of substitution into the N-terminal region, practical and maneuverable synthetic protocols for the N-terminal fragment have been demanded. Standard Fmoc protocols on Fmoc-His(MBom)-SEA linker[9] **5g** incorporated ChemMatrix® resin, followed by on-resin deprotection with TFA–thioanisole–*m*-cresol–1,2-ethanedithiol (EDT)–H_2O (80:5:5:5:5, (v/v)) afforded resin-bound CXCL14 (1-28) SEAlide peptide. Exposure of the resulting resin to 5% (v/v) MPA in 0.2 M phosphate buffer (pH4.0) in the presence of 40 mM TCEP and 50 mM sodium ascorbate at 50 °C for 1 h gave C-terminal His thioester peptide **11** in 17% isolated yield. Having requisite alkylthioster fragment **11** corresponding to CXCL14 (1-28), we next conducted one-pot/sequential three-fragment ligation for preparation of CXCL14, as shown in Scheme 2.

The first NCL of **11** with N-terminal cysteinyl SEAlide peptide **12** in 6 M guanidine HCl–0.2 M HEPPS (pH 6.0) in the presence of 30 mM TCEP at 37 °C for 3 h yielded ligated SEAlide peptide **13** without accompanying a cyclic peptide derived from an intramolecular NCL reaction of **12**. The addition of C-terminal fragment **14** in a 1 M phosphate buffer into the reaction mixture then triggered the second NCL step to allow for the formation of fully ligated product **14** in a one-pot manner through the phosphate-induced NCL of the amide-type SEAlide unit. Resulting **14** was then brought to the oxidative folding step to produce desired CXCL14. One-pot/sequential protocol with the use of alkylthioester and cysteinyl SEAlide in solution with or without phosphate salts is undoubtedly powerful strategy for protein chemical synthesis; however, control of stoichiomeric amounts of employed fragments is sometimes required for the efficient purification of the desired product. From this point of view, direct application of resin-bound amide-type SEAlide peptide to NCL with cysteinyl SEAlide peptide was attempted. As previously mentioned, the resin-bound SEAlide shows similar reactivity for the thiolytic release in both phosphate and HEPPS buffers. Therefore, we hypothesized that the amide-type SEAlide peptide on resin could participate in NCL in an HEPPS buffer whereas the SEAlide peptide in solution could not function as a thioester in this buffer system. This hypothesis prompted us to attempt the alternative N–to–C-directed sequential three-fragment ligation using a resin-bound SEAlide peptide (H-**LYRANA**-SEAlide-resin **15**), a cysteinyl SEAlide peptide (H-**CLYRANA**-SEAlide **16**) and a cysteinyl peptide (H-**CFGRK**-NH_2 **17**) (Scheme 3).

Scheme 2. Synthesis of CXCL14 Using One-pot/sequential NCL



Scheme 3. Sequential NCL Using Resin-bound and Soluble SEAlide Peptides



The first NCL without phosphate salts was achieved between **15** and **16** in 6 M guanidine HCl in 0.1 M HEPPS buffer (pH 6.9) in the presence of 40 mM MPAA[10] and 40 mM TCEP at 37 °C for 12 h to afford ligated SEAlide peptide **18**, as shown in Fig 3, a and b. Although not less than four equivalents of **15** was required for complete consumption of **16**; only the desired peptide **18** was observed as peptide material on HPLC analysis. Because excess released highly reactive MPAA ester as aryl thioester should react with an SEA unit on the resin to regenerate the resin-bound SEAlide, filtering out the resin gave solution containing ligated **18**. In the first step, it is worth noting that different chemical environment of the SEAlide unit in **15** and **16** resulted in the success of the first chemoselective ligation NCL. That is, the resin-bound SEAlide unit of **15** can react with the cysteinyl unit in HEPPS buffer, but not the soluble SEAlide unit of **16**. Next, addition of **17** in 0.5 M phosphate buffer (pH 6.6) in the presence of 40 mM TCEP into the above HEPPS buffer solution of **18** allowed the soluble SEAlide unit of **18** to work as a thioester to give fully ligated peptide **19** in 40 % isolated yield (over two steps) (Fig. 3, c).

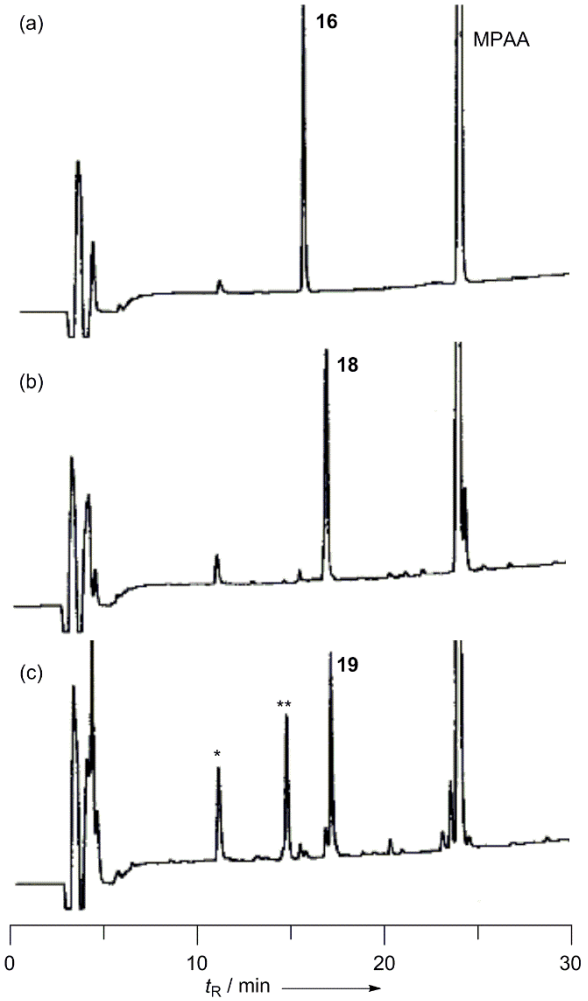


Figure 3. Progress of sequential ligation using resin-bound SEAlide peptide **15**: (a) first NCL (t = 0 h); first NCL (t = 12 h); (c) second NCL (t = 24 h). *non-peptide (derived from buffer); **released SEAlide unit.

Recycling of the filtered-out resin was also examined. After completion of the NCL of the cysteinyl peptide **16** with 10 equivalents of **15** (12 h), the peptide resin was filtered out and regenerated by treatment with 40 mM TCEP and 50 mM sodium ascorbate in 6 M Gn·HCl (pH 3.2) for 2 h at 37 °C. The resulting SEAlide peptide resin was subjected to a second round of NCL with 10 equivalents of **15**, and the attempted reaction was continued for 42 h through to completion. The reason for decrease in reactivity of the resin-bound SEAlide peptide remained to be elucidated. Possible examinations could include the consumption of a hot reaction spot or insufficient regeneration of the used resin. Further investigation into the decrease will be discussed in due course.

Here, reactivity of the resin-bound SEAlide peptide in the formation of a thioester or NCL was elucidated. The SEAlide peptide in solution can participate in NCL only in the presence of phosphate salts, which is not the case for the resin-bound SEAlide peptide. Difference in chemical behavior of SEAlide peptide in solution versus on resin was successfully applied to one-pot/N–to–C-directed sequential ligation using different phase SEAlide peptides.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures and additional experimental data (PDF)

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ACKNOWLEDGMENT

This research was supported in part by a Grant-in-Aid for Scientific Resarch (KAKENHI, 16H02611 for A.O. and 17J03475 for N.N.)

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(7) See Supporting Information Fig. S2

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