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Synthetic Procedure for *N*-Fmoc Amino Acyl-*N*-Sulfanylethylaniline Linker as Crypto-Peptide Thioester Precursor with Application to Native Chemical Ligation

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Supporting Information

ABSTRACT: *N*-Sulfanylethylanilide (SEAlide) peptides **1**, obtainable using Fmoc-based solid-phase peptide synthesis (Fmoc SPPS), function as crypto-thioesters in native chemical ligation (NCL), yielding a wide variety of peptides/proteins. Their acylating potential with N-terminal cysteinyl peptides **2** can be tuned by the presence or absence of phosphate salts, leading to one-pot/multifragment ligation, operating under kinetically controlled conditions. SEAlide peptides have already been shown to be promising for use in protein synthesis; however, a widely applicable method for the synthesis of *N*-Fmoc amino acyl-*N*-sulfanylethylaniline linkers **4**, required for the preparation of SEAlide peptides, is unavailable. The present study addresses the development of efficient condensation protocols of 20 naturally occurring amino acid derivatives to the *N*-sulfanylethylaniline linker **5**. *N*-Fmoc amino acyl aniline linkers **4** of practical use in NCL chemistry, except in the case of the proline- or aspartic acid-containing linker, were successfully synthesized by coupling of $POCl_3$- or $SOCl_2$-activated Fmoc amino acid derivatives with sodium anilide species **6**, without accompanying racemization and loss of side-chain protection. Furthermore, SEAlide peptides **7** possessing various C-terminal amino acids (Gly, His, Phe, Ala, Asn, Ser, Glu, and Val) were shown to be of practical use in NCL chemistry.

INTRODUCTION

Peptide thioesters are indispensable synthetic intermediates for native chemical ligations (NCLs), yielding much success in the chemical syntheses of a wide variety of proteins.[1,2] Practical synthetic procedures, using Fmoc-based solid-phase peptide synthesis (Fmoc SPPS), for peptide thioesters[3−10] have been extensively explored for two main reasons. One is that the method has become increasingly popular because the experimental procedures are simpler, and the synthetic materials more readily available, than those used in Boc SPPS. The other is that optimized synthetic protocols for Boc SPPS of peptide thioesters[11] are incompatible with Fmoc SPPS. In this context, we have developed an *N*−*S* acyl-transfer-mediated synthetic methodology for peptide thioesters in which the use of *N*-Fmoc amino acyl-*N*-sulfanylethylaniline linkers **4** as a key synthetic unit guarantees compatibility with Fmoc chemistry.[7b,8] In the course of our studies on *N*-sulfanylethylanilide peptides (referred as to SEAlide peptides), we found that SEAlide peptides **1** function efficiently as peptide thioesters in the presence of phosphate salts, without preconversion to the corresponding thioester, to participate in NCLs with an N-terminal cysteinyl peptide **2** (Figure 1),[12] and are of great use in kinetically controlled NCL reactions.[6c,13]

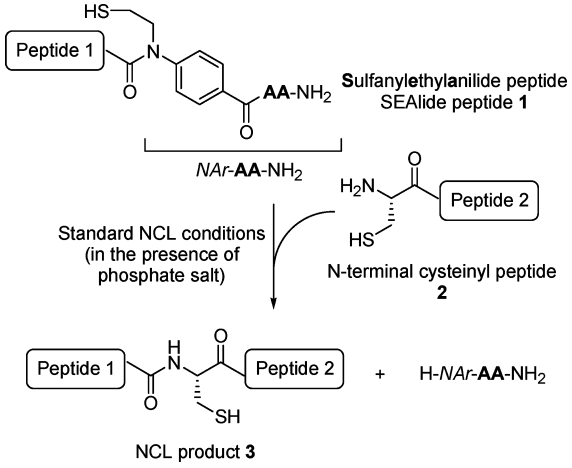

Figure 1. SEAlide peptides can function as peptide thioesters under normal NCL conditions in the presence of phosphate salts.

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We therefore realized that the development of a facile preparative method for *N*-Fmoc amino acyl-*N*-sulfanylethylaniline linkers **4** should lead to a further increase in the usefulness of SEAlide peptides. In this study, the three issues shown in Figure 2, i.e., coupling efficiency, racemization, and stability of side-chain protections, are discussed.[14]

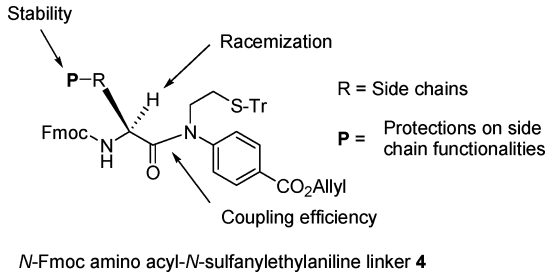

Figure 2. Issues addressed in the preparation of *N*-Fmoc amino acyl-*N*-sulfanylethylaniline linker **4**.

In our previous investigation, the loading of aliphatic Fmoc derivatives of amino acids such as Gly and Ala derivatives on an aniline linker **5** was achieved by coupling Fmoc amino acyl chlorides with sodium anilide **6**, produced by treating aniline **5** with NaH (Method A, Figure 3). Here, the requisite acyl

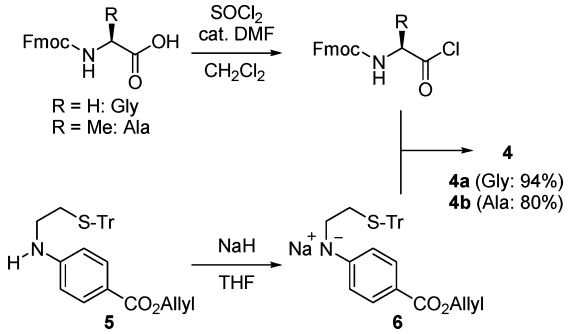

Figure 3. Preparation of Fmoc-Gly- (or Fmoc-Ala)-incorporated aniline linker (**4a** or **4b**) using coupling of acyl chlorides with sodium anilide **6** (Method A).

chlorides were prepared by a standard protocol using $SOCl_2$ in CH_2Cl_2 in the presence of a catalytic amount of DMF. Application of this procedure (Method A) to other Fmoc amino acids, possessing acid-labile protecting groups such as *t*-Bu, Boc or Tr group, is expected to induce loss of the side-chain protections and fail to afford the desired linkers.[15]

In this study, we investigate versatile procedures compatible with the coupling of the aniline linker **5** with naturally occurring proteogenic Fmoc amino acid derivatives. We discuss $SOCl_2$- and $POCl_3$-mediated procedures. The applicability of the resulting aniline linkers **4** to NCL protocols, including the synthesis of SEAlide peptides, is also addressed.

RESULTS AND DISCUSSION

Initially, in addition to Gly and Ala derivatives (**4a** and **4b**), other aliphatic amino acids such as Val, Ile, Leu, and Phe were successfully incorporated to yield the aniline linkers **4** (**4c** Val, **4d** Ile, **4e** Leu, and **4f** Phe) using Method A, although the yield of the Pro-incorporated linker **4g** was medium. These results are summarized in Table 1 (entries 6, 8, 10, 12, and 14), as are the coupling data obtained from other coupling procedures

Table 1. Summary of Coupling of Fmoc Amino Acid Derivatives with *N*-Sulfanylethylaniline Linker 5 under Various Reaction Conditions

entry	Fmoc amino acid	method[a]	product	isolated yield of aniline linkers **4** (%) [racemization (%)]
1	Gly	A	**4a**	94
2	Gly	D	**4a**	65
3	Ala	A	**4b**	80
4	Ala	B	**4b**	75 [2.5]
5	Ala	D	**4b**	67
6	Val	A	**4c**	98
7	Val	D	**4c**	87
8	Ile	A	**4d**	88
9	Ile	D	**4d**	87
10	Leu	A	**4e**	74
11	Leu	D	**4e**	88
12	Phe	A	**4f**	83
13	Phe	D	**4f**	88
14	Pro	A	**4g**	48
15	Pro	D	**4g**	39
16	Ser(*t*-Bu)	modified A	**4h**	89 [0.3]
17	Ser(*t*-Bu)	B	**4h**	48 [33]
18	Ser(*t*-Bu)	C	**4h**	51 [n.d.[b]][c]
19	Ser(*t*-Bu)	C	**4h**	65[d]
20	Ser(*t*-Bu)	D	**4h**	90 [1.8]
21	Ser(*t*-Bu)	D	**4h**	91 [0.4][e]
22	Asn(Tr)	modified A	**4i**	—[f]
23	Asn(Tr)	C	**4i**	88
24	Asn(Tr)	D	**4i**	89
25	Asp(O*t*-Bu)	modified A	**4j**	—[e]
26	Asp(O*t*-Bu)	D	**4j**	68
27	Thr(*t*-Bu)	D	**4k**	84
28	Glu(O*t*-Bu)	D	**4l**	72
29	Gln(Tr)	D	**4m**	93
30	Cys(Tr)	D	**4n**	72 [n.d.[b]]
31	Met	D	**4o**	80
32	Tyr(*t*-Bu)	D	**4p**	86
33	His(Tr)	D	**4q**	41 [23]
34	His(MBom)	D	**4r**	88 [0.4]
35	Lys(Boc)	D	**4s**	80
36	Arg(Pbf)	D	**4t**	95
37	Trp	D	**4u**	92

[a]**Method A**: Fmoc amino acid chlorides, prepared from reaction of Fmoc amino acids with $SOCl_2$ in CH_2Cl_2 in the presence of a catalytic amount of DMF, were reacted with sodium anilide **6** at ambient temperature. **Modified Method A**: Instead of Fmoc amino acids as in Method A, the corresponding triethylammonium salts were used. **Method B**: Fmoc amino acids were coupled with the aid of $POCl_3$ in pyridine solvent. **Method C**: An equimolar mixture of Fmoc amino acids, Et_3N, and $POCl_3$ in CH_2Cl_2 was used for the coupling with aniline **5**. **Method D**: An equimolar mixture of Fmoc amino acids, Et_3N, and $POCl_3$ in THF (5-fold excess over **6**) was coupled with sodium anilide **6** at ambient temperature for 24 h at 0.3 M concentration of activated acyl components. [b]n.d. = not detected. [c]3-fold excess of Fmoc amino acid was used. [d]5-fold excess of Fmoc amino acid was used. [e]Coupling reaction was conducted at 4 °C. [f]No desired products were obtained.

mentioned later. The syntheses of aniline linkers (**4h**, **4i**, and **4j**) possessing the acid-labile Fmoc amino acids Ser(*t*-Bu), Asn(Tr), and Asp(O*t*-Bu), respectively, were examined using a slightly refined version of Method A (referred to as modified Method A), which included the use of triethylammonium salts

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of Fmoc amino acids for the preparation of acyl chlorides with $SOCl_2$. We assumed that the modified conditions would suppress the loss of acid-labile protecting groups to yield the requisite acyl chlorides, and subsequent coupling with the sodium anilide **6** would give the desired materials.[16] The reaction of Fmoc-Ser(*t*-Bu)-OH using modified Method A gave the desired Ser-incorporated aniline linker **4h** in 89% isolated yield, without loss of optical purity (entry 16). However, this was not the case for the syntheses of the Asn(Tr)- and Asp(O*t*-Bu)-incorporated linkers (**4i** and **4j**) (entries 23 and 25). Activation of Fmoc-Asn(Tr)-OH with $SOCl_2$ in the presence of Et_3N afforded an *N*-Tr cyclic imide derivative. Activation of Fmoc-Asp(O*t*-Bu)-OH also failed to yield the corresponding acyl chloride; loss of the *t*-Bu group, probably attributable to formation of a five-membered-ring compound, was confirmed by NMR measurements.[15] Although bis(trichloromethyl)carbonate seems to be a potential reagent of choice, its applicability to the synthesis of the aniline linker **4** remains in doubt.[17]

Next, we investigated the usefulness of $POCl_3$ in the coupling of amino acid derivatives with weakly nucleophilic aromatic amines.[18] The applicability of the $POCl_3$-mediated protocol was examined using Fmoc-Ala-OH as a representative amino acid. On the basis of previous reports in the literature, $POCl_3$-mediated coupling in pyridine solvent (Method B) was employed. The reaction, using a 2-fold excess of Fmoc-Ala-OH and $POCl_3$ over the aniline **5** in pyridine for 1.5 h at 4 °C, afforded the desired aniline linker **4b** in 48% isolated yield. Increasing the amounts of reagents (5-fold excess) raised the reaction yield (75%). However, removal of the Fmoc group of the resulting linker **4b**, followed by coupling with Boc-L-Leu-OH, gave a diastereomeric mixture of dipeptides, indicating racemization of Fmoc-L-Ala-OH during the condensation step using Method B (ca. 2.5% racemization) (entry 4). This problem was more pronounced in the condensation of Fmoc-L-Ser(*t*-Bu)-OH (33% racemization) (entry 17). The extent of racemization observed in the coupling of Fmoc-Ser(*t*-Bu)-OH was determined with high sensitivity by Fmoc-removal from the resulting linker **4h**, followed by condensation with Boc-L-Leu-OH or Boc-D-Leu-OH, and subsequent analysis using reversed-phase HPLC. In this analysis, the major component generated from coupling with Boc-D-Leu-OH corresponds to a D-Leu-L-Ser derivative, an enantiomeric dipeptide of Boc-L-Leu-D-Ser compound derived from racemized Ser material (**ent-4h**). Coupling of Boc-Ala-OH with a weak nucleophilic hetero-aromatic amine in pyridine with the aid of $POCl_3$ at −15 °C for 30 min was reported to induce no racemization. However, our case involves the use of an *N*-substituted aniline, and the larger decrease in nucleophilicity requires harsher conditions (reaction temperature: 4 °C vs −15 °C; reaction time 1.5 h vs 30 min) for the coupling reaction to proceed, leading to the observed racemization. The use of $POBr_3$ in pyridine also did not improve the coupling efficacy.

We next examined whether the excess amount of pyridine is involved in the racemization. Instead of pyridine, an equimolar amount of Et_3N to $POCl_3$ was used in CH_2Cl_2 (Method C).[19] The reaction of an equimolar mixture (3-fold excess over aniline) of Fmoc-L-Ser(*t*-Bu)-OH, $POCl_3$, and Et_3N in CH_2Cl_2 with aniline **5** at 4 °C for 1 h afforded the desired aniline linker **4** in 51% isolated yield, although complete consumption of the starting aniline was not observed (entry 18). It is worth noting that the pronounced racemization, detected in the reaction with $POCl_3$ in pyridine, was completely suppressed (<0.1%). Using similar reaction conditions, the reaction of Fmoc-Asn(Tr)-OH, which is a problematic coupling as mentioned above (modified Method A), proceeded to yield the desired material in 88% isolated yield (entry 23). In the coupling of Ser derivatives, a 5-fold excess of an activated species was used to improve the chemical yield. The attempted reaction of Fmoc-L-Ser(*t*-Bu)-OH with aniline **5** in the presence of Et_3N and $POCl_3$ was incomplete even after 26 h, and gave the corresponding aniline linker **4** in 65% isolated yield (entry 19).

We therefore examined the effects of increasing the nucleophilicity by the use of sodium anilide **6** (Method D). The reaction of a 5-fold excess of the activated species resulting from Fmoc-L-Ser(*t*-Bu)-OH, Et_3N, and $POCl_3$ in THF with the preformed sodium anilide **6** in THF at ambient temperature proceeded to completion within 24 h to furnish the desired material **4h** in 90% isolated yield with 1.8% racemization (entry 20). Furthermore, conducting the coupling reaction at 4 °C suppressed racemization to less than 1%, without loss of coupling yield (entry 21). Encouraged by these results, we used the above reaction conditions (Method D) in the synthesis of proteogenic amino acids incorporating aniline linkers **4**. The results are summarized in Table 1.

As shown in Table 1, the reactions performed using Method D, except for the coupling of Pro and His(Tr) (entries 15 and 33), proceeded with reasonable efficiency to give the corresponding aniline linkers **4** in isolated yields of over 65%. Further investigations aimed at improving the coupling of Pro or His(Tr) were suspended because of the unsuitability of the Pro-thioester for NCL reactions[20] or for reasons mentioned later in the case of His(Tr). Because Cys and His, like Ser, are well-known to have high susceptibility to racemization during coupling reactions with amines, the extent of racemization of the synthesized Fmoc-Cys(Tr)- and Fmoc-His(Tr)-incorporated linkers (**4n** and **4q**) were also examined using Fmoc deprotection followed by coupling with Boc-L-Leu-OH or Boc-D-Leu-OH, as used previously for Ser(*t*-Bu). In the case of Cys(Tr), no racemization was detected (entry 30). However, use of the His(Tr) derivative resulted in 23% racemization (entry 33). Suppression of the loss of optical purity of His during the coupling step with sodium anilide **6** was achieved by employing a newly developed His derivative. Very recently, we reported that a *p*-methoxybenzyloxymethyl (MBom) group on the π-nitrogen of the imidazole ring suppresses racemization during coupling steps more efficiently than does an N^{τ}-protecting group such as Tr.[21] Coupling of Fmoc-His(MBom)-OH using Method D proceeded efficiently to afford the desired anilide linker **4r** in 88% isolated yield with negligible amounts of racemization product (<0.4%) (entry 34). The coupling efficiencies of Gly and Ala using Method D were lower than those using Method A (entry 1 vs 2; entry 3 vs 4). One possible explanation for these results is that acylation of the carbamoyl nitrogen of the less-hindered Fmoc amino acids derived from Gly or Ala occurs under Method D conditions to prevent the desired acylation to give the aniline linker.[22] Subjection of Fmoc-Gly-OH to a reagent mixture consisting of $POCl_3$ and Et_3N in CH_2Cl_2 led to partial formation of the envisioned acylcarbamoyl species, which was also detected in TLC analysis of the reaction mixture of Fmoc-Gly-OH in Method D. On the basis of these results, we concluded that the $POCl_3$-mediated coupling conditions (Method D) are widely compatible with the incorporation of naturally occurring amino acid derivatives, except for Gly and Ala, to the aniline linker **5**. The sterically less-hindered Gly and Ala derivatives are more efficiently incorporated in the linker using Method A; this method is also

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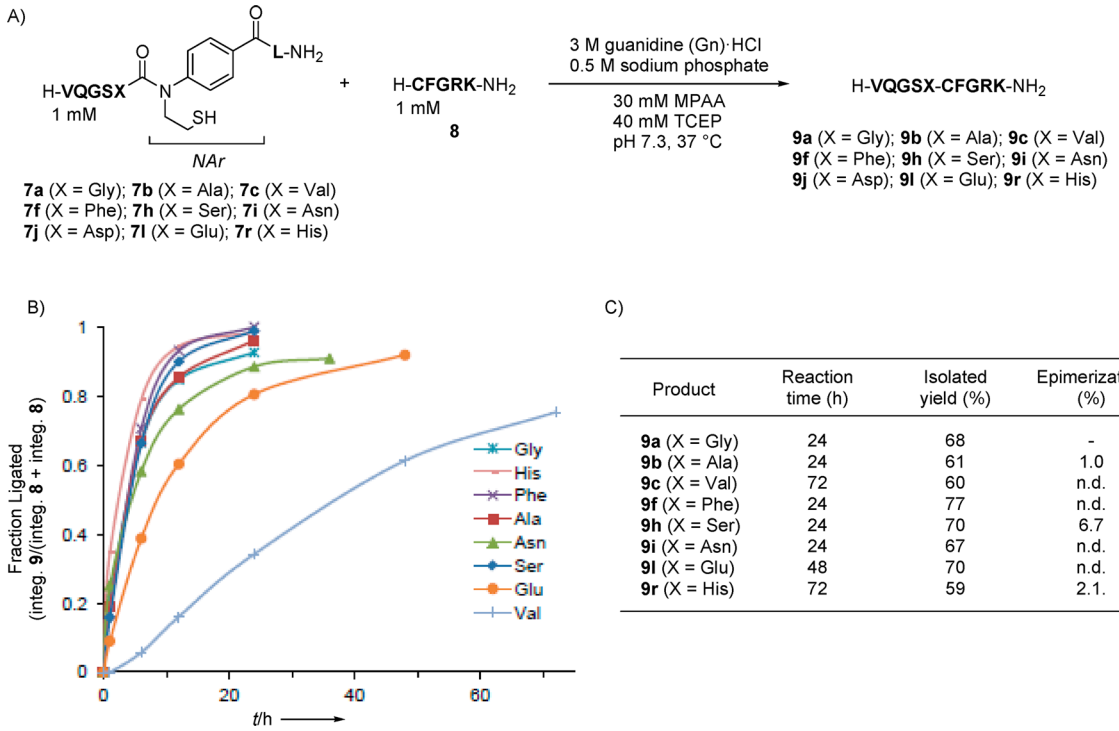

C)

Product	Reaction time (h)	Isolated yield (%)	Epimerization (%)
9a (X = Gly)	24	68	-
9b (X = Ala)	24	61	1.0
9c (X = Val)	72	60	n.d.
9f (X = Phe)	24	77	n.d.
9h (X = Ser)	24	70	6.7
9i (X = Asn)	24	67	n.d.
9l (X = Glu)	48	70	n.d.
9r (X = His)	72	59	2.1.

Figure 4. Summary of NCL reaction of SEAlide peptides **7** with N-terminal cysteinyl peptide **8**. (A) Reaction conditions of attempted NCL reactions. (B) Progress of NCL reactions: the fraction ligated was determined by HPLC separation and integration of ligated peptides **9** (integ. **9**) detected at 220 nm as a fraction of the sum of the unreacted N-terminal cysteinyl peptide (integ. **8**) and integ. **9**. (C) Table for NCL reactions: n.d. = not determined.

excellent for the coupling of Fmoc amino acids without protected side-chain functionalities (entries 6, 8, 10, and 12).

Having established facile synthetic procedures for the *N*-Fmoc amino acyl-*N*-sulfanylethylaniline linkers **4**, we next examined the usefulness of SEAlide peptides **7** with C-terminal diversity by subjecting various SEAlide peptides **7** to NCLs with an N-terminal cysteinyl peptide **8**. Dawson and co-workers reported that analyses of the reaction rates of conventional NCL for **X**-Cys ligation sites, where **X** is any of the 20 naturally occurring amino acids, using model ligations indicated that the amino acids can be listed in descending order of preference as follows: Gly (**G**), Cys (**C**), His (**H**) > Phe (**F**), Met (**M**), Tyr (**Y**), Ala (**A**), Trp (**W**) > Asn (**N**), Ser (**S**), Asp (**D**), Gln (**Q**), Glu (**E**), Lys (**K**), Arg (**R**) > Leu (**L**), Thr (**T**) > Val (**V**), Ile (**I**) > Pro (**P**).[23] Referring to these results, we chose **G**, **H**, **F**, **A**, **N**, **S**, **E**, and **V** as representative amino acids to examine the applicability of SEAlide peptides to NCL. Aspartic acid (**D**) was also added to the list of examined amino acids because NCL at the Asp-Cys site resulted in the formation of a non-negligible amount of the β-backbone isoform.[24]

Model peptides H-**VQGSX**-**CFGRK**-NH_2 (**9**) were evaluated for possible involvement of SEAlide peptides **7** in NCL reactions. The SEAlide peptides H-**VQGSX**-*NAr*-**L**-NH_2 (**X** = **G** (**7a**), **A** (**7b**), **V** (**7c**), **F** (**7f**), **S** (**7h**), **N** (**7i**), **D** (**7j**), **E** (**7l**), and **H** (**7r**)) were synthesized using standard Fmoc-based protocols. Removal of the allyl group of the aniline linkers **4** was achieved using Pd(0) (0.1 equiv) in the presence of *N*-methylaniline in THF to give allyl-deprotected Fmoc amino acyl aniline linkers Fmoc-**X**-*NAr*-OH **10** (**X** = **G** (**10a**), **A** (**10b**), **V** (**10c**), **F** (**10f**), **S**(*t*-Bu) (**10h**), **N**(Tr) (**10i**), **D**(O*t*-Bu) (**10j**), **E**(O*t*-Bu) (**10l**), and **H**(MBom) (**10r**)), which were then coupled on leucyl-Rink-amide-type NovaSyn TGR resin using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) in the presence of diisopropylethylamine (DIPEA), with the aim of achieving high coupling efficiency, followed by Fmoc synthesis.[8] The completed resins were treated with TFA−thioanisole−*m*-cresol−1,2-ethanedithiol (EDT)−H_2O (80:5:5:5:5 or 90:2.5:2.5:2.5:2.5, *v*/*v*) at ambient temperature for 90−120 min. Except for the Asp-containing SEAlide peptide **7j**, all the SEAlide peptides were obtained in high purity using this TFA-based deprotection followed by reversed-phase HPLC purification. Each of the successfully obtained SEAlide peptides (1 mM) was subjected to model NCL reactions with H-**CFGRK**-NH_2 **8** (1 mM) in 3 M guanidine (Gn)·HCl−0.5 M sodium phosphate buffer in the presence of 30 mM 4-mercaptophenylacetic acid (MPAA)[25] and 40 mM tris(carboxyethyl)phosphine hydrochloride (TCEP) (pH 7.3) at 37 °C. The time courses of the reactions are summarized in Figure 4B. The attempted NCLs, except for those with **7c** and **7l**, in the presence of phosphate salts went almost to completion within 24−36 h to yield the desired ligated peptides. Ligation of the Val- and Glu-peptides (**7c** and **7l**) required more time to complete the reaction; however, the desired NCL products (**9c** and **9l**) were obtained in reasonable isolated yields after 72 and 48 h, respectively (Figure 4C). For the NCLs using Ala-, Ser-, or His-peptide (**7b**, **7h**, or **7r**), the extent of racemization was checked by comparative HPLC analysis of the L- and D-amino acid-containing peptides (Figure 4C). Under standard ligation conditions for SEAlide peptides, racemization of Ser-peptide (ca. 7%) was detected, as is the case in other NCL protocols such as the cysteinyl-prolyl-ester ligation.[7l] Suitable conditions for tuning the ligation conditions to suppress epimerization of the Ser-peptide have yet to be found.

Exposure of the Asp-containing peptide resin to a TFA-based reagent cocktail (TFA−thioanisole−*m*-cresol−EDT−H_2O) at room temperature for 120 min gave a mixture of two compounds

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(major:minor = 88:12, HPLC peak area) with the desired mass number but different retention times on HPLC.

The later-eluted component (minor) disappeared on treatment with sodium mercaptoethylsulfonate (MESNA) under neutral conditions (Figure 5). HPLC analysis of the reaction

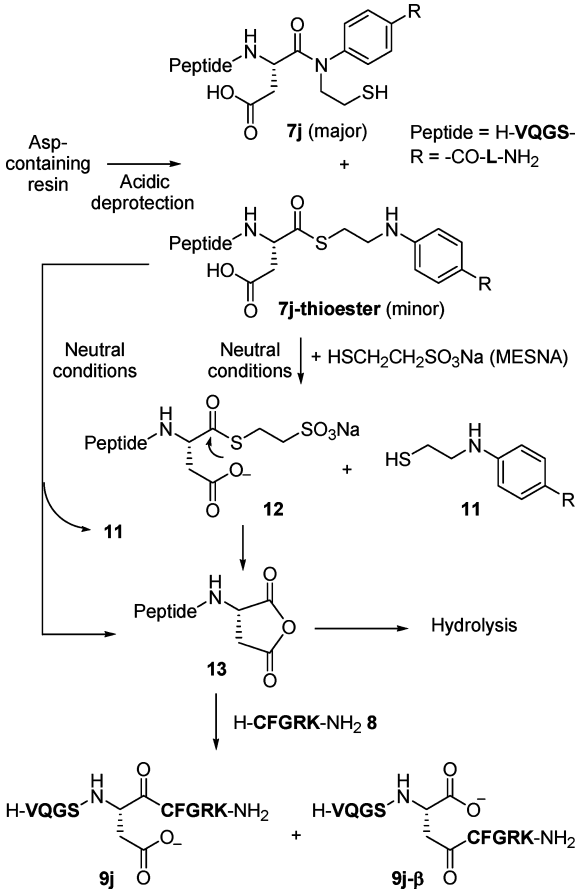

Figure 5. Formation of anhydride from thioester-type peptide **7j-thioester** followed by hydrolysis or generation of α- and β-peptides (**9j** and **9j-β**).

indicated the presence of the hydrolyzed aniline linker **11**, which is formed by thiolysis of the possibly formed thioester-type peptide **7j-thioester** (later-eluted component) with MESNA or direct conversion to anhydride **13** from **7j-thioester** under neutral conditions. Under neutral conditions, the thioester-type peptides **7j-thioester** and **12** were not detected in HPLC analysis because of facile nucleophilic attack of the β-carboxylate anion to thioester moieties leading to formation of **13**. In order to obtain pure amide-type SEAlide peptide **7j**, the early eluted component (major) was fractionated by reversed-phase HPLC using an eluent containing 0.1% TFA. Analytical HPLC showed that the pooled fraction contained a homogeneous peptide **7j**; however, lyophilization of the pooled solution resulted in the formation of a mixture of the anilide **7j** and the thioester-types peptide **7j-thioester**. Such *N*−*S* acyl transfer during TFA-mediated deprotection or lyophilization steps has not been observed for other amino acid-containing SEAlide materials.

These results indicate that the Asp-SEAlide moiety tends to isomerize to the corresponding thioester under acidic conditions, which keep the side-chain carboxylic acid in the undissociated form. This could be rationalized by the fact that the Asp-Pro linkage is susceptible to hydrolysis under acidic conditions;[26] i.e., involvement of the side-chain carboxylic acid functionality as an acid−base catalyst in the *N*−*S* acyl transfer of the Asp-*N*-(sulfanylethyl)aniline linkage via seven-membered ring formation could be attributable to the observed isomerization to the thioester peptide **7j-thioester** (Figure 6). This

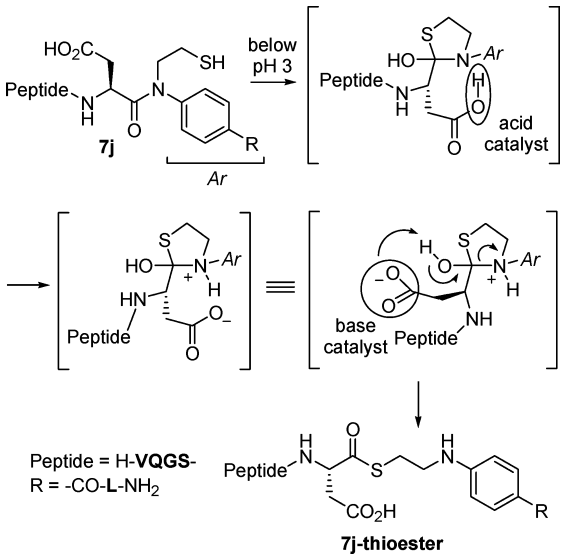

Figure 6. Plausible reaction mechanism for thioester formation from SEAlide peptide **7j** under acidic conditions.

assumption prompted us to speculate that HPLC purification under neutral conditions, to keep the Asp side-chain carboxyl anion form, would be effective for Asp-SEAlide peptide **7j**. HPLC purification of the crude materials containing anilide **7j** and thioester peptides **7j-thioester** with a 10 mM aqueous ammonium acetate (NH_4OAc) (pH 6.1)−CH_3CN system, followed by lyophilization, yielded pure **7j**.

Having obtained pure Asp-SEAlide **7j**, we next subjected this material to model ligation with the N-terminal cysteinyl peptide **8**. However, the attempted reaction using the SEAlide peptide **7j** as a crypto-thioester resulted in failure and gave a mixture of α- and β-peptides (**9j** and **9j-β**) (Figure 5), as reported for Asp thioester peptides.[24] NCL reactions at the Asp-Cys site still need to be addressed.

■ CONCLUSION

Efficient synthetic protocols for the preparation of the Fmoc amino acyl aniline linkers **4** were developed in this study. The developed protocols have wide applicability to naturally occurring amino acid derivatives, which can be appropriately employed in NCL reactions. SEAlide peptides, as crypto-thioesters, have proved to be very useful in NCL chemistry, including one-pot/multifragment condensations operating under kinetic conditions, so a preparative methodology applicable to a wide variety of SEAlide peptides will open up new avenues in the use of SEAlide peptides. Applications of the SEAlide unit to complex protein synthesis and in chemical biology will be reported in due course from our laboratory.

■ EXPERIMENTAL SECTION

Representative Experimental Procedure for Method A. To a solution of aniline **5** (100 mg, 0.21 mmol) in THF (2.0 mL) was added by portions 55% NaH (suspendend in mineral oil)

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(10 mg, 0.23 mmol) at 4 °C. The mixture was stirred for 30 min at ambient temperature. In another flask, Fmoc-Leu-OH (148 mg, 0.42 mmol) was treated with $SOCl_2$ (0.31 mL, 4.2 mmol) in CH_2Cl_2 (1.5 mL) in the presence of a drop of DMF at room temperature. After being stirred at this temperature for 3 h, the reaction was concentrated under reduced pressure to dryness. The residue was added the above-mentioned sodium anilide **6** solution at 4 °C. After 15 h reaction at ambient temperature, the reaction was quenched by the addition of saturated $NaHCO_3$ aqueous solution (saturated $NaHCO_3$ aq). Resulting mixture was extracted with EtOAc. The organic phase was successively washed with saturated $NaHCO_3$ aq, 10% citric acid aq, and brine and dried over $MgSO_4$. After removal of the solvent under reduced pressure, chromatographic purification on silica gel using EtOAc/*n*-hexanes gave desired Fmoc-Leu-incorporated linker (allyl ester) **4e** in 74% (126 mg) isolated yield.

Representative Experimental Procedure for Modified Method A. A mixture of Fmoc-Ser(*t*-Bu)-OH (480 mg, 1.3 mmol) and Et_3N (174 μL, 1.4 mmol) in CH_2Cl_2 (12 mL) was treated with $SOCl_2$ (137 μL, 2.0 mmol) in the presence of a drop of DMF at refluxing temperature. After 2 h reaction, solvent was removed to dryness under reduced pressure. The residue was redissolved with THF (3 mL), and the formed salts were filtered off. To the resulting filtrate was added sodium anilide (0.63 mmol) in THF (6 mL) added at room temperature. After being stirred for 20 h at ambient temperature, the reaction was quenched by the addition of saturated $NaHCO_3$ aq. Resulting mixture was extracted with EtOAc. The organic phase was successively washed with saturated $NaHCO_3$ aq, 10% citric acid aq, and brine and dried over $MgSO_4$. Removal of the solvent under reduced pressure, followed by chromatographic purification on silica gel using EtOAc/*n*-hexanes gave desired Fmoc-Ser(*t*-Bu)-incorporated linker (allyl ester) **4h** in 89% (469 mg) isolated yield.

Representative Experimental Procedure for Method B. To a mixture of Fmoc-Ser(*t*-Bu)-OH (399 mg, 1.0 mmol) and aniline **5** (100 mg, 0.21 mmol) in pyridine (2.0 mL) was added $POCl_3$ (97 mL, 1.0 mmol) at −15 °C. After being stirred at 4 °C for 3 h, the reaction was quenched by the addition of saturated $NaHCO_3$ aq. Procedures identical to those employed in modified Method A afforded desired material **4h** in 48% (85 mg) isolated yield.

Representative Experimental Procedure for Method C. To a stirred mixture of Fmoc-Ser(*t*-Bu)-OH (2.0 g, 5.2 mmol) and Et_3N (0.73 mL, 5.2 mmol) in CH_2Cl_2 (17 mL) were successively added aniline **5** (500 mg, 1.0 mmol) and $POCl_3$ (0.49 mL, 5.2 mmol) at 4 °C. After being stirred at 4 °C for 26 h, the reaction was quenched by the addition of saturated $NaHCO_3$ aq. Procedures identical to those employed in modified Method A afforded 571 mg of desired material **4h** in 65% isolated yield.

Representative Experimental Procedure for Method D. To a solution of Fmoc-Ser(*t*-Bu)-OH (400 mg, 1.05 mmol) in THF (2.4 mL) were successively added Et_3N (146 μL, 1.05 mmol) and $POCl_3$ (98 μL, 1.05 mmol) at 4 °C. Instantly, the resulting solution was added to the sodium amide **6** solution in THF (0.21 mmol, 1.1 mL), prepared according to Method A, at 4 °C with additional stirring at ambient temperature for 24 h. For all other coupling, 0.3 M concentration of Fmoc amino acid species was used. Procedures identical to those employed in modified Method A afforded 159 mg of desired material **4h** in 90% isolated yield.

*Allyl 4-[(Fmoc-L-Val-2-tritylsulfanylethyl)amino]benzoate (**4c**).* White amorphous solid, yield: 87% (831 mg); $[\alpha]^{21}_{D}$ 100.5 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 0.68 (3H, d, *J* = 6.5 Hz), 0.76 (3H, d, *J* = 6.5 Hz), 1.75 (1H, oct, *J* = 6.5 Hz), 2.28 (1H, ddd, *J* = 12.8, 9.0, and 5.5 Hz), 2.55 (1H, ddd, *J* = 12.8, 8.4, and 5.4 Hz), 3.34 (1H, ddd, *J* = 14.2, 8.4, and 5.5 Hz), 3.58 (1H, ddd, *J* = 14.2, 9.0, and 5.4 Hz), 4.05 (1H, dd, *J* = 9.0 and 6.5 Hz), 4.20 (1H, t, *J* = 7.0 Hz), 4.30 (1H, dd, *J* = 10.4 and 7.0 Hz), 4.38 (1H, dd, *J* = 10.4 and 7.0 Hz), 4.85 (2H, d, *J* = 5.6 Hz), 5.33 (1H, d, *J* = 10.4 Hz), 5.34 (1H, d, *J* = 9.0 Hz), 5.44 (1H, d, *J* = 17.2 Hz), 6.06 (1H, ddt, *J* = 17.2, 10.4, and 5.6 Hz), 7.06 (2H, d, *J* = 8.2 Hz), 7.09−7.36 (17H, m), 7.39 (2H, t, *J* = 7.3 Hz), 7.58 (2H, dd, *J* = 7.4 and 2.8 Hz), 7.76 (2H, d, *J* = 7.3 Hz), 8.05 (2H, d, *J* = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 17.2, 19.5, 29.2, 31.5, 47.2, 49.0, 56.4, 65.9, 66.9, 66.9, 118.7, 120.0, 125.1, 125.1, 126.7, 127.0, 127.7, 127.8, 128.4, 129.5, 129.8, 131.1, 131.9, 141.3, 143.8, 144.5, 145.0, 155.9, 165.3, 171.6; HRMS (ESI-TOF) *m*/*z* calcd for $C_{51}H_{49}N_2O_5S$ $[M + H]^+$ 801.3362, found 801.3358.

*Allyl 4-[(Fmoc-L-Ile-2-tritylsulfanylethyl)amino]benzoate (**4d**).* White amorphous solid, yield: 87% (848 mg); $[\alpha]^{21}_{D}$ 114.9 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 0.67 (3H, d, *J* = 6.6 Hz), 0.73 (3H, t, *J* = 7.2 Hz), 0.84−0.98 (1H, m), 1.31−1.45 (1H, m), 1.45−1.56 (1H, m), 2.28 (1H, ddd, *J* = 13.3, 9.0, and 5.4 Hz), 2.55 (1H, ddd, *J* = 13.3, 8.3, and 5.1 Hz), 3.37 (1H, ddd, *J* = 14.3, 8.3, and 5.4 Hz), 3.60 (1H, ddd, *J* = 14.3, 9.0, and 5.1 Hz), 4.08 (1H, dd, *J* = 8.8 and 7.5 Hz), 4.20 (1H, t, *J* = 7.0 Hz), 4.31 (1H, dd, *J* = 10.5 and 7.0 Hz), 4.38 (1H, dd, *J* = 10.5 and 7.0 Hz), 4.85 (2H, d, *J* = 5.6 Hz), 5.30 (1H, d, *J* = 8.8 Hz), 5.33 (1H, d, *J* = 10.5 Hz), 5.44 (1H, d, *J* = 17.4 Hz), 6.06 (1H, ddt, *J* = 17.4, 10.5, and 5.6 Hz), 7.06 (2H, d, *J* = 8.0 Hz), 7.10−7.36 (17H, m), 7.40 (2H, t, *J* = 7.4 Hz), 7.59 (2H, dd, *J* = 7.2 and 2.5 Hz), 7.76 (2H, d, *J* = 7.5 Hz), 8.04 (2H, d, *J* = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 11.2, 15.6, 23.9, 29.2, 38.2, 47.2, 49.0, 55.8, 65.9, 66.8, 66.9, 118.6, 119.9, 125.1, 125.1, 126.6, 127.0, 127.7, 127.8, 128.4, 129.5, 129.6, 129.8, 131.1, 132.0, 141.3, 143.8, 144.5, 145.0, 155.8, 165.3, 171.7; HRMS (ESI-TOF) *m*/*z* calcd for $C_{52}H_{51}N_2O_5S$ $[M + H]^+$ 815.3519, found 815.3549.

*Allyl 4-[(Fmoc-L-Leu-2-tritylsulfanylethyl)amino]benzoate (**4e**).* White amorphous solid, yield: 88% (75 mg); $[\alpha]^{18}_{D}$ 97.4 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 0.37 (3H, d, *J* = 6.0 Hz), 0.72 (3H, d, *J* = 6.2 Hz), 1.08−1.20 (1H, m), 1.32−1.49 (2H, m), 2.30 (1H, ddd, *J* = 12.9, 9.3, and 5.4 Hz), 2.53 (1H, ddd, *J* = 12.9, 8.3, and 5.3 Hz), 3.28 (1H, ddd, *J* = 13.8, 8.3, and 5.4 Hz), 3.61 (1H, ddd, *J* = 13.8, 9.3, and 5.3 Hz), 4.15−4.40 (4H, m), 4.86 (2H, d, *J* = 5.6 Hz), 5.27 (1H, d, *J* = 9.2 Hz), 5.33 (1H, d, *J* = 10.5 Hz), 5.43 (1H, d, *J* = 17.1 Hz), 6.06 (1H, ddt, *J* = 17.1, 10.5, and 5.6 Hz), 7.05−7.35 (19H, m), 7.39 (2H, t, *J* = 7.4 Hz), 7.58 (2H, dd, *J* = 7.2 and 4.8 Hz), 7.76 (2H, d, *J* = 7.4 Hz), 8.06 (2H, d, *J* = 8.1 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 20.8, 23.2, 24.3, 29.2, 42.3, 47.1, 48.9, 50.2, 65.9, 66.9, 66.9, 118.6, 119.9, 119.9, 125.1, 126.6, 127.0, 127.7, 127.8, 128.4, 129.5, 129.8, 131.1, 131.9, 141.3, 143.8, 143.9, 144.5, 144.9, 156.0, 165.2, 172.5; HRMS (ESI-TOF) *m*/*z* calcd for $C_{52}H_{50}N_2NaO_5S$ $[M + Na]^+$ 837.3338, found 837.3304.

*Allyl 4-[(Fmoc-L-Phe-2-tritylsulfanylethyl)amino]benzoate (**4f**).* White amorphous solid, yield: 88% (883 mg); $[\alpha]^{19}_{D}$ 63.8 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 2.21−2.41 (2H, m), 2.71 (1H, dd, *J* = 12.9 and 6.1 Hz), 2.85 (1H, dd, *J* = 12.9 and 8.8 Hz), 3.25−3.45 (2H, m), 4.15 (1H, t, *J* = 7.1 Hz), 4.21−4.40 (1H, m), 4.32 (2H, d, *J* = 7.1 Hz), 4.85 (2H, d, *J* = 5.5 Hz), 5.33 (1H, d, *J* = 10.4 Hz), 5.38 (1H, d, *J* = 8.9 Hz), 5.43 (1H, d, *J* = 17.3 Hz), 6.05 (1H, ddt, 17.3, 10.4, and 5.5 Hz), 6.88 (2H, d, *J* = 7.2 Hz), 7.09−7.34 (22H, m), 7.39 (2H, t, *J* = 7.4 Hz), 7.55 (2H, dd, *J* = 7.2 and 4.4 Hz), 7.76 (2H, d, *J* = 7.4 Hz), 7.88 (2H, d, *J* = 8.1 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 29.0, 39.8, 47.1, 48.9, 52.9, 65.9, 66.9, 66.9, 118.6, 119.9, 125.1, 125.1, 126.7, 127.0, 127.7, 127.8, 128.0, 128.5, 129.5, 129.7, 130.9, 132.0, 132.0, 135.8, 141.3, 143.7, 143.8, 144.5, 155.2, 165.3, 170.9; HRMS (ESI-TOF) *m*/*z* calcd for $C_{55}H_{49}N_2O_5S$ $[M + H]^+$ 849.3362, found 849.3356.

*Allyl 4-[(Fmoc-L-Pro-2-tritylsulfanylethyl)amino]benzoate (**4g**).* White amorphous solid, yield: 39% (66 mg); $[\alpha]^{19}_{D}$ 62.0 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.56−2.07 (4.3H, m), 2.13−2.34 (1.2H, m), 2.42−2.54 (0.5H, m), 3.12−3.34 (1H, m), 3.34−3.51 (2H, m), 3.51−3.72 (1H, m), 3.87−4.05 (1H, m), 4.10−4.40 (3H, m), 4.67−4.81 (2H, m), 5.20−5.30 (1H, m), 5.31−5.41 (1H, m), 5.91−6.05 (1H, m) 6.50−6.78 (1H, m), 6.95−7.57 (22H, m), 7.64−7.74 (2H, m), 7.76−7.88 (1H, m), 7.91−8.10 (1H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 23.3, 24.4, 29.3, 30.2, 31.4, 46.9, 47.1, 47.3, 47.5, 48.5, 48.9, 57.0, 57.5, 65.8, 65.9, 66.8, 67.0, 67.3, 118.5, 118.6, 119.9, 125.0, 125.1, 125.3, 126.6, 126.9, 127.0, 127.0, 127.1, 127.6, 127.6, 127.8, 127.9, 128.4, 128.6, 129.4, 129.5, 129.6, 131.0, 131.9, 131.9, 141.2, 143.8, 144.0, 144.1, 144.4, 144.6, 145.2, 145.7, 154.2, 154.7, 165.2, 165.4, 171.5, 171.9; HRMS (ESI-TOF) *m*/*z* calcd for $C_{51}H_{46}N_2NaO_5S$ $[M + Na]^+$ 821.3025, found 821.3010.

*Allyl 4-[(Fmoc-L-Ser(t-Bu)-2-tritylsulfanylethyl)amino]benzoate (**4h**).* White amorphous solid, yield: 90% (159 mg); $[\alpha]^{20}_{D}$ 52.1 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.06 (9H, s), 2.35−2.54 (2H, m), 3.18−3.28 (1H, br m), 3.32 (1H, t, *J* = 7.8 Hz), 3.42

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(1H, ddd, J = 13.5, 8.3, and 5.2 Hz), 3.54 (1H, ddd, J = 13.5, 8.3, and 5.8 Hz), 4.17 (1H, t, J = 7.0 Hz), 4.30 (2H, d, J = 7.0 Hz) 4.25−4.38 (1H, m), 4.85 (2H, d, J = 5.4 Hz), 5.32 (1H, d, J = 10.4 Hz), 5.36−5.48 (1H, m), 5.43 (1H, d, J = 17.2 Hz), 6.04 (1H, ddt, J = 17.2, 10.4, and 5.4 Hz), 7.05−7.43 (21H, m), 7.57 (2H, dd, J = 7.2 and 3.6 Hz), 7.75 (2H, d, J = 7.4 Hz), 8.03 (2H, d, J = 8.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 27.2, 29.2, 47.1, 49.2, 51.6, 62.5, 65.8, 67.0, 73.5, 118.5, 119.9, 125.1, 126.6, 127.0, 127.6, 127.8, 128.4, 129.5, 129.7, 130.9, 132.0, 141.2, 143.8, 143.8, 144.5, 145.1, 155.5, 165.3, 170.3; HRMS (ESI-TOF) m/z calcd for $C_{53}H_{53}N_2O_6S$ $[M + H]^+$ 845.3624, found 845.3643.

*Allyl 4-[(Fmoc-L-Asn(Tr)-2-tritylsulfanylethyl)amino]benzoate (**4i**).* White amorphous solid, yield: 89% (980 mg); $[\alpha]^{19}_D$ 2.4 (c 0.40, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 2.37 (2H, t, J = 7.4 Hz), 2.50 (1H, br d, J = 15.2 Hz), 2.60 (1H, dd, J = 15.2 and 5.2 Hz), 3.35 (2H, t, J = 7.4 Hz), 4.05 (1H, t, J = 6.3 Hz), 4.17 (2H, d, J = 6.3 Hz), 4.38−4.55 (1H, m), 4.76 (2H, d, J = 5.4 Hz), 5.26 (1H, d, J = 10.4 Hz), 5.37 (1H, d, J = 17.4 Hz), 5.43−5.56 (1H, m), 5.97 (1H, ddt, J = 17.4, 10.4, and 5.4 Hz), 6.70 (1H, s), 6.91 (2H, d, J = 7.9 Hz), 7.06−7.34 (32H, m), 7.38 (2H, t, J = 7.3 Hz), 7.49 (2H, d, J = 6.7 Hz), 7.75 (2H, d, J = 7.2 Hz), 7.89 (2H, d, J = 7.9 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 28.9, 36.0, 39.4, 46.9, 49.3, 49.4, 50.2, 65.6, 66.9, 67.1, 70.4, 74.0, 118.4, 119.8, 119.9, 124.8, 124.9, 124.9, 125.0, 126.5, 126.8, 126.9, 127.0, 127.4, 127.6, 127.8, 127.9, 128.4, 128.6, 129.3, 129.5, 130.9, 131.8, 141.1, 141.1, 141.8, 143.4, 143.5, 143.6, 143.6, 144.3, 144.4, 155.0, 155.6, 165.1, 168.2, 170.2, 172.9, 174.8; HRMS (ESI-TOF) m/z calcd for $C_{69}H_{59}N_3NaO_6S$ $[M + Na]^+$ 1080.4022, found 1080.4039.

*Allyl 4-[(Fmoc-L-Asp(Ot-Bu)-2-tritylsulfanylethyl)amino]benzoate (**4j**).* White amorphous solid, yield: 68% (62 mg); $[\alpha]^{19}_D$ 47.1 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.36 (9H, s), 2.27 (1H, br dd, J = 14.8 and 6.0 Hz), 2.33−2.54 (3H, m), 3.38 (1H, ddd, J = 14.0, 8.4, and 5.4 Hz), 3.52 (1H, ddd, J = 14.0, 9.2, and 4.8 Hz), 4.16 (1H, t, J = 7.0 Hz), 4.21−4.35 (2H, m), 4.40−4.54 (1H, br m), 4.83 (2H, d, J = 5.6 Hz), 5.30 (1H, d, J = 10.4 Hz), 5.41 (1H, d, J = 17.1 Hz), 5.52 (1H, d, J = 9.0 Hz), 6.02 (1H, ddt, J = 17.1, 10.4, and 5.6 Hz), 7.06−7.36 (19H, m), 7.39 (2H, t, J = 7.4 Hz), 7.56 (2H, dd, J = 6.4 and 4.8 Hz), 7.76 (2H, d, J = 7.4 Hz), 8.02 (2H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 27.9, 29.1, 38.2, 47.0, 49.0, 49.2, 65.8, 67.0, 67.0, 81.3, 118.5, 119.9, 125.1, 126.6, 127.0, 127.7, 127.8, 128.1, 129.5, 129.6, 129.9, 131.2, 131.9, 141.2, 143.7, 143.8, 144.5, 144.7, 155.2, 165.2, 169.0, 169.9; HRMS (ESI-TOF) m/z calcd for $C_{54}H_{52}N_2NaO_7S$ $[M + Na]^+$ 895.3393, found 895.3373.

*Allyl 4-[(Fmoc-L-Thr(t-Bu)-2-tritylsulfanylethyl)amino]benzoate (**4k**).* White amorphous solid, yield: 84% (698 mg); $[\alpha]^{23}_D$ 122.0 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 0.89 (3H, d, J = 5.6 Hz), 1.03 (9H, s), 2.20−2.36 (1H, br m), 2.45−2.66 (1H, br m), 3.10−3.34 (1H, br m), 3.51−3.66 (1H, br m), 3.66−3.82 (1H, br m), 4.15−4.30 (1H, m), 4.22 (1H, t, J = 7.1 Hz), 4.35 (2H, d, J = 7.1 Hz), 4.86 (2H, d, J = 5.6 Hz), 5.33 (1H, d, J = 10.4 Hz), 5.44 (1H, d, J = 17.4 Hz), 5.54 (1H, d, J = 9.0 Hz), 6.06 (1H, ddt, J = 17.4, 10.4, and 5.6 Hz), 7.05−7.35 (19H, m), 7.40 (2H, t, J = 7.4 Hz), 7.61 (2H, dd, J = 7.4 and 7.2 Hz), 7.77 (2H, d, J = 7.5 Hz), 8.06 (2H, d, J = 8.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 20.1, 28.1, 29.1, 47.2, 49.4, 56.5, 65.9, 67.0, 67.7, 74.2, 118.6, 119.9, 125.2, 126.6, 127.0, 127.6, 127.7, 127.8, 127.9, 128.2, 129.5, 129.6, 131.2, 131.9, 141.3, 143.8, 144.0, 144.5, 145.4, 156.1, 165.3, 170.0; HRMS (ESI-TOF) m/z calcd for $C_{54}H_{54}N_2NaO_6S$ $[M + Na]^+$ 881.3600, found 881.3580.

*Allyl 4-[(Fmoc-L-Glu(Ot-Bu)-2-tritylsulfanylethyl)amino]benzoate (**4l**).* White amorphous solid, yield: 72% (665 mg); $[\alpha]^{19}_D$ 81.5 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.36 (9H, s), 1.65−1.83 (2H, m), 1.96−2.19 (2H, m), 2.23−2.37 (1H, m), 2.44−2.59 (1H, m), 3.33 (1H, ddd, J = 13.4, 8.8, and 5.9 Hz), 3.57 (1H, ddd, J = 13.4, 9.0, and 6.1 Hz), 4.08−4.24 (1H, m), 4.18 (1H, t, J = 7.2 Hz), 4.32 (2H, d, J = 7.2 Hz), 4.86 (2H, ddd, J = 5.6, 1.5, and 1.3 Hz), 5.33 (1H, dtd, J = 10.4, 1.3, and 1.2 Hz), 5.44 (1H, dtd, J = 17.2, 1.5, and 1.2 Hz), 5.53 (1H, d, J = 8.5 Hz), 6.06 (1H, ddt, J = 17.2, 10.4, and 5.6 Hz), 7.07−7.36 (19H, m), 7.40 (2H, t, J = 7.4 Hz), 7.58 (2H, dd, J = 6.8 and 5.0 Hz), 7.76 (2H, d, J = 7.4 Hz), 8.05 (2H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 27.5, 28.0, 29.1, 30.9, 47.1, 48.9, 51.1, 65.8, 66.9, 80.6, 118.6, 119.9, 125.1, 126.3, 127.0, 127.8, 128.3, 129.5, 129.6, 130.0, 131.3, 131.9, 141.2, 143.7, 143.8, 144.5, 144.6, 155.7, 165.2, 171.1, 171.8; HRMS (ESI-TOF) m/z calcd for $C_{55}H_{54}N_2NaO_7S$ $[M + Na]^+$ 909.3549, found 909.3569.

*Allyl 4-[(Fmoc-L-Gln(Tr)-2-tritylsulfanylethyl)amino]benzoate (**4m**).* White amorphous solid, yield: 93% (1032 mg); $[\alpha]^{19}_D$ 48.3 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.64−1.88 (2H, m), 2.00−2.22 (2H, m), 2.28 (1H, ddd, J = 13.5, 9.0, and 5.3 Hz), 2.46 (1H, ddd, J = 13.5, 8.0, and 5.3 Hz), 3.29 (1H, ddd, J = 14.1, 8.0, and 5.3 Hz), 3.48 (1H, ddd, J = 14.1, 9.0, and 5.3 Hz), 4.06−4.23 (2H, m), 4.24−4.41 (2H, m), 4.84 (2H, d, J = 5.5 Hz), 5.31 (1H, d, J = 10.4 Hz), 5.42 (1H, d, J = 17.2 Hz), 5.62 (1H, d, J = 8.2 Hz), 6.04 (1H, ddt, J = 17.2, 10.4, and 5.5 Hz), 6.47 (1H, s), 7.00−7.43 (36H, m), 7.56 (2H, d, J = 6.6 Hz), 7.74 (2H, dd, J = 7.6 and 2.8 Hz), 7.98 (2H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 27.8, 29.2, 32.7, 47.1, 49.0, 51.3, 65.8, 66.8, 67.0, 70.5, 118.6, 119.9, 125.1, 126.6, 127.0, 127.0, 127.7, 127.8, 127.9, 128.3, 128.6, 129.5, 129.6, 130.0, 131.3, 131.9, 141.2, 141.3, 143.7, 143.8, 144.5, 155.8, 165.2, 170.4, 171.0; HRMS (ESI-TOF) m/z calcd for $C_{70}H_{62}N_3O_6S$ $[M + H]^+$ 1072.4359, found 1072.4370.

*Allyl 4-[(Fmoc-L-Cys(Tr)-2-tritylsulfanylethyl)amino]benzoate (**4n**).* White amorphous solid, yield: 72% (78 mg); $[\alpha]^{18}_D$ 16.8 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 2.06 (1H, dd, J = 12.4 and 6.8 Hz), 2.20−2.34 (2H, m), 2.38−2.51 (1H, m), 3.26−3.50 (2H, m), 4.19 (1H, t, J = 7.3 Hz), 4.22−4.36 (3H, m), 4.86 (2H, d, J = 5.6 Hz). 5.34 (1H, d, J = 10.4 Hz), 5.45 (1H, d, J = 17.1 Hz), 5.38−5.50 (1H, m), 6.06 (1H, ddt, J = 17.1, 10.4, and 5.6 Hz), 6.86 (2H, d, J = 7.4 Hz), 7.08−7.34 (32H, m), 7.39 (2H, t, J = 7.4 Hz), 7.59 (2H, d, J = 7.2 Hz), 7.76 (2H, d, J = 7.4 Hz), 7.95 (2H, d, J = 8.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 29.1, 34.0, 47.0, 49.2, 50.6, 65.9, 66.5, 66.9, 67.1, 118.6, 119.9, 125.2, 126.6, 126.7, 127.1, 127.7, 127.8, 127.9, 128.1, 129.4, 129.5, 129.9, 131.2, 131.9, 141.2, 143.7, 143.8, 144.2, 144.4, 144.5, 155.3, 165.1, 169.6; HRMS (ESI-TOF) m/z calcd for $C_{68}H_{58}N_2NaO_5S_2$ $[M + Na]^+$ 1069.3685, found 1069.3671.

*Allyl 4-[(Fmoc-L-Met-2-tritylsulfanylethyl)amino]benzoate (**4o**).* White amorphous solid, yield: 80% (690 mg); $[\alpha]^{18}_D$ 68.3 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.56−1.78 (2H, m), 1.88 (3H, s), 2.14−2.25 (1H, m), 2.25−2.38 (2H, m), 2.42−2.60 (1H, m), 3.38 (1H, ddd, J = 13.0, 8.1, and 6.5 Hz), 3.55 (1H, ddd, J = 13.0, 8.2, and 6.5 Hz), 4.18 (1H, t, J = 6.9 Hz), 4.25−4.39 (3H, m), 4.85 (2H, d, J = 5.4 Hz), 5.32 (1H, d, J = 10.4 Hz), 5.43 (1H, d, J = 17.4 Hz), 5.48 (1H, d, J = 8.9 Hz), 6.05 (1H, ddt, J = 17.4, 10.4, and 5.4 Hz), 7.03−7.36 (19H, m), 7.39 (2H, t, J = 7.4 Hz), 7.57 (2H, dd, J = 7.6 and 5.6 Hz), 7.76 (2H, d, J = 7.4 Hz), 8.06 (2H, d, J = 7.9 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 15.4, 29.2, 29.7, 32.5, 47.1, 48.9, 51.0, 65.9, 66.9, 66.9, 118.6, 119.9, 125.1, 126.7, 127.0, 127.7, 127.8, 128.2, 129.5, 130.1, 131.3, 131.9, 141.3, 143.7, 143.8, 144.4, 144.6, 155.8, 165.2, 171.3; HRMS (ESI-TOF) m/z calcd for $C_{51}H_{49}N_2O_5S_2$ $[M + H]^+$ 833.3083, found 833.3077.

*Allyl 4-[(Fmoc-L-Tyr(t-Bu)-2-tritylsulfanylethyl)amino]benzoate (**4p**).* White amorphous solid, yield: 86% (773 mg); $[\alpha]^{22}_D$ 25.9 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.31 (9H, s), 2.23−2.42 (2H, m), 2.67 (1H, dd, J = 13.0 and 5.9 Hz), 2.83 (1H, dd, J = 13.0 and 9.0 Hz), 3.24−3.43 (2H, m), 4.16 (1H, t, J = 7.0 Hz), 4.22−4.43 (3H, m), 4.85 (2H, d, J = 5.5 Hz), 5.33 (1H, d, J = 10.5 Hz), 5.36 (1H, d, J = 8.9 Hz), 5.44 (1H, d, J = 17.2 Hz), 6.06 (1H, ddt, J = 17.2, 10.5, and 5.5 Hz), 6.79 (4H, s), 7.09−7.36 (19H, m), 7.40 (2H, t, J = 7.4 Hz), 7.56 (2H, dd, J = 6.8 and 2.4 Hz), 7.76 (2H, d, J = 7.5 Hz), 7.90 (2H, d, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 28.8, 29.0, 39.3, 47.1, 48.9, 52.9, 65.8, 66.9, 67.0, 78.4, 118.6, 119.9, 124.1, 125.1, 125.1,126.7, 127.0, 127.7, 127.7, 127.8, 127.9, 128.1, 129.5, 129.7, 129.8, 130.0, 130.6, 130.8, 132.0, 141.3, 143.8, 143.8, 144.5, 144.5, 154.5, 155.2, 165.3, 171.0; HRMS (ESI-TOF) m/z calcd for $C_{59}H_{57}N_2O_6S$ $[M + H]^+$ 921.3937, found 921.3939.

*Allyl 4-[(Fmoc-L-His(Tr)-2-tritylsulfanylethyl)amino]benzoate (**4q**).* White amorphous solid, yield: 41% (745 mg); $[\alpha]^{20}_D$ 32.9 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 2.15−2.38 (1H, br m), 2.52−2.71 (2H, br m), 2.71−2.87 (1H, br m), 3.17−3.37 (1H, br m), 3.40−3.61 (1H, m), 4.18 (1H, t, J = 7.3 Hz), 4.20−4.35 (2H, m), 4.37−4.49 (1H, m), 4.87 (2H, d, J = 5.4 Hz), 5.34 (1H, d, J = 10.4 Hz), 5.45 (1H, d, J = 17.3 Hz), 6.07 (1H, ddt, J = 17.3, 10.4, and 5.4 Hz), 6.13 (1H, d, J = 8.0 Hz), 6.50 (1H, s), 7.02−7.45 (37H, m), 7.59

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(2H, dd, J = 7.6 and 2.8 Hz), 7.76 (2H, d, J = 7.4 Hz), 8.02 (2H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 29.1, 30.4, 47.0, 49.2, 51.9, 65.7, 66.9, 67.0, 75.3, 118.5, 119.6, 119.8, 125.2, 125.3, 126.5, 127.0, 127.0, 127.1, 127.5, 127.7, 127.9, 128.0, 128.3, 129.4, 129.7, 131.0, 131.9, 135.6, 138.2, 141.1, 142.1, 143.8, 143.9, 144.5, 145.1, 155.7, 165.2, 170.6; HRMS (ESI-TOF) m/z calcd for $C_{71}H_{60}N_4NaO_5S$ $[M + Na]^+$ 1103.4182, found 1103.4187.

*Allyl 4-[(Fmoc-L-His(MBom)-2-tritylsulfanylethyl)amino]benzoate (**4r**).* White amorphous solid, yield: 88% (91 mg); $[\alpha]^{20}_D$ 45.4 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 2.25 (1H, ddd, J = 12.8, 9.2, and 6.0 Hz), 2.44 (1H, ddd, J = 12.8, 8.8, and 6.0 Hz), 2.77 (1H, dd, J = 14.7 and 6.7 Hz), 2.93 (1H, dd, J = 14.7 and 7.9 Hz), 3.20 (1H, ddd, J = 14.0, 8.8, and 6.0 Hz), 3.60 (1H, ddd, J = 14.0, 9.2, and 6.0 Hz), 3.78 (3H, s), 4.07 (1H, d, J = 11.6 Hz), 4.12 (1H, d, J = 11.6 Hz), 4.15 (1H, t, J = 6.9 Hz), 4.24–4.41 (3H, m), 4.77 (1H, d, J = 11.2 Hz), 4.81 (1H, d, J = 11.2 Hz), 4.83 (2H, d, J = 5.5, 1.7, and 1.5 Hz), 5.30 (1H, dtd, J = 10.4, 1.5, and 1.3 Hz), 5.42 (1H, dtd, J = 17.2, 1.7, and 1.3 Hz), 5.50 (1H, d, J = 8.8 Hz), 6.03 (1H, ddt, J = 17.2, 10.4, and 5.5 Hz), 6.70 (1H, s), 6.72–6.81 (1H, br m), 6.84 (2H, d, J = 8.5 Hz), 7.05–7.35 (21H, m), 7.38 (2H, t, J = 7.3 Hz), 7.55 (2H, dd, J = 7.2 and 3.2 Hz), 7.74 (2H, d, J = 7.5 Hz), 7.94 (2H, d, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 27.5, 29.0, 47.0, 48.8, 51.2, 55.2, 65.9, 66.9, 69.0, 72.3, 114.0, 118.7, 120.0, 125.0, 126.1, 126.6, 127.0, 127.7, 127.8, 127.9, 128.1, 129.4, 129.6, 129.7, 129.9, 131.0, 131.9, 138.2, 141.3, 143.7, 144.3, 155.3, 159.6, 165.1, 170.6; HRMS (ESI-TOF) m/z calcd for $C_{61}H_{56}N_4NaO_7S$ $[M + Na]^+$ 1011.3767, found 1011.3791.

*Allyl 4-[(Fmoc-L-Lys(Boc)-2-tritylsulfanylethyl)amino]benzoate (**4s**).* White amorphous solid, yield: 80% (779 mg); $[\alpha]^{21}_D$ 69.1 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.01–1.30 (4H, br m), 1.31–1.52 (2H, m) 1.41 (9H, s), 2.29 (1H, ddd, J = 12.8, 9.4, and 5.4 Hz), 2.53 (1H, ddd, J = 12.8, 9.2, and 5.1 Hz), 2.81–3.05 (2H, br s), 3.31 (1H, ddd, J = 14.5, 9.2, and 5.4 Hz), 3.60 (1H, ddd, J = 14.5, 9.4, and 5.1 Hz), 4.10–4.26 (1H, m), 4.19 (1H, t, J = 7.2 Hz), 4.33 (2H, d, J = 7.2 Hz), 4.37 (1H, m), 4.86 (2H, ddd, J = 6.0, 2.0, and 1.6 Hz), 5.33 (1H, dtd, J = 11.2, 2.0, and 1.2 Hz), 5.38–5.48 (1H, m), 5.44 (1H, dtd, J = 16.8, 1.6, and 1.2 Hz), 6.06 (1H, ddt, J = 16.8, 11.2, and 6.0 Hz), 7.04–7.45 (19H, m), 7.40 (2H, t, J = 7.4 Hz), 7.59 (2H, dd, J = 7.2 and 4.8 Hz), 7.76 (2H, d, J = 7.5 Hz), 8.06 (2H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 22.1, 28.4, 29.2, 29.2, 32.5, 40.1, 47.1, 48.9, 51.3, 65.9, 66.9, 66.9, 79.1, 118.6, 119.9, 125.1, 126.6, 127.0, 127.6, 127.8, 128.3, 129.4, 129.6, 130.0, 131.2, 131.9, 141.2, 143.7, 143.8, 144.4, 144.7, 155.8, 155.9, 165.1, 171.7; HRMS (ESI-TOF) m/z calcd for $C_{57}H_{60}N_3O_7S$ $[M + H]^+$ 930.4152, found 930.4143.

*Allyl 4-[(Fmoc-L-Arg(Pbf)-2-tritylsulfanylethyl)amino]benzoate (**4t**).* White amorphous solid, yield: 95% (1101 mg); $[\alpha]^{22}_D$ 46.3 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.16–1.34 (2H, m), 1.41 (6H, s), 1.43–1.55 (2H, m), 2.06 (3H, s), 2.19–2.33 (1H, m), 2.43–2.51 (1H, m), 2.42- 2.51 (1H, m), 2.48 (3H, s), 2.54 (3H, s), 2.66–2.96 (2H, br m), 2.89 (2H, s), 3.23 (1H, ddd, J = 13.6, 9.0, and 5.4 Hz), 3.63 (1H, ddd, J = 13.6, 9.2, and 5.5 Hz), 4.06–4.20 (1H, m), 4.13 (1H, t, J = 6.4 Hz), 4.21–4.37 (2H, m), 4.85 (2H, d, J = 5.5 Hz), 5.32 (1H, d, J = 10.4 Hz), 5.43 (1H, d, J = 17.2 Hz), 5.92 (1H, d, J = 8.1 Hz), 5.98–6.16 (3H, m), 7.04–7.33 (19H, m), 7.37 (2H, t, J = 7.4 Hz), 7.54 (2H, t, J = 7.4 Hz), 7.74 (2H, d, J = 7.5 Hz), 8.04 (2H, d, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 12.4, 17.9, 19.3, 24.6, 28.5, 29.1, 30.2, 40.5, 43.1, 47.0, 48.9, 50.9, 66.0, 66.9, 67.3, 86.4, 117.5, 118.7, 119.9, 124.7, 125.0, 125.1, 126.7, 127.0, 127.2, 127.7, 127.7, 127.8, 127.9, 128.3, 129.4, 130.2, 131.3, 132.5, 138.5, 141.2, 143.6, 144.4, 144.4, 144.4, 155.7, 156.3, 158.9, 165.1, 171.4; HRMS (ESI-TOF) m/z calcd for $C_{65}H_{67}KN_5O_8S_2$ $[M + K]^+$ 1148.4068, found 1148.4044.

*Allyl 4-[(Fmoc-L-Trp-2-tritylsulfanylethyl)amino]benzoate (**4u**).* White amorphous solid, yield: 92% (852 mg); $[\alpha]^{23}_D$ 82.1 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 2.16 (1H, ddd, J = 12.4, 8.4, and 7.2 Hz), 2.29 (1H, ddd, J = 12.4, 7.4, and 6.9 Hz), 2.93 (1H, dd, J = 13.8 and 5.4 Hz), 3.06 (1H, dd, J = 13.8 and 9.2 Hz), 3.27–3.47 (2H, m), 4.19 (1H, t, J = 7.0 Hz), 4.32 (1H, dd, J = 18.0 and 7.0 Hz), 4.35 (1H, dd, J = 18.0 and 7.0 Hz), 4.51 (1H, ddd, J = 9.2, 8.8, and 5.4 Hz), 4.83 (2H, d, J = 5.5 Hz), 5.34 (1H, d, J = 10.4 Hz), 5.44 (1H, d, J = 17.3 Hz), 5.49 (1H, d, J = 8.8 Hz), 6.06 (1H, ddt, J = 17.3, 10.4, and 5.5 Hz), 6.18–6.52 (1H, br s), 6.76 (1H, d, J = 1.7 Hz), 6.93 (1H, t, J = 7.3 Hz), 7.09–7.37 (21H, m), 7.40 (2H, t, J = 7.4 Hz), 7.58 (2H, dd, J = 7.6 and 3.6 Hz), 7.66 (2H, d, J = 6.9 Hz), 7.71–7.82 (1H, m), 7.77 (3H, d, J = 7.6 Hz) ; ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 29.1, 29.7, 47.1, 48.5, 52.0, 65.7, 66.7, 66.9, 110.0, 110.9, 118.4, 118.6, 119.6, 119.9, 122.2, 123.0, 125.1, 125.1, 126.6, 127.0, 127.4, 127.6, 127.7, 127.8, 129.3, 129.5, 129.6, 130.5, 132.0, 135.9, 141.2, 143.8, 144.3, 144.5, 155.4, 165.3, 171.7; HRMS (ESI-TOF) m/z calcd for $C_{57}H_{50}N_3O_5S$ $[M + H]^+$ 888.3471, found 888.3444.

*4-[(Fmoc-L-Val-2-tritylsulfanylethyl)amino]benzoic acid (**10c**).* White amorphous solid, yield: 80% (76 mg); $[\alpha]^{24}_D$ 82.8 (c 0.40, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 0.75 (3H, d, J = 6.5 Hz), 0.81 (3H, d, J = 6.5 Hz), 1.75–1.94 (1H, m), 2.23–2.38 (1H, m), 2.48–2.65 (1H, m), 3.39 (1H, ddd, J = 13.9, 8.5, and 5.4 Hz), 3.60 (1H, ddd, J = 13.9, 8.8, and 5.2 Hz), 4.11 (1H, dd, J = 9.5 and 7.2 Hz), 4.22 (1H, t, J = 7.0 Hz), 4.34 (1H, dd, J = 10.4 and 7.0 Hz), 4.43 (1H, dd, J = 10.4 and 7.0 Hz), 5.80 (1H, d, J = 9.5 Hz), 7.08 (2H, d, J = 8.1 Hz), 7.10–7.44 (19H, m), 7.63 (2H, dd, J = 7.6 and 2.8 Hz), 7.76 (2H, d, J = 7.2 Hz), 8.09 (2H, d, J = 8.1 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 17.3, 19.4, 29.1, 31.7, 47.2, 49.1, 56.5, 67.0, 67.0, 120.0, 125.1, 125.2, 126.7, 127.1, 127.3, 127.7, 127.8, 127.9, 128.4, 129.5, 131.7, 141.3, 143.8, 144.5, 145.3, 146.8, 156.1, 168.4, 171.7; HRMS (ESI-TOF) m/z calcd for $C_{48}H_{45}N_2O_5S$ $[M + H]^+$ 761.3049, found 761.3057.

*4-[(Fmoc-L-Phe-2-tritylsulfanylethyl)amino]benzoic acid (**10f**).* White amorphous solid, yield: 93% (176 mg); $[\alpha]^{19}_D$ 53.5 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 2.23–2.43 (2H, m), 2.78 (1H, dd, J = 12.9 and 5.9 Hz), 2.91 (1H, dd, J = 12.9 and 9.2 Hz), 3.28–3.45 (2H, m), 4.18 (1H, t, J = 7.0 Hz), 4.25–4.45 (3H, m), 5.86 (1H, d, J = 8.9 Hz), 6.95 (2H, d, J = 7.2 Hz), 7.10–7.35 (22H, m), 7.39 (2H, t, J = 7.2 Hz), 7.59 (2H, dd, J = 7.2 and 1.2 Hz), 7.76 (2H, d, J = 7.2 Hz), 7.93 (2H, d, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 28.9, 39.8, 47.1, 49.0, 53.0, 67.0, 67.1, 119.9, 125.1, 125.2, 126.7, 127.0, 127.7, 127.8, 127.9, 128.1, 128.5, 128.7, 129.5, 129.6, 129.6, 131.4, 132.1, 132.2, 132.2, 135.8, 141.3, 143.7, 143.8, 144.5, 144.6, 155.5, 168.8, 171.3; HRMS (ESI-TOF) m/z calcd for $C_{52}H_{45}N_2O_5S$ $[M + H]^+$ 809.3049, found 809.3044.

*4-[(Fmoc-L-Ser(t-Bu)-2-tritylsulfanylethyl)amino]benzoic acid (**10h**).* White amorphous solid, yield: quant. (187 mg); $[\alpha]^{20}_D$ 45.4 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.08 (9H, s), 2.35–2.55 (2H, m), 3.20–3.34 (1H, m), 3.34–3.50 (2H, m), 3.50–3.65 (1H, m), 4.19 (1H, t, J = 7.1 Hz), 4.32 (2H, d, J = 7.1 Hz), 4.35–4.46 (1H, m), 5.68 (1H, d, J = 8.8 Hz), 7.10–7.35 (19H, m), 7.39 (2H, t, J = 7.3 Hz), 7.59 (2H, dd, J = 6.8 and 4.0 Hz), 7.75 (2H, d, J = 7.3 Hz), 8.06 (2H, d, J = 8.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 27.2, 29.1, 47.0, 49.3, 51.6, 62.5, 67.0, 67.1, 73.7, 119.9, 125.2, 126.7, 127.0, 127.7, 127.8, 127.9, 128.5, 129.1, 129.5, 131.5, 141.3, 143.7, 143.8, 144.5, 145.4, 155.8, 169.3, 169.3; HRMS (ESI-TOF) m/z calcd for $C_{50}H_{49}N_2O_6S$ $[M + H]^+$ 805.3311, found 805.3332.

*4-[(Fmoc-L-Asn(Tr)-2-tritylsulfanylethyl)amino]benzoic acid (**10i**).* White amorphous solid, yield: 83% (160 mg); $[\alpha]^{24}_D$ −17.0 (c 0.40, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 2.28–2.47 (2H, t, J = 6.7 Hz), 2.63–2.86 (2H, m), 3.15–3.46 (2H, m), 4.11 (1H, t, J = 6.7 Hz), 4.23 (1H, dd, J = 10.3 and 6.7 Hz), 4.30 (1H, dd, J = 10.3 and 6.7 Hz), 4.39–4.69 (1H, m), 6.34 (1H, d, J = 7.6 Hz), 6.77 (1H, s), 6.92 (2H, d, J = 7.4 Hz), 7.04–7.34 (32H, m), 7.36 (2H, t, J = 7.4 Hz), 7.55 (2H, dd, J = 13.6 and 7.2 Hz), 7.74 (2H, d, J = 7.4 Hz), 7.82 (2H, d, J = 7.4 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 28.9, 39.3, 47.0, 49.5, 49.5, 67.0, 67.0, 70.7, 119.9, 125.2, 125.2, 126.6, 127.0, 127.0, 127.2, 127.7, 127.8, 127.9, 128.7, 129.4, 129.6, 131.5, 141.3, 143.5, 143.8, 144.2, 144.5, 144.7, 155.6, 168.2, 169.0, 170.3; HRMS (ESI-TOF) m/z calcd for $C_{66}H_{56}N_3O_6S$ $[M + H]^+$ 1018.3890, found 1018.3877.

*4-[(Fmoc-L-Asp(Ot-Bu)-2-tritylsulfanylethyl)amino]benzoic acid (**10j**).* White amorphous solid, yield: 98% (188 mg); $[\alpha]^{23}_D$ 80.3 (c 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.37 (9H, s), 2.23–2.44 (2H, m), 2.44–2.60 (2H, m), 3.29–3.46 (1H, m), 3.46–3.61 (1H, m), 4.17 (1H, t, J = 7.1 Hz), 4.29 (2H, d, J = 7.1 Hz), 4.45–4.61 (1H, br m), 5.77 (1H, d, J = 9.1 Hz), 7.07–7.35 (19H, m), 7.38 (2H, t, J = 7.4 Hz), 7.58 (2H, dd, J = 7.6 and 2.0 Hz), 7.75 (2H, d, J = 7.4 Hz), 8.06 (2H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 28.0, 29.1, 38.3, 47.0, 49.0, 49.3, 67.0, 67.2, 81.5, 120.0, 125.2, 126.7, 127.0, 127.7, 127.8, 128.2, 128.5, 128.6, 129.5, 131.7, 132.1, 132.2, 141.3,

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143.7, 143.8, 144.5, 145.2, 155.4, 169.1, 169.4, 170.1; HRMS (ESI-TOF) m/z calcd for $C_{51}H_{49}N_2O_7S$ $[M + H]^+$ 833.3260, found 833.3247.

*4-[(Fmoc-L-Glu(Ot-Bu)-2-tritylsulfanylethyl)amino]benzoic acid (**10l**).* White amorphous solid, yield: 86% (164 mg); $[\alpha]^{24}_D$ 57.0 (c 1.00, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ = 1.38 (9H, s), 1.66−1.87 (2H, m), 2.00−2.24 (2H, m), 2.25−2.40 (1H, m), 2.45−2.60 (1H, m), 3.30−3.45 (1H, m), 3.58 (1H, ddd, J = 13.4, 8.8, and 5.9 Hz), 4.20 (1H, t, J = 7.2 Hz), 4.16−4.28 (1H, m), 4.35 (2H, d, J = 7.2 Hz), 5.92 (1H, d, J = 8.3 Hz), 7.06−7.37 (19H, m), 7.39 (2H, t, J = 7.4 Hz), 7.61 (2H, dd, J = 7.6 and 2.4 Hz), 7.76 (2H, d, J = 7.4 Hz), 8.10 (2H, d, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 25.6, 28.0, 29.1, 30.8, 47.1, 49.0, 51.1, 67.1, 68.0, 80.7, 119.9, 125.2, 126.7, 127.0, 127.2, 127.7, 127.8, 127.9, 128.3, 128.6, 129.5, 131.8, 132.1, 141.3, 143.7, 143.8, 144.5, 144.9, 155.9, 168.6, 171.3, 172.0; HRMS (ESI-TOF) m/z calcd for $C_{52}H_{51}N_2O_7S$ $[M + H]^+$ 847.3417, found 847.3391.

*4-[(Fmoc-L-His(MBom)-2-tritylsulfanylethyl)amino]benzoic acid (**10r**).* White amorphous solid, yield: 90% (52 mg); $[\alpha]^{20}_D$ 27.8 (c 1.00, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ = 2.16−2.30 (1H, m), 2.38−2.54 (1H, m), 2.81 (1H, dd, J = 14.8 and 6.6 Hz), 2.97 (1H, d, J = 14.8 and 7.7 Hz), 3.24−3.38 (1H, m), 3.52−3.67 (1H, m), 3.73 (3H, s), 4.08−4.26 (1H, m), 4.13 (1H, d, J = 11.6 Hz), 4.20 (1H, d, J = 11.6 Hz), 4.28−4.48 (3H, m), 4.85 (1H, d, J = 10.7 Hz), 4.96 (1H, d, J = 10.7 Hz), 5.63 (1H, d, J = 8.5 Hz), 6.65−6.92 (1H, m), 6.71 (1H, s), 6.83 (2H, d, J = 8.4 Hz), 7.06−7.44 (22H, m), 7.50−7.64 (3H, m), 7.66−7.79 (2H, m), 8.07 (2H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 27.4, 29.1, 47.1, 48.7, 51.6, 55.2, 66.9, 69.5, 72.9, 114.1, 120.0, 125.1, 126.7, 126.8, 127.1, 127.6, 127.7, 127.8, 127.9, 129.5, 129.7, 131.4, 131.9, 137.7, 141.3, 143.5, 143.7, 144.4, 155.4, 159.6, 168.3, 170.7; HRMS (ESI-TOF) m/z calcd for $C_{58}H_{53}N_4O_7S$ $[M + H]^+$ 949.3635, found 949.3661.

Representative Experiment for the Check of Racemization during Coupling Reaction of Fmoc Amino Acid to the Aniline Linker. Fmoc-Ser(t-Bu)-incorporated aniline linker **4h** (50 mg, 0.059 mmol) was treated with 20% piperidine in DMF (0.5 mL) at room temperature. After 10 min, the solvents were completely evaporated off using oil pump. To the residue was added preactivated Boc-L-Leu or Boc-D-Leu derivative, prepared from reaction of Boc-L/D-Leu-OH (16 mg each, 0.065 mmol) with HBTU (22 mg, 0.059 mmol) and DIPEA (10 mL, 0.059 mmol) in DMF (0.5 mL) for 10 min at ambient temperature. After 30 min reaction, a mixture of EtOAc and saturated $NaHCO_3$ aq was added to quench each reaction. The organic layer was washed with saturated $NaHCO_3$ aq, 10% citric acid aq, and brine and dried over $MgSO_4$. After removal of the solvent, each sample obtained from the coupling with Boc-L-Leu-OH or Boc-D-Leu-OH was analyzed by reversed-phase HPLC on analytical column. Because major coupling product (D-Leu-L-Ser dipeptide) resulting from the Boc-D-Leu-coupling corresponds to the epimer of the reacemization product (L-Leu-D-Leu diepetide), extent of the racemization was determined as integration of minor peak correspond to D-Leu-L-Ser dipeptide as fraction of the sum of major and minor dipeptides by HPLC analysis of Boc-L-Leu-coupling sample.

General Experimental Procedure for the Synthesis of SEAlide Peptides 7. To NovaSyn TGR resin (Rink amide type: 0.25 mmol amine/g, 0.40 g, 0.10 mmol) was coupled Fmoc-Leu-OH (100 mg, 0.30 mmol) with the aid of diisopropylcarbodiimide (DIPCDI) (48 mL, 0.30 mmol) and HOBt·H_2O (48 mg, 0.30 mol) in DMF at room temperature for 2 h, followed by Fmoc removal with 20% piperidine in DMF, to give Leu-incorporated resin (H-Leu-NovaSyn TGR resin). The resulting resin was treated with an equimolar mixture of an acid-type *N*-Fmoc amino acyl-*N*-sulfanylethylaniline linker (Fmoc-**X**-*NAr*-OH **10**: 0.20 mmol), HATU (76 mg, 0.20 mmol), and DIPEA (36 mL, 0.20 mmol) at room temperature for 3 h to yield an Fmoc-**X**-*NAr*-incorporated resin. On the resulting resin, standard Fmoc SPPS (coupling with an Fmoc amino acids (3 equiv) using DIPCDI/HOBt·H_2O (3.0 equiv each) (2 h) and Fmoc removal with 20% piperidine in DMF (10 min)) was performed for peptide chain elongation to give a protected peptide resin for a model SEAlide peptide **7**. The completed resin was exposed to a TFA-based reagents cocktail (TFA−thioanisole−*m*-cresol−EDT−H_2O, 80:5:5:5:5 or 90:2.5:2.5:2.5:2.5, (v/v), 50 μL/1 mg of resin) at room temperature for 90−120 min. The reaction was filtered to give filtrate to which was added cold Et_2O to afford a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to give a crude model SEAlide peptide **7** (H-**VQGSX**-*NAr*-**L**-NH_2: **X** = **G** (**7a**), **A** (**7b**), **V** (**7c**), **F** (**7f**), **S** (**7h**), **N** (**7i**), **D** (**7j**), **E** (**7l**), and **H** (**7r**)). The crude materials, except for **7j**, were purified by reversed-phase HPLC on preparative HPLC column using linear gradient using solvent system consisting of 0.1% TFA in H_2O and 0.1% TFA in CH_3CN. For Asp-containing peptide **7j**, linear gradient of CH_3CN in 10 mM NH_4OAc buffer was used.

7a (X = **G**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 18.4 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{32}H_{52}N_9O_9S$ $[M + H]^+$ 738.4, found 738.1.

7b (X = **A**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 18.7 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{33}H_{54}N_9O_9S$ $[M + H]^+$ 752.4, found 752.2.

7c (X = **V**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 20.8 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{35}H_{58}N_9O_9S$ $[M + H]^+$ 780.4, found 780.5.

7f (X = **F**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 23.4 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{39}H_{58}N_9O_9S$ $[M + H]^+$ 828.4, found 828.6.

7h (X = **S**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 17.5 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{33}H_{54}N_9O_{10}S$ $[M + H]^+$ 768.4, found 768.1.

7i (X = **N**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 17.1 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{34}H_{55}N_{10}O_{10}S$ $[M + H]^+$ 795.4, found 795.2.

7l (X = **E**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 18.9 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{35}H_{56}N_9O_{11}S$ $[M + H]^+$ 810.4, found 810.2.

7r (X = **H**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 10 to 40% over 30 min, retention time = 15.0 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{36}H_{56}N_{11}O_9S$ $[M + H]^+$ 818.4, found 818.4.

7j (X = **D**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 10 to 40% over 30 min, retention time = 16.6 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{34}H_{54}N_9O_{10}S$ $[M + H]^+$ 796.4, found 796.2.

7j-thioester Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 10 to 40% over 30 min, retention time = 17.0 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{34}H_{54}N_9O_{10}S$ $[M + H]^+$ 796.4, found 796.2.

Synthesis of N-Terminal Cysteinyl Peptide 8. N-Terminal cysteinyl peptide **8** was prepared by standard Fmoc SPPS protocol on NovaSyn TGR resin (Rink amide type: 0.25 mmol amine/g, 0.40 g, 0.10 mmol) by procedures identical to those reported.[12a]

General Experimental Procedure for NCL of SEAlide Peptides 7 with N-Terminal Cysteinyl Peptide 8. Model NCLs of SEAlide peptides **7** (1.0 mM) with N-terminal cysteinyl peptide **8** (1.0 mM) were performed in 3 M Gn·HCl−0.5 M Na phosphate buffer in the presence of 30 mM MPAA and 40 mM TCEP (pH 7.3) at 37 °C. For each reaction, at each time point 5 μL aliquots were withdrawn and analyzed by analytical reversed-phase HPLC on Cosmosil 5C_{18}-AR-II analytical column (4.6 × 250 mm; detection at 220 nm) with a concave gradient (curve 7 of the Waters 600E) of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min. The extent of ligation was quantified by integration of the ligated products **9** as a fraction of the sum of the unreacted N-terminal cysteinyl peptide and the ligated products.

For each reaction yielding the ligated peptides **9**, a whole reaction mixture was fractionated by preparative reversed-phase HPLC on Cosmosil 5C_{18}-AR-II preparative column (20 × 250 mm) after 24−72 h

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reactions to yield the desired ligated peptide. Epimerization during couplings of **9b**, **9h**, and **9r** was examined by comparative HPLC analyses of crude ligation products with D-amino acid-incorporated reference peptides, which were synthesized by standard Fmoc SPPS.

9a (X = **G**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 12.0 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{43}H_{73}N_{16}O_{12}S$ $[M + H]^+$ 1037.5, found 1037.4.

9b (X = **A**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 12.3 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{44}H_{75}N_{16}O_{12}S$ $[M + H]^+$ 1051.5, found 1051.6.

9c (X = **V**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 10 to 40% over 30 min, retention time = 12.3 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{46}H_{79}N_{16}O_{12}S$ $[M + H]^+$ 1079.6, found 1079.8.

9f (X = **F**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5 to 35% over 30 min, retention time = 18.7 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{50}H_{79}N_{16}O_{12}S$ $[M + H]^+$ 1127.6, found 1128.0.

9h (X = **S**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 12.0 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{44}H_{75}N_{16}O_{13}S$ $[M + H]^+$ 1067.5, found 1067.8.

9i (X = **N**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 12.2 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{45}H_{76}N_{17}O_{13}S$ $[M + H]^+$ 1094.6, found 1094.2.

9l (X = **E**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 10 to 25% over 30 min, retention time = 11.9 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{46}H_{77}N_{16}O_{14}S$ $[M + H]^+$ 1109.6, found 1109.2.

9r (X = **H**). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 16.7 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{47}H_{77}N_{18}O_{12}S$ $[M + H]^+$ 1117.6, found 1117.6.

NCL of pure **7j** with **8** was also conducted under reaction conditions identical to those employed for other SEAlide peptides. After 24 h reaction, HPLC analysis of the reaction showed the formation of two ligated peptides, which had the same molecular weight. Further analysis for determining which peptide is α- or β-peptide was not attempted.

9j (early eluted) Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 19.0 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{45}H_{75}N_{16}O_{14}S$ $[M + H]^+$ 1095.5, found 1095.5.

9j (later eluted) Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5−45% over 30 min, retention time = 19.4 min; LRMS (ESI-Ion Trap) m/z calcd for $C_{45}H_{75}N_{16}O_{14}S$ $[M + H]^+$ 1095.5, found 1095.6.

■ ASSOCIATED CONTENT

Supporting Information

HPLC charts of crude model SEAlide Peptides **7**, of representative NCL of SEAlide peptides **7** with N-terminal cysteinyl peptide **8**, of check of racemization during coupling reaction of Fmoc amino acids to the aniline linker, and of check of epimerization of C-terminal amino acids during NCL. Copies of ^1H and ^{13}C NMR spectra of compounds **4c**, **4d**, **4e**, **4f**, **4g**, **4h**, **4i**, **4j**, **4k**, **4l**, **4m**, **4n**, **4o**, **4p**, **4q**, **4r**, **4s**, **4t**, **4u**, **10c**, **10f**, **10h**, **10i**, **10j**, **10l**, and **10r**. 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.

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